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BIOREMEDIATION OF AQUATIC TOXICANTS:
APPLICATION OF MULTI-OMIC APPROACHES

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ABSTRACT

Aquaculture is one of the most important economic ventures in many countries across theworld. Over the last few decades, there has been increased demand for seafood by consumers, which hasnecessitated the development of novel strategies for enhanced production of aquaculture. However, environmental and health challenges are increasingworldwide which are recognized as significant constraints on aquaculture production andtrade. Indiscriminate use of chemicals in agriculture, and industrial effluents adverselyaffect the aquaculture and related environment. Removing the chemical contaminants, from the environment is a difficult task. Therefore, various strategies to remediate the chemical contaminants are being looked into. In this context, bioremediation or the utilization of microorganisms to clean up these contaminants from apolluted environment has appeared as a promising strategy for removing and/or decreasing the effect of the contaminants. Recent advancements made in this field of research are discussed in this chapter.

Keywords: Bioremediation, Aquatic toxicants, Omics technology, Aquatic pollution

INTRODUCTION

Aquaculture is one of the most important economic venture in many countries across the world. Over the last few decades, there has been a continous decline – in marine fisheries and the increased demand for seafood by consumers (Boopathy 2009), which has necessitated the development of novel strategies for enhanced production of aquaculture. The current worldwide growth rate of the aquaculture business (8.9 – 9.1%/yr) needs to be increased in order to cope with the problem of shortage in protein food supplies (Ponniah and Krishnani 2009). However, environmental and health challenges are increasing worldwide which are recognized as significant constraints on aquaculture production and trade. Indiscriminate use of chemicals in agriculture, and industrial effllents adversely affect the aquaculture and related environment. The fate of these aquatic contaminants

depend largely on the various metabolic activities of microorganisms present in aquaculture ecosystem. Bioremediation is one of the most rapidly growing areas of environmental biotechnology (Fig. 1). The utilization of microorganisms to clean up these contaminants from a polluted environment represents a potential solution to such environmental problems. Microbial bioremediation could be advantageous with the result of formation of completely non toxic end products, which can be beneficial for human health perspectives. The biochemical importance of bioremediators indicate a need for development of reliable methods for identification of environmentally important microorganisms. Adoption of 16S rRNA gene approach and culture independent – nucleic acid based techniques have led to the realization that microbial populations in the natural environments are very diverse. Holistic approaches, such as "omics" like proteomics, genomics, transcriptomics and metabolomics offer new ways of facing the challenges of environmental pollution and has greatly improved the efficiency of bioremediation predictability and reliability. Adoption of nucleic acid methods based on the sequencing of metagenomic clone libraries has provided an insight into the diversity of microbial populations in terms of sequence and the phylogenetic information of an individual clone. A typical metagenomic study combines the potential of genomics and bioinformatics in exploring the collective microbial genomes, isolated directly from the environmental samples. Any single 'omics' approach may not be sufficient to characterize the complexity of fundamental microbial biology. Therefore, integration of multiple layers of information, the multi- 'omics' approach is required to acquire a precise picture of living micro-organisms. This review is extensively focused on aquatic toxicants, their adverse impacts on agriculture, aquaculture and their possible bioremedial measures through multi-omics approaches.

AQUATIC TOXICANTS AND METABOLITES

Aquaculture water can come from one source or a combination of several sources such as ground water, surface water (freshwater, brackishwater and seawater) and alternative source (rain water). Based on salinity, aquaculture is classified as freshwater aquaculture, brackishwater aquaculture and mariculture. In freshwater aquaculture systems, two inorganic forms of nitrogen, unionized ammonia and nitrite are highly toxic to fish when present at higher concentration. High ammonia concentrations are common in ponds with very high feeding rates, high organic matter and also in sewage-fed ponds. The proportion of ammonia increases with increase in pH and temperature of water. This affect adversely in enyme catalysis reaction and membrane stability, increases the oxygen consumption by tissues, damage gills and reduces the ability of blood to transport oxygen. The U.S. Environmental Protection Agency (EPA) has established three kinds of criteria (one acute and two chronic) for ammonia (nitrogen), based on the duration of exposure. The acute criterion is a 1-hour average exposure concentration and is a function of pH. One chronic criterion is the 30-day average concentration and is a function of pH and temperature. Another concern for ammonia problems occurs after a crash in the algae community. Rapid decomposition of dead algae reduces the DO concentration and increases ammonia concentrations.

