75 YEARS OF BIO-SCIENCE RESEARCH IN INDIAN ATOMIC ENERGY PROGRAMME

Editors

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FOREWORD

Dr. Homi Jehangir Bhabha, the father of the Indian nuclear programme, recognised the importance of Bioscience research in Indian Atomic Energy programme. He articulated in his concluding presidential address at the International Conference on Peaceful Uses of Atomic Energy, 20th August, 1955 that "*..the discussion of the genetic effects of radiation clearly showed that we have not enough knowledge on which to base definite conclusions, and that a concerted and massive research effort on this problem is required, before we can be quite sure that no suffering will be caused to future generations through the production of deleterious mutations."* That Dr. Bhabha chose Dr. Gopal-Ayengar, a world-renowned biologist, as one of the founders of the Atomic Energy Establishment at Trombay establishes the perceived importance of Bioscience research in the departmental activities.

Understanding the cytological effects of radiation and radiomimetic substances in biological systems was a major area of research in the initial years. The Bioscience research programs quickly expanded in to the areas of nuclear agriculture, food irradiation, molecular biology, cancer biology and protein crystallography. In addition to providing an understanding of the basic cellular processes, these programmes have contributed to the release of improved crop varieties, development of irradiation-based preservation protocols for food grains and fruits and development of radioprotectors. A significant contribution to the societal benefits of these spin-off technologies derived from nuclear science is a demonstration of the multidisciplinary nature of the department.

I am delighted to know that the Bioscience Group is commemorating 75 years of R&D activities this year. I am pleased to note that as a part of these celebrations, this book entitled "75 years of Bioscience Research in Indian Atomic Energy Programme" is being published. The topics detailed in this book chronicle the gamut of developments that took place in the area of biological research over more than seven decades, attempting to highlight the significant societal implications of the atomic energy programs. I am glad that the chapters provide a historical perspective to the evolution of some of the important R&D activities and at the same time carry a treasure of information relevant for future endeavors.

I compliment all the contributing authors and the editors for their diligent efforts in bringing out this compilation and wish everyone an enriching experience from this book.

25.09.2024

(Vivek Bhasin)

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PREFACE

Right from the inception of Atomic Energy Establishment under the inspiring leadership of Dr. Homi Jehangir Bhabha, Bio-science research has been an integral part of the R&D activities of the centre. Dr. Gopal-Ayengar who was a founding member of the Atomic Energy Establishment laid the foundation stone for Bioscence program which has expanded in size and scope over the years. This book is an attempt to chronicle the seventy-five years of glorious journey of bioscience research and look forward to the future with a greater sense of dedication to achieve the Amritkaal objectives of the Nation. This compendium encompasses an account of the rich legacy of research and development activities by the predecessors, a bird's-eye view of the current activities and a forecast of future directions.

Beginning with a historical perspective of the Bio-Science research in BARC, achievements in important research areas contributing to food security (such as nuclear agriculture and food irradiation and preservation) healthcare (radioprotectors, cancer biology, immunology, diagnostics), environmental research related to agriculture (soil health, bioremediation, waste management) and high impact basic research are covered in this book. In addition, significant contributions have been made towards societal applications through several spin-off technologies. This demonstrates the amalgamation of basic research and its translational potential which are being highlighted in this book. An attempt has been made to structure the articles in such a way that it can be understood by someone who may not have the domain knowledge. Each article provides a historical perspective, an account of current research and a brief account of the way forward. Further, we have attempted to present the chapters thematically and also made an effort to showcase the multidisciplinary nature of the department. We believe this book will serve as an inspirational archive for the younger generations to reflect on the rich legacy and also as a reference guide for future activities.

We express sincere gratitude to Dr. A. K. Mohanty, Chairman, Atomic Energy Commission and Secretary, Department of Atomic Energy and Shri Vivek Bhasin, Director, Bhabha Atomic Research Centre for their support, encouragement and guidance in bringing out this book. The efforts of all contributing authors are greatly acknowledged for timely compilation of the chapters. Special thanks are due to Shri Dhanasekar and Ms. Anusree Dey for design of the cover page. We thank Dr. S. Adhikari, Director, KMG, Shri Manoj Singh, Head, SIRD for providing information from the archives and facilitating the publication of the book. We specially thank colleagues from SIRD, in particular, Shri Sanjay Kumar Singh, Shri Nilesh Pote and Shri Bhusan Chavan for extending all help required to publish the book at a short notice.

While we have made all efforts to make it error-free, we will be highly thankful to the readers to bring to our notice any inadvertent errors that might have crept in.

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About the Editors

Dr. Devashish Rath joined Molecular Biology Division, Bhabha Atomic Research Centre (BARC), Mumbai in 1997 through BARC Training School. Currently, he is Head, CRISPR Biology Group, Applied Genomics Section, BARC and Associate Professor at Homi Bhabha National Institute (HBNI), Mumbai. His current research focus is on basic and applied studies of CRISPR-Cas systems including, CRISPRi, genome editing, study of essential genes as potential drug targets and development of CRISPR-based point-of-care diagnostics.

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DOWN THE MEMORY LANE: SEVEN DECADES OF BIO-SCIENCE RESEARCH

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Two years ago, India completed 75 years since independence, a memorable milestone indeed. As a part of the exercise of taking stock of the nation's achievements and charting a course for the future, developments in scientific research and its applications also need to be reviewed. Department of Atomic Energy (DAE) has been at the forefront of national emergence. Its self-reliant nuclear programme, vision and nurture of talented young engineers and scientists have contributed immensely to India's progress towards strengthening not only its national security but also energy security, health security and food security. Bhabha Atomic Research (BARC) has been spearheading this effort. The vision of our Founder, Dr. Homi Jehangir Bhabha, extended much beyond energy production by harnessing nuclear power. He also emphasized the development of non-power applications of nuclear energy and radioisotopes for industry, agriculture and healthcare along with building a strong directed as well as futuristic basic research programmes. I am very proud to state that the Bio-Sciences/Bio-medical Group of BARC has distinguished itself, in the seven decades of its existence, through its contributions in food security by improving crop productivity, food preservation methods and to healthcare by establishing radioisotope based diagnostics and treatments under nuclear medicine while at the same time the group has consistently pursued quality basic research in radiation biology, biochemistry, molecular and cell biology, biotechnology, food science and agriculture.

The Bio-sciences group has its origins in the Cell Biology Unit of Atomic Energy Commission in Tata Memorial Hospital (TMH) created in 1948 with Late Dr. A R Gopal-Ayengar as its Head. It later became Biology Division of DAE with Dr. Gopal-

Ayengar as Assistant Director in 1952. The laboratories were first housed at the then Indian Cancer Research Centre attached to the Tata Memorial Hospital. Its research activities gathered momentum with cytologists such as Dr. K.C. Bora, biochemists such as Dr. M. B. Sahasrbuddhe, Dr. A. Sreenivasan and food technologists such as Dr. P. B. Mathur joining the team. The laboratories then moved to the Richardson & Cruddas building in Byculla, South Mumbai blossoming into the Biology Group of Atomic Energy Establishment, Trombay with Dr. Gopala-Ayengar assuming charge as its first Director in 1962. Medical Division, which was a separate entity, primarily tasked with healthcare services to DAE employees in Mumbai operated from Sir J. J. Hospital also had a research programme in mammalian radiobiology and clinical research with radioisotopes. It was initially a part of the Biology Group. In 1963 another Division, Biochemistry and Food Technology Division was created within the Biology Group. A National Botanical Garden with an area of approximately 6,06,000 sq. metres was developed at Atomic Energy Establishment, Trombay, later renamed as BARC after Dr. Bhabha's shocking and untimely demise. After some years this garden was excluded from Biology Group. In 1963, Radiation Medicine Centre was created in the TMH complex under the Biology Group. In 1967, the group was designated as Bio-Medical Group (BMG), again, with Dr. Gopala-Ayengar as Director till he retired in 1969. Over the years, changes in the name and the composition of the group were necessitated by structural reorganization from time to time. Nevertheless, the vital programs initiated in the early years remained unaltered.

In 1971 or early 1972 late Dr. K. Sundaram succeeded Dr. Gopala-Ayengar as Director, BMG. Biology Division had then become Biology and Agriculture Division. Bio-Organic Division was also created at that time and Dr. Sundaram held office till 1986. In between during the three-year assignment of Dr. Sundaram in IAEA at Vienna, Shri. N. S. Rao, Dr. V.R. Shah, and Dr. S. M. Sharma were designated Head of the Biology Group, Medical Group and RMC respectively. After Dr. Sundaram's retirement the group was split, late Dr. N. K. Notani became Director of Biology Group and late Dr. M S. Chadha became Director, Biochemical Group. After Dr. Chadha's retirement in 1990, Dr. Notani became Director, BMG. He was succeeded by late Dr. C. R. Bhatia as Director, BMG till he left to become Secretary, DBT in 1992. Dr. D.V. Gopinath who was Director, Health safety and Environment Group also held the charge of Director BMG. In 1994, the group was again split and Prof. P.C. Kesavan, who came from JNU, became the Director of Bio-Sciences Group (BSG). It again became BMG when Dr. Mrs A. M. Samuel took over as Director after Prof. Kesavan retired in 1998. She retired in 2002 and again BSG and Medical Group were created and I became Associate Director, BSG and Dr. B. J. Shankar was designated Associate Director, Medical Group. In January 2006, Medical Division and RMC were again merged with BSG and I was designated Director, Bio-Medical Group. I held office till the end of October 2013. The group was again split and Dr. S. K. Apte became Director, BSG. Dr. S. Chattopadhyay, Dr. V. P. Venugopalan, Dr. S. K. Nayak, Dr. T. K. Ghanty and Dr. A. K. Tyagi were designated successive group Directors of BSG after Dr. Apte's retirement in 2014.

Currently the BSG is headed by Dr. P.A. Hassan as Associate Director. In between there were occasions when Director, BARC, Director Physics Group, BARC and even Director, TMC held charge of Director, BMG. Shri N. S. Rao (Biology Group), Dr. D. S. Pradhan, (Biology/Biochemical Group) Dr. S. M. Sharma, Dr. V. R. Shah, Dr. Usha Desai, Dr. Mrs. Samuel and Dr. B. J. Shankar (All from Med Group), Dr. S. K. Apte, Dr. S. F. D'Souza (BSG/BMG), Dr. S. P. Kale, Dr. S. Chattopadhyay, Dr. V. P. Venugopalan, Dr. S. K. Nayak, Dr. S. K. Ghosh and Dr. T. Ghanty (all BSG) have also served as Associate Directors. The activities of the BSG are today physically located at, Trombay, Kollam, Kerala, BARC-Vizag, Andhra Pradesh and Gauribidanur, Karnataka.

As early as 1927, Prof. H. J. Muller showed that X-rays could cause mutations, i.e., changes in heritable characteristics, in the fruit fly. These effects of radiations were most dramatically revealed to the world at large with the atomic bombing of Hiroshima and Nagasaki Hiroshima in August 1945. The survivors constitute a most extensively and critically studied cohort of radiation exposed population that has been providing important data on the nature and extent of radiation induced damage. In the famous "mega mouse" experiment thousands of mice were exposed to radiation to study induction of mutations, the impact on life span of animals due to external exposure as well as ingestion of radioactive material were undertaken. Similar studies were also initiated globally on other living organisms like plants and microbes. BARC was no exception. United Nations Scientific Committee on Effects of Atomic Radiation (UNSCEAR) was constituted in 1955 and it estimates the exposure of human population from various sources of ionizing radiation, both natural and man-made, e.g., radioactive fall-out from nuclear weapons tests, discharges from nuclear facilities, medical radiation and the ensuing potential health risks like cancer and hereditary effects. India has been a founder member of this now 31 nations scientific committee. Group Directors of BMG/BSG and HSG as well as some senior scientists with domain expertise have represented India in this committee's deliberations. The availability of radiolabelled biomolecules provided the much-needed sensitive tracers to delineate pathways of a myriad biochemical reactions underlying physiological processes and some of them proved very useful as radiopharmaceuticals, diagnostic as well as therapeutic tools. These developments have transformed the conduct of research in biology. The understanding of the effects of radiation continues to improve with better understanding of the naive unexposed, and hence undamaged, living system.

1. Sustainable Agriculture

Dr. Gopal-Ayengar initiated several research programmes. To study radiation's mutagenic effects, he established the Gamma Garden at the Experimental Field Station, Trombay with a 500-curie cobalt-60 source. This was utilised to do induce mutations in ornamental plants. Research on mutation breeding had humble beginnings. In the 1960's, mutants generated by neutron irradiation of Geb 24 rice seeds flowered 21 days earlier, with 15% more yield. Also, superior performance of large pod mutant groundnuts was observed in trials conducted in Trombay, Mandala, Jalgaon, Sai, and Talaja, in

Maharashtra, and in Gujarat. This led to the submission of the proposal for the release of two groundnut mutants, TG-1 and TG-3, to the Central Varietal Release Committee in the 1970's. Mutation breeding experiments using irradiated seeds of different crop plants like groundnut, linseed, pulses, jute, and rice were also undertaken. TG1 (Groundnut) was the first mutant variety developed at Trombay and released for commercial cultivation in 1973. Today, radiation-induced mutation-based crop improvement is a flagship programme of the DAE and so far 62 such mutants of twelve different crops have been developed in collaboration with several agriculture universities and after a series of mandatory trials stipulated by ICAR, they have been gazette notified and released for commercial cultivation by farmers. These include crops like groundnut (16), black gram (8), green gram (9), soybean (2), mustard (9), sunflower (1), pigeon pea (5), cowpea (2), rice (7), jute (1), linseed (1) and sorghum (1). A large number of selections are in different stages of development and trials. They are higher yielding, early maturing, and some show disease and drought resistance. Our contributions are especially significant in the oil seeds, pulses and recently in rice crops. Furthermore, Trombay mutant varieties contribute very significantly to the national breeder and foundation seeds indent.

Recently, the Indian Council of Agricultural Research recommended foliar spray of thiourea for achieving higher yield of Soybean following extensive research done by our scientists in BSG and their collaborators from Agriculture Universities. More importantly this work was backed by exhaustive and excellent basic research at molecular level conducted by our young scientists in Nuclear Agriculture and Biotechnology Division (NA&BTD). The Division also has programmes on soil science (utilization of fertilizers), pesticide residue assessment and integrated pest management. In the 1970s, the mosquito larvicidal properties were demonstrated in garlic extract and the active principles were found to be diallyl disulfide and diallyl trisulfide. This work was published in Science in 1971. Sterile Insect technique and method for Biological Control of insect pests using crystalliferous *Bacillus thuringiensis* and *B. sphaericus* were also developed.

2. Plant Biotechnology

Plant tissue culture and plant biotechnology has been another important area of research in BSG. In the early days, biotechnology research focused on haploid plants generation, protoplast culture, plantlet regeneration from protoplasts, somaclonal variants, micropropagation techniques etc. In the early 1980's, anther and pollen cultures of potato, rye, capsicum, and Physalis species were started at BARC in collaboration with Max Planck Institute, Germany. Protocols were developed for micropropagation of several commercially important plants like several elite varieties of banana, pineapple, sugarcane, sandalwood, and several medicinal plants that produced anticancer, anti-HIV and antimalarial drugs. A bioreactor for cultivation of cultured plant cells was developed and the technology was transferred to Kabra Drugs. The banana micropropagation technology has been transferred to Krishi Vigyan Kendras as well as private entrepreneurs. First transgenic tobacco plants containing kanamycin resistance were

developed in BMG and later transgenic plants containing herbicide resistance gene Bar were also generated. After the year 2000, a spin off technology was developed for biodegradable waste management called Nisargruna Biogas Plant. This is a biphasic bio-methanation technology that uses aerobic predigester followed by anaerobic main digester and processes various kinds of waste like kitchen waste, food waste, cow-dung and abattoir waste. Currently there are more than 300 such installations all over India.

3. Radiation preservation of food and food products

Research on preservation of food grain and food products started under the leadership of late Dr. A. Sreenivasan after the group moved to Trombay and a separate laboratory, FIPLY (Food Irradiation and Processing Laboratory) was built for that purpose. A package food irradiator with a Co-60 source was donated by Atomic Energy of Canada Ltd for this purpose. Later on, this facility was further augmented by installing a Cs-137 source. Work on radiation mediated microbial decontamination and disinfestation of food grain, meat, fish, fruit and vegetables and delayed ripening of fruit (especially mangoes) and enhancement of shelf life has been going on there since then. Combination of irradiation and GRAS chemicals as well as other hurdles technologies like low temperature, moisture control have also been employed. Development of new packaging materials was also undertaken. Several microbiologists, biochemists, food technologists, toxicologists and some chemical engineers have sustained this programme. An extensive study on the wholesomeness of irradiated wheat including genetic toxicology was undertaken and the irradiated wheat was successfully demonstrated as safe for human consumption. In 1995 the Government of India, Ministry of Health accorded clearance for radiation processing of onions and potatoes for prevention of sprouting which would enhance shelf life. First technology demonstration plant for this purpose, "KRUSHAK", was commissioned at Lasalgaon near Nasik in 2002.

On 26 April 2007 the first batch of Alfonso mangoes irradiated at KRUSHAK were air-shipped to USA and there has been no looking back since then. By 2012, a process for preventing browning and extending shelf life of litchi fruits for 45-60 days by a sequential dip treatment using GRAS chemicals was developed by BARC and the technology was transferred to SCRIMAD, Madagascar. FTD's contributions resulted in obtaining a class specific approval of the Ministry of Health for radiation processing of different food items. Over 7 such classes of foods covering nearly 100 food items have been approved after the amended Atomic Energy Act (Radiation Processing of Foods and Allied Products) Rules 2012 have come into effect. BARC also developed a fruit vegetable dip treatment machine for processing litchi and other fruits. A high-throughput plant was set up at NRCL, Muzaffarpur, for processing 15 tons of litchi fruits. By 2020, GRAS preservatives were also approved for treating mangoes to extend shelf life. Based on the success of these programs, as many as 28 food irradiation facilities are commercially operating in India today and the MoFPI has come out with a notification for expression of interest to avail financial support to set up 50 such plants for radiation processing of food.

In the last decade or so several new products have been developed e.g. ready to eat food, food for immunocompromised individuals and defence forces working in remote areas and at high altitudes. Apoptosis in bacteria was investigated. A technology for making banana juice was also developed. A cytotoxic and immunosuppressive red pigment (Prodigiosin) was isolated from small prawns, Jawla. Studies were also carried out to detoxify Salmonella using gamma radiation. A technology for conversion of cane sugar to invert sugar was developed and transferred.

4. Radiation Medicine Centre (RMC)

Steered by late Dr. R. D. Ganatra and Dr. Jeejeebhoy and located in the TMH complex in Parel, RMC was started in September 1963 and it became the first WHO recognized centre for investigation and treatment of thyroid diseases in India using radioisotopes (mainly I-131). It is a pioneer in nuclear medicine in India, Radioimmunoassay, Tc99m labelled radiopharmaceuticals and scintigraphy have been the most dominant diagnostic tools till 2002 when India's first Medical Cyclotron for production of F-18 and PET Scanner became functional there and that has benefitted a huge number of patients of cancer and other diseases. RMC also provides services in nuclear cardiology, radioanalytical clinical services and radioisotope therapy for thyroid and other cancers. Additionally, spoligotyping of clinical Mycobacterial strains of different lineages, development of an ELISA assay for antigens of *Mycobacterium tuberculosis* and studies related to innate immune response to Mycobacterial infections have been carried out at RMC. In recent years Lu-177 based scintigraphy and therapy have been started.

5. Medical Division

This Division has provided quality healthcare to more than 100,000 DAE employees and their families in Mumbai. BARC Hospital in Anushaktinagar is a 390-bed tertiary care facility equipped with ICCU, NICU as well as modern diagnostic equipment like CT and MRI and a state-of-the-art dental care facility. It is supported by zonal dispensaries and occupational (industrial) health dispensaries. The present main building of the hospital was occupied in mid 1970s. An annexe was added many years later. During the recent years several new equipment have been added to augment patient care. Medical Division also has a strong academic programme in 10 specialities leading to DNB.

6. High Level Natural Radiation Areas in Kerala

The study of radiation effects in various biological systems has been the most important mandate of the Bio-science Group. These effects are known to be dose dependent. Globally there is a growing interest in the effects of very low doses of ionizing radiation as these have tremendous implications to the International Council for Radiological Protection (ICRP) prescribed limits of exposure for radiation protection of workers and general public. One of the flagship projects of BMG is the studies on the human population living on the south west coast of Kerala which is rich in monazite sand. This

population is continuously exposed to high level natural radiation at all stages of development. It has a high population density and people have been living there for tens of generations for nearly a thousand years. The external radiation dose varies between 1.5 to 45 mGy. The importance of this area to radiation biology was recognized by WHO in the early 1960s A project called Monazite Survey Project was initiated on Dr. Gopal-Ayengar's initiative in mid1960s. Cytogenetic studies on the population were started by K. P. George. In the 1970s a systematic study of this population was undertaken with respect to house dose, demographic profile, reproductive performance, assessment of cytogenetic parameters etc. Early results with rats and in adult human beings did not show increased genetic damage in the exposed population. A dedicated laboratory (LLRRL) was commissioned in Kollam for this purpose in 1999. Under its aegis, a more extensive study on screening of newborn children was undertaken in which nearly 2,00,000 new-borns have been screened for the incidence of nearly 100 different congenital malformations detectable at birth, still births and twins, Down's syndrome, chromosomal aberrations (stable and unstable, structural and numerical), micronuclei frequency, telomere length etc. None of these showed any significant difference between those born to parents from High Level Natural Radiation Areas (HLNRA) and those born to parents from Normal Level Natural Radiation Areas (NLNRA). A case control study on mental retardation and cleft-lip and cleft-palate also did not reveal any deleterious effect of high natural radiation. The older exposed population from HLNRA shows lesser DNA damage than their age matched NLNRA counterparts, indicating a better DNA repair and a radio-adaptive effect. At the molecular level, studies on DNA mutations based on more than 50 hypervariable loci in human DNA and more than 200 families have also not indicated any change due to HLNR exposure. These studies are regarded as unique and extensive and have drawn the attention of the global low dose researchers and radiation protection community alike. The exposure here mimics the likely continuing exposure scenario after a nuclear accident like the one in Fukushima. In Kerala as well as in China, where a similar high natural background radiation area exists, the excess relative risk of cancer has been found to be marginally negative according to the analysis performed by reputed Japanese epidemiologists. The work done in last two decades in LLRRL has been published in reputed journals in the field and has been taken note of by international bodies like UNSCEAR. This is a stupendous achievement and efforts are on to harness newer developments in genomics to understand global gene and protein expression profiles, epigenetic changes and microRNA mediated gene regulation as well as look for changes in specific genes as indicators of low dose radiation associated effects or lack of them. Correlating individual exposure to health effects as an end point is a daunting task and is likely to be part of future research endeavours.

7. Basic Research

Basic research is very fascinating to most youngsters in biology. Evaluating the effects of radiation on different biological systems has been a topic of interest in bioscience group since its inception. The research programs included cytogenetics (plants, animal &

human), *in vitro* cell systems and molecular studies. In the Biochemistry and Food Technology Division several studies were undertaken to evaluate the effect of ionizing radiation on processes such as carbohydrate metabolism, nucleic acid and protein metabolism and energy metabolism and vitamins. Studies on carcinogenesis were also initiated in mid 1970s. Enzyme immobilization research was initiated during that time which led to larger programme on enzyme biotechnology and has now matured to the stage of development of biosensors for a pesticide and urea. The studies on pathways of genetic recombination in *E. coli*, UV sensitivity of *E. coli*, transformation in *Haemophilus influenzae* and responses to osmotic, salinity and oxidative stress in cyanobacteria, radiation resistance in *Deinococcus radiodurans* and cyanobacteria, use of thermoluminescence (TL) technique to probe photochemistry of photosystem II, and organization of multiprotein complexes in photosynthetic carbon fixation were undertaken in MBD. A proteomic map of *Deinococcus radiodurans* after radiation exposure has been constructed Several critical genes regulating DNA repair and radiation resistance in that organism have been identified. Mechanism of formation of multiple forms of superoxide dismutase under oxidative stress was demonstrated in cyanobacteria. DNA markers for rust and drought resistance in rice were developed. Evidence for the existence of a multienzyme photosynthon complex containing RuBP Carboxylase was gathered.

DNA repair, redox regulation in mammalian cells, radiation protection, apoptosis or programmed cell death in cancer cells, modification of tumor cytotoxicity by tumor microenvironment have been investigated in RB&HSD. A V-D-J recombination mediating enzyme complex in thymocyte nuclear extract was shown using a synthetic substrate. Many genes that regulate these processes were identified. Augmentation of cell mediated immune response was shown in low dose exposed mice but for the first time differences were also revealed based on the genetic background of the animals and the type of antigen and response. Positive bystander effect of radiation exposure was demonstrated in lymphocytes for the first time. Mechanism of radio-adaptive effect in lymphocytes has also been investigated.

Several naturally occurring substances including extracts of medicinal plants were evaluated for their antioxidant and radioprotective actions. One of them, Chlorophyllin is a component of the recently DCGI approved and commercialized radio-modifier tablet, AKTOCYE® which has proved useful in the treatment of haemorrhagic cystitis, a side effect of radiotherapy. An acidic arabinogalactan obtained by activity-based purification of stem extracts of *Tinospora cordifolia* has been shown to be a strong stimulator of macrophages, dendritic cells and B lymphocytes. It protected against endotoxic shock, induced maturation of dendritic cells and enhanced their cytotoxicity to tumor cells, enhanced innate immunity against Mycobacteria and also inhibited growth of *Mycobacterium tuberculosis* in mice.

In mammalian Radiation Biology, radiosensitization of cancer cell by hypoxic sensitizers and hyperthermia and radioprotection by a variety of agents such as caffeine and anaesthetics were thoroughly investigated using mice as well as several cell lines. These

studies were extended to include ion beam irradiation and alfa particle irradiation, the latter using a newly developed BARC Alfa Irradiator. A host of chemicals were also evaluated for their genotoxicity using micronuclei formation, DNA fragmentation (SCGE or Comet assay), gamma H2AX foci etc. Studies on toxic effects of thorium incorporation and methods for its decorporation have been under investigation. Furthermore, these basic research programmes enabled our scientists to establish sensitive, state-of-the-art, high throughput techniques in our laboratories and achieve recognition by their peers elsewhere. These include establishment of facilities for genomics, proteomics, gene expression analysis, transgenics and gene cloning, knock-out and silencing, DNA sequencing, microarray, flowcytometry, confocal microscopy, surface plasmon resonance, transmission electron microscopy, MALDI-TOF spectrometry etc.

8. Bioremediation

A highly radioresistant bacterium, *Micrococcus radiophilus* was isolated in late 1960s in FIPLY. In the mid-1990s work on the highly radioresistant bacterium, *Deinococcus radiodurans* was initiated to understand the mechanism of its extreme radioresistance. *E. coli* and *Deinococcus* were genetically engineered to express a phosphatase gene and were shown to sequester uranium. Bioremediation of tailing ponds near the uranium mining sites using some indicator plants was also carried out. These achievements have resulted in addition to recognition to individual scientists for reporting some significant observations for the first time, in opening up possibilities for useful deployment of technologies or products based on them.

9. Bio-Organic Chemistry

A separate Bio-organic Division was created within the BMG in 1971. It had several organic chemists and some biologists too. Several bioactive natural products were isolated and synthesized. Some of them were evaluated for their insecticidal properties. A success story was about the synthetic pheromones that were used to make pheromone traps for reduction of insect pests like cotton boll worm and sugarcane pests. Plant growth regulator triacontanol and its analogs were synthesized. A convenient method for the preparation of important radiopharmaceutical, namely hexamethylene propylene amine oxime (HMPAO), which had extensive use in cerebral perfusion imaging was developed. In collaboration with BRIT, radiopharmaceuticals for preparation of technetium complexes for brain imaging had been synthesized and the kits made from these were under final stages of evaluation for clinical trials. In recent years this Division has undertaken programmes on anti-cancer drugs and their mechanism of action. Furthermore, ligands for extraction of valuable radionuclides from reactor waste (CMPAO for actinide extraction) and nuclear imaging agent like MIBI (for nuclear cardiology) and PSMA-617(for prostate cancer) have been synthesized and are being used for treatment of patients.

10. Human Resource Development

The long-term mandated research programmes of BMG/BSG could not have been sustained without adequately trained human resources. In the early days the research projects were led by scientists trained for PhD and/or post-doctoral research abroad. In this context, the most important role of BARC training school in initiating as well as sustaining Indian Nuclear Programme in the last seven decades needs to be underscored. Several young graduates from Physics and Chemistry streams of training school joined the BMG in the 1960s and initiated new lines of research in biosciences, especially in radiation biology, immunology and biophysics. The bioscience research in BARC turned a new leaf in 1971 with the introduction of Biology-Radiobiology training programme in BARC training school. During the periods 1971 to 1977, then from 1994 onwards a number of young biologists with varying specializations were recruited through this route. These researchers, along with some of their chemistry and physics counterparts strengthened the research, development and deployment (RDD) programs of not only the Bioscience/ Bio-Medical group but also those of Radiochemistry and Isotope Group, Physics group and Health and Safety Group in BARC. Furthermore, they are actively involved in the RDD projects of our sister institutions like BRIT, IGCAR and RRCAT. The introduction of a large pool of talented researchers over the years, many of whom, through their dedicated and assiduous work brought the quality and productivity to appreciable levels. This also provided the overall sustenance and expanded the scope of research programmes in the Group.

In the field of nuclear medicine, the commencement of two courses viz. Diploma in Radiation Medicine (DRM) for doctors and Diploma in Medical Radio Isotope Technology (DMRIT) for science graduates affiliated to University of Mumbai in 1973 provided a very large trained manpower of clinicians and technicians for large scale utilization of radioisotopes and nuclear medicine in the country. In 1982 the National Board of Examination (GOI) accredited RMC for DNB (Nuclear Medicine). In 2015, MD (nuclear Medicine) course under HBNI replaced the DRM/DNB programme and M.Sc. (NMMIT) and M.Sc (Hospital Radiopharmacy) courses have been introduced under the aegis of HBNI. The impact of these courses on clinical nuclear medicine practice in India has been tremendous.

The Category I and Category II training programmes introduced two decades ago have provided a much-needed technical assistance in the laboratories. Another very significant human resource for the group has been the PhD students joining through BARC-University of Mumbai Collaboration, UGC-CSIR NET and in recent times to a large measure through HBNI's PhD programme. Many BSG/BMG scientists have been recognised as PhD guides for UOM, HBNI and other universities.

11. Research Equipment

Biological research requires several sensitive and expensive equipment and chemicals most of which are imported. Over the decades, the research programs in bioscience group were managed though the import delays and international trade policies, including the periods after India's peaceful nuclear experiments in 1974 and 1998. Even importing a liquid scintillation counter was not easy. Some scientific equipment like a DNA microarray system and image analysis system were built in-house with the help of multidisciplinary expertise. Several advanced instruments including nucleotide synthesizers, DNA sequencer, flow cytometers, MALDI-TOF, NMR spectrometer and transmission electron microscope were procured, through the institutional grants provided from the five-year plan outlays, which made life easier for the younger recruits. In recent years a significant emphasis was placed on patenting and product development and significant progress on that front has also been achieved.

The large and diverse work force of former and present colleagues - both within the bioscience group and in other divisions of BARC/DAE - leaves a credible history behind, placing the bioscience group fully equipped for the imminent challenges and leading to a bright future.

12. Epilogue

An article, "Biology & Medicine: Excitement of Research and Deployment of its Outcome–The Twain Do Meet in BARC" written by the author in BARC Newsletter in 2013 had summarized the then biomedical group achievements and articulated some of the future opportunities and challenges. The sustained progress seen from the collective efforts over the last decade reassures that the present team of biologists are fully equipped for the challenges - with a credible history behind and a bright future ahead.

IONIZING RADIATIONS FOR PLANT MUTAGENESIS: SUCCESS STORY AT BARC, TROMBAY

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Abstract

Ionizing radiation has immense applications in agriculture for crop improvement, crop production and crop protection. Crop improvement is reliant on wide genetic diversity of economic characters towards achieving food and nutritional security. In nature, occurrence of genetic change (mutation) in plants is evolutionarily slow and gradual process. The frequency of such mutations can be increased through ionizing radiations. The radiations cause mutations at chromosomal, gene or DNA level, which may be manifested into desirable characters in crop plants. Since fifties, Bhabha Atomic Research Centre (BARC), Mumbai has been engaged in radiation-based induced mutagenesis to develop improved varieties in cereals, food legumes and oilseeds. Earlier, experiments were undertaken to study radiosensitivity, mutation frequency and cytological aberrations upon induced mutagenesis and its effect on morphological, biochemical and physiological traits in crop plants. Subsequent induced mutagenesis generated large spectrum of mutants in different crops. By identifying promising induced mutants and their utilization in recombination breeding, BARC has developed 62 improved varieties in various crops. These mutants

and mutant derived varieties were released for commercial cultivation across the country in collaboration with State Agricultural Universities and national ICAR institutes. Several of Trombay varieties have been cultivated extensively by the farmers in most of the states and have immensely benefitted by enhancing their income.

Keywords: *Induced mutagenesis, Ionizing radiation, Mutation breeding, Cereals, Food legumes, Oilseeds*

1. Introduction

Mutation is a heritable alteration in the genetic material in an organism, which is the main driving force for crop evolution. Both spontaneous and induced mutations constitute the basis for genetic diversity in crop plants. Genetic diversity for various characters is crucial to crop improvement and enhanced food production, which in turn for ensuring national food and nutritional security. Hugo de Vries in 1901 first identified mutagenesis as a phenomenon of creating genetic variability, which he distinguished from recombination and segregation. Spontaneous mutations were the only source of novel genetic variation till twentieth century, which were used in plant selection for domestication and breeding. After the discovery of the mutagenic potential of X-rays as demonstrated by Stadler in 1928 and 1930 in maize, barley and wheat, radiation based induced mutagenesis was employed as a tool for generating novel genetic variability in plants. In 1928, Gustafsson and Nilsson-Ehle first identified valuable mutations in diploid barley by using X-rays and UV rays. The first commercial mutant variety was produced in tobacco in 1934. Bhabha Atomic Research Centre (BARC), Mumbai has been engaged in the field of crop improvement by using ionizing radiations through mutation breeding since fifties.

2. Induced plant mutagenesis

Mutagenesis is the process of bringing stable genetic changes, which may include, a) **Induced mutagenesis,** wherein mutation is induced by treating the seeds with physical or chemical mutagens; b) **Insertion mutagenesis,** wherein mutation is obtained due to DNA incorporation from transformation or transposon activation and c) **Site-directed mutagenesis** by creating a mutation at a target site by transformation followed by homologous recombination between native DNA and T-DNA. Usually induced mutants occur at random, whose genes mostly are recessive in nature, occasionally with pleiotropic effects and are influenced by genetic background and environmental effects.

3. Physical and chemical mutagens for induced mutagenesis

A mutagen is an agent that cause mutations in DNA sequence. These agents are physical, chemical and biological in nature. Each mutagen type acts differently in the genome. Physical mutagens, generally ionizing radiations, have been effectively employed for inducing mutations in crop plants. Globally, >85% mutant varieties were developed

through irradiation. Ionizing radiation dislodges an electron from its orbit around the nucleus after passing through a plant tissue, thus producing an ion (ionization) and free radicals. Ionizing radiations such as X rays, gamma rays, beta particles, neutron, proton, electron and ion beams have been utilized for induced mutagenesis in crop plants. They bring changes in DNA sequence either by base substitutions (transition or transversion) or by indels (insertions or deletions). Majority of ionizing radiations are emitted from naturally decaying isotopes and can also be produced artificially in reactors and through accelerators.

Using chemical mutagens is a simple and effective method of altering a single base in the DNA sequence. The most commonly used chemical mutagens for crop improvement include Ethyl methanesulphonate (EMS), sodium azide, colchicine, nitrosoethyl urea and N-methyl N-nitrosourea. EMS is the most effective and potent mutagen causing point mutations, loss of small DNA regions and reorganization in other chromosomes, alkylation of DNA to form base pairs of guanine with thymine instead of cytosine, and conversion of GC base pair to AT. For mutation induction, factors like moisture content of irradiated tissue, mutagen type, irradiation dose, dose rate or concentration and treatment duration are crucial.

4. Mutation breeding methodology

Crop improvement is a continuous process of development of improved varieties suitable for different agro-climatic situations. Natural radiations bring new variability in different characters spontaneously at extremely low frequency (one in a million). Using radiations and/or chemical mutagens, mutation frequency can be enhanced to several folds (one in thousand). Mutation breeding includes induction, isolation and stabilization of mutants and their judicious utilization in cross breeding. In mutation breeding, the main goal is to create suitable varieties with increased seed yield and nutrients, earliness, desired seed size and dormancy, tolerance to diseases, insects, drought, salinity, heat etc.

Successful mutation breeding starts with well-defined objectives to generate new genetic variability for nuclear and/or cytoplasmic traits; to improve one or a few traits in popular and well adapted varieties; to break the tight linkages between the traits; to enhance chromosomal translocations in inter-specific crosses and to improve vegetatively propagated crops. To begin with, effective dose for a mutagen has to be standardized for the selected genotype in given crop by treating seeds with different doses of radiation. After the treatment, values on seed germination and seedling growth from different treatments are subjected to Probit analysis to derive the dose close to LD_{50} (dose that brings 50% reduction in germination) and/or GR_{50} (dose that brings 50% growth reduction). For large scale induced mutagenesis, seeds are treated with 2-3 doses of radiation around LD_{50} or GR_{50} values (M_1 generation) using gamma irradiator (**Fig. 1a**). These treated M_1 seeds are sown in the agricultural fields to raise M_1 plants. Seeds (M_2) from the M_1 plants are sown to raise M_2 generation. Fig. 2 shows field view of M_2

generation of rice and groundnut. Usually mutants are identified from M_2 generation onwards.

Fig. 1: (a) Seed irradiation with gamma rays for induced mutagenesis; (b) Crossing of crop mutants to recombine favourable alleles.

Fig. 2: Field view of M_2 generation of (a) transplanting of rice material; (b) groundnut population at Gamma field, BARC, Trombay.

Homozygosity (stable genetic nature) of the induced mutants is ascertained by studying their breeding behavior in subsequent generations. These stabilized mutants are evaluated with the existing varieties over the locations and seasons to find their superiority in yield parameters, suitability and adaptability in the trials carried out by State Agriculture Universities (SAUs) and Indian Council of Agricultural Research (ICAR). Based on the superiority of new mutant, varietal identification committee of ICAR/SAU recommends the suitable mutant for release. Further, Department of Agriculture and Farmers' Welfare, Ministry of Agriculture & Farmers Welfare, Government of India releases and notifies new mutant for commercial cultivation. Sometimes, such mutants are crossed with other

mutant, variety or distant parent to combine the beneficial traits from both the parents (Recombination or cross breeding) (**Fig. 1b**).

5. Ionizing radiation based mutation breeding at BARC

5.1. Basic experiments on plant mutagenesis

Seed irradiation studies were initiated in the fifties at Atomic Energy Establishment, Trombay (AEET), to study radiosensitivity, mutation frequency, cytological aberrations upon induced mutagenesis and their effect on morphological, biochemical and physiological traits by treating seeds of over 50 varieties of rice, maize, sorghum, water melon, groundnut, cotton, foxtail millet and isabgol with X rays, neutrons and gamma rays. Considerable differences for radiosensitivity among these varieties were observed. LD_{50} values ranged from 70 Gy in brinjal to 400 Gy in mustard for X rays and 100 Gy in peas to 550 Gy in mustard for gamma rays. Stimulating effects of low doses of thermal neutrons were also observed. In 1957, thermal neutron irradiation in rice evolved a plant with awned seeds. Chlorophyll deficiency of several types, pigment development affecting various parts of rice, fasciation of stem and branch in groundnut and water melon were also noted. It was also observed in water melon that the ratio of male and female flowers was altered by pile neutrons in favour of female flowers.

Dosimetry studies were conducted for barley seeds by irradiating with fast neutrons at APSARA reactor and gamma rays in gamma cell. Based on LD_{50} values, barley seeds showed increased radiosensitivity with storage time for fast neutron, while there was no such effect with gamma rays. With a view to study the ploidy-dose level effect on the relative biological efficiencies (RBE) of the radiations, seeds of tetraploid and hexaploid wheat were irradiated with X-rays, fast neutrons and thermal neutrons. It was shown that RBE for fast neutron/X-rays was not significantly influenced by the ploidy, but was dependent on the dose level. Further in another study, RBE values for gamma rays/fast neutron ranged from 6.75 in *Phaseolus lunatus* to 33.70 in barley. RBE values had also been determined for specific mutations affecting culm height in rice. Fast neutrons were effective to induce variability for this trait and offer possibility of dwarf mutants with good fertilizer response. Recently, the pulsed electron beam treatment from linear accelerator in wheat showed slightly higher RBE compared to gamma rays. Further with accelerated proton ions from BARC-TIFR Pelletron accelerator facility, significant differences were observed in wheat seedling growth and survival parameters and later for mutation spectrum compared to gamma rays. Based on the survival and growth curves, the thermal neutron from Dhruva research reactor showed considerably high RBE in wheat compared to gamma rays.

5.2. Effective doses of mutagen for induced mutagenesis in different crops

In food legumes, the effective doses for gamma rays (pigeonpea: 100-200 Gy; groundnut, cowpea: 200-300 Gy; mungbean, urdbean, chickpea: 300-400 Gy) and for electron beam (groundnut: 150-200 Gy; cowpea: 270 Gy, chickpea: 300 Gy; urdbean: 400 Gy; mungbean: 500 Gy) have been standardized through radiosensitivity assays. Gamma rays of 300 Gy and electron beam of 250 Gy are suitable for rice and sorghum mutagenesis. In

wheat, LD_{50} and LC_{50} values were 290–315 Gy and 0.90–1.35%, respectively for gamma rays and EMS under laboratory conditions, 240–290 Gy and 0.50–1.1% under field conditions. In another wheat experiment, it was found that 300 Gy and 350 Gy of gamma rays and 200 Gy and 250 Gy of electron beam were most effective in HD2967 and PBW343, respectively. The frequency of yellow rust resistant mutants was higher in electron beam than in gamma rays. Similarly, in chickpea, electron beam irradiation showed higher mutagenic effectiveness and efficiency compared to gamma rays.

5.3. Cytogenetic effects of radiation based mutagenesis

Radiations cause chromosomal aberrations in the first generation of their treatment (M_1). In an earlier experiment in rice, a single quadrivalent was observed in thermal neutron induced awned mutant. In groundnut, X-rays induced irregular association of chromosomes and their separation at anaphase, abnormal spindle development, chromosomal bridges and fragments, polyad occurrence, cytokinesis failure and developmental anomalies in pollen grains. Further studies have reported reciprocal translocations with chain and ring multivalents, inversions with fragments and bridges at anaphase I and II. In groundnut, trisomics, tetrasomics, long chromosomes with altered coiling, cytomixis and pollen mother cells with 15–18 chromosomes were reported. In X-ray-induced dwarf groundnut mutant, asynaptic chromosomes with reduced number of bivalents at diakinesis were observed.

5.4. Inheritance of induced mutant traits

In various crops, radiation induced mutants were studied for their inheritance pattern of some of the mutant traits and are given in table 1. Certain mutants exhibited unusual genetic behaviour such as preferential segregation for leaflet size and type, paternal inheritance for foliaceous stipule and hard kernel and suppressive gene action for disease mimic leaflet in groundnut.

5.5. Development of Trombay varieties through mutation breeding

With basic understandings from earlier radiation induced mutation experiments and in line with national and state crop breeding programmes, BARC had initiated and streamlined mutation breeding programmes for the improvement of cereals, oilseeds and food legumes. Over six decades of consistent efforts in induced mutagenesis had resulted in hundreds of mutants with several desirable agronomic features in these crops at BARC. Such mutants were utilized directly or made inter-mutant or mutant-variety crosses to develop 62 improved Trombay varieties. These varieties have been released and notified for cultivation by the farmers in different states during 1973-2023 in synergistic research collaboration with the ICAR and SAUs (Table 2). Of these, 19 varieties are direct mutants and rest are mutant derivatives and 17 varieties are released in the national ICAR system, while rest are released through SAUs. Some of the desirable traits in these crops are enhanced seed yield, higher nutrient content, earliness, ideal plant type, greater seed size, seed dormancy, wider adaptability and resistance to lodging, diseases and moisture stress. These characters not only advanced the crop productivity but also facilitated the development of new or alternate cropping systems, which in turn generated additional farm income.

Table1: Inheritance of induced mutant traits in different crops

Crop	Mutant trait	Inheritance of genes
Mungbean	Large seed	Incomplete dominance
	Yellow mosaic disease resistance	Single recessive gene
	Lanceolated leaf	Single recessive gene
	Cerrated leaf	Single recessive gene
	Small leaf	Single recessive gene
	Chlorina	Single recessive gene
	Yellow seed	Single recessive gene
Urdbean	Bruchid resistance	Duplicate dominant genes
Cowpea	Connate foliaceous stipules	Duplicate recessive genes
	Subsessile leaf	Single recessive gene
	Monopodial branching	Double recessive genes
	Seed coat pattern (Solid, Holstein and eyed)	Duplicate recessive epistasis (Supplementary genes)
	Determinate plant type	Single recessive gene
	Self-incompatibility	Single recessive gene
Pigeonpea	Compact-dwarf plant type	Single recessive gene
Wheat	Reduced height	Single recessive gene
	High tillering, grassy bunch and clustered panicle	Single recessive gene
Rice	Lesion mimic	Single recessive gene
Sesbania rostrata	Late flowering	Single recessive gene
Chickpea	Elongated organ and large seed size	Incomplete dominance
Groundnut	Dwarf plant height	Single recessive gene
	Gibberellin-insensitive dwarf	Single dominant gene
	Suppressed branches	Single recessive gene
	Imparipinnate leaf	Single recessive gene
	Bifurcated leaf	Triple recessive gene
	Funnel leaflet	Suppressive gene action
	Suborbicular leaflet	Single recessive gene
	Lupinus leaflet	Suppressive gene action
	Dark green leaf	Duplicate recessive gene
	Virescent, chlorina, variegated	Single recessive gene
	Disease lesion mimic leaf	Suppressive gene action
	Foliaceous stipules	Duplicate recessive gene
	Yellow flower	Single recessive gene
	Sequential flowering	Duplicate recessive gene
	Purple, Chocolate testa	Duplicate recessive gene
	Rose testa	Single dominant gene
	Hard seed	Single recessive gene

5.5.1. Food legumes

Food legumes (pulses) comprise an important crops that provide high quality protein supplementing cereal proteins for country's majority vegetarian population. Pulse production in India is 24.49 million tonnes (mt) during 2023-24, where chickpea contributed 11.57 mt, followed by pigeonpea with 3.38 mt and mungbean with 2.91 mt. Genetic improvement of food legumes such as, blackgram/urdbean, greengram/mungbean, cowpea, pigeonpea, chickpea and cluster bean has been undertaken by the BARC through radiation induced mutagenesis and recombination breeding. BARC has developed 24 varieties in pulse crops, which include nine in mungbean, eight in urdbean, five in pigeonpea and two in cowpea (Table 2; **Fig. 3**). Pulse varieties such as TAP-7 in mungbean, TT-6 in pigeonpea and TRC77-4, TC-901 in cowpea are direct mutants, while rest of them are mutant derivatives. In urdbean, mutants, UM-196 and UM-201 were crossed with variety T-9 to evolve three varieties, TAU-1, TAU-2 and TPU-4 for Maharashtra. Similarly in pigeonpea, cross between a fast neutron induced large seed mutant variety TT-6 and ICPL 84008 had led to development of early maturing varieties, TT-401, TJT-501 for Chhattisgarh, Gujarat, Madhya Pradesh and Maharashtra and PKV-TARA for Maharashtra. While in mungbean, an early maturing variety TMB-37 was developed by crossing Kopargaon and TARM-2 and released for Assam, Bihar, Jharkhand, Uttar Pradesh and West Bengal. Trombay pulse varieties like TU-40 in urdbean, TM-96-2, TM-2000-2 in mungbean and TRC-77-4 in cowpea are also suitable for cultivation in rice fallows. Cowpea mutant variety, TC-901 is the first variety in the country suitable for summer. Recently urdbean varieties TJU 130 and TJU 339 are released for Madhya Pradesh and TRCRU 22 for Karnataka. Mungbean variety TRCRM 147 is released for Karnataka. Most of the pulse varieties are with ideal plant type, better seed size and disease resistance.

Fig. 3: Plants of (a) mungbean variety, TRCRM-147, (b) urdbean variety, TJU 339, (c) cowpea variety, TC-901 and (d) pigeonpea variety, TJT-501

5.5.2. Oilseed crops

Indian oilseed production crossed 39.59 mt during 2023-24. Rapeseed-Mustard ranked first by contributing 13.16 mt, followed by soybean (13.05 mt) and groundnut (10.28 mt). Since sixties, BARC has been engaged in mutation breeding of Indian mustard using beta particles, X-rays, gamma rays and developed spectrum of mutations for plant height,

inflorescence, flower morphology, maturity, seed colour, seed weight and oil content. Sustained breeding efforts with these mutants has evolved eight varieties, which are commercialized in different states (Table 2). In mustard, yellow seed coat mutant has more oil, more protein, thinner seed coat and lower fiber compared to brown seed coat parents. First yellow seed coat mutant in India, Trombay Mustard 1 (TM 1) was developed by BARC by treating Rai 5 variety with beta rays from Phosphorus-32 radioisotope. Subsequent recurrent selection in the same mutant has resulted in high yielding variety, TPM 1 with reduced erucic acid for Maharashtra. Earlier years, a direct X ray short mutant with appressed pods and brown seeds, TM 2 and a mutant derivative TM 4 have been released for Assam. TPM 1 and TM 4 are with yellow seed coat. Further, TM 2 was diversified using IC264133 to develop two varieties: Trombay Him Palam Mustard 1 (THPM-1) for Himachal Pradesh and Birsa Bhabha Mustard-1 (BBM-1) for Jharkhand. Similarly, TM 102 was successively recombined with other breeding lines to evolve TAM 108-1 for Maharashtra; TBM-143 (**Fig. 4a**) and TBM-204 for West Bengal.

In soybean, BARC has developed two varieties viz., TAMS 38 (**Fig. 4b**), a gamma ray mutant of JS 80-21 and TAMS 98-21, a cross derivative with superior seed yield, non-pod shattering, resistance to diseases and pests. Both the varieties were cultivated widely by the farmers in Vidarbha region of Maharashtra.

Fig. 4: Plants of (a) mustard variety, TBM-143; (b) soybean variety, TAMS 38; (c) groundnut variety, TAG 73; (d) linseed variety, TL 99

Groundnut is an important food, oilseed and feed crop in our country. At BARC, its mutation studies were started with X-ray irradiation in 1957 and with gamma rays and electron beam in subsequent years. Periodical induced mutagenesis in groundnut had generated gene pool having many divergent mutants. Succeeding breeding efforts using these mutants in recombination breeding has developed and released 16 Trombay groundnut (TG) varieties for cultivation across the country (Table 2). First BARC variety TG 1 was developed in 1973 through X-ray mutagenesis. Its large seed mutant trait contributed immensely in the succeeding TG varieties. Later, another X-ray mutant variety, TG 3 was commercialized for Kerala. Inter-mutant cross variety, TG 17 was developed for Maharashtra. Crosses involving both TG 1 and TG 17 had developed a

mutant derivative, TKG 19A having large seed for Maharashtra. Further, these TG mutants and their derivatives were genetically diversified by other varieties to develop TGS-1 (Somnath) and TG 22 for Gujarat and Bihar, respectively. Recombination breeding involving these mutants and M 13 has resulted in the development of four varieties: TAG 24 and TLG 45 for Maharashtra; TG 39 for Karnataka and Rajasthan and RARST-1 (TG 47) for Andhra Pradesh. Genetic diversification of these mutants was continued by involving more parents for incorporation of newer characters, which has evolved five varieties, TG 26, TG 37A, TG 38, TPG 41 and TG 51 and are released by ICAR for different states. Recently, gamma ray mutagenesis has evolved mutant, TG 73, suitable for Maharashtra and Gujarat (**Fig. 4c**). These TG varieties were with semi-dwarf height, compact plant type, large seed, early maturity, fresh seed dormancy, desired seed and pod type, drought tolerance, high oleic acid, which make them suitable to different seasons and cropping systems.

Linseed is the winter oilseed crop and its oil contains linolenic acid (36–50 %), linoleic acid (18–24 %) and oleic acid (16–24 %). Its oil is non-edible as it develops off-flavours during storage. Varieties with minimal linolenic acid will enable linseed oil for edible purpose. BARC has developed a high yielding variety, TL 99 with 2-5% linolenic acid which was released for commercial cultivation in Assam, Bihar, Jharkhand, Nagaland, Uttar Pradesh and West Bengal. TL 99 is the first Indian variety for edible oil (**Fig. 4d**). In sunflower, gamma ray mutagenesis of zebra stripped seed coat variety, Surya has resulted in high yielding black seed coat variety, TAS 82 with 2-7 % more oil than its parent for Maharashtra.

5.5.3. Cereals

Rice is the vital Indian staple food crop with 136.7 mt production during 2023-24 and is ensuring country's food security. Radiation induced mutation breeding in rice at BARC has led to the development and release of seven varieties (Table 2). In early seventies, a cross between fast neutron mutant TR-5 and IR-8 resulted in breeding line TR-RNR-21, which was released as Hari in 1987 for Andhra Pradesh. India is known for traditional rice landraces having unique grain quality, which has separate niche in the domestic market. Usually, these are late maturing with poor yield and lodging due to tall stature, hence their cultivation is minimal. Gamma ray induced mutagenesis was successfully employed at BARC for improvement of these landraces. Recently, concentrated breeding efforts have resulted in release of five high yielding mutant varieties with improved agronomic traits for Chhattisgarh; Trombay Chhattisgarh Dubraj Mutant-1 (TCDM-1), Vikram-TCR (**Fig. 5a**), CG Jawaphool Trombay (CGJT), Trombay Chhattisgarh Sonagathi Mutant (TCSM) and Trombay Chhattisgarh Vishnubhog Mutant (TCVM). Further, a promising mutant derivative, Trombay Karjat Rice Kolam (TKR-Kolam) has also been released for Maharashtra. These high yielding varieties are lodging resistant due to semi-dwarf stature and with enhanced milling and head rice recovery. TCDM 1, CGJT and TCVM are with aromatic grains like their parents. TKR Kolam has superfine grain and better taste like other Kolam rice varieties. Grains of Vikram-TCR are suitable for puffed rice making, while that of CGJT and TCVM for kheer making.

Sorghum is a climate resilient crop cultivated for grain, fodder and biofuel under drought prone areas with low input conditions. Its cultivation is on 4.0 mha with 4.74 mt production. Gamma ray induced mutagenesis has generated a mutant, TRJP1-5 with superior grain and fodder yield and grain quality, which has been released for Karnataka in 2023 (**Fig. 5c**). It has synchronous maturity, bold and lustrous seeds with better rheological properties (roti making), besides moderate tolerance to charcoal rot, rust and blight diseases. In another experiment, gamma ray mutant for *hurda* (consumed at green stage) type, TAKPS-5 was developed and was released for Maharashtra as Suruchi (**Fig. 5b**). It is early maturing with compact and easily threshable panicles, better *hurda* grain yield. The grains showed more spongy tissues with improved organoleptic properties.

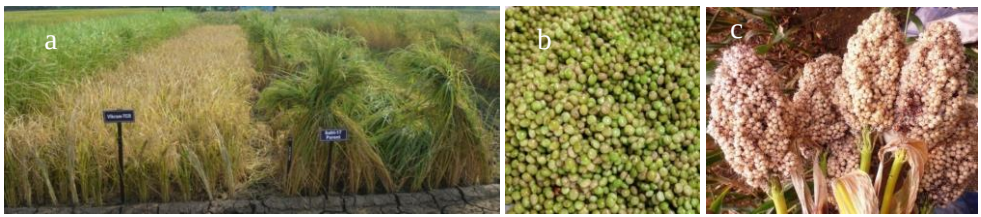

Fig. 5: (a) Field view of rice variety, Vikram TCR, (b) hurda type grains of TKPS-5, (c) panicles of TRJP 1-5 of sorghum

Trombay crops with certain novel mutant characters have been registered with National Bureau of Plant Genetic Resources, New Delhi (Table 3). Of the 20 genotypes registered, 10 mutants belong to groundnut, three to sesame, two each to sorghum and wheat, one each to sesbania, sunflower and urdbean.

5.5.4. Success with Trombay varieties

Trombay mutant varieties have contributed as parental material in respective crop breeding programmes at various state universities. Using Trombay varieties as parents, 14 groundnut and two urdbean varieties were developed in the country. Groundnut varieties include JCG 88, TPT 25, TCGS 894 for Andhra Pradesh; JL 501, GG 34, GG 35, GG 41 for Gujarat; GPBD 5 for Jharkhand, Manipur; Dh 40, R 9251, Dh 232 for Karnataka; JL 501, PDKVG 335 for Maharashtra; GG 21, RG 559-3 for Punjab; GG 21, JL 501, RG 559-3 for Rajasthan; TCGS 894 for Tamil Nadu, Telangana and RG 559-3 for Uttar Pradesh. In urdbean, TAU-1 was used to develop DU-1 for Karnataka and PDKV Blackgold for Maharashtra. Many of the Trombay varieties are being used as check varieties to test new entries in the state and national varietal evaluation trials.

Table 2: Chronological list of released Trombay varieties

Year	No of varieties	Crop	Trombay crop varieties
1973	1	Groundnut	TG 1
1983	2	Jute	TKJ-40 (Mahadev)
		Mungbean	TAP-7
1985	4	Pigeonpea	TT-6, TAT-10
		Groundnut	TG 17
		Urdbean	TAU 1
1987	1	Groundnut	TG 3
1988	1	Rice	Hari (TR-RNR-21)
1991	1	Groundnut	Somnath (TGS 1)
1992	2	Groundnut	TAG 24
		Urdbean	TPU-4
1993	3	Mustard	TM 2, TM 4
		Urdbean	TAU-2
1994	2	Mungbean	TARM-2
		Groundnut	TG 22
1996	2	Groundnut	TKG 19A, TG 26
1997	2	Mungbean	TARM-1, TARM-18
1999	1	Urdbean	TU 94-2
2004	2	Groundnut	TG 37A, TPG 41
2005	2	Mungbean	TMB 37
		Soybean	TAMS 38
2006	1	Groundnut	TG 38
2007	8	Groundnut	TLG 45
		Mungbean	TJM-3, TM 96-2
		Mustard	TPM 1
		Soybean	TAMS 98-21
		Sunflower	TAS 82
		Cowpea	TRC-77-4
		Pigeonpea	TT 401
2008	2	Groundnut	TG 51, TBG 39
2009	1	Pigeonpea	TJT-501
2010	1	Mungbean	TM-2000-2 (Pairy Mung)
2011	1	Groundnut	RARST-1 (TG 47)
2013	2	Pigeonpea	PKV-TARA
		Urdbean	TU-40
2018	1	Cowpea	TC 901
2019	2	Rice	TCDM-1
		Mustard	TBM-204
2020	2	Linseed	TL-99
		Rice	TKR Kolam
2021	8	Rice	Vikram TCR, CGJT, TCVM, TCSM
		Mustard	THPM-1, BBM-1, TAM 108-1
		Groundnut	TAG 73
2022	1	Mustard	TBM 143
2023	6	Urdbean	TRCRU-22, TJU 130, TJU 339
		Mungbean	TRCRM 147
		Sorghum	TRJP 1-5, TAKPS 5

Table 3: Trombay mutant germplasm registered with National Bureau of Plant Genetic Resources, New Delhi

Year	INGR No.	Crop	Name of Trombay germplasm	Trait
2001	1014	Sesbania	TSR-1	Photoperiod insensitivity
2004	4039	Groundnut	TG-18AM	Disease lesion mimic leaf
2004	4040	Groundnut	TGE-1	Early (95 days), foliaceous stipule, high shelling outturn (80%)
2004	4041	Groundnut	Small leaf mutant	Dwarf with small leaflet
2004	4097	Groundnut	Imparipinnate leaf mutant	Imparipinnate leaf with small leaflet
2004	4098	Groundnut	Suppressed branch mutant	Suppressed primary branches
2004	4100	Sunflower	Fasciation mutant	Fasciation and more leaves
2005	5018	Sesame	NM-58	Non-lodging due to stiff stem
2007	7029	Sesame	N-129	Tall seedling with greater initial vigour
2007	7030	Sesame	N-29	Polypetalous corolla
2007	7032	Groundnut	TG-18A	Large pod and seed
2010	10133	Urdbean	Trombay wild urid	Bruchid resistance
2011	11058	Groundnut	TGM-112	White to light orange flower
2013	13011	Groundnut	TGM-167	Gibberellin insensitive dominant dwarf
2013	13025	Groundnut	TGM-38	Sub-orbicular leaflet, erect, compact and dwarf plant type
2013	13026	Groundnut	TGM-51	Funnel leaflet, dwarf plant
2021	21201	Wheat	TAW-33	High seed hardness index
2023	23038	Sorghum	TAKPS-1	High yielding hurda with better sucrose and organoleptic properties
2023	23039	Sorghum	TAKPS-3	Semi-compact, threshable hurda with taste and flavour
2023	23081	Wheat	TAW-41	Spot blotch resistance & terminal heat tolerance

BARC has followed multi-pronged approaches to reach Indian farming community with Trombay varieties. It has undertaken large-scale seed production, participated in *Kisan Melas*, exhibitions of SAUs, conducted field demonstrations and attended the Public Awareness Programme on Peaceful Uses of Atomic Energy (**Fig. 6**). Some Trombay varieties like TAG 24, TG 37A of groundnut, TAU 1 of urdbean and TJT 501 of pigeonpea have made distinct impression and impact across the states due to their consistent better yield, wider adaptability and other desirable characteristics. Farmers have harvested record yields by cultivating these varieties.

Fig. 6: Field visit of farmers to learn about Trombay crop varieties at Gamma field, BARC

Cumulative share in national breeder seed indent for Trombay varieties by different states for 2021-2025 period is extremely encouraging. TG varieties constitute 74.5% of the Maharashtra's cumulative national indent for groundnut; followed by 73.2% of Gujarat and 62.2% of West Bengal's indent (**Fig. 7**). For urdbean, Maharashtra indents 68.7% for Trombay urdbean varieties and in the rest of the states, it ranges from 8.5% for Madhya Pradesh to 37.5% for Tamil Nadu. Similarly, for pigeonpea, Tamil Nadu indents 34.5% for Trombay pigeonpea varieties and in the rest of the states, it ranges from 5.0% for Jharkhand to 17.0% for Chhattisgarh. For recently released Trombay rice varieties, the demand is picking up by the marginal and progressive farmers in Chhattisgarh, where its share is 3.8% for the above period.

As per the demand, more than 2500 tonnes breeder seed of Trombay varieties were supplied to National Institutes; National Seed Corporation, State Seed Corporations of Andhra Pradesh, Bihar, Chhattisgarh, Gujarat, Maharashtra, Odisha, Rajasthan and West Bengal; State Agricultural Departments & Universities; Non-Governmental Organizations and farmers. Apart from this, various SAUs also dispersed hundreds of tonnes breeder seeds of BARC crop varieties. Some of the seed material was further multiplied and distributed horizontally to many farmers in the country. Most of these varieties have considerably benefited thousands of farmers, traders, exporters and other stakeholders.

5.6. Molecular characterization of mutant traits

Among the various radiations, gamma rays are the most extensively used physical mutagen in crop mutation breeding. In cowpea, large seed, small seed and cowpea aphid-

borne mosaic disease resistant mutants were isolated through gamma ray mutagenesis of cv. CPD103. Genomic DNAs from these mutants and parent were subjected to whole genome resequencing. The mutation rate was 1.4×10^{-7} per base pair. Gamma rays brought 88.9% of single base substitutions (SBSs) with an average transition to transversion ratio (Ti/Tv) of 3.51. A > G and T > C transitions were the major transition mutations, while all four types of transversion mutations were identified. Of the total induced variations, 11% were the indels followed by 6.3% small insertions and 4.8% small deletions. Distributed across all 11 chromosomes, only a fraction of SBSs (19.45%) and indels (20.2%) potentially altered the encoded amino acids/ peptides.

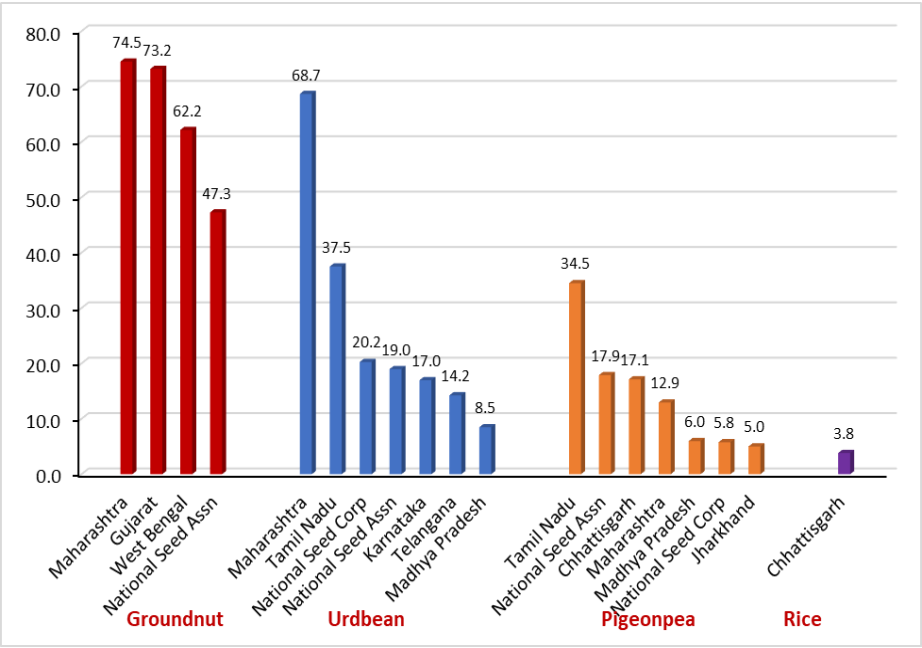

Fig. 7: Cumulative share (%) in national breeder seed indent for Trombay varieties by different states during 2021-2025 period.

Assessment of gamma ray wheat mutant with medium-hard texture found variations in either *pina* or *pinb* or both genes. This suggested that gamma rays brought mutations in loci coding puroindloines. Besides, characterization of gamma ray dwarf mutant of dicoccum wheat showed that dwarfing locus was different from the existing *Rht B1b* of tetraploid varieties. Further locus specific primer analysis of gamma ray GA_3 insensitive semi-dwarf dicoccum mutant showed absence of known wild and mutant alleles, *Rht-B1a* and *Rht-B1b* genes indicating reduced height mutant carried an altered mutation for the dwarf trait. Another gamma ray mutant TAW 41 had lower spot blotch infection and had higher normalised difference vegetation index (NDVI) and chlorophyll content (SPAD) values under terminal heat stress. Expression analysis showed that down-regulation of

genes promoting senescence (TaSAG4, TaSNAC11) and up-regulation of wheat copper binding protein 1 (WCBP1) in the TAW41 exposed to spot blotch and terminal heat stress. In another high temperature stress tolerant gamma ray wheat mutant, HSP20, serine threonine kinase, calcium dependent protein kinase, ATP-binding cassette transporters, SOD, heat shock transcription factor, starch synthase, sucrose synthase and debranching enzyme were upregulated.

A chickpea mutant (*elm*) that showed increased organ and seed size was characterized and a novel, previously uncharacterized gene (*CaEl*) with a role in regulating organ size, early vigor and seedling establishment under salinity stress was identified. The gene LOC101503252, corresponding to *CaEl* is found to be deleted in the mutant resulting in its complete loss of expression. Both cell proliferation and expansion are also affected in the mutant. Transcriptomic profiling identified cell cycle, cell wall organization/biogenesis and related carbohydrate metabolism as major pathways involved in the regulation. The *in-silico* analysis showed that this single copy gene acquired a broader function in regulating cell division and/or proliferation in multiple tissue types. In high oleate groundnut mutant, sequencing of *ahFAD2A* gene detected several point mutations in the coding region. Further analysis of mutant confirmed that the G to A transition is responsible for high oleate trait.

6. Future prospects

Crop improvement is a continuous breeding process, wherein new breeding lines are to be developed in line with future needs of the farmers in various growing conditions. Categorically, a thoughtful merger of mutation and recombination breeding has a greater prospect in the genetic improvement of crop plants, which can be strengthened with genomic tools. Further, mutation breeding needs to be explored for the improvement of inbred lines of the existing, established and popular hybrids in respective crops; nutraceutically important minor millets; unexplored medicinal and aromatic crops; widely adapted land races with rich nutritional and other quality traits; vegetatively propagated crops along with *in vitro* mutagenesis. Additionally, many more crop species need to be experimented for induced mutagenesis with mutagens like electron beam, proton beam, ion beam, neutron or with novel chemical mutagens or their combination. Consequent mutant reservoir in different crops would be ideal genetic resource material for understanding structural and functional genomics of various economic traits. In future, radiation breeding could help to feed billions by promoting crop flexibility amid looming climate change, shrinking water and land resources, growing soil exhaustion and increasing insect resistance.

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EVOLUTION OF STRATEGIES FOR CROP PROTECTION AND PRODUCTION

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Abstract

Nuclear Agriculture and Biotechnology Division (NA&BTD), Bhabha Atomic Research Centre (BARC) has an important programme on crop protection and production strategies, which includes developing sterile insect techniques (SIT), biopesticides for insect pests and plant disease control, and identifying suitable biomolecules for plant growth. The research activities in these areas were primarily focused on understanding the effect of gamma radiation on insects, mechanisms of insecticidal proteins, beneficial fungal strain improvement, developing effective biopesticides and detection of single to multiple pesticides through biosensors. The development of radiation based depolymerised chitosan and polymerized superabsorbent hydrogels that support plant growth and improves soil moisture retention, respectively. Further, the developed technologies were made available to farmers to integrate in the package of practices for crop cultivation towards reducing the negative effect of biotic and abiotic stresses.

1. Introduction and historical background

Crop protection is an important tool for enhancing food production worldwide. The management of insect pests and plant diseases as well as crop modifications are being undertaken through biotechnological approaches. The development of synthetic organic molecules (pesticides and antimicrobial agents) in the mid-1940s revolutionised the pest

control strategies, but it caused negative effects on the environment and human health. However, practices with alternative approaches to the chemical agents were soon developed and continuously improved from the perspective of their application in crop protection and public health. The concept of integrated pest management (IPM) became popular after 1970, and attention was given to use of selective pesticides. Some of the prominent alternative approaches developed were: area-wide management of insect pests viz. Sterile Insect Technique (SIT) and use of hormones, pheromones, biocontrol agents etc. for eco-friendly management of insect pests. The SIT is an environment-friendly and autocidal insect pest control strategy in area-wide integrated pest management (AW-IPM) programme. Basically, SIT for target insect pest requires large scale mass rearing, sterilisation using ionizing radiation and periodic release of sterile males into the target area. The released sterile males would copulate with wild fertile females, leading to no offspring and further results suppression/eradication of the pest population. SIT has been implemented against several insect pests and vectors for prevention, suppression, containment, and eradication.

The use of radiation for the betterment of society was envisaged by Dr. Homi Bhabha with the establishment of Atomic Energy Establishment, Trombay (AEET) in Bombay. The biology research was initiated first on the campus of Richardson and Crudas at Byculla in Mumbai (then Bombay) in 1966 and later at BARC, Trombay. Pest Control Section was created under the leadership of Shri. G. W. Rahalkar, who took the reign of entomological research in BARC. Shri. Rahalkar and his team members, Dr. M. R. Harwalkar, Dr. H. D. Rananavare, Dr. A. J. Tamhankar, Dr. T. K. Dongre and others-initiated research on the effect of radiation on different insect pests. They have contributed to the monumental research work on understanding the impact of radiation on insects, developing mass rearing techniques, identifying orange eye mutant of potato tuber moth (PTM) *Phthorimaea operculella* (Zeller 1873)), SIT for red palm weevil (*Rhynchophorus ferrugineus* (Olivier)), Potato Tuber Moth, paddy stem borer (*Sirphiphaga incertulas* (Walker)), cotton bollworm (*Earias vittella* (Fab.) red cotton bug (*Dysdercus koneiji* F.) and diamond back moth (*Plutella xyleostella* L.), and research on pheromones, hormones and plant extracts for insect pest control. Field studies were carried out in collaboration with State Agricultural Universities (SAUs) and Indian Council of Agricultural Research (ICAR) to demonstrate the feasibility of SIT for insect pest management. Dr. S. V. Amonkar initiated the work on insect bio-control agents, plant extracts and entomopathogens, exploring their potential in insect pest and mosquito control. Some of the prominent insect pathogenic bacteria like *Bacillus thuringiensis* subsp. *kenyae* (Btk) ISPC-1 (H. Dulmage collection of the U.S. Department of Agriculture (USDA) is HD-549), *B. sphaericus* (Bs) ISPC-8 and *B. thuringiensis* subsp. *israelensis* (Bti) ISPC-12 have been isolated and tested against different insect pests and mosquito larvae.

Plant pathogens cause substantial yield loss in several field crops, leading in economic and social adversity. They attack plants, obtain nutrients, and cause disease by releasing effector proteins, enzymes, toxins etc. Currently, fungicides are used frequently to

control these deadly plant pathogens. Moreover, soil-borne plant pathogens are very difficult to manage by using fungicides.

Table 1: Development of sterile insect technique (SIT) for major insect pest management at BARC

Insect pests	Host plants	Yield loss	Status/trials
Potato tuber moth	Potato, tomato, egg plant	60%	Field
Red palm weevil	Coconut & ornamental palms	25-100%	Field
Oriental fruit fly	Fruits and vegetables	30-100	Field cages
Melon fly	Cucurbits	70%	Field cages

Alternatively, usage of biocontrol agents in agriculture is an effective environment friendly approach to control plant diseases in the field. Towards this, different biocontrol agents like bacteria and fungi have been exploited in recent years. *Trichoderma* sp. is a fungus used for seed and soil treatment to manage various plant pathogens. In India, *Trichoderma viride* and *T. harzianum* were extensively commercialized as bio-fungicide for the control of soil-borne fungal diseases like *Fusarium* sp., *Rhizoctonia* sp., *Pythium* sp., *Sclerotium* sp., *Sclerotinia* sp. In BARC, several biocontrol agents have been isolated and tested against economically important plant pathogens. The R&D activities on *T. virens* started during 1990s and was extensively studied under laboratory and field conditions and proved to be effective against *Pythium aphanidermatum*, *Sclerotium rolfsii* and *Rhizoctonia solani*. A substantial amount of work has been done to understand the molecular mechanisms of biocontrol, identify novel genes, gene clusters and genome sequencing. Further efforts were made to develop an improved strain by using gamma radiation, which has been registered and commercialized.

Several technologies have been developed to improve crop productivity and food safety. During the 1980s, Enzyme and Microbial Technology Section was created under the leadership of Dr. S. F. D'Souza. He and his team members initiated work on immobilization of enzymes and microbial cells. Immobilization refers to any physico-chemical technique that immobilizes cells and biomolecules. It offers several advantages, including improved re-usability of precious biomolecules like enzymes, developing continuous bioprocesses, improved stability, and hence allowing its application in harsher environmental conditions. Initially, the focused was on continuous conversion of sucrose to fructose and gluconic acid by immobilized yeast cell multienzyme complexes using gamma radiation. Later, several enzymes and microbial cells were immobilised to develop various bioprocesses including bioremediation of metal ions and radionuclides. Recently, radiation-depolymerised chitosan that can act as a plant growth modulator and radiation-polymerized superabsorbent hydrogels have addressed water scarcity in agriculture to improve productivity. Further, immobilized biocomponents were used for the development of biosensors to detect single to multiple pesticides in food commodities.

2. Crop protection and production research at BARC

2.1. Recent developments in SIT and insect pathology

BARC is actively engaged in the development of SIT for the control of economically important insect pests in India (Table 1). Fruit fly species of Tephritidae family cause significant damage to fruits and vegetables. The population structure and distribution of fruit fly species in India has been studied. The Oriental fruit fly (*Bactrocera dorsalis*) is the most dominant species followed by Peach fruit fly (*B. zonata*), Guava fruit fly (*B. correcta*), Melon fly (*Zeugodacus cucurbitae*) and Pumpkin fruit fly (*Z. tau)*. The population dynamics of fruit fly species in mango and other fruits orchards in and around Dahanu region of Palghar district of Maharashtra was profiled. The performance of sterile males of *B. dorsalis*, *Z. cucurbitae* and *B. correcta* has been assessed in field cage experiments (**Fig. 1**). The sterile males were fully competing with non-irradiated males, which resulted in minimising the egg hatchability and suppressing the further generations. Currently, SIT module is being tested under pilot field conditions in collaboration with agricultural universities and ICAR institutes. Moreover, the tomato leaf miner (*Tuta absoluta* (Meyrick)) is an invasive insect pest of tomato and other solanaceous crops. The feasibility and integration of SIT with other biocontrol agents for this insect is evaluated in collaboration with ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru. The distribution, mass rearing protocols, sterility dose optimisation and performance of sterile males in polyhouse was studied. Inundative release of 150 Gy irradiated male moths at weekly interval was found to be effective in reducing number of live larvae per plant.

Fig. 1: Mass rearing, sterilization and evaluation of performance sterile fruit fly males in the field cages. (A) Mass reared fruit fly pupae; (B) and (C) field cages setup for releasing the sterile males in the presence of fertile males and females to estimate the competitiveness

2.2. Bacterial endosymbionts in pest management

Insects harbor many bacterial endosymbionts either intracellularly or extracellularly, and they play an important role in the biology and fitness of insects. Currently, certain bacterial endosymbionts are being explored for insect pest and vector management. *Wolbachia*, an endosymbiotic bacterium, is a intracellular reproductive parasite and is widely explored as a pest management tool. It has the ability to cause reproductive alterations such as feminization, thelytokous parthenogenesis, male-killing, cytoplasmic

incompatibility (CI) and speciation through reproductive isolation. Association of *Wolbachia* with different fruit fly species (*B. dorsalis*, *B. zonata*, *B. correcta*, *B. scutellaris*, *B. nigrofemoralis*, *Z. cucurbitae*, *Z. tau* and *Z. caudatus*) was characterized by using 16S rRNA, Multi-Locus Sequence Typing (MLST) and *Wolbachia* specific protein (wsp) approaches. The prevalence of *Wolbachia* and other reproductive parasites varies with each fruit fly species and locations. The phylogenetic analysis showed that the majority of *Wolbachia* prevalent in fruit fly samples belong to Supergroup A and B which are parasitic in nature. *Wolbachia* inducing CI could serve as an important control strategy as "Incompatible Insect Technique" (IIT) for the control of insect pests including fruit flies and mosquitoes.

Bacterial communities associated with *B. dorsalis* and *Z. cucurbitae* were assessed by using culture dependent and molecular approaches (16S rRNA and Next Generation Sequencing analysis). We found varied relative abundance of bacterial communities in the gut of mass-reared or wild *B. dorsalis* and *Z. cucurbitae*. Enterobacteriaceae (61-73%) was the dominant family in *B. dorsalis* and *Z. cucurbitae*. Overall, these fruit fly species mainly host *Bacillus, Citrobacter, Enterobacter, Klebsiella,* and *Providencia* species. Gut microbiota associated with fruit fly species could help in developing efficient mass rearing protocol for SIT. Certain gut bacteria of fruit fly species attract the fruit fly adults e.g. cultural filtrate of *Bacillus, Citrobacter, Enterobacter, Enterococcus, Klebsiella, Pseudomonas* and *Raoultella* attracted different fruit fly species adults. Solid Phase Micro Extraction Gas Chromatography **(**SPME-GC-MS) analysis revealed 3-methyl-1-butanol, 2-phenylethanol, butyl isocyanatoacetate, 2-methyl-1-propanol and 3-hydroxy-2-butanone are the abundant chemical compounds in the supernatants of *K. oxytoca* and *C. freundii*. The chemical constituents identified from gut bacteria could be explored as attractants for eco-friendly insect control strategies.

2.3. Insect pathology-identifying novel insecticidal proteins

Various entomopathogenic bacteria have been isolated in BARC and biopesticides based on these bacteria have been developed. Insecticidal activity of these entomopathogenic bacteria is mainly due to the presence of several insecticidal toxins produced during the sporulation. *B. thuringiensis* subsp. *kenyae* ISPC-1 (**Fig. 2A**) produce insecticidal crystal (Cry) proteins during sporulation. Two prominent crystal toxins *viz.*, Cry1Ac17 and Cry2Aa14 from this isolate have been recombinantly expressed, purified and characterised. These are highly active against *Helicoverpa armigera* Hub. and *Spodoptera litura* (F.). Binary toxin, BinA (41.9 kDa) and BinB (51.4 kDa) of *B. sphaericus* ISPC-8 have been characterised and exhibit mosquito larvicidal activity. *B. thuringiensis* subsp. *israelensis* ISPC-12 (**Fig. 2B**) produce intracellular crystal inclusions during sporulation. These include four major, Cry4Aa (134 kDa), Cry4Ba (125 kDa), Cry11Aa (72 kDa) and Cyt1Aa (27 kDa) and two minor, Cry10Aa (78 kDa) and Cyt2Ba (29 kDa) insecticidal crystal proteins. The insecticidal toxicity is attributed to these toxins.

Xenorhabdus and *Photorhabdus* bacteria exhibit a mutualistic symbiosis with entomopathogenic nematodes belonging to Steinernematidae and Heterorhabditidae

families, respectively. These bacteria are known to produce a variety of insecticidal toxins such as Xpt, XnGroEL, Txp40 and XaxAB toxins by *Xenorhabdus* and Tcs, Mcf, PVC and PirAB by *Photorhabdus*. Insecticidal activity towards target insects can score either through ingestion (oral route) or within their circulatory system (hemocoel). We have recombinantly expressed and purified the PirAB toxin and confirmed the insecticidal activity. The PirAB toxins share structural similarities with 3-domain Cry delta endotoxins from *B. thuringiensis*, suggesting a potential shared mechanism of action. On the other hand, Txp40 from *Xenorhabdus* possesses a unique structure with distinct N-terminal and C-terminal domains that enable the formation of a complex. This binding, along with its broad insecticidal activity against various insect pests, makes Txp40 a particularly interesting candidate for developing new insect pest control strategies that might be less susceptible to resistance.

2.4. Insect-plant resistance

Host plant resistance is the most economical and environment friendly means of managing insect pests. Identification of insect pest resistance has been explored through different breeding approaches including transgenics. The banana bunchy top virus (BBTV) is the major viral pathogen is transmitted by banana aphid causing disease in bananas and plantains worldwide. The detection of small interfering RNAs (siRNAs) derived from the ihpRNA transgene sequence in transformed BBTV-resistant plants positively established RNA interference as the mechanism underlying the observed resistance to BBTV. Efficient screening of transgenic plants showed resistant to BBTV infection. In groundnut, bruchid (*Caryedon serratus* Olivier) is a major storage insect pest damaging quality of stored groundnuts and impacting the market value. Screening of bruchid resistance in groundnut is difficult due to environmental variation and occurrence of biotypes. Thus, tightly linked markers or quantitative trait loci (QTLs) identification is required for further selection and pyramiding of resistance genes for stable resistance. Towards which, two common main QTLs were identified for bruchid resistance in groundnut.

2.5. Biopesticides for insect pest management

Several entomopathogens have been isolated and tested against different agricultural insect pests and mosquito larvae at BARC. Among them, *B. thuringiensis* subsp. *kenyae* ISPC-1, *B. sphaericus* ISPC-8 and *B. thuringiensis* subsp. *israelensis* ISPC-12 were found to be highly toxic to insect pests belonging to orders lepidoptera and diptera. Biopesticide based on *B. thuringiensis* subsp. *kenyae* ISPC-1 (AB03NABTD) has been evaluated in collaboration with State Agricultural Universities (SAU) and found effective against pod borer (*H. armigera*) on chickpea and pigeon pea crops. This biopesticide was included in multilocation trials under SAUs and All India Co-ordinated Research Project (AICRP) on chickpea of ICAR & showed effective in control of pod borer. *B. thuringiensis* subsp. *israelensis* (Bti) ISPC-12 is toxic to *Aedes aegypti, Anopheles stephensi*, and *Culex quinquefasciatus* larvae. Granular formulation was developed using spore-crystal powder of Bti ISPC-12 and tested in simulated field conditions as per WHO guidelines. The spore-crystal powder (active ingredient) and formulation is safe to

mammals and other animals as per toxicity tests carried out by Central Insecticide Board (CIB) approved toxicology laboratory. Field studies were carried out in Anushaktinagar, Mumbai and RRCAT township, Indore and resulted a significant mosquito larval population reduction in tested sites.

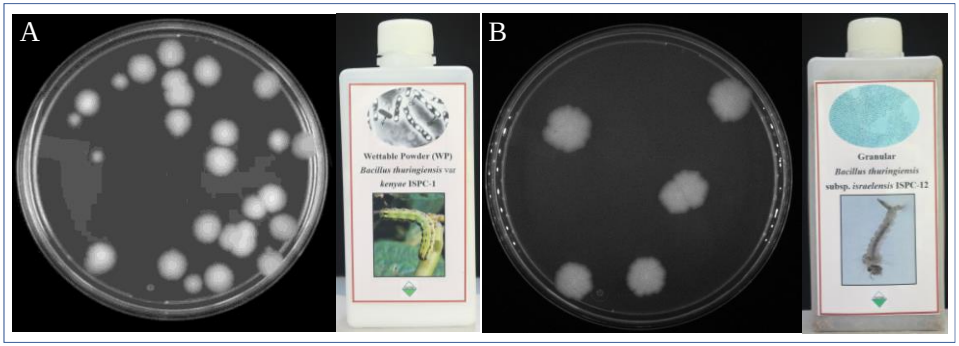

Fig. 2: (A) *Bacillus thuringiensis* subsp. *kenyae* (Btk) ISPC-1 and (B) *B. thuringiensis* subsp. *israelensis* (Bti) ISPC-12 used for characterisation of insecticidal proteins and development of biopesticide formulations

2.6. Biopesticides for plant disease management

2.6.1. Induced mutagenesis of Trichoderma virens

Trichoderma virens is an important beneficial fungus used for the control of soil borne plant pathogens. The potential biocontrol agents must have a higher degree of bioefficacy and should be amenable to mass production. During 2001, genetic improvement of *T. virens* was initiated through radiation-induced mutagenesis. Towards this, wild *T. virens* strain (**Fig. 3A**) was exposed to 1250 Gy gamma rays and different purified colonies exhibiting distinct morphological features have been selected for further research. Different mutant isolates were screened for antifungal activity, antibiosis, secondary metabolite production, *in vitro* and *in-vivo* inhibition of plant diseases. A mutant strain designated as G2 with dark pigments in the medium and brown colour conidia (**Fig. 3B**) was selected for further study. The liquid culture filtrate of G2 strain was shown higher inhibition than the wild type against *P. aphanidermatum*. This G2 mutant isolate has been deposited as a novel mutant strain with microbial type culture collection vide no. MTCC 11567. *T. virens* G2 mutant isolate is producing several secondary metabolites such as viridin and viridiol. Transcriptome analysis of G2 mutant and the wild-type strain revealed upregulation of several secondary metabolism biosynthesis, transport and mycoparasitism-related genes like polyketide synthases, O-methyl transferases, cytochrome P450s, oxidoreductases, glycosyl hydrolases, and MFS transporters in this mutant.

