MICROBIAL CELLS- AND BIOFILM-MEDIATED BIOREMEDIATION

Celin Acharya[*1,3], Y. V. Nancharaiah [2,3] and V. P. Venugopalan[2,3]
[1]Molecular Biology Division
Bhabha Atomic Research Centre
Mumbai - 400085, India
[2]Water & Steam Chemistry Division
Bhabha Atomic Research Centre
Kalpakkam - 603102, Tamil Nadu, India
[3]Homi Bhabha National Institute, Mumbai - 400094, India

[*]Email: celin@barc.gov.in

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

We gratefully acknowledge the valuable contributions from Dr. Hema Rajaram, Ms. Divya T.V. and Ms. Devanshi Khare from MBD, BSG, BARC and Dr. C. S. Misra, Dr. Shyam Sunder R. and Dr. Devashish Rath from AGS, BSG, BARC for the manuscript.