Brackishwater shrimp aquaculture has expanded rapidly worldwide especially in tropical areas, such as Southeast Asia and Latin America. One approach to improve sustainability has been the development of high intensity grow-out systems with no water discharge during the crop cycle (Burford and Lorenzen 2004). A zero-water exchange system also generates toxic ammonia,the major end product of protein catabolism as a result of excess feed and faecal waste (Boopathy 2009; Boyd *et al.,* 1998), which can adversely affect shrimp aquaculture productivity. Ammonia remains in the form of unionized ammonia (NH_3) and ionized ammonia (NH_4^+). Unionized ammonia is a critical water quality parameter and toxic to aquatic life, which adversely affects shrimp yield.

Nitrogenous metabolites such as NO and N_2O produced during the process of denitrification are well known potent greenhouse gases. The nitrous oxide concentration has increased in the atmosphere from 275 ppb in 19th Century to 315 ppb in 21st century, which has mainly been attributed to anthropogenic inputs (Stres *et al.,* 2004). Small N_2O accumulation may cause destructive effects for centuries due to its long half life of 120 years, and its 310 times more global warming potential than carbon dioxide (Trogler 1999).

Aqua-farmers have a great need for effective and economical management method for the treatment of nitrogenous toxicants and metabolites. Understanding ammonia and controlling it is critical in aquaculture systems. Even though practical ammonia management actions may be limited in a large pond aquaculture setting there may be some ways to reduce ammonia levels but others may exacerbate the situation - no method is a complete long-term solution in and of itself. Aeration can be ineffective at reducing overall pond ammonia concentrations due to the relatively small area of the pond being aerated. Application of liming materials could actually make a potentially bad situation much worse by causing an abrupt and large increase in pH. Increasing pH will shift ammonia toward the form that is toxic to fish.

During recent years, many researchers have focused their interest on heavy metals due to their known toxicity and carcinogenicity as they are discharged in small quantities by numerous activities such as rapid industrialization, urbanization and anthropogenic sources into the environment. These have potential adverse health impact on public as well as the aquatic species as they are conservative pollutants which are permanent additions to the aquatic environment. The worst part about these toxicants is that they are not subject to biodegradation or breakdown into simpler forms. For these reasons, the legislations governing the levels of contaminants is becoming progressively more stringent. Various agencies have recommended safe levels of heavy metals for protection of drinking water, fish and other aquatic life (2004). For protection of fish and aquatic life, the safe levels recommended by Tennessee Water Quality Control Board are 0.47 mg/l for Ni, 0.12 mg/l for Zn, 0.065 mg/l for Pb, 0.016 mg/l for Cr(VI), 0.013 mg/l for Cu, 0.002 mg/l for Cd and 0.0014 mg/l for Hg, respectively. Acute toxicities of heavy metals such as Hg, Cu, Cr and Mn have been determined for the fish *Lates calcarifer* using static bioassay tests (Krishnani *et al.,* 2003). This has helped in deriving allowable safe levels of heavy metals for the fish.