2.6.2. Bio-fungicide for plant disease management

The field application of *Trichoderma* spp. requires mass multiplication, which can be done using solid or liquid state fermentation. Solid state fermentation is preferred in India due to low initial investment as well as availability of agro byproducts. In India, tamarind seeds are available as a byproduct and can be stored for a longer time. Further, tamarind seeds contain high organic matter in the form of carbohydrates, proteins, fats and are suitable for fungal multiplication. Tamarind seeds exhibits intrinsic sticking properties and abundant in xylo-glucan. Thus, tamarind seeds can be easily covered with formulation without incorporating sticker. A tamarind seed based solid-state media was developed in-house for mass production of *T. virens* G2 strain. This mutant strain grows profusely and appears brown (conidia colour) within 10 days. A wettable powder bio-fungicide (TrichoBARC) (AB18NABTD) was developed based on *T. virens* G2 strain (**Fig. 3C**) and is currently used for seed treatment.

Fig. 3: (A) Morphological features of wild type *Trichoderma virens* and (B) G2 mutant strain of *T. virens*. (C) Commercial formulation based on mutant strain (G2) of *T. virens* available as "VIREN" for soil borne plant pathogens control

TrichoBARC was found to be effective in suppressing major soil borne plant diseases such as collar rot, seed and root rots and also damping off. During 2015 to 2023, multi-location field trials were conducted with G2 strain in collaboration with SAUs and ICAR and found to be highly effective against collar rot of chickpea. In parallel, toxicity data were generated for this formulation and this technology has been transferred to five industries for commercialization as on August 2024. Recently, Central Insecticide Board and Registration Committee (CIB & RC) approved grant of registration for indigenous manufacturing of above formulation (1% WP) of mutant strain (G2) of *T. virens* for the control of collar rot (*S. rolfsii*). To the best of our knowledge, this is the first mutant biopesticide have been registered anywhere in the world for commercial application. The product ("VIREN") based on this technology has been launched in the market. Recently (2023 - 2024), the G2 strain formulation has been recommended as Package of Practice (PoP) for controlling soil-borne disease of chickpea, cumin and cluster bean by ICAR and Agriculture University, Jodhpur, Rajasthan. Presently, the mutant formulation is being further evaluated at various SAUs and ICAR.

3. Newer biomolecules for plant growth and biosensors for pesticide monitoring

3.1. Plant growth promoting formulation: "Anu-Chaitanya"

Millions of tons of crustacean's shells are cast aside each year by the seafood industry, creating environmental concerns. A major component is chitin (20-30%), a valuable resource exhibiting considerable functional properties that have led widespread exploitation of chitosan as a versatile, high-value bioactive substance. Chitosan and its derivatives are finding exciting uses in a wide range of fields, from cosmetics and pharmaceuticals all the way to agriculture. Unlike chitin, chitosan is soluble in acidic aqueous solutions, significantly expanding its practical applications in agriculture. Although formulation of chitosan nanoparticles can be achieved through traditional methods, it might not be economically viable owing to time extensive, low quality product synthesis and expensive (like using enzymes). Besides, some methods might create harmful chemicals in the process. Therefore, the application of gamma radiation (100 kGy) has been explored for the formulation of chitosan nanoparticles offering a promising and potentially more sustainable alternative (**Fig. 4A**).

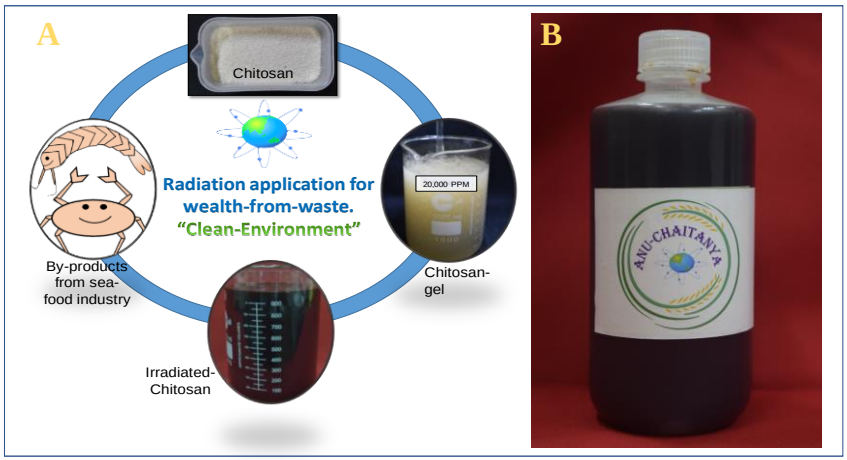

Fig. 4: (A) Application of gamma radiation on chitosan to obtain depolymerized chitosan. (B) "Anu-Chaitanya" product was developed and employed as plant growth promoter on field crops and flowers.

In collaboration with Vasantdada Sugar Institute (VSI), Pune and BARC have developed gamma-radiation based chitosan formulation named "Anu-Chaitanya" (**Fig. 4B**) (AB52NABTD). About 99 % homogeneity in the particle size (30-100 nm) was achieved under 100 kGy dose. In addition, viscosity and turbidity was also reduced post-irradiation. Successful field trials demonstrated improved plant growth potential of Anu-Chaitanya in potato, sugarcane and other crops, including flowering plants like Hydrangea and Chrysanthemum and forage crop like tall fescue.

4. Superabsorbent BARC-Hydrogel (MRIDAMŔT) (मृदाअमृत)

Changes in climate, including global warming, have resulted in irregular monsoon across the globe. The changing rainfall pattern along with prolonged shortage of water and an increase average temperature in the environment have led to drought and arid conditions in many areas. Drought and arid conditions adversely affect the plants growth and its productivity. Moreover, shortage of water availability for routine irrigation is an important aspect contributing towards crop production. In BARC, radiation-polymerised hydrogels were developed to address the water scarcity in agriculture, horticulture and plantations. A radiation polymerized hydrogel known as Superabsorbent BARC-Hydrogel (MRIDAMŔT) (मृदाअमृत) (AB51NABTD) was developed (**Fig. 5**). The production process is eco-friendly in nature as compared to chemically synthesized processes due to the elimination of harmful chemical cross-linkers. This material can absorb and retain water up to several hundred times (>550 times) of its own weight and maintain a slow and sustainable release of water supporting plant growth. Dry granules of BARC-Hydrogel enhance soil properties by retaining more water and improving aeration for plants. Eco-friendly nature of hydrogel formulation helps plants to survive and maintain good health in semi-arid and arid regions and conserve water by reducing the frequency of irrigation. The use of hydrogel in macroporous medium (sandy soil) is very effective to increase the water holding capacity, which significantly improves the plants health and productivity.

Fig. 5: Development of superabsorbent BARC-Hydrogel.

6. Biosensors for detection of pesticides

Due to the extensive use of pesticides in agriculture and other allied sectors, the presence of pesticides in food commodities and their entry into the food chain has become a major concern all over the world. Crop protection and food safety have become crucial for all involved in the value chain, and consumers have to be assured that they are not exposed to an unacceptable level of pesticide residues. BARC has initiated research on the development of biosensor-based technologies for the monitoring of pesticides using different immobilized biocomponents.

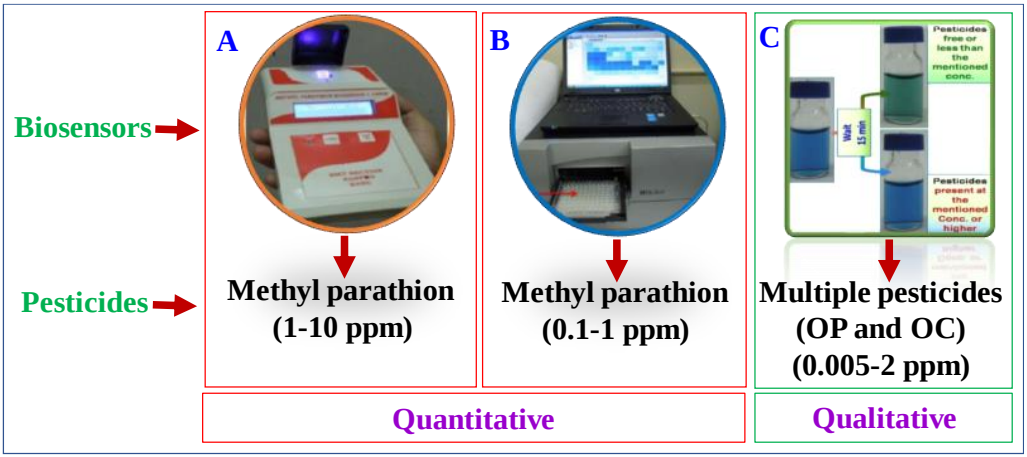

Fig. 6: Development of biosensor-based technology for pesticides detection ranging from single to multi sample analysis. (A) Hand held optical biosensor device for field use; (B) Microplate based biosensor for multiple samples in laboratory and (C) Biosensor Kit (Biokit) for the qualitative detection of multiple pesticides. OP: Organophosphates and OC: Organocarbamates.

The concept of microbial biosensors was established by immobilizing microbial cells on different matrices and associated with various transducers for the detection of methyl parathion pesticide in single to multiple samples. Later, Handheld Optical Biosensor Device (**Fig. 6A**) was developed by employing microbial enzyme in the immobilized form which helps to detect methyl parathion pesticide directly in the field (AB28NABTD). Further, microplate-based biosensor (**Fig. 6B**) was developed to detect methyl parathion pesticide in multiple samples in the lab (AB35NABTD). Moreover, the concept of enzyme-based biosensors was strengthened for the qualitative detection of multiple pesticides (12 types of pesticides including 6 banned pesticides) belonging to the organophosphate (OP) and organocarbamate (OC) groups. This outcome was translated into technology of Biosensor Kit (Biokit) (AB37NABTD) (**Fig. 6C**). The working protocols and results were validated from the pesticide testing laboratory. This technology was certified by State Food Analyst, Assam and FSSAI also recognized Biokit as Rapid Food Testing Kit. These technologies have been transferred to different entrepreneurs (14).

7. Way forward

Insect pests and diseases affect agricultural sustainability and pose a challenge for food security. In addition, climate change is altering pest behaviour and geographical distribution, which may further increase the risk of introducing invasive insect pests and diseases to new areas. Crop protection and production research has consistently included both basic and applied aspects for protecting the crop damage by insect pests and diseases and enhancing the crop yield. Currently, efforts are being made to support

farmers in the transition to achieve the ambitious targets of the 'Lab to Land Strategy'. At BARC, working on new crop protection and production technologies and continuous research support for enhancing crop production and productivity will be carried out at a sustained pace. SIT, implications of bacterial endosymbionts, identifying novel insecticidal proteins to minimize the resistance development in insect pests, developing resistant plant materials, developing suitable biopesticides for insect pest, vector and disease control have been carried out to suppress major pests and disease in crops. The radiation depolymerised chitosan is effective against biotic and abiotic stresses, while radiation-polymerised hydrogel supports moisture retention in the soil during low rainfall or less irrigation facilities. Further, development of biosensors for detection of multiple pesticides from various groups of pesticides was undertaken. These technologies significantly improve plant health and productivity. Peaceful application of nuclear energy for crop improvement and protection through molecular and nuclear techniques like mutation breeding, SIT, microorganism strains and biomolecules improvement will be further explored. Newer areas of research including microbial, molecular, biochemical and information technology methods could support the ongoing programmes which will facilitate in improving methodologies, processes and also enhance cost-effectiveness for crop protection and production technologies.

8. Acknowledgements

Authors gratefully acknowledge the scientists (past and present) of NA&BTD, BARC whose contributions are compiled in this article.

MANAGING BIO-WASTE: A WEALTH FROM WASTE PERSPECTIVE

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Abstract

Managing bio-waste is the concept of enrichment of natural process of degradation in a more controlled way towards utilization of its resources for mankind. Bhabha Atomic Research Centre has developed a bi-phasic bio-methanation plant (Nisargruna) which uses a thermophilic phase of rapid degradation of complex biomolecules into simple organic acids and successive generation of methane in a completely anaerobic environment. The product methane is used directly for cooking or in generating electricity. The byproduct manure is a good source of soil conditioner and improves soil health. This process needs complete segregation of biodegradable waste from general waste. Apart from this, BARC has developed a rapid composting technology based on the use of a single cellulolytic fungal strain of *Trichoderma koningiopsis* for aerobic composting. This technology does not require complete segregation and helps in managing waste into a good quality manure. The article discusses in detail both these processes to generate wealth from waste.

1. Significance of Bio-Waste Management

Waste management has assumed global significance as huge amounts of waste is getting piled up every day in major cities of the world. About half of this waste is biodegradable

and needs to be processed by different technologies. Bio-methanation process offers an excellent way to convert this waste to a good quality fuel and more importantly bio-manure/slurry which can be used for soil health improvement. This manure/slurry will be a good alternative to cow dung for soil health management. Apart from providing manure, this process also generates energy in a sustainable manner. On the other hand, leaf litter decomposition is a complex and time-consuming process spanning over years, if occurring naturally in a forest floor. Such time scales of degradation cannot be afforded in urban and commercial rural areas, where garden and plantation wastes are generated on a daily basis. Such wastes are most often burnt causing fire and environmental hazards. Currently management of dry leaf waste comprises of dumping in land-fills and burning which liberates smoke and carbon monoxide. If left unattended, they can be a medium for spreading of forest and landfill fires. Residual agricultural biomass in the form of stubble and leaves after harvesting of grains or other useful parts of the plants, are another difficult to degrade organic substrate. These residues are often burnt as a no-cost measure to render the field available for the next cropping cycle. BARC has developed two technologies, Nisargruna and Rapid composting to offer promising solutions to the above problem. A few decades of research efforts were made to develop such processes, validate technologies and deploy them in field and/or society. Overview of the exciting journey of these technology development and important milestones are discussed here.

2. Process of Nisargruna plant

Fig. 1: First Nisargruna plant installed at Nursery (opposite to CFB), BARC during 2001

The first Nisargruna plant was installed at Nursery (Opposite to CFB, BARC), in June 2001. The design of the plant is shown in **Fig. 1**. The capacity of this plant was 500 kg per day. It was the first design with minimum infrastructure. At this initiation stage, this was only an illuminating idea which was put into action. After seeing the success of this

idea, more plants were installed at different parts of India. Many changes were made during next design of the plant. The present design of the plant is given in **Fig. 2**. The waste generated in kitchens in the form of vegetable waste, stale cooked and uncooked food, extracted tea powder, waste milk and milk products have been processed in this newer version of plant. In order to accommodate such diverse wastes, two important modifications were made in the conventional design of the biogas plant. First, a mixer/shredder was introduced to process the waste before putting it into the pre-digestor tank. Second, the pre-digestion is accelerated by the addition of hot water and

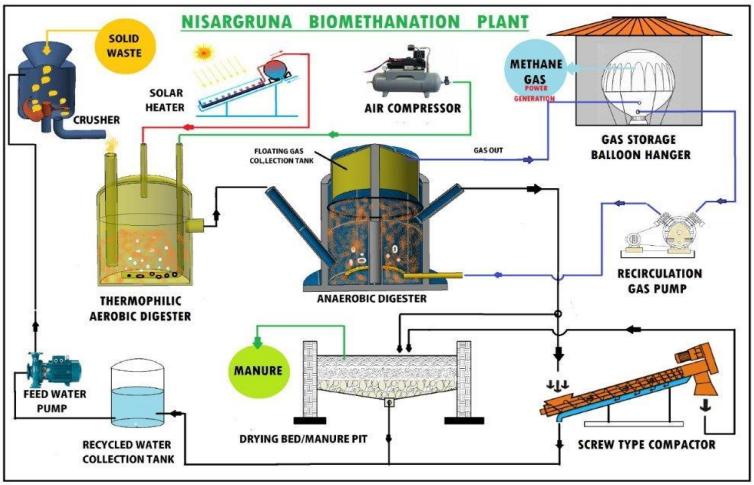


Fig. 2: Design of Nisargruna plant used during recent times with improved parameters

intermittent aeration. The waste is converted into slurry by mixing it with water in a 1:1 ratio. This helps in reduction of substrate size and thereby prevents clogging of the system. In the pre-digester, the slurry is aerobically digested and complex bio macromolecules in food are converted to organic acids. Pre-digestion reactions are exothermic and temperature rises to 40ºC by itself. Hot water obtained using solar energy is added to further raise the temperature to 50ºC. In case of insufficient sunlight during winter, provision is made to use a part of the generated biogas for production of hot water using methane stoves. The high temperature enriches thermophilic microflora present in the waste and helps to achieve faster kinetics in enzymatic degradation of biological macromolecules. The main role of these enriched microflora is to digest fats, proteins and carbohydrates into simple sugars, volatile fatty acids, amino acids and organic acids. The pH of the feed slurry in the pre-digester is around 6.5 to 7.5 and the retention time (often termed as hydraulic time) is 4 days. After the pre-digestion process, the pH reduces to 4.0 - 5.0. The pre-digested slurry is further digested under anaerobic conditions in the main digester for about 25 days by a process called as methanogenesis. The anaerobic bacteria are naturally present in the alimentary canal of ruminant animals (cattle) and such culture is added during the installation of bio-gas plants. The undigested lignocellulosic and

hemi-cellulosic materials along with microbial biomass are then passed on to a settling tank. After about a month, good quality manure is obtained from the settling tanks. Introduction of sand filters at this stage also improves the process of easy drying of the manure and allows reuse of the filtered water either for gardening or for processing back to the plant. Methane and carbon dioxide are the terminal products of this process. Methane is produced from two primary substrates namely acetate and 'hydrogen / carbon dioxide' by acetoclastic ($CH_3COOH \rightarrow CH_4 + CO_2$) and hydrogenotrophic ($CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$) methanogenic archaea, respectively. The difference between Nisargruna technology and classical anaerobic digesters are mentioned in Table 1. Overall biochemical and/or microbiological reactions are summarized as follows:

Solubilization: Facultative anaerobic microorganisms hydrolyse complex materials into soluble monomers. The mechanical mixer helps in reducing the particle size of the waste material for better solubilization. Polymeric biomolecules such as lipids, proteins, and carbohydrates are primarily hydrolysed by extracellular hydrolases secreted by these microbes. Hydrolytic enzymes (lipases, proteases, cellulases, amylases, etc.) convert polymers into monomeric units, which are then utilized by another group of microbes.

Non-methanogenic phase (acidification): The dissolved complex organic substrates are reduced to soluble simple organic acids (mainly acetic acid). Obligate H_2-producing acetogenic bacteria produce acetate and H_2 from higher fatty acids. The fermentative products, ethanol and lactate are also converted to acetate and H_2 by other acetogens and anaerobes.

Methanogenic phase (methanogenesis): Methane producing bacteria reduce acetate to methane and carbon dioxide. Some methanogens ferment the acetic acid to methane and carbon dioxide. While, others reduce carbon dioxide to methane by using hydrogen gas or formate. H_2/CO_2-consuming methanogens reduce CO_2 via formyl, methenyl, and methyl containing compounds with the help of unusual coenzymes, to finally produce methane.

3. Advantages of Nisargruna Technology

Environmental Benefits

Waste Management: Nisargruna effectively converts biodegradable waste into valuable resources, eliminating the need for landfills and reducing methane emissions associated with waste decomposition.

Nutrient Cycling: By mimicking natural processes, Nisargruna ensures the recycling of essential elements like nitrogen, carbon, hydrogen, and oxygen, promoting ecosystem health.

Land Conservation: Decentralized waste management significantly reduces the demand for landfill space, conserving land and reducing transportation costs.

Carbon Reduction: Utilizing biogas as a clean energy source displaces fossil fuels, mitigating greenhouse gas emissions and contributing to the reversal of climate change.

Soil Health: The nutrient-rich manure produced by the process enhances soil fertility, promoting sustainable agriculture and food security.

Economic Benefits

Resource Recovery: Nisargruna generates valuable end products such as biogas and manure, creating economic opportunities and reducing waste disposal costs.

Energy Independence: In rural areas, Nisargruna plants can provide a sustainable and affordable energy source, fostering self-reliance and reducing dependency on fossil fuels.

Table 1: Salient differences between Nisargruna technology and the classical anaerobic digesters

Properties	Conventional anaerobic digesters (gobar gas plant)	Nisargruna biogas plant
Type of waste processed	Mainly cow dung (gobar)	All biodegradable wastes, e.g. kitchen waste, food waste, green grass, animal waste, abattoir waste, etc.
Pre-digestor	Not included	Included
Waste feeding	Direct	After making a slurry through a mechanical mixer
Handling of waste	Direct	Needs segregation
Use of hot water	No usage of hot water	Solar heater is used for getting hot water, which is then mixed in predigestor
Types of bacteria	Only methanogenic	Thermophilic bacteria in predigestor and methanogenic bacteria in main digester
Digestion	Single phase, i.e. anaerobic	Biphasic, i.e. aerobic and anaerobic
Type of manure	Manure is more fibrous and less consistent. Loss of nitrogen is more due to open storage	Good quality and odourless Use as soil conditioner
Processing time	About 45-50 days	About 30 days
Gas output	Methane 50-55%	Methane 75-78%
Scope	Only rural area	Urban and rural area both
Design	Small scale	Suitable for scaling up
Recycling water	Not done	Saves 60% water
Advantages/ disadvantages	More retention time Useful for small-scale use	Less retention time Save on transporting cost of waste. Uses in large scale

Technical Advantages

Efficient Processing: Nisargruna offers a continuous and compact solution for treating large volumes of biodegradable waste without requiring additional space.

Versatility: The technology can be adapted to various waste streams and scales, making it applicable to both urban and rural environments.

4. Various major success stories of Nisargruna technology

4.1. Obtaining an ISO certificate for managing waste at Matheran, Maharashtra

Matheran is a small hill station and a popular tourist destination that is 80 km away from Mumbai. This has been declared as a highly eco-sensitive zone and is home to several rare flora and fauna. There are several hotels and restaurants to cater to a large number of tourists and such establishments produce several metric tons of biodegradable kitchen waste. Being a vehicle free town, horse is the main mode of transportation and sometimes the roads are littered with horse dung. Due to its official eco-sensitive status, hoteliers are not allowed to dump their waste in the vicinity of the town. A five-ton capacity Nisargruna project was installed during 2007. All food waste from hotels and a part of the horse dung has been processed in this plant. The biogas generated in the plant is used to run a 30 kVA generator and the eletricity produced from it lights up the town's street lights. This not only shows the utility of the technology in such an eco-sensitive zone with variable weather conditions, but also proves that such projects can be successful even under municipal settings. Successful operation of the Nisargruna plant in such a new environment has led to awarding of an ISO certification for managing waste.

4.2. NISARGRUNA for community kitchen

Kurudampalyam is a small village, 20 km away from Coimbatore, with most of the villagers residing below poverty line. Coimbatore (rural) district authority installed a 2-ton capacity Nisargruna plant during 2014. Biodegradable waste from the adjoining part of Coimbatore city is delivered to the plant every day. The biogas produced at the plant is supplied to a community kitchen across the road. The community kitchen has 12 biogas burners. Villagers come here with their raw ingredients and cook their meals (**Fig. 3**).

Fig. 3: Nisargruna plant for community kitchen installed at Coimbatore village during 2014

4.3. Nisargruna at the doorstep of software techies

Several corporate houses including Tata Consultancy Services (TCS) have adopted this technology to make their corporate campuses and premises zero waste facility. TCS has Nisargruna plants at many of their campuses including their largest campus at Chennai. The Chennai campus has a 3 MTPD (metric ton per day) plant where the gas is converted to electricity using a biogas generator. TCS, Thane plant is working for more than a decade now.

4.4. Handling Abattoir waste - Nisargruna gave a solution

A MoU was signed between BARC and MCGM, Mumbai for processing slaughter house waste (15-ton capacity) by Nisargruna technology. Deonar abattoir being the biggest slaughter house in Mumbai was selected for setting up the plant. Before setting up of the biogas plant, slaughter house waste was discarded in the dumping ground resulting in pollution of the surrounding area. During the working of this plant for three years, a total of 10,000-ton waste was processed resulting in 400 ton manure and about 55,000 units of electricity. This plant has also saved the expenditure of sending this material to the dumping ground and making the environment clean. Subsequently the technology was implemented at different slaughter houses in Chennai, Rajkot and Bengaluru.

4.5. Large scale deployment of Nisargruna technology to the state of Chhattisgarh

The technology was transferred to Chhattisgarh Biofuel Development Authority (CBDA), Government of Chhattisgarh during 2023. During the first phase, biogas plants were installed at seven locations from tribal districts of Chhattisgarh. The first plant at Janapad Panchyat, Jagdalpur was inaugurated by Shri Bhupendra Baghel, Hon. ex-chief minister on 25th Jan 2023. The plant has a capacity of processing 500 kg cow dung per day. The plant has also successfully completed production of 10 KW electricity per day and it is connected to the grid (**Fig. 4**).

Fig. 4: Nisargruna plant installed at Chhattisgarh and inaugurated by Chief Minister during January 2023

4.6. Modernization of the technology and setting up of a new plant at BARC hospital

Nisargruna plant was initially installed at hospital site during 2006. This plant has been further modernized for enhanced performance last year. The newer concepts like spraying water to the main digester slurry through a high pressure nozzle has been introduced for breaking scum formation. Methane recycling grid has been introduced which has helped in improving methane quality by maintaining complete anaerobic environment. Improved water recycling system has been installed by using screw press system. All these modifications have resulted in better performance of the plant.

4.7. Nisargruna for Swachha Bharat Mission

The technology has been recommended by Swachha Bharat mission of Government of India for making cities clean. The technology was installed by many municipal authorities throughout the country. Further the second phase of Swachha Bharat Mission was launched for rural part of country and Nisargruna technology has been introduced for implementation.

5. Nisargruna technology in the context of climate change:

Nisargruna technology offers a two-fold approach towards curtailing CO_2 emissions from fossil fuel combustion. Firstly, biogas produced through this process serves as a direct replacement for gas or coal in cooking, heating, electricity generation, and lighting. Secondly, the high-quality manure generated by Nisargruna plants reduces the dependency on chemical fertilizers, thereby avoiding CO_2 emissions associated with their production. Moreover, by substituting firewood with biogas, Nisargruna mitigates deforestation and land degradation, indirectly functioning as a carbon sink. While methane, a primary component of biogas, is a potent greenhouse gas, its conversion to CO_2 through combustion within the Nisargruna process is a net benefit when compared to the uncontrolled release of methane from anaerobic decomposition. This is analogous to the sustainable use of firewood, where CO_2 emitted is offset by recent carbon absorption through plant growth. Unlike fossil fuels, biogas combustion does not introduce new carbon into the atmosphere. Nisargruna's potential to generate carbon credits through integrated plant systems underscores its significant contribution to carbon reduction and climate change mitigation.

6. Technological improvement/upgradation: Generation of '*Shesha*'

A novel, compact helical shaped digester cum waste converter made of low-cost PVC pipes, has been developed and deployed for kitchen waste processing during 2021. The name Shesha (शेष) has been given on the basis of the serpentine shape of this digester (its resemblance to the snake *Shesha*) as well as Sanskrit word for waste (**Fig. 5**). The system has been patented with Indian Patent No.531960 very recently. The main advantage of this waste converter includes helical shaped digester made from low-cost PVC pipes which saves major cost of civil construction and MS (mild steel) dome required for conventional designs. It is suitable for skid mounting on a vehicle or wheels required for

processing waste from smaller societies/residential complexes. Also, the design has inbuilt suitability of biogas recycling for methane enrichment and is suitable for online monitoring of process parameters. The overall process includes converting organically rich bio-degradable portion of solid waste to slurry by mixing equivalent quantity of water and it is almost the same as in Nisargruna except the plug-flow system. The undigested lignocelluloses and hemicelluloses then flow out as high-quality organic manure slurry. The pH of this slurry ranges from 7.5 – 8.0. It has been observed that the waste is converted into good quality manure and the gas generation is substantial. All the microbial and biochemical parameters of the waste is achieved at the end of the process. The know-how of this novel design made of PVC pipes has been transferred to several industries. A higher capacity Shesha plant (50 Kg) has been installed at IGCAR campus for catering biowaste (**Fig. 6**).

Fig. 5: Shesha pilot plant installed at Training School Hostel during 2021

Fig. 6: Shesha plant installed at BARC Facility, Kalpakkam during 2023

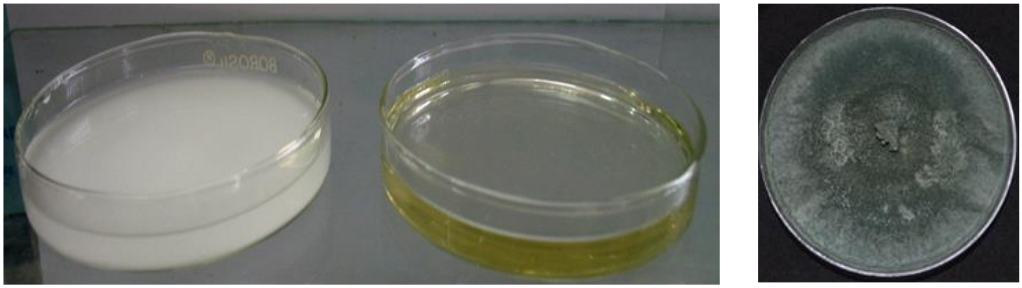
7. Managing solid waste through rapid composting technology

7.1. Genesis of Rapid Composting Technology

Biodegradable organic wastes such as crop residues, agro industrial organic wastes (including animal litter), city garbage and forest litter have wide C/N ratios ranging from 80 to 110, and thus can't be used directly in agricultural soil without converting them into compost. Around 7 to 8 million metric tons of paddy residue are set on fire in open fields between October and November. Thus, air quality in the thickly populated adjoining regions is badly affected. Similar problems are encountered in case of sugarcane and banana plantations where residual biomass poses a problem in disposal. Its treatment in anaerobic digestion (like in bio-methanation plant) as such is not feasible because it requires longer retention period and also forms scum during the initial stages leading to clogging of pipelines. These organic solid wastes generated in different sectors are often indiscriminately dumped on-ground. When organic material such as food and green waste is disposed in landfills, it gets compacted and covered. This cuts off oxygen supply and causes it to break down via an anaerobic process, eventually releasing methane, a greenhouse gas that is 25 times more damaging than carbon dioxide. The implications of such release for global warming and climate change are enormous. Methane is a flammable gas that can cause fire hazards in dumping grounds. This comes with a heavy carbon footprint as well. Composting food scraps and green waste into manure, eliminates many of these problems. Such a process needs effective microbial strains for decomposition.

7.2. Isolation of a cellulolytic fungus, formulation, and its potential use in waste composting

Cellulose is the most abundant natural polymer on the earth and also the structural component of majority of cells. It is also a substrate which is difficult to degrade. Hence it is pertinent to look for an organism that effectively uses cellulose as a carbon source. Tree bark was one such lignocellulose rich niche from where we could isolate at least 10 micro-organisms. However cellulolytic filamentous fungi are the organism of choice given the ease in downstream processing and amenability to solid state fermentation. Considering other criterion for selection like growth under ambient conditions, non-pathogenic to plants/animals and safety in handling at large scales, the genus *Trichoderma* was selected. The cellulose degradation potential of this isolate was checked on 1% cellulose suspension which was solubilized in 10 days under constant shaking (**Fig. 7**). The species was identified as *Trichoderma koningiopsis* by molecular tools. Pilot experiments on leaf degradation were carried out at BARC in drums and further large-scale experiments were managed on diverse substrates at different locations.



CONTROL **TREATED** ***T. koningiopsis***

Fig. 7: Cellulose degradation by rapid composting strain, *Trichoderma koningiopsis*

7.3. Development of culture formulation

The process of formulation development for *Trichoderma koningiopsis* is depicted in the figure below (**Fig. 8**). Briefly, it includes inoculation of pure culture of *T. koningiopsis*, in sorghum seeds, incubation for growth and making a wettable powder (WP) formulation.

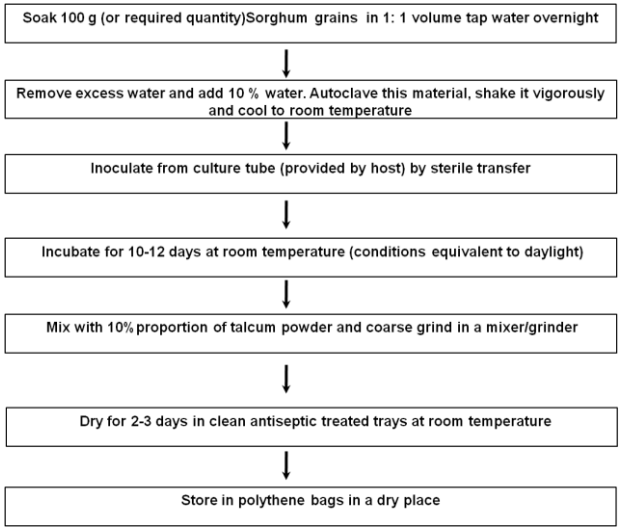


Fig. 8: Flow chart for the development of rapid composting formulation

7.4. Implementation of rapid composting technology and it's success stories

The Rapid Composting Technology (RCT) was implemented for the first time at Kurla Kamgar Society, Mumbai for which the society received the "Clean and Green Society" Award in 2018, organized jointly by Pollution Control Board and Loksatta. At Tata Institute of Fundamental Research (TIFR), Colaba, Mumbai several tons of garden waste comprising of peepul, barrintonia, banyan and almond were processed and the manure obtained was used in the gardens of the same premises. Implementation for kitchen waste

was done at Mahindra Nagar colony, Malad and Homi Bhabha Centre for Science Education and Research (HBCSE, TIFR). The introduction of this technology was particularly useful after the imposition of strict guidelines by Brihanmumbai Municipal Corporation banning collection of biodegradable garbage from institutions and housing societies, thus forcing them to process their waste *in-situ*. Even sending waste out of the premises by private agencies for processing incurred good amount of expenditure. HBCSE has successfully implemented RCT from last five years and composting kitchen waste in–house, till date. This technology has been implemented at DAE colony in Mandala and Electron Beam Centre at Kharghar. Agricultural residues like sugarcane leaves were composted at farmer's fields in Ahmednagar district, Maharashtra. Whole geranium plants after extraction of oil were processed to yield manure at Sangamner. Farmers at Nandurbar district of Maharashtra have adopted RCT for in-situ decomposition of whole banana plants after harvesting of fruits. **Fig. 9** represents a collage of various wastes processed and its implementation at several areas.

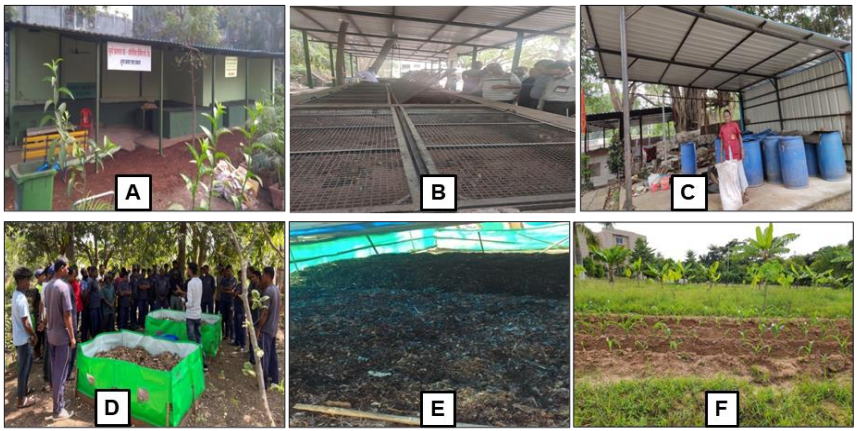


Fig. 9: Collage of different composting sites and different wastes processed

7.5. Technology transfer and Implementation under AKRUTI scheme of BARC

This technology has been transferred to more than 65 agencies including biotechnology and agricultural businesses, institutions, housing societies, non-governmental organizations and agencies working in the field of environment and agriculture. Agri business start-ups have also taken up this process and are establishing themselves in this venture. A list of products launched in the market is depicted in **Fig. 10**. AKRUTI training centre at Tarapur Atomic Power Station (TAPS), Maharashtra has successfully implemented preparation of microbial formulation in-house as well as setting up of facilities for composting of kitchen waste and dry garden leaf waste. AKRUTI centre conducted training programs to create awareness about Rapid Composting Technology amongst local villagers in and around Tarapur as well as employees of TAPS, workers, gardeners, cleaners and other staffs. As a result of this, many residents have started composting at their house hold levels. Many youngsters have also come forward to take

up the license of this technology and become an entrepreneur. A glimpse of the deployment of this technology is narrated in Table 2.

Fig. 10: Various products marketed using BARC-Rapid Composting Technology

Table 2: Implementation of Rapid Composting Technology to process diverse bio-substrates

Substrate	Location	Scale of processing
Whole Coconut leaves (after shredding)	Kurla Kamgar Society, Mumbai	200 kg/month in pits
Peepul, Barrintonia, Almond and banyan leaves	Tata Institute of Fundamental Research	500 kg/month in heaps on the ground
Kitchen waste	Mahindra Nagar Colony	400 kg/day, in pits on the ground
Kitchen waste	DAE Mandala Colony, Trombay	100 kg/day in plastic drums
Kitchen waste	Homi Bhabha Centre for Science Education and Research, TIFR, Mumbai	100 kg/day in plastic drums
Paddy straw	GCNEP, Bahadurgarh	Demonstration in 100 kg heaps
Spent mushroom straw	GCNEP, Bahadurgarh	100 kg in waste collection bins
Whole banana plants	Nandurbar, Maharashtra	Farmers field (*in situ*)
Sugarcane leaves	Ahmednagar, Maharashtra	Farmers field (*in situ*)
Whole geranium plants after extraction of oil	Ahmednagar, Maharashtra	Farmers field (*in situ*)
Mahua Cake	Nandurbar, Maharashtra	In pits
Nirmalya or Floral waste	Anushaktinagar, Ganeshotsav	In drums at the pandal sites
Canteen waste	Imperial College, Bargarh, Odisha	In drums at the canteen site
Elephant grass	DCSEM, Anushaktinagar	Pits dug on the ground with earth removing machines
Nirmalya	Kumbh Mela 2021, Haridwar	In drums and pits

8. Contributions on soil science related techniques at NA&BTD

Application of isotopic techniques for sustainable agriculture in general and their use for soil health improvement in particular has remained an important mandate of bioscience group since its inception. Different radioisotopes were used for studies of nutrient movement in soil and plants. These isotopes act as tracer tools for unlocking the biochemical pathways in plant and soil.

8.1. Use of radioisotopes for soil fertility management:

Radioisotopes are used as a 'tracer' or 'label', which helps to follow the movement of specific nutrients in different layers of soil. This helps in getting information on fertilizer use efficiency, quantifying losses from soil and biological transformations of mineral nutrients in plants. It allows tracing of the translocation routes in the soil and different plant parts. Soil science section (presently plant pathology and microbiology section) played a pioneering role in studies on movement of nutrients in plethora of soils across the Indian sub-continent by using isotopic techniques. Fertilizers enriched with ^{15}N and ^{54}Fe offer excellent methods of understanding their fractionation among different components while ^{32}P and ^{35}S help in dissecting differential uptake of the nutrients by the plant. Effects of crop residue incorporation on soil stabilization and fertility enhancement is determined by the analysis of stable isotopes like ^{13}C and ^{15}N. Studies on labelled fertilizers provided useful information on nutrient requirements of crops, precise knowledge on the type, amount, method and time of application of fertilizer materials best suited for specific soil-crop combination and quantification of biological nitrogen fixation by leguminous crops.

8.2. Development of improved fertilizer formulations:

Characterization of rock phosphate from all over the world was carried out extensively at NA&BTD, BARC. An improved phosphorus fertilizer formulation (Patent No 238485) was developed and commercialized. A zinc fertilizer formulation (Patent No 239929) was made as an alternative to commonly available micronutrient-fertilizers in the market. Both these inventions were further developed as a technology and transferred to industries through Technology Transfer and Collaboration Division (TT&CD).

8.3. Development of soil organic carbon detection kit and multi-nutrient extractants:

Organic carbon is an important parameter of soil health and indicator element for status of soil productivity. In order to make farmers aware about importance of soil organic carbon, an instant field-testing kit has been developed at BARC. This kit analyses the carbon status of soil in minimum time and therefore the farmer doesn't have to rely on other agencies for results. This ultimately helps farmers to decide the nutrient supply to crop which is very critical for better production. The technology has become very popular amongst farmers. The simplicity of the technology was appreciated by IAEA and an impact story was published on IAEA webpage during April 2021 (**Fig. 11**). Taking this technology further for understanding the status of other nutrients in soil, a universal multi-nutrient soil extractant has been developed. This extractant got Indian Patent (No

358936). This was further developed as a technology and transferred to three industries. One product based on this technology is available in the market (Harit Kranti).

Effect of irradiated sewage sludge obtained from SHRI facility, Baroda was evaluated as part of IAEA project on different soil and plant systems. The study revealed improvement in physicochemical properties and organic carbon content in soil without any significant uptake of heavy metals in plant residues.

Overall, the above studies at NA&BTD have contributed in the development of different strategies towards improving soil health for sustainable agriculture.


IAEA
International Atomic Energy Agency

Living in Harmony with Mother Earth: User-Friendly Instant Tool Kit Helps Indian Farmers Determine Soil Organic Carbon

22 Apr 2021
Carley Willis, IAEA Office of Public Information and Communication

(https://www.iaea.org/sites/default/files/styles/original_image_size/public/soil-india-1140x640.jpg?itok=tS3cDvtj)

Carbon-rich minerals, organic materials and living organisms – these various components of soil are necessary to absorb water and breakdown pollutants. Having the right balance of organic matter and living organisms, nutrients are released in the soil, allowing healthy crops to grow. (Photo: S. Mehetre/Bhabha Atomic Research Centre)

"To be a successful farmer, one must first know the nature of soil," ancient Greek

Fig. 11: Soil organic carbon kit developed at BARC

9. Way forward: Future direction of managing liquid and solid waste

Nisargruna and Rapid Composting technologies have played a significant role in processing variety of biodegradable waste generated in different parts of the country. These technologies have been transferred to 225 entrepreneurs (till August 2024) and spread across the country for processing variety of wastes. There are more than 360 plants working successfully throughout the country starting from Leh to Kanyakumari, processing different types of waste and utilizing the bio-gas generated therein for diverse applications. The Rapid Composting technology has been transferred to more than 65 agencies so far. Nisargruna is an excellent decentralized sustainable option for processing biodegradable waste materials generated in various parts of the country. The technology has made significant impact on the solid waste management sector for last two decades. It has showed the way forward for successful bio-methanation option from biodegradable waste. The biogas generated is being used for cooking, street light, electricity generation, compression purpose and gas turbine use. In the near future, challenge remains in exploring such technology at house-hold level to combat the effect of climate change and develop a sustainable model for biodegradable waste management.

Conversion of waste biomass to manure for soil application allows effective carbon sequestration which will go a long way in reducing the alarming levels of atmospheric carbon di-oxide concentration that stands at 425 ppm as opposed to 325 ppm of pre-industrial revolution. The USP of the rapid composting technology lies in the use of single microbial culture to develop a formulation which in turn leads to low capital investment by the industry. Further, large-scale implementation, of this technology requires minimum infrastructure and zero involvement of skilled labour. Strict segregation of non-biodegradable materials is also not mandatory, since they can be separated from the manure at the end of the process. Beyond the environmental issues that are addressed, it opens up avenues for dignified stable employment to people from the marginalized sections of the society like rag pickers in a hygienic set up. This is in direct contrast with the dumping grounds where they forage for useful material in the waste. In agronomic and horticultural practices compost can be used as a very good soil amendment, in the form of natural fertilizer which enhances physical, chemical and biological properties of the soil.

It is pertinent to mention that many nations are signatory to the COP 28 where steps have been taken to achieve carbon neutrality. These technologies owing to their circular nature of utilizing resources will not only aid in reducing carbon emissions but also help in earning carbon credits, thus enhancing their commercial potential. Composting is an oxygen-demanding process which involves the hydrolysis of organic matter into humus. Further, sensor-based automations in aeration and reduction of leachate can decrease the time of processing. This can also help in getting rid of anaerobic pockets within the composting mix improving its acceptability in public. Efforts will be taken up for defining a parameter for assessing compost maturity and reducing leachate production. Future direction of research will involve manufacturing value-added products from compost like humic acid and fulvic acid and a semi-organic fertilizer formulation.

10. Acknowledgements

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ADVANCES IN PLANT TISSUE CULTURE AND BIOTECHNOLOGY RESEARCH AT BARC

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Abstract

Plant tissue culture has several applications in areas such as clonal propagation, crop improvement, molecular farming, transgenic research, and production and isolation of bioactive compounds. BARC has been engaged in varied applications of plant tissue culture research since the 1960s and has contributed significantly to micropropagation, crop improvements, process development and understanding of radiation effects on plant cells and transgenic plants. This has significantly contributed to basic and applied research. This chapter summarizes some of the key achievements of plant tissue culture and plant biotechnology since its inception at BARC, Trombay campus. This chapter includes a snapshot of the recent activities and contributions from the group to acquaint a larger platform of interested research scholars, farmers and the industrial fraternity.

1. Introduction

Somatic cells of plants have the same genetic makeup as that of a fertilized zygote, and it was postulated that all plant cells are totipotent. Since the initial studies of plant cells cultured in the test tube by Haberlandt in 1902 and the discovery of plant hormones like auxins and cytokinins, the field of plant tissue culture has blossomed with successful propagation of plant cells *in vitro* through organogenesis or somatic embryogenesis. Rapid developments in cell and tissue culture and genetic engineering have generated

great interest for plant biologists. As our understanding evolved over the decades, plant tissue cultures have found diverse applications in several fields of plant biology, including:

- Micro propagation for the production of a large number of clonal plants.
- Shoot tip culture for virus elimination.
- Development of somaclonal variants.
- Anther or microspore culture for production of haploids.
- Protoplast culture for somatic cell hybridization, cybridization and gene transfer.
- Development of genetically modified plants through *Agrobacterium*-mediated and direct gene transfer methods.
- Production of secondary metabolites in medicinal plant cultures and manipulation of cultural parameters to enhance secondary metabolites as well as manipulation of metabolic pathways.
- Use of plant cells for precise genome editing and crop improvement.

2. Plant Tissue Culture Work at Bhabha Atomic Research Centre

Developments in cell and tissue culture have generated great interest among plant biologists. Plant tissue culture (PTC) emerged as a tool for a number of basic and applied research problems of relevance to plant biology and crop improvement. The plant tissue culture laboratory in BARC was initiated in 1966 by Dr. S. Narayanaswamy who had vast experience in the Department of Botany, Delhi University. Mr E. W. Rajasekhar joined him and helped him in setting up the lab, which needed aseptic conditions for the culture of plant cells. Mr. Rajasekhar went to work in the UK with Professor H. E. Street, who was a doyen of plant tissue culture. The objectives of the plant tissue laboratory in BARC in the initial years were to study the effect of ionizing radiation on plants, morphogenesis and develop regeneration system in various plants.

2.1. Ionizing radiation and plants

Studies concerning the effect of ionizing radiations on seeds, seedlings and callus cultures of *Petunia inflata*, *Antirrhinum majus* and *Pharbitis nil* were initially done at BARC. The striking differences in growth and development of these three types of tissues on exposure to gamma rays were attributed to be organizational differences. One striking observation noted was the stimulation of seed germination in all three plant species at low doses of radiation. The effects of gamma and ultra violet radiations on the survival and totipotency of haploid tobacco cells was also studied.

2.2. Haploid culture, protoplast culture & inter-kingdom hybridization

In 1966, Dr. Sipra Guha and Dr. S. C. Maheswari from Delhi University reported the development of haploid plants from anther culture for the first-time in *Datura innoxia*. Soon after, some pioneering findings were reported on the anther culture in *Datura metel* and in other plants from BARC. As pollen culture was expected to produce mainly the haploids, there were instances of triploid plants instead of haploid plants. The origin of triploids from haploid pollen grains was a mystery and it was postulated that triploids

arose due to the fusion of one generative and two vegetative nuclei in the pollen grains. Later, this was confirmed by Dr. Sutherland from the UK.

Protoplast isolation and regeneration was one of the most exciting research subjects in plant biology worldwide during the 70s. This inspired researchers to undertake studies on plant protoplasts and somatic cell hybridization. The work on *Datura metel* pollen protoplasts was featured in the prestigious journal "Nature" on the cover page (Nature 246, 223-224; 1973). The first successful inter kingdom hybrid between *Amoeba* and *Atropa belladonna* protoplasts using polyethylene glycol and microsurgery was developed. Although there was no fusion of animal and plant nuclei, the plant nuclei divided inside *Amoeba* cytoplasm, which was confirmed by autoradiography. The plant nuclei disintegrated after one month of culture. This is the first report on successful inter kingdom hybridization between an animal and a plant cell (Cytologia 45: 149-155; 1980).

Successful protocols for protoplast isolation and regeneration from *Santalum album* (first for a forest tree), *Nicotiana tabacum*, *Brassica juncea*, *Tylophora indica*, *Pergularia pallida*, *Arachis hypogaea*, *Sesamum indicum*, *Vigna aconitifolia*, *Vigna mungo* and *Catharanthus roseus* were established and plants regenerated in some cases.

2.3. Plant regeneration and genetic improvement in various crops

Extensive research strides took place in the plant regeneration studies in different (and recalcitrant) plant systems, leading to the development of efficient regeneration systems in several economically important crops by organogenesis and somatic embryogenesis.

2.3.1. Cereals, oil seeds and pulses

Untill the 1980s, cereals like wheat were postulated to be recalcitrant, and the regeneration of plants was difficult. Dr. Ingo Potrykus and his group in Switzerland, even after testing hundreds of media combinations, could not get plant regeneration in cereals. For the first time successful plant regeneration in bread wheat using immature embryos and immature inflorescence cultures was reported from BARC. This was reported by the Press Trust of India in the major National newspapers, and there were discussions in Parliament on plant regeneration in wheat. Later, plant regeneration was reported in major cereals and millets like Rye, Triticale, Durum wheat, Sorghum, Bajra, Ragi etc. and oil seed crops like peanut, soybean and mustard. For the first time, a yellow-seeded somaclonal variant was reported in *Brassica juncea*, which was a recessive mutant developed from the black seeded *B. juncea*. Grain legumes are very difficult to regenerate and were thought to be recalcitrant. Regeneration and development of complete plants were demonstrated in several pulse crops such as chickpea, moong bean, black gram and moth bean.

2.3.2. Horticultural crops

In India, BARC was one of the few institutes to take initiatives in the early 1980s to develop a micropropagation protocol for economically important horticultural crops. Soon, efficient regeneration and complete technological protocols in *Santalum album* (sandalwood), *Morus indica* (sericulture), banana and pineapple were developed. Somatic

embryos are a valuable tool for the establishment of long-lasting regenerable tissue, with varied applications in the genetic improvement of plants. For the first time, somatic embryogenesis from male floral buds was reported in banana. A novel approach of somatic embryos production in two bioreactors was developed at BARC. Research on the generation of superior variants of bananas through the development of somaclonal variants and *in vitro* mutagenesis was carried out. Radiation induced mutations in banana cultivar "Giant Cavendish" resulted in the development of a couple of dwarf mutants which are now in advanced trials in collaboration with National Research Centre for Banana, Trichy (**Fig. 1**). One of the them, named TBM-9 is expected to be notified as a new variety very soon. Similarly, a few promising mutants in pineapple with a small crown, large fruit and high sugar content have been screened, and their detailed assessment is underway.

Fig. 1: Dwarf mutant (TBM9) developed in banana. Wild-type cavendish banana (left) displaying lodging due to bunch weight. Bunch development in dwarf mutant (right) of giant Cavendish

Turmeric and ginger are medicinally important spice crops in India. Callogenesis and direct organogenesis from sprouted shoot buds in both of these crops are standardized. Further, *in vitro* mutagenesis is being carried out to create genetic variability for three specific traits, including enhanced rhizome yield, curcumin/ginegerol content, and disease resistance. Field trials of mutagenized turmeric population resulted in a few potential turmeric lines with a higher rhizome yield (up to 1.5-fold higher than control) and curcumin content (4.5-6.5% against 4.2% in control). Similarly, a few promising mutants for resistance/tolerance against rhizome rot-a major disease of turmeric, have been developed.

2.3.3. Ornamental crops: a new opportunity

There is always demand and necessity of new ornamental crop varieties in the modern and rapidly growing floriculture industry. Genetic variations using induced mutagenesis in such crops are introduced for selected traits, including flower morphology, flower colour, compact growth, variegated leaves and disease resistance. A good number of mutants with desired traits are identified in Chrysanthemum (**Fig. 2**), Gladiolus and Carnation, and field trials of the selected mutant lines are going on to develop them as novel varieties. One of the constraints in commercial cultivation of gladiolus is non-availability of a large quantity of propagules. To overcome these limitations, an efficient protocol for the *in vitro* production/multiplication of cormels in gladiolus was standardized.

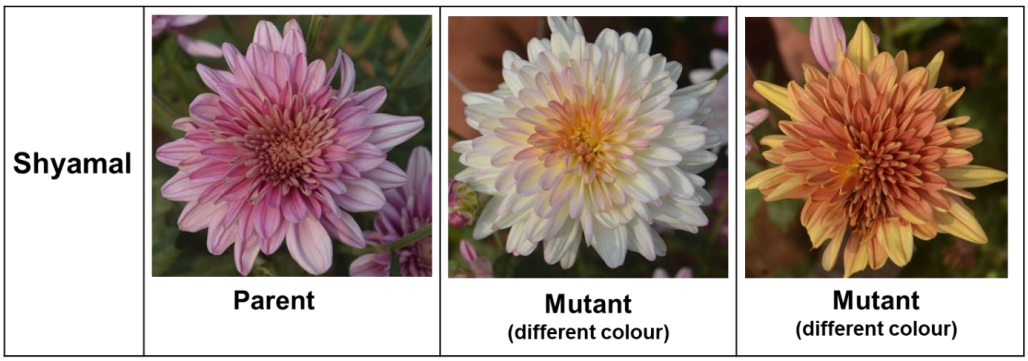

Fig 2: A few promising mutants screened in Chrysanthemum

2.4. Sugarcane

A research program on sugarcane started in the early 2000s, with the idea to employ plant cells cultured *in vitro* for the selection of useful induced mutations at the cellular level. BARC developed a simple and direct method of somatic embryogenesis in sugarcane using segments of immature "inflorescence" of the sugarcane plant. The method was granted an Indian patent (2004, Patent No. 243373). In sugarcane, partial desiccation treatment proved to be beneficial for augmenting growth and plant regeneration in high-dose gamma-irradiated embryogenic callus cultures (**Fig. 3**). This simple and novel

approach can be useful in stimulating the regeneration response of higher dose gamma-irradiated cultures in plants. Studies on *in vitro* mutagenesis using gamma irradiation and *in vitro* selection led to the isolation of several useful mutants for salinity tolerance in popular sugarcane cultivars. Field assessment of a few of them is underway.

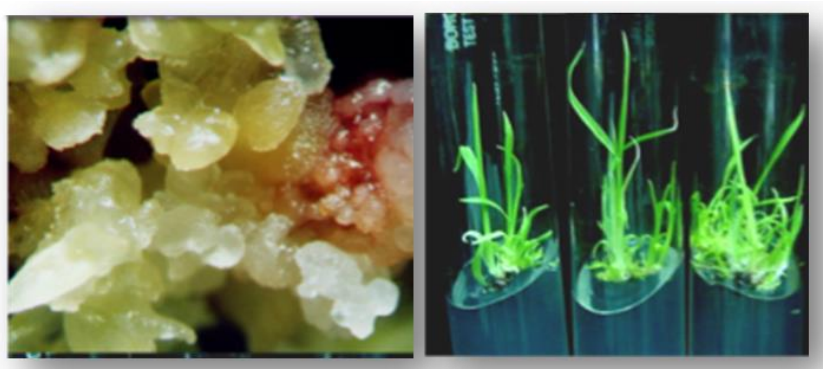

Fig. 3: *In vitro* embryogenesis and plant regeneration in sugarcane

2.5. Synthetic seeds

Somatic embryos and multiple shoots are excellent tools to mass propagate the clonal progeny of vegetatively propagated crops, but they are sensitive and difficult to transport. To overcome it, the concept of synthetic seeds was explored worldwide. BARC demonstrated synthetic seed production and regeneration in mulberry, banana, sandalwood and cardamom.

2.6. Plant secondary metabolites

Medicinal plants constitute an important source for the production of a variety of novel compounds used in therapy. Plant tissue cultures initiated from these medicinal plants offer an excellent source for the production of these important secondary metabolites without the destruction of the entire tree/natural habitat. Research aimed to explore secondary metabolites in tissue cultures of various medicinal plants, like *Tylophora indica, Atropa belladonna, Physalis minima, Catharanthus roseus, Rauwolfia serpentina, Holarrhena antidysenterica, Tinospora cordifolia, Azadirachta indica, Artemisia annua, Psoralea corylifolia, Andrographis paniculate, Taxus baccata* and *Coleus* sp. A bioreactor facility for the plant cells was established and the stepwise procedure of scale up of cultures from flasks to bioreactors was consistently demonstrated.

Work on haploid *Atropa belladonna* cultures showed that tropane alkaloids could be synthesized under tissue culture conditions, contrary to the earlier reports. *Taxus baccata* callus cultures revealed significant levels of 10-deacetyl baccatin III-a precursor for the semi-synthesis of taxotere, a novel anticancer agent with improved solubility than taxol. A two-stage method proved effective for obtaining good growth and production of taxanes. Organized shoots, suspension culture and hairy roots induced by *A. rhizogenes*

in a few medicinal plants were scaled-up in bioreactors and their alkaloid synthesis studied. Realising the importance of Podophyllotoxins and their derivatives in cancer therapy, attempts were made to develop tissue culture methods for the production of these lignans. An alternate plant species namely *Linum flavum*, widely occurring in the European countries, was found to be an ideal source for the lignan 5-methoxypodophyllotoxin (5-MPT). Genetic transformation studies using *Agrobacterium rhizogenes* enhanced the levels of 5-MPT significantly. Later, work focused on *Nothapodytes foetida* and *Ophiorrhiza* sp.- the two endangered Indian medicinal plants and source of prominent anticancer compound- camptothecin (CPT). For enhancing the production of bioactive molecules in the target plants, elicitation, immobilization, and the development of hairy roots cultures are also being carried out regularly. Induced mutagenesis using various mutagens can be one of the effective tools for enhancing secondary metabolites production in medicinal plants. A significant enhancement of CPT production in gamma-radiation treated *Nothapodytes foetida*'s callus culture over control was reported. To enrich the understanding of camptothecin biosynthesis in *Ophiorrhiza rugosa*, tissue-specific "OMICS' based analyses were undertaken. Together with various functional validation strategies, several crucial players of CPT biosynthesis could be identified, and are being used for targeted metabolic engineering of terpenoid indole alkaloid (TIA) biosynthesis.

2.7. Pioneering work in plant transgenic research

Transgenics are valuable tools for trait introduction and for basic research on gene characterization. Consequent to the first report in 1983 on the genetic transformation of tobacco using *Agrobacterium* by Dr. Jeff Schell and his team from Germany, experiments were initiated at BARC for the development of genetically modified plants. Soon transgenic moth bean plants with a bacterial gene (*npt*II) were developed using three different techniques, such as *Agrobacterium*-mediated, PEG-mediated, and electroporation. This was the first report on development of a transgenic pulse crop from protoplasts. Later, c*ry1Ac* gene from *Bacillus thuringiensis* was introduced into chickpea and moth bean by *Agrobacterium* and direct gene transfer using particle gun and transgenic plants with improved tolerance to insect pests were developed. BARC developed transgenic groundnut plants with a foreign gene for the first time. Introduction of a synthetic *cry 1Ac* gene in Indian mustard improved tolerance to insect pests, while the introduction of a chitinase gene from fungus *Trichoderma virens* into tomato enhanced fungal resistance. Similarly, over-expression of an anti-microbial peptide gene (MSI–99, magainin) in banana and tobacco improved disease resistance in both the plants. Work on coat protein genes for disease resistance has been done on Potato and transgenic potato plants were raised and tested at Potato Research Laboratory. Shimla,

Molecular farming is an attractive option for vaccine production using plants system. In this direction, attempts were made and BARC published first report on the genetic transformation of banana cv. Rasthali, an elite Indian variety of banana. At BARC, hepatitis B surface antigen (HBsAg) was expressed in tobacco, tomato, soybean, banana and potato, and its expression was demonstrated in fruits of banana and tomato and

microtubers of potato. These studies encouraged many research institutes in their efforts for development of edible vaccines. The banana transformation system has been followed extensively for the development of transgenic banana plants and on understanding the gene functions in biotic and abiotic stress tolerance, secondary wall deposition, xylem development, biofortification and shoot multiplication. Work on understanding the nature and activities of the native promoters of banana has helped to decipher their basic mechanisms. Fortified banana developed by overexpressing *nicotianamine synthase 2* (*OsNAS2*) gene of rice in banana (an India-Australia technology transfer project) showed higher accumulation of Fe (17 times) and Zn (12 times) in the selected transgenic lines compared to control. Contained advance field trials of the promising transgenic banana lines are being carried out at National Research Centre for Banana (NRCB), Trichy.

Transgenic research as a tool has also been demonstrated in the field of phytoremediation. To enhance the potential of plants to degrade organic pollutants, two genes namely a human cytochrome P450 and a fungal glutathione S-transferase, were introduced into tobacco, and transgenic plants were shown enhanced degradation of radiolabelled pesticides. Introduction of a fungal Zn transporter and a fungal Cu transporter into the plants led to enhanced removal of Zn and Cu, respectively from soil and solution. Further, plants expressing a Ni-Co transporter gene could take up high levels of radioactive cobalt compared to control plants. However, these transgenic plants could not be evaluated under the field conditions due to restriction on field testing of genetically modified plants, but efforts are going on to translate these findings.

3. Spinoff technologies

Developing efficient micropropagation system is a prerequisite to achieve significant progress in *in vitro* mutagenesis. At BARC, micropropagation technologies for some of the important crops were developed for their varietal improvement program. Tissue culture technologies for the vegetative propagation of commercially important horticultural crops such as banana (AB10NABTD**)**, pineapple (AB44NABTD), ginger (AB49NABTD), turmeric (AB40NABTD), and the medicinal plant *Ophiorrhiza rugosa* (AB01NABTD) were developed and transferred to several end-users. These methods have several advantages over traditional propagation approaches, including efficient generation of a large number of clonal and disease-free plantlets that can be disseminated to farmers for cultivation.

4. International/National collaborations

BARC had an Indo-FRG bilateral program during the 1980s and many BARC Scientists visited laboratories in Germany. Scientists from abroad (Russia, Cuba and Syria) were trained in the lab. The plant tissue culture lab was involved in an Oil Palm Multiplication project by Department of Biotechnology, Govt. of India (GOI) and a Cotton Mini Mission project of GOI. Under a DBT-BIRAC Indo-Australian Biotechnology project, the genetic constructs received from Queensland University of Technology, Australia,

were transferred in Indian banana cultivars for iron bio-fortification. Promising banana lines with significantly elevated iron content are being tested under controlled field trials at National Research Centre for Banana (NRCB), Trichy. Scientists with expertise in plant tissue culture participated in expert missions on *in vitro* mutagenesis in crop plants, and in several International Atomic Energy Agency (IAEA, Vienna) sponsored Coordinated Research Projects on radiation induced mutations including, an ongoing project on potato.

Collaborative work with other national research organizations and universities was a major ongoing activity, and accordingly several MoUs were signed. Productive research outcome was achieved from these collaborations. Students and faculties from all over India were trained in plant biotechnology on a regular basis.

5. Precise genome editing in plants: the way forward

Precise genome editing is the way forward for the genetic improvement of crops. As per the recent DBT guidelines, genetically modified plants, free of transgene insertion with limited editing of bases, do not fall under preview of regulations for transgenics, and can be released as mutants. With an experience of several decades in efficient regeneration and transformation systems in different crops, BARC took early initiatives on the entablement of protocols for gene editing of crops using tissue culture and CRISPR-Cas9 based systems. Recently, researchers at BARC reported editing of SNAC67 gene, a master regulator of stress-induced senescence, and its roles in salicylic acid dependent senescence induction in banana plants. Further, silencing lipoxygenase genes led to an improvement in resistance of banana plants towards *Fusarium oxysporum*. Similar efforts are going on in other important crops.

6. Acknowledgements

Generous inputs from senior ex-members of the group, including Prof. Susan Eapen, Prof. V. A. Bapat, Prof. P. Suprasanna, Prof. T. R. Ganapathi & Prof. Roja Gopalakrishnan, are sincerely acknowledged. Past and present colleagues of the Plant Biotechnology Section, NABTD, and Landscape & Cosmetics Section, A&SED, are sincerely thanked for their invaluable contributions to research programs.

FOOD IRRADIATION: HISTORICAL PERSPECTIVE AND STATUS IN INDIA

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ABSTRACT
Food irradiation has emerged as a pivotal technology in the realm of food preservation and is gaining increased importance in food and agricultural sectors. The technology uses ionizing radiations emanating from either radioactive or machine sources in facilities designed for this purpose. It can be effectively utilized for the extension of the storage life of agricultural commodities, and elimination of harmful microorganisms, insects, and parasites for mitigating risk of foodborne illnesses. It is also used to destroy quarantine pests to overcome barriers in international trade. The technology is well entrenched in the historical developments and understanding of the sources of ionizing radiation, and their biological effects including those on agricultural commodities and foods. Its commercial application for large scale public use was approved by the national and international health regulatory authorities only after carefully examining a large volume of scientific evidence vouching for the overall safety of the radiation processed food for the consumption by human being. India being one of the world's largest producers of agricultural and horticultural commodities incurs significant post-harvest related economic losses annually. Food borne illnesses also cause huge burden on Indian economy. For preventing these losses, the country needs to integrate food irradiation technology with its vast food supply chains for improving food safety and security.

1. Introduction

Radiation processing of food, also called food irradiation, is an important emerging technology for integration in country's food supply chains, for extending shelf life and improving its safety. Ionizing radiations are the most effective means to destroy harmful insect pests, parasites and microbes in food and agricultural commodities. Ionizing radiation can also be used to control certain physiological changes including ripening as well as senescence in various fruits, vegetables, and sprouting in bulb and tuber crops. The dose dependent beneficial effects of ionizing radiation do not compromise the nutritional and sensory attributes of food commodities. The commercial adoption of the technology by the industry started only after decades of scientific research, safety assessment of irradiated food, and stringent global regulatory oversight approved its use for ensuring food security, food safety, and facilitating international trade. India, being one of the world's largest producers of a diverse variety of agricultural and horticultural crops incurs significant post-harvest related economic losses annually. For preventing these losses, the country needs to integrate food irradiation technology with its food supply chains, making it an important component of its food safety and security strategies. Thus, food irradiation technology will not only help reduce post-harvest losses in agricultural and horticultural commodities, but also help their export to countries meeting stringent quality as well as quarantine standards in international trade. The active role that the government has played in promoting research and development in food irradiation, establishing regulatory framework, setting up of commercial technology demonstration units, and promoting entrepreneur development, underscores its commitment for leveraging this technology for the benefit of farmers as well as entrepreneurs, both in domestic and global markets, ultimately benefiting the public at large.

2. Historical developments

2.1. Early discovery of radiation and its potential uses

In 1895 and 1896, Rontgen and Becquerel discovered ionizing radiation X-rays, and Gamma rays, respectively. Shortly thereafter the bactericidal and therapeutic properties of these radiations were discovered. The whole idea of utilizing ionizing radiation for food preservation quickly gained importance, primarily due to its ability to penetrate deeply and effectively destroy spoilage organisms and harmful pathogens in the food. In the early year of 20^{th} century, initial patents were granted in the United Kingdom and the United States, and Germany for the using ionizing radiation for food preservation. Food irradiation was primarily a part of the global efforts to explore biological effects of ionizing radiation during first half of the twentieth century. X-rays were shown to kill eggs, larvae as well as insects, in tobacco leaves, and *Trichinella*, a parasite in pork. In 1953 the then US president Eisenhower announced his famous 'Atoms for Peace' program that gave a much-needed boost for exploring peaceful uses of ionizing radiation including food irradiation in the R&D institutions around the world. In India this journey started in 1954 under the great leadership of Dr. Homi Jehangir

Bhabha, the founder of India's Atomic Energy Program.

2.2. Start of coordinated research program

In the year 1950, Atomic Energy Commission of the United States (USAEC) launched a coordinated research program focused on using the ionizing radiation for food preservation. Under this program it started using spent fuel rods obtained from different nuclear reactors as a source of ionizing radiation. Soon it found several limitations of using spent fuel rods. These primarily included the presence of mixed radiation, including dangerous neutrons, in spent fuel. Also, precise dose delivery and dosimetry posed numerous challenges. It led to the development and use of cobalt-60, an artificially produced radioisotope from neutron irradiation of cold cobalt-59, that mainly emitted highly penetrating gamma rays of reasonable energy. Subsequently, in the early 1960's USAEC generated Cobalt-60 sources which were provided to various academic institutions in the USA including University of California at Davis, the Massachusetts Institute of Technology, Boston, and University of Florida at Gainesville. Soon after, a Cobalt-60 Marine Products Development Irradiator was developed at the National Marine Fisheries Services at Massachusetts, with an installed capacity of 235 kCi. This was followed by the development of a grain Irradiator at the Entomological Research Centre in Georgia with 35 kCi of cobalt-60. An important role was played by the United States Armed Forces in the primary stages of research in radiation processing of foods. To accomplish this, the US Army Natick Laboratories obtained a 1.3 MCi Cobalt-60 source for the purpose of food irradiation. At the same time 18-kW linear electron accelerator was also utilized for the same purpose. After 1960, as an alternative to frozen or canned military rations, the U.S. Army concentrated majorly on development of sterile meat products using high dose gamma irradiation. Similar facilities including a grain irradiator, an advanced cesium-137 based marine product irradiator, and a cobalt-60 food package irradiator, were procured by Dr. Bhabha and installed in the Food Irradiation Processing Laboratory of the Food Technology Division at BARC. Even today cobalt-60 remains the work horse of food irradiation, although the usage of X-rays and electron beam is finding favor with the modern industry.

2.3. Spread of radiation technology

Research on food irradiation was initiated in several countries including Belgium, Germany, Canada, Russia, France, The Netherlands, Poland, United Kingdom, and India after the successful demonstration of useful food applications in the United States. However, the concerns raised in certain quarters related to the wholesomeness and safety of food treated with ionizing radiations for human consumption became a major obstacle in its commercialization. Hence, marketing of gamma radiation processed foods was not permitted by the health authorities in these countries. This led to initiation of several safety and wholesomeness evaluation research programs in various laboratories around the world spanning more than a decade of seventies and eighties of the last century.

3. Establishing safety along with wholesomeness of irradiated foods

Wholesome food means food conducive to the general human nutrition and well-being. This also implies that any deleterious substance is absent in food. Therefore, wholesomeness of food treated with ionizing radiations had to be evaluated for any physical, chemical, and biological, changes affecting microbiological, toxicological, and nutritional safety. Laboratories around the world including India spent a lot of money, time and efforts in establishing that food processed by ionizing radiation was safe for human consumption. These studies included, studies on radiological safety, safety of chemical changes, safety related to the changes, if any, in residual microflora, *in vitro* studies, animal studies (short as well as long term), and finally, studies involving human volunteers, generating huge volume of well documented data.

3.1. Radiological safety

The induction of radioactivity in foods treated with ionizing radiations and risk to the environment due to the food irradiation facilities were two major radiological concerns. Experiments with high dose irradiated foods clearly demonstrated that either gamma rays originating from cobalt-60 (1.25 MeV) or X-rays with energies up to 7.5 MeV or electrons accelerated up to 10 MeV, did not induce radioactivity in the atoms of the food material. Also, as the food during processing with the ionizing radiation never comes in contact with the radio isotope, there cannot be any radioactive contamination in the treated food. Radioactive cobalt-60 is used inside sealed stainless-steel pencils from which only gamma rays emanate. When foods were irradiated by kilocurie cobalt-60 source, no radioactivity could be detected in 24 elemental food constituents when analyzed by advanced scintillation counters. It was observed that X-rays up to 7.5 MeV did not induce radioactivity in food items and hence, USA extended the usable energy of X-rays from 5 MeV to 7.5 MeV. Regarding the risk to the environment, the facilities for radiation processing that use Co-60 would need replenishment and transport every 12 years. The transport of cobalt-60 and also, the operation of facilities using cobalt-60 is conducted under a well-regulated national and international protocol, and thus never causes any risk to the population or environment around these facilities.

3.2. Safety of chemical changes

It is observed that based on the properties and storage conditions of the irradiated commodities, the free radicals formed in these foods due to irradiation disappear. The process of irradiation with the prescribed doses has a minimal effect on the basic composition of foods that cannot be easily identified by either sensory evaluation or by any advanced instrumentation. Minor losses in some radiation sensitive vitamins may be observed. Nevertheless, these losses are often within the range allowed even under conventional processing techniques.

3.3. Safety of residual microflora

There are number of detailed studies regarding microbiological aspect of irradiated foods in terms of residual microflora. All the studies confirmed that radiation processed food does not cause any hazardous changes in the residual microflora for example heritable

gene mutations, or making toxigenic or pathogenic microbes more toxigenic or virulent.

3.4. Short-term and long-term animal studies

To evaluate the wholesomeness of irradiated foods, animal feeding experiments were carried out in a number of small and large animal species in various laboratories around the world. These studies were expensive and at the same time most time consuming. In separate experiments, rats fed with freshly irradiated wheat did not induce dominant lethal mutations in these animals. In another experiment, Swiss male rats were fed with irradiated (25 kGy) and unirradiated diet for about 8 weeks and these rats were then mated with normal females. The females were tested for dominant lethal mutation in the mid-term pregnancy period. None of the females showed any dominant lethal mutation during the testing. Moreover, there was no pre as well as post implantation lethality in the rats fed on irradiated diet. Even the fertility of mice remained unchanged after consumption of irradiated diet. Wistar rats were fed with irradiated and unirradiated wheat diets within the period of 24 hours of irradiation, and the cytological analysis of the bone marrow cells in metaphase was carried out. No significant difference was observed in the frequency of polyploid cells in these Wistar rats indicating no genotoxic effect. Considering all the data of short term as well as long term animal feeding studies with various irradiated foods and number of species of laboratory animals it was concluded that there was no genotoxic effect of irradiated foods in these animals. Mutagenicity testing studies reiterated the findings. Even the human volunteers who were fed irradiated foods have shown no adverse effect, indicating safety of irradiated foods. In fact, radiation processed food is being consumed by both astronauts and cosmonauts during their space mission.

3.5. Reassurance of safety at BARC

Even though the issue of safety and wholesomeness of irradiated food is settled for all times to come, recent studies at FTD, BARC continue to inspire confidence. In these studies, two gene loci, tk-/+ (thymidine kinase), and hprt+ (Hypoxanthine Phosphoribosyl transferase) of human lymphoblast thymidine kinase heterozygote (TK6) cell lines were sub-cultured for 100 generations in various foods irradiated to a high dose of 25 kGy. Similarly, subculturing of *E. coli* MG1655 (wild type) cells for 3000 generations was carried out in a medium comprising of irradiated food. Various analysis including comet assay, micronucleus test, Ame's test as well as DNA sequencing along with restriction digestion profiling of phagemid DNA present in *E. coli* cells that were grown in medium containing food treated with gamma radiation were also carried out. Results indicated no induction of mutagenesis during long-term subculturing in medium having food treated with ionizing radiation. No changes were observed in the profiles of micronucleus as well as comet in the sub-cultured cells. There was no difference in the restriction digestion profile and randomly amplified polymorphic DNA in these cells. DNA sequences also showed no changes confirming non-occurrence of silent mutation in the genome. Further, studies were done using high dose (10-70 kGy) irradiated goat meat, chicken, fish and shrimp samples and enumerating mutation frequency after long-term sub-culturing of *E. coli* MG1655 cells till 1500 generations in

the media containing 1% irradiated food and till 250 generations in the media containing 5and 10% irradiated food. Human lymphoblast cells (TK6) cells were grown till 156 generations in the medium containing 2% irradiated food. Another study used 'Differential loss of Plasmid Antibiotic Resistance' (DPAR) assay and subsequently analyzing the sequence of tetracycline resistance gene of pBR322 plasmid that was isolated from the *E. coli* cells cultured on irradiated food medium for 1500 generations. Same cells were subjected to RAPD analysis of the entire genome. All these studies showed no induction of any mutation and no change in nucleotide sequence suggesting non-existence of silent mutations. Thus, the above studies provided strong evidence of absence of any mutagenic effect due to high dose gamma irradiated foods, and thus endorsed their genotoxic safety and highly credible molecular evidence supporting the safety of food treated with ionizing radiations.

3.6. Investigations in human volunteers

In China, number of studies on healthy human volunteers were carried out at Shanghai Institute of Nuclear Research. The studies included diets including about 70 food items. These food items were irradiated using a Cobalt-60 irradiation source and the radiation doses used were from 1 kGy to 8 kGy. After irradiation, the food products were stored for different time period ranging from one week to 6 months. Staple food items including flour and rice were stored for about 50 and 180 days, respectively. Unirradiated or irradiated diets were fed to the volunteers for the period of 90 days. Peripheral blood lymphocytes from these volunteers were used for chromosomal preparation to score numerical as well as structural aberrations. No significant differences were observed in the occurrence of polyploid cells in the volunteers fed with either unirradiated or irradiated diets, both before or after feeding.

4. Contributions of IAEA, FAO & WHO

4.1. Joint FAO/IAEA/WHO Expert Committee on Food Irradiation

In the year 1970, two agencies namely, International Atomic Energy Agency (IAEA), Vienna, and the Food and Agriculture Organization (FAO), Rome came together to form an International Food Irradiation (FIP). The project initially involved 19 countries with the project headquarter at Karlsruhe, Germany. Five more countries joined this group later. Subsequently, The World Health Organization (WHO), Geneva also joined this project. Various food items including rice, wheat, fruits, spices, meat and fish were irradiated with gamma radiation and evaluated for chemical changes as well as used for animal feeding studies. The Joint FAO/IAEA/WHO Expert Committee on Food Irradiation (JECFI) convened series of meetings in the year 1970 and 1976 for analyzing the emerging data, and evaluation of wholesomeness and safety. In the last meeting in the year 1980, the committee inferred that the foods irradiated at an overall average dose of 10 kGy does not pose nutritional or microbiological hazards. They further confirmed that food commodity irradiated up to average dose of 10 kGy does not require any toxicological testing as no toxicity is generated at this dose. Based on the findings of

JECFI, in the year 1981, the World Health Organization published a document titled "Wholesomeness of Irradiated Foods" in which the organization reiterated the above claims.

4.2. International Consultative Group on Food Irradiation (ICGFI)

In 1984 the three UN agencies (FAO, IAEA and WHO), and the 19 founding member states signed a declaration in 1983, establishing ICGFI with the major objective to assess global advances in the field of food irradiation and to serve as a central source of guidance on the various application of food irradiation for IAEA member countries. Its top priority was to promote public awareness about food irradiation and objectively discuss the process through publications addressing its safety and effectiveness. It also focused on different aspects required to commercialize food irradiation as well as legislative aspects of irradiation facilities. Additionally, emphasis was also given on the process of organizing training courses for the persons involved in the plant operations, food inspectors and other key stakeholders and regulatory control of these facilities. In the year 1995, the total number of ICGFI member states increased to 44.

5. Major applications of food irradiation

5.1. For ensuring food security

Major technological benefits of radiation processing that can help control food losses include, sprout inhibition in bulbs, tubers, and rhizomes, insect disinfestation in grains and grain products, delay in the process of ripening as well as senescence in fruits and vegetables, and destruction of microorganisms causing spoilage of foods. These benefits can be classified into low, medium, and high dose applications, based on the specific dose requirements. These objectives are fulfilled using recommended doses ranging from 0.1 to 10 kGy, for different classes of foods. India houses one of the major domestic markets of the world with vast amounts of fruits, vegetables, cereals, pulses, spices and seafoods are acquired, and distributed throughout the country after the storage for the variable period. Due to improper storage conditions, grains of the value of crores of rupees are lost due to damage caused by insect infestation and associated issues. Radiation processing offers a solution for storing the bulk and consumer-packed products, aiding in retail marketing and stockpiling. In order to control the huge losses if India wishes to process even a small portion of the total production using radiation, numerous new facilities would be required in the future.

5.2. For improving food safety

There are several studies in India documenting incidences of food-borne pathogens in different food samples. Prevalence of Salmonella in various food samples, mainly poultry meat and seafood. Salmonella load in raw poultry is a cause of major concern to the regulators around the world including India. The incidence of Campylobacter with seasonal variation in food has also been documented. Other important pathogens studied in great detail in food included, Yersinia, Listeria and E. coli. Apart from the major food-borne pathogens, incidence of many emerging pathogens like Cronobacter, Enterobacter,

Klebsiella, Shigella and Aeromonas. Radiation processing can be deployed as one of the critical steps in Hazard Analysis and Critical Control Points (HACCP) in food industry along with good manufacturing practices (GMP) and good agriculture practices (GAP). Rapid detection of pathogens enables quality control professionals to meet quality standards. The traditional detection methods for microbiological analysis are time intensive and tedious. In FTD, approaches based on PCR have been developed for detection of Salmonella, Aeromonas and E. coli. These methods can detect even a single bacterium in food after a short period enrichment. Escherichia coli is a fecal indicator, among which some of the virotypes are pathogenic, making its detection and prevention in food crucial. Newer methods developed recently focus mainly on detection of pathogenic E. coli. In these studies, a certain fraction (7.3%) of E. coli isolates tested positive for different virulence genes including aggR, lt, stx1, eaeA, ipaH, and stx2. Among others 2 isolates showed presence of toxicity marker stx genes.

The radiation dose required to eliminate population of the given pathogen, called 'radicidation' dose, has been determined for all important food-borne pathogens in FTD. The earliest work was on Vibrio and Salmonella. The decimal reduction dose, D10, was determined for Salmonella Typhimurium, Listeria monocytogenes and Aeromonas inoculated in a variety of minimally processed fruits and vegetables. Gamma radiation has been shown to effectively kill various human pathogens in food products, yet its efficacy varies depending on the pathogen, its growth form, stage, and the nature of the attachment surface. For Shigella spp. and Aeromonas spp., gamma radiation demonstrated no significant difference in sensitivity between planktonic and glass-associated biofilm cells, but a significant increase in resistance was noted for carrot-associated biofilms. Klebsiella pneumoniae, an opportunistic pathogen, was completely eliminated from mixed sprouts, poultry, and fish samples with a 1.5 kGy dose of gamma radiation, demonstrating no recovery during storage. Similarly, a 1 kGy dose effectively eradicated Campylobacter from poultry meat, ensuring its safety. Additionally, gamma radiation improved the microbiological quality of minimally processed pineapple by eliminating Salmonella Typhimurium, thus highlighting the prospects of radiation treatment in ensuring food safety, especially in food for immunocompromised patients.

5.3. Meeting phytosanitary requirements

A Final Rule pertaining to the 'Irradiation Phytosanitary Treatment for Imported Fruits and Vegetables' was issued by The Animal and Plant Health Inspection Service (APHIS) of the USDA in the year 2003. At the same time, India, along with New Zealand and Australia also amended their quarantine regulations to include radiation processing as a quarantine treatment for processing the tropical fruits meant for export. World Trade Organization (WTO) has issued clear incentives to the traders who adopted radiation processing as an SPS measure in the international trade through the Agreements on Sanitary and Phytosanitary Practices and Technical Barriers to Trade. Thus, in the global trade, radiation can be used for the hygienization of the product as well as for overcoming the quarantine barrier. International organizations such as International Plant Protection Convention and Codex Alimentarius Commission administer the agreements under their

standards and recommendations. Thus, WTO member states would experience improvement in international trade of horticultural produce using radiation processing. For export purpose, the food can undergo radiation processing mainly to achieve hygienization, shelf life extension and overcoming quarantine barrier. Globally due to extended shelf life, trade in fresh agricultural commodities is increasing steadily among the countries. India exports various food commodities including onion, Basmati rice, spices, meat, poultry and seafood. Radiation processing can benefit the export of bulk commodities by restructuring the cost and it can also assist in selling the value-added packed commodities in the retail market directly.

6. International regulatory approvals

In 1958 the Soviet Union was the first country to grant clearance to potatoes for sprout inhibition, and later to disinfestation of grains treated with ionizing radiations for human consumption. In 1960 Canada granted clearance for sprout inhibition in potatoes and later to irradiation of onions. USFDA too granted approval in 1963 for the processing of wheat and wheat products for insect disinfestations. In 1984 the Codex Alimentarius, under the umbrella of FAO and WHO, published the 'Codex General Standard for Food treated with ionizing radiations and recommended International Code of Practice for the Operation of Radiation Facilities'. Codex reiterated that the foods irradiated up to 10 kGy average dose presents no microbiological as well as nutritional issues. It also identified usable ionizing radiation sources, provided dose and energy limit guidelines as well as global GMP standards recommendations for operating gamma irradiation facility. Both USA and Canada recorded gamma irradiation under their respective legislations that regulated additives in food. Later in the year 1989, irradiation was recategorized as a physical process by Health Canada whereas, USFDA continues with its earlier stand

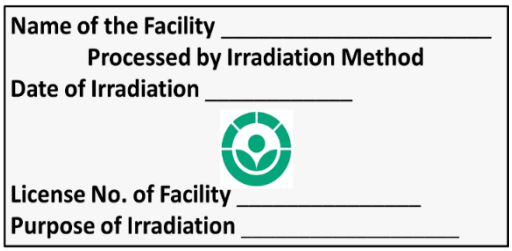
6.1. Labelling of food treated with ionizing radiation

The JECFI also suggested that there was no valid scientific reason for identifying the food treated with ionizing radiations with a label at the retail level when similar labelling is not required for the other commonly used processing methods (WHO, 1981). However, The Codex Alimentarius Commission wanted that the Codex member states follow uniformity in labelling for the facilitation of international trade. The labelling committee recommended that usage of an international logo or '*Radura*' can be optional, it should have a statement 'treated with ionizing radiation'. In most of the countries the irradiated food that is sold in prepacked or bulk form is easily identified by the logo of *'Radura'*. The package should contain one of the statements including 'Irradiated', 'Treated by Irradiation', 'Treated with Irradiation'. Additional statements explaining the benefits of irradiation may also be used. The idea of using a label is to provide consumers the choice of selection. The '*Radura*' logo is a symbol of quality rather than warning. On the ingredients list, if the finished product contains irradiated ingredients $\geq$ 10%, then the product has to be described as "irradiated". The finished products containing < 10% irradiated ingredients or spices, do not have labelling compulsion. As per the Indian regulations, the foods treated with ionizing radiation must

have a label with the treatment name written close to the product name. Moreover, the label of radiation processed food should have 'RADURA' symbol in green color as given below:

7. Food Irradiation in India

In India, R&D in nuclear sciences started in 1954 at the Atomic Energy Establishment,



Trombay, which was subsequently called Bhabha Atomic Research Centre (BARC) after the name of the founder. In 1961 research in radiobiology was initiated. In 1967, a first of its kind in this part of the world, a Food Package Irradiator (FPI) was installed for carrying out the R&D in food irradiation at the Food Irradiation and Processing Laboratory, at the Food Technology Division, BARC. For according regulatory approval for irradiated foods, a National Monitoring Agency (NMA) was established by the Government of India in the year 1987 for considering different aspects of food irradiation. In 1992, the committee approved irradiation of onion and potato for sprout inhibition, spices for hygienization while frozen seafood for export. Subsequently, under the Prevention of Food Adulteration Act (PFA), Rules (1954), the proposal was draft notified. In the year 1994, the draft notification for both export as well as domestic consumption was approved and published vide GSR No. 614(E) dated August 9, 1994. In 2003, quarantine regulations were amended by the Ministry of Agriculture in order to include radiation processing as a quarantine measure to facilitate international trade. In 2006 a framework equivalence work plan was signed between USDA-APHIS and MOA (GOI) for export of mangoes. This enabled export of about 150 tons of irradiated mangoes to the USA in the year 2007 from the KRUSHAK irradiation facility, a technology demonstration unit at Lasalgaon, dist. Nashik, which got approval from USDA-APHIS that year. In June 2012, a notification for class-wise approval for radiation processing of food items was issued vide G.S.R 158 dated June 26, 2012 under Atomic Energy (Radiation Processing of Food & Allied Products) Rules, 2012. In 2016, a class wise approval for irradiation of foods was gazette notified by the Government of India under the Food Safety and Standards (Food Products Standards and Food Additives) Sixth Amendment Regulations, 2016. The regulations stress on: -(a) Approval of facilities - No irradiation facility shall be used for the treatment of food unless such facility -has been approved and licensed under the Atomic Energy (Radiation Processing of Food and Allied Products) Rules, 2012; complies with the conditions for approval,

operation, license and process control prescribed under the above said rule; and carries out irradiation in accordance with the provisions of this rule. -(b) Besides irradiated food will not exit the irradiation plant if it has not been irradiated as per the requirements of this rule and the certificate of irradiation displaying both the dose as well as purpose of irradiation is furnished by the irradiation plant. The irradiation will strictly adhere to the dose limits, the approved radiation source, and the specified conditions for every category of food for processing by radiation, under the Atomic Energy (Radiation Processing of Food and Allied Products) Rules, 2012.

8. Conclusion

Early discoveries of ionizing radiations and their properties set the stage for their exploitation in the fields of medicine, food, and industry, with pioneering research in the United States. The establishment of international bodies such as the IAEA, FAO, and WHO provided a structured approach to evaluating and standardizing food irradiation practices, ensuring that the technology is both safe and effective. The checkered history of food irradiation ultimately reflects convergence of international cooperation in achieving common R&D goals, establishing safety and wholesomeness of irradiated food, and preparing and harmonizing international regulatory frameworks and SOPs. The country's progress from early research and development initiatives to developing a comprehensive regulatory framework demonstrates its commitment to reducing food spoilage, providing safe food to consumers, and meeting international trade standards. The establishment of rigorous safety protocols, combined with advancements in detecting foodborne pathogens and assessing the genotoxic safety of irradiated foods, underscores India’s proactive stance in ensuring both the quality and safety of irradiated food products in the country.

9. Acknowledgement

Endeavours and contributions from the former and current colleagues of Food Technology Division towards the progress and advancements made in the food irradiation program is highly acknowledged.

APPLICATIONS AND COMMERCIAL DEPLOYMENT OF FOOD IRRADIATION TECHNOLOGY IN INDIA

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Abstract

India produces enough food grains annually for its growing population, but also incurs significant post-harvest storage losses. Food security and safety are the twin challenges that face the country's food and agricultural sector. These challenges demand modern technological interventions. Radiation processing being a physical process offers an eco-friendly and effective alternative. Bhabha Atomic Research Centre (BARC) has a comprehensive research programme in the domain of food and agriculture. R&D work pertaining to the utilization of ionizing radiation for shelf life extension of agricultural produce and allied food products has been extensively carried out for last 7 decades. Protocols for shelf-life extension have been standardized and effectively demonstrated in several food and agricultural commodities. Commercial deployment of the technology for diverse applications is now feasible. Multiple value-added benefits include reducing post-harvest losses, pest-disinfestation, microbial hygienization, phytosanitary treatment to meet export requirements, as well as shelf-life extension of ready-to-eat/ ready-to-cook processed food commodities. Large scale trials with potato and onion have been successfully demonstrated to the

concerned stakeholders. The export of Indian mangoes to the USA is made feasible using irradiation as a phytosanitary treatment. Protocols for treatment of other exportable fruits and vegetables are also worked out. Industry driven research and deployment of the technology in public-private partnership (PPP) mode is essential for accruing widespread societal benefits. It is essential to understand the key challenges and hurdles faced by the stakeholders in the adoption and propagation of the radiation technology at commercial level. A coherent and concerted approach involving various stakeholders is required for the wider adoption and dissemination of radiation technology for improving food security and safety

1. Introduction

Food irradiation is an effective food preservation modality. It is being practiced in more than 70 countries world-wide including India. Indigenous ability and expertise in cobalt-60 production in nuclear power reactors ensures its regular supply and sustainability for the food irradiation program in India. Growing awareness among the stakeholders has resulted in a steady rise in the number of food irradiation plants being commissioned in the country. Standard operating procedures including proper dose delivery, pre- and post-harvest storage and transport conditions, have now been developed in FTD for a broad range of food products. A concept of lab to land has also been developed for public outreach of the food irradiation program in the country. Under this concept, initially a semi-commercial scale trial in the tune of 15 to 30 tons of potato and onion was successfully accomplished with a private agency through Expression of Interest (EoI). Subsequently commercial trials with approx. 1200 tons of onion for long-term preservation have been successfully demonstrated along with NCCF and Department of Consumer Affairs. Several fruit products preserved through irradiation are also being marketed through the technology licensees further highlighting the importance and acceptance.

2. Applications of Radiation Processing

2.1. Sprout inhibition in bulb and tubers

Sprouting is a natural process by which tubers, bulbs, and rhizomes germinate under favorable environmental condition to give rise to shoots for growth. In general, it does not initiate immediately after harvest but starts after a certain duration termed as the dormancy period. The dormancy period, varies in different crops and may continue for several weeks or even months. The sprouting is suppressed during dormancy by the expression of a hormone, abscisic acid. After dormancy period, depending on the environmental conditions including relative humidity, temperature and light exposure the sprouting initiates. The level of expression of phytohormones such as cytokinins and gibberellins play important roles in breaking the dormancy and promote sprouting. Sprouting during storage is considered as one of the most obvious manifestations of

quality deterioration. This is accompanied by associated issues including weight loss, reduction in nutritive value, bulb softening and loss of processing qualities. Ionizing radiation mediated Inhibition of sprouting in stored tubers, bulbs, and rhizomes isone of the important low dose applications of radiation processing. The radiation treatment during dormancy period at very low doses (<100 Gy) leads to inhibition of sprouting in onion, potato, garlic, ginger, and shallots. The mechanism of radiation-induced inhibition of sprouting was thought to be due to an effect on the metabolism of endogenous growth hormones as well as on nucleic acids. Gamma radiation was believed to inactivate the meristematic tissue and inhibit cell division (mitosis) in buds. It was also believed that irradiation suppresses nucleic acid synthesis in the meristem (as a result of suppression of oxidative phosphorylation and ATP synthesis. However, recent studies in a commercial trial on Indian potatoes have shown that genes responsible for the biosynthesis of abscisic acid were up-regulated and those related to its catabolism were observed to be down-regulated in radiation processed potatoes. Additionally, the genes implicated in the biosynthesis of phytohormone auxin were significantly down-regulated in radiation treated potatoes. On the contrary, irradiated potatoes displayed retention of processing quality attributes such as cooking and chip-making qualities, which was believed to be due to enhanced expression of invertase inhibitor. In another recent study in onion, the prime odor volatiles known for pungency and total soluble phenolics were reported to increase in irradiated onion during storage. Inclusion of radiation processing at commercial level may gradually replace the use of commonly utilized chemical sprout inhibitors like isopropyl N-(3-chlorophenyl) carbamate (CIPC) and maleic hydrazide (MH) which are known to leave chemical residue in the commodity that are harmful to health as well as environment.

2.2. Insect disinfestation

Cereal crops serve as the staple food for a large section of population around the globe, providing macronutrients, micronutrients, and functional ingredients. Cereals and legumes constitute a major source of carbohydrates and proteins, in the Indian vegetarian diet. Cereals are rich in sulphur containing amino acids and deficient in lysine, while the reverse is true for legumes. Hence, a proper mixture of cereals and legumes is important. Moreover, legumes are cheaper source of protein compared to animal proteins. It is therefore important to maximize availability of both cereals and legumes in the country as a part of the food security strategy. However, insect infestation of these commodities during storage and transport results in significant losses and quality deterioration. Radiation technology can help increase their shelf life and improve their quality.

Rice, wheat and wheat, the most commonly consumed cereals in India, have a shelf life of 3-4 months. Infestation and proliferation of insects of *Coleoptera* and *Lepidoptera* species are very common. Fumigation with fumigants like ethylene dibromide, phosphine and methyl bromide is used even by the large warehousing agencies. However, fumigation does not eliminate all the metamorphic stages of insects and thus requires repeated treatments, resulting in toxic residue built-up in the products. Fumigants are being gradually phased out worldwide owing to their environmental and health concerns,

thus highlighting the necessity of deploying radiation technology. Moreover, pre-packed products are not amenable to fumigation. On the contrary, irradiation of packaged food commodities serves as a green, safer and an end-to-end solution.

Radiation processing at a low dose (<1 kGy) is efficient in destroying all metamorphic stages of insects and sterilizing the adults of many known species of granary insects, thus preventing their reproduction and proliferation. Owing to high penetration of the gamma rays, the radiation treatment can effectively be used to disinfest pre-packed commodity. The process does not affect the wholesomeness, nutritional quality, and chapati making or cooking quality of grains (**Fig. 1A, B**). The shelf life of these products can be more than 12 months. The treatment in wheat improved the functional attributes by decreasing the gelatinization with concomitant increase in water absorption and better extensibility. The specific loaf volume wasF significantly higher in breads prepared by straight dough lean formula from 'maida' prepared from irradiated wheat. Sensory acceptability was higher for bread as well as chapatis made from irradiated wheat. Wheat, rice, rava and milled products including whole wheat flour (*Atta* and *Maida*) are also approved for radiation preservation under the FSSR (Food Product Standards and Food Safety Standards) Regulations (FSSAI, 2016).

A

Non-irradiated 'Chana Dal'

Irradiated (D_{min} 650 Gy) 'Chana Dal'

B

Non-irradiated 'Wheat'

Irradiated (D_{min} 650 Gy) 'Wheat'

Fig. 1: Insect disinfestation by radiation processing in (A) Chana dal and (B) Wheat

2.3. Nutritional quality improvement of grains

Flatulence-producing oligosaccharides, stachyose and verbascose were observed to undergo degradation at varying rates during 0-4 days of germination with associated accumulation of sugars (which are relatively more metabolizable) n gamma irradiated (<1 kGy) legumes *viz*. mung, Bengal gram, horse beans (val), horse gram, cowpeas and rajma. Thus, radiation treatment (1 kGy) of the ragi (Finger Millet) prior to malting processes reduced further viscosity which helped in preparation of weaning foods with higher malt concentration causing increase in its energy density. High dose irradiation of soybean improved its functional properties by increasing protein solubility and improving emulsification and gelling properties. Reduction of beany flavor in the tofu prepared from irradiated soybean increased the acceptability of patties prepared by incorporating the soy tofu. The functionality of the legumes (chickpea and kidney beans) in terms of cooking time was also improved at higher dose (10 kGy) without affecting their antioxidant properties.

Gluten free (GF) multi grain flour

Gluten is a structural protein commonly found in cereals including wheat, barley & rye. It is responsible for the dough viscoelastic properties, which is essential for the quality of the end product. Although a lot of benefits are associated with gluten, however, in certain individuals it may trigger an unwanted immune response called Celiac disease (CD). CD is a chronic inflammatory autoimmune disease that is known to occur in genetically predisposed individuals owing to an immune response to the protein gluten. CD is a multi-system disorder, where some of its clinical manifestations include diarrhea, weight loss, mal-absorption, abdominal pain, bloating, anorexia and vomiting. In view of this, ICMR has recommended the gluten free (GF) diet for the management of complications associated with celiac disease (CD). Developing new gluten free food products often comes with various bottlenecks such as cost, availability, texture-taste, palatability and dietary compliance. Therefore, with increased incidence of CD, short fall in the dietary fibre consumption and rising demand for multi grain food commodities in India, technology for 'Gluten free (GF) multi grain premix' has been developed at BARC using irradiated dietary fibre and multigrain.

In the present technology, gamma radiation processing as a cost-effective tool (with an ease for bulk processing of the material) to bring these desired properties. Thereby, increasing the fibre fortification levels in GF food products. In addition to this, the combinations of grains constituting the multi grain premix for the present deployed technology were judicially selected not only for its low cost but also for better nutritional and sensory aspects. Currently, this product is being marketed by the technology licensee.

2.4. Microbial decontamination

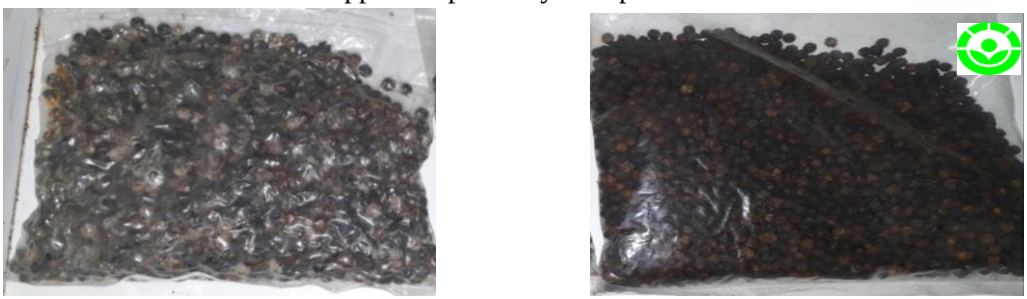
Legume sprouts and sweet corn kernels are nutritious foods and consumed for health benefits, however, being high (>50%) moisture commodities, making the produce unsafe for consumption due to high microbial load. Combination treatment involving sonication / blanching, antioxidant dip and gamma irradiation ensured microbial safety of these

processed products and also led to enhanced shelf life of mung, chickpea and sweet corn for 35 days and lucerne up to 21 days at 4-6°C. During storage, various quality parameters including physical, biochemical, nutritional & organoleptic attributes were found to be retained. Interestingly, anti-nutritional factors (phytate, trypsin inhibitors, cyanogenic glycosides &oxalate) were observed to be significantly reduced.

2.5. Spices and dehydrated products

In general, most spices get heavily contaminated during sun drying with microbes including pathogenic bacteria of public health concern including Salmonella, *Escherichia coli*, *Clostridium perfringens*, *Bacillus cereus*, &toxigenic molds. Mold spores grow under humid storage leading to formation of mycotoxins. Intra and inter country (Japan) transportation studies, confirmed quality retention in irradiated spices during transport & storage. Fumigation of spices with methyl bromide and ethylene oxide leaves carcinogenic residues. Recently, export consignments of fumigated spices have been rejected and returned by importing countries. Moreover, fumigants deplete atmospheric ozone layer. Hence, radiation processing is the most effective alternative for microbial decontamination of the spices. Gamma irradiation in the dose range of 6-14 kGy is required for microbial decontamination and extension of shelf life of these products (**Fig. 2**).

The first commercial-scale gamma irradiator for food processing in India was the Radiation Processing Plant, BRIT, Vashi (RPP) which was commissioned on January 1, 2000. It is ISO 9001:2015, ISO 22000:2018 and ISO 13485:2016 certified facility which is also enlisted in the list of approved plants by European Union.



Non-irradiated Black pepper **Irradiated Black pepper**

Fig. 2: Microbial decontamination of black pepper by gamma irradiation.

2.6. Overcoming quarantine barriers

Radiation processing was included as a phytosanitary measure through a regulation ‘Plant Quarantine (Regulation of Import into India) Order, 2003 notified by the Ministry of Agriculture & Co-operation, Government of India in the year 2004. This inclusion facilitated the signing of a Frame Work Equivalence Work Plan agreement in 2006 between United States Department of Agriculture-Animal and Plant Health Inspection Services (USDA-APHIS) and the Ministry of Agriculture & Co-operation, Government of India, which resulted in use of radiation as a phytosanitary treatment of mango for

export to USA. Radiation processed mangos were exported to four countries (USA, Australia, South Africa and Malaysia). Quantum of mango exported in 2024 through air-route is approx. 3000 Tons. Besides, through sea-route also a trial commercial shipment containing 16 Ton of Kesar mangoes, processed as per the BARC developed protocol, was successfully shipped to USA in 2022. Upon arrival at port in USA, the mangoes were found in excellent physical condition by the USDA-APHIS, US-FDA & US-custom's and border protection force. This mode of shipment through the sea-route is advantageous as it is cost-effective and more quantum of processed mangoes can be exported, and the said technology has been advertised at the BARC website for commercial deployment.

3. Value Addition

3.1. Ayurvedic and medical products

Radiation processing of ayurvedic herbs, formulations, and medicines ensures their microbial safety assurance without affecting their functional properties. Most studies indicated a dose of ≤10 kGy to be sufficient in eliminating most microbes. However, for the complete microbial hygienization a dose of ≤ 25 kGy is required. Health foods, dietary supplements and nutraceuticals as well as body care and cleansing products can also be radiation processed.

Radiation is also effective in sterilizing packaging material used in aseptic processing of foods and pharmaceuticals. Radiation sterilization (at a dose of 25 kGy) is being used to sterilize medical products and their packaging, both thermoplastics and thermosets.

3.2. Guar gum

Dietary fibre is a nutritive component considered quite important for human health. Among these, guar gum (polygalactomannan derivative) is a promising soluble dietary fibre obtained from seeds of *Cyamopsis tetragonalobus*. Its molecular weight is from 2000 to 3000 kDa. It is widely used in various food products as thickener. Even at low concentrations, it can have very high viscosity in aqueous solutions. Further, for guar gum de-polymerization, gamma radiation is found to be advantageous alternative method.

3.3. Animal feeds

Animal feeds are also prone to insect infestation resulting in their short storage life, besides being a source of diverse pathogenic microbes. Gamma radiation is an effective alternative to heat or ethylene oxide. Treatment ease of packaged products prevents recontamination during transport and storage. Exposure to doses ≤ 10 kGy is effective in ensuring this. Animal feeds are also irradiated for insect disinfestations for meeting quarantine requirement for export to various countries.

3.4. Cut flowers

Fresh cut flowers require quarantine treatment for export/import. Methyl bromide is used for disinfestation of cut flowers, however, irradiation is a superior and effective alternative. A combination of modified atmosphere packaging (MAP) and irradiation

followed by storage at 5 to 15°C is very effective in extending shelf life of cut flowers while retaining its freshness.

4. Flesh foods

Fish, meat and their products though important for a healthy diet, but these are highly vulnerable to microbial spoilage that leads to quality losses during post-mortem storage. Therefore, it is important that proper preservation approaches are incorporated to such products to ensure their microbial safety with significant shelf-life extension.

In our country, in general, meat and fish are sold either fresh or in frozen forms. However, fresh meat & fish have very short shelf life. Frozen flesh food is expensive, and has poor texture, and consumer acceptability. India is major exporter of frozen fish, buffalo and goat meat. However, if these products could be exported in the chilled state it would not only save energy but also increase their export potential. In this context, radiation processing offers a cost-effective alternative to the currently practiced preservation methods that can make this possible. Our studies have shown that fresh buffalo and goat meat could be stored at 0-3°C for 30 days when irradiated at 4 kGy, while the non-irradiated samples are spoiled within 3-6 days. Irradiated meat was organoleptically acceptable and microbiologically safe. Diverse range of ready-to-cook meat products (mutton mince, chicken mince, chicken chunks, and chicken legs) are available in Indian supermarkets. However, they have limited market due to their short shelf life. Faecal coliforms were eliminated by irradiation treatment. When these samples were irradiated (2.5 kGy), followed by cold storage(0-3°C) they were safe for consumption up to twenty-one days. Irradiated samples had relatively lower counts of *Staphylococcus* spp and the organoleptic quality was found to be retained in these irradiated samples stored at chilled temperatures. Similarly, whole fresh & marine water fish under chilled condition showed an increased shelf-life from 9 to 15 days in whole fish varieties (Silver Pomfret, Indian Oil Sardine, Gold-spot Mullet) when processed with 4 kGy and stored at 4 °C. Further shelf-life extension of 20-25 days was obtained when whole fish varieties, Indian Mackerel, Seer fish and Hilsa, were irradiated 4 kGy and stored at 1 °C. Radiation processing and chilled storage of processed fishery products such as dispersion coated steaks from Seer and Rohu fish resulted in shelf-life extension from 5-10 days as compared to un-irradiated fish steaks which had only 1 day of shelf-life. Radiation processed whole fish and processed fish steaks resulted in significant reduction in microbial counts thus extending shelf-life of the products and acceptability on the basis of its biochemical and organoleptic quality during entire storage period (**Fig. 3**).

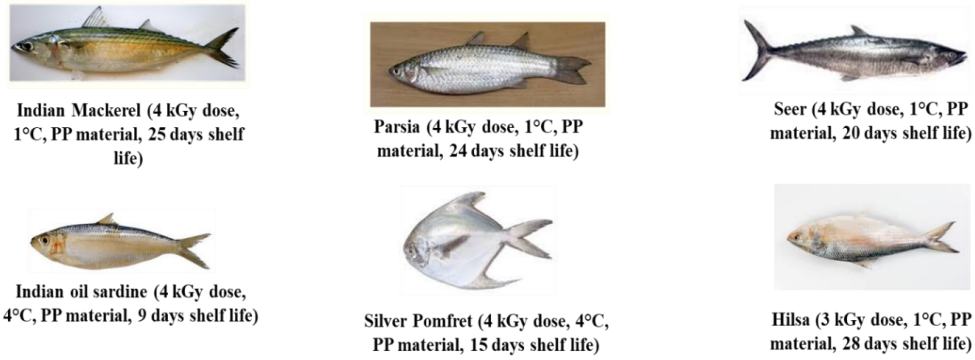


Fig. 3: Preservation of packaged fish samples for 20-25 days using radiation processing (3-4 kGy) and cold storage (1 ± 0.5°C).

A successful market trial of whole Hilsa, mackerel, and seer fish was conducted at low temperature irradiator at the Board of Radiation and Isotope Technology (BRIT), Vashi, Navi Mumbai. Effect of radiation processing on the shelf-life and microbial safety of some ethnic Indian processed meat products during chilled storage was also investigated. Besides, ready to eat shelf stable processed meat products which could be stored at ambient temperature for 1 year were also developed using high dose of irradiation (25 kGy). Additionally, various value-added fish products including Intermediate-Moisture (IM) Shrimp, were developed using radiation processing. These products were amenable to storage at either chilled or ambient temperature. Value addition of fish is done in order to utilize the valuable fishery resources sustainably and reduce post-harvest losses.

5. Relief food

There is a demand for nutritionally adequate and microbiologically safe shelf-stable food products which can be deployed during relief operations under adverse conditions including natural calamities. A product 'Stuffed Baked Food (SBF)' has been developed using radiation technology, which is stable for 8 months at ambient temperature. The product was well acceptable after storage while retaining its quality attributes. SBF can also be useful for others like defence personnel, school lunch programme, expeditions, and astronauts. This ready-to-eat (RTE) meal was supplied through a technology licensee to National Disaster Response Force (NDRF) as well as to the natural calamity affected people at Kullu district of Himachal Pradesh.

6. Processed fruit products

Syzygium cumini ('Jamun') is a highly perishable and seasonal fruit, limiting its availability and trade. A shelf-stable, chemical preservative & additive free, microbiologically safe product has been developed through the utilization of radiation

processing. This technology has already been advertised and the product is being marketed by a licensee on various online and offline channels.

Strawberry is a seasonal, non-climacteric fruit which is prone to rapid spoilage and microbial growth. A secondary product using strawberry pulp has been developed incorporating a a sweetener as well as an anti-browning agent to reduce the extent of drying induced sourness and discoloration, respectively. The packed and radiation processed (≤ 5 kGy) product (strawberry roll) is shelf stable for 9 months under ambient conditions. Physical, biochemical, functional (antioxidant and antimutagenic) and organoleptic properties (including flavor compounds) are either retained or even enhanced in the processed product even after storage. The product has been commercially available on both online and offline mode.

Processing of fresh fruits (mango, banana, papaya, pineapple, and apple cubes) into intermediate moisture (IM) a ready-to-eat (RTE) product is a suitable option to control their post- harvest losses as these products are shelf stable, retain nutritional value, convenient to use, and can be stored at ambient temperature. Intermediate moisture foods (IMF) are defined as shelf-stable products having water activities of 0.6-0.84 and moisture content in range from 15- 40% & are edible without subjecting to rehydration. The peeled and diced fruits of uniform size are blanched, osmotically dehydrated using concentrated sugar (> 30%) solution bringing down the water activity to about 0.9. Further dried either by hot air, infrared, or food dehydrators (around 60°C) to 35-40 % moisture gave a slightly firm and chewy texture. After drying the cubes are cooled down to ambient condition to prevent condensation inside the packaging. The packed fruit cubes in airtight containers or vacuum-sealed bags can maintain their moisture content and freshness. Since such fruit cubes with 0.72 water activity cannot prevention growth of fungus, the further microbial decontamination by gamma irradiation is recommended for shelf-life extension of IM fruit cubes. Gamma radiation dose range of 2-5 kGy was used for microbial decontamination of intermediate moisture (IM) fruits. Processing ensured microbiological safety, nutritionally adequacy, as well as organoleptic acceptability. The product prepared is of high calorie value on dry weight basis compared to the unprocessed fresh fruit. The products are safe for consumption and can be stored at ambient temperature storage condition for more than 6 months.

7. Large-Scale Trials

7.1. Onion

Onion grown during *rabi* season is amenable to storage as compared to Kharif variety. However, onion prices rise significantly when the onion stocks of rabi variety get depleted and arrival of kharif variety is awaited. A seasonal trend is noted for onion prices which usually peak during the lean period spanning from July-October and later display a dip during April & May. Therefore, technological interventions specifically in the onion sector are very much essential not only in ensuring a sustained supply of quality produce during the lean period but also in effectively controlling the price

fluctuation to a greater extent. There are several interlinked factors that affect the long-term storability of onions. Primarily, high humidity coupled with tropical high temperatures serve as deteriorating factors for the stored onions resulting in weight loss (~35-40%), fungal contamination & rotting, and sprouting. Onions that are kept in routine and traditional storage facilities are vulnerable to this spoilage affecting their long-term extended preservation.

Through the R&D efforts at the BARC, a SOP has been developed that maintains the overall quality of onions stored for extended duration (7.5 months). Through an integrated approach involving radiation technology and specific storage conditions, radiation processed (Dose: D_{min} 60 Gy) onion can be practically stored for 7.5 months with minimal weight loss (≤ 10%) and retention of quality attributes. Through the R&D efforts undertaken at BARC, 15 &30 Tons and subsequently 1200 Tons commercial storage trials were undertaken in 2023 in association with National Cooperative Consumer's Federation of India Ltd. and Department of Consumer Affairs, Ministry of Consumer Affairs, Government of India. In 2024, the newly constructed cold storage at KRUSHAK is being fully utilized by a FPO (Farmer's producer organization) for the storage of 250 tons of radiation processed 'Rabi' onions (**Fig. 4**).

Fig. 4: Onion specific cold storage facility for technology demonstration commissioned at KRUSHAK, Lasalgaon

7.2. *Potato*

In general, chemical sprout suppressants including propan-2-yl (3-chlorophenyl) carbamate (CIPC) or chlorpropham are routinely used at commercial level for the purpose of sprouting inhibition during long-term storage of potatoes. However,

herbicides are restricted and recently banned by European Union. For demonstration of the efficacy of radiation technology at the commercial level, a storage trial (with ~28 tons potato) was undertaken with a industry. Three different potato cultivars ('Santana', 'Frysona' and 'HYSM') were utilized in this trial. Properly suberized potatoes were subjected to low dose (D_{min}: 73 Gy) gamma radiation processing followed by cold storage (in a cold storage facility, Mehsana, Gujrat) at 14°C and RH > 90% and CO_2 level 2500 ppm. During storage, the non-irradiated potatoes manifested complete sprouting within 100 days. On the contrary, radiation treated potatoes retained the quality attributes without any sprouting even till 7-8 months of storage depending upon the potato cultivars. Irradiated potatoes were suitable for table consumption as well as for industrial processing. After 8 months, these irradiated potato samples were further successfully channelized to a processing industry for the manufacturing of end products including chips and French fries (**Fig. 5**).

The above commercial trials contribute to the 'Operation Greens' scheme of the Government of India for integrated development of Tomato, Onion and Potato (TOP) value chain.

Non-irradiated potato (7 months of storage) **Irradiated potatoes (7-8 months of storage)**

Fig. 5: Commercial trial for potato irradiation and storage

8. Future Prospects

Currently radiation processing can be considered as the most effective modality to control storage and quarantine pests in agricultural & horticultural produce. It can therefore play a significant role in strengthening national food security, and international trade. India being the major producer of grains but the climatic conditions of the country are highly non-conducive for their ambient storage. A huge number of radiation facilities with high throughput handling capacity are required to set up. The recent market trials have proven that well informed consumers are keenly interested in buying irradiated food. It has been suggested that the trade organizations, government agencies, and university extension departments can utilize digital and social media platforms to endorse

the multifarious advantages of radiation technology. Radiation is also a very effective food safety tool, as it can penetrate deep and kill hidden microbes in packaged food.

Future focus is required on combining and integrating irradiation with complementary modalities for improving storability, safety as well as quality of food. Overall, the success of this technology predominantly depends upon the cooperation amongst various stakeholders including the processors, retailers, policymakers, regulators, and scientists.

Strategy for pan India deployment of radiation technology requires:

a) Adequate financial planning and outlay for pan India deployment

b) Encouraging innovative design and development in material handling equipment, logistics warehousing for high throughput applications

c) Encouraging design and development in indigenous machine sources for high throughput applications and multitasking

c) Developing a unified national policy framework involving relevant stakeholders for easy adoption and wider dissemination of the technology

d) Planning outreach activities and awareness programs among stakeholders

9. Conclusion

Radiation processing of food being a physical method offers a superior alternative to other food preservation modalities. The technology is safe and approved under Indian food regulations. There are a number of operational food irradiation facilities existing in the country, but this number is quite small considering the volume of annual agricultural output and its storage. A highly focused and coherent unified policy framework is required involving various stakeholders in food and agriculture enterprise for a wider adoption and spread of the food irradiation technology. Continuous R&D would be required for developing SOPs for conservation of several nutritionally important but highly perishable underutilized foods. For enhanced commercial adaptability, large scale trials with concerned agencies need to be promoted to establish techno-commercial feasibility. A market driven R&D can catalyze transition of this advanced yet unexploited - technology to an indispensable processing tool in the national food supply chain.

10. Acknowledgement

Endeavors and contributions from the former and current colleagues of Food Technology Division towards the progress and advancements made in the food irradiation program is highly acknowledged.

FOOD IRRADIATION FACILITIES AND RADIATION MEASUREMENTS – JOURNEY, CHALLENGES AND FUTURE

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Abstract
Radiation processing technology is now available commercially for integration with the supply chains in Indian agricultural and food sectors. Dosimetry techniques and dose measurements are the two most essential components of any food irradiation system both in R&D and commercial use. Selecting appropriate dosimetry technique and dose measurement method are fundamental to the efficacy of various applications that have been developed over the years since the inception of the food irradiation program at BARC. Accurate delivery of the standardized doses to the food products is governed by a reliable dosimetry system and its traceability to the national and international standards.

1. Introduction

Radiation processing of food or food irradiation is a technology that can help reduce food spoilages, enhance safety and promote trade. Irradiation facilities are the backbone of the food irradiation program. Since the start of R&D in early 1960's a number of irradiation facilities have been procured and installed at the Food Technology Division, BARC. These facilities include small experimental irradiators for R&D as well as irradiators that allow large scale irradiation of food and agricultural commodities for studying techno-

economic feasibility. The effectiveness of processing of food by ionizing radiation depends on proper delivery of absorbed dose and its reliable measurement. It is important that the dosimetry techniques and dose determination are carried out as per the nationally and internationally accepted protocols. For exportable commodities the process may also require approval of a mutually accepted dosimetry protocol between the importing and exporting countries. Several dose measurements procedures have been used to get reliable quantification of radiation doses. Finding a single dosimeter system covering the entire range of applicable doses likely to be used in food processing, 20 Gy to 25 kGy, is a challenging proposition. Different dosimetry systems may be required for different dose range used for processing of food. External environmental influences such as the temperature during dose measurement, and accuracy in a rather narrow dose range, are the other problems that confront dosimetry. Therefore, attempts have been made to explore and design novel materials for food irradiation dosimetry.

2. Food irradiation facilities

R&D at Food Irradiation Processing Laboratory (FIPLY) at FTD, BARC started with the laboratory scale experiments using small imported irradiators like Gamma Cell 220 (AECL, Canada), later replaced with the indigenous Gamma Cell 900, and Gamma Chambers 5000 supplied by BRIT, BARC. These irradiators are used even today for exposing various experimental materials including food, to gamma rays at various doses, and also needed for standardizing dosimetry protocols for industrial food irradiation facilities.

In the year 1967, a panoramic wet storage type cobalt-60 gamma irradiation facility called Food Package Irradiator, the first plant in India, and in this part of the world, along with a demineralized water (DM) plant for the water to be used in source storage pool, was procured from Atomic Energy Canada Ltd (AECL). FTD also has a portable cesium-137 gamma irradiator. A dry storage type grain irradiator with a screw type grain conveyor system (AECL) was installed in FIPLY, however this facility is now not in operation (**Fig 1**).

For carrying out the food processing, various food, plant machinery, instruments, safety systems etc. were procured, installed, operated and maintained by the trained personnel of the division. A small engineering workshop was set up for development, fabrication and maintenance of various food plant machinery and gadgets. Irradiation facilities in FIPLY were also used for the R&D on sterilization of medical products before the ISOMED plant in BARC was commissioned into operation.

Fig. 1: A dry storage type grain irradiator with grain lifting conveyor system (collaboration with AECL, Canada, currently the facility is not in operational state).

2.1. Food Package Irradiator

The Food Package Irradiator (FPI) is the workhorse for all major scale-up studies on food irradiation since 1967. After every 5-6 years, the radiation source replenishment work is also undertaken to keep the sources usable for covering applications in all dose ranges. This facility houses a split source frame for variable dose delivery, and therefore, suitable to cater to a wide spectrum of commodities. The current concept of multitasking irradiation facility has actually evolved from this facility. Because of more than 5 decades of service, various components of the facility gradually became unserviceable leading to difficulties in plant operation. Efforts were made to revamp the detailed design of the existing mechanical and electrical components in 2019-2020. Based on the information and operational experience the refurbishment of the facility was planned without altering existing civil structures. Several technical discussions were conducted and the following major scope of works were finalized in collaboration with DRHR, BARC to modernize the facility. The scope included a) Replacement of the old product handling system with upgraded design, b) Replacement of the entire source drive systems including source lifting ropes as per the present AERB safety standard (AERB/RF/SS-6), and c) Up-gradation of control console by replacing relay-based logic with PLC-SCADA interface. Several advanced safety interlocking systems such as ozone delay interlock, exit conveyor interlock, radiation interlock at DM plant and watch dog timer for product safety are now installed. The structural integrity of the source storage pool was assessed for the first time since commissioning ensuring safe storage of source. The facility has

now been refurbished and deployed for regular routine operation after complying all the safety requirements from the regulatory agencies of BARC since 2022 (**Fig.2a, b, c & d**).

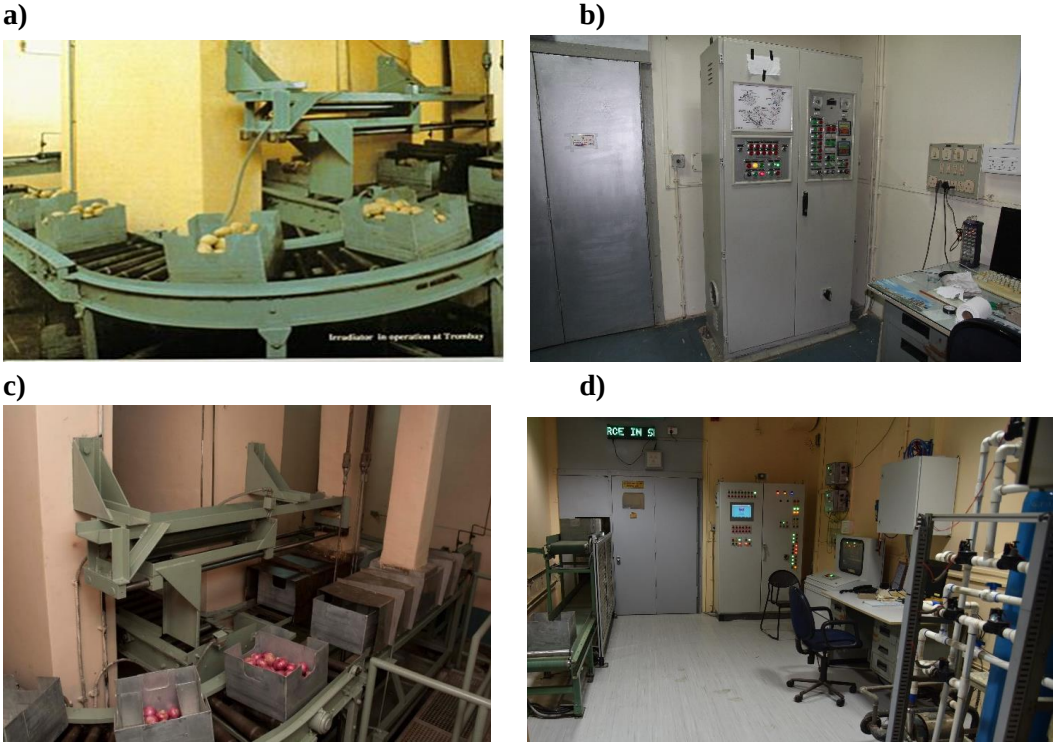

Fig. 2: (a) Food Package Irradiator (FPI) during installation in 1967 and (b) control room during 2004, (c) Food Package Irradiator - Upgraded (FPI -U) during 2023- 2024, (d) Refurbished control room of FPI-U in 2024.

2.2. Cesium Irradiator

FIPLY houses one of the oldest cesium-137 based marine product mobile irradiator (Brookhaven National Laboratory, USA). The facility was installed in 1968 and recently refurbished with the new control console (**Fig. 3**). The current source strength (cesium-137) of the irradiator is 30 kCi. It was designed to be portable primarily for on board fishing and simultaneous irradiation on large ships. The control mechanism of the cesium irradiator has been refurbished and various detailed engineering drawings of the facility were reconstructed and digitized for future reference.

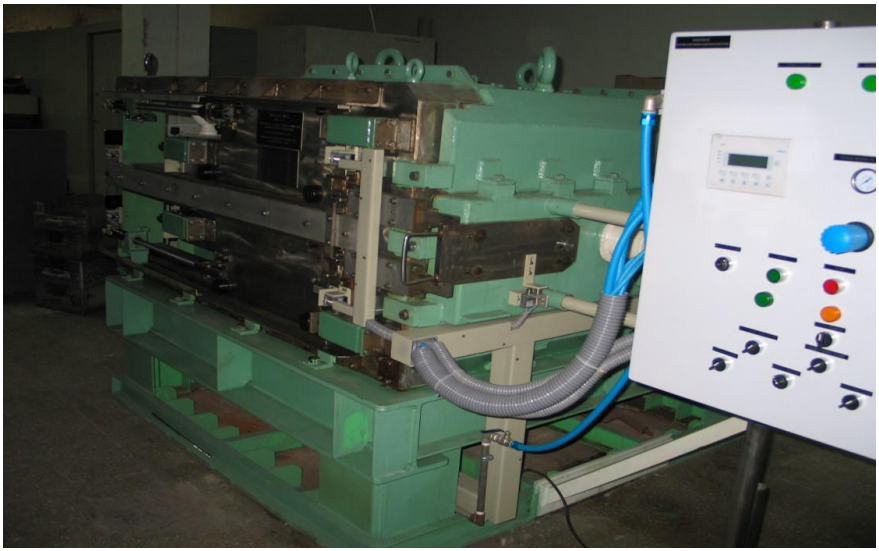

Fig. 3: A batch type Cesium-137 Gamma Irradiation facility in collaboration with the Brookhaven National Laboratory, USA (1968)

2.3. KRUSHAK Irradiator

In 1994, Government of India gave clearance for the commercial radiation processing of certain foods including potato, onion and some other agricultural commodities. FTD, BARC initiated the process for identifying land and setting up a 30 T/ d technology demonstration facility for potato and onion (POTON) irradiator, later named KRUSHAK, (Krishi Utapdan Sanrakshan Kendra), at Lasalgaon in Nashik district of Maharashtra. The POTON irradiator was designed, and built indigenously through the collaborative effort of several divisions of BARC, but majorly DRHR, TSD and CED. Before commissioning, the name POTON was changed to KRUSHAK, and the facility was dedicated to the nation by the Late Prime Minister Shri Atal Behari Vajpayee in 2002. For a few years KRUSHAK was managed and commercially operated by the engineers and scientists of FTD (**Fig. 4a & b**). Several collaborative experiments were undertaken with the local farmers and traders on onion and other agricultural commodities. Consequently, this operational experience gave practical inputs for handling of commodities, regulatory, operational and administrative experience. Since then, the expertise of FTD has been continuously utilized in installation, operation, dosimetry and safety of the operation of various irradiators across the country. During 2006-07, the KRUSHAK facility was upgraded for phytosanitary treatment of fruits specially to overcome quarantine trade barriers enabling export of Indian mangoes to USA. KRUSHAK became the first cobalt-60 gamma irradiation facility in the world, outside US, to be certified by USDA-APHIS for phytosanitary treatment of mango. In order to make the facility multi-tasking, various modifications were carried out in the control systems relating to conveyor speed and in product box dimensions. Concept of movable

lead shield was introduced for the first time to attenuate the radiation dose to process low dose requiring agricultural commodities. Further to ensure adequate dose delivery in the low dose range with lead shield, several experimental dose measurement exercises were carried out. Currently, the facility is capable of processing bulbs and tubers for control of sprouting, insect disinfestation of cereals, pulses, their products, spices and quarantine treatment of fresh fruits and vegetables. Since 2007, KRUSHAK has been successfully processing Indian mangoes for quarantine requirement of export to USA and other countries. Since then, the facility is being operated under PPP mode.

a) **b)**

Fig. 4: a) KRUSHAK irradiator, BARC at Lasalgaon, Nashik, (b) Mango irradiation at KRUSHAK for export to USA since 2007.

2.4. Small Experimental Irradiators

There are four Gamma Chambers housing cobalt 60 sources to carry out lab-scale R&D activities not only in food and biological samples but for radiation sensitivity testing of various equipment of departmental interests. The first gamma cell (Gamma cell-220) was received from the Atomic Energy Canada Ltd in 1967 (**Fig. 5a**) and still maintained in full operation for various research works. In 1995 a Gamma Chamber 900 and subsequently in 2005 another Gamma Chamber 5000 of BRIT, DAE were commissioned (**Fig. 5b & c**). In order to cater to the R&D need, a new Gamma Chamber 5000 (BRIT, DAE) has also been installed with enhanced dose rates in 2017 and deployed for utilization (**Fig. 5d**).

Today India has 28 food irradiation facilities, 5 in Government sector and 23 in private sector. Further, a greater number of commercial irradiation facilities are expected to come up in various parts of the country showing good prospects for radiation processing of food in the country.

a)

b)

c)

d)

Fig. 5: (a) Gamma Chamber (Gamma Cell 220) of FTD, BARC commissioned in 1967 in collaboration with AECL, Canada, (b) Gamma Cell 900 designed by BRIT, DAE commissioned in 1995, (c) Gamma Chamber 5000 of BRIT, DAE commissioned in 2005, (d) Gamma Chamber 5000 designed by BRIT, DAE commissioned in 2017.

3. Radiation measurements-past and present

3.1. Dosimetry activities

It is the responsibility of a food irradiation facility to ensure delivery of the required dose of radiation, uniformly and within the legal limits prescribed under the relevant regulations, adequate to achieve the desired objective. In view of this, a Food Dosimetry Section was created within FTD to standardize the dose requirement of various foods for meeting various technological objectives set by food scientists. A dosimetry laboratory was specially created to monitor and administer absorbed dose (maximum and minimum dose) that a food can tolerate and without compromising its nutritive and sensory quality. Each food has a specific minimum and maximum dose tolerance. The dosimetry systems used then were Fricke (for low doses) and ceric-cerous (for higher doses) dosimeters. The product box size was reduced to one-third of its original size to accommodate all the products ensuring uniform distribution of the absorbed dose. The periodical dosimetry was carried out with each product to obtain accuracy and precision in desired dose delivery. In order to carry out lab-scale experiments, a cobalt-60 based gamma chamber (Gamma Cell-220) was used, and the dose output of the machine was estimated periodically. Collaborative programs with other divisions like RPD were also carried out to gain experience.

3.2. Current R&D in dosimetry

3.2.1. Experimental routine dose measurements

In radionuclide-based irradiation facility, quantification of radiation is essential to characterize the facility in operational qualification (OQ), dose distribution evaluation in exposed commodities during performance qualification (PQ) programs and routine dosimetry during processing to ensure the desired objectives of the technology. The energy imparted to the product in a radiation facility is influenced by the optimized dwell time or conveyor speed. Dose also depends on the bulk density of the process load. Time to deliver the same dose to a product becomes longer as the bulk density increases. These relationships should be established during facility qualification. In view of this, a detailed experimental dosimetry is carried out in Food Package Irradiator-Upgraded (FPI-U) periodically using food as medium and employing Fricke or ceric cerous sulphate dosimetry systems. The dose distribution profile inside the product box is experimentally measured to find out the positions of the D_{max} and D_{min} positions and the dose rate of the facility during shuffle-dwell movement of the conveyor. The dose outputs of all the gamma chambers (4 Nos) are also periodically measured for precise dose delivery to all the experimental samples.

Dose mapping experiments of the product trays of the cesium-137 facility were also carried out to find the dose distribution profile and dose uniformity ratio using Fricke dosimetry system. The improved dose uniformity was also experimentally determined with modified irradiation geometry. The operational approval of the refurbished facility for R&D applications is under process.

Industry interest in the deployment of linear accelerators (LINAC) for food irradiation is gradually increasing. Absorbed dose to food commodity is the function of several operational parameters of the LINAC. In order to assess the feasibility of a 10 MeV, LINAC for food irradiation, alanine-EPR dosimetry system was used to measure the process parameters at Electron Beam Centre (EBC), APPD, BARC, Kharghar. Experiments were performed with different foods such as mango, potato and semolina in one sided and two-sided irradiation geometries. The dose distributions (0.25 to 1 kGy) were evaluated in each set of trials. The depth dose pattern in food and scan width of the electron beam were observed suitable for large scale irradiation of food. Subsequently, to determine the dose uniformity with statistical variation, an experiment with packets of semolina was conducted. The dose uniformity ratio was observed to be within 2.2 indicating that the facility is suitable for commercial food irradiation. Table 1 shows the list of dosimeters available for routine dosimetry and to ensure traceability.

Table 1. Dosimetry systems and their useful dose ranges

Compound	**Applicable for**	**Measurement method**	**Dose Range (Gy)**
Radiochromic Film	Electron/ Photons (γ and X-rays)	Spectrophotometer	1 - 1.5 $\times 10^5$
Polymethyl methacrylate	Photons (γ and X-rays)	Spectrophotometer	10^2 - 1.5 $\times 10^5$
Radiochromic Optical wave guide	Photons (γ and X-rays)	Spectrophotometer	1 - 10^5
Dichromate	Photons (γ and X-rays)	Spectrophotometer	2 $\times 10^3$- 5 $\times 10^4$
Ethanol chlorobenzene solution	Photons (γ and X-rays)	Spectrophotometer, colour titration, high frequency conductivity	10 - 2 x10^5
Alanine	Electron/ Photons (γ and X-rays)	Electron Paramagnetic Resonance spectrometer	1 - 10^5
Calorimetric	Electron	Calorimeter	10^2 - 5 $\times 10^4$
Cellulose tri acetate	Electron/ Photons (γ and X-rays)	Spectrophotometer	5$\times 10^3$ - 3 $\times 10^5$
Fricke solution	Photons (γ and X-rays)	UV spectrophotometer	20 - 4 $\times 10^2$
Ceric-Cerous Sulfate	Photons (γ and X-rays)	Potentiometry	5 $\times 10^2$ - 5 $\times 10^4$

3.2.2. Development of novel dosimetry systems

The dose uniformity inside the product box of any radiation facility is strongly dependent on the bulk density of the process load. During the routine operation of a radiation facility, the dimension of the product box and the source pencil distribution pattern cannot be altered. To observe the role of bulk density of the product on absorbed doses in a cobalt 60 based radiation facility (FPI-U), a range of bulk densities (0.01 to 0.8 g/cc) were prepared using different materials. D_{max}, D_{min} and Dose Uniformity Ratio (DUR), the three most crucial parameters exhibited significant variation with the change in bulk densities of the product.

There has been a continuous requirement to prepare simple, stable and accurate dosimeters for food irradiation dosimetry. For perishable foods, irradiation is carried out at sub-ambient temperatures like chilled (2±1 °C) and frozen (-20±2 °C) and therefore, radiation doses are required to be measured at these low temperatures. In view of this, solid state dosimeters could be the most suitable options in comparison with aqueous systems. Passive dosimeters such as thermoluminescent (or photoluminescent) solid state systems are simple and already established as useful options for personnel monitoring application. However, these systems show multiple and changing luminescence peaks with increasing dose. Moreover, in the higher doses for food irradiation application, these dosimeters exhibit nonlinear response because of filled traps and/or trap competition/trap damage. In order to address this problem two thermoluminescent phosphors ($CaSO_4$:Dy and $CaSO_4$:Dy, Bi) have been studied by pre-irradiation thermal treatments and established their efficacy in measurement of food irradiation dose at sub-ambient temperatures. The results showed that the polycrystals as host matrices of the dosimeters showed limitations in the high dose measurements and amorphous systems such as glass could be of paramount importance for this application because of their inherent inertness toward radiation. Several studies have recently been carried out demonstrating utility of thermoluminescence of borophosphate, aluminosilicate, and lithium borate glasses as promising candidates to measure food irradiation doses. One potential glass system, lithium borate doped with dysprosium has been recently developed in collaboration with GAMD, BARC.

In electron beam irradiation, thin film dosimeters are useful. The radiochromic dyes have shown their potential as reliable dosimetry systems since a long time because of their simple mode of operation and reliability. In order to develop radiochromic thin film dosimeter, a basic study has been conducted to optimize the appropriate ingredient of a dosimetric formulation of leuco crystal violet as the leuco dye and 2,2,2-trichloroethanol as sensitizer in acetonitrile solution. In order to make the dose evaluation tool cheaper, the response of the leuco crystal violet film was measured using two image analysis methods – single and a novel double channel readout. It was shown for the first time that the dose measurement of leuco crystal violet-based film can be carried out using an inexpensive desktop scanner-based color analysis.

There is a need to develop simple and cost-effective dosimeter as an import substitute for low dose phytosanitary application of food. An indigenous dye-based dosimeter has been

developed and validated. This new system has immense potential of deployment for the measurements of low dose radiation processing of food. This dosimeter is an aqueous system used for measuring gamma radiation doses in cobalt-60 irradiation facilities and may not be suitable for electron beam facilities. The technology has recently been made available on BARC website for further dissemination to the users.

The adequate dose delivery depends on source configurations, product geometry and conveyor speed to ensure uniform dose distribution. Mathematical models to estimate the dose profile inside the product box are required for various functions: 1. to optimize several plant-running parameters, 2. to optimize precise dose delivery, 3. to plan adequate source pencil distribution, 4. to assess the process control capabilities of any commercial irradiation facilities. In this regard, simulation of radiation absorbed doses in the food products are also being carried out using Monte Carlo N-Particle Transport (MCNP) code.

4. Current challenges and future possibilities

4.1. Irradiation facility design

A major challenge in designing food irradiation facility is finding the optimized design for making a multitasking facility capable of delivering all the required dose ranges in food applications (20 Gy to 25 kGy). In addition, the facility should operate in a cost-effective manner both in capital investment during construction, and O&M expenditure. The majority of the food and agriculture produces amenable for radiation processing are seasonal in nature. In view of running the facility throughout the year with a viable commercial goal the facility should be designed in a way that different commodities can be processed with different technological objectives. In order to achieve this, innovative plant design are required e.g a) split source frame configuration to obtain a wide range in dose delivery, b) product conveying system with wide range of speed control to ensure absorbed dose delivery with accuracy and operational safety, c) the source lifting assembly needs innovative design to reduce the transit dose, d) control system with reliable programmable logic control and user-friendly interface, e) the safety interlock systems with redundant features to ensure both the operational and product safety. In comparison with traditional food processing practices the radiation installations require higher capital investment in civil construction. In order to address this, several novel ideas are used such as a) small modular irradiator (SMI) with adequate process rate, b) mobile irradiator with accelerator as source of ionizing radiation with adequate safety and security, c) innovation in civil design and material usage without compromising with radiological safety, d) innovative design of the category III type land based irradiator where source is stationary in water pool and the water-proofed product boxes would go into the water for adequate dose delivery. This novel concept would help in reducing considerable cost involved in the construction of irradiation cell with 1.5 to 3 m thick concrete wall.

Accelerator based systems have certain advantages over radionuclide-based irradiators. However, there are some limitations too. These are mainly related to poor penetration of

electrons, and their conversion efficiency to X-rays. In view of these, there is a huge scope of research in designing accelerator-specific product conveying system to achieve desired process rates, both in one-sided and two-sided irradiation geometries. An exclusive design for product handling in free-flow grain irradiator would also be required. A novel accelerator-based low energy beam technology is also being currently explored globally for surface decontamination of food commodities. The efficacy of surface treatment using low energy electron beam for food such as spices, herbs, or grains has already been reported. A deeper understanding on the interactions of low energy electron beam (LEEB) and low energy X-ray (LEEX) with foods is required.

4.2. Dosimetry challenges

Measurements of radiation dose at sub-ambient temperature has always been a challenging task. Development of amorphous systems with suitable dopants is therefore of importance for this specific application. Thermoluminescence is a potential and cost-effective option.

Electron accelerators are becoming popular in the field of radiation processing technology. Radiochromic thin film capable of measuring high radiation dose is therefore essential. Commercially available films are imported mainly for medical use. These are not readily usable for food dosimetry. Hence, indigenous development of these films for food use is of interest. Radiation processing of food using LEEB and LEEX will be of interest in certain application areas of food industry. Measuring surface dose and its penetration depth will be a challenging dosimetry problem. Thin foil dosimeters could be potential candidates, but a dedicated research effort would be required.

5. Conclusion

Considerable efforts have been put to design and construct various food irradiation facilities catering to the need of basic and applied R&D, scale-up trials, and subsequent commercial deployment. Significant research has been carried out in the past six decades to develop and standardize dosimetry and dose measurement techniques in food products to achieve uniform dose delivery and establish its traceability. There is an ample scope to conceptualize, design and develop novel need-based food irradiation facilities considering the growing interest of the entrepreneurs in deployment of the technology in India's food and agricultural sector. Design and development of novel radiation dosimetry systems will remain a major endeavor.

6. Acknowledgement

Endeavors and contributions from the former and current colleagues of Food Technology Division towards the progress and advancements made in the food irradiation program is highly acknowledged.

RADIOBIOLOGY RESEARCH WITH FOCUS TO HUMAN HEALTH AND CANCER RADIOTHERAPY

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Abstract

This Chapter gives a brief account of research contributions of 'Radiobiology Research Group' at Bhabha Atomic Research Centre (BARC) since early years of Indian atomic energy program. The major focus of our research consists in understanding the mechanisms of ionizing radiation damage at various levels of biological organizations such as cellular, molecular, membrane, and at tissue levels with relevance to radiation-mediated cell death for cancer radiotherapy and protection of normal cells/tissues from radiation damage. Research contributions have made significant contributions in advancing knowledge on the formation, characterization and involvement of primary free radicals from water radiolysis, reactive oxygen species, and their reactions with DNA and components of plasma membrane in triggering the cascade of structural and signalling mechanisms for determining the radio responses of normal and tumour cells. These results are published in reputed journals and mechanisms of radiation oxidative damage, role of apoptotic sensitivity,

involvements of ROS in radiation damage in cells enabled us to predict the tumour radiosensitivity. In our efforts to selectively kill tumour cells without affecting normal cells, we developed liposome-based nanosized vesicles to envelope anticancer drugs into surface engineered formulation and demonstrated targeting and enhanced toxicity of loaded cargo by many folds to the experimental tumour and succeeded in overcoming radioresistant tumors. To further improve upon therapeutic outcome and overcome radioresistance, we experimented electro-radio-chemotherapy technology for more efficient drug delivery to intended target with considerable success in *in vitro* and in animal tumour models. Taking note of emerging new knowledge in radiobiology, we turned to study the mechanism of 'Non-targeted Effects' of low (gamma, X-rays, electron) and high-LET radiations (viz. alpha particles and proton) such as bystander effect, abscopal effect, adaptive responses, radiation hormesis and low dose radiation effects in cell and animal models, which have received wide recognition among peers. These lines of research are actively continuing and newer research is initiated to stay ahead in the field. To aid and support DAE's three-stage nuclear power program, 'Nuclear Radiobiology Research' was initiated and developed to evaluate radiological and chemical toxic effects of Thorium and Uranium in cells and animal models and develop technologies/approaches for decontamination of internalized radionuclides. In summary, research of our Group has made sustained progress in basic radiobiology research and applications earning the credit of some patents. It firmly foreseen that active low dose radiobiology research may open new vistas for advancing knowledge and staying competitive among professional groups and exploring newer health applications for better serving the society.

1. Preamble

The atomic energy program of our country has been founded on the premise of peaceful applications including electricity generation by nuclear method and health improvement programs such as cancer treatment. The program was started under the Chairmanship of Dr Homi J. Bhabha, a famous nuclear physicist, with the formation of Atomic Energy Commission (AEC) by the Government of India in January 1953. It is pertinent to recall that revolutionary discoveries of X-ray by W. R. Rontgen of Germany in 1895 and radioactivity by Henry Becquerel of France in 1896 had generated enormous excitements often claiming that new discoveries may prove panacea for all existing problems in the world such as shortage of electricity for industrialization, daunting problem of hunger and poverty and treatment of diseases to save life. The promises made by scientists and policy makers seemed sound and achievable but a few adverse health effects came to notice demanding urgent attention. Within a few years of the new discoveries, the discoverers and researchers encountered some ill effects of atomic radiations on their health e.g., skin burns, rashness, inflammation and blisters raising alarms on the harmful

effects of radiation necessitating the urge to understand the biological effects of radiation giving birth to a new field of research called radiation biology. Over the years, intensive research on mechanisms of radiation action on living and non-living matters revealed that X-rays and the radiations emitted from radioactive substances were either charged particles such as alpha, beta particles or electromagnetic waves such as gamma rays. Both these types of radiations produce ionizations and excitations in the medium through which they travel and therefore they were called ionizing radiations. In later years, studies on biological effects of radiation became a front-line research project in most of the advanced institutes and atomic energy establishments. In early 20th century, it became known mainly from the results on fruit flies reported by US and German Scientists that atomic radiations were capable to cause mutations in the living organisms. Further, during WWII, dropping of atomic bomb on Hiroshima and Nagasaki in 1945 caused enormous loss of human life and destruction of property created enormous horror and negative image in the mind of public. In the following years, there was unusual push to intensify and accelerate radiobiological research to assess radiation effects on health and environment. In this backdrop of observed harmful effects of atomic radiation, especially induction of cancer in the bomb exposed Japanese population, radiobiology research found re-focus by the scientists and policy makers of atomic energy to ensure safety standards and protection of health of researchers, nuclear workers as well as general public. Indian atomic energy program was no exception and Dr. Bhabha invited Indian scientists with expertise and experience from advanced countries to build laboratories for studies on biological effects of radiation on microbial, mammalian and plant systems at the laboratories in Bombay then called Atomic Energy Establishment (AEE), Trombay. The scientists were apt and quick to foresee the urgency and need for radiation biology research in nuclear program for power production. Dr. Bhabha foresaw the need for building research and development laboratories in physics, chemistry and biology at AEE. Dr. A. R. Gopal-Iyengar who obtained his Ph.D. Degree from University of Toronto, Canada and was the Chief Cytologist at Tata Memorial Hospital, Parel, Bombay, was invited to head the then Biology Division at AEE. Radiobiology program was aimed to investigate the basic mechanisms of radiation effects and emerging applications. The major focus of radiobiology program was to understand the mechanism of ionizing radiation-induced damage to biomolecules, model membrane systems, cells and animal tissues with relevance to radiation risk assessment and improvement of cancer radiotherapy. Under the dynamic leadership of Dr. Iyengar close to 2 decades, the radiation research programs at Biology Division progressed continuously and made significant contributions matching with the advances made from western countries and in alignment with the guidance of International Atomic Energy Agency (IAEA), Vienna. The radiobiology research and technological developments at this Centre received appreciation and recognition from international scientific community. Professional expertise and laboratory facilities have grown over the years and research based applications have culminated into identified technology groups dedicated to serve the nation and benefit to society, namely, radiation induced mutant development in agriculture (now Nuclear Agriculture & Biotechnology Division), food sterilization by

radiation for avoiding spoilage and prolongation of shelf life (Food Technology Div.), radiation insect sterilization for insect control, soil chemical analysis for detection and quantification of radionuclide in soils, biological effects of radiation on living systems to advance knowledge on radiation damage mechanisms and their modifications (radiosensitization and radioprotection) applied to cancer radiotherapy and radioprotection including free radical mediated damage to cellular biomolecules e.g. DNA, lipids and others (Radiation Biology & Health Sciences Div.) and radioisotopes applications in nuclear medicine for diagnosis and therapy (Radiation Medicine Centre) of patients. It was in 1970s that deep interest grew to understand the health effects on population especially for cancer risk assessment and other ailments to people residing in high background radiation areas (HBRA) in Kerala and a research laboratory was established in Kollam. Dr. Gopal Iyengar directed the biology and radiobiology research program for almost two decades until his superannuation in 1970. More recently, the radiobiology research program has been further expanded and diversified in frontline research areas such as high LET radiation effects on cellular systems, non-targeted radiation damage, evaluations of effects of radionuclides after internal contamination with actinides (Thorium, Uranium, etc.) and tumor-targeted nano formulations. Some of the current radiobiology research programs such as dose-response assessment in low dose ranges, thorium research and technology are aimed to advance knowledge and develop expertise matching with the progress and developments in advanced laboratories of the world.

2. Biophysical, Molecular Radiobiology and Free Radical Research

In the early 20th century, it was widely known that nuclear radiations and X-rays cause ionizations and excitations in the molecules of the medium in which they travel. Further, extensive research suggested that ionizing radiations produce free radicals either after directly interacting with the cellular molecules such as DNA, proteins, lipids or cellular molecules or they can cause indirect damage by depositing the energy in water leading to formation of short lived radicals namely, $^{\cdot}OH$, e^{-}, O_2^{-}, H atoms (called primary radicals produced after water radiolysis). The central concept of radiobiology was evolving with the fact that IR-induced free radical-mediated events were primarily responsible for radiation injury. In 1940s, it was mostly agreed that the free radicals formed following radiation action were either chemically scavenged (or repaired) by free radical scavengers (such as GSH or cysteine) to be restored to their original structure (RH or H_2O), or if O_2 was present, primary as well as secondary free radicals formed of critical biomolecules could react to form peroxyl radicals ($ROO^{\cdot}$), which was thought to fix the damage and enhance radiobiological damage. This line of argument helped to explain the dramatic effect of O_2 on the observed enhancement of IR-induced tissue injury that was universally noted and commonly called "Oxygen Effect" in radiobiology. Noting the developing scientific discussions on the roles of IR induced free radicals in biological damage, the then Director, Biological Research Group, Dr. Iyengar, was prompt to build a team of researchers in 1960s to investigate radiation generated formation of free

radicals and their reactions with biological macromolecules such as DNA. The Research Group was named Physical Radiobiology Section within Biology Division of AEE. Dr. Bam Bahadur Singh, a physicist from 3rd batch of Training School was made the Head of this research program. He built a very active research team consisting of biophysicists, chemists and biologists, who were trained for research in nuclear science and technology. This team was dedicated to detect, identify and characterize the radiation induced free radicals and understand the molecular mechanisms in radiobiological damage to cells and tissues (**Fig. 1**). Molecular radiobiology was considered frontline and hot subject the world over and this research group earned the reputation of leader in India. The research publications on identification and characterization of free radical mediated damage to DNA, adenine, thymine, guanine, cytosine in aqueous alkaline-frozen systems following gamma irradiation using electron spin resonance (ESR) technique drew due attention of international scientific researchers. The group grew from strength to strength and made seminal contributions on understanding the mechanisms of ionizing radiation produced electrons and hydroxyl radical reactions with DNA and its constituents. This research program further evolved and explained the chemical mechanisms of radiosensitizers and radioprotectors. Taking note of active research and publications, this research team was invited by IAEA for expert consultations and participation in coordinated research programs (CRP) in chosen topics for many years. The early research and subsequent sustained progress in radiation biophysics laid firm foundation for the detailed understanding of radiation generated free radical reactions with cellular biomolecules, molecular mechanisms of radiation damage in biological systems with relevance to cellular injury and modification of radiation damage with special emphasis on relevance to radiotherapy and radioprotection.

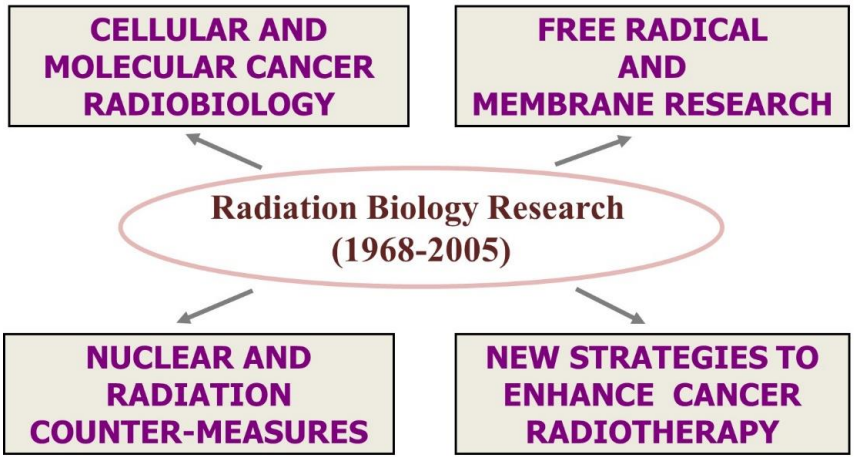

Fig. 1: Early radiation biology research during 1970s-2005

Extensive radiobiology research in 1920s showed that X-rays induced mutations in fruit flies (Drosophila). Prof. H. J. Muller (1927) provided the first evidence of genetic effects of radiation. In following years, enormous interest was generated in radiobiological

research concerning paradoxical findings that radiation caused diseases mainly cancer (carcinogenesis) as well as it can be used to kill cancer cells (cancer radiotherapy) opening up many new directions of research in radiobiology with regard to radiation safety and risk assessment. Studies on basic mechanisms in radiobiological research on bacterial and mammalian cells with relevance to improvement of cancer radiotherapy, search for drugs and molecules which could be effective radiosensitizers, developing new strategies for overcoming radioresistance of tumor have been the main thrust of our group in the years that followed. After superannuation of Dr. B. B. Singh in 1998, Dr. (Mrs) A. M. Samual, a reputed Nuclear Medicine physician and Head, RMC, took over as Director, Biomedical Group, BARC who galvanised radiobiology research activities in terms of human resources, equipment and laboratory modernization and upgradation. It is satisfying to note that our continued original research publications have been widely cited by peer research groups and our research Group at BARC is considered lead radiobiology research group of India by the global scientific community. It may be noted that after the death of Dr. Bhabha, AEET has been named Bhabha Atomic Research Centre as a mark of honour to him for his leadership role in Indian Atomic Energy Program.

By 2000s, our group diversified and expanded the research programs in relevant and frontline areas of radiobiology in sync with the contemporary research in other countries. Research programs were redesigned and refocused to accept new challenges in advancing the knowledge on assessment of molecular mechanisms of radiation induced oxidative stress in normal cells, tumour cells and tissues involving free radicals and reactive oxygen species (ROS) as mediators of radiation damage by employing fluorescent probes and electron spin resonance spin labelling techniques to address fundamental questions in emerging radiobiology using cells in culture, tissues and animal models. Apart from studies on DNA as a target of radiation effects, our research team began focusing attention to exploring the mechanisms and consequences of membrane oxidative damage following irradiation of normal and tumour cells *in vitro*. To understand the role and relevance of membrane damage in the radiation mediated cell death following gamma radiation, research program was undertaken using phospholipid vesicles (liposomes) as model membrane system to investigate the effect of ionizing radiation on biophysical properties of membrane such as membrane fluidity, permeability, lipid damage, etc. Gamma radiation-induced changes in the liposomal membrane permeability were monitored by measuring the leakage of pre-encapsulated 6-carboxyfluorescein fluorescent probe, and alterations in lipid bilayer fluidity were determined by 1,6-diphenyl-1,3,5-hexatriene fluorescence polarization. The changes in permeability and fluidity in the bilayer were found to be dependent on the radiation dose in a biphasic manner. These results were interpreted in terms of lipid bilayer fluidization after exposure to doses up to 1 kGy, however, rigidization was observed in the lipid bilayer at higher doses probably due to cross reactions of lipid radicals in the bilayer model. Notably, it was for the first time that our group reported these findings in radiation research field establishing a relationship between alterations in membrane permeability and fluidity after irradiation. Radiation-induced changes in the permeability of the

liposomes after exposure to gamma radiation and their modification by antioxidants postulated the role of free radical mechanism in the membrane damage, which offered new insights for the modification of cellular radiosensitivity for radioprotection of normal cells and cancer radiotherapy. In view of the seminal research findings and its relevance in radiobiology research, scientists from Germany came forward to develop Indo-German Collaborative research in this field with Prof H. Diehl from Germany.

During 1990s, it was considered a challenging task to search for new compounds and probable drugs, which could sensitize tumour cells to ionizing radiation and develop strategies to overcome radio resistance of tumour cells because many drugs under investigation as radiosensitizers had failed in clinical trials. Our Research Team took up the challenge and started exploring the possibility of antioxidants (AO) as radiosensitizers and use of pulsed electric field to overcome radioresistance of tumour cells by permeabilization of plasma membrane of these cells. During 1980s, electroporation, a membrane-associated biophysical technique was used. Electroporation is a membrane-associated bio-physical technique, which involves transient increase in the permeability of the plasma membrane by the application of external electric field of high voltage and of short duration. Electroporation causes non-selective plasma membrane permeabilization due to structural modification of the membrane (formation of micropores), which allows entry of impermeable molecules into the cell interior and offers a controlled method for incorporation of drugs/antibodies (**Fig. 2**). This technique was found to be highly useful in the area of cancer therapy, where the main challenge was to overcome resistance of tumor cells toward uptake of anticancer drugs and/or radiation. Combination of electroporation with anticancer drugs led to the emergence of a new field called ***electro-chemotherapy***.

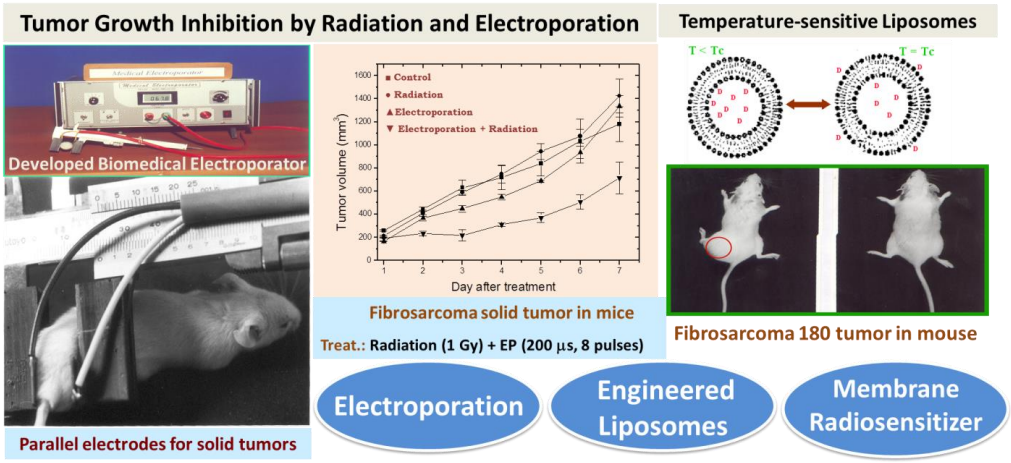

Fig. 2: A synergy was discovered between radiation action and electroporation with the objective to enhance anticancer drug delivery and cancer radiotherapy. Right panel shows the application of temperature sensitive liposomes for drug delivery in fibrosarcoma tumor.

Electroporator Technology Transferred

A biphasic electroporator (2005) was designed and developed and its technology was transferred to M/s D. S. Electronics, Mumbai. Using this technology, a synergy between electro-chemotherapy and radiation was explored for cancer treatment. Radioiodine is a highly effective therapy applied at RMC for treatment of thyroid cancer owing to the presence of membrane transporter for iodine in thyroid cancer cells. However, in clinic it was observed that a subset of thyroid patients was refractory to radioiodine uptake. Therefore, during 2000-2003, we have explored the application of electroporation to enhance the radioiodine uptake in thyroid cancer cells. It was one of the highly cited research contributions from our laboratory. Further, the synergistic cytotoxic effects of anticancer drug (doxorubicin) and γ-radiation in combination with electroporation was successfully demonstrated in the fibrosarcoma tumor model of Swiss mice. Electroporation being a tumor-targeted modality was found to be significantly useful for reducing the dose of chemotherapeutic drugs and radiation for treatment of solid cancer in patients.

3. Radiation Damage to Membranes and Cells

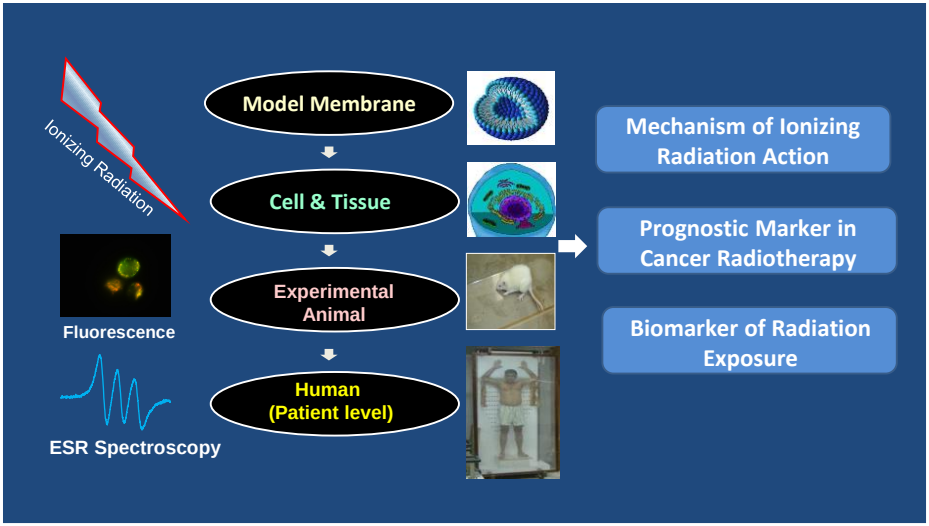

Fig. 3: Ionizing radiation triggers a cascade of free radical-mediated reactions leading to membrane oxidative damage, which was investigated in various membrane systems (liposomes, cells, tissues, tumor and cancer patient's samples) with implications to (i) understand the mechanism of radiation action and (ii) membrane damage as a predictive/prognostic marker of cancer radiotherapy and biomarker of radiation exposure.

Radio-oxidative damage to the plasma membrane of cells and its consequences in the mechanism of cell death has been investigated, which received significant attention of radiation scientists during 2000-2007 (**Fig. 3**). Various fluorescence probes (cis-parinaric acid, DPH, DCH-FDA, etc.) were employed to determine changes in the permeability

and fluidity of the plasma membrane, intracellular level of reactive oxygen species and lipid peroxidation in mouse thymocytes following gamma-irradiation within a low to moderate radiation dose range (a few cGy to 10 Gy). These studies have shown a correlation between radiation-induced membrane changes, ROS generation and apoptotic cell death in moderate dose range. These observations highlighted the distinct mechanisms of low dose radiation effects at cellular and membrane levels.

Further research was taken up to modulate radiation-induced membrane oxidative damage, generation of ROS, and apoptosis by natural antioxidants. The inhibition of apoptosis by membrane-localized antioxidants such as eugenol, isoeugenol, and alpha-tocopherol was more effective than the cytosolic antioxidant such as ascorbic acid. It was inferred that damage to membrane played a significant role in radiation-induced apoptotic death, which was markedly modified by membrane-localized antioxidants. Thus, it has been demonstrated that membrane oxidative damage was initiated by radiation-induced ROS, which could be modified by structure modulating factors such as cholesterol or membrane-specific antioxidants viz. tocopherol. Post-irradiation permeability changes in thymocyte membrane suggested propagation of initial events with the passage of time. Results have shown that fluorescence probes give a good account of chemical and structural alterations in membranes. Furthermore, it was suggested that measurement of membrane injury may provide an indicator of radiation exposure. These studies provided the evidence that membrane damage may initiate or contribute to processes leading to induction of cell death. Further extension of these studies was found to have considerable implications in basic radiobiology and in clinical cancer radiotherapy.

4. Classical Radiobiology Research: Mechanisms and Perspectives

Gamma radiation-induced tumor induction in thymus and its suppression by pre-exposure to low dose irradiation (10-30 cGy) has been investigated in Swiss albino mice. These studies showed that a single dose of whole-body γ-irradiation (3 Gy) induced thymic lymphoma (TL) after 3-4 months followed by shortening of the life span of tumor-bearing animals. These findings have been extended to detailed investigations on the mechanisms of radiation-induced occurrence of tumor and its modification by antioxidants and low dose exposures prior to tumor-causing radiation dose. Further studies have confirmed that pre-exposure of animals to low doses of radiation significantly suppressed the growth of the lymphoma tumor. Radiation-induced tumor induction was found to be dependent on the age of animal at the time of irradiation. The younger age mice showed greater sensitivity to radiation-induced TL. In addition, radiation-mediated tumor induction was found to be gender-dependent. In irradiated female mice, the TL incidence was significantly higher and the growth of tumor in terms of weight and size was more aggressive than in males of the same age. Moreover, mice with higher age groups at the time of irradiation showed substantial decrease in TL incidence and its aggressiveness. Interestingly, these effects were more conspicuous in males than in females. It was further observed that the post-irradiation feeding of animals with antioxidants resulted in a significant decrease in TL incidence, and the prevention in

TL incidence was more in animals fed with curcumin (55%) than with ascorbic acid and eugenol (20%). These results have provided a significant insight about radiation-induced TL incidence and its modification by antioxidants. These studies and in-house developed radiation tumor models have great potential to develop low dose radiation research program with relevance to cancer treatment by low dose therapy or radon exposure at BARC. Whole body exposure of animals to sub-lethal doses (1-5 Gy) was observed to cause a dose-dependent increase in ROS and consequent apoptosis in thymocytes of irradiated animals, which was found to be inhibited by antioxidants such as vit-E, curcumin, ascorbic acid and eugenol. This project revealed the role of gamma-radiation generated ROS in cell/membrane oxidative damage and also the role of apoptosis in the mechanism of radiation-induced lymphoma tumor in mice. These studies further revealed a correlation between the magnitude/kinetics of DNA damage in peripheral blood leukocytes of mice exposed to whole body gamma irradiation (3 Gy) and aggressiveness of thymic lymphoma.

5. Basic Radiobiology Studies on Tumor Cells for Cancer Radiotherapy

The discoveries of X-rays and radioactivity at the end of 20^{th} century brought revolution in diagnosis and treatment of many diseases including cancer. Early radiobiological studies showed that ionizing radiations could kill the rapidly proliferating cells. In fact, physicians have quickly applied radiations such as X-rays and gamma rays for treatment of cancer patients. However, further studies revealed that radiation does not distinguish between normal and tumor cells thereby imposing a limitation on the dose of radiation for therapy. Most often, due to unacceptable adverse effects of therapeutic radiation on normal tissues, treatment of cancer by single large dose of radiation has to be discontinued. Based on the observations on radiation cellular effects, it was suggested to employ the strategy of fractionated doses in the clinical settings taking into account the repair of DNA damage. Extensive studies on the mechanism of cellular damage by radiation revealed the intracellular generation of ROS. These radiobiological results allowed the utilization of observed higher oxidative stress status of tumor cells compared to corresponding normal cells in causing selective radiotoxicity in tumor cells. Therefore, we have actively pursued exploring higher radiation killing of tumor cells in presence of herbal pro-oxidative flavonoids. Research from our group has shown the promise of several plant-based compounds in combination with radiation to selectively kill tumor cells while sparing the normal cells. It is emphasized that future research challenges lie in gaining the deeper insight in the mechanisms of radiation-induced damage on normal and tumor cells for developing novel protocols for effective treatment of cancer patients.

There exists enormous prospect for screening and evaluation of herbal/plant products for developing effective radiosensitization and radioprotection relevant to nuclear research program. Research was focused on the mechanism of activity of variety of anticancer and antioxidative agents, viz. Betulinic acid, Diospyrin, Eugenol, (EU), Ellagic acid (EA), Plumbagin, Triphala (TPL), Tocopherol Succinate (TOS), Thymoquinone, Silibin and Arachidonic acid on normal and cancer cells with view to design effective protocols in

practical radioprotection and cancer radiotherapy. This project was mainly focused on studies on the mechanism of cytotoxic effects in cancer cell lines and their mouse tumor models. Results have shown that these agents produced radio-sensitizing action involving oxidative damage, membrane alteration, damage to DNA and apoptosis induction. It has been found that cytotoxic effect was induced by initiating membrane oxidative damage and by triggering intracellular generation of ROS by γ-radiation in combination with phytochemicals like TPL, EA, diospyrin, and TOS in tumor cell lines viz. Ehrlich Ascites (EAC), Human cervical (HeLa), breast (MCF-7, T47D), human and mouse fibrosarcoma (HT1080 and WEHI164) and lung cancer cells. It was concluded that modulation of membrane peroxidative damage and intracellular ROS may help in achieving efficient killing of cancer cells vis-à-vis normal cells, which may provide a new approach for developing effective cancer treatment.

A research project was initiated in collaboration with Radiation Oncology and Hyperthermia Division of Nanavati Cancer Hospital, Mumbai to test whether membrane oxidative damage and associated apoptosis could be a predictive marker of radiotherapy in cervical cancer patients. The plasma membrane fluidity and intracellular SOD with relation to apoptotic death in cervical carcinoma cells of cancer patients after radiation therapy was evaluated. Cells from biopsies of cancer patients (stage IIIB) prior to and 24 h after radiation dose of 2 Gy were examined. Plasma membrane fluidity and SOD activity showed significant decrease but percentage apoptotic cells, as determined by Annexin-V/PI and TUNEL assays, were found to be increased by two folds after radiotherapy. This project validated our findings of radio-oxidative membrane damage and consequent induction of apoptosis in cancer patients. Hence it was suggested that decrease in DPH polarization in membrane, reduction in SOD activity and increased apoptosis in cervical cells of cancer patients treated with radiation may be consequent to oxidative damage induced by reactive oxygen species, which may have potential implications in developing predictive protocol in cancer radiotherapy. An inverse correlation was observed between membrane fluidity/SOD level and apoptosis in cervical carcinoma cells. On the other hand, a positive correlation was observed between intracellular calcium level and apoptosis. These results suggested that changes in membrane fluidity, SOD and calcium level were involved in the mechanism of radiation-induced cervical cell apoptosis. Moreover, apoptotic sensitivity of these cells after the first dose of radiation treatment showed a direct correlation with the radiation treatment outcome in patients after completion of radiotherapy (50-70 Gy), suggesting that apoptotic index may serve as a basis for prognosis in radiotherapy of stage-III cervical carcinoma patients.

In another collaborative research with Department of Radiation Oncology, Tata Memorial Hospital, Mumbai, radiobiological studies were performed in the relapsed/refractory Non-Hodgkin's Lymphoma (NHL) cancer patients undergoing low dose total body irradiation (LDTBI). These patients were treated to a total 200 cGy at 10-20 cGy per fraction with five fractions a week. LDTBI has shown a good efficacy in NHL patients; however, it was important to determine the haematological effects following LDTBI to establish its efficacy and safety. This study demonstrated that

LDTBI is a well-tolerated treatment in patients with good clinical response. However, in non-responding patients with progressive disease, LDTBI exacerbates the haematological parameters. The crab's jaw shaped pattern was observed in polymorph and lymphocyte count, which indicated that LDTBI works by altering immune mechanisms and apoptosis. Radiobiological analysis was performed by measuring Superoxide dismutase, Catalase, ROS and apoptosis in the lymphocytes of NHL. These initial results were highly encouraging, which initiated further evaluation of LDTBI by assessing haematological profile, clinical response, survival and patient's quality of life in combination with radiobiological studies to understand the cellular and molecular basis of 'crab's jaw patterns in LDTBI radiotherapy of NHL cancer patients. Developments of biomarkers for prognosis of metastasis and radiotherapy outcome are warranted for better management of cancer patients. Transcriptomics studies of radio-resistant lung cancer cells and its correlation with lung cancer patients TCGA data base identified cancer stem cell gene signatures as prognostic marker for responders to radiotherapy. In collaborative study with Tata Memorial Hospital, Mumbai, serum biomarkers (VEGF, IL-8 and MMP-9) in metastatic lung cancer patients showed their ability to predict metastasis occurrence. In another study with Nanavati Hospital, Mumbai, prognostic efficacy of serum HSP90 beta was studied in head and neck cancer patients subjected to hyperthermia therapy, which showed complete response in patients with lower HSP90 beta.