BIOREMEDIATION

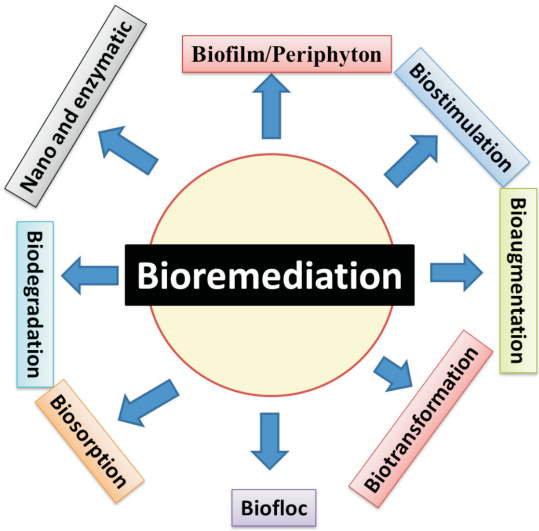


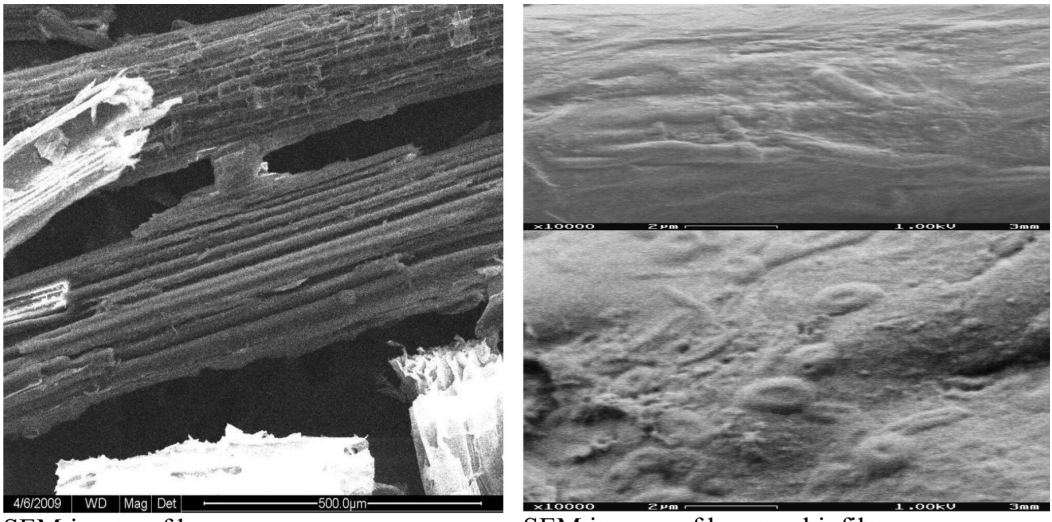
Fig. 1. Types of bioremediation

Biofilm/periphyton Based Bioremediation/Plant Aassisted Bioremediation

Provision of artificial feed accounts for nearly 50–60% of the production cost and more often is beyond the reach of poor farmers. Further, only 15–30% of nutrient input is converted into harvestable products in most feed-driven, pond production systems, the remainder being lost to the sediments, effluent water and the atmosphere (Gross *et al.*, 2000). In recent years, efficient utilization of agro-wastes has been increasing. Substrates provide sites for epiphytic microbial production consequently eaten by fish food organisms and fish. Fish easily exploit the sessile forms of bacteria colonized on the surface of substrates as compared to free planktonic forms. In this direction, adoption of microbial biofilm – based on agrowaste-periphyton in freshwater aquaculture system has the capacity to increase the productivity by conversion of nutrients into harvestable products (Mridula *et al.,* 2003; Azim *et al.,* 2001; 2002; Keshavanath *et al.,* 2001; Ramesh *et al.,* 1999, Umesh *et al.,* 1999, Wahab *et al.,* 1999).

Organisms such as algae, zoogleal, filamentous bacteria growing on aquatic macrophytes and other submerged substrates/surfaces are termed as periphyton, which has more than one role in aquaculture (Keshawanath *et al.,* 2001). Periphyton grown on these substrates are excellent natural food for certain fish species and support enhanced fish production. Periphytic bacteria in the biofilm play a key role in the production of enzymes, degradation of organic matter and environmental toxicants. Hence, periphyton improves production and water quality as well (Azim *et al.,* 2001; 2002; Keshawanath *et al.,* 2001). This process is often referred to as plant assisted bioremediation or periphyton based bioremediation. India is an agricultural country and generates considerable amount of

agricultural waste/byproducts such as sugar cane bagasse, paddy straw, rice husk, wheat corns, coconut husk, ground nut husk, crop wastes, peanut hulls, fertilizer wastes etc. Bagasse is a complex native cellulosic fibrous waste left after extraction of juice from cane sugar. This is an attractive agricultural by-product for a pond supplement because of its low cost and general availability across shrimp-growing latitudes. One potential use of bagasse is as a feedstuff for shrimp, as this forms a potential base for feeds when applied to extensive shrimp cultures, and has no adverse effect on water quality. Bagasse is a biodegradable substrate, which harbours higher periphytic biomass than non degradable ones. This could be because biodegradable substrates provide a better surface structure for periphytic species to attach to, or they may leach nutrients beneficial for the growth of periphyton, predominantly consisting of bacteria. In bagasse treated ponds, biofilm acts as a biofilter with the result of significant increase in production (Fig. 2). However, further research is needed to determine the optimal ways to produce natural biota, principally micro-algae and phytoplankton, and optimize the nutritional composition. It would also be beneficial to determine the role of natural biota in supplying the other nutritional requirements of the shrimp, and ultimately to determine the effect of the natural biota on shrimp growth.



SEM image of bagagsse SEM images of bagasse biofilm

Fig. 2. Scanning electron microscopic images

For the advantage of the artificial substrates on shrimp growth, various researchers have carried out experiments in an intensive freshwater and brackishwater aquaculture (Arnold *et al*., 2005, 2006; Ballester *et al*., 2007; Bratvold and Browdy, 2001; Burford *et al*., 2004a; Kumlu *et al*., 2001; Tidwell *et al*., 1998, 1999; Thompson *et al*., 1999, 2002;; Zarain-Herzberg *et al*., 2006). They obtained different results and gave different recommendation for improvement of water quality, addition of natural food supplement, limited reproduction of pathogenic bacteria which provide refuge for shrimp to escape any negative behavioural interactions and adding living space. However, there is not an agreement

about the predominant factor among those factors. Ballester *et al*. (2003; 2007) determined that growth and survival of shrimp were not enhanced in the presence of floated cages that had their biofilm periodically removed, suggested that the importance of using substrates for shrimp is not related to space but to the availability of food provided by biofilm formed on the substrate. While recent study by Zhang (2010), who studied the effects of artificial substrates on the spatial distribution of shrimp in the intensive culture condition, suggested that the difference of the shrimp growth and survival were affected mainly by living space added with the addition of artificial substrates. Therefore, a better understanding of the effect of artificial substrate on shrimp performance is necessary.