6. Paradigm Shift in Radiation Biology: Non-targeted Radiation Effects

DNA is considered as primary target of the cellular response to radiation and presumably no radiation effect would be expected in cells that receive no direct radiation exposure through nucleus. The dogma dominated in classical radiobiology was challenged by several radiobiologists, which showed that not only DNA but cytoplasm, membrane and mitochondria act as targets of radiation. Seminal discoveries showed that plasma membrane, which was otherwise considered merely as cell boundary, act as radiation signalling hub. These discoveries made paradigm shift in radiobiology. Joining the global radiobiologists, studies were initiated to investigate the role of membrane as target of radiation effects. Owing to simulation to cellular structure and ease to prepare a desired composition, liposomes, a bilayered phospholipid membrane with aqueous core, were used as a model membrane. The dose dependent and oxidative stress associated damage was found in liposomes prepared with unsaturated fatty acids sensitive to ionizing radiation. Cholesterol is one of the major ingredients of cellular membrane (~30 %) and is known to vary in different cell organelles. While mitochondria, peroxisome and endoplasmic reticulum membranes are cholesterol poor, plasma membrane is enriched with cholesterol. Lipid rafts and caveolae (the signaling hubs harboring receptors) are highly enriched with cholesterol. Moreover, due to structure cholesterol is known to affect the rigidity of membrane. Hence, liposomes with varying concentrations of cholesterol were prepared to understand how cholesterol content can modify the membrane and thus cellular radiosensitivity. Enrichment of cholesterol resulted in increased rigidity of liposomal membrane, which was correlated with formation of lipid

oxidative products after radiation exposure (1999). Moreover, liposomes prepared with membrane localized antioxidants like eugenol resulted in prevention of membrane damage (2004). These results were further extended in immature mouse thymocytes, which are sensitive to oxidative damage and apoptosis after radiation treatment.

Experimental evidence generated in many laboratories including ours reveal the fact that radiation effects also occur in cells or populations, where cells do not encounter direct radiation exposure. Such non-targeted effects of radiation can be either localized to neighbouring cells, known as "radiation-induced bystander effect (RIBE)" or extended to distant tissues/organs [radiation-induced abscopal effects (RIAE)]. Even though, radiation-induced bystander effect (RIBE) was discovered in 1970s and radiation-induced abscopal effect (RIAE) in 1930s, the studies pertaining to cancer radiotherapy are limited in literature. The non-targeted radiation effects (NTRE), encompassing RIBE and RIAE, are highly relevant during cancer radiotherapy due to several reasons. (i) The tumor mass is surrounded by normal tissues and thus their mixed boundaries provide an excellent platform for bystander interaction, when the tumor is targeted by radiotherapy. (ii) In some situations, tumors are located very close to critically important organs like lungs, brain, thus partial tumor irradiation is performed which provide possibility of bystander interaction between irradiated and non-irradiated regions of the same tumor. (iii) Majority of the cancer patients are diagnosed with metastasis at critical sites or spread at multiple locations in the body. Generally, radiation oncologists are able to treat only the primary site of tumor, which in-turn might affect the distant metastatic/micro-metastatic tumors. Hence, the magnitude, nature (damaging/protective) and direction (unidirectional/bidirectional) of interaction of irradiated and bystander cells/distant tissues and subsequent fate of these cells would govern the clinical outcome of cancer radiotherapy.

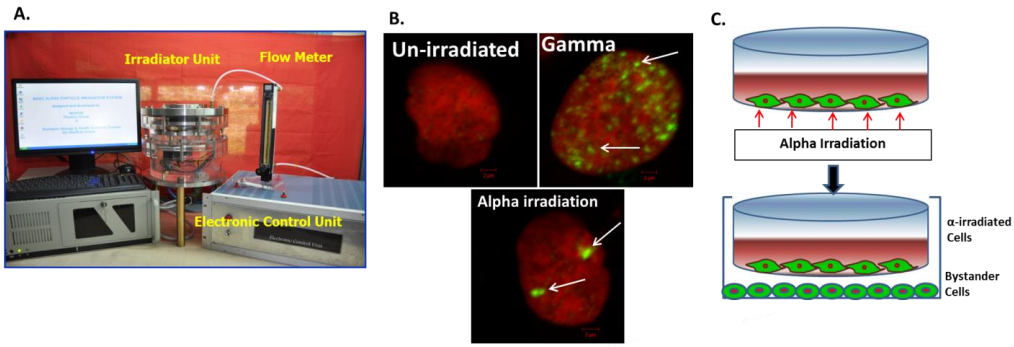

Fig. 4: (A) In-house developed alpha-particle irradiator (BARC Bio-alpha) in collaboration with TPD, BARC to develop 'Alpha Radiobiology Research Program' at BARC. Its technology has been transferred to M/s Ande Mechatronics, Mumbai through TTCD, BARC. Technology incubation for new compact Bio-alpha is done to M/s General datum Product Design Pvt. Ltd., Hyderabad. (B) Alpha particle-induced DNA damage (gamma-H2Ax foci) in comparison to gamma radiation in human lung carcinoma cells (A549). (C) Scheme depicts co-culture experiments to investigate the mechanism of alpha-radiation-induced bystander effect in normal and cancer cells.

To understand the mechanism of radiobiological response of normal and tumor cells following alpha particle exposure, an alpha-particle irradiator was designed and developed in collaboration with Technical Physics Division, BARC (**Fig. 4**). This led us to develop alpha radiobiology research in India, which has relevance to address key questions for targeted alpha radiation therapy program of BARC for cancer treatment. In addition, it also helps in investigation of bystander effect and radiological effects of alpha-emitting radionuclides in normal cells following internal contamination. The development of this research facility has generated potential research opportunities especially the characterization of alpha-particle-induced clustered DNA damage and the mechanism of consequent cellular response in a variety of cell and tissue models. Depending on the experimental settings, cell types and exposure doses, 'BARC Bio-alpha' will enable us to contribute towards space radiobiology, high-LET radiobiology for cancer therapy and scientific basis for radiation protection models, bystander effects as well as nuclear radiobiology research.

Multiple experimental models/strategies were employed to develop the non-targeted radiation research. In one of the seminal works simulating cancer and normal cell proximity during charged particle therapy, human lung cancer and normal fibroblast cells were co-cultured, where nuclei of either caner or normal cells were selectively irradiated with 500 protons using proton microbeam (3.4 MeV) facility at National Institute of Radiological Sciences (NIRS), Japan followed by measurement of DNA double-strand break in irradiated/bystander cells. Transmission of DNA damaging signal was observed from the proton irradiated lung cancer cell to bystander lung cancer cell. It was interesting to observe that the magnitude of DNA damage in the irradiated lung cancer cells was attenuated, when human normal fibroblasts were placed neighbouring to these irradiated cells. The damaging bystander effect was abrogated, when gap junction between irradiated and bystander cells was blocked. These findings for the first time established the bidirectional and rescuing bystander effect between lung cancer and normal cells after proton microbeam irradiation. The factors/cytokines released from the irradiated-cancer cells are also known to contribute to the bystander effect. In this direction, cancer cells of different tissue origins (breast, lung, fibrosarcoma, colon and brain) showed variation in secretion of cytokine profile when irradiated either with acute or fractionated doses of gamma radiation. Furthermore, the conditioned medium from the irradiated lung cancer cells showed toxicity to bystander lung cancer cells.

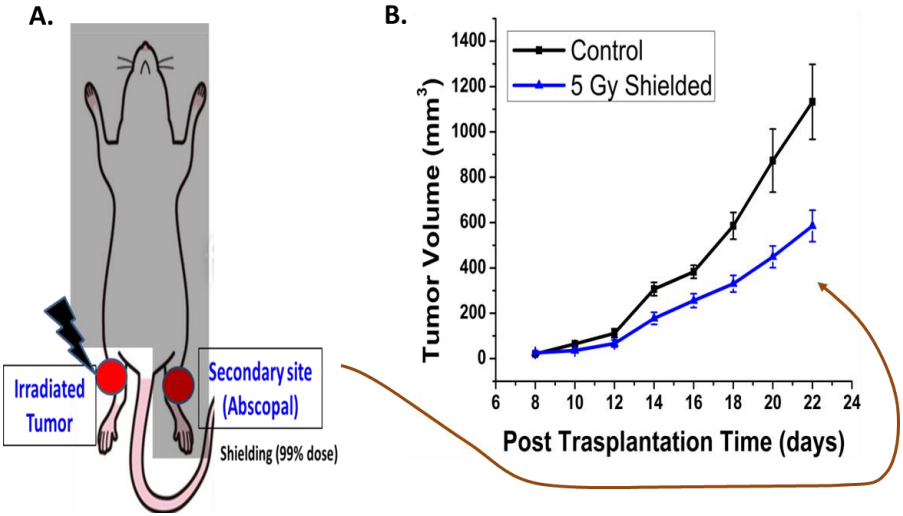

Fig. 5: A. Development of mouse tumor model to investigate the tumor suppressive effect on second un-irradiated tumor by gamma-radiation-induced abscopal effect from primary irradiated tumor. B. Tumor growth kinetics of radiation shielded (un-irradiated) fibrosarcoma tumors of control and 5 Gy irradiated tumor

Studies were extended at animal level to demonstrate and understand the NTRE results in mouse fibrosarcoma tumor models (**Fig. 5**). For this, following strategies were employed. **(i) Co-implantation of lethally-irradiated tumor cells with bystander cells:** In this approach, only a fraction of tumor cells was irradiated with lethal high doses of gamma radiation (15 Gy), mixed with bystander cells followed by implantation of the cell mixture in mice for the measurement of tumor growth. It was interesting to observe that a fraction of high dose irradiated tumor cells inhibited the growth of bystander tumor cells and thus developed into smaller tumors. The inhibition of growth of tumor was found to be associated with secretion of anti-tumor proteins/factors from the irradiated cells, which resulted in cell death of bystander cancer cells as well as decrease in the process of angiogenesis during tumor progression. **(ii) Partial tumor irradiation:** For this a cone irradiator (designed by DRHR, BARC and dosimetry by RPAD, BARC) for Cobalt-60 teletherapy irradiator, was used to irradiate only part of mouse tumor. The study showed that compared to control, significant tumor inhibition was observed, when only ~10 % volume of tumor was irradiated. **(iii) Non-targeted radiation effects at distant tumors:** In this, we have studied the possibility to control the distant tumors, when only primary tumor was irradiated. Such studies have gained attention of researchers as well as clinicians as they can be exploited to enhance radio-immunotherapy of metastatic tumors and prevent post-irradiation tumor recurrence. To simulate NTRE at distant tumors, mouse fibrosarcoma tumors were developed in both the hind limbs. While one of the tumors was irradiated with gamma radiation, the other tumor and animal body parts were shielded. Decrease in tumor growth in non-irradiated shielded tumor was observed when tumor in another leg was irradiated, which was more prominent at higher doses than

conventional therapeutic dose (2 Gy). The directly irradiated tumors showed expression of immunogenic cell death markers. To enhance the NTRE in distant tumors, radiation in combination with CpG-ODN (cytidine phosphate guanosine-oligonucleotides), an immunomodulatory oligo, was administered in the irradiated tumor after delivering radiation dose to the tumor. CpG-ODN in conjunction with radiation resulted in better tumor control. The tumor growth-inhibitory effects were mediated through increase in immunomodulatory markers and induction of apoptosis in the shielded tumors. It was interesting to observe that in these animals freshly transplanted tumor cells didn't produce tumors suggesting long lasting non-targeted radiation effects.

Gap junction or cellular synapses are the major direct cell-to-cell contact modality for intercellular communication in normal and tumor microenvironment (TME) conditions. In addition to these modalities of communication, intercellular bridges widely referred to as tunneling nanotubes (TNTs) or shedding of independently migrating viable cell fragments (VCFs) referred to as microplasts or cytoplasts in cancer cells/tissues have gained attention of researchers as emerging modes of cellular interaction. TNTs are actin-based membrane bound cytoplasmic bridges formed between donor and recipient cell, through which wide variety of cellular cargos and organelles can be directly transported to distantly connected cells (up to few hundred microns). Cellular communication through TNTs have known to govern several processes during cancer pathogenesis and chemo-resistance. For instance, the drug resistant phenotypes were found to spread through TNTs in the cancer population by intercellular transfer of ABC transporter P-gp, mitochondria or miRNA (2023). In our laboratory, TNTs and microplast formation were studied in human breast cancer cells treated with macrophage conditioned medium. Mitochondria, vesicles and cytoplasm could be transferred from parent cell body to microplasts through connecting TNTs. Microplast formation was inhibited in the presence of cell migration inhibitor, cytochalasin-B. Metalloproteinases (MMP) activity localized in vesicles in the cell body as well as in microplasts suggests their potential role in the process of invasion.

7. Research on Thorium Toxicity and Radiotoxicity: Cell and Animal Models

Dr. Bhabha has envisioned Thorium-based nuclear research programme for DAE. Therefore, it was imperative for radiation biologists to carry out fundamental research towards understanding the radiobiological effects of Thorium and other relevant radionuclides in human cells and experimental animals. By 2002, Atomic Energy Research Institutes of several nations (Japan, France, US and UK) have conceived such research programs and developed dedicated Departments/Institutes for development of *Nuclear Radiobiology Research* (**Fig. 6**). India has the largest Thorium reserve and operating sand beach mineral separation plants under Indian Rare Earth Limited (IREL, DAE) at Manavalakurichi, Aluva, Udyogmondal and Chavara for separation of various minerals including monazite (Th ore) and Orissa Sand Complex (OSCOM) is a dedicated facility of IREL for extraction and purification of Thorium from monazite. In addition, DAE has developed the long-term plan for advanced nuclear reactor technologies for

Thorium utilization. Therefore, a need for establishing '***Thorium-centric nuclear radiobiology research***' program at BARC was recognized. The potential implications of Thorium biology research are **i)** fundamental high-LET radiobiology, **ii)** targeted alpha cancer therapy and **iii)** development of novel decorporation strategies for management of internal nuclear contamination.

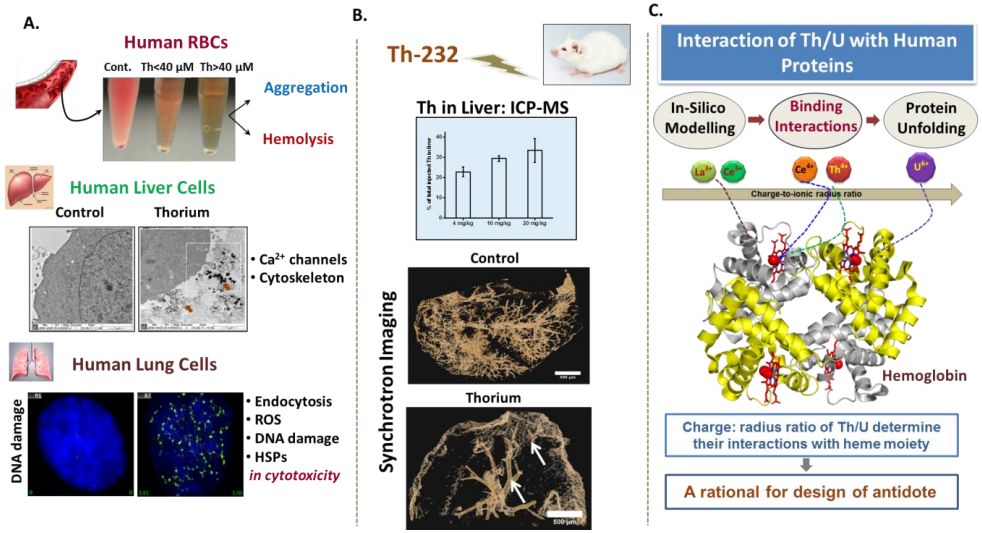

Fig. 6: An overview of nuclear radiobiology research highlighting the radiobiological responses of Th-232 in (A) human cells and (B) target organ (mice liver) as well as (C) the mechanism of interaction of Thorium with human blood protein (hemoglobin)

7.1. Understanding the mechanism of radiobiological response to Thorium

In RB&HSD, research has been pursued to understand the basic mechanisms of Thorium interaction with human cells and proteins and its consequences. A decade-long research activities revealed crucial answers for **i**) the mechanism of effects of Thorium in human cells; **ii**) the mechanism of cellular internalization of Thorium; **iii**) fundamental aspects of the binding of Thorium to protein and their functional consequences; and **iv**) major target organs of Thorium and their early and late chronic effects. Understanding these aspects of Thorium at organ, cell and molecular levels using biophysical, biochemical, microscopic, spectroscopic and computational approaches led to the rational design and development of antidotes for removal of Thorium and mitigation of its associated radiological and chemical effects (**Fig. 6**). Thus, the Bio-Thorium Research Program at BARC has significant implications for developing India's capability for efficient utilization of Thorium with adequate human health and environmental protection.

Research was primarily focused on understanding the mechanism of toxic effects of Thorium in human cells viz. red blood cells, lung cells, liver cells, and bone, which represent the target organ/sites of Thorium accumulation/toxicity in human/animal models (**Fig. 6**). Our experimental data revealed that radiobiological response to Thorium depends on the cell type, chemical forms, concentration and exposure time of Thorium.

Briefly, in human lung cells, Th-dioxide (colloidal) was found to be more toxic than the Th-nitrate at equivalent metallic concentration of Thorium. This was found to be due to higher uptake of Th in cells exposed to its dioxide form as compared to the Thorium uptake from its nitrate form. Moreover, transmission electron microscopy in combination with confocal microscopy revealed the mechanism of Thorium internalization, which was found to be via clathrin/caveolin-mediated endocytosis following Th-dioxide exposure as compared to membrane perforation in cells exposed to Th-nitrate. Following internalization, Thorium induces oxidative stress, DNA damage response and proteotoxic stress, which play major roles in determining cytotoxicity. Following transmigration through air-blood barrier in lung, Th gets circulated via blood and finally accumulates in liver and skeleton. Our studies on human liver cells have identified the role of cytoplasmic calcium in Thorium uptake, suggesting the possible application of calcium modulators for minimizing Th internalization. Using ultrasensitive analytical techniques, we have identified cytoskeleton as the major intracellular target of Thorium. Interestingly, effect of Thorium on human red blood cells (RBCs) was found to be determined by Th:RBCs ratio. Lower Th-to-RBCs ratio caused aggregation due to neutralization of surface negative charge. However, at higher Th-to-RBCs ratio, RBCs undergoes cell lysis (hemolysis) through colloid-osmotic mechanism. The mechanistic understanding of Th-induced cytotoxicity has significant implications to develop rational approaches for mitigation of Th-toxicity.

During circulation in blood, Thorium ions can also interact (in addition to RBCs) with soluble proteins such as albumin/globulin in blood plasma as well as haemoglobin localized on RBC's membrane. Using biophysical tools, the binding sites of Thorium ions in human serum albumin and haemoglobin proteins were investigated. Interestingly, Th ions were found to perturb the structural and functional integrity of Fe-containing heme of haemoglobin. This was due to the similar charge-to-ionic-radii ratio of Th with Iron. This led to understand the binding of Th at Fe-binding sites in iron transport/storage proteins (e.g. transferrin, ferritin, catalase, etc.). Thorium interaction with haemoglobin was further investigated in the environmentally-important aquatic midge, *Chironomus*, which may serve as bioindicator of Th contamination in aquatic systems. Importantly, our spectroscopic data determined the ability of actinide ions including Thorium to unfold the proteins with significant alteration in their functionally-important conformations. In this direction, further research is being pursued using computational modelling and biochemical approaches to characterize the bio-coordination of Thorium and other actinide ions with relevant proteins, which have been identified as a molecular target of toxicity.

Extensive studies in experimental mice/rat models were carried out to identify the major sites of Th accumulation and underlying mechanism of toxicity at cellular and molecular levels. Liver, skeleton and spleen were found to be the major target organs of Thorium following its administration. We have determined the biodistribution, histological and functional changes as well as alterations in gene expression in organs/tissues after 6 to 12 months of Thorium exposure. Our recent analysis of gene expression and system biology

approaches highlighted the involvement of β-catenin/Myc driven signalling pathways in Thorium-induced oncogenesis in mice.

Since inhalation can be a potential route of radionuclide exposure in occupational and accidental scenarios, a facility dedicated to simulate inhalation route of exposure in animals (nose-only inhalation exposure facility) was developed, which led to inhalation nuclear toxicology research at BARC for understanding the response of lung tissues to chronic and acute doses of Thorium and other radiometals/toxicants/materials. Recently, using this facility, effect of Th aerosols exposure was investigated in Swiss mice, which revealed alterations in the expression of functionally-essential lung surfactant proteins. This facility will be used for evaluation of newly designed and synthesized chelating agents for development of rational decorporation therapy for various radionuclides relevant to Indian Nuclear Fuel Cycle.

7.2. Technology Development for Decorporation of Internalized Thorium

Design and development of effective mitigation approaches for internal contamination with Thorium and other radiometals in human and environment is the need of the hour. Presently, there is no FDA-approved antidote for treatment of human subjects in case of Thorium contamination. DTPA (Ca/Zn salt of diethylenetriamminepentacetate) is the only available agent recommended for Plutonium, Americium and Curium contamination. Our experience from animal experiments suggested inability of DTPA to remove Thorium from liver and skeleton. Therefore, further research was pursued to design Th-specific rational antidote. Our learning experience for the mechanism of Th interaction at cell/protein levels has provided significant clues about the biological ligands (proteins/DNA, etc.) with which antidote (chelator) need to potentially compete for Th decorporation. In this direction, multidisciplinary approaches were adopted such as **i)** rationally-selected existing molecules; **ii)** rationally-designed new agents and **iii)** improvement of organ distribution profile of chelators using drug delivery systems. Our efforts on screening of rationally-selected existing molecules have identified L52S (a hepatoprotective formulation) for its potential of Th decorporation in comparison to DTPA as a reference agent (**Fig. 7**).

Further research has been extended to gain deeper insights about the cellular and molecular mechanisms of radiobiological responses of cells/tissues to Thorium and other heavy metal radionuclides. These biological investigations would lead to the identification of sensitive and specific biomarkers of Thorium exposure/effects. Extensive efforts are underway to develop rational and novel decorporation agents/therapy for removal of internalized Th/other radiometals from lungs, surface wounds and other vital organs based on the understanding of mechanisms of their toxicity and interaction. Fundamental understanding of mechanisms of Th interaction/toxicity would help in optimizing the combination of chelating agent and pharmacological inhibitor of Th-specific toxicological processes for enhanced decorporation and mitigation of toxicity.

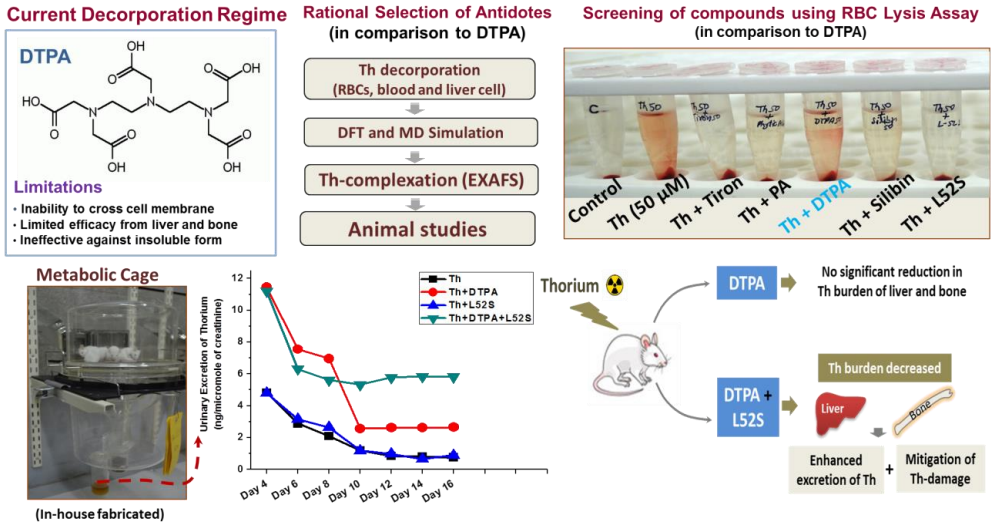

Fig. 7: Screening of rationally-selected agents for Thorium decorporation and mitigation. Top right panel shows the relative efficacy of selected agents for preventing hemolysis as a measure of Thorium chelation in comparison to DTPA. Lower left panel shows in-house fabricated metabolic cage for determination of Thorium decorporation efficacy in mice. L52S, a lead candidate was found to significantly enhance Th excretion efficacy of DTPA through urine and mitigated Th-toxicity in liver.

8. New Approach to Improve Cancer Radiotherapy: Nanomedicine

The most challenging part for any modality of cancer treatment is the tumor-specific delivery of anti-cancer drug or radiation dose for maximizing the damage to tumor cells, while sparring the surrounding normal tissue. Although, around 60 % of solid tumors are treated with Chemo-Radio Therapy (CRT), its non-tumor-specificity severely hampers its therapeutic efficacy, resulting in dose-limiting toxicities and subsequent development of treatment resistance. Thus, a holistic approach that can simultaneously improve the selectivity of anti-cancer drugs to the tumor, minimize their adverse health effects and augment the efficacy of radiotherapy would be desirable by clinicians for achieving better therapeutic outcomes. In this direction, nanotechnology has great potential to contribute by facilitating targeted delivery of the anti-cancer drugs to the tumor site, as well as by, augmenting and facilitating the application of multi-modal therapies including hyperthermia and targeted CRT. Hyperthermia therapy (HT) has been used in clinics as an adjuvant to CRT to enhance its therapeutic efficacy. HT involves heating the tumor in the range of 40-43°C, which can either kill the cancer cells directly or sensitize them for subsequent CRT. In clinics, conventional HT is applied using infra-red, microwaves, high intensity focused ultrasound, sauna bath or water bath. However, conventional HT suffers the limitations of in-sufficient and non-homogenous heating of tumors often leading to development of thermo-tolerance during subsequent HT sessions.

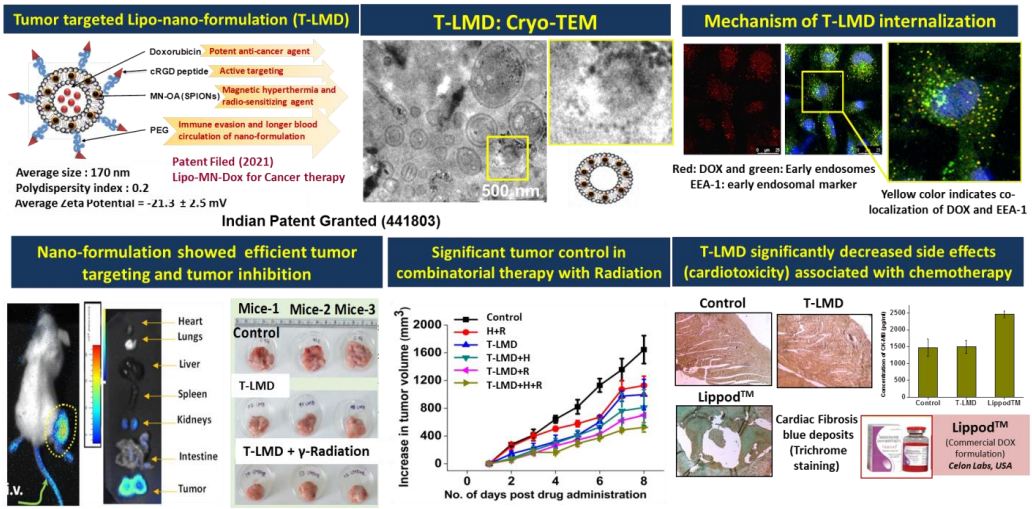

Fig. 8: Top left panel shows the patented design of tumor-targeted liposomes encapsulated with magnetic nanoparticles and anticancer drug (T-LMD) for multimodal chemotherapy, radiotherapy and hyperthermia therapy of cancer. Lower left panel shows specific targeting of tumor by T-LMD, which enhances the effect of gamma radiotherapy. Lower right panel shows minimal-to-no side effects of T-LMD in cardiac tissues in comparison to commercially available liposomal-dox formulation (Lippod^{TM}).

Thus, development of alternate and more efficient hyperthermia modalities with ability to induce nano-heating effects at cellular/molecular level becomes vital. Photothermal therapy (PTT) and magnetic hyperthermia therapy (MHT) are two such modalities with superior hyperthermia efficacies and better therapeutic abilities as compared to conventional HT. In this direction, our laboratory has been working since the year 2008 towards development of rationally designed nanoparticles for improvement of therapeutic efficacy of CRT. One of the first designs of nanoparticles developed in our laboratory comprised of super-paramagnetic iron-oxide nanoparticles (SPIONs) coated with oleic acid for delivering MHT to cancer cells. In MHT, SPIONs generate heat under the influence of an alternating current (AC) magnetic field (AMH) predominantly by Néel or Brownian relaxation. Unlike CHT, in MHT, SPIONs can be functionalized using tumor-targeting surface ligands to specifically internalize inside the tumor cells and distribute to cellular compartments sensitive to heat, such as plasma membrane. Thus, we developed oleic acid coated SPIONs termed as 'MN-OA' which can specifically target the cell membrane owing to its hydrophobic nature. We have demonstrated the membrane localization and significant anti-cancer efficacy of MN-OA in combination with MHT in cancer cells and animal tumor model. Moreover, MN-OA induced hyperthermia also resulted in significant enhancement of radiation induced DNA damage in cancer cells and animal tumor models. For the first time, our laboratory reported the role of HSP90 modulation in the mechanism of radio-sensitization and tumor growth inhibition after treatment with MN-OA, wherein interaction of MN-OA with intra-cellular HSP90,

probably via hydrophobic interactions, resulted in down-regulation of its down-stream client proteins playing important role in cell survival, cell cycle progression (AKT, CDC2, CyclinB1) and DNA repair (RAD51, CHK1 and BRCA1). Moreover, the role of mitotic catastrophe as an alternate cell death mechanism induced by MN-OA in combination with γ-radiation was also demonstrated.

For further improving the tumor targeting of MN-OA and to impart multi-modal tumor therapy, we designed a liposomal nano-formulation termed as 'T-LMD'. These liposomes were co-encapsulated with MN-OA and doxorubicin (DOX) in the bilayer and core of the liposomes, respectively (**Fig. 8**). Further, to impart tumor-specificity, they were functionalized with cyclic RGD (cRGD) peptide, which enables the targeting of αvβ3 integrin receptor over-expressing tumor cells (including triple negative breast carcinoma, melanoma, glioblastoma, ovarian and cervical cancers, etc.) as well as tumor neo-vasculature. In vitro evaluation showed significantly higher cyto-toxicity of T-LMD (average size of ~170 nm) as compared to commercial nano-formulation of liposomal-DOX (Lippod^{TM}), in cancer cells of skin, breast, lung and brain origin. Importantly, the cyto-toxicity of T-LMD was significantly lower in normal lung epithelial cells as compared to cancer cells. Moreover, T-LMD showed significant radio-sensitization of murine fibrosarcoma cells predominantly via activation of JNK mediated pro-apoptotic pathway. Furthermore, recent findings in our laboratory established ferroptosis induction by T-LMD in triple negative breast carcinoma cells and its tumor model in mice. T-LMD was found to be internalize in MDAMB-231 cells by clathrin and caveolin mediated endocytosis, followed by induction of lipid and cytosolic reactive oxygen species (ROS), damaging the cell's plasma membrane and mitochondria, ultimately culminating in increased DNA double strand breaks and cell death by ferroptosis. Release of immunogenic damage associated molecular patterns (DAMPs: HMGB-1) was also observed in culture supernatant of MDAMB-231 cells treated with T-LMD, suggesting the ability of T-LMD to activate anti-tumor immunity, a desired phenomenon reported to improve the therapeutic efficacy of CRT.

Most importantly, bio-distribution studies of T-LMD in live mice showed ~2-9 folds higher accumulation in fibrosarcoma tumors as compared to other off-target organs (Liver, spleen, kidney, heart and lungs), suggesting their superior tumor targeting ability. Moreover, in combination with radiation (R) or MHT (H) or both, T-LMD showed ~ 3-fold higher tumor growth inhibition compared to single treatments alone. Another important limitation for most of the clinically used anti-cancer drug is their systemic toxicity which severely hampers their therapeutic efficacy and negatively affects the quality of life in cancer patients. However, the nano-drug (T-LMD) developed in our laboratory showed significant inhibition of DOX induced cardio-toxicity in mice as suggested by in-significant induction of cardiac fibrosis studied by trichrome staining and immuno-fluorescence detection of phospho-Smad-3, which is one of the mediators of TGFβ-induced fibrosis in heart tissue. Moreover, serum levels of early cardiac damage marker, CK-MB were also found to be unaffected by T-LMD treatment as against Lippod^{TM} which showed significant increase in serum CK-MB levels as compared to

control (2022). These results suggest in-significant toxicity of T-LMD in healthy mice re-emphasizing its clinical potential as a targeted and multi-modal anti-tumor agent. Currently, toxicological studies in higher rodents and pharmaco-kinetic studies are being pursued to further facilitate the clinical translation of T-LMD.

9. Future Research in Radiation Biology

Radiobiology is a rapidly progressing field, and it opens new areas of research and brings new challenges to researchers. Intensive research in the past years has brought new knowledge to the forefront. Undisputedly, radiation damage to DNA continues to occupy central space in the basic research as well as applications but radiation damage to cell membrane and its role in the cell death remains inadequately investigated. Using microbeam technology, it may be important future task to determine the relative or exclusive role of radiation membrane oxidative damage or specific radiation targeted damage in cytosol in cell death process. It is important to understand and quantify the effects of High Linear Energy Transfer (HLET) radiation on human health with relevance to space exploration (Space Radiobiology). Further, the discovery of transmitting radiation effects from irradiated cells to unirradiated cells called bystander phenomenon has generated many new questions to address the implications to cancer therapeutic as well as risk as estimation task. Intensified research in future would allow assessment of impact on health. A new emerging area in radiobiology is the multifaceted role of Reactive Oxygen Species (ROS) in the oxidative mechanisms of survival and death of tumour cells. It is an active area of current radiobiology research how tumour cells form their strategy to survive anticancer therapies by cleverly manipulating intracellular balance of ROS and antioxidative capacity (AOC). It will be important to identify and control radiation sensitive genes and their implications in cellular and individual radiosensitivity. From the point of developing new technologies based on low dose radiation, it would be warranted and may prove highly rewarding to advance knowledge in low dose radiobiology with relevance to low dose health benefits and contributions to acceptance or denial of Linear-No-Threshold (LNT) model of radioprotection and radiation risk assessment goals for public health.

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GENESIS AND FUTURE OF BIOLOGICAL RESEARCH IN HIGH-LEVEL NATURAL RADIATION AREAS OF KERALA, INDIA

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Abstract

High level natural radiation areas (HLNRA) in India encompass a stretch of 55 km long and 0.5 km wide coastal belt along the Arabian sea, which extends from Kollam district to Alappuzha district of Kerala. The monazite containing beach sand contributes to 20-30 times higher background radiation in these areas compared to the global average of 2.4 mSv/year. In 1959, WHO's expert committee on radiation highlighted this area to investigate long-term biological effects of ionizing radiation. Early studies, during 1960-75 were focused on investigating genetic effects on black rats, radioactivity in food chain, demographic survey and aerial dosimetry. In 1975, "Monazite Survey Project" was started by Bio-science group, BARC to initiate studies involving epidemiology, cytogenetic analysis, cellular/molecular responses and cancer incidence in human populations. These investigations revealed no significant health risks and there was no increase in cancer incidence in the human population residing in HLNRA of Kerala. Current research focuses on radio-adaptive mechanisms,

genomic/epigenomic responses and addressing the low-dose radiation risk debate.

1. Introduction

Over millions of years, all forms of life on the earth have evolved in a radiation rich environment. We all are exposed to different types of radiations through natural as well as man-made sources. Although, naturally-occurring background radiation is the main source of exposure for living beings but the incidence of Hiroshima and Nagasaki atomic bombings in 1945, brought to the fore the urgent need to understand the long-term biological and health effects of ionizing radiation in human population. There are few areas around the world where the levels of natural background radiation from terrestrial and cosmic sources are significantly higher than the world average of 2.4mSv/year. These geographical places are termed as high-level natural radiation areas (HLNRA). Some of the predominant HLNRA's around the world are Guarapari (Brazil), Yangjiang (China), Ramsar (Iran) and Kerala (India). In early 1950's, the Karunagapally Taluk of Kollam district in Kerala was identified as one such area which had 2-20 times higher levels of background radiation due to thorium-232 containing monazite beach sand. The coastal zone from Sakthikulangara in Kollam district in the south to Purakkad in Alappuzha district (north) is a 55 km long and 0.5 km wide stretch skirted by Arabian sea and western ghats.

The large deposits of monazite sand in this coastal belt were discovered as early as 1909. The main sources of monazite are beach boulders and sedimentary rocks which include khondalites, chamockites, gneiss and granites present in the granulitic terrain of southern India mostly in parts of Tamilnadu and Kerala. They are transported to the sea by many lakes (e.g. Ashtamudi) and rivers (e.g. Kallada) flowing through this terrain and also some sort of panning action deposits the heavy metallic sand along the seacoast (UNSCEAR 2017 report).

The HLNRA of Kerala coast came to highlight through the WHO's expert committee report on "Effects of Radiation on Human Heredity" in the year 1959. The WHO committee mentioned that "Only in the light of more knowledge can decisions be taken to define more accurately the maximum amount of exposure which may be accepted by individuals and populations without risk of serious harm." At the same time, they noted that "Kerala area of India is one untapped source of information, which might be profitably investigated" (WHO technical report, 1959 and UNSCEAR 2017 report).

Dr. Homi Bhabha, the founder-architect of India's Atomic Energy program being the visionary leader envisaged the importance of biological research in nuclear establishment of our country and recruited Dr. A.R Gopal-Ayenger as first biologist to Head, Biology Division in Atomic Energy Establishment, Trombay. Dr. Gopal-Ayenger carried out pioneering work in establishing low dose radiation program and was a member of WHO expert committee on radiation which in its first report brought out a comprehensive plan with a section titled "The Kerala Project" for the study of long-term consequences to human population exposed to continuous doses of high-level natural radiation for several

generations. The committee further observed that "Such is the present status of knowledge of the somatic and genetic effects of chronic low-level exposures that any proper investigation of areas of high natural radiation is certain to contribute to the fund of biological knowledge and the ultimate specification of the genetic risks accruing from increasing exposure to ionizing radiations" (WHO technical report, 1959 and UNSCEAR 2017 report).

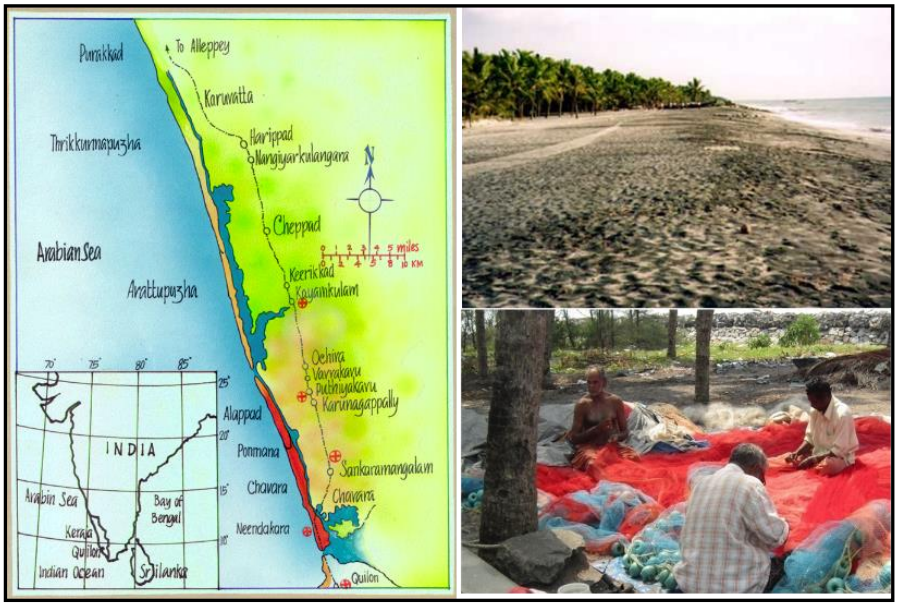

Fig. 1: Geographical representation of High-level natural radiation areas at Kerala coast in southwest India (left). The monazite containing beach sand and fishermen sitting on it (right).

2. Historical perspective:

2.1. Pioneering work on genetic effects of chronic radiation in HLNRA, Kerala:

The pioneering work to understand the genetic effects of chronic low-level radiation in Kerala was carried out on black rats, scientific name; "Rattus rattus L" by noted British geneticist Dr. Gruneberg in collaboration with Dr. Gopal-Ayenger and his team with the support of radiobiologist Dr. L.H. Gray. The work was started in the autumn of 1961 in a makeshift laboratory at Sree Narayana College at Kollam with the support of then principal Dr. M. Sreenivasan and Dr. S. Sivaprasad, a Professor of Zoology.

The black rats were chosen for this seminal study because they were the only mammals other than humans to exist in large numbers, Initially, it was thought to include one more mammal species-the bandicoots in the study, but due to insufficient numbers, it was later decided to exclude them. The study was carried out in a total of 896 black rats, out of which 438 rats were captured from house-holds of eight villages of Neendakara,

Puthenthura, Kovilthottam, Panmana, Cheriazhiekal, Alappad, Srayikkadu and Aziekal which were located near black sand beaches and 458 rats were caught from eight villages which were far from the coastal area and not exposed or migrated from high radiation areas. The radiation exposure dose on the coast was 7-8 times greater than the control value, although dosimetry was not very accurate during those days but the study was designed with great accuracy, precision and finesse.

Dr. Gruneberg and his team carried out detailed measurements of dental structure, minor and major changes in skeleton structure and reproductive pattern in these black rats. Analyzing the results with refined statistical procedures, Dr. Gruneberg and his team concluded and wrote in an epilogue that "There is no evidence for any consistent and systematic difference between the strip and control populations which might be reasonably attributed to radiation". He further stated "In view of the absence of any strong and consistent pattern of difference, the only possible conclusion seems to be that if there is any effect of radiation it is masked by the variation already existing within both areas from population to population." They summarized the absence of any genetic effects of exposure to high natural radioactivity in the black rat of Karunagapally with four possible scenarios. 1. Gamma rays at extremely low dose rate do not produce mutations. 2. Mutations were nullified by natural selection. 3. There might have been genetic change but those were masked by environmental change. 4. Accidents of sampling might have obscured a real effect (Gruneberg et al, 1966, A.P. Jayaraman 2005).

The studies on black rats of Karunagapally were further taken up by Mohapatra et al. at Utkal University, Bhubaneshwar, Orissa. During 1982-1986, they caught 345 black rats; 135 from Chavara,125 from Chatrapur and 85 from Bhubaneshwar and carried out cytogenetic studies on the bone marrows of these rats and discovered paired dot-like structures called double-minutes in the black rats captured from radioactive zone. They concluded that "the double minutes are gene-amplifying elements which cook up extra protein to counter the cell from harmful assaults and the appearance of double minutes in the progenitive tissue of rats is supposed to defend the animal from the background radiation shock."

In 1972, Dr. Gopal-Ayenger and his team conducted a demographic survey for various reproductive parameters such as fertility index, sex ratio and infant mortality in 70,000 human residents of these areas. No significant difference in these parameters were reported in residents of HLNRA in comparison to residents of adjacent normal level natural radiation areas (NLNRA).

2.2. Pioneering work on radioactivity in food chain in HLNRA, Kerala:

In another pioneering work during 1970's, Dr. Gopal-Ayengar and his colleagues carried out a dietary survey to collect data on the quantity of diverse foodstuff eaten by four hundred families of twenty-three south west coastal villages in Kerala and measured the profile of radioactive inputs in these food items. The study revealed the signature of the food style of Kerala coastal villages. They observed that cereals, roots and tubers and flesh foods make up most of the diet followed by milk and milk products. Dr. Gopal-

Ayenger and his team assiduously estimated daily intake of radioactivity by an individual through their dietary habits. They found that alpha and gamma activity was highest in fish and lowest in milk and coconut, respectively. Beta activity was highest in lady's finger and lowest in rice. Potassium 40 was maximum in plantains and minimum in rice. Tapioca is eaten in Kerala as energy source to meet the short supply of cereals during draught conditions. In studies to measure the uptake of natural radioactivity, it was observed that thorium content of tapioca was proportional to the thorium content of the soil and potassium was also found in high measure in tapioca (Mistry et al, 1970 and A.P. Jayaraman 2005). The seminal findings of these pioneering works prompted multiple questions and to answer these questions a new project "The Monazite Survey Project" was established in BARC.

2.3. Monazite Survey Project established in Kerala:

In 1975, Monazite Survey Project (MSP) was initiated by Bio-science/Bio-Medical group, BARC with an outstation lab set up at Medical College Health Unit, Neendakara, Kerala. Mr. K.P George was designated as first Officer-In-Charge of the laboratory. The project was started with a goal to study the biological and health effects of high background radiation in human population. The major works envisaged under the projects were dosimetry survey, Health audit and demographic survey of the population, Cytogenetic studies in newborn and adult population to detect karyotype anomalies and chromosomal aberrations in HLNR and NLNR areas, Cytogenetic studies among occupational workers from various DAE units, Cytogenetic studies on plants and rodents among others. A country wide survey of outdoor natural background radiation levels reported highest air-kerma in the monazite areas of Kerala. The studies showed the average per capita dose received by the population of this area was about four times the normal background radiation levels.

In 1976, Kochupillai et al. published a study in Nature, where they reported higher prevalence of Down's syndrome in the HLNRA population. However, the study was highly criticized for its shortcomings in design and interpretation and a strong rebuttal was written by Dr. Sundaram, then Director, Bioscience (Medical) Group and was published in the same journal.

Subsequently, Bhabha Atomic Research Centre, Mumbai signed a Memorandum of Understanding (MoU) with Department of Health & Family Welfare, Directorate of Health Services, Govt. of Kerala in 1986 to carry out collaborative studies in human population of normal and high-level natural radiation areas of Kerala coast. The major studies initiated under this MoU were cytogenetic analysis among newborns to estimate the frequency of chromosome aberration and karyotype anomalies, newborn survey to identify major congenital malformations and /or genetic disorders. The MoU is continued since then.

In 1988, the field laboratory of MSP was shifted from Neendakara to a rental space in Indian rare earth limited (IREL) campus, Beach Road, Kollam. The MSP project received major momentum and thrust under the leadership of Dr. Anil Kakodkar, then Director, BARC and Dr. P.S. Chauhan, then Head, Cell Biology Division, BARC &

Project Manager, MSP. Dr. Kakodkar inaugurated the new building of Monazite Survey Project (MSP) on September 4, 1999 at Kollam, Kerala, in the presence of Dr. A.M Samuel, then Director, Bio-Medical group. Dr. Kakodkar rechristened MSP as Low-Level Radiation Research Laboratory (LLRRL). At the same time, an independent section Low Level Radiation Research Section (LLRRS) was also created in Bio-Medical group, BARC at Trombay and Dr. M. Seshadri was made Section Head and Project Manager.

Fig. 2: Dr. Anil Kakodkar, then Director, BARC, inaugurating the new building of Monazite Survey Project (Low level Radiation Research Laboratory) at Kollam, Kerala

In his inaugural address, Dr. Kakodkar appreciated the importance of the studies being carried out at MSP and their significance to the people of the monazite belt. He also emphasized that the facilities at MSP (LLRRL) should be continuously updated so that people from other center's in the country will be encouraged to interact with BARC scientists to seek answers to the basic biological questions pertinent to the exposure of human population to continuous low-level radiation. He desired that LLRRL should become a unique centre for research in this area of life sciences and health sciences (BARC Newsletter 1999). Over the years, multiple collaborative studies with different universities and research institutes have been carried out at LLRRS Mumbai and LLRRL, Kollam. In 2010, for the first time in India, 7th International conference on high levels of natural radiation and radon areas was organized by Bio-Medical Group, BARC in association with International Committee on High Level Natural Radiation and Radon Areas (ICHLNRRA) and Indian Association for Radiation Protection (IARP) and Indian Aerosol Science and Technology Association (IASTA) at Mumbai, India.

2.4. Health effects of high background radiation in HLNRA, Kerala:

In 1990's, Regional Cancer Centre (RCC), Trivandrum in collaboration with BARC, initiated an epidemiology study to investigate health effects of HBR on Karunagapally population. A cohort of 385,103 residents of the Karunagapally taluk was selected for the epidemiological survey. During 1990-97, house to house surveys were carried out to record indoor and outdoor dose and document personal information on sociological and demographic factors including lifestyle, diet, tobacco chewing and alcohol consumption. In total, personal information on 359,619 subjects in 71,674 households which constituted 93% of the population was collected. (UNSCEAR 2017 report).

First cancer registry was established in Karunagapally in 1990. Dr. M. Krishnan Nair and his team from RCC Trivandrum established a sub-cohort of 173,067 individuals from six panchayats (Alappad, Chavara, Neendakara, Panmana, Oachira and Thevalakkara) and followed them up for more than 2 decades to investigate cancer incidence rate in these individuals. Recently, the results of above study were published and it was reported that "there is no elevated risk of cancer associated with high background radiation exposure in this cohort (Amma et al. 2021)". Moreover, the excess relative risk (ERR) of cancer excluding leukemia was -0.05/Gy (95% CI:-0.33, 0.29).

3. Current status of biological studies at LLRRS, BSG and Future direction:

For the last 3 decades, scientists at LLRRS, Mumbai and LLRRL, Kollam have carried out extensive investigations on biological and health effects of HBR in Kerala population. Multitude of studies involving epidemiology, cytogenetics, and molecular biology have been carried out in blood samples collected from HLNRA and NLNRA population. The prevalence of congenital malformation/birth defects has been analyzed in more than 2,10,000 newborns, chromosomal abnormalities have been analyzed in 27,295 newborns as well as in adult population. DNA damage studies using multiple biomarkers such as gamma-H2AX, telomere length, micronuclei, *HPRT* gene mutation, excess PCC fragments, and DNA strand breaks (comet assay) have been carried out. High-throughput transcriptomics and proteomics analysis have been done to understand the molecular mechanisms of radio-adaptive response in Karunagapally population. These studies did not reveal any adverse effect of chronic high background radiation in human population. Moreover, better and more efficient DNA repair mechanism were shown to be active in HLNRA population. All these studies have been published in reputed peer-reviewed journals. The importance of these studies is reflected in several reports of United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR), where these publications have been included. The ongoing and future studies are being focused on deciphering the mechanism of radio-adaptive response and healthy ageing in HLNRA population. Large-scale multi-omics studies are being planned to investigate transgenerational changes at genomic and epigenomic level in multiple organism models to elucidate the effect of high-level natural radiation on living beings. These studies will provide in-depth insights into genetic changes in response to chronic low dose radiation

exposure and also might answer the linear no threshold (LNT) conundrum at low dose radiation.

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IMMUNOLOGY RESEARCH: INTERCONNECTED ENDEAVOURS IN RELATION TO LOW DOSE RADIATION, NATURAL PRODUCTS AND CANCER

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Abstract

Immune response is central to our existence, and after perturbation, be it physical, chemical, or biological, the first responders will be the cells of the immune system. Over the years, immunology research at BARC has been focused on the following areas: immune alterations following radiation exposures, immunomodulators, basic research on cancer-immune system interactions, and epigenetic changes in immune cells. Radiation exposure can be too low or high doses, acute or chronic. Diagnostic procedures are the primary source of low dose acute exposures, and nuclear workers or people living in high background radiation areas are exposed to low dose fractionated or chronic exposures. Acute high doses could be from accident scenarios, and high dose fractionated exposures are usually administered to the tumors in cancer patients. Changes in immune responses can occur in each of these scenarios, and these changes could also reflect inter-individual variations. Over many years, we have studied different aspects of immunological alterations following radiation exposure. Extensive research has been carried out on immunomodulators derived from both natural products and synthetic chemicals. For example, G1-4A, a polysaccharide

isolated from *Tinospora cordifolia*, MAMPDM, a red pigment isolated from *Serratia marcescens*, and several others. Immunomodulatory effects of synthetic chemical inhibitors discovered through fundamental research were also investigated, for example, COX-2 inhibitor NS-398 and TGF-β inhibitor SB431542. Apart from the cancer cells, the tumor microenvironment comprises several other cell types, like infiltrating immune cells, fibroblasts, and pericytes. We have been studying the relationship between cancer and immune cells like dendritic cells, macrophages, B cells, and T cells to identify immunosuppressive mechanisms employed by tumor cells and develop strategies to overcome them. Many immune cells have several subtypes, and based on the surrounding microenvironment, cells can differentiate from one type to another, this process is regulated by epigenetic changes, such as DNA or histone modifications or miRNA. For example, miR365 was found to negatively regulate IL-6 secretion; EPZ004777 and FG2214 were identified as epigenetic inhibitors of Treg cells. Understanding the intricate interplay between immune cells and tumor cells is crucial for developing effective cancer immunotherapies. By targeting specific epigenetic modifications, or microRNAs, researchers hope to enhance anti-tumor immune responses and improve patient outcomes.

1. Preamble

The immune system is the backbone of our daily defense against apparent and invisible threats. These can include a variety of agents like microbes, their products, transformed cells, ionizing radiation, pollutants, and diverse chemicals. Immunosuppression is associated with exposure to acute high doses of radiation. But the dose-effect relationship for immune responses at lower doses has been found to be different, and even stimulation has been reported. The Bhabha Atomic Research Centre (BARC), Department of Atomic Energy, emphasizes research into the consequences of low doses of radiation exposure. This is especially relevant in the light of the linear-no threshold (LNT) model of radioprotection, which is used to evaluate the risk of different stochastic health effects due to exposure to ionizing radiation. The LNT model states that exposure to radiation, regardless of how small the dose, is an increased cancer risk for humans. However, animal data on low-dose exposures are contradictory. Interestingly, epidemiological studies in high-natural-background radiation areas and nuclear power workers have shown decreased cancer mortality compared to controls. Therefore, we investigated the effects of low-dose radiation on the immune system in radiosensitive and resistant mouse strains and the associated signaling mechanisms. Another important area of research is the identification of novel immunomodulators. Cells of the immune system are differentiated quiescent cells and serve as the human body's defense mechanism. The signaling mechanisms are extremely sensitive, and any slight alterations might trigger an alert. This could result in the activation of diverse immune cells to varying degrees. This could impact their proliferation or differentiation, causing changes in their cytokine

secretion pattern. Cytokines are soluble mediators that circulate in the blood and reach all organs. Most of these effects are transient, and the cytokines remain elevated for a short time before returning to their resting condition. There are other regulatory mechanisms in the body that ensure that immune activation is temporary. However, in situations such as malignancies, both the immune system and the inflammation that occurs in the microenvironment play important roles in cancer growth and progression. In the early stages of cancer, the immune system can exert control and fight against the cancer cells. Several immune cells, like dendritic cells, macrophages, NK cells, and T cells, are involved in the detection and elimination of the transformed cells with the help of soluble mediators like tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), perforins, and granzymes. However, if tumor cells proliferate rapidly, the immune cells are unequipped to stop them. In addition, tumors generate a variety of immune evasion and immunosuppressive strategies to promote their proliferation. At this stage, the tumor cells completely overwhelm the immune system. At BARC, we have been studying each of these research areas to better understand the underlying mechanisms and find targets that can be blocked by drugs in order to restore the immune system. Additionally, we have also studied immunomodulatory agents of plant origin, microbial origin, and synthetic chemicals identified through mechanism-based investigations.

2. Brief history and scope of immunology activities

In the early 1960s, it emerged that the hematopoietic system was highly vulnerable to the deleterious effects of ionizing radiation, and death in irradiated animals could be prevented by reconstitution with syngeneic bone marrow cells. Since hematopoietic stem cells are also the progenitors of the cells of the immune system, it was also recognized that immunological responses would be adversely affected by exposure to ionizing radiation. The science of immunology was still in its infancy in the 1960s, and the pivotal role of the lymphocyte was beginning to be established. In the Bio-Medical group of BARC, studies on the immune system were initiated by Dr. K. Sundaram and Dr. G. P. Phondke around this time. Their initial work was related to the biophysical characterization of the lymph node cells (LNC) from normal and *S. typhi* immunized Wistar rats in terms of their electrophoretic mobilities (EPM) as measured using the technique of 'Cell Electrophoresis'. It was shown for the first time that immunization produced a reduction in the EPM of LNC. Interestingly, the heterogeneity among lymphocytes in terms of T cells and B cells was being reported in the literature around this time, and in our studies too, such a heterogeneity was demonstrated in LNC in terms of their mean EPM, with T cells being more electronegative than B cells. The technique of cell electrophoresis was extensively harnessed in the subsequent years to monitor cell-mediated immune phenomena like delayed type hypersensitivity, allograft rejection, and the development of spontaneous lymphoblastic leukemia in AKR mice.

Studies on the electrokinetic properties of normal (unimmunized), immune, and malignant lymphocytes from rats and mice led to two interesting lines of research involving interactions of lymphocytes with ligands like antibodies and a polyclonal lectin

mitogen, Concanavalin A (Con A). In the first set of investigations, heterologous antilymphocyte sera (ALS) were prepared against normal (ALS-N) and immune lymphocytes (ALS-I). It was demonstrated that the sera against nonimmune lymphocytes (ALS-N) suppressed the humoral as well as cell mediated responses in rats only when administered prior to immunization with a specific antigen (sheep red blood cells-SRBC) and not after antigen exposure. On the other hand, administration of sera against immune lymphocytes (ALS-I) specifically suppressed the humoral and cellular responses when administered after antigen exposure due to the presence of anti-idiotypic/anti-clonotypic antibodies. This was the beginning of the later extensive studies on immunomodulation, which till today, involve several different contrivances or agents like acute and fractionated ionizing radiation, plant-derived natural products, antioxidants, and drugs.

Until monoclonal responses of T and B cells could be evaluated experimentally, responses to polyclonal mitogens served as a model for understanding the events associated with antigen-induced stimulation of lymphocytes. A requirement for such stimulation was the induction of redistribution of their receptors on lymphocytes after interaction with such ligands. Cell electrophoresis was used to study the redistribution of receptors to the polyclonal T cell mitogen Con A in the splenic lymphocytes of AKR mice. The EPM increased in conditions favoring redistribution of ligand-receptor complexes at mitogenic concentrations and decreased at supra-mitogenic concentrations of Con A. A detailed assessment of this phenomenon led to the demonstration, for the first time, of two sets of receptors for Con A on the surface of normal, healthy lymphocytes. One that underwent redistribution, like the formation of clusters, patches, and caps, which led to an increase in EPM. The second set appeared consequently. Using Con A labeled with two different fluorochromes and fluorescence microscopy, these two sets could be vividly seen. Since at that time Con A-induced enhanced agglutination was being described as a characteristic of malignant cells, similar studies were performed on the lymphocytes of malignant AKR mice. They showed the presence of only one type of receptor, which showed inhibition of lateral mobility at supra-mitogenic concentrations. It was proposed that the Con A-receptor interaction profiles of normal and leukemic cells could represent those of mature and immature T cells. Since the thymus is an organ in which maturation of T lymphocytes takes place during development, similar studies were carried out on adult thymus cells and hydrocortisone-resistant thymocytes, which supported such a proposition.

In the mid-1970s, studies in tumor immunology were also initiated by Dr. P. K. Ray and colleagues. It was observed that mouse tumor cells (fibrosarcoma) treated with the enzyme neuraminidase, which removed surface sialic acid residues, rendered the tumor cells susceptible to the cytotoxic action of lymphocytes and autologous serum. Even the normal cells thus treated were found to be susceptible to lysis by normal sera, as the treatment with neuraminidase probably exposed xenogeneic neoantigens. Immunotherapy of murine fibrosarcoma by immunization with neuraminidase-treated cells initially yielded positive results. Another aspect of the tumor immunology work was related to the curative effect of BCG administration on tumor growth in mice.

In the early 1980s, a detailed investigation of the effects of whole-body irradiation on delayed type hypersensitivity (DTH) was undertaken to decipher the radiosensitivity of naïve lymphocytes, antigen-presenting cells, DTH effector T cells, and the cells migrating at the site of the inflammatory response. While the naïve cells were found to be most radiosensitive, the effector T cells were shown to be most radioresistant. Thus, radiation effects on various cellular players in a specific immune response to a single antigen were evaluated. In the same system later, the effect of fractionated exposure to low-dose whole body gamma radiation was also studied.

In collaboration with Dr. R. S. Kamat's group at Haffkine Institute Mumbai, it was established that the route of antigen administration influenced the outcome of the immune response to *Mycobacteria*. While intradermal administration of the *Mycobacteria* induced a strong delayed type hypersensitivity response, intraperitoneal administration of the same antigen was immunosuppressive. It was further shown that this suppression was on account of the activation of $CD8^+$ T cells, which competed with the $CD4^+$ effector T cells for the cytokine interleukin-2 (IL-2).

3. Immunomodulation by low dose radiation and bystander effects

Exposure to both acute and fractionated whole-body low dose (<50 cGy) ionizing radiation exposures (LDR) alters immunological markers in mice. It remained unclear, however, if the immunological responses elicited by LDR would be universal and not influenced by genetic background. Many proteins, including p53, are activated in response to radiation, but the significance of p53 in the context of activation-induced apoptosis in LDR-induced immunomodulation was not understood. To answer these problems, two different strains, viz., C57BL/6 and BALB/c mice, were irradiated (4 cGy every day for 5 days a week, amounting to a total dose 20 cGy) to evaluate physiologically important functional responses. Delayed type hypersensitivity (DTH) and spleen cell polyclonal mitogen response were chosen as endpoints, and the antigens used for DTH were *Mycobacterium vaccae* or dinitrofluorobenzene (DNFB) and concanavalin A (Con A) as the mitogen for spleen cell response. Low-dose irradiated C57BL/6 mice had significantly increased Con A-induced spleen cell proliferation and suppressed DTH response to antigens as compared to sham-irradiated controls. In contrast, low-dose irradiated BALB/c mice had suppressed Con A-induced spleen cell proliferation and increased DTH response to antigens. The increase in Con A induced proliferation was primarily seen in $CD4^-(CD8^+)$ T cells in C57BL/6 mice, along with a decrease in p53-expressing cells and decreased apoptosis **(Fig. 1)**. A reverse pattern was observed in BALB/c mice spleen cells. Hence, it was concluded that, following LDR exposure, changes in the immune response are dependent upon the antigen, type of response, and mouse strain employed. These results also highlighted the important role of p53 and activation-induced apoptosis. The expression of several proteins involved in cell cycle and apoptosis was also studied. Increased expression of cyclins D and A, proliferating cell nuclear antigen (PCNA), and a decrease in caspase activity were observed in Con A-stimulated spleen cells of C57BL/6 mice **(Fig. 1)**. Further, it was confirmed that the

decrease in apoptosis was not due to changes in the expression of death signaling molecules like Fas or FasL but because of an increase in mitochondrial stability.

Apart from cell cycle changes, the responses of macrophages and T cell subpopulations were also evaluated in low dose irradiated C57BL/6 mice. Macrophage function was enhanced with low-dose radiation, as seen by increased nitric oxide release and phagocytosis. With respect to T cell subpopulations, there was activation of $CD8^+$ T cells as seen by increased expression of early activation marker CD69, proliferation response to alloantigens in a mixed lymphocyte reaction (MLR), and cytotoxicity response against tumor cells. Such an effect of LDR on $CD8^+$ T cells was demonstrated for the first time. These investigations also found that fractionated exposures resulted in a stronger long-term recovery response after challenge radiation dose, whereas acute radiation exposure resulted in a short-term, immediate adaptive response.

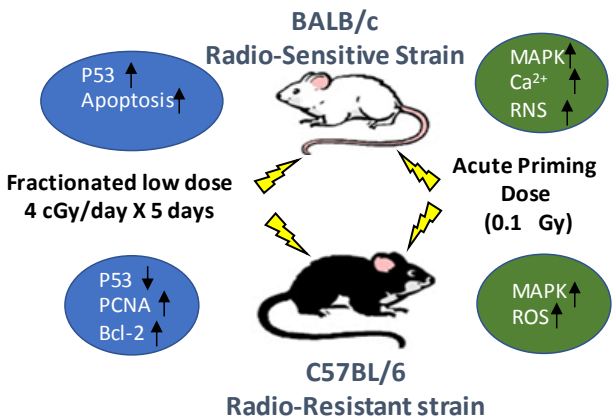

Fig. 1: Schematic representation of different pathways upregulated in radiosensitive and radioresistant strains of mice exposed to acute (0.1 Gy) or fractionated (0.2 Gy) low doses of radiation

Since spleen cells from LDR-irradiated mice showed an increased proliferation response, we explored if comparable effects could be found in low dose irradiated spleen cells. The transfer of conditioned medium (ICM) from such *in vitro* irradiated lymphocytes to unirradiated lymphocytes increased the proliferation response to Con A, with the highest increase observed with 0.5 Gy ICM. Treatment with ICM increased the generation of reactive oxygen species (ROS) as well as the release of nitric oxide (NO), along with increased expression of markers CD25 and cyclin D. Pre-treatment with ICM also resulted in an adaptive response to a challenge dose of radiation in lymphocytes. These studies demonstrated that soluble factors produced by irradiated lymphocytes activated a reactive oxygen/nitrogen species-mediated signaling cascade through medium transfer in control lymphocytes not exposed to radiation. This in turn resulted in enhanced mitogen

response as well as radio resistance in the control lymphocytes, which could play a significant role in radiation-induced immunomodulation.

Recent research has expanded on these findings to gain a deeper understanding of the nature and mechanism of adaptive responses (RAR) caused by prior exposure to low-dose radiation. After irradiating C57BL/6 and BALB/c mice with a low priming dose (PD, 0.1 Gy) or a high challenge dose (CD, 2 Gy) at a 4 h interval (P+CD) in the combination group, the inhibition of mitogenic responses in splenic lymphocytes was examined. The radio-adaptive response was evaluated in terms of DNA damage, early activation markers CD69 and CD71, cytokines IL-2, IFN-γ, and proliferation. The radiosensitive strain of mice, BALB/c, had a transient adaptive response 24 hours after CD, which was found to be due to LDR induced hyperactivation of MAPK signaling pathways in lymphocytes. These results, along with abrogation of the adaptive response by ERK and p38 inhibitors, indicated that LDR-induced MAPK signaling was responsible for the radio-adaptive response. On the other hand, the radioresistant strain, C57BL/6 mice, showed no RAR, either transient or late. However, MAPK activation was observed in spleen cells from both strains after exposure to LDR. Therefore, upstream signaling molecules such as reactive oxygen and nitrogen species (ROS, RNS) and calcium levels were assessed. LDR exposure increased intracellular calcium (Ca^{2+}) and nitric oxide (NO) in lymphocytes of BALB/c mice, while intracellular reactive oxygen species (ROS) levels were increased in lymphocytes of C57BL/6 (**Fig. 1**). In BALB/c mice, NO inhibition and calcium chelation abolished RAR. In both BALB/c and C57BL/6 mice, *in vitro* stimulation of spleen cells with a combination of NO donor and Ca^{2+} ionophore generated an adaptive response after 2 Gy, mirroring the action of PD, demonstrating their critical function in RAR. These data imply that low-dose radiation-induced differential activation of Ca^{2+} and NO signaling together with MAPK was responsible for differing RAR with respect to the immune systems of BALB/c and C57BL/6 mice.

4. Immunomodulation by an acidic arabinogalactan from *Tinospora cordifolia*

Medicinal plants are a rich source of several compounds, and many of them are immunomodulatory in nature. In the initial studies, alkaloid-rich extracts of leaves of *Tylophora indica* (Anantmul) used in Ayurveda for treatment of asthma and catarrh were studied and were found to be highly toxic to lymphocytes at high concentrations but at very low concentrations enhanced the T cell mitogenic response. In another study, oral administration of the crude extracts obtained from dried stems of *Tinospora cordifolia* (Guduchi), a well-known Indian medicinal plant, to mice increased antibody response against *Streptococcus pneumoniae* vaccine and sheep red cells. It was also mitogenic to mouse spleen cells *in vitro*. In an innovative effort, an immunomodulatory large-molecular-weight-polysaccharide from *Tinospora cordifolia* was purified based on its biological activity. This compound, named G1-4A, is an acidic arabinogalactan and was found to act on mouse B cells and increase B cell proliferation as well as antibody production (**Fig. 2a**). Studies into the underlying mechanism of action of G1-4A in B

cells, revealed an increased expression of early activation marker, CD69, Akt, ERK, JNK, IKK phoshorylation, IκB degradation, and NF-κB nuclear translocation. The PI3K inhibitor Ly294002, the mTOR inhibitor rapamycin, and the NF-κB inhibitor plumbagin all suppressed G1-4A-induced B cell proliferation, confirming the involvement of this signaling cascade. In addition, antibody mediated neutralization of the TLR4-MD2 complex also suppressed this increased B cell proliferation and IκB degradation, indicating that theTLR-4 receptor could be the binding site for G1-4A on B cells. In addition to B cells, G1-4A also activated macrophages via ERK and NF-κB-mediated signaling, resulting in increased phagocytosis (**Fig. 2b**). These experiments indicated that G1-4A is a TLR4 agonist with non-microbial origins with potential applications as an immunomodulator and adjuvant.

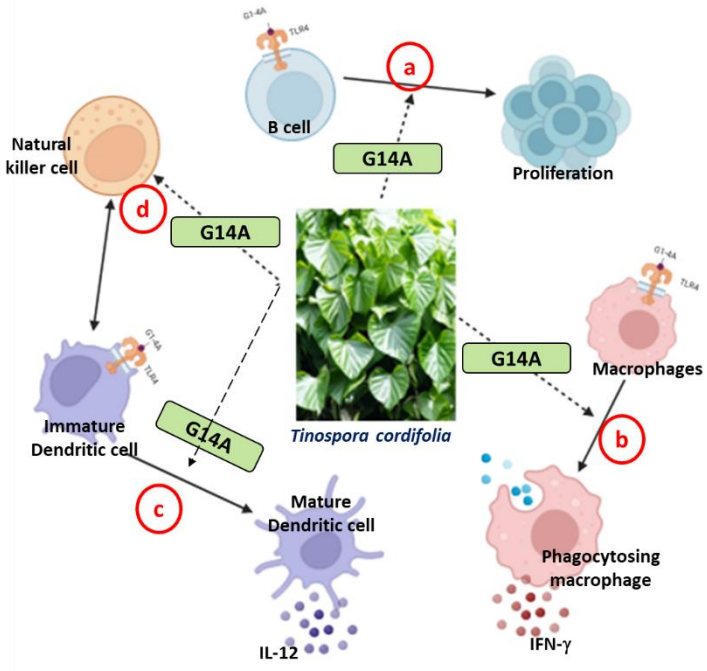

Fig. 2: G1-4A, a polysaccharide derived from *Tinospora cordifolia* has distinct effect on different immune cells like (a) B cells, (b) macrophages, (c) dendritic cells and (d) NK cells

Next, studies were focused on the application of G1-4A to treat diseases like tuberculosis, endotoxin-induced septic shock, and cancer. Being a TLR4 agonist, treatment of *Mycobacterium tuberculosis* (MTB)-infected RAW264.7 macrophages with G1-4A resulted in enhanced expression of co-stimulatory molecules, secretion of nitric oxide, and several proinflammatory cytokines. Consequently, intracellular survival of drug-sensitive as well as multi-drug resistant strains of MTB was decreased upon

treatment of macrophages with G1-4A. This was partially explained by the fact that G1-4A stimulated TLR4-MyD88 signaling, resulting in increased NO generation. These effects were observed *in vivo* also. Similar to this, the lungs of G1-4A-treated MTB-infected BALB/c mice had a significantly lower bacillary burden along with an upregulation of pro-inflammatory cytokines and nitric oxide synthase in the lung tissues. Increased T_{H1} and decreased T_{H2} cytokines were observed in infected mice treated with G1-4A. Additionally, compared to individual isoniazid (INH) or G1-4A, the combination treatment demonstrated superior protection against MTB, indicating the possibility of G1-4A as an adjuvant treatment. Our findings imply that G1-4A regulation of host immunity may improve the therapeutic effectiveness of currently known anti-mycobacterial prescription drugs, providing a compelling strategy for developing new tuberculosis treatments.

G1-4A pre-treatment offered complete protection in a lipopolysaccharide (LPS) model of sepsis in mice, prompting studies to investigate the mechanism of this protection. G1-4A treatment modestly increased pro-inflammatory cytokines TNF-α and IL-1β, whereas LPS increased the levels of these cytokines in serum several times more. So, following LPS challenge, G1-4A pre-treated mice showed considerably lower TNF-α, IL-10, and higher levels of TNF-RII, IL-6, IL-1β, and IFN-γ in serum as compared to mice treated only with LPS. G1-4A also affected the nitric oxide release by murine and human macrophages. Thus, G1-4A appeared to induce'resistance' to septic shock by modulating the cytokines and nitric oxide levels. These studies revealed that G1-4A conferred protection against endotoxin-induced sepsis.

As powerful antigen presenting cells, dendritic cells are crucial for the development of an adaptive immune response to malignancies. Dendritic cells interact with the cancer cells, process the cancer antigen, and prime and activate effector T cells. DC are known to become more immunogenic with maturation, which is stimulated by microbial products such as lipopolysaccharide (LPS). We also investigated the possibility of G1-4A as a maturation agent of murine bone marrow-derived dendritic cells (BMDC) and the possibility of using these G1-4A-treated DC's in cancer immunotherapy in preclinical models. G1-4A induced dendritic cell maturation, allowing them to activate cytotoxic T cells capable of killing cancer cells (**Fig. 2c**). Tumor lysate pulsed G1-4A-treated DC was administered in a preclinical lymphoma model, and the results of both preventive as well as therapeutic tumor challenge experiments showed a decrease in tumor burden. Aside from their function as antigen-presenting cells, DCs have direct cytotoxic effects against tumor cells. We discovered that G1-4A-treated BMDC killed tumor cells multiple times more efficiently via a nitric oxide-mediated mechanism. These data demonstrate that G1-4A-treated mBMDCs develop a killer phenotype during maturation and may be a safe non-microbial origin maturation agent for use in DC-based immunotherapy of tumors. In addition, these G1-4A-activated dendritic cells also activated natural killer (NK) cells by crosstalk in NK cell co-culture systems with either *in vitro* G1-4A-matured BMDC or splenic DC purified from G1-4A-administered mice. In addition, G1-4A also directly activated NK cells in DC-depleted splenic cells and purified NK cells (**Fig. 2d**).

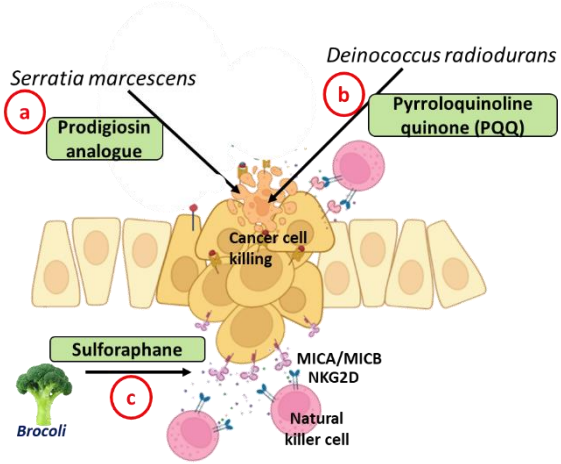

Fig. 3: Anti-tumor effects of natural products isolated from (a) *Serratia marcescens* (b) *Deinococcus radiodurans* (c) Broccoli

To summarize, G1-4A treatment activates B cells, macrophages, dendritic cells, and NK cells and has the potential to be used as an immunotherapeutic drug. However, since the purification procedures may not be that cost-effective, we tested the repeated dosage effects of polysaccharide-rich stem extract (PRE) of *T. cordifolia* and found it to have comparable effects to pure polysaccharide G1-4A. Hence PRE therapy also has the potential to be used as an immunotherapeutic adjuvant.

5. Anti-tumor and immunosuppressive effects of natural products and synthetic molecules.

The anti-tumor effects of several other natural compounds and synthetic agents chosen based on the deregulated pathways in the cancer microenvironment were investigated. These studies are summarized below:

(a) A new prodigiosin analogue was isolated as a red pigment from an organic solvent-tolerant strain of *Serratia marcescens* and identified as 2,2'-[3-methoxy-1'amyl-5'-methyl-4-(1"-pyrryl)] dipyrrylmethene (MAMPDM). MAMPDM had significant cancer cell cytotoxic activity and reduced the proliferation of cancer cell lines, inducing necrotic and apoptotic cell death in murine fibrosarcoma, S-180 cells, and lymphoma EL-4 cells, respectively (**Fig. 3a**). This might be advantageous for eliminating tumor cells that are deficient in the apoptotic pathway and thus resistant to conventional therapy. The effect of MAMPDM was also investigated in splenic lymphocytes stimulated with mitogen Con A. MAMPDM treatment resulted in increased IL-2 secretion, apoptosis, and decreased CD71 expression, proliferating cell nuclear antigen (PCNA), and cyclin D, resulting in the conclusion that MAMPDM selectively inhibited pro-mitogenic signaling but not pro-apoptotic signaling.

(b) The immunomodulatory effects of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), present in Plumbago zeylanica (also called Chitrak) roots, were also investigated. Plumbagin inhibited lymphocyte proliferation by modulating cellular redox, resulting in glutathione depletion and an increase in reactive oxygen species.

(c) The water-soluble sodium-copper salt chlorophyllin (CHL), an analogue of the natural pigment chlorophyll, was identified to be an antioxidant that protects lymphocytes against oxidative stress and radiation-induced apoptosis. Mice treated with chlorophyllin developed splenomegaly due to increased lymphocytes and macrophage infiltration. Additionally, increased peritoneal exudate cells (PEC) obtained from CHL-treated mice also showed elevated phagocytic activity. Administration of CHL to mice immunized with the antigen sheep red blood cells (SRBC) significantly increased both T and B cell responses.

(d) Pyrroloquinoline quinone (PQQ), an antioxidant and redox co-factor, obtained from *Deinococcus radiodurans*, was also studied for its cytotoxic potential. Treatment with PQQ led to depletion of key cellular antioxidant glutathione, resulting in increased intracellular reactive oxygen species (ROS) and apoptosis of U937 cells (human promonocytic leukemia) (**Fig. 3b**). The cellular redox status was found to have a significant impact on PQQ-induced cytotoxic activity. An increase in intracellular GSH promoted PQQ-induced apoptosis, while depletion changed the manner of cell death to necrosis. Our results showed that modulating intracellular GSH can be an effective technique for increasing the cytotoxicity of quinones such as PQQ.

(e) The anti-tumor effects of sulforaphane (SFN), present in Broccoli, were investigated in combination with ionizing radiation (IR) (**Fig. 3c**). Higher doses of SFN induced cytotoxic effects. But lower concentrations (10 μM) of SFN up-regulated natural killer group 2, member D (NKG2D) ligands, increasing tumor cell sensitivity to natural killer (NK) cell-mediated death. Blocking this with an anti-MICA/MICB antibody prevented this effect. Increased cellular glutathione levels with N-acetyl cysteine treatment abrogated SFN induced effects such as expression of MICA/MICB and increased susceptibility of lung and breast cancer cells to NK cell mediated killing. Our findings showed that SFN can be used as an immunomodulatory adjuvant in cancer therapy.

(f) Cancer progression is associated with an evolving interaction between the immune system and the tumor cells. Many of the soluble molecules that promote immunosuppression in the microenvironment have been identified, but the means by which the tumor impacts the bone marrow progenitors is unknown. We identified that the tumor cell-derived prostanoids affected the distant progenitor cells, inhibiting the expression of transcription factor Zbtb46, which is specific to the classical dendritic cell DC (cDC) lineage-specific and therefore influenced its development. Tumor-induced DC dysfunction was abrogated when tumor cells were treated *in vitro* with the COX-2 inhibitor NS-398. Tumor-bearing mice treated with NS-398 developed immunocompetent DC and had a lower tumor burden. The absence of such an effect in SCID mice supported the hypothesis that NS-398's effects were attributable to immunomodulation. These results illustrate that Zbtb46 expression is a marker of

immunocompetent DC and show that COX-2 inhibitors may be effective in cancer immunotherapy.

(g) Soft tissue sarcomas (STS) have a largely unexplored immune milieu, and understanding it is crucial for designing immunotherapy approaches. Murine fibrosarcoma triggered the development of B regulatory cells (Breg) with $CD19^{+}CD25^{+}PD\text{-}L1^{hi}$ phenotypes that secreted TGF-β. These tumor-evoked Bregs decreased the proliferation of T cells in response to anti-CD3/CD28 stimulation, which was reversed by SB431542, a small-molecule inhibitor of TGFβ receptor type I. When tumor bearing mice (TBM) were administered SB431542, the number of Treg cells reduced significantly with the restoration of the T cell proliferation response. Additionally, this treatment significantly reduced the tumor load. Our findings indicate that the tumor-induced Breg cells inhibit immunity through a TGFβ-mediated mechanism. Immunotherapy drugs targeting the Breg-Treg axis may thus have potential benefits in soft tissue sarcomas.

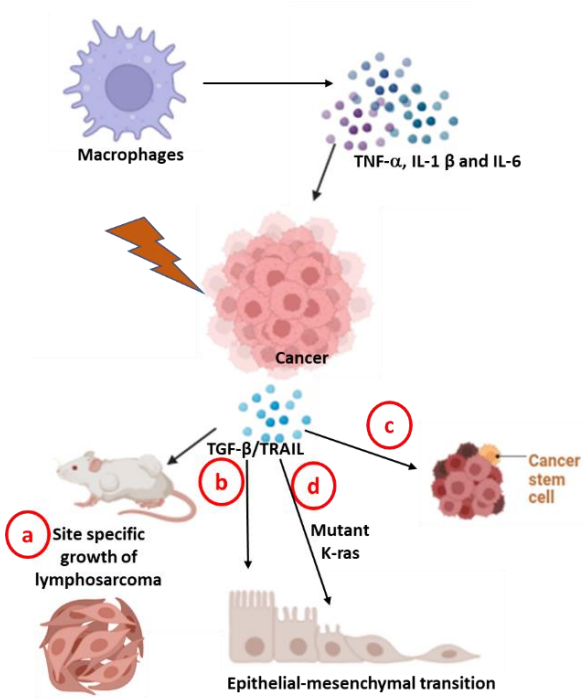

Fig. 4: Pro-tumor effects of TGF-β resulting in (a) site specific growth of lymphosarcoma (b) macrophage mediated epithelial-mesenchymal transition (EMT) (c) enrichment of cancer stem cells (d) TRAIL induced EMT responses in K-ras mutated cancer cells

6. Pro-tumor effects of immune cells and cytokine microenvironment

Earlier studies demonstrated that after intraperitoneal (i.p.) transplantation, ascitic lymphosarcoma (LS-A) showed rapid progression leading to host mortality in Swiss mice. However, this was found to be site dependent, and the tumors showed spontaneous regression after subcutaneous (s.c.) transplantation. Studies were undertaken to understand this mechanism. Studies conducted *in vitro* revealed that at increasing cell densities, neutralization with an anti-TGF-β antibody greatly suppressed LS-A proliferation. Mice with i.p. ascites tumors exhibited increased serum TGF-β1, reduced hemoglobin levels, transferrin receptor (CD71) expression, splenic cellularity, and compromised T cell mitogen response. However, these changes were not found in mice with spontaneous regression of s.c. transplants. These studies indicated that tumor growth sites and the host immune system have a significant impact on tumor progression. These findings suggest TGF-β1-secreting human tumors may have comparable pathophysiological consequences in the host based on their anatomical location (**Fig. 4a**).

Within the tumor microenvironment, macrophages have pro-tumor effects. They can also affect tumor cell proliferation, invade normal tissues, and disseminate to both local and distant locations. We studied the impact of monocytes and macrophage conditioned media (MϕCM) on breast cancer cell proliferation and migration. Twenty-four-hour monocyte and macrophage conditioned media were collected. Pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, present in high concentrations in the macrophage-conditioned medium, stimulated TGF-β1 synthesis in tumor cells, increased CREB phosphorylation, epithelial-mesenchymal-transition (EMT) responses, and migration (**Fig. 4b**). Proteomics studies of these conditioned media identified MϕCM to be enriched in integrin and matrix metalloproteinases. The gene signature identified by macrophage-tumor interactions was significantly associated with mutation, deletion, amplification, and differential expression of some prominent candidate genes in the TCGA database. These genes together formed a 15-gene signature found in >60 % of samples in TCGA database and were linked to high breast cancer risk and poor overall survival ($p < 0.05$). These findings emphasize the crucial role of macrophage signaling in breast cancer. Prognostic indicators based on tumor-macrophage interaction could thus be beneficial for tracking cancer progression.

Breast cancer is a very heterogenous disease with several clonal populations, and the tumor microenvironment affects the plasticity of cancer stem cells. In MCF7 cells and mammospheres, MϕCM-activated ERK/TGF-β1 signaling led to EMT and the enrichment of cancer stem cells (CSC) (**Fig. 4c**). It was discovered that these enriched stem cells exhibited both mesenchymal ($CD44^+$ $CD24^-$ cells) and hybrid ($ALDH1^+$) properties. At the single cell level, the hybrid E/M state was characterized by elevated expression of the mesenchymal marker vimentin (M) and the epithelial marker claudin-1 (E). These effects could be reversed by either inhibiting TGF-β1 synthesis with MEK inhibitor PD98059 or downstream signaling with TGF-βR1 inhibitor SB431542. Prior to implantation in SCID mice, a number of CSC and EMT markers were monitored in the cancer cells. There was no growth benefit of MϕCM-treated cells in SCID mice, and

evaluation of CSC and EMT markers in cells recovered from the tumor showed reversal. The ERK /TGF-β signaling leading to CSC enrichment was abrogated by either removing the MϕCM or antibody-mediated neutralizing of pro-inflammatory cytokines present in MϕCM. This underscores the significance of the requirement of continuous signaling for maintenance of cancer stem cells. Thus, ERK/TGF-β1 signaling plays a major role in MϕCM-induced EMT and CSC plasticity, both of which are completely reversible.

TRAIL (tumor necrosis factor-α-related apoptosis-inducing ligand), also called Apo2L or TNFSF10, is an important cytokine that regulates cell survival and death in the tumor microenvironment. Bioinformatics analyses revealed that the expression of TRAIL had different impacts on disease-free survival in adenocarcinoma (AC) and squamous cell lung carcinoma (SCC), two distinct forms of lung cancer. Genomic analysis showed that AC had considerably higher KRAS mutation rates along, with enhanced TRAIL expression and metastasis, whereas SCC had more TRAIL gene amplifications. *In vitro* studies showed that TRAIL only stimulated ERK phosphorylation in AC cell lines that have mutant KRAS (**Fig. 4d**). This in turn led to enhanced migration, which was abrogated by the MEK inhibitor PD98059. The effects of TRAIL-induced migration were accentuated when combined with ionizing radiation exposure. These results advance our knowledge about TRAIL signaling in metastasis, which is important for creating strategies to convert these signals into pathways that promote apoptosis.

7. Immune involvement in radiotherapy and chemo/radio resistance

Several types of lymphoid and myeloid tumor cells are known to be more resistant to radiation-induced apoptosis than normal lymphocytes. Our research identified that tumor cells have greater inherent radio resistance than normal lymphocytes due to enhanced levels of antioxidants and lower production of reactive oxygen species (**Fig. 5a**). Alterations in mitochondrial membrane potential and cytoplasmic Ca^{2+} concentration were detectable in lymphocytes even at a dose of 1 Gy, while no such variations were observed in tumor cells. After 1 Gy irradiation, approximately 65% of spleen cells died within 24 h. However, under the same conditions, the tumor cells EL-4 and P388 failed to undergo cell death and instead accumulated in the G2/M phase. This constitutive radio resistance of EL-4 cells was found to be due to the activation of the Nrf-2/ERK pathway. In response to radiation, EL-4 cells altered their thiol redox circuits, GSH, and thioredoxin. Pharmacological ERK and Nrf-2 inhibitors significantly increased radiosensitivity of EL-4 cells. Unirradiated lymphoma cells accumulated Nrf-2 in the nucleus, with an increase in the expression of its downstream genes. Interestingly, Nrf-2 nuclear translocation was blocked by MEK inhibitors, indicating that ERK plays a role in basal and radiation induced Nrf-2 activation in tumor cells. This was confirmed by a further increase in radiosensitivity in ERK/Nrf-2 double knockdowns as compared to individual knockdowns. Importantly, EL4 cells lacked even basal-level NF-κB

expression, a protein known to be constitutively active in many tumors. As a result, NF-κB inhibition did not affect EL-4 radiosensitivity.

In addition to natural or inbuilt radio resistance, cancer cells also acquire resistance during radiotherapy. Identifying the underlying mechanisms of therapy-induced radio resistance and activated pathways will result in more effective combination therapies. Breast cancer cell lines were subjected to 6 Gy that was followed by a 7-day recovery period. These cells (D7-6G) demonstrated enhanced proliferation and apoptosis. The cytokine known to induce such dual effects, transforming growth factor β, all its isoforms 1-3, along with their receptors R1, and R2, were expressed at higher levels in these cells. TGF-β downstream transcription factors Zeb1, Snail, and HMGA2 were also enhanced. These cells also displayed a phenotype that had hybrid epithelial-mesenchymal (E/M) characteristics, with increased motility and expression of E/M markers. When challenged with radiation, these cells exhibited resistance to killing and had an increased proportion of cancer stem cells (**Fig. 5b**). SB431542, a TGF-βRI inhibitor, significantly reduced the proliferation of D7-6 G cells. Thus, blocking TGF-β signaling can be a promising way to combat radio resistance produced by radiation exposure.

We also explored the potential of these cells to form tumors in severe combined immunodeficiency (SCID) mice and used proteomic techniques to characterize these tumors. Larger tumors with a shorter latency period were produced by these radioresistant cells (**Fig. 5c**). Expression of TGF-β isoforms, downstream genes pSMAD3, Zeb1, Snail, HMGA2, hybrid epithelial/mesenchymal phenotype, motility, and cancer stem cells were all increased in these tumors. Radioresistant breast cancer cells showed enhanced TGF-β signaling and increased metabolism with both oxidative phosphorylation and glycolysis. We also studied the effects of prolonged treatment of breast cancer cells with the TGF-βR inhibitor SB431542 on radiation-induced signaling. Radioresistant cells had higher levels of TGF-β1 and TNF-α signaling as seen by enhanced phosphorylation of SMAD3, NF-κB, and ERK. Pre-treatment of radioresistant cells with the TGF-βR inhibitor SB431542 lowered phosphorylation of SMAD3, and increased proliferation, apoptosis, and motility. Downregulation of TGF-β downstream genes, Snail and HMGA2, and hybrid E/M phenotype, was also observed. TGF-β independent effects were also observed, whereby SB431542 treatment itself led to increased expression of some E/M genes. This was most likely caused by increased phosphorylation of pSTAT3 and CREB1 genes in addition to enhanced production of cytokines IL-6 and IL-10. These findings indicate that primary signaling pathways in radioresistant cells are mediated by TGF-β/pSMAD3 and TNF-α/pNF-κB and that prolonged SB431542 treatment may result in TGF-β/Smad3 independent effects.

We investigated the influence of tumor necrosis factor-α (TNF-α), a pro inflammatory cytokine, and insulin-like growth factor 1 (IGF-1), a growth factor, present in the tumor microenvironment, on the response of lung cancer cells to radiation. A bioinformatics analysis of 982 lung cancer patients showed that increased TNF-α expression was linked to a lower risk of cancer growth, while IGF-1 overexpression was linked to a higher risk. TNF-α treatment reduced cell motility and increased radiosensitivity, by activating the

MAP kinases such as stress-activated protein kinases (SAPK), jun amino-terminal kinases (JNK), and p38 kinases (**Fig. 5d**). IGF-1 treatment increased mitotic index, reduced DNA repair, and caused abnormal chromosomal segregation, resulting in increased cell proliferation and motility. Collectively, these findings show that the cytokines and growth factors in the tumor microenvironment influence radiation therapy by activating many signaling pathways.

In addition to radiotherapy, cytokines in the tumor microenvironment also influence the effectiveness or failure of molecular- targeted therapies. We investigated the effects of TRAIL and IGF-1 on sirtinol cytotoxicity. Sirtinol or SIRT1 knockdown increased the expression of death receptors DR4 and DR5 and sensitized A549 cells to TRAIL mediated cell death. This was found to be iNOS-mediated, caspase-independent, with classical characteristics of necroptosis (**Fig. 5e**). Inhibiting iNOS increased caspase activity and altered the manner of cell death to caspase-mediated apoptosis. IGF-1 reduced sirtinol cytotoxicity and increased cell survival by preventing ligand-induced IGF-1R downregulation and therefore activation of the PI3K-AKT pathway (**Fig. 5f**). To summarize, these findings indicate that the tumor microenvironment influences drug cytotoxicity and that combination therapy with drugs that inhibit the IGF-1 pathway and increase TRAIL signaling may improve anticancer efficacy.

Despite being the most commonly used cancer treatment, little is known about how radiation affects dendritic cell development. We found that in tumor-bearing mice exposed to localized irradiation, the tumor-induced suppression of splenic and bone marrow-derived DC (BMDC) function was reversed. This was not due to the effect of radiation on tumor cells, because DC derived from normal mice exposed to whole-body irradiation (WBI) also showed higher immunocompetence. This increased immunocompetence was also observed when DC were generated from *in vitro* irradiated progenitor cells. It was shown that this effect was related to STAT5/Zbtb46 signaling, which was mediated by the irradiation-induced apoptotic bodies (ABs). The involvement of this pathway was demonstrated by the reversal of these effects upon annexin-bead-mediated depletion of these ABs. In addition, these DCs generated from irradiated progenitors (IP) were resistant to the suppressive effects of tumor conditioned medium (TCM). For the first time, we demonstrated that ABs formed due to IR exposure at specific doses can enhance DC's capacity to activate the immune system. This may affect the choice of appropriate IR dosages for cancer patients undergoing radiotherapy.

8. Epigenetics in immune cells

The functional plasticity of immune cells in the tumor microenvironment (TME) determines the fate of tumor progression. There are various factors that regulate the characteristics of immune cells in the TME, among which epigenetic regulation plays a major role in modulating immune-immune and immune-tumor cell interactions. In general, epigenetic modifications are alterations in gene expression without altering the primary DNA sequences. Histone modifications, DNA methylation, and translational regulation by non-coding RNAs that have the ability to activate or suppress target gene

expression are examples of these epigenetic alterations. Epigenetic modifications in the tumor cells and immune cells can reprogramme the TME and influence tumor progression and metastasis. Tregs are one of the major immunosuppressive cells in the TME. In the tumor milieu, the cytokine transforming growth factor-β converts naïve T cells into Treg cells, which is largely regulated by epigenetic modifications.

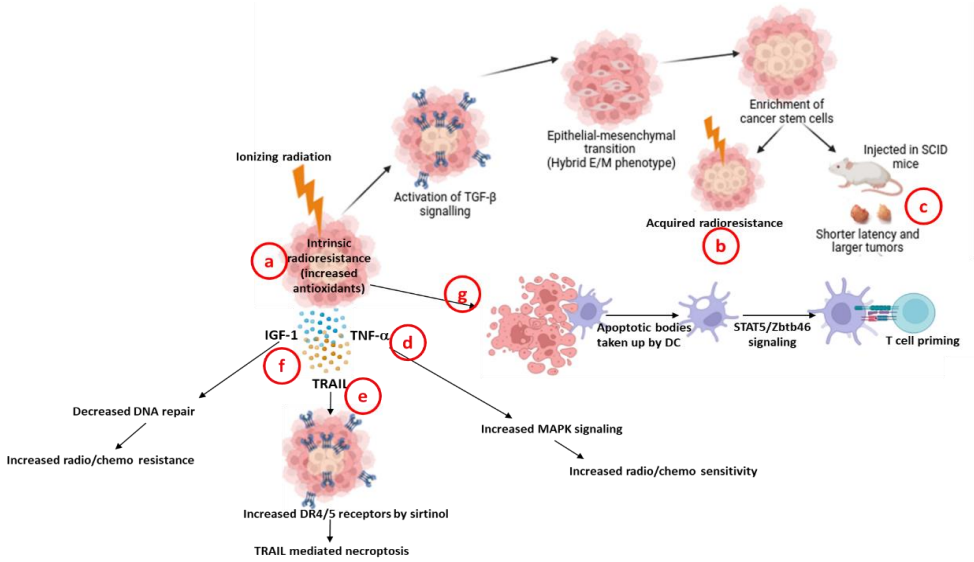

Fig. 5: Radio resistance and immune system: Cancer cells have (a) intrinsic radio resistance or that which is (b) acquired during the course of therapy (c) radioresistant cells grow rapidly in SCID mice. Presence of cytokines like (d) TNF-α and (e)TRAIL increase radio/chemo sensitivity whereas (f) IGF-1 increase radio/chemo resistance. (g) Irradiated apoptotic bodies released from the tumor cells can be taken up by dendritic cells resulting in their activation.

We have extensively studied the epigenetic modifications involved in regulatory T cell (Treg) generation. We generated a detailed epigenetic landscape (DNA methylation and 14 histone modifications) of TGF-β-induced changes in three regulatory regions of the Foxp3 gene, the master transcription factor of Treg generation. By validating in physiologically relevant conditions, we found out that increased levels of histone methylation H3K4me3 and decreased histone methylation H3K27me3, along with increased acetylated H3K27 or decreased DNA methylation, are indispensable for TGF-β induced Foxp3 gene expression and Treg generation and form an epigenetic signature for Treg cells. Currently, there are some epigenetic drugs that are approved for clinical use in hematological malignancies. While the anti-tumor effects of these drugs are demonstrated, how these drugs reprogram the immune cells in TME is unknown. To identify potential epigenetic drugs that can reprogram Tregs in the TME, we developed a

screening strategy using TGF-β-mediated immune responses as an endpoint. We identified two compounds, EPZ004777 and FG2214, that could inhibit TGF-β-induced Treg generation by reversing the epigenetic signature of TGF-β in the Foxp3 gene locus. While these two drugs show promise in the *in vitro* studies, further preclinical and clinical studies are required to establish their efficacy and suitability as potential immunotherapeutic agents, with ongoing research efforts aimed at this goal.

MicroRNAs are among the major regulators in the TME. It is well known that the dysregulation of microRNAs contributes to oncogenic transformations leading to onset and progression of cancer. In 4T1 murine mammary cancer, dendritic cell dysfunction is mediated by the tumor-derived prostaglandin PGE2, which acts through EP2/EP4 receptors present on the immune cells. We have identified that PGE2-induced DC dysfunction is mediated through IL-6/STAT3 signaling. Dysregulation of microRNAs can also exert immunosuppressive functions in the TME. Our studies on dendritic cell dysfunction in the TME identified the differential regulation of multiple microRNAs in dendritic cells generated in the presence of tumors. For example, mmu-mir-155-2p, mmu-mir-146, and mmu-mir-365-5-2p. Using synthetic mimics and inhibitors of the above miRNAs, we observed that miR 365 negatively regulates PGE2-induced IL-6 generation in DCs, and use of miR 365 mimics could reverse PGE2 induced DC dysfunction. Our studies show novel insights on the potential use of miRNA regulators as immunotherapeutic agents in cancer treatment.

9. Summary and Way forward

The focus of immunology research at BARC has been in the following areas: (a) low dose radiation studies; studies so far have been carried out in animal systems, which have revealed changes in several markers but also have shown that this could vary between different strains. Future work will be focused on studying the effect of low dose diagnostic exposures in cancer patients, with particular emphasis on DNA damage response and immune response. (b) Research on *Tinospora cordifolia:* studies so far have clearly established the mechanism of action of G1-4A, the polysaccharide from *T. cordifolia*, to activate antigen presenting cells and NK cells, preclinical studies on animals also have shown three potential areas of application: as an adjuvant to treat tuberculosis, sepsis, and cancer. In the future, efforts will be undertaken to prepare formulations that can be used to treat these diseases\conditions. (c) Research on several immunomodulators and anti-cancer compounds, of both natural and synthetic origin: Amongst the various immunomodulators studied, chlorophyllin has been prepared as a formulation and approved as a food supplement/nutraceutical. Other promising molecules like COX-2 inhibitors or TGF-β inhibitors will be taken up for their potential use as an adjuvant in cancer chemo/radio/immunotherapy. (d) role of immune mediators in radio/chemoresistance: studies so far clearly confirm the role of cytokines like IL-6 and TGF-β in radio resistance, so efforts in the future will be directed to identify the prognostic value of TGF-β/IL-6 in radiotherapy and the application of TGF-β/IL-6 inhibitors or siRNA to overcome radio resistance in cancers. (e) epigenetic changes in

immune cells: studies so far show that miR-365 negatively regulates IL-6, a proinflammatory cytokine involved in several diseases. Future studies will be focused on developing miR-365 mimics that can abrogate IL-6 and therefore can be used in inflammatory disorders.

In addition, our expertise in understanding the biology of differentiation and reprogramming of immunosuppressive cells, especially regulatory B cells and regulatory T cells, would provide greater insight into potentiating them as beneficial therapeutics. Our epigenetic screens have identified two molecules, EPZ004777 and FG2214, as potential inhibitors of T regulatory cells. Application of these compounds or related compounds in inflammatory disorders will be tested. Autoimmune diseases caused by uncontrolled immune activation are often associated with the dysfunction of these regulatory cells and currently do not have any effective treatment against them. With our understanding of these immunosuppressive cells, identifying ways to improve their differentiation and functions in such conditions may provide potential benefits to patients with autoimmune diseases. The research in this area may also help in developing effective treatment modalities, which are lacking as of today.

Overcoming chemo and radiotherapy resistance is a major challenge in cancer treatment. Our recent studies have shown increased fatty acid metabolism associated with chemo and radioresistance in human cancers. This metabolic reprogramming of cancer cells in the TME will be studied in depth to identify novel molecules to reverse them and thereby improve the clinical outcome. Extensive studies in this field are necessary to identify more promising TME modulators that can be potential therapeutics either on their own or as adjuvants to chemo/radio/checkpoint blockade therapy.

Most of the conventional cancer treatments also destroy the immune system, which is replenished by the hematopoietic stem cells. However, recent studies have shown that the soluble mediators secreted by cancer cells can affect the distal progenitors also, affecting their ability to differentiate into functional cells. These defective progenitors may thus have a role in inducing immunosuppression and poor outcomes with immunotherapeutics in the majority of the patients. Another future prospect is to carry out research on the effect of the cancer derived soluble mediators on the hematopoietic stem cells and approaches to prevent their negative impact on differentiation of progenitor cells.

While extensive studies on immunomodulation in tumors and the development of advanced immunotherapies are ongoing globally, the role of co-morbidities as contributory factors remains poorly understood. Co-morbidity is defined as the co-existence of a disorder in addition to a primary disease of interest. The interaction of co-morbidities such as diabetes, cardiovascular diseases (CVD), obesity, and other non-communicable diseases is one of the major challenges in cancer treatment and survival. The involvement of these factors in delaying diagnosis and affecting the efficacy of treatment is being studied globally. But whether and how they affect the immune interactions in the TME and their role in the outcome of cancer immunotherapeutics is still unclear. Considering our country's growing cancer incidence and increasing CVD, diabetes, and hyperlipidemia, it has become the need of the hour to delineate the

interactions of these diseases with each other. Our major focus of research in the coming years would be to study this aspect, particularly diabetes and obesity, in modulating tumor biology. With the current preliminary research, it is evident that obesity in fact negatively modulates anti-tumor responses in at least some strains of mice. These studies can be extended to include drug-induced or dietary reprogramming to manage these diseases with better anti-tumor responses.

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JOURNEY OF FREE RADICALS AND ANTIOXIDANT RESEARCH IN BARC

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Abstract

This book chapter describes the contributions of the Bhabha Atomic Research Centre (BARC) towards understanding the role of free radicals and antioxidants in biological systems. Free radicals, which highly reactive molecules, not only play crucial roles in multiple cellular processes such as signalling and immune responses but also contribute to oxidative stress, which can lead to diseases. This chapter highlights the pioneering research carried out in BARC in the field of free radical biology, focusing on their impact on radiation-induced damage, which is particularly relevant to the Atomic Energy Program of DAE. Additionally, extensive research has been carried out using antioxidants which were shown protect against oxidative damage to vital biomolecules, ensuring cellular function and reducing radiation induced damage. The modern facilities at BARC enabled the study of free radical lifetimes and the development of effective antioxidant strategies, crucial for development of radiation countermeasures. The chapter also explores the development of radioprotective molecules, such as AKTOCYTE and Sanjeevani rice, designed to boost immunity and improve the outcome of cancer therapy. Looking ahead, the Free Radical Biology Program at the Department of Atomic Energy (DAE) is set to further

advance the understanding of oxidative stress and biological effects of radiation for possible therapeutic applications.

1. Introduction

The study of free radicals and antioxidants has been a cornerstone of research at the Bhabha Atomic Research Centre (BARC). This book chapter explores the profound journey and contributions of BARC in free radical and antioxidant research. Free radicals, with their high reactivity, play crucial roles in various biological systems, influencing processes ranging from cellular signaling to disease pathogenesis. A better understanding of oxidative stress and developing effective antioxidants is paramount, especially within the context of atomic energy programs where radiation-induced oxidative damage can have significant implications. The journey of free radical research in BARC has been marked by pioneering approaches and innovative discoveries. BARC has modern facilities and infrastructure for studying free radicals and measuring their lifetimes. Antioxidants, essential for maintaining redox balance, have been a focal point of this research. This work has far-reaching applications, impacting the safety and efficiency of Nuclear Energy Programs. Looking ahead, the Free Radical Biology Program at the Department of Atomic Energy (DAE) is poised to explore deeper into this vital area, aiming to enhance our understanding and application of these critical biological entities.

2. Role of free radicals in biological systems

Free radicals are highly reactive molecules containing unpaired electrons which makes them unstable and prone to participate in chemical reactions. While they are often associated with damage and disease, free radicals also play essential roles in biological systems related to various biological process and function such as cellular signaling, metabolism, homeostasis, immune system function, antioxidant defense system regulation, adaptation to environmental stressors, development and aging etc. Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide (O2•-) serve as signaling molecules in cellular pathways. They regulate several biological processes such as cell division, differentiation, apoptosis, and immune responses. Low levels of ROS can induce hormetic responses, where cells activate defense mechanisms and adapt to stressors, leading to improved resilience and longevity. Phagocytes (neutrophils and macrophages) produce ROS to destroy invading pathogens, including bacteria, viruses, and fungi, through a process known as the respiratory burst. ROS are natural by-products of mitochondrial respiration. They participate in metabolic signaling, regulating processes such as energy production, nutrient sensing, and thermogenesis. Nitric oxide (NO•), a reactive nitrogen species (RNS), acts as a signaling molecule in blood flow regulation and neurotransmission.

ROS play a role in apoptosis and act as signaling molecules that trigger programmed cell death in response to stress or damage, and as executioners of apoptosis by damaging

cellular components. ROS regulate transitions between cell cycle phases by altering the activity of cyclins and cyclin-dependent kinases (CDKs). Cells activate antioxidant defense mechanisms in response to oxidative stress induced by environmental factors viz radiation, pollutants, and toxins. Free radicals are generated during exposure to ionizing radiation, leading to DNA damage and cellular stress. Cells activate repair mechanisms and antioxidant defenses to mitigate radiation-induced damage. ROS are also involved in embryonic development, tissue remodeling, and organogenesis by regulating cell proliferation, differentiation, and morphogenesis. The accumulation of oxidative damage over time contributes to aging and associated disorders. However, moderate levels of oxidative stress can activate stress response pathways that promote longevity and health span.

3. Importance of studies on oxidative stress and antioxidants in Atomic Energy Program

The inception of free radical research can be traced back to efforts to comprehend the indirect effects of radiation exposure, particularly following the atomic bomb explosions in Hiroshima and Nagasaki in 1945. The catastrophic impact of these events prompted scientific inquiry into the biological consequences of radiation. Radiation has long been a mystery to the general public, often met with a sense of uncertainty and skepticism. Today, ionizing radiation finds wide application beyond nuclear power generation, extending into numerous therapeutic, industrial, and other fields. It also plays a key role in developing high-yielding crop varieties and extending the shelf life of food products. Radiotherapy has emerged as a key mode of cancer therapy wherein radiation specifically destroys cancerous cells with minimal harm to healthy tissues surrounding the tumor. However, normal tissue toxicity is still a liming factor in radiotherapy and hence a better understanding of the mechanisms of radiation toxicity is important to identify compounds that can act as radioprotectors. One key area of research in this regard is the study of role of free radicals in radiation-induced damage. In this context research on free radicals and antioxidants are relevant to the Atomic Energy Program. This research aims to develop strategies to protect individuals who are at risk of radiation exposure, whether in medical, industrial, or research settings. By advancing our understanding of how free radicals contribute to radiation damage and how antioxidants can counteract this damage, scientists can improve safety protocols and develop novel treatments to prevent or alleviate radiation-induced injuries.

4. Genesis and journey of research on free radical reactions

In recent years, there is increasing awareness about one’s health. In this respect, the possible role of free radicals in human health and its prevention is of immense interest. Free radicals as well as the agents that keep them in check, namely antioxidants have attracted lot of attention from both health professionals as well as basic scientists involved in biomedical research. Free radical biology has many potential applications both in disease prevention and therapy, apart from radioprotection. In this section we will

explore the genesis and journey of free radical reaction studies carried out at BARC during different time periods.

4.1. Studies before 1995

Although free radical research was initiated as early as 1945, the progress of research in this area in our country was quite slow. The initial studies at BARC centered around spin trapping using electron spin resonance and pulse radiolysis in the 1970s.. Biochemical studies started much later. In 1980s assays for free radical damage in the form of lipid peroxidation were developed and publications using different rat tissues during some physiological states were published in standard biochemical journals such as BBA, BBRC and FEBS Letters. Pioneering studies carried by different scientists became the foundation to initiate research programs for deeper understanding of the role of free radicals in radioresistance of cancer cells, discovery of redox active agents, development of radioprotectors and radiosensitizers in BARC.

4.2. Studies between 1995 to 2003

Later on assays and methods were developed for evaluating DNA, lipid and protein damage and combined with pulse radiolysis and other techniques. Further, collaborations within Biomedical Group and with scientists from Chemistry Group, Radiochemistry Group besides other Institutes from both India and abroad, led to several publications. These studies also involved natural products for possible prevention of damage in relation to human diseases such as cancer, diabetes, and radiation damage. The collaborations with other institutes include Indian Institute of Technology, Mumbai; Amala Cancer Centre, Thrissur; University of Pune; ACTREC, Navi Mumbai and many other institutes of repute. The Institutes from abroad include, Kyoto Prefectural University of Medicine, National University of Singapore and Palm oil research Institute of Malaysia. These collaborative studies also lead to publication of good reviews on free radicals, antioxidants, radioprotection, natural products for prevention of human disease and these received many citations. In 2001, scientists from BARC who were working in this formed the Society for Free Radical Research, India (SFRR-India). Annual meetings of this society, involving many scientists working in the same field were conducted. This also promoted interaction between many groups working on free radicals in our country and abroad. During this period several research projects were initiated which are described below;

4.2.1. Research on radioprotection and radiosensitization

Cancer, a major cause of illness and death, is often treated with radiotherapy, a highly effective approach. However, it is crucial to protect healthy cells from radiation damage during such treatments. Hence there is a need to understand the mechanisms of radiation damage and its possible prevention by compounds such as antioxidants that can act as radioprotectors.

The currently used radioprotectors have numerous downsides such as high cost and toxicity etc. Novel approaches are being worked upon to identify newer radioprotectors. In this aspect, natural products have many advantages such as being non-toxic and have

proven therapeutic effects. There are several studies at BARC in this aspect that have examined the radioprotective properties of natural compounds like caffeine, curcumin, chlorophyllin, vanillin, Troxerutin, tocotrienol besides extracts from mushrooms, *Asparagus racemosus* and *Phyllanthus amarus.*

BARC has contributed significantly to the field of the radiosensitization for better tumor control. A radiosensitizer is a compound that enhances the cytotoxic efficacy of radiation, thereby improving the outcome of radiotherapy. Radiosensitization is traditionally achieved through (i) suppression of intrinsic radioprotective elements, (ii) transformation of the radiosensitizer into a cytotoxic agent via radiolysis, (iii) blocking of DNA repair mechanisms, (iv) use of thymine analogs, and (v) mimicking oxygen. Inhibition of the thioredoxin system can augment ionizing radiation-induced oxidative stress and potentiate cytotoxic effects. Scientists from BARC extensively worked on Sanazole (AK-2123), a hypoxia sensitizer, and showed that it enhances gamma-radiation-induced apoptosis in murine fibrosarcoma and anaerobic yeast. Sanazole is currently in advanced stages of clinical trials. In recent research, scientist from BARC demonstrated that thioredoxin (Trx) and thioredoxin reductase (TrxR) play a critical role in reducing oxidized cysteine thiols across various proteins, thereby influencing cellular redox balance, survival, proliferation, DNA synthesis, transcription factor activity, and apoptosis. TrxR is particularly vital for sustaining a redox environment conducive to tumor growth, survival, and therapy resistance. Additionally, researchers from BARC have shown that dimethoxycurcumin (DIMC), a structural analog of curcumin, can significantly enhance the effectiveness of radiation therapy in killing cancer cells by specifically targeting the thioredoxin system. In our recent study, we utilized current research strategies and identified that the clobetasol propionate (FDA-approved anti-psoriatic drug) can be repurposed as a promising radiosensitizer for Keap-1 mutant lung cancers. At present, several research activities are ongoing towards the radiosensitization of cancer cells using small molecules or natural compounds.

4.2.2. Advances in instrumentation and methodology and free radical research (upto 2003)

There are various methods for estimating free radical damage to biomolecules and determination of the antioxidant properties. There is great advancement of methodologies in recent years that has helped in the progress of research on free radicals. Measurement of damage to lipids include estimation of thiobarbituric acid reactive substances (TBARS) by spectrophotometry and spectrofluorometry; spectrophotometric assays for lipid hydroperoxides and conjugated dienes; HPLC analysis for 4-hydroxynonenal; HPLC and ELISA assay for isoprostanes; gaschromatography assay for exhaled gases and fluorescence assay for lipid-DNA adducts. For measurement of endogenous antioxidants such as glutathione, protein thiols, superoxide dismutase, glutathione peroxidase and catalases spectrophotometric assays were developed. For measurement of DNA damage gel electrophoresis, comet assay, HPLC and GC-MS assays were also developed. Physico-chemical and radical scavenging methods involve studies using pulse radiolysis, cyclic voltammetry, singlet oxygen detection by germanium diode,

stopped flow spectrophotometry, different spectrophotometric scavenging assays, electron spin resonance (ESR) etc.

4.3. Studies after 2003

The journey of research on free radicals and antioxidants, initiated and propelled by our esteemed seniors and colleagues, has evolved significantly since 2003 within the Bio-Science group at BARC. This evolution marks a profound advancement in our understanding of these critical biological entities. Initially focused on fundamental mechanisms, the research has now permeated almost every division of the Bio-Science group and extended to various other groups, demonstrating the interdisciplinary nature and wide-reaching implications of this work. One key area of exploration involves elucidating the molecular mechanisms by which anticancer drugs exert their effects. Free radicals play a dual role in this context: they can induce cancerous mutations, but they also participate in the mechanism of action of certain chemotherapeutic agents. By understanding how free radicals generated by an anticancer drug, researchers can design more effective treatments with reduced side effects, potentially enhancing patient outcomes.

Radiation-induced mutation breeding is another significant area where free radical research is pivotal. The role of free radicals in DNA damage and repair mechanisms is crucial for developing improved strategies for inducing beneficial mutations in crops, thus contributing to agricultural advancements and food security. In the field of food science, investigating the antioxidant capacity of irradiated foods is vital. Irradiation, used for food preservation, can lead to the formation of free radicals. Understanding how antioxidants in food can neutralize these radicals ensures the safety and nutritional quality of irradiated food products. Furthermore, the modulation of immune responses by antioxidants is a growing research focus. Free radicals are involved in immune signaling pathways, and antioxidants can influence these processes. By deciphering these interactions, there is a potential to develop novel therapeutic approaches for immune-related disorders.

4.3.1. Advances in instrumentations and methodologies and free radical research (2003 onward)

After 2003, the Bioscience group made significant progresses in the study of free radicals and antioxidants research by acquiring advanced instruments and establishing state-of-the-art facilities. Notable acquisitions include flow cytometer, multi-mode plate reader, confocal microscope, high-throughput cell analyzer, live cell imaging, and a hypoxia facility. These tools enabled sophisticated analyses and experiments that were previously unattainable, facilitating groundbreaking research in this field. The availability of free radical-specific probes, coupled with the advanced instrumentation at their disposal, empowered the Bioscience group to conduct high-precision and high-throughput studies. This led to a surge in high-quality research output, with numerous articles published in prestigious, high-impact journals. The integration of these advanced tools allowed researchers to observe deeper into the mechanisms of oxidative stress, explore the role of antioxidants in various biological processes, and develop new therapeutic strategies to

combat oxidative damage. In this way, Bioscience group's research extended beyond the country and establishes a robust global presence. Our findings contributed significantly to the scientific community's understanding of free radicals and antioxidants, influencing research agendas both in India and internationally. This period marked a transformative phase for the Bioscience group, positioning it at the forefront of antioxidant and free radical research, and cementing its reputation as leaders in the field.

5. Facilities and Infrastructure to study Free Radical Generation and Lifetime Measurements

Free radical reactions can be studied by a variety of techniques that can be used to detect, quantify, and characterize these highly reactive short-lived species. Most widely used techniques include electron paramagnetic resonance (EPR) spectroscopy, which measures the magnetic moments of unpaired electrons to identify and quantify radicals and study their environments; pulsed laser photolysis, which generates excited states and radicals with a pulsed laser to investigate reaction kinetics and lifetimes, pulse radiolysis technique which utilizes high-energy radiation to generate and monitor radicals, offering insights into reaction kinetics and mechanisms (**Fig. 1**).

Fig. 1: EPR spectrometer facility at FIPLY

Chemiluminescence is another technique that utilizes detection of light emitted from radical-involved reactions, particularly in biological and environmental systems. To decipher the mechanism of a free radical reaction, it is also equally important to quantify

the end product of such reaction. Here chromatographic technique like HPLC coupled to mass spectrometry (MS) plays an important role in identifying and quantifying the reactions induced by free radicals. Additionally, computational methods, like density functional theory (DFT) and molecular dynamics (MD), often complement the experimental results to predict and analyze radical properties. The stopped-flow spectrometer is an important indispensable tool in the study of persistent free radicals, providing rapid mixing, high temporal resolution, and versatile detection capabilities. It enables detailed kinetic analysis and mechanistic insights, contributing significantly to our understanding of free radical chemistry with various biologically important molecules like proteins, DNA and low molecular weight compounds that exhibits antioxidant/radioprotection properties. Each technique offers unique advantages, often requiring a combination of methods to fully understand the nature, behavior, and reactivity of these important chemical species.

5.1. Advancement in the instrumentation and basic understanding of free radical research

There has been substantial progress in the understanding of reactions of biologically relevant free radicals, by utilizing both biochemical studies and monitoring their free radical scavenging abilities. Biochemical techniques help to evaluate the efficacy of a molecule. However, in the development of new antioxidants and radioprotectors, it is important to have a deeper insight into the probable reactions taking place in the cellular milieu, which can be deduced from the kinetic parameters and lifetime of the intermediates. In this context, rapid kinetic methods such as pulse radiolysis have proven highly effective for directly investigating the interactions between antioxidants and free radicals with brief lifetimes, ranging from a few microseconds to seconds. Pulse radiolysis technique utilizes radiation chemical protocols where desired free radicals can be generated, quantified and their interactions with potential protective compounds can be monitored by different detection techniques like absorption, conductivity, EPR, Raman, polarographic technique.

The pulse radiolysis facility in Chemistry Group, BARC has a pulse linear electron accelerator (LINAC) of 7 MeV energy coupled to absorption-based detection. The LINAC was procured from Radiation Dynamics, UK in the year 1986 and the optical detection system and the software for kinetic analysis were developed indigenously. The LINAC, which is currently 38 years old, is still the main working instrument driving the radiation chemistry group in BARC. Most of the components, except the waveguide, have been upgraded by the competent team of RPCD and the current machine is capable of generating 7MeV electrons in pulses of 25, 50, 100, 200, 500 ns as well as 2 µs pulse width. The LINAC allows controlled generation of free radicals, which allows monitoring their reactions on different time scales from ns to µs, revealing detailed mechanisms, identifying transient species and monitoring their formation as well as decay kinetics. In the year 2023, to improve its operational efficiency furthermore, an application software using for the 7MeV LINAC accelerator was developed which features a user-friendly GUI for intuitive operation. It incorporates soft interlocks and

precise timing optimization using a delay generator. Additionally, the program includes both soft and hardware-based safety interlocks to ensure safe operation. The pulse radiolysis facility has contributed tremendously in collecting the large databases and facilitating structure-activity relationship studies, optimizing the chemical structures of protective compounds for enhanced efficacy. The work on Radiation Chemistry is highly appreciated and acknowledged in the international arena.

Similar experimental support one gets by utilizing stopped flow spectrometer which is utilized to understand the reaction of persistent free radicals and molecular oxidants like hydrogen peroxide, peroxynitrite, hypochlorite, etc. with antioxidants. Using stopped flow spectrometer the reaction kinetics and mechanism of various natural and synthetic antioxidants with peroxynitrite has been evaluated and documented in scientific journals. Similarly, the reaction of curcumin and its derivatives with superoxide radical has been studied.

5.2. Advancement in instrumentation, multidisciplinary approach and collaborations

In the last 25 years, the Radiation Chemistry section of the Chemistry group, BARC has contributed immensely towards antioxidant research. Initially, a number of naturally occurring and synthetic compounds belonging to phenols and non-phenol functional group were studied for their ability to scavenge free radical using pulse radiolysis. These diverse compounds were synthesized in house (Chemistry Group) and some were procured through various national and international collaborations. Our research has explored various natural molecules such as curcumin, folic acid, eugenol, isoeugenol, dehydrogingerone, resveratrol, embelin, sesamol, bergenin, ferulic acid, caffeic acid, cinnamic acid, plumbagin, bakuchiol, ellagic acid, vanillin, chlorophyllin, and capsaicin. These investigations have identified the vulnerable sites for free radical and the factors that enhance the stability of phenoxyl radicals. These studies were published and were well cited in scientific community and one of the natural compound, chlorophyllin is now established as radioprotector. In addition to the pure compounds, plant extracts and herbal formulations enriched with phenols were also evaluated. Extracts of plant products like *Phyllanthus amarus Linn, Phyllanthus emblica (amla),* T*rigonella foenumgraecum, Plumbago zeylanica, Momardica charantia Linn, Glycyrrhiza glabra, Acacia catechu, Terminalia chebula*, *Terminalia arjuna, Cysalpinea digyna, etc.* have been examined for antioxidant activity. While initial findings in this area are promising, the complexity of formulations and the influence of various components, which depend on processing and environmental conditions, have limited these studies to screening purposes rather than detailed evaluation of reaction mechanisms. In last decade a new program on developing selenium based radioprotector was initiated in Chemistry Group, BARC. The main challenge for developing selenium based radioprotectors is their toxicity in presence of redox active agents. The radiation chemical studies played a pivotal role in exploring the role of structure and substitution on the reactivity of selenium compounds towards free radicals and molecular redox agents. This research led to the design of selenium compounds having moderate reactivity and stability under in vivo redox conditions and identifying the lead compound diselenodipropanoic acid (DSePA). During the

investigation, it was observed that certain phenomena take place within picoseconds timescale and hence the current nanosecond facility is not sufficed to venture in the short time window. In view of this a new facility is being developed in RPCD, Chemistry Group in collaboration with RRCAT, Indore to build a picosecond electron accelerator facility where picoseconds electron pulses were initially planned to generate at the photocathode electron gun by using a femtosecond laser.

6. Critical role of antioxidants in maintaining the redox balance

Antioxidants are molecules that are key to maintaining the redox balance in biological systems. Redox balance refers to the equilibrium between oxidants (such as free radicals) and antioxidants in the body. This balance is essential for proper cellular function and overall health. Various critical role of antioxidants in maintaining redox balance includes; neutralizing ROS, protecting biomolecules from oxidative damage, regenerating other antioxidants, modulating cellular signaling pathways etc. ROS, including $O_2^{\bullet -}$, $^{\bullet}OH$, and H_2O_2, are natural by-products of cellular metabolism. However, excessive ROS production, due to factors like environmental stressors or metabolic dysfunction, can lead to oxidative damage. Antioxidants act as scavengers, neutralizing ROS and preventing their harmful effects. Enzymatic antioxidants (Superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx)) and non-enzymatic antioxidants (vitamin C, vitamin E, glutathione (GSH), flavonoids) play key roles in ROS detoxification. ROS can damage lipids, leading to lipid peroxidation, a chain reaction that produces harmful lipid hydroperoxides and aldehydes. This process damages cell membranes and can lead to cell dysfunction or death. ROS can also oxidize proteins, altering their structure and function. This can disrupt enzymatic activity, impair signaling pathways, and contribute towards age-related diseases such as neurodegenerative disorders. ROS-induced damage to DNA, such as base modifications and DNA strand breaks, can lead to mutations, genomic instability, and increased risk of cancer. Some antioxidants can regenerate other antioxidants, for example, vitamin C can regenerate oxidized vitamin E, while glutathione can recycle vitamin C and repair oxidized proteins. Enzymatic antioxidants such as glutathione reductase and thioredoxin reductase play key roles in maintaining the reduced state of antioxidants like GSH and thioredoxin, ensuring their availability for ROS scavenging. Antioxidants help in regulating redox signaling by modulating ROS levels and protecting against excessive oxidative stress. Moderate ROS levels can activate cellular defense mechanisms, such as the upregulation of antioxidant enzymes and heat shock proteins, leading to cellular adaptation and improved stress resistance. Redox imbalance, characterized by excessive ROS production or antioxidant deficiency, results in oxidative stress. Oxidative stress contributes to the pathogenesis of numerous diseases, including cardiovascular diseases, neurodegenerative disorders, cancer, and metabolic disorders. Oxidative damage to biomolecules impairs cellular function and can lead to cell death or senescence. Chronic oxidative stress accelerates aging by promoting cellular senescence, telomere shortening, and the accumulation of damaged

macromolecules. Oxidative damage is linked to various age-related conditions, including Alzheimer's disease and age-related macular degeneration.

7. Application and impact of antioxidant research in Nuclear Energy Program

Antioxidant research has significant applications in various fields, including radioprotection within the nuclear energy program. The impact of antioxidants in this context is multifaceted, encompassing protection against ionizing radiation, mitigation of radiation-induced damage, and enhancement of cellular repair mechanisms. One of the major application and impact of antioxidant research in nuclear energy program is development of radioprotector. Antioxidants can mitigate the harmful effects of ionizing radiation by scavenging free radicals generated during irradiation. This includes ROS such as •OH, and O2•-, which are produced by the radiolysis of water molecules. Antioxidants protect biomolecules, including DNA, proteins, and lipids, from oxidative damage caused by these harmful radicals. By preventing oxidation and maintaining the integrity of cellular components, antioxidants help in preserving cellular function and reduces the risk of radiation-induced mutations and cell death. Antioxidants can be used to protect radiation workers in nuclear reactors and facilities from the harmful effects of chronic low-dose radiation exposure. Supplementation with antioxidants may help reduce the risk of radiation-induced health problems, such as oxidative stress-related diseases and cancer. In the event of a radiation emergency, such as a nuclear accident or radiation spill, antioxidants can be given to affected individuals to mitigate acute radiation injury and support recovery. By reducing oxidative stress and DNA damage, antioxidant supplementation can improve the health and well-being of workers in the nuclear energy sector, thereby enhancing occupational safety. Antioxidant-based radioprotection strategies have the potential to safeguard public health in the aftermath of nuclear accidents by minimizing the health risks associated with radiation exposure. Thus, antioxidant research is needed to identify and develop novel antioxidants with enhanced radioprotective properties, as well as formulations optimized for specific applications in nuclear energy programs. Determining the optimal dosage and delivery methods for antioxidant supplementation in radiation protection contexts is essential for maximizing efficacy while minimizing potential side effects.

8. Free Radical and Antioxidant Research for Society

Free radical and antioxidant research at the BARC has led to the development of products such as AKTOCYTE and the medicinal rice variety Sanjeevani. These innovations serve as adjuvants and immunity-boosting products with potential applications in cancer treatment and overall health enhancement. AKTOCYTE is a Chlorophyllin-based formulation designed to offer radioprotection to normal cells while sensitizing cancer cells. It also aids in the regeneration and repair of damaged tissues following radiotherapy. This dual action makes it a promising candidate for enhancing the effectiveness of cancer treatments and improving patient outcomes. AKTOCYTE has received approval from the Food Safety and Standards Authority of India (FSSAI) for

marketing as a nutraceutical. Additionally, it is currently at an advanced stage of clinical trials for use in cancer patients, further underscoring its potential therapeutic benefits. Sanjeevani rice is a medicinal variety of rice developed for its immunity-boosting properties. The research has resulted in the creation of three products derived from this rice: Sanjeevani Kalk, Sanjeevani Bar, and Sanjeevani Instant product. These products are designed to enhance immune function, contributing to overall health and wellness. The development of Sanjeevani rice and its related products highlights the innovative use of traditional crops in modern health applications. Thus, AKTOCYTE and Sanjeevani rice, represent significant contributions to both cancer therapy and general health promotion.

9. Future directions of free radical biology program in the department of atomic energy

The Department of Atomic Energy (DAE) plays a pivotal role in various domains, including nuclear energy, agriculture, food security, nuclear medicine, and basic radiation research. Incorporating a Free Radical Biology Program within the DAE holds immense potential for advancing our understanding of oxidative stress, radiation biology, and radioprotection. Some of the future directions for the Free Radical Biology Program within the DAE may be, integration of free radical biology with radiation research, to investigate the molecular mechanisms underlying radiation-induced oxidative stress, application of free radical biology in nuclear medicine. Other than this discovery and validate biomarkers of radiation exposure and oxidative stress for use in bio-dosimetry and radiation emergency preparedness. We can also utilize state-of-the-art spectroscopic, imaging, and omics technologies to investigate free radical biology and oxidative stress in nuclear settings. Foster collaborations with other research institutions, universities, and industry partners to leverage expertise in free radical biology, radiation biology, and nuclear science for mutual benefit.

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RADIATION-INDUCED SIGNALING: EXPLORATIONS OF PAST, PRESENT AND THE FUTURE

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Abstract

This book chapter delves into the birth and evolution of radiation signaling research at Bio-Science Group (BSG) of Bhabha Atomic Research Centre (BARC), tracing its origins from the discovery of X-rays by Wilhelm Roentgen in 1895 to the intricate molecular studies of radiation-induced pathways, as known today.

The chapter emphasizes the contributions of BSG scientists in elucidating the role of signal transducers like PI-3K and PKC in radiation response, the differential responses of these kinases to varying doses of radiation, the role of MAP kinases in determining cell fate, and the exploration of natural compounds like curcumin in modulating radiation-induced signaling.

The chapter also explores the impact of fractionated irradiation on cellular signaling, particularly in cancer cells, revealing mechanisms behind radioresistance and potential strategies for radiosensitization. Additionally, it covers the research on charged particle-induced signaling, including the differential effects of heavy ions, carbon ions, protons, and oxygen beams on cellular pathways and DNA damage responses.

The chapter underscores the transition from basic radiation biology to the complex study of molecular signaling, highlighting the evolution of research

at BARC and its contributions to the field. The discoveries made by the Bio-Science Group at BARC continue to influence the understanding and application of radiation therapy in oncology.

1. Introduction

Radiosensitivity of tumors and normal tissues varies considerably among individuals; for treatment with same radiation dose to tumors in patients, some experience more severe reactions than others, while few will experience unacceptable late reactions. Using simple laboratory tests for identifying radiosensitive individuals in advance who are particularly sensitive to radiation would be of immense benefit for clinical treatment.

Irradiating cells under experimental controlled conditions and being able not just to predict fate of the cells such as growth, death, resistance or mutation but also to correlate these biological end points to the molecular and cellular alterations is the ultimate goal of radiation biologists. However, many times these correlations are often difficult to make because of the cell specific pathways which are activated leading to different end points in different cells. Understanding effects of radiation on signaling networks in search of molecular targets which could predict or alter the sensitivity of the cells to radiation would be an advantage.

Possibility for the future may be to screen patients who are to undergo radiation therapy for the presence of genes or proteins that may confer radiosensitivity or radioresistance and then decide the course of treatment to avoid post radiotherapy complications in radiosensitive patients.

2. Importance of Radiation-Induced Signaling

Radiation-induced signaling studies are essential for understanding the complex biological responses to ionizing radiation (IR), which has significant implications for medicine, environmental safety, and radiation protection. These studies focus on how cells detect and respond to radiation exposure, leading to various cellular outcomes such as DNA repair, apoptosis (programmed cell death), senescence (cell aging), and carcinogenesis (cancer development). Here are some key aspects highlighting the importance of these studies:

2.1. Understanding DNA Damage and Repair Mechanisms

Radiation can cause direct damage to DNA or produce reactive oxygen species (ROS) that indirectly damage cellular components. In addition to ensuing repair pathways activation, DNA damage sensors and other survival pathways are also activated which affect the final outcome of cellular repair or death. Understanding these signaling pathways activated in response to DNA damage is crucial for developing strategies to protect normal cells during radiation therapy and to enhance the repair of radiation-induced damage in various medical and industrial applications.

2.2. Enhancing Cancer Treatment

Radiation therapy is the cornerstone of cancer treatment. By studying radiation-induced signaling, researchers can identify targets to improve the efficacy of radiation therapy. For instance, inhibiting certain signaling pathways that promote cancer cell survival can enhance the sensitivity of tumors to radiation, making the treatment more effective. Additionally, understanding these pathways can help in minimizing the side effects on normal tissues, thereby improving the overall therapeutic index.

2.3. Radioprotection and Mitigation

Understanding the signaling pathways involved in radiation response can lead to the development of radioprotective agents that can be used to protect individuals from the harmful effects of radiation exposure. This is particularly important for people working in high-radiation environments, such as healthcare workers, nuclear power plant employees, and astronauts. These studies can also aid in developing strategies to mitigate the effects of accidental or intentional radiation exposure.

2.4. Biomarker Development

Research in radiation-induced signaling can lead to the identification of biomarkers for radiation exposure and response. These biomarkers can be used for early detection of radiation-induced damage, monitoring the effectiveness of radioprotective measures, and assessing individual susceptibility to radiation. This has important applications in personalized medicine, where treatments can be tailored based on a patient's specific biological response to radiation.

2.5. Advancing Basic Science

These studies contribute to the broader field of cell biology by providing insights into fundamental processes such as signal transduction, gene regulation, and cellular stress responses. The knowledge gained can have far-reaching implications beyond radiation biology, informing research in areas like cancer biology, immunology, and developmental biology.

In summary, radiation-induced signaling studies are critical for advancing our understanding of how living organisms respond to radiation at the cellular and molecular levels. The insights gained from these studies have wide-ranging applications in improving cancer treatment, enhancing radioprotection, safeguarding public health, and advancing basic biological research.

3. Purpose and Scope of the Chapter

The purpose of this chapter is to provide a comprehensive overview of the pioneering research conducted at the Bhabha Atomic Research Centre (BARC) in Mumbai on radiation-induced signaling. This chapter aims to chronicle the evolution of scientific understanding in this field, highlighting key discoveries and experimental breakthroughs from the early years to the present day. It explores the various mechanisms by which different forms of ionizing radiation, including gamma rays, protons, and heavy ions, interact with cellular components to initiate complex signaling pathways. The scope of

this chapter encompasses detailed discussions on the contributions of notable researchers, the methodologies employed in their investigations, and the implications of their findings for both fundamental biology and clinical applications. By examining past achievements, current advancements, and future directions, this chapter seeks to provide readers with an in-depth appreciation of the significance of radiation-induced signaling research at BARC and its impact on the broader scientific and medical communities.

4. Birth and Evolution of Radiation Signaling

In 1895, Wilhelm Roentgen named the rays which blackened photographic film at the end of the tube as 'X' rays, with 'X' representing the unknown. Soon after, in 1898, first biological effects of X-rays were known, when Henry Becquerel observed skin damage and ulceration around the area of his vest pocket where he kept the radium. These results were further confirmed by Pierre Cardin in 1901 and thus, the field of Radiation Biology; study of action of ionizing radiation on living beings, was born. Within few years, X-rays were used to treat cancer and other diseases.

Early studies on radiation and living systems primarily focused on radiation-induced cell death. These studies were mainly conducted on microorganisms because there was no simple, effective technique for large-scale colony production from single mammalian cells, unlike the plating and colony-forming techniques available for bacteria. In the 1950s, Marcus et al. developed a similar plating-based assay to estimate the reproductive potential of mammalian cells in a petridish. This breakthrough provided ample scope for *in vitro* studies on mammalian systems and is still considered the gold standard for assessing radiation-induced cell killing. This development generated lot of enthusiasm among radiation biologists, who could now study the effects of radiation on mammalian cells grown in plates and elucidate mechanisms such as damage to DNA, membranes, and other biomolecules. Evidences then started accumulating towards DNA being principal target for the biological effects of radiation, including cell killing, carcinogenesis, and mutation. Studies on DNA damage and repair therefore, took the center stage; however, cells from IR-sensitive ataxia telangiectasia patients showed that double strand break (DSB) repair was not sufficient to prevent IR hypersensitivity. Subsequently, upregulation of genes and ensuing signal transduction process after radiation were explored. In the late 1980s, it was discovered that radiation increased the expression of early response genes, which were previously known to be activated by physiological inducers such as growth factors.

These early response genes, being transcription factors, could induce the expression of many other proteins responsible for cell division and growth. Investigations were then carried out to identify the kinases involved in early gene expression from damaged DNA. In the early 1990s, it was reported that Mitogen-Activated Protein Kinases (MAPK) and tyrosine kinases, which are directly activated by growth factors or mitogens, were also activated by free radicals and radiation.

The discovery of kinase activation and early response gene induction by radiation transformed the field of radiation signaling, which had previously focused on understanding the mechanisms of radiation-induced killing. This led to a new era of research on cytoplasmic and proliferative signaling after radiation, making it plausible to study differential cell sensitivity and the development of radioresistance in cancer cells. Many radiation biologists worldwide began investigating the effects of radiation on cellular signaling networks and how these networks influenced the final outcomes. The scientists at the Bio-Science Group of BARC were also inspired by this enthusiasm and sought to uncover the key to the cell's radiation response.

5. Early Years of Radiation Signaling Work at Bhabha Atomic Research Centre

Early work on two main signal transducers known at that time, phosphoinositide kinase (PI-3K) and protein kinase C (PKC) activity in response to whole-body irradiation laid the foundation for understanding the molecular intricacies of radiation's impact on living tissues. The study published in 1999, revealed that alterations in PI3K pathway could be detected as early as 15 minutes post-irradiation. This was a significant finding, as it indicated that the initial changes in diacylglycerol (DAG) levels and PKC activation were crucial early events that could potentially trigger tumorigenesis. These early alterations highlighted the potential for manipulating tumor responses to radiotherapy.

In 2001, deeper insights into activation of signaling molecules following whole-body gamma irradiation in mice model were attempted. The work showcased the differential responses of tyrosine kinase and PKC to varying doses of radiation. It was shown that while tyrosine kinase responded sharply to lower doses (10 cGy), PKC required higher doses (3 Gy) for activation. This nuanced understanding underscored the complexity of the whole organ's response compared to single-cell studies, emphasizing the importance of considering whole-animal responses in radiobiological research.

In subsequent years, dose-dependent expression of PKC isozymes in mouse lymphocytes after gamma irradiation was investigated. In an early work conducted in 2004, the activities of three main cytoprotective kinases, tyrosine kinase, PKC, and MAP kinase, in *ex vivo* and *in vivo* settings were compared at different doses. Another study revealed a dose-dependent differential response in PKC activity and highlighted the importance of MAP kinase activation at higher doses *in vivo*. These works highlighted significant differences in isozyme responses to *in vivo* and *ex vivo* irradiation. These insights were crucial for developing strategies to manipulate tumor responses to radiotherapy, considering the whole animal's response rather than relying solely on single-cell data.

With observed *in vivo* and *in vitro* differences in activation of kinases and various reports on radiation- induced activation of both cytoprotective and cytotoxic kinases it was becoming evident that activation of the kinase *per se* was not the deciding factor of the fate of the cell and that there was fine tuning of networks. Elegant studies done in 2004 showed that duration of activation of kinases and their chronology of activation in the cells could play an important role. These studies done in radioresistant liver cells showed early and transient activation of cytotoxic kinases which probably was in response to

stress and for the immediate requirements of the cell and did not further feed into cytotoxic pathways; however, the cytoprotective kinases such as ERK which showed prolonged activation led to final outcome of liver cell survival.

Similar studies were performed to understand the relative roles of two major DNA damage sensors known at that time, Poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PK). Another study published in 2004 provided clues into how these enzymes, both vital for DNA repair, shared their responsibilities for recognizing DNA damage after radiation. It was observed that at high doses, when probability of DNA DSBs is high, DNA-PK is more active while PARP is switched off, which could be safe bet for cells as high activation of PARP leads to cell death. At high doses therefore, early sensing and transducing DNA damage was assigned to DNA-PK so that repair prevails.

Modulation of Signaling Factors

As radiation-induced cytoprotective/proliferative pathways were being discovered, the hunt was on to find natural compounds which could modulate their activation so as to achieve radio-sensitization for desired clinical outcome of radiotherapy. Scientists at BSG also delved into this area of testing effect of natural phenolic compounds on radiation-induced kinases. Their work demonstrated that compounds like curcumin, ellagic acid, and quercetin could inhibit PKC activity, with curcumin and ellagic acid being particularly effective which could thereby prevent the development of radioresistance in tumor cells. Most of the radiation-induced studies done till then were on mouse lymphocytes as both *ex vivo* and *in vivo* irradiation studies could be easily done with these cells. However, there was need to study radiation signaling in tumor cells for which fibrosarcoma cells were chosen to generate tumors in mice which could be later excised and made in to single cell suspension and reinjected if the need be. This tumor model therefore, supported studies on both *ex vivo* and *in vivo* irradiation of tumor cells.

Nuclear factor-kappaB(NF-kB), an important transcription factor with pleiotropic role was also found to be induced by radiation during early 90s. The BSG team thus, looked at the modulation of NF-kB in addition to other cell survival factors like PKC, and ERK in fibrosarcoma induced in mice. Their results were promising: while PKC isoforms were unaffected, both ERK and NF-kB were significantly inhibited by curcumin and nicotinamide. This suggested potential new strategies to enhance tumor cell killing and prevent radioresistance.

Another modulator which was very intriguing was Nitric Oxide (NO). NO was initially identified as a vasodilator generated by endothelial cells and later was found to be produced by macrophages during anti-pathogenic and anti-tumor response. These important initial studies led to explosion of NO research which had started revealing the importance of this diatomic molecule in biological systems as a signaling molecule. Just like reactive oxygen species which were known by then to be important signaling mediators in living systems, reactive nitrogen species were also researched if they could act in a similar way. Studies were therefore done to explore modulation of radiation-induced signaling in presence of nitric oxide**,** as ROS generated by radiation could lead to

RNS in presence of NO which could then modify proteins differently. Tyrosine moieties of MAP kinases, one of which is phosphorylated for the kinase to be active was looked for their nitration under NO rich environment after irradiation. The study revealed that NO donor could inhibit radiation-induced tyrosine phosphorylation of kinases; however, it did not have any effect on cellular function as assessed by phagocytic efficiency of these phagocytic cells. These studies were intriguing and supported the idea that nitro group could probably do the job of phosphor group on tyrosine. Estimating activation of signaling kinases via phosphorylation in a pathway thus needed a revisit in context of high nitric oxide rich environment inside the tumors which contained many different cells including phagocytes.

The transition from basic principles of radiation biology to intricate studies of cellular signaling pathways underscores the evolution of research at the institution. The early focus on understanding radiation's biological effects expanded into a detailed exploration of molecular mechanisms, driven by the relentless curiosity and dedication of BSG scientists.

The above-mentioned studies were done on single doses used in the therapeutic range (2Gy - 4Gy). However, during radiation therapy, radiation is delivered not in single but in multiple repeated doses to kill tumor cells while limiting the dose to normal cells. Studies were therefore, designed to simulate this mode of radiation delivery akin to the regimen used in cancer radiotherapy and then look at the activation or upregulation of signaling components which could answer the development of radioresistance in tumors due to repeated multiple doses.

6. Navigating Radioresistance: The Role of Fractionated Irradiation Signaling

In a pivotal study published in 2007, scientists at BSG explored the effect of fractionated doses of Co-60 gamma-irradiation on a murine fibrosarcoma model. Role of the three major MAP kinases: p44 MAP kinase, p38 MAP kinase, and stress-activated protein (SAP) kinase was probed. These kinases are crucial in determining whether a cell will survive or undergo apoptosis after radiation exposure. The study showed that fractionated irradiation elicited an adaptive response, characterized by sustained activation of the prosurvival p44 MAP kinase over seven days. This was balanced by increased activation of the proapoptotic p54 SAP kinase shortly after irradiation. Interestingly, the dual specificity phosphatase PAC1 was also induced, potentially acting as a feedback regulator of p44 MAP kinase. These findings highlighted the potential of targeting p44 MAP kinase to enhance the efficacy of radiotherapy.

Building on this foundation, the role of the Rad52 gene in fractionated irradiation-induced signaling in A549 lung adenocarcinoma cells was explored. The study compared the effects of fractionated doses of gamma-irradiation with acute doses and found that A549 cells exhibited increased radioresistance when the 10 Gy dose was delivered fractionally. Microarray analysis revealed the upregulation of DNA repair and cell cycle arrest genes in cells exposed to fractionated irradiation. Key DNA repair pathway-associated genes, including DNA-PK, ATM, Rad52, MLH1, and BRCA1, were intensely

activated. Moreover, phospho-p53 translocated to the nucleus, indicating an active DNA damage response. Remarkably, silencing the Rad52 gene in fractionated A549 cells rendered them radiosensitive, underscoring the crucial role of Rad52 in mediating radioresistance.

Comprehensive studies on fractionated radiation-induced radioresistance and the subsequent activation of signaling pathways were also conducted utilizing an isogenic cell line developed through repeated irradiation of the lung cancer cell line A549 and a pivotal role of the transcription factor Nrf2 in promoting radioresistance was uncovered. These findings highlighted the Nrf2 pathway as a potential target for radiosensitization, presenting a promising avenue in the fight against resistant lung cancer subpopulations.

The cumulative efforts of the Bio-Science Group at BARC underscore a profound understanding of fractionated irradiation-induced signaling. These studies not only unravel the molecular intricacies of radiation response but also pave the way for potential therapeutic strategies. By targeting specific signaling pathways and genes, such as p44 MAP kinase, Rad52 and Nrf2 researchers aim to enhance the sensitivity of cancer cells to radiotherapy, thereby improving treatment outcomes.

7. Chronicles of Charged Particle Induced Radiation Signaling Work at BARC

The Bhabha Atomic Research Centre (BARC) has long been at the forefront of radiation research, pioneering advancements that bridge physics and biology. Within this hallowed institution, the Bio-Science Group embarked on a journey to unravel the mysteries of charged particle-induced radiation signaling, a field teeming with promise and challenges. The team's research has significantly advanced our understanding of how different forms of ionizing radiation, such as heavy ions, carbon ions, protons, and oxygen beams, influence cellular signaling pathways and DNA damage responses. Through meticulous research and relentless pursuit of knowledge, they illuminated pathways that hold the potential to revolutionize cancer therapy and enhance our understanding of cellular responses to high-linear energy transfer (LET) radiation.

7.1. Early Explorations: NF-kappaB and ERK Pathways

In 2004, a pivotal study examining the effects of heavy ion irradiation on the expression of NF-kappaB and extracellular signal-regulated kinase (ERK) in cells was published. Heavy ion irradiation was found to be more biologically effective than gamma radiation due to its propensity to cause clustered DNA damage. The study revealed that both NF-kappaB and ERK, which are critical for cell survival and act as anti-apoptotic factors, exhibited fluctuating levels post-irradiation. This suggested that the enhanced biological effectiveness of heavy ions might be due to altered signaling patterns, potentially leading to increased mutagenicity and inhibition of apoptosis.

7.2. Delving Deeper: Carbon Ion Irradiation

Further expanding on the above work, another study published in 2005 investigated the effects of carbon ion irradiation on signaling pathways. The work focused on the expression of p44/42 MAPK and NF-kappaB, which are crucial for cell survival. It was

found that the responses to high and low doses of carbon ion irradiation were markedly different. Specifically, the expression of p44/42 MAPK varied with the dose, and its inhibition by wortmannin, a DNA-PK inhibitor, indicated a complex interplay between DNA repair mechanisms and signaling pathways. Notably, NF-kappaB expression was higher at 1Gy compared to 0.1Gy and was not affected by DNA-PK inhibition. These findings underscored the need for further investigation into the time-dependent nature of cellular responses to high LET radiation.

7.3. Cytoprotective Pathways after Proton, Gamma and ROS

In 2009, differential activation of mitogen-activated protein kinases (MAPKs) in murine macrophage cells following exposure to gamma and proton radiation was explored. This study demonstrated that the activation patterns of ERK, JNK, and p38 kinases varied significantly between proton ions and X-rays. High LET radiation resulted in a prolonged but marginal activation of the prosurvival ERK pathway and a significant activation of the proapoptotic p38 pathway. This research highlighted the distinct cellular responses elicited by different types of radiation and emphasized the importance of the MAPK signaling cascade in determining cell fate post-irradiation.

7.4. Proton Beam and Lung Adenocarcinoma Cells

The quest to differentiate the biological effects of proton beams from gamma radiation continued with a study published in Cancer Investigation in the year 2010. It was found that proton beams were more cytotoxic to A549 lung adenocarcinoma cells than gamma radiation, leading to distinct signaling outcomes. The proton beam-induced cell death was linked to the upregulation of pro-apoptotic Bax and downregulation of anti-apoptotic Bcl-2, revealing crucial insights into the mechanisms driving proton beam therapy's effectiveness.

7.5. Carbon Beam and DNA Damage Response

A 2011 study further investigated the differential signaling responses to gamma and carbon beam irradiation in A549 cells. Despite both radiation types activating the same repair molecules, such as ATM and BRCA1, the study found significant differences in the nature and extent of DNA damage responses. Carbon beams, characterized by high LET, induced early-phase apoptosis and did not activate the prosurvival ERK pathway, unlike gamma radiation. This suggested that the distinct macromolecular complexes formed by high and low LET radiation could explain the differential cellular responses observed.

7.6. Oxygen Beam Induced Signaling Differences

In the same year 2011, another study compared the effects of oxygen ion and gamma irradiation on A549 cells. Oxygen beams were found to be significantly more cytotoxic, inducing efficient DNA repair only in cells exposed to gamma radiation but not in those exposed to oxygen beams. The study highlighted the significant activation of sensor proteins ATM and ATR, as well as Chk1, Chk2, and p53, in oxygen beam-irradiated cells. This comprehensive analysis provided valuable insights into the unique signaling pathways activated by different types of high LET radiation

7.7. Comprehensive Analysis: Proton vs. Gamma Irradiation

In 2015, an extensive study was conducted on the biological effects of proton and gamma irradiation on human non-small cell lung carcinoma cells (A549). Various biological endpoints, including gene expression, cell cycle, cell death, epithelial-mesenchymal transition (EMT), and cancer stem cell traits were investigated. Proton beams were found to be twice as cytotoxic as gamma radiation, inducing higher and longer cell cycle arrest, and affecting a broader range of genes. Proton irradiation also reduced cell adhesion, migration ability, and the population of cancer stem cell-like cells, indicating its potential biological advantages in cancer therapy. Their studies corroborated the radiation biologist's apprehension of use of 1.1 RBE for protons which were found be more cytotoxic to cells due to activation of various pathways that were triggered. Protons therefore, did not just have a physical dose deposition advantage but also a biological advantage in terms of lower metastatic potential and stemness of the irradiated cells. Hence, there use in treatment of pediatric tumors could all the more be justified and preferred and that considering their higher toxicity, the RBE of 1.1 could be revisited so as to reduce the dose to pediatric patients while achieving the desired tumor cell kill.

The Bio-Science Group at BARC has thus, made monumental strides in understanding charged particle-induced radiation signaling. Their work has unveiled the intricate signaling pathways activated by different types of radiation, providing a foundation for developing more effective cancer therapies. Through their dedication and pioneering research, they have illuminated the path toward a future where radiation therapy can be tailored to maximize its therapeutic potential while minimizing adverse effects. The legacy of their discoveries continues to inspire and guide the scientific community in the quest for breakthroughs in radiation biology and oncology.

While the impact of radiation-induced signaling within cells was being meticulously studied to understand its effects on cellular fate, intriguing discoveries were made regarding the communication between irradiated and nearby unirradiated cells. A dedicated team in the Bio-Science Group was trying to uncover the mysteries of how cells not directly exposed to radiation could still experience profound biological changes, thanks to signals from their irradiated neighbors.

8. The Unseen Messengers: Unraveling the Bystander Effect

The journey began in the year 2006 with studies on bystander effects of gamma radiation on murine lymphocytes. Irradiated conditioned medium (ICM) from gamma-irradiated lymphocytes was used to observe its effects on unirradiated lymphocytes. The results were startling. Unirradiated lymphocytes exposed to ICM showed increased proliferation, elevated levels of reactive oxygen species (ROS), and enhanced expression of proliferation markers such as CD25 and cyclin D. This finding suggested that soluble factors released from irradiated cells initiated a cascade of signaling events in non-irradiated cells, potentially leading to increased radioresistance.

Building on this foundation, in the following year, gap junction-independent signaling was found to be responsible for the observed bystander effects in human erythroleukemia cells (K562). The medium from irradiated cells, rich in signaling molecules and stable free radicals, activated critical proteins like NF-kappaB and p21 in bystander cells, leading to apoptosis and other stress responses.

These studies were further extended to explore intercellular communication between different cell types. It was found that irradiated lymphoma cells (EL-4) and macrophages (RAW 264.7) could also induce bystander effects. Interestingly, this signaling was highly dependent on the activation of inducible nitric oxide synthase (iNOS) and the subsequent production of nitric oxide (NO), emphasizing the role of specific signaling pathways in mediating these effects.

Meanwhile, the differential responses of normal and cancer cells to bystander signaling was being investigated by separate teams in BSG. A study published in the year 2011 highlighted that low-dose and high-dose gamma-irradiated conditioned medium (ICM-L and ICM-H, respectively) from human leukemic cells induced varying levels of apoptosis in both normal lymphocytes and cancer cells. The dose and dose rate of radiation played crucial roles in determining the extent of the bystander response, with high-dose ICM inducing more significant apoptosis in both cell types.

The complexity of these interactions was further elucidated and cytokine profiles of various tumor cell lines following acute and fractionated doses of gamma radiation were analyzed. It was revealed that the radiation-induced cytokine response varied significantly across different tumor types, influencing the survival and growth of bystander cells. This nuanced understanding suggested that radiation therapy's effectiveness could be modulated by targeting specific cytokines involved in bystander signaling.

Study conducted in 2015 focused on the role of ataxia-telangiectasia mutated (ATM) protein in bystander signaling between human monocytes and lung adenocarcinoma cells. The study demonstrated that ATM activation was crucial for the bystander effect, especially in maintaining cell survival and DNA repair mechanisms in both directly irradiated and bystander cells. Suppressing ATM with siRNA significantly reduced the bystander effect, highlighting its potential as a therapeutic target.

The culmination of these studies led to a profound understanding of how bystander effects could be harnessed in cancer therapy. In a groundbreaking *in vivo* study using a mouse fibrosarcoma tumor model, it was demonstrated that irradiated tumor cells could inhibit the growth of unirradiated bystander tumor cells. This effect was mediated by soluble factors such as cytokines and proteins like VEGF, Rantes, and PDGF, which were differentially expressed in the supernatants of irradiated cells. These findings provided a deeper insight into the damaging and protective aspects of bystander effects, offering new avenues for enhancing the efficacy of radiotherapy.

Through meticulous research and collaboration, the Bio-Science Group at BARC has significantly advanced our understanding of radiation-induced bystander effects. Their work underscores the intricate web of intercellular communication and the potential for

manipulating these signals to improve cancer treatment outcomes. The unseen messengers within cells continue to reveal their secrets, paving the way for novel therapeutic strategies in the fight against cancer.

9. Modern Era: Innovation and Future Directions

Navigating early technological limitations and safety concerns, research at BARC has evolved significantly in both knowledge and techniques. Initially, studies focused on estimating the activation of kinases or receptors were limited to enzyme assays, gel electrophoresis and Western blotting, where only individual proteins could be analyzed using specific antibodies and needed elaborate procedures and dedicated rooms. However, with advancements in omics technologies, it became possible to simultaneously examine the activation of multiple genes and proteins within a cell. DNA damage and repair studies, once reliant on lengthy methods like PFGE and comet assays, have been streamlined with quicker techniques such as gamma H2AX estimation using confocal microscopy or flow cytometry. The roles of signaling proteins can now be directly assessed through specific inhibition using si/shRNAs, which effectively reduce their levels in situ. Additionally, the availability of high-throughput systems enables the simultaneous study of the radioprotective or sensitizing effects of various inhibitors within a single assay. Furthermore, live-cell imaging systems at Bio-Science Group allow for the automatic monitoring of cell growth, freeing up researchers' time to stay updated with the latest developments in radiation signaling.

The modern era of radiation signaling research at the BARC has been marked by significant strides in innovation and the exploration of future directions in radiobiology. As the foundational principles of radiation-induced cellular damage and signaling pathways have been established, current research has shifted towards more complex and integrative approaches. These advancements aim to enhance our understanding of radiation's effects on biological systems and to develop novel strategies for improving therapeutic outcomes, particularly in cancer treatment.

One of the most promising areas of research has been the exploration of charged particle therapy, such as proton and carbon ion therapy, which offers superior precision in targeting tumors while sparing surrounding healthy tissues. Studies at BARC have demonstrated that high LET radiation, including heavy ions and protons, induces distinct cellular responses compared to traditional low LET radiation like X-rays. These findings have led to a deeper understanding of how different types of radiation affect signaling pathways, including those involved in DNA repair, apoptosis, and cell cycle regulation. The research has revealed that proton beams, for example, are more cytotoxic than gamma rays, potentially offering a biological advantage in reducing metastatic potential and cancer stem cell traits in tumors.

In addition to exploring new types of radiation, BARC scientists have also focused on the modulation of radiation-induced signaling pathways to enhance radiotherapy's effectiveness. This includes investigating the role of natural compounds, such as

curcumin and quercetin, in sensitizing tumor cells to radiation while protecting normal tissues. By targeting specific signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway, researchers aim to overcome radioresistance in cancer cells, a major challenge in current cancer therapy.

Another innovative direction in BARC's research has been the study of fractionated radiation doses, which mimic the clinical delivery of radiation in multiple sessions rather than a single high dose. This approach has provided insights into how cells adapt to repeated radiation exposure, leading to the activation of survival pathways that contribute to radioresistance. The identification of key genes and proteins involved in this adaptive response, such as Rad52 and Nrf2, has opened up new avenues for developing targeted therapies that can disrupt these pathways and improve the efficacy of fractionated radiotherapy.

Looking to the future, the integration of advanced technologies, such as genomics, proteomics, and bioinformatics, is expected to play a crucial role in further unraveling the complexities of radiation signaling. These tools will enable a more comprehensive analysis of how radiation affects cellular networks and may lead to the discovery of new biomarkers for predicting treatment responses and outcomes. Additionally, the development of personalized radiotherapy approaches, tailored to the unique genetic and molecular profiles of individual patients, holds great promise for improving the precision and effectiveness of cancer treatment.

The modern era of radiation signaling research at BARC is characterized by a commitment to innovation and a forward-looking approach to addressing the challenges of cancer therapy, mainly radioresistance.

SIXTY GLORIOUS YEARS OF CYANOBACTERIAL RESEARCH IN BARC

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Abstract

Tradition of cyanobacterial research in BARC has been quite old, spanning nearly six decades of excellent scientific contributions in terms of high-quality basic research as well as of several appropriate technologies. It is a pleasure to recall some of these, the discoverers and some of their significant publications of that time. Sixty years is a long-time to remember and in doing so, I may have missed some of the important contributions inadvertently. This article attempts to take stock of some of the notable research carried out in MBD, by about a dozen researchers including myself, which has led to recognition of BARC, as the top-most laboratory for modern state of art cyanobacterial research in India in the last 5 decades. It has also earned a comparable reputation globally.

1. The Beginning

In BARC, research on blue-green algae (as cyanobacteria used to be referred to before 1980s) started in the late 1960s when late Dr. Joseph Thomas (my mentor, research supervisor and Boss) joined the then Biology Division of the Bio-Medical Group. One of his previous expertise was in mass cultivation of green algae and he used it to set up fermenter-based mass cultivation systems for the green alga *Chlorella* and the blue-green alga *Anabaena*. His major achievement in this field was also to establish the first *continuous culture* system for *Anabaena* at BARC. Later he also developed methods for growing two blue green algal isolates from Trombay, *Anabaena* L-31 and *Nostoc*-4, on

huge plastic sheets overlayed with a thin layer of soil, that made their harvesting easier. This mass cultivation technique came in handy for later evaluation of nitrogen biofertilizer potential of these cyanobacterial strains using ^{15}N-labeled Urea and NH_4NO_3 in the 1980s, in collaboration with Rashtriya Chemicals and Fertilisers (RCF) and NOCIL. This aspect of Dr. Thomas' research earned him lot of attention and considerable fame nationally.

However, what really caught attention of youngsters like me joining BARC in the early 1970s were his outstanding contributions to the basic biology of heterocyst differentiation in *Anabaena*. Heterocysts in *Anabaena* filaments not only looked special – they were shown to be special using a unique equipment Absorption Microspectrophotometer, that existed in the division that time, and could record absorption spectra of individual cells *in situ*. These studies revealed that heterocysts lacked the Photosystem II (PSII) pigments and therefore would not photoevolve O_2 like the remaining photosynthetically active vegetative cells (Nature 228: 181-183, 1970). That they were indeed microaerophilic was shown soon thereafter, by Dr. Thomas with another senior colleague Dr. K.A.V. David (Nature New Biol. 238: 219-221, 1972) using tetrazolium blue and nuclear emulsion-based microscopy. Later, after the procurement of a fluorescence microscope in the group, it became possible to demonstrate that fluorescence from Phycocyanin (the most important component of PSII) was also absent in heterocysts. This established heterocysts as the sole sites of aerobic nitrogen fixation (oxygen being highly toxic for N_2 fixation) in cyanobacteria. There was one more paper in Nature (by late Prof. R.N. Singh of BHU, Varanasi), published 2 years before my birth, which reported reclamation of highly saline alkaline sodic soils using naturally occurring populations of cyanobacteria around Varanasi (Nature 165: 325-326, 1950). The work was highly impressive because of its sheer simplicity and yet high quality, and truly reflected a case of *appropriate technology* which was the buzzword at that time. Charmed by these 3 Nature papers on cyanobacteria, I decided to join Dr. Thomas' group in the Biophysics Section of the then Biology & Agriculture Division (B&AD). When I narrated the saline soil reclamation story and my aspiration to experiment with it further to Dr. Joseph Thomas, he immediately agreed to allow me to work on this aspect, in spare time.

2. Scientific Contributions of first 3 decades

When I joined the laboratory in 1974, my senior colleagues Dr. David was continuing to work on heterocyst differentiation, and Dr. Tonina Fernandes, was busy discovering factors that trigger sporulation in *Anabaena torulosa*, and obtaining UV-induced mutants of cyanobacteria. Another colleague Nagaraja, from the previous (15^{th}) batch of BARC Training School Biology & Radiobiology discipline, was devising methods to isolate heterocysts using density gradient centrifugation. His methodology, though never published, was used by us every year to prepare heterocysts (as pure photosystem-I samples) for Prof. Govindjee (of Photosynthesis fame) who used to visit Biophysics

Section in summer to do fluorescence and thermoluminescence-based investigations in the mid-1970s.

During my deputation on an IAEA fellowship, I chose to work with Prof. W.D.P Stewart on CYANOBACTERIA, in the University of Dundee in Scotland, U.K. I must admit it was here that I really received my core training in cyanobacterial physiology and biochemistry. I worked there for 15 months on a new pathway of electron donation to nitrogenase in the heterocysts of *Anabaena*. This publication (Proc. Roy. Soc, London B 200: 1-25, 1978) remains one of my most cited paper which also earned my mentor the Fellowship of Royal Society in 1977. We published 2 more papers on the regulation of this pathway and purification of major components of the pathway.

Before I returned to India, my Ph. D. guide, Dr. Thomas, went on Sabbatical Leave to Prof. Peter Wolk's laboratory in Michigan State University, USA, to do the most significant work of his career – discovery of the pathway of ammonia assimilation in *Anabaena* using the cyclotron-generated but very short-lived radioisotope of nitrogen (^{13}N) at MSU-DOE-Plant Research Laboratory at Michigan USA. This work identified the glutamine synthatase-glutamate oxoglutarate aminotransferase (GS-GOGAT) as the principal pathway of assimilation of nitrogen fixed in heterocysts (J. Biol. Chem. 251: 5027-5034, 1976) and led to several important research questions, such as how heterocyst pattern is regulated, or why they don't differentiate in nitrogen-supplemented media, and others.

Around that time, David and I initiated an interesting piece of work. We noticed that reduction of acetylene to ethylene, which was the routine assay for nitrogenase activity, was exponentially enhanced several-fold if the assay was carried out for more time than 30 min, resulting in erroneous over-estimates of nitrogen-fixed in field experiments, where prolonged incubation with acetylene was a norm. Using enzyme kinetics approach, we showed that this was due to conformational changes occurring in nitrogenase complex upon prolonged incubation with acetylene. This was first ever demonstration of conformational changes in nitrogenase in any organism (Biochem. Biophys. Res. Commun. 82: 39-45, 1978; Biochem. Biophys. Res. Commun. 83: 1157-1163, 1978) and immediately caught attention of N_2 fixation community. For us this was even more special since such conformational changes *in vivo* were demonstrated using cyanobacteria as the model system.

We followed this work with another interesting paper on acetylene reduction assay. In this nitrogenase activity assay, wherein a great access of acetylene is used as substrate, while the reduction product ethylene was detected within 30 seconds by gas chromatography, one had to wait for 5 min for acetylene to exit the column before the next injection could be made. This meant a tediously long day for researchers who had 100s of such samples to be assayed each day, such as in agricultural research in the field. With the help of Dr. Ashok Bannerjee (Bio-organic Division), we shortened the analysis time to less than a minute by completely precipitating acetylene as silver acetylide with the help of ammoniacal silver nitrate solution (Appl. Env. Microbiol, 39: 1078-1080, 1980) before analysis. This became instantly popular among field workers on nitrogen

fixation. An additional note put in the paper stated that such assay vials should be washed as soon as possible else it turned the glass vials black, and in case the precipitate dried up there was small risk of a mini-explosion upon friction. The warning note was appreciated even more than the paper itself, and made the life of researchers significantly easier. The aforesaid three papers gave us a lot of popularity in years to come.

Meanwhile another enthusiastic young researcher Rakesh Tuli had joined our laboratory (from 19th batch of BARC TRaining School) and started working on regulation of glutamine synthetase (GS) – Dr. Thomas's favourite subject at that time. He painstakingly purified glutamine synthetase from *Anabaena* using conventional ion exchange, molecular sieve and affinity chromatography – today's rapid technique of expression of recombinant His-tagged proteins and their purification using NiNTA affinity chromatography was not available then. He biochemically characterized GS and studied its regulation in detail, including adenylylation/deadenylilaton based inactivation/activation of the enzyme. Later he was deputed to the laboratory of Prof. Robert Haselkorn at the University of Chicago where he cloned the *glnA* gene – the first recombinant DNA work in our group. His elegant work was published in couple of excellent publications. Dr. H. S. Misra who joined Dr. Tuli's laboratory a little later did some work on the non-heterocystous filamentous cyanobacterium *Plectonema boryanum* and its diurnal regulation of nitrogen fixation and assimilation. Later this groups interest in cyanobacteria somewhat dwindled and their attention shifted to plant molecular biology.

My old interest on cyanobacteria-based remediation of saline soils was revived in the late 1970s and early 1980s. We found a novel and unique requirement of trace quantities (micromolar levels) of sodium for normal growth and metabolism of two filamentous, heterocystous (*Anabaena torulosa and A.* sp. strain L-31) and one filamentous non-heterocystous (*Plectonema boryanum*) cyanobacteria (Curr. Microbiol. 3: 291-293, 1980; J. Gen. Microbiol. 130: 1161-68, 1984). Upon severe starvation of Na^+ growth came to a complete halt due to inhibition of the vital processes of both photosynthesis and nitrogen fixation in cyanobacterial strains. Effects of Na^+ deficiency were bacteriostatic in nature – photosynthesis (measured by photochemical reactions as well as carbon fixation), nitrogen fixation (estimated as C_2H_2 reduction or total Kjeldahl nitrogen) and growth resumed within minutes of re-addition of Na^+ ions. The effect was irrespective of combined nitrogen sources and was observed even in nitrate or ammonium-supplemented media, wherein nitrate assimilation and ammonium ion recirculation were significantly inhibited. Sodium deficiency also lowered aerobic respiration and reduced intracellular phosphate pools and ATP levels (J. Biosci. 6: 771-794, 1984). Such dependence on an otherwise unwanted cation (Na^+) was not known before for any mesophile.

In order to understand the molecular basis of such Na^+ dependence, two *out of box* experiments were carried out. Purification of nitrogenase was attempted from 1500L of *Anabaena* culture grown under Na^+-starved as well as Na^+-supplemented conditions. This experiment was performed over a 3 month visit to the AFRC Unit of nitrogen fixation at Brighton, U.K. aided by the award of a short-term fellowship from the Nuffield

Foundation U.K. The enzyme purified from Na^+-supplemented cultures showed nitrogenase activity while that purified from the Na^+-starved cultures did not. When subjected to ESR spectroscopy (to detect the 3.7*g* unique signal from the MoFe protein or dinitrogenase), the signal was detected in both preparations. This was a strong indication that nitrogenase was synthesized in Na^+-starved condition, but was inactive.

To address this issue, another elegant experiment was carried out by way of detecting presence of Fe (an active component of nitrogenase active site, the FeMoco) in nitrogenase proteins synthesized by Na^+-supplemented and Na^+-starved cultures. Cells were radio-labelled with ^{59}Fe, proteins were extracted and electrophoretically resolved anaerobically by SDS-PAGE. Then 1 mm gel slices were cut and radioactivity was monitored in each slice. Activity bands of the expected molecular mass which were absent in NH_4^+-supplemented media were identified as dinitrogenase and dinitrogenase reductase bands. Both the Na^+-starved cultures as well as the Na^+-supplemented cells exhibited the bands corresponding to both the nitrogenase proteins. This clearly established that Na^+ deprivation did not affect nitrogenase synthesis but adversely affected nitrogenase activity. Recovery of activity within minutes of Na^+ re-addition amply supported this result. A limitation of available ATP, caused by reduced phosphate uptake, thus appeared to result in loss of nitrogenase activity during Na^+ starvation.

For reclamation of saline soil, the relevant cyanobacteria need to accumulate Na^+ and yet be salt tolerant. The relationship between Na^+ uptake and accumulation and salt tolerance, if any, was therefore investigated in detail. Na^+ transport was measured, using ^{22}Na or ^{24}Na, in two differentially salt tolerant *Anabaena* strains along with membrane electrogenesis (Eur. J. Biochem. 154: 395-401, 1986). Voluminous work on both the influx as well as the efflux of Na^+ revealed that curtailed Na^+ influx and high efflux resulting in low intracellular concentration of Na^+ was responsible for higher salt tolerance. Conditions which reduced the Na^+ influx, such as high external K^+, alkaline pH and presence of combined nitrogen enhanced the inherent salt tolerance of *Anabaena* strains further (Appl. Env. Microbiol. 53: 1934-1939, 1987; Plant Physiol. 89: 204-210, 1989). These studies also provided clues for enhancing nitrogen fixation in saline environment, without genetic manipulation.

However, the above-mentioned work pointed out that the basis of cyanobacterial salt tolerance was Na^+ exclusion, and not Na^+ accumulation, and this raised serious questions about the ability of these microbes to remove Na^+ from saline soils. Detailed analysis of cellular distribution of Na^+ showed that while *Anabaena* strains did remove Na^+, much of it remained adsorbed in the extracellular mucilaginous sheath and did not enter the interior of the cells. Subsequent to the death and decay of *Anabaena* cells, such as during mulching in rice fields, the sequestered sodium was released back in to the soil. Thus, late Prof. R. N. Singh's reclamation success was somewhat short-lived, i. e. during the cropping season Na^+ would remain cell-bound and rendered unavailable thereby supporting better crop growth. But the method cannot offer a permanent solution to the problem of soil salinity. Indeed, this conclusion was supported by the experiments of late Dr. G, S. Venkataraman and his colleagues at IARI, New Delhi, who practiced such

bioremediation of saline soils, but had to do fresh inoculation of cyanobacteria in every cropping season. Dr. Venkataraman was my Ph. D. examiner and as my results were contradictory to his group's practices, we had a lot of argument in my Ph. D. viva wherein he graciously agreed with my findings and granted me a Ph. D. in 1985. Much later, we published these results in a prestigious soil journal (Plant and Soil 189: 205-211, 1997), which finally brought the curtain down on this exciting story.

3. Cyanobacterial research in the last 3 decades

Around late 1980s, our group seemed to somewhat disintegrate with Dr. Thomas quitting BARC (to join as a Vice President in SPIC), Dr. Tuli departing to greener pastures in NBRI, Lucknow and Dr. David choosing to work on an entirely different but interesting aspect of PS-II as a second target of UV in the unicellular cyanobacterium *Anacystis nidulans*. This gave us a chance to reorganize our activities around my choice subject - the molecular basis of stress responses in *Anabaena* strains - which I and my colleagues continued working with till my superannuation in 2014. We were initially joined by (a) Dr. Arvind Bhagwat who had been doing some fascinating work on Rhizobium-legume molecular interactions leading to nodulation and symbiotic nitrogen fixation with Dr. Thomas, and (2) Dr. Tonina Fernandes, who had been busy carrying out field work with Dr. Thomas around that time. A couple of students also joined me for their Ph.D. work on stress response of *Anabaena*. Together we initiated a strong program of stress proteomics in *Anabaena*.

To visualize stress-induced proteins immediately following short or long exposure to stress we devised the [^{35}S] methionine pulse radiolabeling technique for proteins and resolving them by 5-15% gradient SDS-PAGE. This gave the best resolution of *Anabaena* proteins. The first excellent publication using this methodology was on detection of salinity stress-induced proteins in two differentially salt tolerant *Anabaena* strains (J. Bacteriol. 171: 909-915, 1989) The importance of this paper lied in the fact that this was the first demonstration of *stress-induced gene expression in cyanobacteria through transcriptional ac*tivation which showed how salt-sensitive and salt tolerant strains responded to salinity within minutes of exposure to as low as 1-2 mM NaCl. The paper was not only appreciated for its scientific content but also for the quality of protein resolution in *Anabaena*. It was followed up with another paper using 2-dimensional resolution of radio-labelled proteins in *Anabaena* for the first time to show that different stresses (salinity, osmotic and heat stresses) evoked induction of some stress-specific proteins as well as some proteins that were commonly induced by all stresses (J. Bacteriol. 171: 5187-5189,1989) These two papers, published back-to-back, provided important leads for later work on (a) cloning of salinity stress-induced genes, and (b) testing the possibility of cross protection against multiple stresses by pre-exposure to one of them.

In 1989-90 while on my post-doctoral assignment to Prof. Robert Haselkorn's laboratory, we used our finding of transcriptional activation of salinity stress-induced genes to design a strategy to fish out genes differentially expressed during exposure to stress, by a

subtractive RNA hybridization procedure (Plant Molec. Biol. 15: 723-733, 1990). This enabled us to clone multiple salinity stress-induced genes (in a single experiment) from a cosmid library of *Anabaena* that was constructed for the purpose. The technique became a trend setter. The second lead from the aforesaid J. Bacteriol papers was also used to design experiments where pre-exposure to a sub-lethal level of one stress (for example, heat shock) not only enhanced tolerance to lethal dose of the same stress (heat shock) but also to a totally unrelated stress (such as salinity or purely osmotic stress triggered by sucrose). These experiments established the concept of cross protection by stresses, which is common in nature where exposure to multiple stresses occurs successively.

The publication of the above-mentioned studies and methodology, led to a spree of analysis of proteins induced by a variety of stresses across many different organisms, such as salt tolerant fungi and rhizobia, pesticide degrading bacteria, salt tolerant rice varieties and even mammalian cell proteins induced by radiation stress, by many workers (both from other divisions of BARC and from quite a few non-DAE institutes as well). Impressed by the power of this technique, Department of Science & Technology persuaded us to take up a project on Stress Proteomics of *Anabaena* strains and supported us with a major grant. We expanded studies on identification of ionic and osmotic stress-induced proteins (Appl. Env. Microbiol. 59: 899-904, 1993; J. Bacteriol. 176: 5868-5870, 1994), potassium starvation-induced novel proteins in *Anabaena* (J. Biosci. 29: 153-161, 2004), heat-shock proteins (Arch. Microbiol. 179: 423-429, 1993), and oxidative stress proteomics (Proteomics 14: 1895-1904, 2014; J. Proteomics 127: 152-160, 2015). These studies led to important projects pursued by new training school graduates, KSKRAs and students who joined us for their Ph.D. subsequently.

Dr. Alahari discovered a novel and obligatory requirement for potassium in two *Anabaena* strains and in non-heterocystous *Plectonema boryanum* (Ind. J. Biochem. Biophys., 31: 267-279, 1994). Potassium is normally needed for maintenance of intracellular pH and turgor pressure in bacteria, in addition to role in some steps of protein synthesis. In *Anabaena*, pleiotropic effects of potassium deficiency on growth, photosynthesis, nitrogen fixation and nitrate assimilation were revealed (Microbiol. 154: 1557-1563, 1998). The effects were quickly reversed upon re-addition of K^+, almost like the effects of Na^+ deficiency reported earlier. In particular, potassium starvation resulted in synthesis of nearly a dozen new proteins, which were not known until then and were termed the potassium deficiency proteins, or PDPs. Using immunodetection approach, some of them were also identified as Kdp proteins, which constitute a potassium-dependent ATPase (Kdp-ATPase) in bacteria, and their regulation was elaborated in *Anabaena* for the first time (J. Bacteriol. 183: 5778-5781, 2001).

This work was subsequently followed up to find very exciting things about the Kdp-ATPase in *Anabaena*. While details of this work are elaborated in a latter article, the key elements of the work deserve a mention here itself. Unlike *E. coli*, *Anabaena* was found to contain two *kdp* operons (Appl. Env. Microbiol. 71: 5297-5303, 2005). Both had the structural genes (*kdpABC*). One of them had a truncated *kdpD* – the transmembrane sensor, but lacked *kdpE* – the cytosolic regulator of *kdp* expression, and did not express

kdp. The other one lacked both *kdpD* and *kdpE*, but possessed interesting genes that looked like another two-component regulatory system (comprising of a sensory kinase, SK; and response regulator, RR) in the neighborhood, and expressed *kdp*. The very fascinating work involving protein engineering of truncated *kdpD* of *Anabaena* with the C-terminal half of the *kdpD* of *E. coli* to obtain a chimeric *Anacoli* KdpD protein (J. Bacteriol. 187: 4921-4927, 2005), and to investigate who and how regulated the functional *Anabaena kdp* operon (Arch. Biochem. Biophys. 474: 65-71, 2008) has been nicely reviewed recently (J. Biosci. 32: 559-568, 2007).

Another important aspect that was followed by younger colleagues was characterization of heat-shock response (HSR) in *Anabaena*. While the HSR had been characterized in the unicellular cyanobacterium *Synechocystis* earlier, not much was known about it in the filamentous, heterocystous, nitrogen-fixing *Anabaena* strains, especially its regulation. They were instrumental in cloning the two major heat shock chaperone genes/operon, *groESL* and *cpn60*, (Biochim. Biophys. Acta 1519: 143-148, 2001; Microbiol. 154: 317-325, 2008**)** and elucidating their contribution to the thermotolerance of *Anabaena* (Arch. Microbiol. 179: 423-429, 2003). For the cpn60, this work also demonstrated that the differential expression of this gene by nitrogen status and heat proportionately influenced thermotolerance of *Anabaena*. Presence of the negative regulatory element CIRCE upstream of both the chaperone genes and its regulation by the HrcA was demonstrated. A *hrcA* null mutant constitutively synthesized high levels of both Cpn60 and GroES and GroEL and notably enhanced thermotolerance. The most creditworthy finding was the regulation of *groESL* operon in *Anabaena* both by heat and light (Ach. Microbiol. 192: 729-738, 2010**)**. Further, identification and cloning of a stress induced heme-erythrin DNAse and its regulation in *Anabaena* (Arch. Biochem. Biophys. 505: 171-177, 2011) was demonstrated. Subsequently with more younger colleagues joining the attention of this group shifted to DNA repair genes and enzymes of *Anabaena*. Details of their work and significant findings on HSR and DNA repair in *Anabaena* are elaborated in an article that follows.

In 2004, the Department of Biotechnology wrote to Director, BARC requesting that our laboratory undertake work on genetic transformation of *Anabaena* by developing new tools and techniques for the purpose of enhancing nitrogen fixation potential of cyanobacteria. While they funded us well, they set up for us quite a challenging task. With the induction of a very able Research Associate (Dr. Anjali Parasnis) and a new dedicated JRF (Akhilesh Chaurasia) we set out to (1) develop an integrative expression vector for *Anabaena* (2) design a protocol for its transformation, and (3) construct some recombinant strains with higher nitrogen fixation capabilities. The strategy used to design the vector was as follows : (a) identification of an innocuous integration site in the genome wherein there were no genes whose function could be impaired upon integration (termed the F region) (b) the 900bp 'F' sequence was divided roughly in to two halves (F1 and F2) which flanked the cloning site for desired gene(s) (c) placement of an eco-friendly, light-inducible *Anabaena* promoter preceding the cloning site (d) incorporation of a suitable antibiotic resistance (neomycin resistance, Nm^r) gene with its own promoter

following the cloning site, for selection in *Anabaena*, and (e) inclusion of the entire cassette (F1-promoter-cloning site-Nm^r gene-F2) in a suicide vector [pBluescript IISK (+)] to ensure its non-replication and integration in *Anabaena*. Several trials and errors yielded a suitable electroporation buffer good for *Anabaena* electro-transformation with high efficiency. The vector was electro-transformed in to *Anabaena* and evaluated for its integration in the desired site, proper functioning of the light inducible promoter and overexpression of the candidate gene (*hetR* – which is the master regulator of heterocyst differentiation in *Anabaena*). Indeed, the transformed cells showed double the number of heterocysts and 3 to 4-fold higher rates of nitrogenase activity for a prolonged time compared to wild type *Anabaena* cells. That is how, the first integrative expression vector for *Anabaena* was created successfully (J. Microbiol. Methods 73: 133-141, 2008).

Availability of such a vector was quite a boon for strain improvement for field applications. Non-transferability of the transgene by lateral or horizontal gene transfer was an essential pre-requisite for this. Genome integration of the transgene ensured that. Several laboratories in India and a couple of them abroad requested for the vector and used it for their specific purpose. Our laboratory also constructed several agriculturally important improved strains of *Anabaena* over the next 5-6 years. Such strains were endowed with desired *cis* or *trans*-genes and constitutively over-expressed *groESL* for enhanced heat and salt tolerance (Appl. Env. Microbiol. 75: 6008-6012, 2009) or *hetR* for enhanced heterocyst frequency and nitrogen fixation (Appl. Env. Microbiol. 77: 395-399, 2011) or *linA* for pesticide lindane (γ-HCH) biodegradtion capability (Bioresource Technol. 149: 439-445, 2013) to *Anabaena*. A biosensor for detection of pesticide lindane was also designed (Anal. Chem. 84: 6672-6678, 2012). These developments more than fulfilled the DBTs mandate. The technology also paved the way for identification of oxidative stress tolerance genes and their integrative over-expression for increased stress tolerance in years to come.

The next target was tolerance to oxidative stress, which was central to all stresses since reactive oxygen species were invariably produced under all stress conditions. To start with, attention was paid to cloning and overexpression of superoxide dismutase (*sod*) genes and catalase (*kat*) genes. Two newly joined KSKRAs undertook the responsibility and did some commendable work. One of them chose to clone and overexpress the manganese and iron dependent MnSOD and FeSOD of *Anabaena* while the other targeted the peroxiredoxin (*prx*) genes of *Anabaena*. It was shown that the overexpression of MnSOD enhanced oxidative stress tolerance of *Anabaena* in nitrogen-fixing conditions, while that of FeSOD was beneficial under nitrogen-supplemented conditions (Plant Mol. Biol. 77: 407-417, 2011). Thus, the contribution of the two SODs to oxidative stress tolerance was nitrogen status dependent. An elegant paper also demonstrated N-terminal processing of membrane-targeted MnSOD and formation of multiple active molecular forms of this enzyme in different cell compartments from a single protein precursor (FEBS J. 280: 4827-4838, 2013). The membrane-targeting of MnSOD was found to be absolutely essential and offered a distinct physiological

advantage to *Anabaena* exposed to oxidative stress (Plant Mol. Biol. 88: 503-514, 2015). Two equally elegant papers revealed that a novel glutaredoxin domain containing peroxiredoxin (Biochem. J. 442: 671-680, 2012) and an unusual Mn-catalase (Environ. Microbiol. 14: 2891-2900, 2012) confer significant protection from oxidative stress to nitrogen-fixing *Anabaena* cultures. These and subsequent outstanding contributions have been elaborated in the following article.

Around the year 2002, and then onwards, we developed a new interest in *Deinococcus radiodurans*, the most radioresistant bacterium in this world. Its genome had just been sequenced in 1999 and opened up immense possibilities of doing basic research on its radiation and oxidative stress tolerance and DNA repair, and biotechnological research on its possible applications in nuclear industry, particularly in bioremediation of nuclear waste. We did both quite successfully for a decade publishing over a dozen excellent papers on the molecular basis of its radioresistance as well as on engineering this microbe for bioremediation of uranium from acidic/alkaline radioactive waste. But pertinent to the present discussion were the striking similarities between this heterotroph and *Anabaena* in terms of their radiation, desiccation and oxidative stress tolerance right up to molecular level of organization of certain operons (kdp) and their regulation. Possibilities of using photautotrophs like *Anabaena* for biotechnological exploitation were any day far more attractive than heterotrophic *Deinococcus*. Therefore, the excitement generated in *Deinococcus* trickled down to cyanobacteria as well and their radiation/desiccation tolerance and proteomics and utilization for uranium recovery were explored in some detail.

Thus, the last, (but not the least) of the stresses to be examined was ionising radiations from ^{60}Co. Earlier UV tolerance and mutagenesis was investigated in cyanobacteria by Dr. Tonina Fernandes, but nothing was really known about response to γ-rays in the hetercystous nitrogen-fixing cyanobacteria. It was found that *Anabaena* cultures exhibited very unusual resistance to ionizing radiation. No adverse effects were observed on the diazotrophic growth and nitrogen fixation in *Anabaena* exposed up to 5kGy dose of γ-rays. Higher doses affected growth and N_2 fixation, which however recovered after a short lag (J. Biosci. 35: 427-434, 2010). Such high radioresistance, as well as desiccation resistance, emanated from its phenomenal DNA repair capabilities (Photosynth. Res. 118: 71-81, 2013) which were quite comparable to those of *Deinococus radiodurans*. Equally interestingly, proteomic response of *Anabaena* to desiccation and radiation showed considerable overlap (The Protein J. 37: 608–621, 2018) as has been reported for *Deinococcus*. Addition of low concentration of ethanol during irradiation drastically reduced DNA damage *in vitro* and also protected live cultures of *Anabaena* from radiation damage (J. Biosci. 43: 15-23, 2018).

With the joining of another KSKRA, a new relevant venture was undertaken to explore the possibility of uranium removal from sea-water by selected resident marine cyanobacteria. For this, a *Synechococcus elongatus* strain BDU75042 (a unicellular cyanobacterium) was procured from the National Facility of Marine Cyanobacteria (NFMS) at Tiruchirapally. The microbe was found to remove 72% or 53.5 mg U g^{-1} dry

weight from test solutions containing 100μM U at the sea-water pH of 7.2. All the bound U was detected in the exopolysaccharide sheath using EDXRF. FT-IR spectroscopy showed that amide groups and deprotonated carbonyl groups were involved in U sequestration (Biores. Technol. 100: 2176-2181, 2009). Another brackish-water strain, *Anabaena torulosa*, similarly sequestered 48% or 56 mg $g^{-}1$ U in 30 min and 65% or 77.35 mg $g^{-}1$ U in 24h from same concentration of U (Biores. Technol. 116: 290-294, 2012**)**. Interestingly, in *A. torulosa*, the sequestered U was found to be bound to novel surface-associated polyphosphate bodies (Metallomics 5: 1595-1598, 2013). Of course, the capability of *S. elongatus* BDU 75042 to remove U from actual sea-water, where the U concentration is only 13 nM, was low and it could remove only 3 mg U g^{-1} dry weight. Impressively, from RO water (which contains 21 μM U) obtained from Desalination Division of BARC, the cyanobacterum could remove 13.3 mg g^{-1} U in 24h. Obviously, therefore, the inherent U removal capabilities were higher than sea-water concentrations and could be enhanced further by suitable manipulations, in future.

4. Future Perspective

Cyanobacterial research has had a glorious past in our country, and 4-5 national laboratories (including ours) have been globally well acclaimed in the past for the research carried out on blue-green algae. The advent of recombinant DNA technology in MBD, BARC gave us an edge over other laboratories in the country, and today MBD is recognized as a premier institution of cyanobacterial research in India. We should continue to work on these organisms particularly since (1) the interest of other institutes and universities in this area is on a decline in India, and (2) cyanobacteria are fascinating organisms which have served as model systems for studying all kinds of biological phenomena - from nitrogen fixation and photosynthesis to stress responses and circadian rhythms to biotechnologies for production of vitamins, pigments and many other natural products. Important leads have emerged from past work on cyanobacteria in MBD (for example, radio-resistance and oxidative stress tolerance) and these must be explored further, in view of their importance to the department. Outstanding quality basic research has been the strength of MBD and we should build on it further. Also, applications are a need of the hour and those of great relevance to DAE programs (such as uranium sequestration from sea-water) must be pursued vigorously. Now that the expertise is available in rDNA technologies right up to the CRISPR technology in the division, genome editing should be the way forward. I am hopeful that MBD will continue to pursue excellent programs on cyanobacteria and continue our glorious tradition in this field for few more decades.

STRESS RESPONSES IN THE NITROGEN-FIXING CYANOBACTERIUM *ANABAENA*

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Abstract

The ancient organisms, cyanobacteria, also thought to be the progenitors of plant chloroplasts, have exhibited various overlapping mechanisms while dealing with myriad environmental stresses. The filamentous nature of the nitrogen-fixing cyanobacterium, *Anabaena*, makes it an interesting model for study of stress responses as several unique features need to be incorporated as against what is observed in the unicellular bacteria. This chapter will take the reader on a journey of what was discovered and established as mechanisms for overcoming heat stress, oxidative stress, nutrient-starvation and DNA damage due to exposure to γ-radiation, in *Anabaena*.

1. Introduction

Having inhabited the Earth billions of years ago, cyanobacteria have learnt to acclimatise themselves to various environmental challenges, be it temperature, radiation, desiccation, salinity, heavy metal exposure etc. The major modes of damage across various stresses are the damage to proteins, membrane and/or production of reactive oxygen species (ROS) leading to damage to DNA and several physiological processes. Many of the stresses have been extensively studied in the filamentous cyanobacterium, *Anabaena* (*Nostoc*) PCC 7120 which has been considered as a model system among the filamentous cyanobacteria owing to its ability to be genetically manipulated. Presence of multiple genes encoding proteins with similar functions, absence of certain genes and pathways,

presence of global transcriptional regulatory proteins, several *cis*-elements contributing to transcriptional and post-transcriptional regulation and protein processing have all contributed to the unique ability of *Anabaena* to combat various stresses. Some of these mechanisms have perpetuated to the modern-day bacteria during the course of evolution, while some have been lost in bacteria, but observed in plants, further indicating the close association of cyanobacteria with plants. A brief glimpse into the world of *Anabaena* while combating the various abiotic stresses is detailed below.

2. Novel Insights into the Heat Shock Response of *Anabaena*

Proteins, be it structural or functional proteins, contribute to the normal functioning of all physiological processes and adaptability when exposed to sudden changes in growth conditions that could be even detrimental to organism i.e. upon perceiving stress. This would require these proteins to maintain their structural and functional integrity, and that's where the magical proteins i.e. chaperones and proteases play an important role. While the chaperones, as the name indicates, guide other proteins to maintain their integrity through assisting in correct folding, proteases, on the other hand, degrade the irreversibly misfolded proteins so that their unwarranted accumulation does not result in cellular toxicity and their degradation also allows for the availability of the basic building blocks i.e. the amino acids for synthesis of new proteins. Since, these proteins were identified in response to heat stress; they have been classified as Heat Shock proteins (HSPs). *Anabaena* has a plethora of HSPs which have been well characterised in other bacteria, but differs in having multiple genes encoding HSPs with similar functions, exemplified by the presence of at least 5 DnaK (Hsp70) encoding genes and two GroEL (Hsp60) encoding genes.

2.1. Multiplicity of Hsp60 proteins in Anabaena

While most bacteria have a single Hsp60 protein with the exception of *Rhizobium* and *Bradyrhizobium*, which have 4-5 genes encoding for this protein, most cyanobacteria possess two Hsp60 encoding genes, the proteins differing in their molecular mass by at least 2 kDa and with distinct C-terminal regions. This difference refers to the presence of a 'GGM' tail which is a signature of all bacterial Hsp60 proteins, but is present only in the larger of the two Hsp60 proteins in cyanobacteria. In general in cyanobacteria, the ~59 kDa GroEL is part of a bicistronic *groESL* operon, while the ~61 kDa Cpn60 is encoded by a monocistronic gene. The 59 kDa GroEL protein exhibited chaperone activity comparable to other bacterial Hsp60 proteins, however, GroES and ATP has not been found to be essential for the chaperone activity. On the other hand, both GroES and ATP aided the chaperone activity of the 61 kDa Cpn60 protein, which was found to be 10-fold lower than that of GroEL. This could also be due to the differences in their substrate specificity. Despite the presence of two *hsp60* gene, deletion mutants were not found to be viable further reiterating that they could be targeting different substrates for folding. Indeed, though the use of recombinant *Anabaena* strains, wherein only one of the two Hsp60 proteins was overexpressed, it was shown that the 59 kDa GroEL was essential under nitrogen-fixing conditions and involved in maintain the integrity of

nitrogenase and photosynthetic apparatus. The 61 kDa Cpn60 protein, on the other hand, was found to be essential under nitrogen-supplemented conditions, contributing to the stability of nitrate reductase and the photosynthetic apparatus. During the course of evolution, the mesophilic bacteria retained only one *hsp60* gene, as part of a bicistronic operon with its co-chaperone *groES* or *hsp10*, with certain essential features of the 2nd Hsp60 protein incorporated in the single *hsp60* gene.

2.2. Regulation of hsp60 genes

Regulation of these *hsp60* genes in cyanobacteria also differed from what was well known in other gram negative bacteria, wherein the *hsp60* genes were under the positive regulation of a special sigma factor (σ32). Cyanobacteria, on the other hand, followed the model established in gram positive bacteria *Bacillus*, wherein they were found to be negatively regulated by the dimeric HrcA protein which bound to an inverted repeat element known as CIRCE. Additionally, several *cis*-regulatory elements in the form of direct and inverted repeats were also found to be involved in the intricate regulation of these genes in *Anabaena* and *Synechocystis*, in response to not only heat stress but also light stress. The intricate regulatory network may have been essential to well regulate the levels of these proteins in response to the environmental conditions.

3. K^+ starvation and the K^+-dependent ATPase (Kdp-ATPase) from *Anabaena*

Potassium (K^+), the chief intracellular cation, is critical for life in all cells. The very high intracellular concentration of K^+ echoes the primeval strategy of living cells to accrue K^+ and exclude the sodium ions. The elevated concentration of K^+ is maintained by a set of K^+ influx or efflux transporters whose activities are modulated in such a manner that there is no wasteful cycling of K^+ across the membranes. When many bacteria are subjected to potassium starvation or osmotic up-stress, the high affinity Potassium-dependent Adenosine triphosphatase (Kdp-ATPase), a P-type ATPase, is synthesized. The *E. coli* Kdp-ATPase (K_m= 2μM) is capable of decreasing the K^+ content in the growth-medium to 100 nM or lower. In effect, the Kdp-ATPase is an efficient potassium scavenging enzyme that is produced when cellular requirement of K^+ cannot be met by other K^+ uptake proteins.

The *E. coli* Kdp-ATPase complex (encoded by the *kdpFABC* operon) is made up of four protein subunits viz. KdpF, KdpA, KdpB and KdpC. The KdpF subunit provides stability, whereas the KdpA polypeptide binds to and carries potassium into the cell. Interestingly, the KdpB subunit, which closely resembles the other characterized P-type ATPases, contains the evolutionarily conserved phosphorylation site, while the KdpC is required for the assembly of the whole complex. Unlike KdpF, KdpA, KdpB and KdpC proteins are indispensable for Kdp-ATPase activity *in vivo* in *E. coli*. The Kdp-ATPase, was the first P-type ATPase to be discovered from any prokaryote. In all other P-type ATPases, the central sub unit that gets phosphorylated is also responsible for the transport of the ion. But in Kdp-ATPase, the ATP hydrolysis is carried out by KdpB, and consequently KdpB is phosphorylated, while the actual transport of K^+ is carried out by the KdpA.

3.1. Identification and structural organization of the kdp operons in Anabaena

In response to potassium starvation, three strains of *Anabaena* (i.e. *Anabaena torulosa*, *Anabaena* sp. strain PCC 7120 and *Anabaena* L-31) produced a 78 kDa polypeptide that cross-reacted with the *E. coli* KdpB antiserum, indicating the presence of Kdp homologs in *Anabaena*. To decipher the structural organisation and regulation of *kdp* genes in *Anabaena*, *kdp* operons from *Anabaena* sp. strain L-31 (henceforth referred to as *Anabaena* L-31) were identified and sequenced. A strategy that employed PCR (employing degenerate primers), followed by Southern blotting and chromosomal walking was utilized to accomplish this objective.

Surprisingly, not one but two, distinct *kdp* operons were found in *Anabaena* L-31 (termed *kdp1* and *kdp2*). The *kdp1* operon (GenBank accession no. AF213466) showed 5 open reading frames (ORFs) viz. *kdpA1*, *kdpB1*, *kdpG1*, *kdpC1* and *kdpD* whereas the *kdp2* operon (GenBank accession no. AY753299) contained 4 ORFs i.e. *kdpA2*, *kdpB2*, *kdpG2* and *kdpC2*. Although *kdpF* was absent from both the *kdp* operons, an additional ORF, *kdp*G (encoding hydrophobic protein), was observed between the *kdpB* gene and the *kdpC* gene in *kdp1* as well as *kdp2*. An unusually truncated *kdpD* ORF was observed downstream of *kdpC1*, while no such ORF was present in *kdp2*. The *kdpD* protein (365 amino acids) showed similarity only to the KdpD-N terminal domain of *E. coli* KdpD; while the critical C-terminal histidine kinase domain, which is responsible for phosphorylation reaction was absent in this KdpD. No *kdpE*-like gene was found downstream of the two *kdp* operons. Thus, analysis of the deduced amino acid sequence suggested that KdpATPase encoded by both the *Anabaena* L-31 *kdp* operons was structurally similar to the other bacterial KdpATPases. However, the genes encoding the regulatory proteins (KdpDE) were distinctly different from those present in other bacteria (such as *E. coli*).

3.2. Induction of the kdp operons in response to different stimuli

When subjected to K^+ limitation (< 50 μM K^+ in medium), expression of only *kdp2* (not *kdp1*) expression was detected as a 5.3-kb transcript on Northern blots, indicating that *kdpA2B2G2C2* genes constituted a polycystronic operon. The *kdp2* expression was seen after 1h of K^+ starvation and maximal expression occurred by 3h of potassium deprivation. When 5 mM potassium was added to the K^+-starved cells, the *kdp2* expression ceased within 30 m. In response to K^+ limitation, a 78 kDa cross-reacting polypeptide, corresponding to *Anabaena* L-31 KdpB, was observed only in K^+-starved cells. The KdpB polypeptide was detected exclusively in the crude membrane fractions while none was observed in the cytosol. When K^+ was re-added to KdpB expressing *Anabaena* L-31 cells, the content of cross-reacting KdpB polypeptide decreased with time. These results demonstrated that neither the *kdp* transcript nor the Kdp-ATPase proteins (KdpB) were stable in presence of K^+. Unlike *E. coli* and *Salmonella typhimurium*, addition of common salt did not induce *kdp* in *Anabaena* L-31. Moreover, pH of the medium, heat-stress or presence/absence of combined nitrogen in the growth medium also did not affect *kdp* expression. Notably, strong induction of the *kdp2* operon was observed in response to desiccation stress. Thus, unlike the enterobacterial *kdp*

operons, the cyanobacterial *kdp* is may not play a role in overcoming salinity, but are likely to enhance survival of *Anabaena* during K^+ starvation or desiccation.

4. Molecular basis of the Oxidative Stress Resistance in *Anabaena*

4.1. Functional aspects of superoxide dismutases from Anabaena

Being a photosynthetic organism, electron transport chain of both photosynthesis and respiration contribute to the generation of intrinsic ROS in cyanobacteria. Additionally, exposure to various environmental stresses induces ROS production and this can cause damage to proteins, lipids, membrane and DNA inducing cell death. The major ROS generated include superoxide radical ($O_2^{\cdot}$), H_2O_2, OH radical etc. Of these, the $O_2^{\cdot}$ generated is accentuated due to photosynthetic activity and needs to be tackled upfront to prevent damage to the photosynthetic apparatus. *Anabaena* has two superoxide dismutases (SODs), which are involved in the dismutation of $O_2^{\cdot}$ to H_2O_2. The need for two SODs, one present solely in the cytosol i.e. FeSOD, and the other distributed in the thylakoid lumen and the cytosol i.e. MnSOD in *Anabaena,* as against only the cytosolic SODs in other bacteria was exemplified when the distribution pattern of these SODs was monitored. Of the two SODs, MnSOD was found to be essential under nitrogen-fixing conditions and FeSOD under N-supplemented conditions. This also explained why the non-nitrogen-fixing cyanobacteria primarily had only the cytosolic FeSOD. The unique feature of SODs which was unearthed in *Anabaena* was the targeted cleavage of MnSOD, its localisation and formation of homo and heterodimers.

The 30 kDa *Anabaena* MnSOD was characterised by a signal peptide (~ 3 kDa) and a linker peptide (~3 kDa) preceding the catalytic unit (~24 kDa). Owing to the signal peptide, the MnSOD could anchor onto the thylakoid membrane, and thereafter could face two fates. Either the signal peptide cleaves off releasing the 27 kDa protein into the cytosol, wherein the linker peptide is further cleaved to generate the 24 kDa protein. Thus, in the cytosol, along with the 22 kDa FeSOD monomer, two monomeric forms of MnSOD i.e. 24 and 27 kDa are available resulting in the formation of 5 homo and heterodimeric species all of which exhibited superoxide activity. The other fate involved translocation of the 30 kDa MnSOD into the thylakoid lumen, wherein the signal and linker peptide get cleaved sequentially to generate the 27 and 24 kDa forms resulting in three dimeric species of active SOD. A similar distribution of SODs was also observed in plants, but the mechanism was unknown, and was demonstrated for the first time in any cyanobacteria. The understanding of the mechanism of SOD processing opened new doors to the possibility of such processing existing for other proteins as well in cyanobacteria, which was earlier thought to be restricted to eukaryotes.

4.2. Role of Mn-catalases and peroxiredoxins from Anabaena

The end product of SOD enzyme activity is hydrogen peroxide (H_2O_2). Although, not very toxic by itself, H_2O_2 gives rise to the most deleterious ROS, the hydroxyl radical, which can damage all biomolecules in its vicinity at diffusion-controlled rates. H_2O_2 is rapidly detoxified by two classes of proteins, catalases and peroxidases. The genome of *Anabaena* PCC 7120 shows the presence of two Manganese-containing, Mn-catalases,

[Alr0998 (named as KatA) & Alr3090 (named as KatB)] while the commonly present iron-containing Heme-catalases are absent. As their name indicates, the Mn-catalases have the metal 'Mn' (instead of Fe-containing heme) at their active sites, and hence, these proteins are also referred to as pseudocatalases or alternative catalases in literature. Along with these two Mn-catalases, 10 genes encoding different classes peroxiredoxins (Prxs, also called as thiol peroxidases) are present in the genome of *Anabaena* PCC 7120. Prxs have a thioredoxin fold-containing thiol–specific antioxidant (TSA) domain, and Prxs in general, have two catalytic cysteine residues at their active site. Incidentally, glutathione peroxidases and ascorbate peroxidases that are present in animal and plant systems respectively, are absent in *Anabaena* PCC 7120.

The catalases dismutate H_2O_2 directly into water and molecular oxygen, whereas peroxiredoxins require the help of a reductant (e.g. thioredoxin, glutaredoxin etc.) to reduce H_2O_2 into water, without the concomitant generation of O_2. Initial experiments with different strains of *Anabaena* showed an inherent lack of catalase activity under control conditions of growth or on exposure to H_2O_2, the substrate of catalase enzyme. However, when overexpressed in *Anabaena* PCC 7120, the KatA was able to defend *Anabaena* from oxidative stress mediated by H_2O_2 or methyl viologen. These results proved that Alr0998 was indeed a functional protein, capable of decomposing H_2O_2 when adequately present in the cyanobacterial cells. Strangely, despite being present in a mesophilic bacterium, the KatA protein was quite thermostable and remained active even after exposure to 80^{o}C.

The absence of catalase activity raised a pertinent question: Do the catalase genes play any role in the physiology of *Anabaena* PCC 7120? Cross-protection experiments with different stresses showed the surprising role of the Mn-catalase Alr3090 (KatB) in overcoming salinity and consequently, other oxidative stresses in *Anabaena*. The salt-treated wild-type *Anabaena* PCC 7120 cells showed remarkable resistance to H_2O_2 as compared to the corresponding cells not pre-treated with NaCl. Subsequent analysis showed induction of the KatB protein in response to NaCl was responsible for this protective effect. The ability of salt to protect *Anabaena* from H_2O_2 was lost in the *katB* mutant, indicating that the KatB protein was indeed responsible for this defensive effect. The *katB* mutant of *Anabaena* was very sensitive to the oxidative effects of salinity, and unlike the wild-type, completely lysed on the subsequent exposure to H_2O_2. These results clearly showed the importance of KatB in the stress physiology of *Anabaena* PCC 7120. Analysis with *katB* promoter-*gfp* fusion showed the absence of *katB* expression in the heterocysts (cells that fix nitrogen), but not vegetative cells of *Anabaena* PCC 7120. It should be noted that, to fix nitrogen, heterocysts maintain a low intracellular concentration of oxygen. Apparently, during the course of evolution, *Anabaena* have ensured that catalases, which liberates O_2 as a bye-product, are not expressed in the heterocysts, thereby maintaining oxygen-free environment for nitrogenase to function.

The his-tagged protein KatB was over-produced in *E. coli* and purified to near homogeneity by affinity chromatography. The KatB protein was an efficient, thermostable catalase, that was insensitive to inhibition by azide. The KatB protein was

crystallized and its structure was determined by X-ray crystallography. Notably, KatB is the only Mn-catalase to be crystallized from any photosynthetic organism. Structural analysis showed KatB to be a compact hexameric protein (i.e. it was a trimer of dimers) whose active site was distinct from that of other previously characterized Mn-catalases. The KatB active site showed Glu_4His_2 coordination geometry with two terminal water ligands, and resembled the active site of the bacterioferritin/ruberythrin proteins. Surprisingly, crystallographic as well as biochemical analysis showed the involvement of the 2nd N-terminal residue of KatB (F-2) in maintaining stability as well as activity of this protein. KatB variants with smaller residues (V/A/G) at the second position were less compact, showed reduced catalytic activity and were more sensitive to denaturation than the corresponding KatB variants with larger residues (Y/W/F) at the same position. The F-2 residue was essential for maintaining intersubunit interactions that provided stability to the hexamer. Also, F-2 interacted with other residues (near the active site) and helped in the formation of the hydrophobic pocket that kept the active site together. Hence, only residues that could sustain activity (i.e., F/Y/W) were naturally selected at the 2nd position in Mn-catalases during the course of evolution.

As catalases are not constitutively expressed in *Anabaena*, which proteins are responsible for detoxification H_2O_2 that is normally produced during photosynthesis or aerobic respiration? Apparently, in the absence of catalases, our research has shown the peroxiredoxins to play a vital role in eliminating the intracellularly produced H_2O_2. Among the different peroxiredoxins present in *Anabaena*, the typical 2-Cys peroxiredoxin (denoted as Alr4641 in *Anabaena* PCC 7120), is the major peroxiredoxin that is constitutively expressed under control conditions of growth. The Alr4641-specific antiserum easily detected this protein the control (i.e. unstressed) cellular extracts of *Anabaena* PCC 7120 on Western blots. Moreover, different stresses such as H_2O_2, methyl viologen, salt and gamma radiation were able to transcriptionally activate expression of this gene over the basal levels. Biochemically, Alr4641 showed dual function i.e. not only it functioned as thioredoxin-dependent peroxidase, but it also showed chaperone function. Interestingly, between the two, only the peroxidase activity was dependent on the presence of the catalytic cysteine residues. The *al4641* promoter was active in vegetative cells as well as heterocysts of *Anabaena* PCC 7120. Overexpression of Alr4641 in *Anabaena* PCC 7120 conferred tolerance to externally added H_2O_2.

To carefully assess the role of Alr4641 in *Anabaena* PCC 7120, the *alr4641* gene was knocked down using the CRISPR-interference approach employing dCas9 and the *alr4641*-specific sgRNA. The knockdown strain (An-KD4641, wherein the Alr4641protein content decreased by ~85%), although viable, grew slower than the corresponding control cells. An-KD4641 displayed inherently higher levels of ROS, suggesting that these filaments were under oxidative duress. The knockdown strain showed disrupted thylakoid structure, diminished photosynthetic parameters, and completely lost its viability when exposed to moderate doses of H_2O_2. Hence, 2-Cys-Prx seems to be the dominant Prx that is required to sustain redox homeostasis in varied photosynthetic systems, ranging from cyanobacteria to chloroplasts, which lack sufficient

catalase expression. Furthermore, as catalase genes are not expressed in heterocysts of *Anabaena*, Alr4641 may play a crucial role in detoxifying H_2O_2 in these specialized cells.

5. DNA Repair and the unusual role of LexA in Multiple Stress Tolerance of *Anabaena*

One of the first proteins, with a probable role in DNA damage to be worked on was a protein, Alr3199 identified as a highly induced heat shock protein. Characterisation of this protein identified it as a hemerythrin DNase with the ability to bind 4 Fe atoms per protein molecule thus playing a role in Fe-homeostasis, and an additional role in regulating DNA degradation in view of its Nickase/ DNase activity. Its neighbouring gene, *Alr3200* was also identified as a DNase involved in radiation tolerance of *Anabaena*. This started the journey for identifying the pathways involved in radiation tolerance and DNA repair in this organism.

5.1. DNA repair genes and mechanisms

Several abiotic stresses, predominantly radiation and desiccation stresses can induce damage to DNA in the form of adduct formation, single and double strand breaks, which if left uncorrected would be lethal to cells. Several cyanobacterial species including *Anabaena*, *Nostoc*, *Chroocodiopsis* exhibit high to both γ-radiation and desiccation. *Anabaena* (*Nostoc*) PCC 7120 exhibits an LD_{50} of 6 kGy and D_{10} of 12 kGy of γ-radiation, making it comparable to the highly radioresistant *Deinococcus*. However, unlike the well-studied mechanisms of DNA repair in *Deinococcus* as well as the radiosensitive *E. coli*, the genes involved in DNA repair mechanisms were unexplored in *Anabaena*. *In silico* analysis revealed the absence of primary proteins (RecB and RecC), SbcA, SbcB required for the RecBCD pathway of Homologous Recombination (HR) and Ku proteins for Non-Homologous End Joining (NHEJ) Pathway. While the absence of RecBCD pathway was also shown for *Deinococcus*, the NHEJ pathway was found to be active in it. Based on the annotated genes in the genome, *Anabaena* was speculated to be dependent on RecF pathway of HR and ESDSA (Extended Synthesis Dependent Strand Annealing), Single Strand Annealing (SSA) and Micro-Homology mediated End Joining (MHEJ) pathway. The major genes in these pathways had to be characterised to know their functional involvement and the feasibility of these pathways of DNA repair in *Anabaena*, which was an unexplored field not just in this organism but in cyanobacteria as a whole.

The very first task in this direction was the identification of a full length Single Strand DNA Binding (SSB) protein, since both the annotated SSB proteins were truncated and lacked the C-terminal region required for interaction with other DNA repair proteins. Based on *in silico* analysis along with biochemical and physiological characterisation, one of the hypothetical proteins, All4779 was identified as the full length SSB, which also contributed significantly to the radiotolerance of *Anabaena*. Ortholog of this protein was found across all cyanobacterial species. The truncated SSB proteins could possibly be involved in DNA replication or recombination processes, as they were not found to

contribute to DNA repair. In the absence of RecBCD complex, the suppressor proteins, SbcC and SbcD were expected to play an important role. Functional characterisation of SbcC and SbcD proteins of *Anabaena* revealed that they could independently contribute to the radiotolerance of *Anabaena*. This is unlike other bacteria, wherein they contribute to DNA repair only when they form a complex (SbcCD) and not independently. It has been speculated that these proteins could be participating in SSA and MHEJ based on their predicted interacting protein partners. The RecA protein which is central to any DNA repair pathway was found to be an essential gene, but expressed at very low levels. Enhancing its expression rendered the *Anabaena* cells radiosensitive.

In general, most DNA repair proteins are expressed at very low levels in *Anabaena*. This was also observed for the proteins central to the RecF pathway, namely RecF, RecO and RecR proteins. Of these, the initiation codon of *recR* was wrongly annotated in the database and with use of *in silico* and expression analysis, the correct initiation codon was identified as 'GTT' present 267 bases upstream of the annotated initiation codon. 'GTT' is a rare initiation codon and has been mentioned as a probable initiation codon only for one gene in *Streptomyces*, though not proven. Presence of the anticodon for 'GTT' in the anticodon arm of fMet-tRNA of *Anabaena* 7120 led to the speculation, that though rare, 'GTT" may function as an initiation codon for more number of genes and not just *recR*, but this needs to be extensively explored.

All three genes (*recF*, *recO* and *recR*) were found to be essential as they could not be deleted; however, knockdown mutants of *recF* and *recR* were viable. Functional analysis of these proteins through modulation of their levels revealed that individual changes in their levels affected the radiotolerance of *Anabaena*, and these proteins would need to interact in a correct proportion to enhance the radiotolerance, which is currently being investigated. All three genes were found to be regulated through an array of elements, which included negative regulation by LexA, positive regulation by NtcA at transcriptional level, role of DNA heptamer repeats in post-transcriptional and translational regulation and that of non-canonical Shine-Delgarno sequence in translational regulation. Involvement of DNA heptamer repeats in gene regulation was established for the first time in bacteria through the studies on their role in regulation of *recF* and *recO* gene expression.

5.2. The global regulator LexA

LexA, which has been extensively studied in *E. coli*, was always considered as a SOS-response regulator in bacteria, involved in the negative regulation of DNA repair genes. However, in cyanobacteria, LexA was first identified as a positive regulator of certain C-metabolism genes and later shown to be involved in negative regulation of two DNA repair genes. However, no specific binding LexA-binding box was identified and showed variation based on the genes regulated. This could have been because of the small set of genes identified to be regulated by LexA in cyanobacteria. Detailed studies on LexA in *Anabaena* 7120 in our laboratory showed that its autoproteolytic activity was independent of activated RecA, unlike that in other bacteria, and was dependent on the availability of nucleophile as in alkaline pH conditions. Physiological evaluation of LexA

overexpressing strain of *Anabaena* revealed its involvement in regulating tolerance to various abiotic stresses, such as γ-radiation, oxidative, C-starvation and heavy metal stress through regulation of genes involved in alleviation of these stresses. While majority of the genes were found to be negatively regulated by LexA, ~10% of the genes were found to be positively regulated, indicating that LexA could function both as a repressor and as an activator protein, and also the consensus LexA-binding box was identified which could cater to all genes identified to be regulated by it. Further analysis of the presence of the AnLexA-Box across the genome identified its presence upstream of several photosynthetic genes and involved in its regulation, and was though to mediate the redox poise. Thus, LexA was identified as a global regulator of stress response in *Anabaena*, adding to the tally of other identified global regulators NtcA and FurA.

6. Future Perspectives

Over three decades of research on stress biology in *Anabaena* revealed cross-talk between stress proteins across various abiotic stresses, intricate linking of some of these proteins with major physiological processes, presence of global regulators and several *cis*-acting regulatory elements. This knowledge is being further expanded to fully understand the role of nucleoside kinases, protein kinases, resolves, and proteins with disordered structures (IDPs) through a mix of omics approaches and relating them not only to their growth but to the major physiological processes, whose functioning is crucial. Moreover, how most of the oxidative stress-responsive genes or the *kdp* operons are regulated is not completely understood, and this aspect can be a fruitful line of research in the future. The deeper understanding of these physiological processes and protein functions would also throw some light into the course of evolution of these proteins from the ancient cyanobacteria to modern day bacteria and plants.

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BASIC AND APPLIED STUDIES ON MICROBIAL SYSTEMS: CONTRIBUTION TO RESEARCH PROGRAMMES IN BARC

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Abstract
This article provides a bird's eye view of long-standing programs in BARC employing microbial systems. These systems served as ideal models to address fundamental questions and to develop various applications. Microbial model systems were adopted early since the commencement of bioscience research in BARC. Over the years, these programs encompassed radiobiology, genetics, DNA recombination and repair, recombinant DNA technology, and stress biology. More recently, along with high class basic research, applications and translational aspects have gained traction. The programs have evolved to assimilate cutting edge technologies like next generation sequencing, omics, CRISPR-gene-editing and high-end imaging etc. Microbial research in BARC which carries a rich legacy of pioneering research is poised to take up new challanges through an interdisciplinary approach.

1. Introduction

Microbes are the natural choice for studying many fundamental processes of life. They provide the simplest yet elegant models for investigating most complex biological phenomena. The ease with which they can be cultivated requiring simple lab

infrastructure and their amenability to rapid, direct and detailed analysis of problems of wide interest makes them ideal model systems. The value of prokaryotic research is quite apparent now, and as many visionaries foresaw, because of the fundamental unity in conservation of the molecular processes throughout the biological kingdom. Many groundbreaking discoveries that have earned Nobel Prizes, such as restriction enzymes, transposons, DNA polymerases, genetic code for protein synthesis and genetic recombination, originated from fundamental microbial research. These innovations have not only deepened our understanding of molecular biology but also revolutionized fields like genetic engineering, biotechnology, and medicine, showcasing the immense value of studying microorganisms.

Studies employing bacteria and bacterial genetics started early in the life cycle of establishment of department of atomic energy. The bacterial research can be traced back to fifties and early sixties and have gone from strength to strength since then. No wonder bacterial research is of historical as well as contemporary interest. At BARC, the history of bacterial research can be divided into three eras; the first representing the early foundational studies employing bacterial model systems to study radio-sensitization using various radio-sensitizers and -modifiers and examination of post-irradiation biochemical processes. Then the natural progression to the second era where the power of genetics and molecular biology approaches was harnessed and finally the evolution to the modern era with the adoption of advanced genetic manipulation, next-generation sequencing and omics approaches. As the evolution was gradual and natural there is significant overlap between these eras and this chapter attempts to capture a glimpse of the glorious history of basic and applied microbial research made over seven decades without straining to be strictly chronological.

2. Early foundation of microbial research

The foundation of bacterial research within the atomic energy establishment was laid in its early years. In the fifties and early sixties, studies were initiated under the leadership of Dr. R. Gopal-Ayengar to understand the basis of radio-sensitivity in microorganisms. These studies which continued in the seventies and well into eighties mostly utilized microbial model systems such as *Escherichia coli* (*E. coli*), *Micrococcus radiodurans* (*M. radiodurans*) and *Hemophilus influenzae* (*H. influenzae*) for investigations of effects of radiation on biological systems. These studies were aimed at using microbes to understand the effects of gamma radiation on cellular components and the eventual outcome in terms of survival. Further, microbial models provided facile systems to test various radio-sensitizers such as iodoacetic acid, ascorbate, N-ethyl maleimide to name a few, which were studied for their capacity to cause radio-sensitization in microbes. Various hypotheses to explain the radio-sensitization in terms of hydroxyl radicals produced, repair capacity of the microorganisms and the interaction of the sensitizing chemicals to different cellular components like membrane, DNA and proteins were evaluated. Some of the notable findings of the various studies carried out in this area are summarized below.

In a breakthrough paper published in the journal Science in 1968 it was demonstrated that Iodine atoms are incorporated in bacterial membrane proteins when cells are irradiated in the presence of iodoacetic acid labelled with iodine-131. Such atoms are produced on reaction of iodoacetic acid with the gamma ray-induced hydroxyl radicals in the surrounding medium. This was one of the significant studies that formed the basis of investigation, in the later years, of incorporation of I-131 in higher cellular systems. Irradiation of cells in the presence of I^{131} showed a decrease in cell radioactivity with increasing concentration of ascorbate present during irradiation over a broad concentration range. It was hypothesized that ascorbate reduced the sensitizing effect by scavenging the iodine atoms which would have otherwise reacted with the cells. The sensitization by 2,2,6,6-tetramethyl-4-piperidone-N-oxyl (TAN) decreased with increasing concentration of ascorbate, but no net protective effect was noticed. Based on absorption studies and employing ESR signals an interaction between the two molecules was hypothesized and the effect of this interaction in net sensitization or protection was quantified.

E. coli B/r cells γ-irradiated under anoxic conditions and in the presence of N-ethyl maleimide showed enhanced damage in terms of their colony-forming ability. It was shown that irradiation leads to binding of N-ethyl maleimide with cellular macromolecules, particularly proteins. Binding of NEM to non-specifically to all amino acid residues was shown with in vitro irradiation of bovine serum albumin in aqueous solution. It was hypothesized that the binding of N-ethyl maleimide with cellular proteins may lead to inactivation of enzymes involved in post-irradiation biochemical processes giving rise to enhanced damage. A reaction between N-ethyl maleimide and amino acid radical was suggested. It was shown that N-ethyl maleimide reacts with amino acids in the absence of radiation also, which could partly explain the residual sensitization noticed in cells treated with N-ethyl maleimide. Using results obtained with ESR it was shown that part of the sensitizing effect of NEM was due to its electron affinic property. Similar studies were extended to recombination deficient mutants of *H. influenzae* to assess the role of recombination in cell protection from killing by gamma irradiation.

It needs to be placed on record that fifties were the nascent years of radio-biology research. Following the bombings of Nagasaki and Hiroshima worldwide interest emerged to understand the effects of radiation on biological systems. This was the period when radiobiology departments began to be established in atomic energy institutions and highly reputed universities in the west. It was during this period many pioneering studies on investigation of the mechanisms of radio-sensitization in microbes were carried out and it is a matter of pride that radio-biology research carried out in atomic energy establishment/ BARC contributed in no insignificant terms to these efforts.

3. Contribution of microbial research to advancement of genetics

The microbial genetic research which started in late sixties and seventies, was initially centered around studies of genetic transformation in *H. influenzae* but later expanded into investigations of recombination and repair pathways in *E. coli*, *Deinococcus radiodurans*

and cyanobacteria. Transformation was discovered by Fred Griffith in 1928. At the time, it was not known that DNA was the genetic material. It was not until 1944 that Avery, MacLeod and McCarty provided the proof that the transforming principle was DNA. This study along with the landmark study of Hershey and Chase in 1952 were pivotal in establishing DNA as the genetic material. While during fifties and sixties transformation was an actively studied area of research, the fate of DNA upon entry, processes of DNA degradation, recombination and integration were hardly understood. Several researchers, including M. S. Fox and Sol H. Goodgal pioneered the use of ^{32}P-labeled DNA to demonstrate the physical uptake of DNA in transformation.

The Hemophilus studies in BARC were led by Dr. N. K. Notani, who had been trained in the laboratories of R. A. Brink at University of Wisconsin–Madison and S. H. Goodgal in the University of Pennsylvania. Brink was a renowned plant geneticist and also doctoral advisor to Esther Lederberg a pioneer in bacterial genetics and molecular genetics. *H. influnzae* was an excellent choice for genetic studies because of its natural ability to take up external DNA. The research focused on elucidating molecular events following the entry of the DNA inside bacterial cell. Radioactive labelling of DNA facilitated quantitative measurements which proved invaluable for understanding molecular mechanisms of transformation. The fate of genetically marked, 32P-labeled, heavy transforming deoxyribonucleic acid (DNA) as opposed to 3-H-labelled host DNA was examined by sedimentation through sucrose gradients. Some of the notable findings were to show that transformation proceeds with insertion of single-stranded segments of donor DNA which displaces the resident homologous DNA. Further it was shown that either strand of DNA could transform. A method of digitonin lysis was developed to easily separate intracellular donor DNA from the resident DNA. This technique was utilized profitably to track the fate of donor DNA during transformation and subsequent recombination. Also, these techniques were combined with electron microscopy for direct visualization of the DNA. Two strains deficient in genetic recombination were studied by this method and also equilibrium density-gradient centrifugation to demonstrate that the two mutants were blocked in two different steps.

These approaches were extended to study the fate of phage DNA. A major finding was to show that after the entry of phage DNA into wild-type cells, the DNA is degraded at early times, but later some of the fragments are reassembled, resulting in molecules that sediment faster than the monomer length of phage DNA. This study demonstrated that phage DNA is fragmented after entry into host cell but is reassembled by recombination to form concatenates. Another significant finding was to demonstrate the requirement of rec-gene expression for chimeric plasmids.

Apart from the genetic studies, with the advancement of molecular tools, genetic engineering approaches were introduced and various cloning vectors were constructed in eighties. With gradual adoption of recombinant DNA technology, the foundation for transgenic research was laid. A plasmid borne larvicidal crystal protein gene from *B. thuringiensis* subsp. *kurstaki* was cloned in *E. coli* and high level of gene expression was demonstrated. Transgenic *E. coli* cells produced large irregular bodies which were

purified by sonic disruption of cells and were shown to be highly toxic to the larvae of the insect pest *Spodoptera litura, Helicoverpa armigera* (Gram pod borer) and *Bombyx mori* (Silkworm). While megaplasmids of Rhizobia were known, researchers in BARC were able to identify relatively, smaller-sized plasmids form Rhizobia and characterized the *nif* and *nod* genes important for nitrogen fixation. Similarly, efforts were made to characterize *nif* and *hut* operons of *Klebsiella pneumoniae*. A small endogenous plasmid from the Cyanobacterium *Plectonema boryanum* was characterized. These initial efforts paved the way for establishing advanced molecular biology research in DAE. Many of the studies listed above particularly those on bacterial transformation were indeed pioneering and fetched due recognition from national and international scientific community.

4. Pathways of Recombination and Repair

Faithful transmission of genetic material from one generation to the next is considered a sine qua non for preservation of life. The discovery of DNA as the genetic material and its replication as the basis of heritability triggered an avalanche of studies on processes that maintain its stability. Armed with the knowledge of deleterious effects of radiation and chemicals on DNA and tools of classical bacterial genetics, scientists were first to elucidate repair pathways in *E. coli*. The isolation of repair sensitive mutants in *E. coli* greatly facilitated the discovery of photo-repair of UV induced pyrimidine dimers, nucleotide excision repair and post-replication-repair (dependent on homologous recombination) all reported between 1962-1968. It was discovered that recombination serves as one of the most important mechanisms involved in DNA repair which ensures transmission of correct genetic information to offspring from bacteria to man. Soon after it was realized that the process of replication, recombination and repair are intricately linked and carefully orchestrated to maintain genomic integrity.

As pathways of repair and recombination were being elucidated in late sixties and seventies, a strong research program emerged in BARC and a glorious chapter was written with a flurry of exciting discoveries in this area of research. This burst of brilliance culminated in four back-to-back publications in the journal Nature with three of them appearing in 1977 alone. Remarkably, S.K. Bhattacharjee was the lead author in two of these publications and sole author in another. It is interesting to note that while these studies were focused on related aspects of 'light or dark repair' of UV induced DNA damage or pathways important in recombination and repair, three very different microbial model systems were used. The first paper which appeared in 1976, reported the variations in sensitivity to near-ultraviolet irradiation of amoebae grown in 12 h light/12 h dark and those grown in complete darkness. The study showed that in the dark-grown cells, as well as the S phase of the light-grown cells, the repair mechanism against the induced lethal damage might be lacking or non-functional. This study assumed significance as little was known of the light or dark repair of UV damage in eukaryotes. The second paper used cyanobacteria, believed to have been precursors to eukaryotes, as model system to study DNA repair. The study reported the existence of a very efficient

repair system against damage induced by UV in *Anacystis nidulans* and went on to demonstrate that the repair system was either inhibited or rendered less effective under aerobic conditions in the presence of light. This work was extended in the third Nature paper where further physiological evidence for the existence of a dark-repair (or protective) system in this organism was presented. It was also shown that a protein which was unstable in the light, appeared to be responsible for the resistance against lethal damage by ultraviolet light.

Around the same time, Mahajan and Datta, utilized a genetic approach to study the nature of intermediate products utilized by RecBCD and RecF pathways of recombination in *E. coli*. These pathways are important for the repair of DNA damage induced by gamma and UV radiation respectively. It is to be noted that like DNA repair, most of the recombination pathways were originally discovered in *E. coli*. Mahajan and Datta used results from conjugation crosses of *E. coli* K 12 to demonstrate that the viable recombinants produced by the RecBC and the RecF pathways were significantly different in terms of the density of genetic exchanges present in them. These experiments built upon the previous work of Dr. Mahajan carried out in University of Pennsylvania, where a mathematical formulation of *E. coli* conjugation system visualized the recombinational process as consisting short regions of recombination (RRs), each of which may contain several genetic exchanges. Increase in the probability of initiation of the RRs was reflected in increased values of two-point recombination frequencies (Rl) between pairs of markers, while increase in the mean number of exchanges per RR, was reflected in increased values of R2(1), which was a measure of the frequency of additional exchanges close to a selected exchange. The study used R1 and R2 values to measure differences in final products of RecBCD and RecF pathways. With remarkable foresight this work proposed a model which postulated that the RecBC pathway preferentially promotes integration of double-stranded donor DNA segments which may have short single-stranded ends, whereas the RecF pathway mostly promotes integration of single-stranded donor segments.

Another notable study that needs mention is the investigation of the role of polynucleotide phosphorylase (*pnp*) in repair of UV damage. The involvement of DNases in recombination repair pathways was well established but it was difficult to envisage a role for RNases. The study stemmed from the observation that deletion of *pnp* conferred increased UV sensitivity in *E. coli*. By analysing various DNA repair pathway mutants and employing classical genetic approaches an interaction between recombination repair pathway and *pnp* was established. Pulse labelling and examination of nascent DNA synthesis showed that restoration of replication fork and replication re-start were unaffected in *pnp* mutant. Interaction of the repair helicase RecG with PNPase was shown at genetic level. Subsequently, studies at other labs with Bacillus corroborated these findings.

In addition to various prokaryotes, the single-celled fungi *Saccharomyces cerevisiae* served as a suitable eukaryotic model to study repair pathways particularly to study the effect of environmental stress on radiation response. Altered UV and gamma radiation

response under various stress conditions, such as osmotic shock, heat shock, and mild chemical treatments was studied. The investigations of stress-inducible DNA repair in *Saccharomyces cerevisiae* identified a general response enhancing repair and a particular response where the DNA damage may act as a signal for enhancement of the DNA repair.

5. Microbial Stress biology research

While studying a genetic conjugational cross in *E. coli*, low yield of recombinants at lower non-optimal temperature led to serendipitous discovery of a cold sensitive mutant MD1157. This sparked investigations into the linkage between cold stress tolerance and recombination pathways. While a linkage could be not be firmly established, these studies gave a fillip to research in cold stress tolerance in *E. coli*. At that time, heat stress response in bacteria and other organisms had been well studied but molecular mechanisms governing cold stress tolerance were less known. The original mutation in MD1157 mapped to a gene *gicA* (growth in cold), which was later renamed as *cspE* (cold shock protein). Interestingly, though *gicA* from MD1157 was cloned and a mutation in the promoter was identified the cold sensitive phenotype could not be complemented with a wild type allele. Contemporaneous, to these studies Masayori Inouye at Rutgers University identified a highly cold inducible gene *cspA*. Analysis of the genome sequence showed that *E. coli* K-12 strains had eight other genes, including *cspE*, with high homology to *cspA* and the gene family was called 'CspA' family.

The quest to understand how *E. coli* responds to cold stress began with a seemingly simple question: why does this microorganism possess not just one, but nine different cold shock proteins (CSPs)? The presence of these highly conserved proteins suggests an ancient and essential role in the survival of this enteric organism—a role that may include functions still hidden from our understanding. Though these RNA/DNA binding proteins seem redundant at the first glance, they are not all regulated in the same way. This hints at a complex, finely tuned system where these proteins play distinct roles in helping the bacterium cope with different environmental stresses. The RNA chaperone activity of these proteins seemed to be crucial for cold-stress as well as virulence. One of the key players in this regulatory network is CspE, a nucleic acid-melting protein. Studies in BARC showed that CspE is regulated by Ribonuclease E post-transcriptionally through temperature-dependent secondary structures, demonstrating a quick and dynamic response of *E. coli* to the cold environments.

It is worth mentioning that while CSPs were cloned, purified and characterized *in vitro* across labs, cold sensitive mutants in any gene of *cspA* family could not be obtained. The *in vivo* role of CSPs was a matter of scientific debate for a long time and it is in this context the subsequent studies carried out in BARC assume significance. Studies in BARC led to identification of loss-of-function alleles of CspC in related strains of *E. coli* K-12. Sequencing of *cspC* alleles showed that the gene suffered mutations frequently and by diverse molecular processes which included deletion, transposon insertion and point mutations. Occurrence of *cspC* mutations in independent strains suggested that they had

an evolutionary advantage. A combination of genetic crosses and competitive fitness studies demonstrated that loss of CspC conferred a selective growth advantage to the cells. Significantly, the selective advantage was manifested irrespective of the molecular mechanism that led to loss of *cspC* function.

The research in cold-shock response extended further with the finding that the cyclic AMP receptor protein (CRP), traditionally known as a master regulator of metabolism, contributed to cold adaptation. Its unexpected role in cold shock gene regulation, particularly in governing the expression of CspD, a bacterial toxin, and CspE revealed that its function encompassed more than just metabolism. When CRP is absent, *E. coli* struggles to grow in cold conditions, emphasizing its vital role in the survival of this bacterium at low temperatures. Further studies highlighted that the role of CRP in stress response goes beyond cold adaptation. It was shown to play a significant role in helping *E. coli* withstand antibiotic treatment by regulating MqsRA, the toxin-antitoxin pair, which is crucial for the formation of persister cells—tough, resilient cells that can survive antibiotic exposure causing recurrent infections. These findings suggested that CRP is central to the *E. coli* overall stress response strategy. Notably, other labs have continued to build on our work, further exploring CRP involvement in antibiotic persistence.

In a parallel approach that focused beyond the CSP-centric narrative, cold sensitive mutations were identified and studied in *E. coli*. One such mutation identified in MD1157 strain, *gicD1*, which was extremely interesting because of its association with UV and gamma sensitivity was investigated intensely. It was demonstrated that *gicD* locus was allelic to *infB* gene coding for translation initiation factor IF2 and this led to identification of a novel mutation in IF2. The mutation completely abolished the streptomycin resistance by *rpsL31* mutation in S12 ribosomal subunit showcasing the temperature dependent fine-tuning of the tripartite interaction between ribosome, IF2 and streptomycin in *E. coli*. As MD1157 carried both *gicA1* and *gicD1* mutations their individual contribution to the cold sensitive phenotype was clarified. Employing a series of genetic crosses and construction of genetic backgrounds devoid of one or the other mutation and sequencing of the alleles it was demonstrated that cold sensitivity in MD1157 was primarily governed by the *gicD1* mutation and the *gicA1* mutation in *cspE* had little contribution.

In addition to research on bacterial persistence, significant progress has been made in understanding the antibacterial mechanisms of various common and important antibiotics. It was found that antibiotics like ciprofloxacin kill bacteria by damaging their DNA and inducing oxidative stress through the production of reactive oxygen species (ROS). It was discovered that common antioxidants, such as glutathione (GSH), ascorbic acid, and N-acetylcysteine (NAC), can protect bacteria from this oxidative damage. The studies revealed that antioxidants neutralize ROS, shielding bacteria from the full impact of antibiotics. Furthermore, GSH lead to activation of bacterial "efflux pumps," which help bacteria expel antibiotics from their cells, thereby increasing their resistance to ciprofloxacin. This means that these antioxidants when taken along with antibiotics might actually hinder the effectiveness of the antibiotics. In stark contrast, while GSH

protected bacteria from ciprofloxacin and aminoglycosides, it made them more susceptible to the β-lactam class of antibiotics. This finding indicates that antioxidants may have different effects depending on the type of antibiotic used. Utilizing advanced techniques like transcriptomic profiling, researchers discovered that GSH triggers multiple stress responses in bacteria, aiding their survival in challenging conditions. This research demonstrates that while antioxidants are beneficial for human health, they may inadvertently contribute to antibiotic resistance in bacteria. This insight could have significant implications for the future use of antioxidants with antibiotics.

Another model organism that generated worldwide interest for its extraordinary resistance to gamma radiation was *Deinococcoccus radiodurans*. This bacterium can reassemble its genome even after it is completely shattered by gamma irradiation. Interestingly, a highly radio-resistant member of Deinococcaceae family, *Micrococcus radiophilus* later renamed as *Deinococcus radiophilus*, was first isolated in BARC by N. F. Lewis in Bombay duck (*Harpadon nehereus*) in 1971. Lewis along with his co-workers in BARC went on to record extreme UV resistance of this organism as well as reported presence of characteristic pigments of lycopene family. They proceeded to deposit the culture with the National Collection of Type Cultures, Colindale, London. However, it was the publication of the genome sequence of *Deinococcoccus radiodurans* in 1999 that created a renewed vigour in the scientific community to unravel molecular mechanisms underpinning its extreme resistance to radiation. Immediately after the publication of genome sequence, in around year 2000, programs were started in BARC to explore the potential of this organism for remediation of low-level radioactive waste as well as to understand the basis of radioresistance. Extensive genetic and biochemistry studies were carried out on repair pathways, signalling and protein complexes involved in such pathways. A series of studies involving a proteomic approach demonstrated protein recycling during post irradiation recovery which was first time reported from BARC. Further it was shown that a common set of proteins are involved in response to gamma radiation and desiccation exposure.

In summary, these findings revealed the intricate link between various stress responses offering new insights into how these bacteria adapt and survive under different adverse conditions. This research opens up new avenues for further exploration, hinting at the complex interplay between metabolism, stress adaptation, and bacterial resilience.

6. Journey of CRISPR-Cas

In recent years CRISPR/Cas based systems have emerged as the most advanced and very powerful technology for genome manipulation. The rapidly emerging CRISPR-Cas toolbox has ushered in a revolution of sorts with versatile applications in all areas of biomedical sciences. Since the first demonstration of the potential of type II *S. pyogens* CRISPR/Cas9, by Jennifer Doudna and Emmanuelle Charpentier labs in 2012, for genome editing, innovative applications of this technology are continuously emerging. In addition to gene editing, CRISPR-Cas tools have expanded into genetic/epigenetic regulations, imaging and genome-scale screens. CRISPR-Cas tools are also facilitating

new discoveries in food, medical and plant biotechnology research. Exciting avenues such as gene therapy, creation of transgenic models, development of new age anti-microbials, diagnostics and vaccine development are being explored with renewed vigour. The Bioscience group realized the potential of this technology as early as 2014 and programs were launched with an overarching goal of establishing the CRISPR technology in-house. Several approaches to engineer natural CRISPR components in various host systems and exploit them for a broad range of biological problems were explored as described below.

CRISPR/Cas systems can introduce double-strand breaks in target DNA, which, if not repaired, can be fatal to the bacteria, making them promising candidates for antimicrobial strategies. This feature was used to demonstrate the antimicrobial action of CRISPR-Cas9 in *Mycobacterium* which was further extended to targeted killing of drug resistant mycobacteria. As gene knockouts are difficult to achieve in Anabaena, Cas9-based system was engineered to develop a gene silencing tool for Anabaena PCC 7120. This tool was used to knockdown a specific gene which was crucial to prove its role in maintaining redox homeostasis. This demonstrated the potential of this tool in advancing cyanobacterial research. It was observed that extending the applications of Cas9 was constrained by cellular toxicity it conferred in different species of bacteria. Investigation of Cas9 toxicity showed that expression of *cas9* caused plasmid instability and reduced frequency of genetic transformation. In order to utilize the full potential of CRISPR-Cas systems, alternative Cas effectors with complementary features were explored. Research in BARC and few labs elsewhere have pioneered the use of type I Cascade for various applications. Additionally, type V Cas12 systems were explored for specific applications. Engineering of these systems, however, requires careful consideration of parameters to select optimal crRNA and effector properties. In-depth exploration of influence of attributes such as GC content of crRNA, secondary structure of crRNA, strand bias, target location and extent of off targets has been carried out through experimental and computational approaches. The findings provided a 'practical guidebook' for determining the Cas effector that would be most optimum for a particular application to achieve expected outcome.

Research in BARC contributed to the first demonstration of type I Cascade-based CRISPRi in any model system. Its utility in investigating difficult to study essential genes was demonstrated in *E. coli*. Employing the Cascade-based CRISPRi an uncharacterised essential gene *racR* was shown to be a negative regulator of toxins, Ydas and YdaT. Further, Cascade-based CRISPRi was extended to *Salmonella* species another important microbial model system. Another significant milestone achievement was to develop CRISPR-based gene silencing in *Deinococcus radiodurans.* It is worthwhile to mention that even though the organism carries a multiploid genome, knockdown efficiency of around 90% was achieved. The expertise in type I system was utilized to develop a CRISPRi vector with several useful features such as easy targeting and inducible, reversible, multiplexed and titrable gene silencing in *E. coli* and *Salmonella*. It is the only vector available with such advanced features for bacterial CRISPRi.

Further extending the applications of CRISPR technology, a type V CRISPR/Cas12 system was employed to investigate essential genes in *M. smegmatis*. The CRISPRi screen revealed several essential genes including a conserved gene cluster involved in cell wall synthesis. Extensive characterization of MSMEG_0311, a gene in the cluster identified it as a potential drug target. The CRISPR-based in house capabilities were extended to manipulate higher organisms. In a significant feat, the expression and interference phases of type IE CRISPR system was reconstituted in the yeast, *Saccharomyces cerevisiae*. CRISPR-based antiviral and gene editing potential was demonstrated as proof of concept. For eukaryotic applications vectors were designed for mammalian and plant genome editing. A challenging task of generating *rag1* gene knockout mouse was accomplished in collaboration with IISER, Pune. The CRISPR-based transgenic mice technology is available only in highly advanced laboratories and select countries across the world. This endeavour supports the department's need for transgenic mice required for disease modelling, cancer research and drug screening.

In response to global SARS-CoV-2 pandemic, a CRISPR-based sensitive detection method for molecular diagnosis of the pathogen was established. Towards point-of-care application, particularly in low resource settings, the assay was coupled with a portable, battery-operated device, CRISPR-Cube which was developed in collaboration with EmA&ID, for rapid and visual detection of the results. The assay was validated on patient samples and the technology was transferred to the industry. This versatility of this platform technology has been demonstrated by single copy detection of Mpox virus in contrived samples, highlighting its potential for broader application in infectious disease diagnostics.

In the ten years since inception of the work in the department, a strong expertise in CRISPR-Cas technology has been developed for addressing various biological problems of fundamental and applied nature as showcased by quality publications and technology transfers. The endeavor to adopt, adapt and improvise the technology for a plethora of applications will continue. It can be anticipated that in future the power of CRISPR-technology will be widely harnessed in almost all programs of Bio-Science group.

7. Future Outlook

The foundation of the journey of microbial research in BARC was erected on strong pillars. Along the way it imbibed new ideas and generally set an illustrious standard of academic scholarship. As we enter the modern era of research, the strive for excellence to remain in the forefront continues. Building upon the core strength of microbiology, genetics, biochemistry, molecular biology and genetic engineering, the new era ushers next-gen approaches that includes omics, informatics, genome engineering and super-resolution imaging that seeks to break the barriers of magnification and limits of resolution. It is also the time to see biology in action, live inside the cells, to catch molecular machines in the act.

CRISPR-Cas tools and next generation sequencing have already hugely impacted all the areas of bioscience research and technology. It is likely that innovations in sequencing

technologies will continue to increase the throughput and will play a bigger role in all areas of research necessitating increased adoption of powerful bioinformatics tools. More and more CRISPR-based gene therapies are likely to cross clinical trials and reach clinics. The two complementary technologies together provide a very powerful combination for genome scale interrogation of structure and function. For instance, it is becoming increasingly common to employ large-scale CRISPR screens for identifying new drug targets for cancer. Together these technologies are likely to usher in an era of personalized medicine. The tools and approaches like AI, machine learning, high throughput sequencing, omics, CRISPR-gene-editing, high-end imaging place unprecedent power in the hands of researchers to venture and explore unchartered territories. The traditional boundaries between biological, physical and chemical science are increasingly getting blurred as different scientific disciplines collaborate and coalesce to create new science and technologies.

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MICROBIAL CELLS- AND BIOFILM-MEDIATED BIOREMEDIATION

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Abstract

Molecular Biology Division at Bio-Science group (BSG), BARC is involved in understanding the cellular and molecular interactions of uranium and other heavy metals with various microbes and exploring the utility of such important interactions/mechanisms in bioremediation. This article showcases some of the key findings of various uranyl/heavy metal interaction mechanisms researched in the division since last two decades. Also, the efforts for large scale biodegradation of Tributyl phosphate (TBP) and unveiling the mechanism of biodegradation is discussed here. Aligning with the requirement of DAE, the biology research group at Water & Steam Chemistry Division (WSCD), Chemistry Group, BARC, is engaged in the field of biofilms and biofouling control research and is a frontrunner in this area in the country. Besides catering to biofouling control in power plants and allied units, it has contributed towards development and deployment of innovative biofilm-based biotechnologies for bioremediation and wastewater treatment.

1. Introduction

Since its commencement, the DAE has emphasized on basic research in Biology and has undertaken various research programmes to develop strong basic groups in the area of genetics, molecular biology, microbiology and biochemistry. Basic research in molecular biology in DAE originated with the fundamental work on genetics and molecular studies of microorganisms. Gradually, the biological research programmes oriented towards the work to support the issues relevant for DAE. In that context, research on microbial bioremediation of uranium was undertaken in BSG, BARC to understand the mechanisms of microbial interactions with uranium that can be used for alleviating uranium contamination. Cellular and molecular mechanisms were also explored for mitigating toxicity of heavy metals other than uranium. Research was undertaken to understand the mechanism behind the microbial degradation of TBP that is used for extraction of uranium and plutonium from spent nuclear fuel. Studies were done to explore the utility of biofilms by WSCD, Chemistry Group, BARC and to develop an efficient system for safe management of effluents generated in nuclear fuel cycle operations. The cooling water system in nuclear power plants provides a conducive environment for biofouling organisms to colonize and thus severely impacting the operational efficiency of the cooling water systems. Research work was undertaken by WSCD for mitigating of biofouling at Madras Atomic Power Station, Kalpakkam. In the following sections, highlights from all the aforesaid work are presented.

2. Microbial interactions with uranium important for bioremediation

Uranium is known to be naturally occurring radioactive element present in the Earth's crust, and is endlessly released into the environment from various geochemical activities including weathering of rocks and minerals. Also, anthropogenic activities such as U-mining and milling operations and nuclear fuel processing, use of phosphate fertilizers and other industrial applications generate substantial quantities of U containing waste. Uranium has no biological role and is mostly known for its chemical toxicity rather than its radiotoxicity. While there are many ways to dispose of uranium containing waste/solution, microbial bioremediation is desirable as it is more eco-friendly than other methods. Having evolved billion-plus years ago on the planet, bacteria have evolved diverse means to disarm toxic effects of uranium by sequestering or mineralizing it while resisting the radioactivity. There has been little exploration on such mechanisms in the country. A multidisciplinary approach including recombinant DNA technology, genomics, transcriptomics and proteomics, advanced imaging, speciation modeling, absorption and X-ray diffraction spectroscopy was taken up while researching on these mechanistic aspects. Some of the major activities related to uranyl interactions with bacteria are described here.

2.1. Uranium bioprecipitation by recombinant and natural bacterial strains

R & D work in the area of uranium bioprecipitation started in early 2000 in Molecular Biology Division (MBD) of BSG, BARC. The work was initiated with a non-specific

acid phosphatase encoding *phoN* gene from *Salmonella typhi*. Phosphatase enzyme is known to catalyze the hydrolysis of phosphate esters under acidic, neutral and alkaline conditions depending on pH for their optimal activity in the presence of organophosphate substrates. The phosphate ions released from the hydrolysis interact with uranyl resulting in uranium precipitation in the form of insoluble and stable uranium phosphate minerals thereby limiting availability and toxicity of uranium. With the motivation of using microbes for the treatment of radioactive waste under high radiation stress, a non-specific acid phosphatase encoding *phoN* gene of a local isolate of *Salmonella enterica* serovar Typhi was cloned and expressed successfully in highly radioresistant *Deinococcus radiodurans* strain R1. The recombinant *Deinococcus* strain expressed PhoN protein and competently precipitated uranium (>90%) from uranyl (0.8 mM) solution in presence of 5 mM β–glycerophosphate within 6 h at pH 5. Additionally, it was observed that the engineered strain maintained its ability for uranium bioprecipitation following exposure to 6 kGy of ^{60}Co gamma rays. To address the biorecovery of uranium from alkaline nuclear waste generated from uranium mining and nuclear fuel processing activities, enzymatic bioprecipitation of uranium using alkaline phosphatase was undertaken. In this context, a *Sphingomonas* sp. strain, BSAR-1, exhibiting high alkaline phosphatase was isolated. Alkaline phosphatase gene, *phoK*, from BSAR-1 was cloned and subsequently overexpressed in *E. coli*. The *E. coli* strain EK4 overexpressing *phoK* exhibited 13 times higher extracellular PhoK activity than BSAR-1. Further it was observed that the recombinant strain precipitated >90% of input uranium (0.5 to 5 mM of uranyl carbonate) in < 2 h from alkaline solutions at pH 9 with a loading capacity of 3.8 g U/g dry weight. This was much faster than the BSAR-1 which precipitated similar amount of uranium under similar condition in >7 h loading only 1.5 g U/g dry weight. After assessing the potential of PhoK for uranium bioprecipitation at alkaline pH, the work was further extended to explore the bioremediation capability of PhoK under high radiation environment. The *phoK* gene was cloned into the radioresistant bacterium *Deinococcus radiodurans*. The resulting recombinant strain, *Deino-PhoK* displayed very high PhoK activity and bioprecipitated U very efficiently. At low uranyl concentrations (1 mM), the strain precipitated > 90 % of uranium within 2 h while a high loading capacity of around 10.7 g U/g of dry weight of cells was achieved at 10 mM U concentration. The Deino-PhoK cells retained its functionality even after exposure to high radiation dose (~15 kGy).

To enhance the bioremediation potential of *D. radiodurans* cells, an attempt to display proteins relevant to bioremediation was undertaken using surface layer proteins, Hpi and SlpA. It was shown that the Hpi protein, which forms a covalently cross linked array on cell surface was a good vehicle for surface display by fusing proteins such as metallothionien and phosphatase to it. Additionally, it was shown that Hpi contributed to a net negative charge on cell surface which enabled efficient biosorption of positively charged uranyl ion on cells as well as the isolated Hpi layer. Using metallothionien fused to the Hpi protein and displayed on cell surface, improved Cd biosorption was demonstrated. The study also revealed the possible location of the SlpA protein in the

complex deinococcal cell envelope. Using biochemical experiments, it was shown that an N terminal domain of SlpA, SLH interacted with the peptidoglycan layer, contributing to a basic understanding about cell wall organization in *D. radiodurans*. However, both SLH and SlpA turned out to be poor candidates for surface display. Alternatively, efficient uranium precipitation could be demonstrated with novel biomaterial constituting a phosphatase fused to the SLH domain and immobilized on peptidoglycan which is a robust polymer.

It has been observed that the microbes residing in uranium contaminated environments constitutively express phosphatases and precipitate uranium. Presence of high concentrations of radionuclides and heavy metals in uranium enriched sites have been shown to impose selective pressure on bacteria, leading to evolution of native bacterial communities resistant to site-specific levels of contamination. In one such instance in our laboratory, an environmental bacterial strain *Chryseobacterium* sp. PMSZPI was isolated from sub-surface soil of uranium ore deposit at Domiasiat site in Meghalaya, India that tolerated high concentration of U exhibiting a minimum inhibitory concentration (MIC) of 4 mM U. The genome of PMSZPI was sequenced using Illumina sequencing that showed the presence of large number of prospective adaptive and metal tolerant determinants including metal resistance, efflux, transporters, antibiotic resistance, DNA repair, oxidoreductases, motility, phosphatases, CRISPR/Cas systems, polysaccharide synthesis and protein secretion systems. The strain expressed high acid and alkaline phosphatase activities and competently precipitated uranium (~93–94%) from 1 mM uranyl solutions and 5 mM β–glycerophosphate at pH 5, 7 and 9 by 24 h of U exposure loading up to ~225.5 mg U g^{-1} dry wt. In an attempt to showcase its biotechnological application, the biomass of *Chryseobacterium* sp. strain PMSZPI was immobilized in calcium alginate beads and investigated for U(VI) biomineralization in batch and column set-up. Under batch mode, the fresh or lyophilized cells entrapped in alginate beads demonstrated effectual U precipitation under acid and alkaline conditions. The maximum removal was observed at pH 7 wherein ~98–99% of uranium was precipitated from 1 mM uranyl carbonate solution loading ~350 mg U/g of biomass within 24 h. Retention of phosphatase activity without any loss of uranium precipitation ability was observed for alginate beads with lyophilized biomass stored for 90 d at 4°C. Continuous flow through experiment with PMSZPI biomass immobilized in polyacrylamide gel exhibited U loading of 0.8 g U/g of biomass at pH 7 using 1 L of 1 mM uranyl solution.

2.2. Biosorption/polyphosphate mediated sequestration of uranium

One of the known uranyl-microbe interactions is biosorption in which various functional groups or ligands such as carboxyl, hydroxyl, amide or phosphoryl groups are available on the cell surface for uranium binding in both living and dead cells. Most of the uranyl binding or sorption studies for uranium have been performed under acidic pH wherein uranium exists as uranyl cation, UO_2^{2+}. Uranium sequestration studies were attempted using unicellular marine cyanobacterium, *Synechococcus elongatus* strain BDU/75042 at pH 7.8 from uranyl carbonate solutions. Uranium exists as stable carbonato complexes of uranyl ions i.e. $[UO_2(CO_3)_2]^{2-}$ or $[UO_2(CO_3)_3]^{4-}$ in aquatic environments like sea or pond

water. This strain could remove 72% of uranium within 1 h from 100 µM uranyl carbonate under phosphate limited condition with a maximum adsorption capacity of 124 mg U g^{-1}dry wt of biomass. The bound uranium could be fully desorbed using 0.1N HCl. The extracellular polysaccharides (EPS) containing amide and deprotonated carboxyl groups were involved in interaction with uranium. The binding kinetics suggested monolayer adsorption on the cell surface which fitted into the Langmuir adsorption isotherm. Eco-friendly option was developed as further expansion of the work for uranium recovery from simulated sea water. Seawater is one of the largest resources of U comprising of 4.5 billion tonnes of U. Long term experiments carried out with continual exposure of *Synechococcus* to simulated sea water (~30 L) at regular intervals, showed a loading of 2.9 mg U/g in 4 weeks. Apart from this unicellular cyanobacterium, a marine filamentous, heterocystous cyanobacterium, *Anabaena torul*osa which was isolated from saline paddy fields of Trombay, Mumbai has also been studied for uranium sequestration. *A. torulosa* cells showed biphasic mode of uranium binding-initially fast binding 48% uranium by 30 min (56 mg Ug^{-1} dry wt.) and then gradual phase, binding 65% uranium with loading of 77.35 mg U g^{-1}dry wt. in 24 h from 100 µM uranyl carbonate solutions at pH 7.8.

Polyphosphates (PolyP) are short and long chain polymers of orthophosphates linked to each other through high energy phosphoanhydride bonds like ATP. Poly P in microbes has been shown to bind uranium and other metals intracellularly limiting uranium toxicity. We demonstrated for the first time the uranium sequestration by distinct surface associated polyphosphate bodies (SAPBs) in cyanobacterium, *Anabaena torul*osa. Uranium exposure in phosphate deficient medium up to 5d demonstrated extensive chlorosis, cell lysis, akinete formation followed by hydrolysis of polyP in *Anabaena torul*osa that precipitated uranium in the form of uranyl phosphate mineral. Further exposure to uranium resulted in induction of alkaline phosphatase in akinetes and regeneration of *Anabaena torul*osa filaments. Polyphosphate rich ($PolyP^+$) and deficient ($PolyP^-$) cells were generated by altering the concentrations of phosphate in growth medium. $PolyP^+$ cells showed increase in phosphate by ~6-7 times as compared to wild type cells. Accumulation of polyphosphate in *Anabaena torul*osa provided significant tolerance towards the U toxicity probably binding U within the polyphosphates.

A uranium mine bacterial isolate, *Chryseobacterium* PMSZPI revealed gliding motility owing to the presence of Type IX secretion system (T9SS). It formed spreading colonies on soft agar (0.35%). The gliding motility was found to be inhibited in presence of uranium leading to lesser colony spreading. However, an increased amount of biofilm formation was observed in PMSZPI cells that limited uranium toxicity. Entrapment of uranium in the biofilms U exposure was observed in PMSZPI cells.

2.3. Tools standardized for uranium detection

Uranium detection is very important for our studies. The spectrophotometric method using Arsenazo III has been standardized and is the mostly used in our laboratory for detection of uranium. The uranium-arsenazo-III complex is stable for more than 3 weeks with constant absorbance. Beer's law was found to be in agreement to a uranium

concentration of 200 μg g^{-1}. Very low concentrations of uranium (ppb levels) were detected using inductively coupled plasma-mass spectrometry (ICP-MS). In our studies regarding uranium biorecovery from simulated sea water containing 3 μg L^{-1} uranyl carbonate at pH 7.8 using marine cyanobacterium, *Synechococcus elongatus*, ICP-MS was used to determine U concentration in ppb concentrations. Energy dispersive X-ray fluorescence (EDXRF) spectroscopy was used for confirmation of uranium association with the microbial cells. Uranium loaded cells of marine cyanobacteria, *S. elongatus* (53.5 mg U g^{-1} dry weight) and *A. torulosa* (77.35 mg U g^{-1}) when analyzed with energy dispersive X-ray fluorescence (EDXRF) spectroscopy confirmed the association of uranium with bacterial cells displaying L X- rays at 13.1 keV(UL_l), 13.6 keV (UL_β), 17.2 keV(UL_α,) and 20.2 keV (UL_γ). Bacterial cell surfaces are composed of various functional groups which have been reported for uranium complexation and can be studied using Fourier transform infrared (FT-IR) spectroscopy. Most of the bound uranium in *S. elongatus* was found to be associated with the extracellular polysaccharides (EPS) which on further investigation with Fourier transform infrared (FT-IR) spectroscopy suggested the amide groups and the deprotonated carboxyl groups on the EPS were possibly involved in uranyl adsorption. The filamentous cyanobacterium *A. torulosa* cells on incubation with 100 μM uranyl carbonate at pH 7.8 until 120 h exhibited poly-P mediated extracellular uranyl precipitation. The identity of the precipitated uranium was characterized as U(VI) autunite-type mineral by X-ray diffraction (XRD) analysis. The fluorescence spectroscopy of bioprecipitated U associated with 120-h U-exposed *A. torulosa* cells recorded with an excitation wavelength of 400 nm revealed fluorescence peaks at 505, 526, 550, and 575 nm, characteristic of chernikovite/meta-autunite. X-ray absorption near edge structure (XANES) analysis showed that absorption edge position in the bioprecipitated sample was consistent with uranium in a +6 oxidation state, i.e., U(VI). The extended X-ray absorption fine structure (EXAFS) analysis of the bioprecipitated U sample in *A. torulosa* showed features and distances for U-Oax, U-Oeq, and U-P consistent with that of a meta-autunite-like uranyl phosphate mineral. In another case, XRD patterns of the uranium-loaded cells of *E. coli*-PhoK and Deino-PhoK confirmed the presence of uranyl hydrogen phosphate hydrate also known as chernikovite.

The uranium speciation is significant in context of its bioavailability and its toxicity and that is the criterion for understanding the uranyl interactions with microbial cells. During the course of phosphatase mediated precipitation in *Serratia* cells, the chemical speciation of aqueous U(VI) in the presence of nitrate and carbonate salts of uranium at pH 2-10 was determined using Visual MINTEQ modeling software. At pH 5, U speciation was controlled by positively charged UO_2^{2+} and UO_2–acetate$^+$ ions whereas at pH 7, positively charged hydroxide ions like $(UO_2)_3(OH)^{5+}$ and $(UO_2)_4(OH)^{7+}$ and negatively charged UO_2 $(CO_3)_2^{2-}$ and $UO_2(CO_3)_3^{4-}$ were prevalent. In contrast, speciation of U(VI) was dominated by negatively charged $(UO_2)_3(OH)^{7-}$, $UO_2(CO_3)_2^{2-}$ and $UO_2(CO_3)_3^{4-}$ at pH 9. Electron microscopy techniques like Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) were employed to observe

morphological changes due to uranium exposure and localization of bound or precipitated uranium on the exterior and interior of the microbial cells respectively. Scanning electron microscopy based imaging, coupled with Energy Dispersive X-ray (EDX) spectroscopy identified the presence of novel surface associated polyphosphate bodies (SAPBs) in the filamentous cyanobacterium *A. torulosa* and the interaction of such SAPBs with uranium. Visualization of uranium precipitation at pH 5 in *Chryseobacterium* PMSZPI with TEM revealed the intracellular location of needle like structures corresponding to uranyl precipitates, while the uranyl precipitates were found to be membrane bound as well as extracellular at pH 7 and the precipitates were predominantly extracellular at pH 9.

3. Bioremediation of other heavy metals

Apart from uranium, efforts have been made to understand the various microbial interactions with heavy metals that have led to the alleviation in metal toxicity and mobility and can be proposed for application for bioremediation of toxic metals in contaminated environments.

3.1. Bioremediation using exopolysaccharides (EPS)

Microbial mats primarily comprising of cyanobacteria have long been known to be associated with dry rock surface and rocks in river stream and involved in binding of several heavy metals. This is largely contributed by the exopolysaccharides (EPS) present on the cell surface of these microbes. Cyanobacteria are unique in this aspect as their EPS are heteropolymeric and carries higher negative charge due to the presence of uronic acid, making it a suitable substratum for remediation of positively charged heavy metals. While multi-microbial mats in the form of granules have been successfully used in waste water management as discussed in the subsequent sections, the use of single cyanobacterial strain for this purpose had not been examined, but has lot of potential. Work was initiated in this respect in 2016 in MBD, using the filamentous cyanobacterium, *Nostoc muscorum*, capable of forming biofilms naturally in the absence of any external stress. The biofilms generated from axenic cultures of *N. muscorum* (Nm) were found to be closely knit with strongest adherence to glass surface compared to other materials. The Nm biofilms exhibited high ability bind Cd over a wide pH range of 5-10 and concentration range of 1-100 ppm through the binding of Cd to functional groups, such as carbonyl and hydroxyl present on its cell surface. This ability was retained upon multiple exposure to Cd as well as when waste water effluents were used indicating high sustainability of the Nm system. It also exhibited the ability to bind multiple heavy metals (Cd, Ni, Pb) simultaneously without compromising on the binding affinity of individual metals. Atomic Force Microscopy (AFM) revealed enhanced formation of EPS on the cell surface of Nm biofilms upon exposure to heavy metals, which was further confirmed through the identification of functional groups involved in binding using X-ray photoelectron spectroscopy (XPS) and Fourier Transform-Infra red Spectroscopy (FTIR). To further enhance the potential of cyanobacteria for metal remediation, it was proposed to enhance the production of EPS as well as make designer

EPS through introducing additional negative charge on the polysaccharides or changing the composition through genetic manipulation of another closely related cyanobacterium, *Nostoc* PCC 7120. At present enhanced EPS production has been demonstrated through the overexpression of ExoD protein which also resulted in enhanced tolerance to Cd. Increase in negative charge on EPS was achieved through overexpression of ExoV and Alr0658 resulting in the introduction of pyruvyl and uronyl groups respectively on EPS. This also resulted in enhanced metal tolerance and the potential of use of the modified EPS directly for metal bioremediation is being currently explored.

3.2. Intracellular metal sequestration via metallothioneins (MTs)

Microbes employ various mechanisms to combat heavy metal stress. One such mechanism is intracellular sequestration of metals with metal binding proteins/peptides like metallothioneins (MTs). Metallothioneins are metal-inducible cytosolic proteins that are rich in cysteine residues. Metals bind to these proteins via the sulphydryl groups forming metal-thiolate clusters. The role of MTs in metal detoxification have been explored extensively. MTs were discovered in eukaryotes and initial studies were limited to eukaryotes, but presently considerable research has been done in prokaryotes.

Prokaryotic MT, SmtA, a metallothionein from *Synechococcus* PCC 7942 was shown to bind to metals like zinc and cadmium by cysteine and histidine residues. SmtA interacted with uranyl ion, UO_2^{2+} via glutamate and aspartate residues. There have not been adequate studies on prokaryotic MTs other than SmtA. We characterized a putative prokaryotic metallothionein, NmtA from *Anabaena* PCC 7120. NmtA was shown to provide protection against cadmium toxicity when overexpressed in the native strain. The metal inducible nature of *nmtA* was also studied in the presence of metals like cadmium, zinc and copper. The inducible expression was deciphered by transcriptional regulator, AzuR (Alr0831). We demonstrated that AzuR bound to upstream element of *nmtA* ORF in the absence of metal. This DNA-protein binding was inhibited in the presence of divalent metal cations. The overexpression of *azuR* in *Anabaena* resulted in downregulation of *nmtA* expression confirming the negative regulation of *nmtA* expression by AzuR. Downregulation of *nmtA* led to metal sensitivity in *Anabaena*. NmtA protein immobilized in magnetic nanoparticles exhibited superior binding to uranium and cadmium that could be removed from contaminated solutions using a magnet.

3.3. Metal efflux by heavy metal translocating P_{IB}-ATPase

Heavy metal translocating P_{IB}-ATPases efflux out the heavy metal ions (Cd^{2+}, Zn^{2+}, Pb^{2+}, Cu^{+} and Ag^{+}) across the cell membrane and play an important role in metal resistance and cytoplasmic metal homeostasis. The P_{IB}-ATPases have been reported to be horizontally transferred among bacteria indicative of their significance in their adaptation and survival in uranium/heavy metal enriched sites. The transcript of P_{IB}-ATPase present in *Chryseobacterium sp.* PMSZPI, a bacterium isolated from uranium ore deposit in India was found to be upregulated under heavy metal (Zn, Cd) stress conditions. The recombinant P_{IB-2}-ATPase protein from PMSZPI was expressed and purified from membrane fraction of *E.coli* that showed stimulation of ATPase activity in the presence

of Pb, Zn and Cd. *In-vivo* metal tolerance and intracellular metal accumulation studies suggested that P_{IB-2}-ATPase mediated the transport of Zn and Cd providing increased resistance towards zinc and cadmium toxicity to *E. coli* cells overexpressing the P_{IB-2}-ATPase protein. The amino acids important for functioning of the protein were determined by creating site-directed mutants of conserved residues like Cys^{318}, Cys^{320}, Lys^{620} and Asp^{641} present in the trans-membrane metal binding sites. Our study showed the loss of *in-vitro* ATPase activity of the purified mutant proteins, reduction of *in-vivo* metal tolerance and increased metal accumulation in recombinant *E. coli* cells overexpressing mutant proteins as compared to WT P_{IB-2}-ATPase protein indicated the importance of these residues in functioning of the protein.

4. Biodegradation of Tributyl phosphate (TBP)

Tributyl phosphate (TBP) is used in large volumes in PUREX (plutonium uranium reduction extraction) process for the extraction of uranium and plutonium from spent nuclear fuel. Over time, TBP is subjected to chemical and radiolytic degradation, which progressively reduces its extraction efficiency. The TBP waste generated must be treated and disposed as waste. The physico-chemical processes for TBP degradation are harsh and have the disadvantage of generating secondary waste. Therefore, development of an eco-friendly process which could allow complete degradation of TBP has always been desirable. Over years several labs reported isolation of microbes that could degrade TBP. However, most of these microbial species either mineralized TBP partially or displayed sequestration of TBP. Among these, the best TBP mineralization reported in literature was 2 mM in 3 days by mixed culture of *Pseudomonas*. Many of the strains could tolerate only very low levels of TBP. Given the low mineralization ability and low tolerance to high TBP concentrations these species were deemed unsuitable for most practical uses.

4.1. Isolation of an efficient TBP degrading bacterium and development of scaled-up processes for TBP bio-degradation

In an effort to find an efficient bio-degrader of TBP, a bacterial strain (*Sphingobium* sp. RSMS) was isolated from Radioactive Solid waste Management Site (RSMS) in BARC. The bacterium, named as *Sphingobium* sp. RSMS, was found to degrade and utilize TBP as well as dibutyl phosphate (DBP) and use them as the sole source of carbon and phosphorous for its growth. *Sphingobium* sp. RSMS strain was found to degrade 30 mM TBP in 3 days under lab conditions which is ~15 times more efficient than the best reported strain. To demonstrate the potential of this bacterium, TBP biodegradation was optimized and the process was scaled up to 30 L volume in collaboration with FTD, BARC. The optimized process achieved degradation of 30 mM TBP in 3 days which was identical to lab-scale efficiency. Similarly, in collaboration with colleagues in ChEG, BARC a 205-liter scale-up in a stirred tank reactor was successfully carried out under non-sterile conditions and at ambient temperature to economize the process. To rule out the flow non-idealities such as dead zones computational fluid dynamics (CFD) modelling of the process in 205 L stirred reactor was used to visualise the flow patterns

and also to identify the optimum values of operating parameters. In this setup, the strain effectively utilized TBP for growth and degraded of 21 mM of TBP over 15 days. This degradation level represented roughly 70% of the TBP removal achieved in laboratory-scale experiments. These two are the most efficient TBP biodegradation scale-up processes reported so far.

Though after decades of research, many TBP degrading bacterium were isolated by different labs across the world, no information was available on the pathway of degradation, the enzymes involved and the genetic determinants of TBP biodegradation. For the first time, the intermediates and products formed in the TBP degradation were identified in BARC using gas chromatography and spectrophotometry and a biochemical pathway of degradation was proposed. The degradation pathway involves initial formation of DBP from TBP, with the release of butanol. DBP is then further degraded to release two butanol molecules and inorganic phosphate. Based on this involvement of phosphoesterases was hypothesized and presence of phosphoesterase activity was demonstrated in this bacterium.

4.2. Comparative genomic approaches to elucidate genetic basis of TBP degradation by RSMS strain

A spontaneous mutant (SS22) which neither utilized TBP/DBP nor released any intermediates/products degradation, suggesting that the whole pathway of TBP/DBP degradation was affected, was isolated. To elucidate the genetic basis of TBP/DBP degradation a comparative genomics approach was taken. The whole genome sequencing as well transcriptomic studies were carried out. The genome sequence revealed that both RSMS and SS22 have two chromosomes and three plasmids (pRSMS1, pRSMS2 and pRSMS3) each but pRSMS1 plasmid of SS22 had a region deleted presumably carrying TBP/DBP degradation genes. The RNA seq analysis of the wildtype and the mutant corroborated the deletion. Among the 32 genes present in RSMS but deleted in the SS22, a metallophosphoesterase (designated as MpeA) was identified and investigated for its role in TBP/DBP degradation. Purified MpeA protein could hydrolyze DBP and monobutylphosphate (MBP), the intermediates of TBP degradation pathway. This is the first report of identification of a gene involved in TBP bio-degradation pathway in any organism. Overall, these studies generated new knowledge and also facilitated development of scaled-up processes for TBP bio-degradation attesting the potential for further development.

5. Biofilm-based bioremediation of wastewater

Like many other industries, water is used in different phases of nuclear fuel cycle operations from mining to fuel fabrication and spent fuel reprocessing. These operations generate effluents containing organic and inorganic contaminants, which include chelating agents (e.g., nitrilotriacetic acid (NTA)), solvents (e.g., tributyl phosphate (TBP), ammonium, nitrate and radionuclides. Treatment of these low-level radioactive effluents is essential prior to environmental discharge. An R&D programme on biofilm-

based bioremediation was initiated in 2004 at Biofouling and Biofilm Processes Section of WSCD with an objective to explore beneficial uses of biofilms and to develop an efficient biological treatment system for safe management of effluents generated in nuclear fuel cycle operations. The vast experience of biofilm R&D on characterization, accrued as part of biofouling control research, was useful for developing and deployment of innovative biofilm-based bioremediation strategies. Apart from effective treatment, efficient separation of biomass from the treated wastewater is essential for field application of bioremediation technologies. Moreover, robust system is needed for treating wastewater containing toxic pollutants and high-strength wastewater. To align with these, a treatment system based on aerobic granular sludge (also referred to as microbial granules (bio-granules or bio-beads) was chosen for investigations, with emphasis on granule cultivation, characterization, optimization, bioreactor development and bioreactor operation.

5.1. Bio-granules for sustainable wastewater treatment

Biological treatment is an important component of wastewater treatment plants (WWTPs) installed for removing soluble and particulate pollutants from domestic and industrial wastewaters. Conventionally, wastewater treatment is achieved using activated sludge (flocs), a mixed microbial community that feeds on the biodegradable organic substrates present in the wastewater. Due to the diffuse (floccular) physical structure and poor settling characteristics of the activated sludge, secondary clarifiers are essential for achieving separation of activated sludge and the treated wastewater. Although activated sludge based WWTPs are widely used in different parts of the world, it has major limitations, including poor nutrient removal (particularly, nitrogen and phosphorus), requirement of large land footprint and recirculation of liquid/sludge. Since these drawbacks are related to poor settling properties of the flocs, advancements in this field have attempted to improve solid-liquid separation by using membrane, biofilm-growth or dense biomass particles. Membrane based biological treatment (i.e. membrane bioreactor (MBR)) methods have been developed to address the drawbacks of activated sludge process. These systems have improved sludge-treated wastewater separation, treatment efficiency and provided compact WWTPs. However, MBRs are not widely implemented due to high capital costs, high energy costs and membrane biofouling problems. Therefore, biofilm-based treatment systems have been developed, resulting in substantial reduction in the land footprint and improvement in biological treatment efficiency. Biofilms are defined as substratum-associated mixed microbial communities formed through self-immobilization in a self-produced extracellular polymeric substances (EPS) matrix. Moving bed biofilm reactor (MBBR) and membrane aerated biofilm reactors (MABR) are examples of biofilm-reactors applied in large scale WWTPs. However, sloughing of biomass from the biofilms is a concern in biofilm-based systems as it can deteriorate the quality of treated wastewater.

In order to improve biological treatment and biomass-treated wastewater separation, aerobic granular sludge (AGS) was reported in 1997 for the first time in sequencing batch reactors. AGS refers to compact and dense microbial biomass, distinct from activated

sludge in terms of microbial community, EPS matrix and settling properties. It is similar to biofilm-type microbial growth, but without a substratum and is also referred to as aerobic microbial granules or bio-granules. Initial work in this area in WSCD was focussed on cultivation of bio-granules under different bioprocess conditions, including bioreactor operating conditions and type of carbon substrate in the feed. For example, formation of bacteria-laden granules was investigated for removing various organic and inorganic pollutants of interest to industrial wastewater including those of nuclear fuel cycle operations. These studies have showed that bacterial granules can be developed for removing different organic (i.e. TBP, *n*-butanol, dibutyl hydrogen phosphate, 2,4-dinitrotoluene, nitrilotriacetic acid, *p*-nitrophenol, textile dye and acetonitrile) and inorganic contaminants (i.e. ammonium, nitrate and phosphate). It was also evident that granules are a better choice for biodegradation and biotransformation of recalcitrant or toxic pollutants present in the industrial wastewater including effluents of nuclear fuel cycle operations. Many of these studies on cultivation of granules under different process conditions, effect of reactor operating parameters and removal of contaminants were performed in lab-scale bioreactors under defined conditions using simulated wastewater. Nevertheless, these studies have identified optimum parameters for granulation and potential applications of granules in treatment of domestic and industrial wastewaters. These studies have ultimately led to development of bacteria-laden granules-based technology (i.e. hgSBR technology) for wastewater treatment detailed in the subsequent section.

In order to further improve environmental sustainability, R&D on algal-bacterial granules for bioremediation was initiated. The algal-bacterial granules cultivated from autochthonous halophilic organisms have demonstrated effective carbon, nitrogen and phosphate removal under saline conditions indicating potential use for treating saline effluents. This work showed that algal-bacterial granules can be cultivated in photo-bioreactors under different process conditions, for simultaneously removing BOD, COD, nutrients and emerging contaminants. Further research on algal-bacterial granules is in progress for developing energy-neutral wastewater treatment technologies with low carbon footprint and greenhouse gas emissions.

5.2. hgSBR technology

To reduce the start-up periods during wastewater treatment, *de novo* development of bio-granules was attempted, where no pre-formed flocs or granules are used as inoculum. This new approach relied on cultivating functional granules directly from water or wastewater-borne microbes. It eliminated the long acclimation periods involved in adopting activated sludge for treating saline wastewater. Furthermore, *de novo* granulation of water/wastewater-borne microbes was enhanced by introducing a small amount of granular activated carbon (GAC) particles. To protect the innovation, the new granulation method for wastewater treatment was applied in 2019 for securing an Indian patent which was granted in 2021. To demonstrate the use of bio-granules-based sewage treatment, pilot-scale plants have been set up for treating real-sewage under tropical climate conditions (https://www.ndtv.com/india-news/nuclear-engineers-fighting-water-

pollution-with-sewage-treatment-plant-1768223, accessed on 28 June 2024). The pilot scale studies have been successfully completed during 2015 to 2019. The lab- and pilot-scale studies showed that the new strategy helps in achieving efficient treatment of wastewater including removal of nitrogen, phosphate and coliform bacteria from the wastewater.

The technology is referred to as hybrid granular sequencing batch reactor (hgSBR) for wastewater treatment (https://www.barc.gov.in/technologies/sbr/index.html, accessed on 28 June 2024). Since 2020, the hgSBR knowhow was made available to private partners for deployment in WWTPs. Several private companies have signed technology of transfer (ToT) agreement with BARC for deployment of hgSBR for sewage treatment. Currently, 27 private companies have signed transfer of technology (ToT) and partnered with BARC for commercialization of hgSBR technology. Efforts have been already made in this direction and the technology has been implemented for several full-scale sewage treatment plants. Presently, the treatment capacities of the installed hgSBR plants are ranging from 5 m^3/day to 1500 m^3/day (equivalent to sewage arising from 10 to 3200 households). The plants are in operation for treating domestic wastewater at different places including Kalpakkam, New Delhi, Shirdi and Surat. Several plants are under construction in Mumbai, Shirdi, Ghaziabad and Trivandrum.

The reduction in number of tanks, their sizes, equipment and re-circulation flows would make the granular sequencing batch reactor more attractive over other mainline treatment systems. The reduction in land footprint and costs are huge (70% and 30%, respectively), as compared to plants based on conventional activated sludge process. However, the land footprint and costs are about 20% lower as compared to existing activated sludge-based sequencing batch reactors. Despite these benefits, bio-granules can offer efficient biological treatment along with effective nitrogen and phosphate removals, due to their resilient and robust metabolism.

6. Biofouling phenomenon and its mitigation

Biofouling is the undesirable attachment of microorganisms, algae and small animals on surfaces submerged in water. This is a natural process that occurs on a wide range of structures such as ship hulls, underwater equipment and industrial pipes that are exposed to water (particularly, seawater), leading to significant economic and environmental impacts. This phenomenon occurs due to the natural propensity of organisms to settle and grow on submerged surfaces, facilitated by the presence of nutrients, dissolved oxygen, light (in the case of algae) and suitable substrata.

6.1. Biofouling

The biofouling process begins as soon as a clean surface is exposed to water; the process starts with formation of what is commonly referred to as the "conditioning film", which is the spontaneous deposition of a complex layer of organic molecules naturally present in the water. This formation of an organic *conditioning film* is followed by the attachment - initially reversible and subsequently irreversible - of different types of microorganisms, among which bacteria are the most common. With passage of time, more complex

organisms such as fungi and higher invertebrates are also recruited to the community, making the film very complex in its constitution. The initial biofilm, which largely consists of microorganisms, serves as a foundation for the attachment of larger organisms, such as hydroids, barnacles, mussels, oysters, ascidians, seaweeds etc. It has been shown that there is considerable amount of physical and chemical interaction happening between the primary film and the subsequent settlement and attachment of larval forms of the macrofouling organisms. The macrofouling growth can often be quite massive, reaching a thickness of several inches, which can cause significant damage to the structure and affect its intended function. Even though there are hundreds of types of macrofouling organisms, the most troublesome ones in the marine environment are usually barnacles and mussels - both having tough calcareous shells.

Biofouling is a significant problem in several maritime industries, including the shipping industry, offshore oil industry and shore-based power generation industry. In shipping industry, biofouling increases hydrodynamic drag, leading to decreased fuel efficiency and increased operational costs. Additionally, the colonization of ship hulls by invasive species can facilitate their spread to new ecosystems, causing ecological imbalances and economic harm. In water treatment facilities, biofouling can impair the performance of membranes and filters, reducing the efficiency of water purification processes and necessitating frequent maintenance. In aquaculture, biofouling can compromise water quality and the health of cultivated organisms, leading to reduced yields and economic losses. Electrical power plants located along seacoasts draw massive amounts of seawater for condenser cooling. The seawater intake lines, water distribution pipes and heat exchangers are prone to severe biofouling, unless appropriate control measures are continuously employed. The problems due to biofouling include flow reduction, heat exchanger tube blockage (sometimes as high as 60-70% or more) and fouling-induced corrosion of metals and alloys used in the cooling water systems.

Overall, biofouling is a multifaceted issue that requires a comprehensive approach to address its economic, environmental and ecological implications. Research efforts aimed at development of innovative antifouling technologies are necessary to mitigate the negative impacts of biofouling and to ensure the efficient and sustainable operation of marine and freshwater-based cooling water systems. Understanding the mechanisms driving biofouling is essential for developing effective mitigation strategies. Factors influencing biofouling include surface roughness, material composition, water chemistry and ambient environmental conditions. Microbial communities play a crucial role in biofouling, with bacteria often serving as primary colonizers, followed by the attachment of larger organisms.

As mentioned earlier, there is considerable amount of interaction between larval forms and the pre-existent microbial biofilm (which includes bacteria, fungi and microalgae) on the exposed surface. Understanding the complex interactions between microorganisms and larvae of macrofouling organisms is crucial for developing effective antifouling strategies. Recent research has highlighted that, apart from chemical interactions, surface texture, wettability and topography play important role in biofouling. This has also led to

the possibility that bio-inspired designs could be used as templates for surface modification to deter biofouling.

6.2. Biofouling control

Numerous approaches have been employed to control biofouling, ranging from physical methods such as hull cleaning and surface coatings to chemical treatments and biological control agents. Chemical treatments include the use of injectable biocides as well as use of toxic or non-toxic coatings that prevent the attachment of organisms. Injectable biocides are commonly used in systems where seawater or freshwater is drawn using a pipe or culvert. Antifouling paints or coatings are employed where a fixed surface (such as ship hull or an offshore oil platform) is exposed to seawater. In the cooling water systems of power plants, therefore, injectable biocides are more appropriate. Several biocides such as chlorine, chlorine dioxide, ozone, isothiazolinones, quaternary ammonium compounds and hydrogen peroxide are available, but the most commonly used ones are halogens or their compounds, which are easily available and relatively economical to use. More recently, nanotechnology is also being employed in the development of novel antifouling materials with enhanced durability and efficacy.

As can be seen from the above, biofouling poses significant challenges to various industries, impacting operational efficiency, environmental sustainability and economic viability. Despite ongoing efforts to combat biofouling, its management remains a complex and dynamic challenge. It is imperative that the problem be approached in an interdisciplinary manner, drawing upon expertise from marine biology, microbiology, materials science and engineering. The need for collaborative approaches to address this pervasive issue has been felt by the department and therefore efforts have been going on in this direction. By fostering interdisciplinary research and innovation, inroads have been made to develop effective strategies to mitigate biofouling and to ensure the long-term integrity and performance of submerged structures and systems.

6.3. Biofouling research work done in Water & Steam Chemistry Division

R&D work in the area of biofouling and its control was initiated in the early eighties in the erstwhile Water & Steam Chemistry Laboratory (WSCL) of BARC. The laboratory, located at Kalpakkam near the Madras Atomic Power Station, Tamil Nadu was mandated with the objective of undertaking systematic studies on the marine biofouling related issues encountered in nuclear power plants, especially at Madras Atomic Power Station, Kalpakkam (on the east coast) and at Tarapur Atomic Power Station, Tarapur (on the west coast). In the following decades, this activity was enlarged in its scope and intensity and the scientific effort was extended to cover the entire gamut of operational and environmental issues related to use of natural water bodies as source of water for power plant cooling and receptacle of thermal effluents released from power plants.

The studies have shown the propensity of the Kalpakkam site to support heavy marine biofouling on all types of surfaces. It was also shown that the cooling water system, including the intake tunnel and associated pipelines) form a conducive environment for biofouling organisms such as barnacles and mussels to colonize and thrive. The intensity of fouling in various parts of the cooling water system was assessed either using remotely

operated vehicles, or when opportunity was presented, during the maintenance shutdowns of the plant. As chlorination is primarily used as the fouling control measure, the effect of chlorine as a biocide has been studied using model fouling organisms. Apart from this, the impact of chlorine residuals and chlorination by-products (such as trihalomethanes) in the outgoing water on non-target organisms such as phytoplankton and benthic invertebrates has also been studied in detail.

Apart from power plant biofouling control, research efforts have been expanded with an objective to develop novel methods for biofouling prevention. As part of it, imidazolium ionic liquids were extensively studied for prospective applications in biofilms and biofouling control. It was shown that long alkyl-chain imidazolium ionic liquids at milli- and micro-molar concentrations have significant anti-biofilm activity against phototrophic biofilms, indicating that ionic liquids may find application for biofilm control in recirculating cooling water systems employing cooling towers. In fact, these studies revealed that selected ionic liquids exhibit strong antimicrobial, antifungal, antibiofilm and anti-larval activities suggesting prospective applications. Interestingly, the attachment of barnacle larvae was prevented using non-toxic concentrations of these compounds offering possibilities for designing environment friendly antifouling methods. Recent work has also successfully identified natural and semi-synthetic natural compounds for preparing antimicrobial, antifungal and antibiofilm formulations.

Use of injectable antifouling biocides, when used in once-through cooling water systems, present an environmental concern, because they are released into the receiving water body along with the outgoing water. The issue could be exacerbated by the presence of elevated temperature in the effluents, exposing organisms to combined chemical and thermal stress. In this context, extensive studies have been carried out at Kalpakkam on the impact of thermal effluents released from condensers on the planktonic and benthic communities in the outfall zone. Water & Steam Chemistry Division (WSCD) served as the nodal laboratory to coordinate the research activities carried out under a multi-institutional Thermal Ecology Studies (TES) project piloted by the Board of Research in Nuclear Sciences, DAE.

Considering the potential environmental implications of chemical biocide based antifouling methods, studies were initiated to control settlement and growth of marine organisms on surfaces with the help of surface modification and nanotechnology. Synthetic hybrid nanocomposites have been developed and successfully tested at laboratory and limited field scale, which showed the inherent antifouling properties of such materials. Chemically mediated surface immobilization of a polysaccharide degrading enzyme led to successful prevention of microbial fouling on ultrafiltration membranes. Such findings have practical applications for water purification and wastewater treatment. Apart from this, investigations showed the intricate interactions between bacterial biofilms (the pioneer colonizers on surfaces immersed in water) and the larval stages of fouling invertebrates. Better insights into the bacterial-larval interactions may, hopefully, lead to development of non-toxic methods that deter marine biofouling. Apart from biofouling, biocorrosion (more accurately described as

microbiologically influence corrosion (MIC)), has also been a subject of research in cooling water systems. Studies on MIC in freshwater cooling systems have indicated the significant role played by different types of bacteria such as iron oxidizing bacteria, nitrate reducing bacteria and sulphate reducing bacteria.

7. Future prospects

An overview of the up-to-date activities of bacterial uranium detoxification mechanisms highlighting examples of uranium bio-precipitation and sequestration in native and recombinant bacterial strains has been provided here. While extensive work has been done, the molecular mechanistic insights behind uranium resistance conferred upon microbes needs to be further explored. The fundamental understanding of such mechanistic aspects could envisage to field scale uranium bioremediation application. Similar attempts are being undertaken for unveiling the microbial interactions with other heavy metals vital for bioremediation. The current and future R&D on biofouling is directed towards understanding biofilm biology, interactions and development of novel biofouling control methods, prospective research on anti-larval and antibiofilm compounds and their impact on cooling water treatment and thermal ecology studies. The knowledge accrued on biofilm biology and biofilm control would be utilized for development and deployment of innovative biotechnologies for applications in healthcare, water and wastewater treatment. The ongoing R&D on microbial biofilms and bio-granules is aimed towards developing high-impact technologies for bioremediation and wastewater treatment.

8. Acknowledgements

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CHEMISTRY AND BIOLOGY OF NATURAL PRODUCTS AND THEIR APPLICATIONS FOR HEALTH BENEFITS

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Abstract

The Bio-Organic Division traditionally possesses a rich expertise in organic chemistry that can further be classified into two branches namely natural product chemistry and organic synthesis. Difficult to isolate, low abundant molecules present among a pool of very similar compounds makes the task of isolation of a single compound from natural resources challenging and exciting. Natural product isolation from important plants was done based on their use in Ayurveda or other forms of alternate medicine for different ailments. Biological activity based isolation was also carried out to identify and isolate the active component from a myriad of different molecules. The expertise in synthetic chemistry was exploited to successfully design reactions to synthesise important molecules that were present in minute quantities in natural sources making the isolation process unviable. Combinatorial chemistry approach paved the way for structure function analysis later. This chapter gives the reader the journey/evolution of the biological research over 2 decades that was carried out exploiting the expertise in chemistry and explains chronologically how the two remain intertwined till this date. Thus this chapter explains the contribution of research focussed on different ailments like oxidative stress in chronic

diseases, radiation injury, gastric ulcer, cancer, cardiovascular disease, antibiotic resistance and targeted therapy.

1. Preamble

Modest beginnings of research cooperation between chemistry and biology for health benefits started post 1995 in our Division. The research on natural products to harness their health benefits picked up pace in 1997. Our group was known for its prowess in natural product chemistry and synthetic organic chemistry. The very modest infrastructure i.e. a UV-Vis spectrophotometer coupled with a few reagents and test tubes gave birth to a research programme focussing on translating the expertise in chemistry to biological research focussed on human health related research. Initial period of research i.e. during the period 1997 to 2005 predominantly involved *in vitro* assays assessing the free radical scavenging property/antioxidant property of molecules of diverse nature derived from natural sources mainly botanical in nature. The antioxidant nature of the molecules/plant derived concoctions was investigated with analysis of an extension towards their ability to protect biological macromolecules against gamma radiation or Fenton reaction induced damage. During this period, *in vitro* free radical scavenging assays, assays to evaluate damage to macromolecules like lipid and DNA and kinetic measurements using pulse radiolysis dominated the research methods. Extensive research on Fenton reaction, in extension with the biochemistry of iron and gamma radiation to analyse the ability of different natural products to protect biological targets resulted in deciphering the mechanisms of sacrificial antioxidants or regenerative antioxidants. Some of the natural products that were evaluated for antioxidant and radioprotective properties were *Myristica malabarica*, *Swertia decussata*, *Ginger officinalis*, *Piper betel* etc. Activity based isolation of bioactive molecules from different medicinal plants was carried out in the period between 1997 to 2003.

2. Mechanistic evaluation of the redox properties of natural products

2.1. Folic acid

In regard to studies of antioxidant molecules and their biological relevance, the ability of the physiologically important molecule folic acid to scavenge different free radicals was reported for the first time. Folic acid was seen to scavenge different radicals very efficiently. In the reaction of thiyl radicals with folic acid, it was observed that folic acid not only scavenged thiyl radicals but also repaired thiols at physiological pH. In the lipid peroxidation study, in spite of the fact that folic acid is considerably hydrophilic, it was observed to significantly inhibit microsomal lipid peroxidation. A suitable mechanism for oxidation of folic acid and repair of thiyl radicals by folic acid was proposed after extensive research. Further, due to the interest in the Fenton chemistry, the interaction of folic acid with iron and hydroxyl radicals generated by Fenton reaction was studied. A detailed analysis revealed the participation of freely diffusible hydroxyl radicals in the oxidative degradation of secondary amines including folic acid. Based on direct

evidences employing kinetic measurement techniques, the involvement of a hydrogen abstraction mechanism in the reaction was shown unambiguously. Given the high cellular concentrations of free iron and H_2O_2, it was postulated that the toxic hydroxyl radicals may play a major role in oxidative degradation of folic acid in the cellular systems. Given the multitude of important biochemical reactions in which folic acid is involved in the body, the knowledge emanating from this study was pivotal to evolve strategies to overcome depletion of folic acid and supplementation of it through external means.

2.2. Diketones

In pursuing the interest in the health benefits of bioactive molecules from natural resources, four ginger (*Ginger officinalis*) derived diketones and the popular molecule curcumin from turmeric (*Curcuma longa*) were investigated for their ability to interact with different biologically relevant radicals and protection of macromolecular targets inside the cells. The study revealed the role of additional phenoxy hydroxyl group in curcumin *vis á vis* the diketones from ginger, a reason to exhibit higher activity in protecting against iron mediated lipid damage. The observation that one of the ginger derived diketone, dehydrogingerdione possessed comparable activity to curcumin in iron independent lipid peroxidation assay, lead to the discovery that it had higher affinity to lipid peroxide radical, and possessed superior antioxidant activity compared to physiological antioxidants like vitamins E and C. A synergistic behaviour between the dehydrogingerdione and vitamic C was established, through chemical repair of dehydrogingerdione by vitamin C. Additionally, the study also revealed the important contribution of phenolic and the methylene group in 1,3 diketones in their antioxidant activity.

2.3. Polysaccharides

Diverging from phenolic molecules, research focussing on complex polysaccharides and their ability to protect molecular targets against free radical induced damage was undertaken. An arabinogalactan from *Tinospora cordifolia*, a plant credited with multitude of health benefits was evaluated for its ability to scavenge different radicals and protect lipids and proteins against free radical induced damage. Further, this polysaccharide was shown to protect DNA against gamma radiation induced damage. In order to decipher that it is not a general property of all polysaccharides to exhibit such a protective ability, starch and guar gum were employed to show that the antioxidant and radioprotective properties are unique only to certain polysaccharides. To decipher the contribution of individual monomeric sugars and the type of branching patterns affecting the antioxidative and radioprotective properties in polysaccharides, different polysaccharides were investigated employing radiation protection and antioxidant assays. Employing three different polysaccharides, from three different medicinal plants, our study revealed arabinose, xylose and rhamnose to be major contributors to the antioxidant activity. Interestingly, galactose and mannose did not have any role in the antioxidant activity. The behaviour of one polysaccharide OSP from the medical plant Tulsi (*Ocimum sanctum*) was investigated elaborately due to its differential ability to protect macromolecular targets against iron induced damage vis á vis gamma radiation

induced damage. Our study revealed OSP prevented the deleterious effects of iron by binding to ferric and ferrous ions and rendering them inactive to redox reactions generating free radicals. Later the *in vitro* findings were validated at the cellular level employing mouse fibroblasts cells. The iron induced cell death in fibroblasts was effectively prevented by OSP. During this period multiple medicinal plants were investigated for their biological relevance employing different *in vitro* assays, leading to activity based isolation of individual molecules from them.

2.4. Stilbenes

Stilbenes are a class of phytochemicals that were investigated for different biological activities worldwide. The most important breakthrough discovery in this class of molecules came in 1997, when resveratrol (the well-studied stilbene) was reported for its cancer prevention properties. Due to the low abundance of resveratrol in natural sources like skin of red grapes, peanuts and others, the organic chemists in our group devised novel methods to synthesise stilbenes, notable among which is the Low valent titanium mediated McMurry coupling. A structure activity relationship study on the antioxidant activity of the stilbenes was taken up and in the course of this investigation stilbenes were found to act as DNA damaging agents in presence of metal cations. Mechanistically, this study identified the structural elements required for the DNA cleavage activity, metal ion specificity, and the free radicals involved in the process and generation of double strand breaks in DNA by the stilbenes.

2.5. Modulation of iron by natural products

Fenton reaction is extensively used in free radical research and a variety of modified Fenton reactions gives clue regarding the mechanistic aspects of the molecule under investigation. Extensive analysis of modified Fenton reactions based assays was carried out to investigate the iron modulatory properties of different molecules. Different berberine class of molecules were investigated thoroughly to dissect their ability to prevent free radical induced damage not only due to their ability to scavenge the radicals, but also due to their ability to complex iron, interfere with the redox cycling of iron, thus preventing its ability to induce damaging free radicals. Similarly, a polysaccharide (OSP) from the medicinal plant *Osmium sanctum* was shown to scavenge free radicals generated by Fenton reaction. However, its extraordinary ability to prevent Fenton induced damage could not be explained by free radical scavenging alone. Detailed investigation revealed OSP complexed both ferrous and ferric forms of iron, reduced ferric to ferrous, did not allow ferrous to generate free radicals. Later this interesting property of OSP was extended to cellular level investigations by successfully preventing iron induced fatality to mouse fibroblast cells.

3. Establishment of animal cell culture and a step forward towards cell biology research

In the period starting 2005, the infrastructure to conduct biological research improved with establishment of a cell culture laboratory and obtaining a set of cancer cell lines

from National Cell Culture repository of National Centre for Cell Science, Pune. Further enhancement in capacity occurred when our group procured flow cytometer in 2010 and confocal microscope in 2014. Concomitant to the discovery of pro-oxidant activity of phenolic compounds during the course of investigation on their antioxidant/radioprotective property provided a genesis towards exploitation of such a property towards killing cancer cells. Once the free radical chemistry behind the action of different molecules was evaluated, with the improvement in the facilities to biological research, the findings were extended to biological systems especially cancer cells in culture. The sensitization of cancer cells by natural products, induction of cell death in cancer cells and the mechanisms underlying such process took a foot hold post 2005. In this period detailed analysis of the free radical chemistry behind berberine, bakuchiol, coralyne etc. was also taken up. It was established that coralyne, synthetic congener of the natural protoberberine alkaloid berberine, possesses DNA photonicking property. Extending the findings, biological studies revealed that compared to coralyne alone, coralyne and UV-A (termed CUVA) efficiently killed cancer cells irrespective of the p53 status of the target cells. Further studies also revealed, in association with UV-A, coralyne, but not related molecules berberine and jatrorrhizine induced significant nicking of plasmid DNA *via* an Oxygen-independent photo-chemical processes. The DNA photo-nicking by the combination of CUVA was primarily caused by the coralyne aggregates without any significant contribution from the DNA-intercalated coralyne monomer. The DNA damaging property of stilbenes which was established *in vitro* was extended to cell culture studies to evaluate their anti-cancer property and the role of DNA damage.

4. In vivo studies to cure ulcer through natural products

Parallel to the research on the free radicals, our work also indulged in evaluating the health benefits of natural products in terms of protecting against NSAID induced adverse effects especially the gastric ulcer. Under the group of such investigations, the following class of molecules were evaluated namely stilbenes, malabaricones, catechins etc. employing mouse models of research. Expertise to handle small rodents like mice and rats in the animal house facility was developed. Withdrawal of blood, excision of tissues from different organs for histopathological and immunological analysis to understand the progress of disease/prognosis after treatment in animal models was a remarkable path forward in terms of capacity building. Notable achievements in this period are healing of indomethacin induced stomach ulcers by epigallocatechin gallate and establishment of the role of COX-independent pathways in this process. On the same lines investigations on the ulcer healing properties of black tea, theaflavins, stilbenes and *Piper betel* derived allylpyrocatechol were carried out and published.

As part of evaluating the healing properties of natural products, we developed an incisional wound model in rats to the evaluate wound healing capability *Piper betel* extract. We prepared various formulations of Piper betel extract (in paraffin oil, petroleum jelly and hydroxyethyl cellulose) and evaluated their ability to heal excisional

wounds in rats. We found that a formulation containing 5 % crude Piper betel extract in a 2 % hydroxyethyl cellulose base gave significantly superior wound healing activity compared to soframycin. In order to determine the principal active component responsible for wound healing, we prepared crude Piper betel extracts depleted of 4-allyl-pyrocatechol (APC, the principal active component of Piper betel extracts as reported previously by our laboratory and others). The depleted extract showed no significant wound healing activity, thus establishing APC as the principal component responsible for wound healing activity of Piper betel extract.

5. Role of organic chemistry and its contributions to biological research

Extensive contributions by the natural product chemists who isolated active molecules from bulk quantities of plants by solvent extraction formed the backbone of the natural products research. Considering the low natural abundance of different bioactive molecules identified by the research carried out with the focus on health benefits, expertise in synthetic organic chemistry was brought to the fore for the efficient synthesis of selected molecules for further evaluation. The cost of isolation of these natural molecules from their parent sources is cumbersome, environmentally unfriendly and are not cost effective. Further due to the similar nature of the molecules, it was technically challenging to isolate them from natural resources where they are present as a cocktail. Hence it was prudent to device methods using synthetic organic chemistry to obtain them in larger quantities from simple precursors using clever chemistry.

5.1. Synthesis of malabaricones:

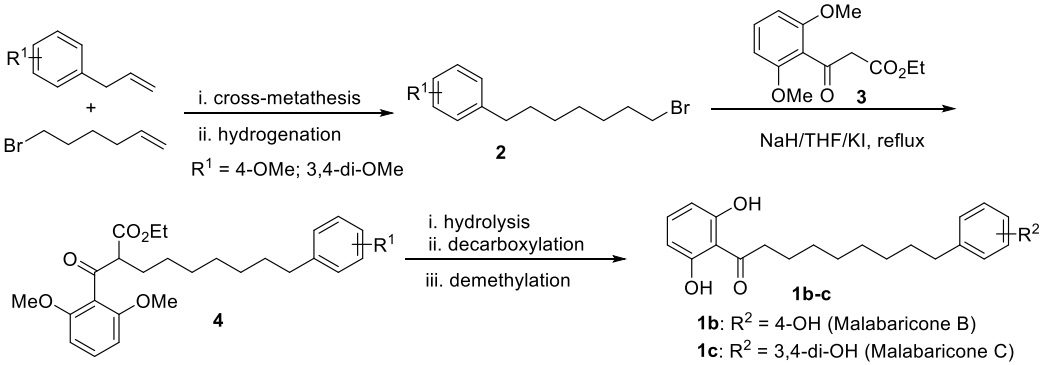


Fig. 1: Complete synthesis of low abundance natural products malabaricone B and malabaricone C via cross-metathesis strategy (Journal of Natural Products 2017, 80, 1776)

To this end, a novel, efficient and practical route for synthesis of malabaricones was developed. Olefin cross-metathesis strategy was successfully utilized as the key steps for total synthesis of malabaricone B and C. The ω-arylheptylbromide, which is one of the key building blocks of the synthetic protocol was achieved via successful cross-metathesis reaction of allylarenes and 6-bromo-1-hexene followed by chemoselective

hydrogenation of double bond in presence of bromide. 2,6-dimethoxyphenyl-β-keto ester was then subjected to successful C-alkylation reaction with ω-arylheptylbromide, in presence of sodium hydride as base and potassium iodide (KI) as additive to afford the corresponding β-keto ester as the product. Malabaricone B and malabaricone C were then obtained by subsequent alkaline hydrolysis, *in-situ* decarboxylation and demethylation. The bio-activities of the synthesized compounds were found to be similar with the compounds isolated from natural product. This synthetic protocol was employed towards total synthesis of all other member of the malabaricone family including malabaricone A and malabaricone D. The methodology developed has various advantages, such as higher yield of product, minimum number of steps and simpler reaction conditions.

5.2. Synthesis of stilbenes

Similar to malabaricones, hydroxystilbenes also occur naturally in several plants in very low amounts. Detailed biological evaluation of promising hydroxystilbenes as well as structure function studies of such molecules was hampered due to the challenges in isolating them as mentioned above. To meet the need of various hydroxystilbene derivatives towards different biological applications, an easy and convenient protocol for synthesis of this important class of molecules was established. Using Low valent titanium (LVT) reagents to mediate reductive coupling of two carbonyl compounds was supposed to be the most convenient way to achieve this important class of molecules. However, when suitably substituted phenolic aldehydes were reacted with LVT reagent [TiCl3-Zn-THF] desired hydroxystilbenes were formed along with corresponding dihydro-compounds, by in-situ reduction of the stilbenic double bond. In contrast, it was shown that when phenolic ketones were used as substrates, stilbene compounds were formed as the sole product. This work established that phenolic aldehydes are not suitable starting materials to obtain hydroxystilbenes by LVT mediated reactions. Since, use of free hydroxyl-group promoted in-situ-hydrogenation of the product methoxy-substituted benzaldehydes were used as the starting materials in this LVT method to obtain corresponding methoxy-stilbenes, which were subsequently de-methylated to their hydroxy-counterparts by using BBr3. Thus, our research proved that LVT promoted method could give easy access to biologically important polyphenolic stilbenes in two steps.

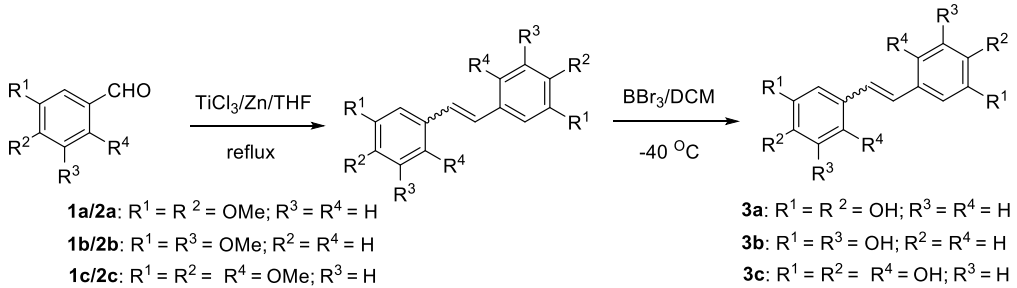
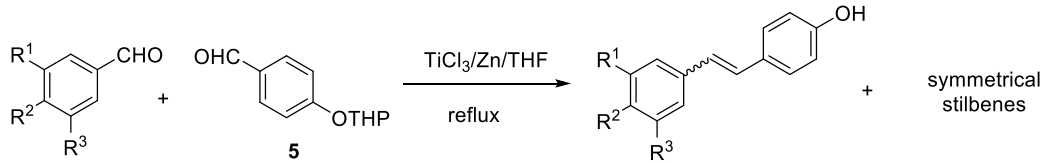


Fig. 2: Synthesis of hydroxystilbenes from methoxy-substituted benzaldehydes employing low valent titanium method (Indian Journal of Chemistry B 2004, 30, 1934)

Along with polyhydroxy stilbenes, many partially methylated hydroxystilbenes also possess important pharmacological activities but it was realized that above method was unsuitable for synthesis of this class of molecules. Due to the interest in LVT reagents, several modifications of this reagent were formulated by our research group to perform different organic transformations which were earlier not reported. Utilizing this understanding, an alternative synthetic route to access partially methylated hydroxystilbenes by using tetrahydropyranyl (THP) protected phenolic benzaldehydes was successfully developed. Here, methoxy substituted phenolic benzaldehydes/phenolic benzaldehydes were initially pyranylated, followed by LVT mediated reductive coupling in presence of [TiCl3-Zn-THF]. During this process, in-situ removal of the THP group occurred while the methoxy group remained intact to provide the corresponding partially methylated hydroxystilbenes. Thus, a library of different stilbene molecules was synthesised successfully to purity. These molecules were evaluated for different biological activities and structure-function relationship studies.



4a: $R^1 = R^3 = OMe$; $R^2 = H$

4b: $R^1 = OMe$; $R^2 = OTHP$; $R^3 = H$

6a: $R^1 = R^3 = OMe$; $R^2 = H$

6b: $R^1 = OMe$; $R^2 = OH$; $R^3 = H$

Fig. 3. Synthesis of partially methylated hydroxystilbenes employing low valent titanium method. (Indian Journal of Chemistry B 2004, 30, 1934)

5.3. Synthesis of conjugated natural molecules for targeting cellular organelles in cancer

Apart from the above-mentioned success stories where organic chemistry and cell biology held hands together to further the quality of research, recent demands to test novel ideas provided a platform for collaboration. Intense research in biology employing cancer cells revealed the molecular targets of selected natural products/synthetic congeners. We hypothesised that targeting these molecules to specific compartment of a cancer cells would increase the efficacy by several folds. The challenge provided to the organic chemists was to combine two molecules into one i.e. molecule A (targeting molecule) that carries molecule B (the drug) to a specific organelle inside the cancer cell so that efficient killing of the cancer cell occurs. In this regard, we synthesised mitochondria targeting stilbenes and malabaricones, which showed 10-20 fold higher efficacy in killing cancer cells in various preclinical models. For the first time, a hypothesis driven design and synthesis of lysosome targeting stilbenes and endoplasmic targeting BOIDPYs was carried out, that is specifically effective against different forms of pancreatic cancer. It may be noted that pancreatic cancers are very difficult to treat and exhibit a high rate of fatality. The continuous effort in this direction has allowed us to

establish the molecular basis of sensitization of pancreatic and other cancers through organelle targeting.

The molecules thus synthesised and characterized as pure were employed in structure-function studies, pharmacological profiling, animal studies and decrease the cost of obtaining them in enantiomeric purity. The valuable contributions from collaborating partners i.e. organic chemists and cell biologists thus resulted in testing new hypotheses that led to good quality research.

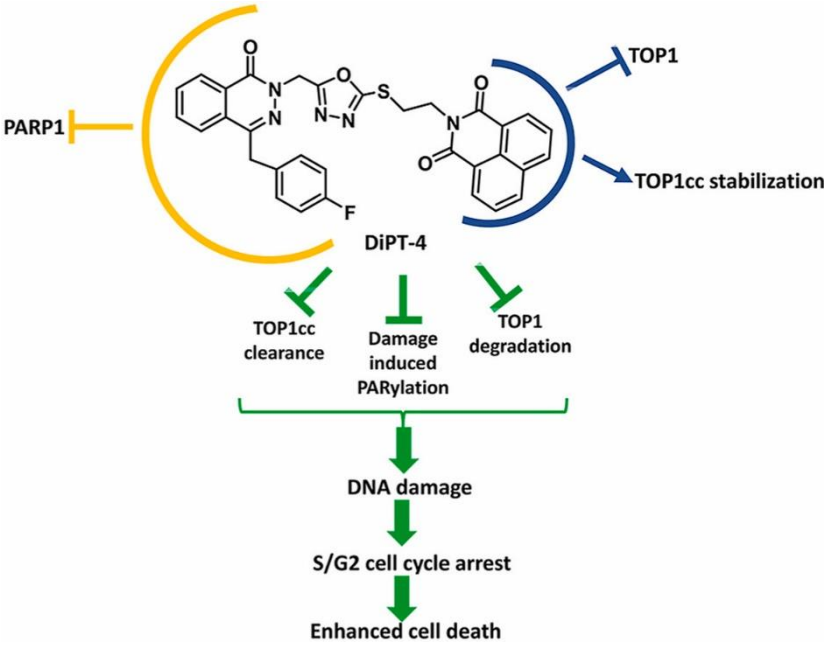


Fig. 4: Design, synthesis and development of a dual inhibitor of Topoisomerase 1 and poly (ADP-ribose) polymerase 1 [PARP1] for efficient killing of cancer cells (European Journal of Medicinal Chemistry 2023, 258, 115598)

6. Combating antibiotic resistant bacteria employing natural products

Considering the potential of phenolic compounds to act as helper compounds or as standalone drugs to counter antibiotic resistant bacteria, resveratrol was established as an antibacterial molecule. Contrary to the popular belief in the existing literature, research from our group found no role of diffusible reactive oxygen species in the antibacterial activity of resveratrol. Further, we established DNA damage is a late event in the bacterial cell death induced by resveratrol. A comprehensive structure function correlation encompassing a multitude of parameters established important structural elements that are mandatory for a successful antibacterial stilbene. Dimer stilbene (DS) was found to be a superior antibacterial molecule than resveratrol in this study. Further studies on the antibacterial potential of DS revealed that it synergizes with antibiotics that target protein synthesis. Detailed analysis employing different stilbenes revealed this

property of synergizing with antibiotics targeting protein synthesis is not unique to DS alone, but is a common property among antibacterial stilbenes. The proof of concept experiment was also demonstrated in an animal infection model employing Swiss mice. In a different study on this topic, hydroxychavicol derived from *Piper betel* was shown to damage Fe-S proteins, engage in redox cycling of oxygen radicals, damage bacterial membrane and DNA leading to cell death in bacterial cells. A time kinetics revealed hydroxychavicol induces oxidative stress, membrane damage and DNA damage in that order. Finally, the membrane damage induced by hydroxychavicol results in entry of hydrophobic antibiotics into difficult to treat gram negative bacteria, augmenting our arsenal against antibiotic resistant bacteria.

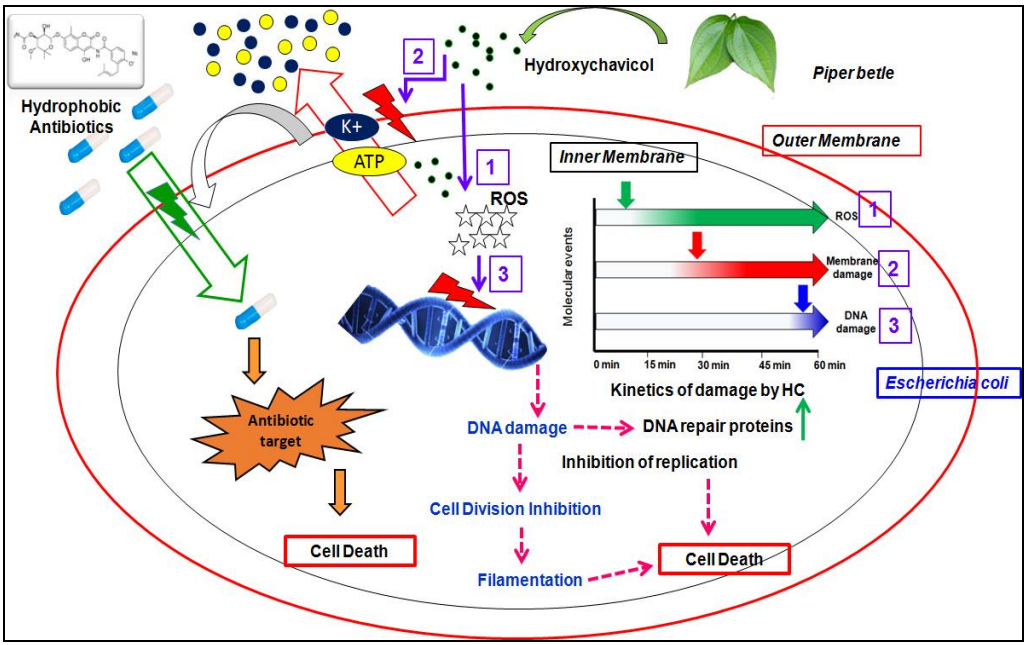


Fig. 5: Hydroxychavicol derived from *Piper betel* leaves induces ROS mediated macromolecular damage in bacterial cells and aids entry of hydrophobic antibiotics into bacterial cells leading to killing of antibiotic resistant bacteria (Biochimie 2021, 180, 158)

7. Improving cardiovascular health through phytochemicals

Cardiovascular disease (CVD) is a disease of heart, kidney, brain and blood vessels, accounting for 1.5 million deaths annually in India. Chronic hypertension (HT) is the insidious culprit and a major risk factors of CVD prevalence. Uncontrolled HT leads to a pathological process to the heart and vasculature, known as cardiovascular remodelling that involves change in the size, shape and function of the heart and vessels. We evaluated the ability of the natural molecule allylpyrocatechol (APC) isolated from Piper betel to remodulate cardiovascular properties in a volume overload, high salt model of hypertension in rats. APC (10 mg/kg b wt) significantly attenuates hypertension in rats. It

corrected the atrial electrical conduction irregularities as well improving the ventricular contractility and pumping efficiency. It reduced organ hypertrophy and fibrosis improving the cardiac and renal functions. In addition, it improved the smooth muscle functions of aorta thus improving its vasoreactivity in response to increased haemodynamic load.

In another study, malabaricone C, another natural product exhibited excellent antihypertensive and antihypertrophic activity superior to curcumin. In addition, Mal C improved the vascular flow and vascular reactivity by protecting the endothelial layer of the blood vessel reducing its oxidative damage. It also reduced cardiac, adrenal and renal hypertrophy indicating better organ functions. It also helped in depolarization potential of cardiomyocytes and papillary muscles indicating significantly better electric profile, contractility, valve function and pumping function. It reduced potent vasoconstrictors like endothelin and anti-diuretic hormone like vasopressin leading to reduced haemodynamic load. It brought down the organ and blood oxidative stress levels. Thus, our studies in animal models proved the potential benefits of natural products mentioned above in improving heart health.

8. Upsetting the redox balance in cancer to induce cell death

Recognizing the importance of the skewed redox balance in cancer cells compared to non-cancerous cells, we hypothesised that any molecule that could disturb the antioxidant- prooxidant balance in cancer cells would potentially be cytotoxic to them. Considering the ability of some natural products from botanical sources like malabaricones to modulate the redox balance inside cells, it was postulated that these molecules could be cytotoxic to cancer cells while sparing the normal cells. Malabaricone C (mal C), a promising molecule from the group of malabaricones isolated from the plant *Myristica malabarica* was capable of inducing oxidative DNA damage leading to cell death in different cancer cell lines. Mal C induced single strand breaks as well as the lethal double strand breaks in the DNA followed by p38-MAPK activation, imbalance in BAX/BCL2 ratio leading to mitochondrial dysfunction in lung cancer cells. Intriguingly, in the breast cancer cells treated with mal C intracellular Ca^{2+} release, calpain activation, lysosomal membrane permeabilization (LMP) were found to be the critical events leading to cell death. In another interesting piece of work, mal C treated cancer cells exhibited higher amount of intracellular reactive oxygen species (ROS) while addition of thiol antioxidants sensitized the cancer cells to death instead of protecting them. This apparent anomaly was solved by proving that thiol antioxidants recycled mal C from oxidized to reduced state and generated more and more site specific ROS in the process. Additionally, the thiol antioxidants also lead to S-glutathionylation of key transcription factors (p53 and p65) leading to abrogation of their protective role against ROS induced cell death. We also showed that the sister molecule malabaricone B (mal B) induced cell death in cancer cells of different tissue origins, independent of the p53 status in them. It is also important to note that mal C and mal B were nontoxic to normal cells at the concentrations that were cytotoxic to cancer cells. Based on the

promising results on the work on malabaricones, recently mal C was tagged to triphenyl phosphine to target it to mitochondria. Targeting promising anticancer molecules to specific organelles inside cancer cells is expected to increase the efficacy of these molecules.

Investigation of the redox active hydroxychavicol isolated from *Piper betel* against pancreatic cancer revealed, this not only induces extensive DNA damage but also forces the cells into mitotic catastrophe. The cell death by hydroxychavicol was induced by JNK pathway-dependent, caspase-mediated apoptosis. Hydroxychavicol also inhibits migration and invasion of pancreatic cancer cells via a generalized repression of genes involved in endothelial mesenchymal transition.

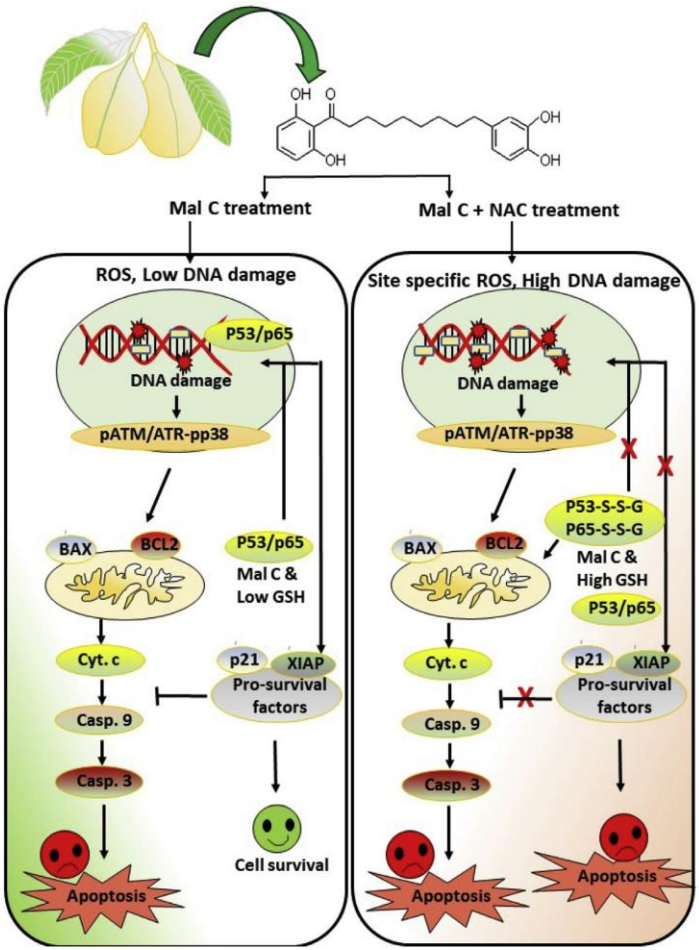


Fig. 6: Natural product malabaricone C reprogrammes redox sensitive proteins p53 and NF-κB in response to thiol antioxidants and induces cancer cell death in vitro and in vivo (Free Radical Biology and Medicine 2020, 148,182)

9. Understanding the fundamental processes in cancer cells and exploiting its Achilles heel

Cancer cells fundamentally differ from a normal cell in many ways. Key differences that could determine the fate of the cancer cell's survival include rapid replication of the genome, cell cycle check points, repairing the damages to the DNA etc. Due to mutations in some of the pathways, the cancer cells are more reliant on/addicted to alternate pathways for their survival. We realised that it is important to unravel such addictions of the cancer cell and induce damages that is repaired by the pathway carrying the mutation. At the same time if the alternate pathway is also blocked by an inhibitor the damage cannot be repaired and results in cell death. This process is called "synthetic lethality". Diverse examples of this concept exist that have the potential to keep different cancers at bay substantiated by a few examples below.

In a study to overcome the limited use of PARP inhibitors in cancer therapy due to their inability to work well in homologous recombination (HR) proficient cancers, we postulated the use of stilbene resveratrol as a chemosensitizer. The resistance to PARP inhibitor in cancers is, at least in part, due to activation of autophagy. Considering resveratrol is a modulator of autophagy, we evaluated the mechanisms behind the ability of resveratrol to enhance the efficacy of PARP inhibitors. Our work established resveratrol induced dysregulation of cell cycle and enhanced PARP inhibitor talazoparib -induced double strand breaks (DSBs), leading to mitotic catastrophe. We also found that resveratrol attenuated fusion of autophagosome and lysosome though induction of lysosomal-membrane-permeabilization (LMP) preventing autophagy and overcoming resistance to PARP inhibitors. Our investigation on the efficacy of different stilbenes *vis-a-vis* resveratrol as potential anti-cancer agents resulted in the identification of dihydroxystilbene (DHS) as a more potent anti-cancer agent than resveratrol. This was established using neuroblastoma tumor model and a melanoma model in mice.

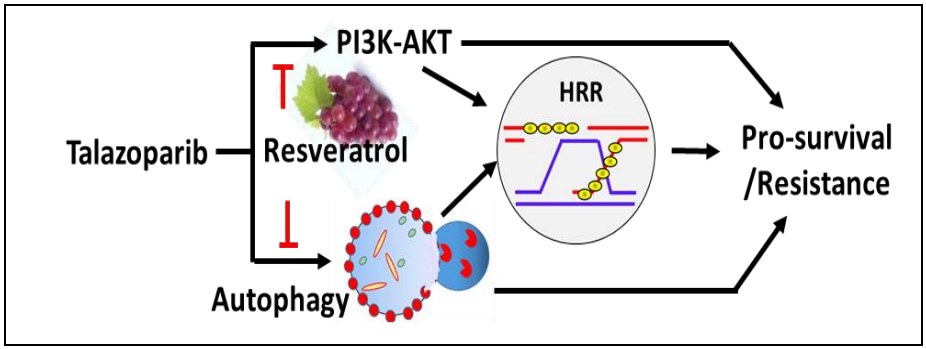


Fig. 7: Natural product resveratrol overcomes resistance to anticancer drug talazoparib by inhibiting participation of talazoparib induced autophagy and PI3K-AKT in homologous recombination repair pathway (Biochemical Pharmacology 2022, 199, 115024)

Replication stress is a phenomenon defined as the state of cells when the entire genome is unable to be replicated and as a consequence the progress of the cells through S phase is impaired. It has been reported previously that stilbenes like resveratrol cause replication stress. We observed PARP inhibitors and stilbenes (for e.g. DHS) induce replication stress individually in cancer cells. However, when combined, these two (talazoparib and DHS) molecules acted synergistically where in the cancer cells exhibited significantly higher DSBs, incurred through extensive damage at replication forks. The cells treated with the combination spent protracted time in S phase and are unable to overcome the replication stress, triggering replication catastrophe and ultimately cell death. The proof of this concept was also successfully demonstrated in ovarian cancer model in SCID mice.

Post 2020, our group has devoted its resources completely on research on cancer especially in its response to radiation, radiopharmaceuticals, chemotherapeutics to develop targeted therapeutics and precision medicines. Mechanistically, research is heavily focussed on understanding the differences that exist in fundamental molecular processes like DNA damage repair pathways, replication stress, autophagy, mitochondria-lysosome homeostasis, cell cycle defects between a cancer cell and normal cell. The thorough understanding emanating from such studies help us employ "targeted-natural products" that interfere with such processes and kill cancer cells. Successful molecules/combinations thus identified are tested on appropriate preclinical models to establish the efficacy of the treatment.

10. Conclusion

As explained above, the research in our group was predominantly Chemistry and Biology of free radicals prior to 2000. In the next two decades, our objectives to exploit phytochemistry to augment health benefits in terms of disease cure made progress in leaps and bounds in addressing diverse diseases like cancer, cardiovascular disease, gastro intestinal ulcer, antibiotic resistant bacterial infections to mention a few. Remarkable feats were achieved in terms of understanding diseases and identification of potential drugs to cure them. True to the spirit of scientific pursuit, the outcomes of the research are credited to the team work especially the diverse expertise brought to the table by "organic chemists" and "biologists" – by "Bio-Organic Researchers". The scientific programme that has undergone the evolution as explained above continues to work on human health related aspects, augmenting the research with understanding fundamental processes inside a cell. This basic science driven research forms a main pillar towards developing strategies/drugs to combat different diseases.

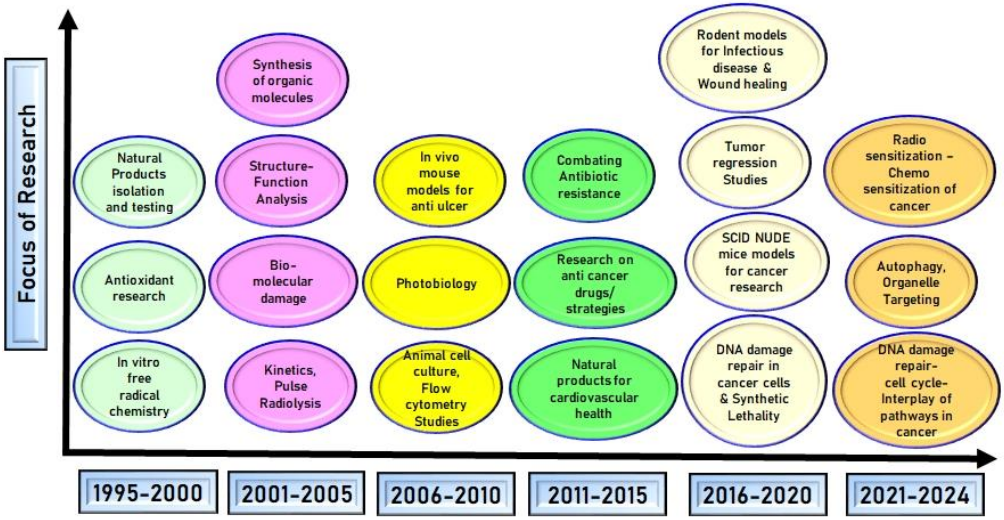


Fig. 8: The journey depicting evolution of scientific work between 1995 to 2024

11. Acknowledgements

The authors wish to acknowledge the contributions of the scientists to the above mentioned research programmes, whose unwavering enthusiasm, hard work, foresight, creativity, perseverance and leadership resulted in successful achievement of the objectives.

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ORGANIC SYNTHESIS FOR HEALTHCARE AND SOCIETAL BENEFITS

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Abstract

Since decades, Bio-Science group in BARC has been instrumental in contributing to the healthcare department and societal benefits via various routes. In this aspect, synthetic organic chemistry has immensely contributed towards the development of diverse bioactive molecules via environment friendly green routes, and their applications in the fields of agriculture, bioremediation, catalysis, radiopharmaceutical ligands, targeted chemotherapeutics and advanced drug intermediates. These research activities have led to different in-house developed products which have been used very regularly in agricultural and clinical practices. This book chapter provides glimpses of the activities in the field of synthetic organic chemistry over the last few decades and acknowledges the efforts and hard work of all the members of the group who has worked with sincerity and dedication towards achieving their goals related to DAE activities, societal benefits, national interests, novel science and modern technologies.

1. Introduction

Since its inception, synthetic organic chemistry in Bio-Science Group, BARC has played an indispensable part in DAE (Department of Atomic Energy) activities through its contribution in high quality basic research and development of organic and bio-materials. Organic multistep syntheses have been instrumental in development of novel strategies to synthesize highly modular synthetic building blocks which are applied in various research projects, from macromolecular and materials science to chemical biology. The

division's research group has pioneered and mastered the multistep organic syntheses in BARC through its highly motivated and well-organized research projects, many of which have found applications towards healthcare and societal benefits.

Over the years, the mandate of the research activities in the division has been motivated by the contemporary departmental and societal needs. During the 70´s, the research focus was mainly directed towards better crop protection strategies and plant biotechnology, leading to the development of several plant-based formulations/compounds for the purpose, with some spin-offs like anti-hepatitic herbal preparations, anti-cancer agents etc. However, with the growing societal need of insect pheromones in the next decade, the research focus was diverted to the syntheses of insect pheromones. In India, our division was one of the first few laboratories to initiate enantiomeric synthesis (1984). This paradigm resulted in major and pioneering contributions from the division in developing short and economical routes for chiral drugs and other bioactive molecules. Several synthetic strategies, including bio-catalysis, organo-catalysis, transition-metal catalysis, solid phase synthesis etc. were developed for this purpose. These strategies were used to synthesize several multi-functional intermediates with multiple stereocenters, and a diverse array of molecules such as macrolides, lactones, spiroketals, fused furanomacrolides, iminosugars, hydroxystilbenes etc. The expertise in organic multistep synthesis was further extended to the in-house synthesis of radiopharmaceutical ligands/carriers, which, in turn, led to the in-house development of several clinically used diagnostic and therapeutic nuclear medicines.

2. Asymmetric Synthesis

The undeniable role of stereocenters in determining the bioactivities of molecules is well documented and warrants the search for new synthetic tools to instil chirality in a molecule. One of the biggest challenges in the asymmetric syntheses of complex organic molecules lies in the design and development of short, simple and flexible strategies. Hence, fundamental and original contributions have been invoked in chemical asymmetric syntheses, by designing several new chiral catalysts as well as synthetic strategies for enantioselective reactions.

To this end, reusable natural sources like terpenoids, steroids, and sugar derivatives (especially (*R*)-cyclohexylideneglyceraldehyde from mannitol) have been used both as chiral pool materials and templates for the enantiomeric syntheses of multitude of target molecules as well as designing new asymmetric synthetic reactions. Formulaion of (i) asymmetric dihydroxylation (ADH) of homoallylic alcohols without the need of expensive ADH reagents; (ii) tuning of diastereoselectivity of Barbier-type reactions by suitable choice of metal-solvent combinations were among some notable achievements in chiral template driven enantioselective reactions. Optimization of chiral template driven enantioselective reactions viz. (i) asymmetric dihydroxylation (ADH) of homoallylic alcohols without the need of expensive ADH reagents; (ii) tuning of diastereoselectivity of Barbier-type reactions by suitable choice of metal-solvent combinations were among some notable achievements. Using either Barbier or Reformatsky or Grignard protocol,

diastereoslective addition of alkyl- and allylic organometallic reagents, in particular, to enantio-pure (*R*)-cyclohexylideneglyceraldehyde was optimised with respect to the metals, solvents, reagents, and additives. These new synthetic protocols were further explored for the enantiomeric syntheses of various enzyme inhibitors, immunosuppressants, chemotherapeutics, nucleosides, herbicidal agents etc. Different bioactive molecules like the anti-AIDS drug (AZT) and its precursors (2,5-disubstituted tetrahydrofurans) and/ or congeners (3′*S*,5′*S*)-isodideoxynucleosides (2-C-branched 2-deoxypentofuranoses), (-)-prelactone (polyketide macrolide with a δ-lactone moiety), octalactin lactone (a polyketide marine metabolite), hapalosin (multidrug-resistance reversing depsipeptide), SPIKET-P (tubulin-binding anti-cancer agent), (*S*)-2-cyclohexyl-2-phenylglycolic acid (component of anticholinergic, oxybutynin), sulfobacin A (an antagonist for von Willebrand factor (WF) receptor) etc. were synthesized in high-yielding protocols (**Fig. 1**). A chiral template-directed enantioselective construction of tertiary carbinols also formed the basis of several medicinal compounds. In a significant study, an asymmetric synthesis of herbarumin III, a phytotoxic macrolide, was developed using (*R*)-cyclohexylideneglyceraldehyde as the chiral template. This report (*Tetrahedron Asymmetry* 2006, *17*, 325-329) was well appreciated globally and was among the most cited paper in 2006-2009. In addition, interest in aliphatic polyhydroxy acids as immunomodulatory, anti-inflammatory, and anti-neoplastic agents prompted to develop the first asymmetric synthesis of oxylipin, an established and important mediator during inflammation, using the same chiral template. Chiral material driven synthesis was further extended to the synthesis of *β*-hydroxy derivatives of L -glutamic acid, L -glutamine and L-proline, useful for peptide/protein studies. A divergent asymmetric synthesis of iminosugars was also accomplished starting from D -glucose. Other chiral templates like citronellal had also been used in asymmetric synthesis of medicinally important trans/cis octahydroacridines, and most importantly for trogodermal (a pheromone, used against stored grain, khapra beetle).

In another innovative effort, it was demonstrated for the first time that cyclopentylmagnesium bromide (CPMB) acts exclusively as an inexpensive and safe reducing agent with aryl and alkyl aldehydes/ketones/esters, and was used for the diastereoselective reduction of various cyclic/polycyclic ketones including steroids, flavanones and terpenes. The reagent was also used for substrate-controlled reduction of chiral oxygenated ketones to furnish chiral alcohols. Notably, CPMB functioned as a normal nucleophile to transfer the cyclopentyl moiety to the ketones in the presence of $ZnCl_2$ (catalytic). This strategy was further utilized for the synthesis of a medicinally important compound (+)-*α*-conhydrine (a hemlock alkaloid) and also a key chiral segment of the muscarinic receptor antagonist (*S*)-2-(cyclopentyl-2-phenylglycolic acid (better efficacy than the widely used drug oxybutynin, highlighting the importance of chiral drugs).

Along with that, desymmetrization of prochiral anhydrides was also investigated as a strategy for stereoselective organic syntheses. Compared to the conventional methods of diastereoselective differentiation where costly and irrecoverable chiral reagents are used,

this unique method using the lithium salt of Evans' oxazolidinone could be efficiently employed for the economic and operationally simple desymmetrization of σ-symmetric anhydrides on a preparative scale. This method was further applied to the stereoselective total synthesis of (1)-preussin via desymmetrisation of σ-symmetric 3-dimethyl(phenyl)silyl substituted glutaric anhydride.

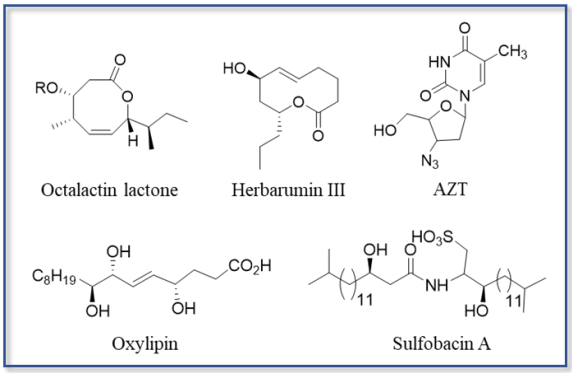

Fig. 1: Some representative bioactive molecules synthesized in the laboratories

In a seminal work, highly regio- and stereoselective vinylogous Horner-Wadsworth-Emmons (HWE) reaction (**Fig. 2**) of aldehydes with allylic phosphonates generated in situ from α-cyanovinylphosphonates, yielding stereochemicallypure 1,3-dienes with a trisubstituted double bond, was developed (*Angew. Chem. Int. Ed.* 2007, *46*, 2348-2348). In a similar line, Horner-Wadsworth-Emmons reaction was utilized to construct reactive dendralenes, which finally led to the syntheses of highly functionalised cyclohexenes with very high regio- and stereoselectivity.

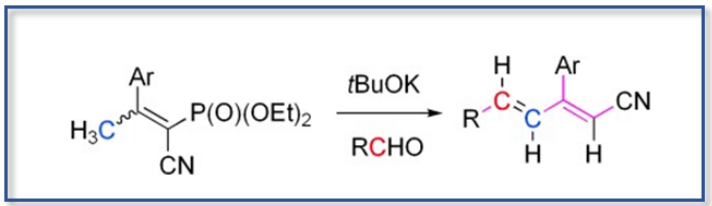

Fig. 2: Vinylogous HWE route to densely substituted 1,3-butadienes

Pioneering contributions were also made in introducing several new concepts for tuning reactivity of the low-valent-titanium (LVT) reagents using various additives (inorganic salts like NaCl vs CsCl; s vs p-donors like (polyaromatic hydrocarbons, I_2, NaCl, CsCl etc.), that helps in carrying out McMurry coupling of carbonyls (i) up to pinacol stage & controlling the stereoselctivity [stereoselective construction of pinacols using covalently-linking ligands/auxiliaries at low temperatures (*J. Am. Chem. Soc.* 1996, *118*, 5932-5937)]; (ii) demethoxylation of aromatics (for phenthrene synthesis), (iii) carrying out McMurry coupling at low temperature for better diasterecocontrol, and/ or proceeding to

alkene stage (for substituted diarylalkanes, hydroxystilbenoids, benzopyrans etc.). In particular, the new LVT reagents, containing LVT or LVT-alkali metal salts or I_2 [generating Ti-Grignard reagents] were found to be hyperactive in single electron transfer (SET) processes, which was useful for carbon-heteroatom bond cleavages at a faster rate and at a low temperature in high yields. In addition, several synthetic utility of low-valent titanium (LVT) reagents were also demonstrated. One such effort was an efficient and short synthesis of benzofurans from ortho-aroyloxyacetophenone using $TiCl_4$-Zn-THF reagent via intramolecular reductive deoxygenation. A remarkable application of LVT reagents for synthesis of polycyclic aromatic hydrocarbons (PAHs) from ortho-alkoxy aromatic aldehydes/ketones was established. It was found that pyridine-stabilized LVT has the highest potential to increase the yield of PAHs from *ortho*-alkoxy aromatic aldehydes. Utility of the LVT reagents were further explored for deprotection of allyl, benzyl and propargyl ethers as well as cleavage of N-allyl/benzyl/aryl/propargyl bonds. In continuation, this method was also utilised for the synthesis of substituted diarylalkanes, hydroxystilbenoids, benzopyrans etc.

Continuing our endeavour towards development of new synthetic methodologies, copper(II) bromide was found to be an effective catalyst for the imino Diels-Alder reaction, conjugate addition of indoles to α,β-enones, conjugate addition of pyrroles to α,β-unsaturated, monobromination of aromatic compounds and deprotection of tert-butyldimethysilyl ethers. In addition, copper(II) bromide was utilized in efficient one-pot synthesis of phenyl substituted benzo[b]furans from styrlphenols. Further, antimony(III) chloride ($SbCl_3$), a group 15 metal containing Lewis acid, was utilised as a catalyst for direct alkylation of electron-rich arenes/heteroarenes with benzylic alcohol under microwave-irradiation. The ortho-alkenyl phenols thus obtained were utilized in synthesis of biologically important functionalized oxygen-heterocycle, 4-phenylchroman. Later, $SbCl_3$ was utilised for solvent-free Friedel–Crafts reaction of phenols with mandelic acids to yield 3-aryl benzofuran-2(3*H*)-ones, which were further utilised for the syntheses of highly functionalized 3-substituted-3-arylbenzofuran-2(3*H*)-ones. Use of camphor-10-sulphonic acid (CSA) as a organo-catalyst to realize different organic transformations was also explored. In a notable effort, CSA was efficiently used as a catalyst for solvent-free direct three component one-pot Mannich-type reactions to afford *β*-amino ketones with good diasteroselectivity. Later, application of this methodology towards synthesis of 4-aminochroman was successfully accomplished. Synthesis of another important class of oxygen heterocycle, 14-aryl/alkyl-14H-dibenzo[a,j]xanthene was also realized by using CSA as catalyst.

Basic research with the stereoelectronic effects of organosilicon compounds in directing regio- and stereoselectivities of organic transformations lead to several complex biologiocally relevant natural products and their congeners. Stereoselective total synthesis of (+)-preussin (antifungal), (+)-carpamic acid (a medicinal alkaloid), fagomine (glycosidase inhibitor) etc. were achieved using this strategy. A one-pot self-regulated approach for the synthesis of amides/peptides based on two reduction–oxidation (redox) reactions was developed from azidotrimethylsilane and alkyl azides/*α*-azido acid

derivatives, and was applied for the synthesis of methionine enkephalin, an endogeneous neuropeptide. In addition, a highly regio- and stereoselective Heck reaction of iodoarenes with vinylated malonates, generated in situ by fluoride-induced protiodesilylation of alkenylsilanols/disiloxanes was developed, and was used for short synthesis of a structurally challenging lignan, (±)-matairesinol, a potential therapeutic for muscle contraction, muscle spasms and other disorders such as epilepsy. Silyl alkylidenes and related compounds were also utilised as a Michael acceptor to construct functionalised and enantiomerically enriched *β*-silyl ketones, vinyl silanes, styrenes, cyclopropanes, cyclobutanes. Additionally, a silicon directed Baeyer–Villiger oxidation on solid-phase was explored to achieve the synthesis of a *β*-silylethanol anchoring group. Efficient and short syntheses of enantiomerically pure enantiomers of 2,6-dioxabicyclo[3.3.0]octane-3,7-dione were achieved from the bis-silylated adipic acid derivatives using Fleming-Tamao oxidation.

The versatile use of organosilicon compounds led researchers to look for improved and green synthetic methods for its synthesis. Towards this, very recently, a method involving visible-light-induced organophotocatalyzed ring-opening followed by remote Giese addition of tertiary cycloalkanols with *β*-silylmethylene malonates was developed under mild reaction conditions, and was utilised for the synthesis of several structurally and electronically challenging organosilicon derivatives.

3. Green Protocols

3.1. Organocatalysis

Organocatalysis is the field wherein small organic molecules efficiently and selectively catalyse organic transformations in metal-free environments. To this end, several organocatalysts were designed to achieve many difficult organic transformations which led to the easy, short and enantioselective syntheses of many bioactive molecules with varied stereochemistry.

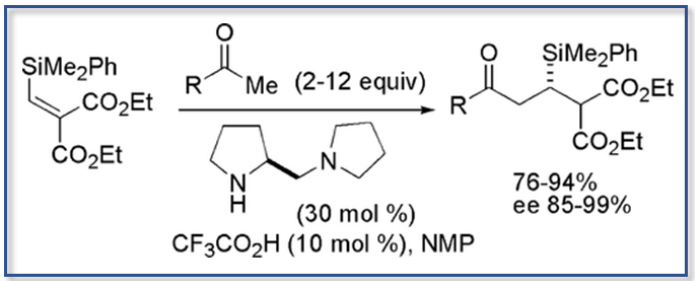

Fig. 3: Organocatalytic conjugate addition of alkyl methyl ketones to a *β*-silylmethylene malonate

The organocatalytic enantioselective direct conjugate addition of alkyl methyl ketones and enolizable aldehydes to dimethyl(phenyl)silylmethylene malonate was reported for the first time (**Fig. 3**). Synthetic manipulation of the resultant products led to the formal

and total syntheses of various *O*-and *N*-heterocyclic natural products [(+)-preussin, (*S*)-hexadecanolide, (–)-tetrahydrolipstatin, (+)-simplactone B, (+)-γ-caprolactone, (+)-methylenolactocine, (-)-quercus lactone, etc.]. Enantioenriched organosilanes bearing a nitro group were also synthesized, which gave access to the nootropic drug (*R*)-oxiracetam, sila-analogue of PAR-2 agonist AC-264613, (*R*)-N-benzyl-4-hydroxy-pyrrolidin-2-one etc.

Incorporation of a silyl group into known drugs /bioactive molecules is known to improve their biological properties. Towards this, highly enantioselective asymmetric catalytic Michael addition of 4-unsubstituted pyrazolin-5-ones to β-silylmethylene malonates was disclosed on applying a chiral H-bonding organocatalyst to incorporate the target organosilanes appendage with a pyrazole moiety.

An alternative reaction concept for the cycloaddition reactions of enone and nitrodienes was explored *via* an endo [4 + 2] "on water''cycloaddition reaction of enones and nitrodienes. In a similar line, a novel organocatalytic asymmetric formal [3+2] cycloaddition of 3-isothiocyanato oxindoles and arylidene malonates was also disclosed using a cinchona derived tertiary amine-thiourea-based bifunctional organocatalyst. This allowed access to pharmaceutically important and highly functionalized 3,2'-pyrrolidinyl spirooxindole derivatives.

A biomimetic asymmetric organocatalyzed decarboxylative aldol reaction of β-ketoacids with α-ketophosphonates yielded biologically important γ-carbonyl tertiary α-hydroxyphosphonates with a hydroxyl and phosphonate bearing chiral quaternary centers. Further to construct a chiral pyrazolone derivative with all-carbon quaternary stereocenters, a one-pot synthesis of 4-monosubstituted pyrazolones was achieved with high yields and stereoselectivities via a primary amine organocatalyzed conjugate addition of 4-monosubstituted pyrazolones to enones.

Apart from that, the increasing importance of Deuterium-labeled biologically active compounds because of their better metabolic stability and bioavailability, led the researchers to develop methods for D-labelling of organic molecules at specific positions. A highly regioselective and enantioselective direct Michael addition of methyl-d3 alkyl ketones to dimethyl(phenyl)silylmethylene malonate, catalyzed by (*S*)-*N*-(2-pyrrolidinylmethyl) pyrrolidine/trifluoroacetic acid/ D_2O combination was achieved with high yield and isotopic purity, and was utilised to synthesize dideuterated silylated tetrahydropyran-2-one, which is an advanced intermediate for *gem*-dideutero (-)-tetrahydrolipstatin and (+)-δ-hexadecanolide syntheses.

3.2. Biocatalysis

A systematic research on biocatalysis was initiated in India, in the early 90's that was furthered to make remarkable contributions in organic syntheses. Creation of new and small chiral intermediates with multiple stereogenic centers and high functional density using inexpensive, operationally simple, scalable and novel enantioselective strategies has been one of the happy hunting grounds since then. These chirons are instrumental in the syntheses of a platter of bioactive compounds *viz*. macrolides, polyhydroxy compounds, plant-growth regulators, pheromones, marine sponge-metabolites and many

more natural products often displaying impressive anti-cancer, antiviral, antifungal, bacteriostatic and vaccine adjuvant activities.

Using commercially available isolated enzymes (lipases)

A lipase-catalysed protocol for synthesis of medium to large ring macrolides even containing chemically fragile 1,4-dienoic compounds was devised. This was extended to control stereogenicity of multiple stereogenic centers by a single macrolactonization step. The researchers also introduced the concept of inter-facial lipase activation by carrying out reactions with immobilized substrates rather than in solution phase that also helped in acetylating hydrophilic molecules like sugars and glycerols. Further, it was demonstrated that the enantioselectivity of the lipase catalysed acylation can be tailored by changing the nucleophile. A few examples are discussed below:

Our tryst with enzymatic protocols dates back to 1993 with lipase catalysed acetylation of some pentose and hexose sugars in organic solvents. The primary motivation of the work stemmed from the utilities of the products for the manufacture of low calorific sweeteners, biosurfactants etc. The existing enzymatic methodologies of sugars acylation in polar solvents suffered from poor product solubility and enzyme deactivation. These limitations were innovatively offset by immobilising the carbohydrates on an inert support when the regioselective acetylation could be accomplished with porcine pancreatic lipase (PPL) in an environmentally safe, hydrophobic media. A highly regioselective esterification strategy for the aliphatic ω, α-dicarboxylic acids in n-butanol, and a chemoselective esterification of a saturated acid moiety in presence of a conjugated acid function were accomplished. Around the same time, the enzymatic resolution of alkyn-3-ols *viz.* racemic 1-octyn-3-ol and 1-nonyn-3-ol in non-aqueous media were established, since the acetylenic unit in such compounds can serve as an intermediate for conversion to alkaloids, prostaglandins, pyrethroids, pheromones, vitamins, steroids and antibiotics. Likewise, a lipase catalysed transesterification strategy for the resolution of alkan-2-ols was formulated after extensive screening of enzymes, solvents and optimization of reaction conditions.

Several of the above enzymatic strategies paved the way for efficient enantiomeric synthesis of a host of bioactive compounds of diverse skeletons (**Fig. 4**). Some examples include ferrulactone II (insect pheromone) and (2*E*)-9-hydroxydecenoic acid (mandibular gland secretion of queen bees, *Apis mellifera L.*)**,** (*R*)-patulolide (anti-microbial macrolide), (*R*)- phoracantholide I and few enantiomers of the antifungal and antibacterial principles of *Sporothrix* species, wherein PPL (porcine pancreatic lipase)-catalysed enantioselective reactions provided the key steps. Similar lipase catalysed protocols were applied for the synthesis of an array of marine sponge components *viz.* (4*E*,7*S*)-7-methoxytetradec-4-enoic acid (antimicrobial), 1-tert-butyldimethylsilylpenta-1,4-diyn-3-ol, (15*E*,*R*,*R*)-duryne, (*S*)-eicos-(4*E*)-en-1-yn-3-ol (cytotoxic), (2*R*,5*Z*,9*Z*)-2-methoxyhexacosa-5,9-dienoic acid and the hydroxy acid segment of schulzeines B and C.

In a remarkable effort, a novel lipase-catalyzed protocol was formulated for the simultaneous enantiocontrol of three stereogenic centers in a flexible acyclic system to

achieve an enantioselective synthesis of tetrahydrolipstatin (anti-obesity). The first asymmetric synthesis of (6*S*,13*R*)-6,13-dihydroxytetradeca-(2*E*,4*E*,8*E*)-trienoate (component of *Mycosphaerella rubella*) and diastereomers of pinellic acids were also developed using an efficient lipase-catalyzed acylation strategy. Later, syntheses and biological evaluation of all possible stereoisomers of a 16-membered macrolide antibiotic (-)-A26771B was achieved *via* lipase (Novozym 435®) catalysed acylation routes. Herein, the lipase was used both for kinetic resolution of a methylcarbinol $CH_3CH(OH)$ moiety and its chemoselective acrylation (using a non-traditional acyl donor, ethyl acrylate) over a secondary allylic alcohol unit. Similarly, a lipase catalysed acylation was used to accomplish the asymmetric synthesis of the C22-trihydroxy fatty acid component of the 42-membered antiviral macrodiolide, macroviracin A

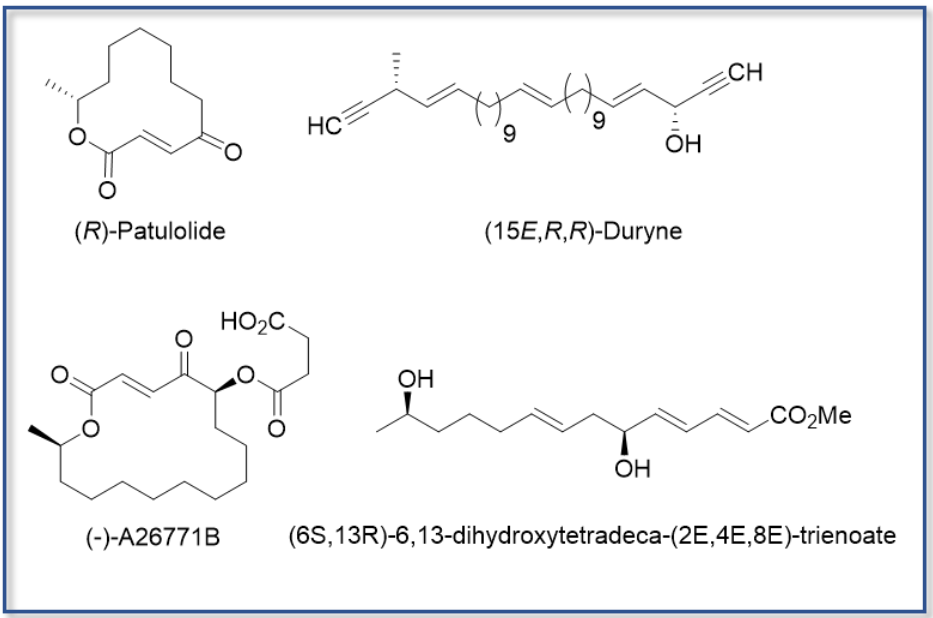

Fig. 4: Some representative bioactive molecules synthesized using biocatalytic route

With the ongoing quest for divergent synthesis of potential anti-cancer and immunomodulatory agents, researchers identified a common structural motif hept-6-ene-2,5-diol found in several bioactive compounds *viz*. decarestrictine (cholesterol biosynthesis inhibitor), pyrenophorol (antimicrobial), acremodiol (antimicrobial), clonostachydiol (cytotoxic), stagonolide (phytotoxin) and nonenolide (anti-malarial), which were synthesized in all its enantiomeric forms using two Novozyme-435® catalysed acylation steps (**Fig. 5**). Similarly, a ceramide trafficking inhibitor HPA-12 was synthesized using an efficient and highly enantioselective lipase-catalyzed acylation in a hydrophobic ionic liquid, [bmim][PF_6]. In continuation of the endeavours to synthesize bioactive compounds, few bioactive diarylheptanoids (from *Alpinia* officinarum) were

synthesized using *PS Amano Lipase* catalysed kinetic resolution, and their antiproliferative properties were tested in-vitro, where one of the compounds showed impressive anticancer effect.

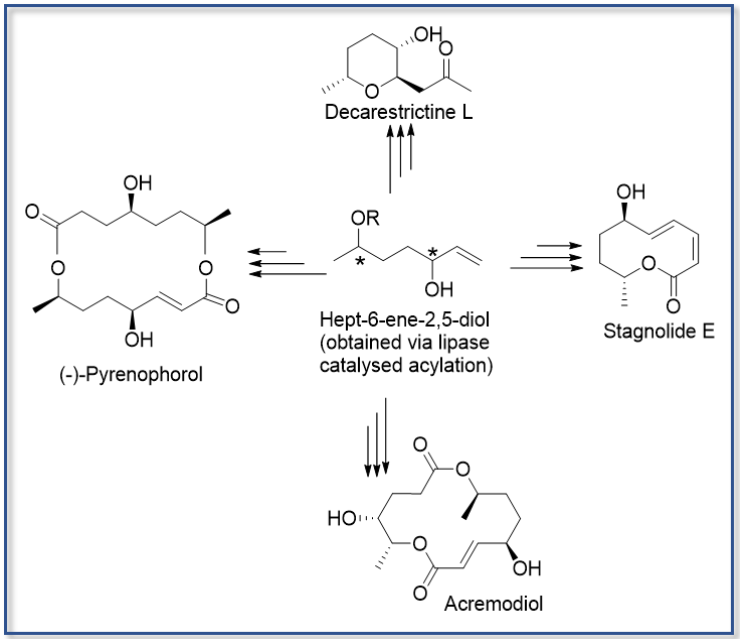

Fig. 5: Divergent synthesis of potential anti-cancer and immunomodulatory agents developed.

Using whole cell enzymatic reactions and potential uses of spent biomass

Unlike the commercially available lipases/proteases, use of whole cell systems offers an economical alternative biocatalytic protocols, especially with cofactor-requiring enzymes (oxido-reductases, oxynitrilase etc.). The microorganisms are available with a wide variety, show broad substrate specificities and possess built-in co-factor regenerating systems. The whole cell plants and microbial systems were extensively used for asymmetric reduction of ketones and hydrolysis of secondary carbinol esters by tuning the enantioselectivity via subtle structural modifications of the substrates. The reaction rates and selectivities were correlated with steric and electronic parameters of the substitutions of the aryl/alkyl and heteroaryl/alkyl substrates. *Rhizopus arrhizus* mediated microbial reduction of a series of arylalkanones, with varying spacer length between the aryl and carbonyl groups, were investigated for the first time, which demonstrated an 'enantio-switch' in the course of microbial reduction. Moreover, the spent (dead and dried) *Rhizopus arrhizus* biomass, obtained from microbial reduction of aryl alkanones, was further evaluated as a potential biosorption material for treatment of waste water containing toxic azo dyes *viz*., amaranth, fast red A, congo red, tartrazine, metanil yellow

and sunset yellow FCF (**Fig. 6**). On a similar line, *Rhizopus arrhizus* biomass was also demonstrated for the removal of dye erythrosine B from aqueous solution (**Fig. 6**).

Fig. 6: Biosorption of Erythrosine B by *Rhizopus arrhizus* biomass.

Plausible applications of biosorption in nuclear waste management motivated us to carry out sorption studies of various radionuclides *viz*. ^{233}U, ^{239}Pu, ^{241}Am, ^{144}Ce, ^{147}Pm, $^{152/154}Eu$ and ^{95}Zr from aqueous nitrate medium using the dead *Rhizopus arrhizus* biomass. The biomass showed selective adsorption capacity for U/Pu and trivalent actinides depending on the pH of the solution. Later on, the said biomass was immobilized by crosslinking with 15% formaldehyde/0.1 M HCl solution, leading to better mechanical strength, rigidity and chemical stability. This showed a sorption capacity of 65 mg/g for Am and U ions, and hence could be utilized for the removal of radionuclides from radioactive waste effluents. As a follow-up study, a highly radiolytically stable biosorbent based on wild-type *R. arrhizus* biomass was employed for separation of Am^{3+} and Eu^{3+}.

3.3. Room Temperature Ionic Liquids

Room temperature ionic liquids (RTILs) are regarded as a greener alternative to traditional volatile organic solvents. BSG has pioneered the use of an inexpensive and hydrophilic RTIL, [bmim][Br] for activation of metals (Ga, In, Bi metals) without the need of any acid, additive or additional energy source (microwave irradiation, heating and ultrasonication) that facilitated Barbier-type allylation/crotylation reactions of aldehydes and even ketones. The activation of Ga was shown to follow a novel mechanism, involving a Ga-NHC intermediate via an unprecedented oxidative formation of [bmim] diradicals. The first report of the synthesis of a Ga-NHC complex with the imidazole-containing RTIL under a hydrous, aerobic and moderate condition has far reaching consequences in organic synthesis. With the aim of tuning the reaction diastereoselectivity of asymmetric crotylation, a *syn*-selective crotylation strategy of aldehydes in [bmim][Br] was established. This strategy was further utilized for the syntheses of C1–C9 segment of dictyostatin, the octadienoic ester fragment of the

desoxycryptophycins, and (+)-*cis*-aerangis lactone. Subsequently, the In metal-catalysed Barbier type allylation of aldehydes in [bmim][Br] was reported with detailed mechanistic studies, which was recognised as a VIP publication by European Chem. Soc. (*Eur. J. Org. Chem.* 2018, 1333-1341). Most importantly, the Barbier type carbonyl allylation could accomplished using catalytic (for In), sub-stoichiometric (for Ga) and stoichiometric (for Bi) quantities of the metal as well as reusing the RTIL several times – key attributes towards development of a green synthesis. With substantial knowledge and experience in handling RTILs, a Bronsted acidic RTIL, [bmim][HSO_4], was synthesized and used for the targeted α-regioselective Barbier-type carbonyl crotylation, yielding α-homoallylic alcohols. The department's contribution in activation of metals by RTIL has been highly appreciated by peers.

4. Organics for Agriculture

Since the beginning, the department has been actively involved in research and development of molecules which play important roles to improve agricultural productivity *viz*. pheromones or plant growth regulators etc. Plant agents like garlic oil was found to destroy aphids, cabbage-white butterfly caterpillars, and *Colorado beetle* larvae, and hence was envisaged as an effective larvicide and pesticide. In one of the earliest ventures (*Science* 1971, *174*, 1343-1344), researchers were able to isolate the hitherto unknown components *viz*. diallyl sulphide, diallyl disulphide, diallyl trisulphide and diallyl tetrasulphide, present in garlic oil. This was accomplished via steam distillation using garlic cloves. These components were tested for larvicidal activity, and it was found that diallyl disulphide and triallyl disulphide alone or in mixture even at a concentration of 5 ppm were highly effective. Later, a plant was set up for the large-scale syntheses of the garlic oil components for field applications.

India is the second largest producer of silk globally and sericulture is an important industry in our country. With the aim to increase silk productivity and decrease the production costs, plant-based moulting hormone (MH) preparations were developed that promote silkworm maturation. The low-cost preparation enhances silkworm larvae maturity, generating better quality of reelable silk in a shorter time-span. Notably, the preparation, which is an import substitute for the silk industry was commercialized (MoU between BARC and Indian Agriculture & Sericulture Institute, Mysore (under IACR)).

To meet the never-ending demand for food and tackle the paucity of cultivable land due to global population burst, plant growth regulators (PGR) play a significant role. In this context, the first synthesis of the racemic and enantiomers of 3-methylnonacosanol (PGR from Indian medicinal shrub *Lowsoni ainermis*, traditionally used for treatment of jaundice, leprosy, skin diseases and enlargement of liver and spleen) and its PGR bioassay *vis-à-vis* the stereochemistry was accomplished in the late nineties.

Pheromones

In view of their extreme potency, species-specificity, non-toxicity, biodegradability and requirement of very small concentrations, insect pheromones offer an eco- and

environment friendly pest management strategy sparing the beneficial insects. However, implementation of a successful insect pheromone technology demands a viable synthesis, development of a slow releasing formulations followed by field trials.

Usually pheromones are medium to long chain aliphatic alcohols or their derivatives, having one or several double bonds where the chain length, functionalites as well as the numbers, positions and geometries of the double bonds confer them the species-specificity. Using the core competence in acetylenic chemistry and Wittig reaction, coupled with the expertise on multistep organic synthesis, syntheses of various insect pheromones were accomplished.

For example, syntheses of 6,8-dioxabicyclo[3.2.1]octanes, *viz*. (±)-frontalin and (±)-brevicomins, the aggregation pheromone components of bark beetles, were achieved from pent-4-en-1-ol and pent-4-yn-1-ol. Likewise, synthesis of gossyplure, the pink bollworm pheromone, was achieved using a stereocontrolled Wittig reaction.

The production of sweet potato is often affected by the ubiquitous sweet potato weevils (SPW), rendering the roots unfit for consumption. Pheromones offer an eco-friendly way to manage this pest. To accomplish that, a practical synthesis of the sweet potato weevil pheromone was developed using a *cis*-selective Wittig reaction. Further, an integrated pest management (IPM) strategy was developed using a combination of weevil-free planting material, re-ridging, mass trapping of males through sex pheromone traps, preservation of biocontrol agents, disposal of harvest residues and early harvesting (**Fig. 7**). A number of experiments and observations were carried out over a period of 10 years, and was successfully used for pest management in farmers' fields across India.

Fig. 7: Field use of in-house synthesized sweet potato weevil (SPW) pheromone, A. SPW pheromone dispenser and trap, and B. Catch of SPW in a pheromone-baited trap

India is the second-largest producer of cotton worldwide. However, the production gets hampered due to the presence of several cotton pests, which are difficult to control using conventional pest management methods. BSG steered the idea of using cotton pest pheromones in pest management by accomplishing a practical synthesis of the cotton pest pheromones, particularly the pheromones of one of the major pests *viz*. American

ballworm. In field trials using the mating disruption technique, the pheromone could effectively control the cotton pest. Variations in dose led to the use of this pheromone in both mating disruption and mass trapping techniques, making it one of the most useful pheromones for cotton pest management. These are regularly used in Northern India for cotton pest management.

Until the early 80s, it was believed that biological recognition of pheromones was dictated by alkyl chain length, number, position and geometry of the double bonds present in the molecule, and not by the stereochemistry present in the molecule. Later on, it was observed that the stereochemistry (orientation in three dimensional space) of a chiral pheromone also plays a very important role in its activities. Over the years, a large number of pheromones were isolated from various insects, and the chirality present in them showed profound impact on their physiological action. Hence, synthesis of chiral pheromones *viz*. 4-dodecanolide, trogodermal (dermestid beetle), (*R*)-japonilure (sex pheromones of Japanese beetle) were accomplished using chiral templates like (*R*)-2,3-isopropanedioxyglyceraldehyde, (*R*)-pulegone etc. (*R*)-2,3-cyclohexylideneglyceraldehyde was used as a divergent chiral template for the syntheses of (6*S*)-acetoxy-(5*R*)-hexadecanolide, an oviposition deterring pheromone of the mosquito *Culex pipensfatigans*, which is a possible vector of filarial diseases.

Chirality due to methyl branching is abundant amongst several insect pheromones, many of which are of economic significance. To synthesize such chiral pheromones, a convenient chemoenzymatic protocol was optimised, starting from the castor oil-derived, 10-undecenoic acid, and was utilised for the synthesis of the gypsy moth sex pheromone. Using the same precursor, syntheses of the pheromones of the destructive pests of cash crops and fruits *viz*. (11*Z*)-hexadecenal, (3*Z*,13*Z*)-octadecadienyl acetate and its 3*E*-isomer and (2*E*,13*Z*)-octadecadienyl acetate were realised in good yields. In addition, chemoenzymatic synthesis of a pheromone antipode of stored grain pests (khapra beetle) was accomplished via a baker's yeast mediated asymmetric carbonyl reduction. Similarly, enantioselective chemical and chemoenzymatic approaches were also employed to synthesize pheromones of cucujid grain beetles, rice moth, southern corn rootworm, spice pest *Dichocrocis punctiferalis*, square-necked grain beetle, bark beetle, *Crematogaster* ants, *Drosophila mulleri* flies, cotton pests, peach tree borer and cherry tree borer etc.

Asymmetric synthesis using biocatalysts was also exploited successfully in the synthesis of insect pheromones. To this end, enzymatic acylation of 2-alkanols was extensively studied by optimizing the effects of solvent hydrophobicity, degree of conversion, alkyl chain length and presence of unsaturation on the course of the reaction. Using this optimized protocol, syntheses of (6*R*)-6-methyl-3-octanone, the alarm pheromone of *Crematogaster* and *Myrmecine* ants, were accomplished in a more practical and enantioselective way.

5. Organics for Health

5.1. Radiopharmaceutical Ligands

A radiopharmaceutical (RPh) delivers a radionuclide to a diseased site using a targeted vehicle, resulting in precise diagnostics or therapy. Unlike conventional drugs, they do not show pharmacological effects as such, and are used in trace amounts. Diagnostic RPhs use γ or positron emitters for imaging *via* SPECT or PET, ^{99m}Tc being the most commonly used radionuclide. PET-CT is more sensitive than SPECT-CT, using positron emitters like ^{18}F and ^{68}Ga. Therapeutic RPhs use β- or α-emitters to treat diseases, especially metastatic cancers. Theranostics combines diagnostics and therapy, as exemplified by [^{68}Ga/^{177}Lu] DOTATATE for tumors and [$^{123/131}$I]mIBG for neuroblastoma. The researchers have pioneered the in-house syntheses of several targeted vehicles, called radiopharmaceutical ligands, and in collaboration with Radiopharmaceuticals Division (RPhD), BARC; Board of Radiation and Isotope Technology (BRIT) and Radiation Medicine Centre (RMC) helped to develop affordable radiopharmaceuticals, which, after proper validation and DAE-Radiopharmaceutical Committee (DAE-RPC) approval, were provided to hospitals pan-India through BRIT. Synthesis of several radiopharmaceutical ligands such as the import substitute cardiac diagnostic precursor, tetrakis(2-methoxyisobutylisonitrile)Copper(I) tetrafluroborate ([Cu(MIBI)$_4$]BF$_4$) and prostate-targeting ligands (PSMA-617 and PSMA-11) have been accomplished (**Fig. 8**).

[Cu(MIBI)$_4$]BF$_4$ is a precursor of [^{99m}Tc(MIBI)$_6$]BF$_4$, a myocardial perfusion imaging agent for evaluating and risk stratifying patients with known or suspected coronary artery disease. As a cationic complex, it passively diffuses through capillary and cell membranes and is captured by myocyte cells in the heart. Myocardial perfusion imaging, primarily used to diagnose ischemic heart diseases, relies on [^{99m}Tc(MIBI)$_6$]BF$_4$ in over 75% of cases. The radiopharmaceutical freeze-dried kit containing [Cu(MIBI)$_4$]BF$_4$, is radiolabeled with ^{99m}Tc using a ^{99}Mo/^{99m}Tc generator for clinical use. A multistep organic synthesis of the key component, [Cu(MIBI)$_4$]BF$_4$ and its purification was accomplished, and delivered to BRIT for preparation of kits. In view of its increasing demand in hospitals performing MIBI scans, the synthesis of [Cu(MIBI)$_4$]BF$_4$ was modified and scaled up to 10 g per batch. This is used to make approximately 7000 kits per year (BRIT code: TCK-50), and benefits over 25000 patients in more than 65 hospitals pan India.

Diagnosis and treatment of prostate cancer, which accounts for nearly 10% of all male tumors, is one of the most challenging jobs in radiomedicines. Clinically, ^{68}Ga-PSMA-11 and ^{177}Lu-PSMA-617 are used for diagnosis and therapy for prostate cancer, respectively. However, due to their high cost and irregular availability in India, a cost-effective synthetic route is warranted. The challenging tasks of synthesizing PSMA-617 and PSMA-11 where accomplished using commercially available amino acid derivatives. In collaboration with BRIT, the ligands were radiolabelled and evaluated for their efficacy. Finally, both the in-house developed radiopharmaceuticals were approved by RPC for clinical use. The in-house made ^{177}Lu-PSMA-617 KIT (BRIT code: LUM-5) has been in clinical use, and caters >4000 patients in different Indian hospitals. This is one of the

most important advancement for treating metastatic castration-resistant prostate cancer (mCRPC) using radiopharmaceuticals.

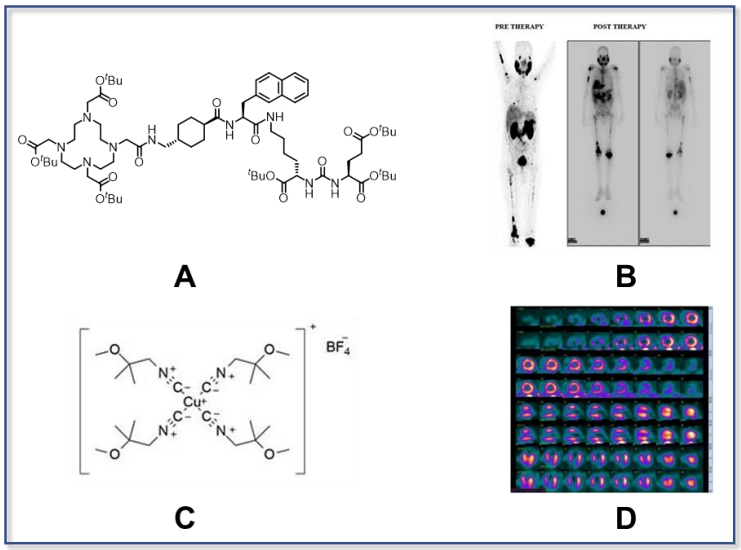

Fig. 8: Chemical structure and clinical use of in-house synthesized radiopharmaceutical ligands. A. PSMA-617; B. Prostate cancer patient images before and after treatment with in-house developed ^{177}Lu-PSMA-617; C. [Cu(MIBI)$_4$]BF$_4$ and, D. Myocardial scan images using in-house developed [^{99m}Tc(MIBI)$_6$]BF$_4$

With an endeavour to further develop in-house synthesis of ligands for other diagnostic and therapeutic radiopharmaceuticals, a protocol for an efficient and economically viable synthesis of three [^{18}F]FLT precursors was indigenously developed using a chiral template (*R*)-2,3-cyclohexylideneglyceraldehyde-directed asymmetric reaction followed by thymidine attachment. [^{18}F]FLT is widely used as a PET radiotracer for disease diagnosis. In addition, mIBG hemisulfate, used as a ligand for [$^{123/131}$I]mIBG preparation, used for imaging and therapy of neuroblastomas, was synthesized using an in-house developed protocol. Similarly, hynic-PSMA, a ligand used in the preparation of [^{99m}Tc]hynic-PSMA, used for SPECT diagnosis of prostate cancer has also been synthesized.

5.2. Targeted therapeutics

Of late, development of targeted therapeutics has become one of the cornerstones in medical sciences, mainly with the aim to deliver chemotherapeutics exclusively at the disease site, so as to reduce the drug dose and generate no/less side effects. Towards this, promising contributions have been made to formulate new compositions as anti-cancer chemotherapeutics and targeted therapeutics for iron overload diseases. Iron-overload has been proven to be detrimental for patients having β-Thalassemia, hereditary hemochromatosis etc. The group's scientists have been instrumental in synthesis and evaluation of targeted iron chelators designed for iron overload therapy. Imidazole based

vectors with varied hydrophobicities and zwitterionic carbon nanodots were synthesized and were coupled separately with an FDA approved chelator, Deferoxamine®, to achieve targeted iron chelators with improved characteristics. Regarding targeted chemo-therapeutics, *N*-succinyl chitosan-based hydrogel beads, stabilized with glycopolymeric network (NSC/Glc-gel) was developed and evaluated for controlled release of the anticancer drug doxorubicin, specifically in a tumor microenvironment (**Fig. 9A**). Recently, a GSH-responsive, Doxorubicin loaded, self-sufficient, metal-organic framework (MOF) based anti-cancer agent chemotherapeutic has been synthesized as a combined chemo-chemodynamic agent, and evaluated *in vivo* in a rodent model (**Fig. 9B**). NET-expressive-neuroblastoma targeting anti-cancer chemotherapeutic has been synthesized and screened for their targeting potential.

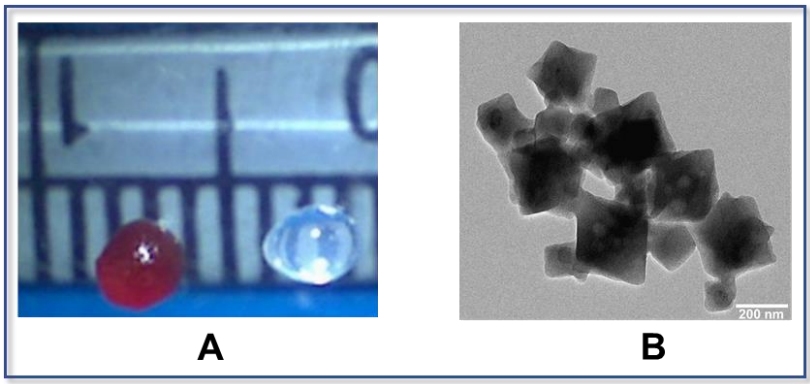

Fig. 9: In-house synthesized drug delivery agents. A. Doxorubicin® loaded NSC/Glc-gel beads, and, B. TEM images of doxorubicin loaded Cu-MOF

5.3. Advanced Drug Intermediates

Although, India is one of the largest manufacturers of bulk drugs, and contributes to approximately 3.5% of the global manufacturing of bulk drugs, it is heavily dependent on other countries for the import of advanced intermediates and key starting materials. One such intermediates, *o*-tolylbenzonitrile (OTBN), an advanced drug intermediate for the -sartan group of drugs, used for treatment of hypertension and heart failures, is extensively imported in a very high volume (>200 MT/year costing >250 million INR/year). In an effort to curb import dependency, and promote local bulk manufacturing of OTBN, a unique method was developed involving the preparation of an organometallic Grignard reagent followed by utilizing a cross-coupling methodology to selectively heterocouple two chemically differentiated aromatic partners using a novel *in-situ* generated catalyst. Our accomplishments for OTBN synthesis include (i) developing an economical, industry-friendly synthetic route using Suzuki-Miyura coupling, (ii) recovery of costly solvent, (iii) avoidance of heavy and toxic palladium metal catalyst, (iv) low amount of waste generation and (v) minimum environmental pollution. Till date, this popular technology named "*A process for synthesis of o-tolylbenzonitrile (OTBN), an*

advanced intermediate for anti-hypertensive -sartan group of drugs, [CH38BOD]" has been transferred to multiple private entrepreneurs for bulk manufacturing.

6. Way Forward

In modern changing era, the national requirements in areas of organic chemistry will gradually grow both in nuclear science programmes and material science (for human benefits). The way ahead will be primarily motivated by excellence in global research in bio-organic chemistry and will be dedicated to modern science and departmental relevance, especially in the field of targeted therapeutics and radiopharmaceutical ligands/carriers. This warrants extensive basic research and development with culmination of multi-disciplines to identify and deliver novel molecules of relevance. By tailoring the molecular structures of the target molecules, one can achieve better targeting of anti-cancer chemotherapeutics with minimal side effects, particularly for those cancers with high prevalence and high mortality rates. Along with that, newer radiopharmaceutical ligands will increase the spectrum of treatments currently offered via nuclear medicine, and will pave the way for improving diagnostic and therapeutic potentials. Solution to challenges like population growth and complexity of modern diseases (including life-style related) seem daunting, and synthetic organic chemistry is bound to play a pivotal role in achieving these solutions. The gathered experience in organic chemistry is indeed and will always be an inexhaustible platform for designing new molecular structures and assemblies, aimed at satisfying the ever-growing societal needs.

7. Acknowledgement

The authors are grateful to all the people (current and retired) whose inputs and suggestions have enriched the chapter and helped compile the group's activities spanning over the last few decades.

RESEARCH AND DEVELOPMENT OF TASK SPECIFIC FUNCTIONAL ORGANIC MOLECULES

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Abstract

Over the years, research in the area of organic chemistry has contributed immensely towards the development of functional organic molecules for various departmental projects and societal benefits. Expertise of organic synthesis is being utilized to design and syntheses of numerous small molecules as well as supramolecules to carry out various task specific functions. Syntheses of ligands useful for actinides and lanthanides extraction in back end fuel cycles always remain as one of the major activities. Fluorescent organic molecules also are being used for many applications such as liquid dye lasers, fluorescent gamma dosimeters, sensors for chemo/bio analytes, bio-imaging agents and photosensitizers for photodynamic therapy of cancers. Similarly, research in the areas of supramolecular host-guest interactions and molecular electronics are also carried out towards the advanced technological developments. In this chapter, research activities carried out in these areas are summarized.

1. Introduction

The activities in the area of organic chemistry are wide spread from developing organic reaction methodologies to their applications for final products required in departmental projects as well as for societal benefits. In this chapter, specific examples of development of these task specific organic molecules are presented. Custom made organic molecules

are always in high demand especially in nuclear science and technology. Thus organic chemistry research is always focused towards the design and development of tailor made molecules for various projects of DAE. For example, in back end fuel cycle, organic ligands are indispensable for actinides and lanthanides separation from nuclear wastes. Thus, synthesis and development of small molecule based organic ligands useful in backend fuel cycles remain as one of the major activities. During the beginning of 21st Century, two new activities based on supramolecules and fluorescent molecules were started which were also driven by the departmental requirements. Slowly these novel molecules were also used for developing hi-tech molecular systems as well as for societal applications, as summarized in this chapter. The intricate R&D work involved during the innovations are not discussed in detail, rather this chapter is focused mainly on the applications of the synthesized molecules.

2. Synthesis of organic ligands for back end fuel cycle

2.1. Small molecule based ligands

In the back end nuclear fuel cycle, one of the most challenging tasks is trivalent actinides (An) and lanthanides (Ln) separation. Thus, numerous ligands containing S and N donor atoms are checked for the separation studies of Ln/An. Four separation techniques are mostly used in different partitioning strategies of back end nuclear fuel cycle: (1) U and Pu partitioning from spent nuclear fuel, (2) heat-generating fission products separation, (3) trivalent An and Ln co-extraction and (4) trivalent An separation form the trivalent Ln. Amongst these, the separation of An from Ln is an urgent and important problem for waste management and processing of fuels from the nuclear plants. Over the years, organic chemistry research has been significantly contributing toward design and synthesis of various extractants to support back end fuel cycle.

In 1985-1988, Horwitz group (Argonne National Laboratory, USA) developed the Trans Uranium Extraction (TRUEX) process for the removal of long-lived alpha emitters from transuranic (TRU) waste solutions using various phosphine oxide ligands. In the early 1990s, similar work was carried in collaboration with the Fuel Reprocessing Division (FRD) and Radiochemistry Division (RCD). Major contribution i.e. partitioning of actinides from high level radioactive wastes, were initiated by synthesising organophosphorus ligands like carbamoyl methylene phosphine oxide (CMPO), TBP etc (**Fig 1**). CMPO was quite efficient for An extraction, but its selectivity was poor. In 1992, a slightly modified three-step procedure [from the reported method by Horwitz group] for CMPO synthesis in substantial quantities was developed. CMPO and TBP mixture was used as the extractant to avoid the formation of third phase hurdle, which could reduce the alpha activity of HAW and HLW solution substantially (~4 nCi/ml). The R&D activities on synthesising new extractants/ligands are continuously pursued over the years. In early 2000, extensive works were carried out on the development of several amides/diamides (e.g. N,N′-dimethyl,N,N′-dibutylteradecylmalonamide (DMDBTDMA)) (**Fig. 1**) based organic ligands as alternate extractants in the back end of nuclear fuel cycle. The major challenge was faced for purification of the long chain

amides by distillation under very high vacuum as well as maintaining optimum temperature to avoid degradation of amide. To meet the growing demands of extractant, these ligands were synthesized and delivered in multi-kilogram scale.

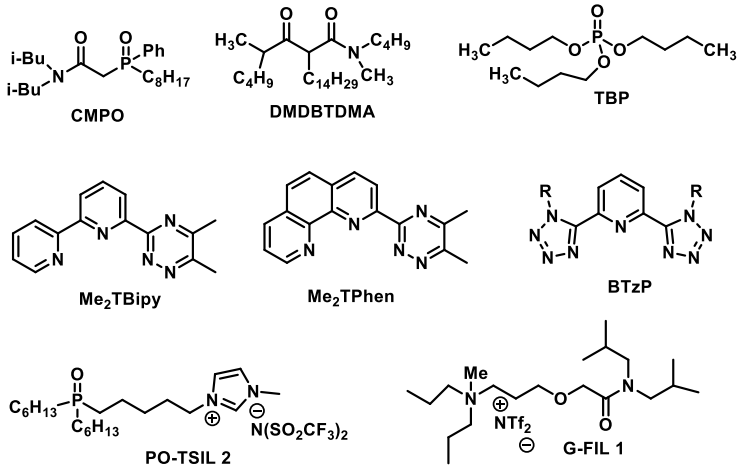


Fig. 1: Chemical structures of different small molecule based ligands

In early 2010, development of new classes of extractants such as heterocyclic based, ionic liquid based, solid supported resin based etc. was started for Ln/An separation. Towards this, heterocyclic based terdentate 'N' donor ligands with different structural rigidity, *viz*. 5,6-dimethyl-(1,2,4)-triazinylbipyridine (Me_2TBipy) and 5,6-dimethyl-(1,2,4)-triazinylphenanthroline (Me_2TPhen) (**Fig. 1**) were designed and synthesized and their Ln^{3+} complexation studies were done in collaboration with Radiochemistry Division (RCD). In continuation, another class of pyridine-triazole based terdentate multiple 'N' donor ligand, 2,6-bis(1H-tetrazol-5-yl)pyridine (H_2BTzP), was developed and its complexation behaviour with trivalent americium, neodymium, and europium were also evaluated.

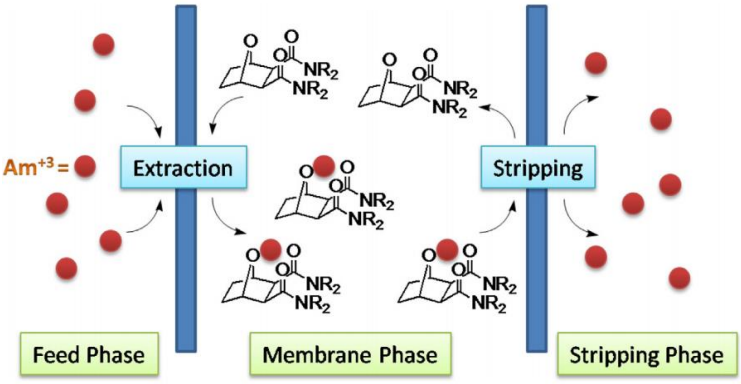


Fig. 2: Transport Am(III) from HNO_3 medium across a supported liquid membrane.

During this investigation, (N,N,N',N'-tetra-2-ethylhexyl)7-oxabicyclo[2.2.1]heptane-2,3-dicarboxamide (OBDA), a new class of conformationally constrained diamide, was developed. It showed selective transportation of Am(III) from HNO_3 medium across a Supported Liquid Membrane (SLM) with good transport rate, diffusion coefficient over other fission products (**Fig 2**). Utilizing this same concept, oxa-bridged tricyclo-dicarboxamide (OTDA) was synthesized which showed selective extraction of tetravalent actinides relevant to the Plutonium Uranium Redox EXtraction (PUREX) process. In continuation, liquid membrane based on OTDA was also prepared which showed promising separation of Pu^{4+} from nuclear waste with high selectivity. Ruthenium (Ru) is one of the most hazardous fission products of nuclear waste, although ^{106}Ru has application in radiotherapy. Besides, Ru compounds are also highly useful in catalysis and analytical applications in various fields. In this context, dipropylmethyl-2-(N,N-diiso butyl) acetamidoammonium iodide impregnated Amberlite XAD-4 resin was investigated for Ru sorption from nitric acid medium (**Fig. 3**). This method could be useful for Ru separation from waste solutions. Studies towards utilizing ionic liquid (IL) as an extractant in the back-end fuel cycle were also carried out. A glycolamide-functionalized ionic liquid was designed and synthesized which was used for trivalent Ln(III) and Ac(III) extractions from low acid feed solutions (in collaboration with RCD). Task specific ionic liquid (POTSIL) based on trialkyl-phosphine oxide and NTf_2^- counter anion was developed for the extraction of UO_2^{2+} and Pu^{4+} from acidic feed solutions. The trialkyl-phosphine oxide based ionic liquid was materialized into an inert polymeric material XAD-7 to obtain a Solvent Impregnated Resin (SIR). This SIR was used for sorptions of both U(VI) and Pu(IV). Another new class of triaryl-pyridine/diaryl pyridine (TAP/DAP) amide based extractans were designed and materialized for extraction in back end fuel cycle. The 3,3′-bis (2-oxydialkyl acetamide) triaryl pyridine based room temperature ionic liquids (RTIL) diluents are also developed for selective Pu(IV) extraction from nitric acid solutions.

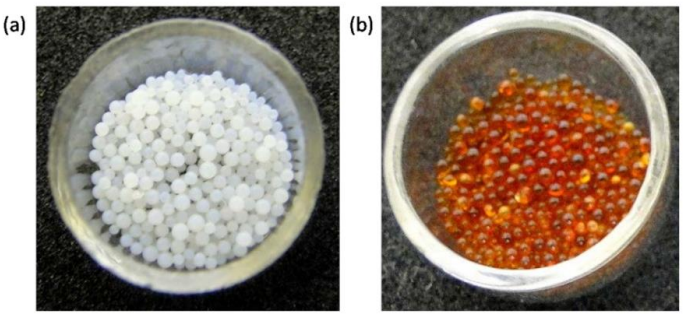


Fig. 3: Amberlite XAD-4 resin beads; (a) before and (b) after Ru impregnation

In another effort in collaboration with Fuel Chemistry Division, a novel deep eutectic solvent (DES) based on alkyl triphenyl phosphonium salt and decanoic acid was

developed, which showed promising results in selective sequestration of Pu(IV). This novel hydrophobic DES was synthesized using a facile and green mechanochemical route, employing alkyl triphenyl phosphonium bromide and decanoic acid (DA) as the hydrogen bond acceptor and donor respectively. The formation of strong hydrogen bonds led to exceptionally high binding energy of DES, which imparted its stability and made it useful for radioactive waste management. Additionally, a technique for decontaminating radioactive surfaces by employing a strippable gel (RADGEL), composed of a deep eutectic solvent and polyvinyl alcohol, was used for radioactive surface decontamination, with the goal of enabling reuse of the surface post-decontamination. The fabricated gel was able to decontaminate various contaminated surfaces upto 99%. In another effort, pyridinium and methyl ammonium based macroporous bifunctional anion exchange resin was prepared by chemical modification of Reillex^{TM} 402 poly(4-vinyl pyridine) which showed efficient separation of plutonium(IV) and neptunium(IV) from acidic solution.

2.2. Supramolecular ligands.

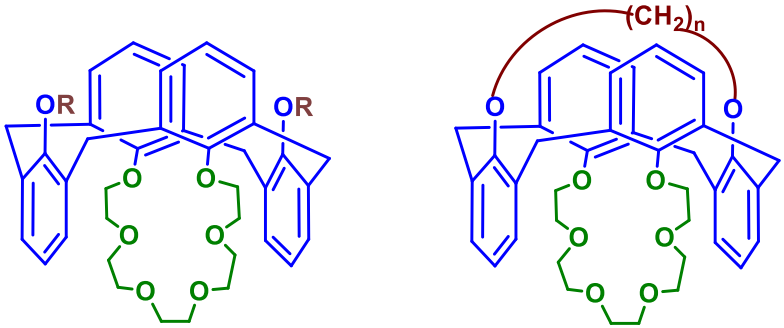
Calix[n]arenes (n = 4, 6, 8), a class of cyclic oligomers, are highly important in supramolecular chemistry because of their hydrophobic cage-like structure with choisable/tunable polar functional groups at the rims. Hence these are potentially useful supramolecular ligands for selective metal extraction. Since early 2000, extensive research work was carried out towards design and synthesis of various calix[4]arene and calix[6]arene derivatives as host molecules. Selective alkylation of the phenolic OH groups of calixarenes is essential for their subsequent functionalization to tailored molecules having pre-organized structures with specific recognition abilities for metal ions or molecules. Hence, a new methodology was developed using ultrasound irradiation for quick access to a series of 4-*tert*-butylcalix[6]arene mono-alkylethers in higher yields as compared to the conventional refluxing procedure (*Tetrahedron*, 2002, *58*, 5287–5290.). Some of these calix[6]arene derivatives were used for the syntheses of molecular hosts for departmental requirements.

Bis-calixarenes attracted considerable attention because of their multi-cavity structure for guest complexation and the probability of higher molecular recognition property due to possible co-operative and/or allosteric effects. Synthesis of such compounds suffers from challenges of protection/deprotection of chemically similar phenolic groups prior to intermolecular coupling. Therefore, efforts were made to realize a practical and efficient synthesis for this important class of molecules. A singly bridged *bis*-calix[6]arene was synthesized by coupling two 4-*tert*-butylcalix[6]arene units and one diethyleneglycol ditosylate molecule. Complexation studies revealed that the synthesized singly bridged *bis*-calix[6]arene is more selective for K^+ ion and has the ability to discriminate between different alkali metal ions.

The safe handling and disposal of high level radioactive waste (HLW) is one of the major challenges faced in nuclear industry. ^{137}Cs producing high energy γ radiation (661.9 keV) with a long half-life ($t_{1/2}$ = 30.1 years) constitutes the major heat source in these wastes. Therefore, separation of radioactive cesium from the nuclear waste is essential to minimize the health hazards associated in management of nuclear waste along with its

safe storage and ultimate disposal. Also, the isolated ^{137}Cs is an alternative radiation source for sterilization of blood, medical accessories, food etc. Among different calix[4]-crown ethers, 1,3-dioctyloxycalix[4]arene-crown-6 (**A**, **Fig. 4**) in its 1,3-alternate conformation is well known for its high efficiency in selective extraction of cesium ions (K_{Cs+}/K_{Na+}> 33000) from highly acidic nuclear waste in the presence of even large concentrations of sodium ions. Conventional route for synthesis of compound **A** involves 1,3-distal dialkylation of phenolic-OH groups in calix[4]arene followed by attaching the polyether linkages to the remaining phenolic OH group using cesium carbonate (Cs_2CO_3) as a template. But, one of the major challenges involved in the synthesis of such a molecule is in the first di-alkylation step, which usually is very sluggish for longer chain (> C5) alkyl halides, requires long time (5–6 days) even under refluxing conditions, provide the products in moderate yields along with unwanted side products. For the first time, it was shown that use of microwave irradiation (MWI) could furnish the desired 1,3-dialkylated calix[4]arenes (in cone-conformation as the predominant/sole product) in substantially increased yields (71-85%) within a very short reaction time (0.5–2.5 h), eliminating the undesired side reactions.

The synthetic utility of this microwave-assisted protocol was also elaborated for base catalysed partial etherification of the phenolic-OH groups in calix[4]arene/4-tert-butylcalix[4]arene with different electrophiles. The 1,3-di-*n*-octyloxycalix[4]arene synthesized by this protocol was further utilized towards the synthesis of 1,3-di-*n*-octyloxycalix[4]arene-crown-6 (**A**, **Fig. 4**) in 1,3-alternate conformation using Cs_2CO_3/pentaethyleneglycol ditosylate in acetonitrile solvent. The solvent extraction studies done in collaboration with Radiochemistry Division (RCD), suggested that calix-crown ligand **A** can be effectively used for selective recovery of cesium ion from the acidic feed solutions including high level nuclear wastes (HLW).



A; R = C_8H_{17} **B; n = 14, 10, 8**

Fig. 4: (A) Chemical structures of cesium ion selective 1,3-di-octyloxycalix[4]crown-6 (B) 1,3-cyclodialkyloxycalix[4]arene-crown-6

In a related, but significantly more challenging effort, design and synthesis of some 1,3-cyclodialkyloxycalix[4]arene-crown-6 (**B**, **Fig. 4**), with similar functionality like

compound **A**, but having more rigid structure was also accomplished. Using these 1,3-cycloalkyloxy bridged calix[4]arenecrown-6-ethers (**B**) as ionophores, liquid membrane based Ion Selective Electrodes (ISE) for determination of cesium ion content in HLW were developed in collaboration with Radioanalytical Chemistry Division (RACD). The best response was obtained for the 1,3-cyclotetradecyloxycalix[4]arenecrown-6 (**B**, n = 14) where the ISE showed low detection limit (3.7×10^{-8} M Cs^{+}) with quick response time (< 20 seconds). In addition, other calixcrown compounds such as, di-hydroxycalix[4]arene-crown-6, tetra-*tert*-butyl-dimethoxycalix[4]arene-crown-6 and 1,3-dioctyloxycalix[4]crown-6 were also synthesized and investigated for their use as ionophore in polyvinylchloride (PVC) based liquid membranes ISE for detection of cesium ion. It was found that ISE developed with 1,3-dioctyloxycalix[4]crown-6 which was highly selective for cesium ion as compaed to other alkali, alkaline earth and transition metal ions in the pH range 4 to 11. The lifetime (10 months) of the electrode was the highest amongst the membrane based Cs-ISE reported till then.

The studies were extended to synthesize homocalixarenes also which are metacyclophanes having structures similar to that of calixarenes but having at least one bridging group between the phenyl rings larger than a methylene group. Hence, they possess bigger cavity sizes and therefore have the ability to accommodate larger guests. In parallel to the host-guest interaction studies with different metal ions, supramolecular interactions of different calix[4]arene, calix[6]arene, *bis*-calixarene, homocalixarene and calix[4]pyrrole derivatives with C_{60} and C_{70} fullerenes were also investigated by UV-Visible, fluorescence, NMR and theoretical studies that provided newer insights in the host-guest (supramolecular chemistry) interactions. The resultant publications in a host of high impact journals are often referred even today.

3. Design and development of organic fluorescent dyes for diverse applications

During the early 2000, research and development of organic dyes was started. The primary focus was to design and synthesis of fluorescent laser dyes for departmental requirements. As per the requirement of Laser & Plasma Technology Division (L&PTD), boron dipyrromethene (BODIPY) based laser dyes were synthesized in multi-gram scales, which were used by the user groups for further applications in various departmental projects. Two technologies ("Laser Dye Pyrromethane 567" and "Laser Dye Pyrromethane PM597") related to the synthesis of BODIPY based laser dyes were transferred to a private company. In continuation, extensive research work was done to develop new highly photostable laser dyes.

Due to better solubility in water and reasonably large portal cavity, cucurbit[7]uril (CB[7]) (**Fig. 5**) appears as a promising host for organic laser dyes to enhance their service life used by L&PTD. The high cost of CB[7] prompted us to develop an indigenous template mediated protocol for its high yielding synthesis of CB[7] during 2013-2014. Thus, a high yielding (up to 20%) and gram scale synthesis of CB[7] was developed wherein organic dyes and small molecules were used as the templates. Extensive host-guest interactions studies with several organic laser dyes using

sophisticated spectroscopic techniques, established that a BODIPY dye in association with CB[7] could be used on continuous runs for isotope separation.

Rhodamine B is another important dye used as active medium in dye laser research. However, aggregation and poor photochemical stability in water restrict its use in aqueous dye laser studies. It was demonstrated that encapsulation of rhodamine B inside CB[7] cavity is able to suppress its aggregation to improve its fluorescence efficiency and photochemical stability dramatically. The synthesized CB[7] was successfully utilised in improving laser performances of Rhodamine B based aqueous dye laser system in collaboration with Laser and Plasma Technology Division (L&PTD). Together, these findings established CB[7] as an efficient modifier in aqueous dye laser systems as it improved the photochemical parameters of active medium immensely.

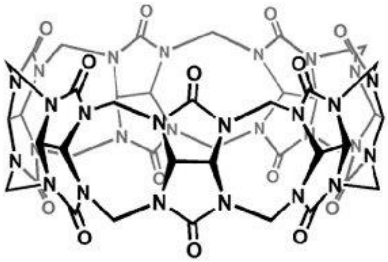


Fig. 5: Three dimensional structure of cucurbit[7]uril (CB[7])

Fluorescence based molecular probes are quite versatile considering their rapid response, easy setup and high sensitivity. These rely on changes in fluorescence colour or intensity during interactions with analytes. The first fluorescent chemosensor was reported in 1867 by Goppelsröder F. *et. al.* where they demonstrated detection of aluminium ions (Al^{3+}) using fluorescent morinchelate. During the late 1970s and early 1980s, Sousa, Bousa-Laurent, de Silva, Tsien, Czarnikand and others carried out pioneering investigations focusing on the development of fluorescent chemosensors. Since then, this field witnessed an exponential growth, thanks to its wide range of practical applications.

Contributions in this area were started since 2009-2010 with a number of synthesized dual chromophoric systems (**1**, **Fig. 6**) made of fluorescent BODIPY and pyrene dyes. One of those synthesized dyes was employed in FRET (Förster resonance energy transfer) based sensing of Fe^{2+} ions. Soon after this report, research interest was germinated within the group to develop BODIPY based fluorescent materials for sensor applications. Initially, research interest was focussed on developing new organic fluorescent materials as pH sensors, given that pH plays a crucial role in biological and chemical systems. In 2013, the imino-BODIPY (**2**, **Fig. 6**) based fluorescent materials for pH sensing was developed.

Copper and iron ions have play crucial roles in variety of physiological processes due to their catalytic and oxidative properties in living organisms. On the other side, their

excessive accumulation can cause oxidative stress and may lead to neuro-degenerative diseases. They are involved in the formation of reactive oxygen species (ROS) that eventually damage the structures and functions of lipids, nucleic acids, and proteins. In view of these physiological importance, a number of fluorescence sensors such as the BODIPY-acetylacetone derivative (**5**, **Fig. 6**) and 1,10-phenanthroline tethered BODIPY (**6**, **Fig. 6**) were developed as Cu^{2+} ions sensors. In another effort, a BODIPY-TEMPO conjugate (**7**, **Fig. 6**) was synthesized for the detection of Fe^{2+} ions present in intracellular labile iron pool. A kit was also developed utilizing the similar concept which is available for technology transfer ("A Kit for Detection of Intracellular Iron (Fe^{2+}) in live cells"). In continuation, the solvent driven aggregation-deaggregation properties of the BODIPY nanoparticles were tactfully exploited in the accurate estimation of alcohols in different solvent mixtures. A technology based on this concept was developed which is available for technology transfer ("Accurate estimation of alcohol content in alcoholic products through easy synthesis of BODIPY dye").

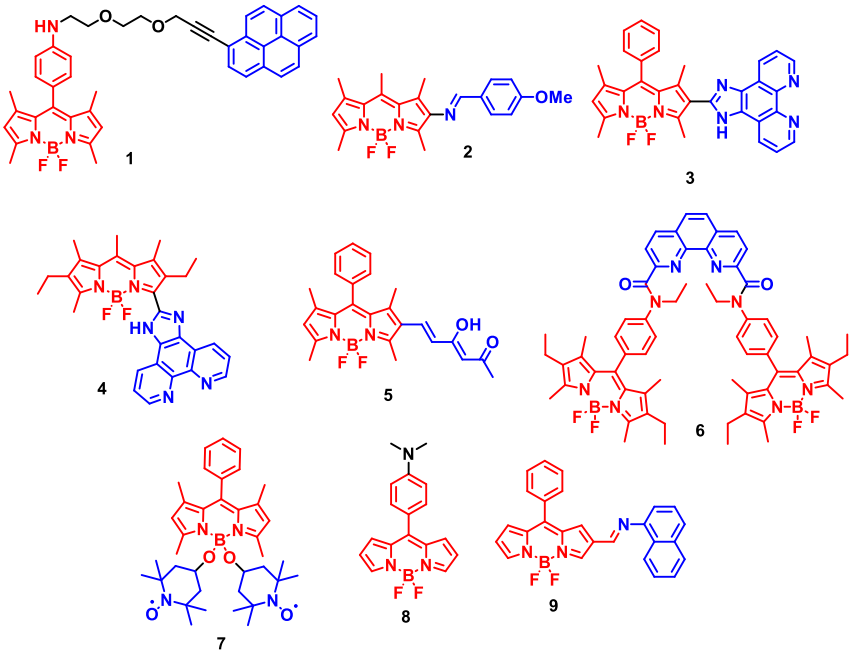


Fig. 6: BODIPY based fluorescence chemo-sensors

As a part of the chemical sensing work, a new project on development of gamma dosimeters was taken up during 2020. Scarcity of the highly sensitive, low cost and easy to use gamma dosimeters were the major motivation to develop fluorescent dye based gamma dosimeters for practical applications. Several fluorescence based gamma dosimeters were developed using the amino- and imino-BODIPY based dyes (**8** and **9**, **Fig. 7**). These dosimeters are highly sensitive, low cost, easy to use and can be used as

colorimetric as well as fluorometric dosimeters. Most importantly, dynamic range of these dosimeters can be tuned by changing the dyes concentrations which are useful for measurement of absorbed gamma doses in food and blood irradiation processes.

Efforts were also made to develop fluorescent materials for the sensing of other classes of health-related important bio-molecules. In this area, water-soluble BODIPY (**10**, **Fig. 7**) nanoparticles were developed which were used for the detection of both bovine and human serum albumins. The BODIPY based nano material was further used to monitor formation of insulin oligomer useful for the early detection of diseases like type II diabetes. In a related effort, a BODIPY based salicylaldimine Schiff base (**11**, **Fig. 7**) and its corresponding boron complex were successfully shown to detect amyloid fibrils of lysozyme that may enable early detection of amyloid fibrils linked neurological disorders like Alzheimer's disease, Parkinson's disease etc. Recently, a distylrylamino BODIPY molecule (**12**, **Fig. 7**) was judiciously designed and synthesized for developing surfactant mediated detection of bio-medically important analytes like protamine (antidote of blood clotting agent heparine) and spermine (cancer marker). In another work, Kumujian-C, a β-carboline based fluorescent probe was synthesized starting from L-tryptophan which was successfully employed for detection of sulfite in real samples.

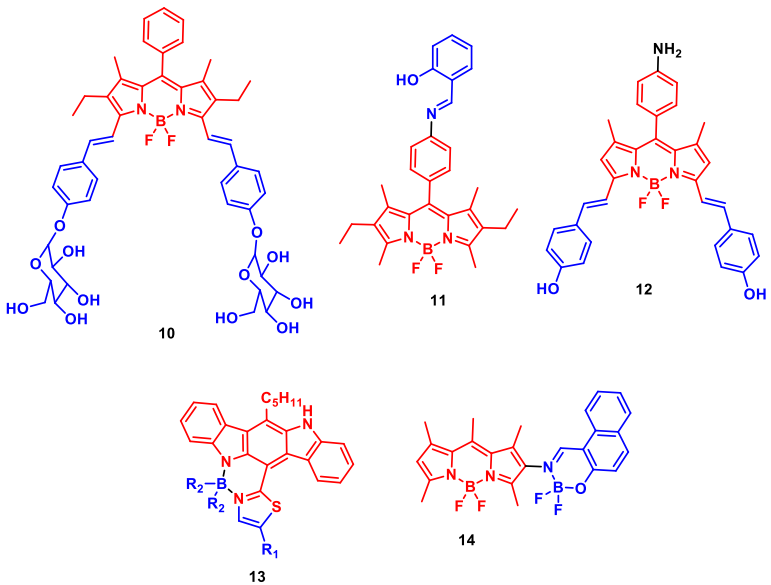


Fig. 7: BODIPY based fluorescent molecular sensors for biomolecules and photodynamic therapy of cancers

Fluorescence imaging is a highly sensitive and selective technique for biomolecules detection in organisms. Small molecular probe-based fluorescent imaging is indispensable because of its excellent selectivity and rapid response towards specific species in living systems. In this contribution, BODIPY and an indolo[3,2-b]carbazole-based boron complexes were synthesized that showed specific localization in different

cellular organelles in different cell lines. The earlier mentioned probes like BODIPY-phenanthroline conjugate (**6**), BODIPY-TEMPO conjugate (**7**) and Kumujian-C are able to detect Cu^{2+}, Fe^{2+} and sulphite ions respectively by cellular imaging.

Besides cellular imaging, organic dyes are also utilized as photoinduced therapeutics in photodynamic therapy of cancer. In this type of therapy, on photoirradiation, BODIPY based triplet photosensitizer populates its triplet excited state through an efficient inter system crossing (ISC) from its singlet excited state which subsequently generate ROS *viz*.. singlet oxygen ($^1O_2$) inside cells, thereby killing cancer cells. In this area, BODIPY-O-glucoside conjugates were well studied as photodynamic agents. Recently, a series of BODIPY-helicenes dyes (**13**) with twisted structures were developed which act as efficient heavy-atom-free triplet photosensitizers showing promising potential as photodynamic agent. With the continuation of this work, a BODIPY-naphtholimine-BF_2 dyad (**14**) was developed which targets both endoplasmic reticulum and lipid droplets, and may be useful for dual imaging, while its photosensitizing ability may be highly useful for killing of cancer cells. This is an example of next generation photosensitizers (PS) which shows "organelle-targeted-PDT" and provides new paradigm in the field of precision medicines to address the current challenge for treating pancreatic ductal adenocarcinoma (PDAC) (*JACS Au*, 2024, *4*, 2838–2852). Many of these sensing and PDT works are published as cover page articles and cover profile in various reputed journals.

4. Development small molecules for organo-electronics. Development of "Molecular Electronics" is an amalgam of physics, chemistry and engineering ideas. One of the ultimate goals of "Molecular Electronics" in nanotechnology is to build electronic devices using individual organic molecules. During the last two decades, BSG, in collaborative efforts with Physics Group contributed immensely towards the synthesis of various potential organic molecules for the development of "Molecular Electronics" as an alternative to silicon-based microelectronics. Porphyrin, pyrene, perylene and BODIPY molecules and their derivatives have been found to be good candidates for hybrid nanoelectronics such as molecular diodes, resonant tunnel diodes, molecular transistors etc.

For this, new class of molecules such as alkenyl (C-6 and C-11 chains) porphyrin, pyrene, perylene, BODIPY (σ–π systems) (**15-17**, **Fig. 8**) were designed and synthesized which were electro-grafted on H-terminated Si surfaces to form the respective monolayers. The I–V characteristics of the monolayers showed excellent rectification behaviour with very high rectification ratio (RR = 1000–5000) or negative differential resistance (NDR) with very high peak-to-valley ratio (PVR). Another interesting work was demonstrated based on donor–acceptor bilayers consisting of fullerene (C_{60}) and tetraphenyl porphyrin derivative that acted as molecular diode and showed very high (∼1500) current rectification ratio. In continuation, σ–π–σ systems based on di-O-alkylated porphyrins with phenyl and fluorophenyl groups were designed and synthesized. The monolayers of the porphyrin with fluorophenyl groups were more

compact and showed a ten-fold PVR relative to those devoid of the fluorine atoms in the porphyrin moiety.

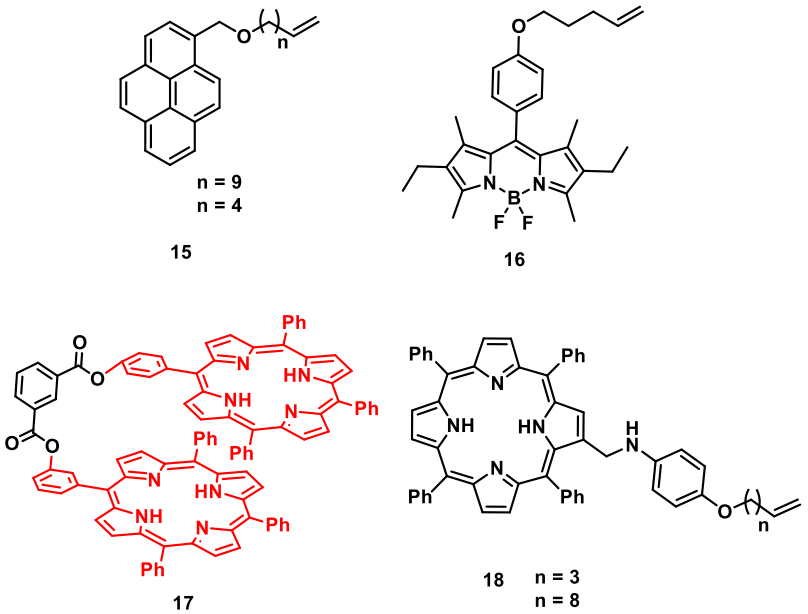


Fig. 8: Small molecules developed for molecular electronics applications

Further R&D on porphyrins resulted in the development of a new bis-porphyrin molecule (bis-TPP) (**17, Fig. 8**). The bis-porphyrin films were highly selective in sensing Cl_2 molecules with fast response (3 s) and recovery (~8 min) times, and sensitivity in the 10–500 ppb range. In another work, the bis-TPP and its Zn complex (Zn-bis-TPP) were used to develop two chemiresistive room temperature NH_3 gas (5–40 ppm in air) sensors with high selectivity, reproducibility and reversibility.

Another porphyrin (**18**, **Fig. 8**) based molecular rectifier was developed wherein a porphyrin (A) and an aniline (D) moiety were linked through a suitable spacer and electro-grafted onto Si-surfaces using the C-6/C-11 alkenyl chain at the aniline derivative as the linker to construct the respective MMS heterostructures. The C-11 containing molecular assemblies exhibited a 10^5 times higher rectification ratio (RR) than its C-6 congener, possibly because of the compact packing (*Chem. Sci.*, 2016, *7*, 1548-1557).

5. Sugar acrylamides: Tunable materials for manifold applications.

D-glucose, D-mannose, and D-galactose, three common naturally occurring hexoses, were chemically converted in to their monoacrylamide derivatives and a bisacrylamide (Glc-bis, crosslinker) from D-glucose (**Fig. 9A**). The applications of these acrylamide constituents, studied in collaboration with Chemistry Group, were demonstrated in

various fields, which include controlled drug delivery, cell imaging, bio-sensing, nanoparticle preparations etc. Homogeneous carbohydrate gels (**Fig. 9A**) made from these sugar constituents were found to be bio-compatible and hence their application in stimuli-controlled drug delivery (**Fig. 9B**) was explored in conjugation with *N*-succinyl chitosan.

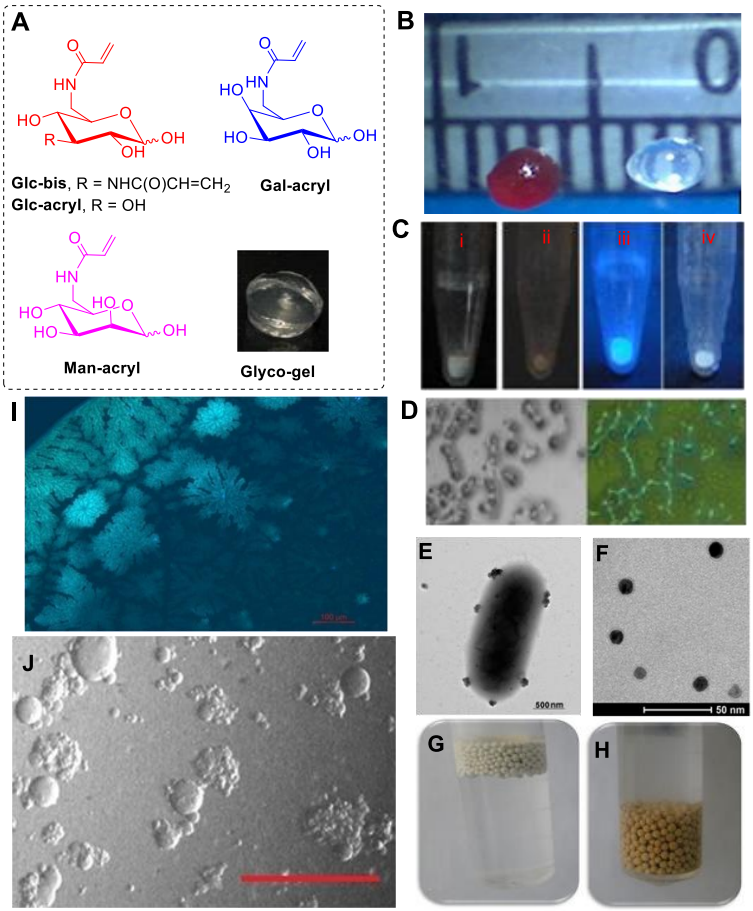


Fig. 9: (A) Structures of acrylamide derivatives of hexoses and swollen gel made from Glc-bis and Glc-acryl; (B) Optical microscope image of doxorubicin (DOX) loaded (red) and unloaded (transparent) swollen Glc-gel beads; (C) Photographic image of solid sample of Glc-acryl and Glc-bis under white light (i and ii); under UV (iii and iv); (D) Fluorescent image of Glc-acryl; (E) TEM image of the *E. coli* after treatment with Glc-bis@AuNPs; (F) TEM image of Glc-bis capped AuNPs; (G) Photographic image of commercial resin before glyco-conjugation and (H) after glyco conjugation; (I) Fractal formation observed with galacto-acrylamide; (J) Agglutination of microspheres (scale bar = 4 cm) upon interaction with *E. coli* bacterial cells

Detection of pathogenic bacteria and lectins are another important area that has been explored using the acrylamide templates. Depending on the carbohydrate units present in them, the sugar acrylamides displayed selectivity to different lectins. Another interesting property of sugar acrylamides is the inherent fluorescence exhibited by them due to Aggregated Induced Emission (AIE) (**Fig. 10C** & **10D**) phenomenon. To date, the afore mentioned acrylamides are the smallest sugar derivatives that display AIE phenomenon. This property was utilized to detect pathogenic *E. coli* bacteria (**Fig. 1E**) and lectins, *via* "*turn-on-turn-off*" mechanism in conjunction with sugar capped gold nanoparticles. The sugar acrylamides-based nanoparticles (**Fig. 9F**) displayed exceptional stability, even on storage at room temperature for months without any aggregation. The glycopolymers, made from these acrylamides, were functionalized on a commercial resin (**Fig. 9G & 9H**) and were used for the capture, detection and quantification of pathogenic bacteria and lectins. This system can sequestrate bacteria from a bulk solution without any volume reduction using external means. Formation of fractals (**Fig. 9I**) is another interesting structural characteristics observed with these small molecules. More recently, a system, based on silver loaded microspheres (**Fig 9J**) were constructed that could detect (visual observation), quantify and kill pathogenic bacteria. The outcomes clearly demonstrated the potential of the synthetic sugar acrylamides as tunable materials for multiple applications.

6. Conclusions

Over the years, organic chemistry research lead to the development of various classes of functional materials useful for different departmental projects as well as societal benefits. These includes (i) synthesis of ligands useful for Ln/An separation, (ii) development of new ligands for backend fuel cycles, (iii) synthesis of laser dyes in multi-gram scale, (iv) development of photostable laser dyes, (v) synthesis of organic supramolecules for metal extraction and laser applications, (vi) development of small organic molecules for opto-electronic applications like chemo/bio-sensing, molecular electronics, dosimetry etc, and, (vii) development of small molecules for diagnostics and therapies. Extensive research was pursued for these developments during the past years. All these resulted in new technologies for laser dyes syntheses and healthcare applications, along with high quality publications in various reputed journals. "High quality basic research towards the development of deliverables for departmental applications as well as societal applications" remains the goal of the research in organic chemistry since decades and the efforts and endeavour will continue with full vigour in the future years to come.

7. Acknowledgements

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BARC's JOURNEY IN STRUCTURAL BIOLOGY

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Abstract
This article provides a detailed account of BARC's pioneering journey in structural biology research. Beginning in the 1960s with neutron diffraction studies, the focus expanded in the 1970s to include X-ray crystallography of macromolecules. Over the years, BARC has established state-of-the-art facilities to investigate key biological processes at the molecular level through X-ray crystallography. Looking forward, the group is poised to target human disease and environmental challenges through structural biology research.

1. Pioneering Structural Biology Research at BARC

The twentieth century witnessed transformative changes in biological research. Advancements in technology enabled researchers from other disciplines such as physics and chemistry to study life systems, leading to the emergence of several new disciplines. One such discipline was 'Molecular Biology', where different cellular processes are explained in terms of involvement of specific molecules. These molecules, both large and small, chemically interact to drive biological functions. The next level of advancement was the emergence of the discipline 'Structural Biology' which enables biological processes to be understood at the atomic level, thereby rationalizing 'specificity' which is a hallmark of living systems. Unraveling how biological molecules such as proteins, DNA, cofactors, drugs, etc. bind to each other in discharging their functions is key to advancing rational molecular design for use in medicine, agriculture, and bioengineering. In keeping with the tradition of being at the forefront of research, BARC initiated

programs in Neutron Physics Division (NtPD) to characterize structures and interactions of biological molecules at the atomic-level by using physical techniques of diffraction. The radiation used in these diffraction experiments was neutrons and X-rays respectively for structures of small molecules such as amino acids, nucleotides, etc. and large molecules such as proteins, nucleic acids, etc. BARC with its nuclear reactor as a source of neutrons, was in a unique position in the country to undertake the task of neutron diffraction. The neutron crystallography was initiated at Trombay in the early sixties, while the X-ray crystallography of large molecules began later, in the late seventies. Incidentally, BARC was only the second institution in the country to venture into the challenging multi-disciplinary task of determining three-dimensional structure of a macromolecule such as a protein molecule. Some of the key developments in BARC around these activities over the years are described briefly below.

Neutron diffraction techniques at BARC, like those at other institutions world-wide, were in their infancy during the 1960s, with a strong emphasis on building instruments and developing crystallography software. Over the years, the Trombay group has maintained a position at the forefront and employed neutron diffraction to address a wide range of critical scientific questions. Among their notable contributions were the determination of crystal structures of several amino acids using neutron diffraction and the precise characterization of hydrogen bonds, which are crucial for intermolecular recognition and biological specificity.

The infrastructure setup for X-ray diffraction studies of biological macromolecules was made under the leadership of Dr. R. Chidambaram & Dr. K. K. Kannan in mid-1970s. It included: 1) a rotating anode X-ray generator procured from M/S Elliot, UK, 2) an optical scanner for digitizing X-ray diffraction patterns, 3) a precession camera, 4) an oscillation camera (Arndt-Wonacott), 5) a cold cabinet for use in protein extraction and purification, 6) fraction collector and 7) equipment for gel-electrophoresis, etc. The computer software required for macromolecular crystallography was modified to run on the PDP computers. A driver software was developed to drive the computer-controlled microdensitometer used to digitize oscillation films. Later, the software were installed on the Norsk-Data (ND-500)-system of computers that became available at that time in BARC. The graphics software used in the visualization of protein models (FRODO) and also in the interpretation of experimentally obtained electron density maps into atomic coordinates for the protein molecule, were installed on a PDP8-controlled Vector General graphics work station. This was a major advancement over the use of a stack of Perspex sheets for visualization of electron density maps (**Fig. 1**). A computer program called FRDICT was developed to create molecular dictionary for any molecule required by FRODO to enable visualization and real-time manipulation of that molecule in the graphics system. The Vector General computer graphics setup was employed to analyze the electron density of the C-subunit of an insect virus, the Black Beetle Virus. Further, computer codes were written to include stereochemical restraints in the least-squares refinement protocols and the crystal structure of triclinic hen egg-white lysozyme was refined to a low R-factor.

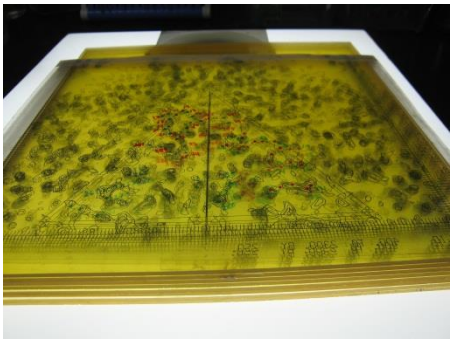

Fig. 1: A typical stack of perspex sheets used to visualize contoured electron density maps in 3-Dspace before the advent of computational graphics (until the 1980s)

2. Expanding Research and Building a National Facility

The first protein pursued at BARC for three-dimensional structural studies was Carbonic Anhydrase (**Fig. 2**), an enzyme that catalyses the reversible reaction between carbon dioxide (CO_2) and water (H_2O) to produce bicarbonate (HCO_3^-) and protons (H^+), which is involved in many physiological processes. Binding of sulfonamide drugs to the human enzyme were explored crystallographically, as a step in the design of better inhibitors of carbonic anhydrase. The crystal structure of carbonic anhydrase complexed with the product bicarbonate was determined, and this work identified active-site residues and also gave insight into the molecular mechanism of this enzyme. This structure identified presence of a hydrogen bond network involving a histidine residue and water molecules as essential for the activity of this enzyme.

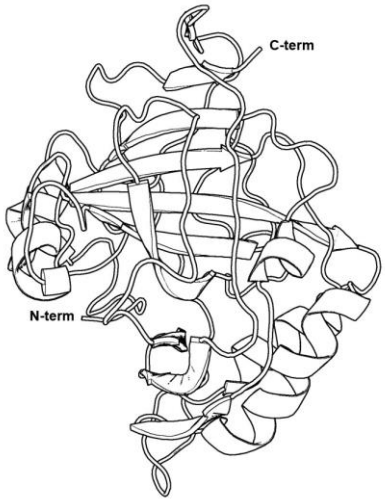

Fig. 2: A cartoon representation the 3D structure of Human Carbonic Anhydrase showing beta-sheet and helical regions

There were several other notable achievements of 1980s and 1990s. The presynaptic neurotoxin-phospholipase Notechis II-5, isolated from the venom of the Australian tiger snake, was the first protein to be crystallized and characterized within India. Further, carbonic anhydrase II was purified from erythrocytes of buffalo blood procured from the slaughter house and crystallized. This was the first enzyme structure determined from India using only the in-house facilities. Since X-ray crystallography of macromolecules is a challenging task because of its multi-disciplinary nature and was being pursued only in very few developed countries at that time, two institutions in India, Indian Institute of Science (IISc), Bangalore and Bhabha Atomic Research Centre, Trombay, were chosen for extra support to pursue this line of research: Therefore, the National Facility for Macromolecular Crystallography was setup at BARC and several experimental facilities needed for this activity were created with partial funding from the Department of Biotechnology, Govt of India. While IISc adopted the stand of creating infrastructure exclusively for diffraction data collection, BARC adopted a different strategy. We preferred to set up a self-sufficient wholesome facility that would cater to all aspects of this work, including the gene cloning, protein expression and purification, crystallization, diffraction data collection and computation. Such an approach allowed us to independently pursue any research project of our interest without seeking extra-institutional collaboration. The equipments procured and installed as a part of the Facility included DNA synthesizer (**Fig. 3a**), High-density fermenter for bacterial cell culture, Controlled temperature incubator, FPLC system with different types of separation columns (**Fig. 3b**), Peptide synthesizer, RAXIS-II imaging plate system for diffraction data collection (**Fig. 3c**) and Silicon graphics work stations for molecular modeling and electron density interpretation. Human carbonic anhydrase, an enzyme naturally found in red blood cells, was recombinantly over-expressed in E. coli bacteria by graduate student Arun Mohanty to produce large quantities for crystallographic studies. Notably, this was the first gene to be cloned in India for crystallographic work, and it was the result of a collaboration between the molecular Biology Division (MBD) and NtPD, BARC. In collaboration with MBD, crystallographic studies of a multi-enzyme complex containing plant RuBisCO was undertaken. Single crystals of the complex, isolated from spinach leaves and harboring RuBisCO bound to RuBP, were successfully grown and characterized. X-ray diffraction data were collected to a resolution of 2.5 Å at the Photon Factory synchrotron in Japan. Structural analysis confirmed the canonical L_8S_8 subunit arrangement of RuBisCO within the complex and revealed well-defined electron density for the other component enzymes and the bound RuBP substrate.

3. Advancing Structural Biology: From HIV to COVID-19 and Beyond

The facilities made available under the National Facility for Macromolecular Crystallography enabled to undertake several other projects including the plant-based toxins gelonin and saporin, HIV-1 protease, drug-resistant mutants of HIV-1 protease, HIV-1 protease substrate interactions, etc. Ribosome inactivating proteins, Gelonin and Saporin, have a potential to be conjugated with suitable antibody to form immunotoxins

for targeted killing of cancer cells. Gelonin was crystallized and diffraction data were collected on the rotating anode X-ray generator. The structure showed two cysteine residues, Cys44 and Cys50, within the protein sequence form a disulfide bond, rendering them unavailable for conjugation with antibodies. Based on structural analysis, a region of the molecule involved in intra-dimer interactions was proposed as a suitable site for introducing a cysteine residue to enable antibody conjugation and the subsequent production of immunotoxins. Saporin, which has a potential to be used as a more effective immunotoxin, was also purified and crystallized.

(a)

(b)

(c)

Fig. 3: Photographs of (a) DNA Synthesizer (1995) (b) FPLC System(1995) (c) RAXIS-II imaging plate system mounted on rotating anode X-ray generator (1996)

HIV-1 protease, a crucial enzyme for the virus's survival, has long been a target for drug development against AIDS. However, capturing the enzyme in action with its natural substrates proved challenging. At BARC, a breakthrough could be made by discovering that the protease "flaps" adopt a closed conformation even without a bound ligand (**Fig. 4**). This finding, combined with substrate soaking and X-ray diffraction on numerous crystals, allowed to obtain the first-ever crystal structures of an active HIV-1 protease complexed with true substrates, not just analogs. This groundbreaking research, published in PNAS, revealed a critical finding: a low-barrier hydrogen bond between the catalytic aspartate residues. Additionally, these studies provided evidence for the tetrahedral intermediate, a key step in the proposed catalytic mechanism. Six reaction intermediates were successfully trapped in protein crystals, enabling the proposal of a detailed mechanism of action for the enzyme. Further, neutron diffraction studies helped elucidate the protonation state of the trapped tetrahedral intermediate. These studies also delved into mutations in HIV-1 protease that arise under clinical conditions, conferring resistance to three major FDA-approved drugs: saquinavir, nelfinavir, and ritonavir. By determining the crystal structures of these drug-resistant mutants, both liganded and unliganded, it became possible to elucidate the molecular mechanisms of resistance. This work provided valuable insights for overcoming these resistance pathways.

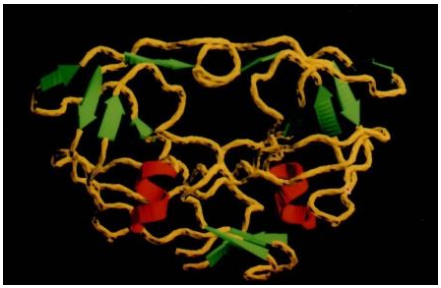

Fig. 4: Ribbon diagram of unliganded HIV-1 protease in 'closed' flap conformation

Subsequently, structural studies were conducted on a variety of other proteins to elucidate their structural and functional details. These investigations have encompassed proteins with diverse biological roles. PSP94, a semen protein associated with prostate cancer, was structurally characterized, revealing a unique dimeric architecture. In the realm of microbial enzymes, structural studies on phosphatases, including PhoN and AphA from Salmonella and SPAP from Sphingomonas, provided insights into catalytic mechanisms and facilitated protein engineering for bioremediation applications. The structure of Translin was determined to explore its potential involvement in RNA binding. To identify potential radioprotective agents, the Kelch domain of Keap1 was structurally characterized. Additionally, the structural elucidation of KatB, a manganese catalase essential for Anabaena survival, was undertaken. The mechanism of action of drFrnE, a protein from Deinococcus radiodurans, was investigated through structural analysis. In the field of photosynthesis, the structures of phycoerythrin, phycocyanin, and allophycocyanin, components of the phycobilisome, were determined to understand light-harvesting processes. Within the context of mosquito control, the structure of Cqm1, a receptor for mosquito larvicidal toxins, was elucidated. The human UVSSA protein, involved in DNA repair, was characterized structurally to map its nucleic acid binding activity. Nanobodies targeting human thyroglobulin were developed and structurally characterized, demonstrating their potential for therapeutic applications.

With the outbreak of COVID-19 pandemic in 2020, it was decided to extend our expertise on HIV protease to the proteases of SARS-CoV-2, like the Papain-like protease (PLro) and the Main protease (Mpro). These two proteases are essential for viral replication and are considered as attractive drug targets. High-throughput screening of a compound library was employed to identify PLpro inhibitors. Notably, compound ATA emerged as a potent candidate, exhibiting low micromolar inhibition of PLpro. Detailed characterization using enzymatic assays, isothermal titration calorimetry (ITC), and other biophysical methods confirmed its inhibitory activity. Furthermore, in vitro studies demonstrated significant antiviral potential against SARS-CoV-2. In vivo evaluation in a Syrian hamster model of SARS-CoV-2 infection revealed that oral administration of ATA significantly reduced viral loads in throat swabs. Independent structural analysis of Mpro crystallized with the peptide substrate revealed covalent attachment of the product peptide in the active site. In a separate line of investigation, Ceftazidime and Sennoside

A were identified as potential inhibitors of RNA binding by the SARS-CoV-2 Nucleocapsid (N) protein. Multi-dimensional NMR spectroscopy confirmed their inhibitory activity and delineated the binding sites. NMR titration experiments demonstrated concentration-dependent chemical shift perturbations, signifying ligand interaction.

The structural biology facilities have been further augmented through the acquisition and installation of advanced instrumentation. The facility's capabilities were initially expanded with the commissioning of a microfocus sealed tube X-ray diffraction system, followed by the installation of a high-flux liquid gallium metaljet X-ray source coupled with a Pilatus pixel detector. To enhance efficiency in the crystallization process, high-throughput screening and imaging systems were integrated. Complementing these structural biology tools, a comprehensive suite of biochemical and biophysical instrumentation was added, including but not limited to isothermal titration calorimeter, differential scanning calorimeter, differential scanning fluorimeter, circular dichroism spectropolarimeter, Fourier-transform infrared spectrometer, surface plasmon resonance system, dynamic light scattering system, and spectrofluorimeter.

Concurrent with the development of in-house capabilities, a dedicated protein crystallography beamline was established at the INDUS-II synchrotron, RRCAT, Indore. This beamline enables high-resolution diffraction studies on macromolecular crystals, including proteins, DNA, and their complexes. The beamline offers precise energy tunability in the range of 5 - 20 keV, facilitating single and multi-wavelength anomalous diffraction experiments. With data acquisition times ranging from 5 to 30 seconds per frame, the beamline serves as a valuable resource for researchers across the nation. Infrastructure for remote usage of synchrotrons abroad for diffraction data collection was set-up at HBNI, Anushaktinagar.

4. Way forward

Building upon the foundation in structural biology, ongoing exploration of intricate molecular mechanisms underlying critical diseases and environmental issues will be continued. The structures and functions of key proteins will be elucidated to develop innovative therapies and sustainable solutions. Studies on SARS-CoV-2 enzymes and Plasmoredoxin are aimed at developing new antiviral and antimalarial drugs, while research on cancer targets MTHFD2 and KRAS focuses on uncovering novel cancer therapies. Additionally, the complex interplay between the microbiome and cancer drug metabolism is being investigated to optimize treatment modalities. Research into the structural properties of enzymes, such as terephthalases, aims to engineer solutions for plastic degradation. These efforts are intended to translate findings into tangible benefits for human health and the environment.