Biostimulation

Biostimulation involves the addition of electron acceptors, nutrients, or electron donors to increase the number or stimulate the activity of indigenous biodegradative micro-organisms. For biostimulation purposes, an important choice criterion is the substrate cost, which, combined with the interest in byproducts recycling, has been leading to an increasing search for cheap and available potential biofilm carriers. The very low cost of this lignocellulosic material is a real advantage that renders it a suitable alternative for the remediation of ammonia. In India, large amount of solid wastes are produced by agriculture-based industries, mainly sugarcane bagasse from distilleries and sugar industries. Sugarcane bagasse, the residue obtained after crushing the sugarcane to extract the broth, is the most abundant lignocellulosic residue (Pandey *et al.,* 2000). Although most of bagasse have been employed in the own sugarcane industry to generate energy. There is a surplus of this agro-industrial waste and several alternatives for its utilization have been evaluated, among which bagasse as cell support in different bioprocesses (Sene *et al.,* 1998). Bagasse consists of approximately 50% cellulose, remaining hemicellulose and lignin and 2.4% ash. Because of its low ash content, bagasse offers numerous advantages in comparison to other crop residues such as rice straw and wheat straw, which have 17.5% and 11.0% ash contents, respectively. Bagasse can be used as the source of carbon (energy), and it can also be used as an inert solid support. Over the years, a large number of micro-organisms were cultivated on bagasse including bacteria, yeasts and fungi (Pandey *et al.,* 2000). Santos *et al.* (2008) has used bagasse as alternative low cost biomaterial for the immobilization of *C.guilliermondii* for fermentation application. Krishnani *et al* (2006a; 2006b; 2010; 2013) have successfully demonstrated the use of lignocellulosic bagasse material as a biostimulator to maintain ammonia and nitrite in coastal shrimp aquaculture which leads to increase in shrimp production through the enhancement of autotrophic nitrifying bacteria. Krishnani and Kathiravan (2010) have further demonstrated through field trials and employing various molecular tools to quantify ammonia oxidizing bacteria (AOB's) in bagasse biofilm. Translation into improvements in shrimp growth and production efficiency has been established. The sugarcane bagasse has been proposed and investigated as an alternative, abundant and economical biostimulator. Bagasse as biodegradable substrate has significant role in Ca^{++} ion exchange mechanisms combined

with biostimulation through supporting the biofilm mode of growth of nitrifying consortia. A zero-water exchange system is an environment friendly alternative to conventional aquaculture for producing high-density shrimp. However, this system also generates toxic nitrogenous metabolites such as ammonia and nitrite, which adversely affect shrimp yield. A number of natural products, waste materials have been tested for biostimulation capacity in shrimp aquaculture. A biostimulator developed from bagasse has been demonstrated for bioremediation of ammonia, chromium and nitrite in shrimp aquaculture. Biostimulator as a biodegradable substrate has significant role in Ca^{++} ion exchange mechanisms combined with biostimulation through supporting the biofilm mode of growth of nitrifying consortia, which has been substantiated by quantifying *ammonia monooxygenase* gene (*amoA)* by real-time PCR. Innovative way of using bagasse and its efficacy as biostimulator has been exploited. Integration of bagasse-biostimulation technology (@ 10 kg biostimulator/ hectare shrimp pond) in coastal aquaculture for 2-3 months led to 29-52% ammonia removal and 4-28% higher shrimp production in the aerated culture ponds. This is due to a biofilm mode of growth of nitrifying consortia and periphytic growth onto bagasse. Biostimulation technology is simple, cost effective without much technical sophistication. Integration of this technology in zero water exchange could be advantageous for water savings and reduced risk of contamination which leads to a better environmental control.

Bioaugmentation

In situations, where indigenous degraders cannot rapidly degrade recalcitrant chemicals, bioaugmentation may be the only means for successful bioremediation. Bioaugmentation involves the addition of indigenous laboratory grown microorganisms capable of biodegrading the target contaminant or serving as donors of catabolic genes. Nakano *et al.* (2008) developed microbial consortium to remove nitrogen from aquaculture through the coupling of ammonia-oxidation using *Nitrosomonas* spp., and denitrification using *Pseudomonas* sp. and *Alcanivorax* spp. Diep *et al*. (2009) isolated *Pseudomonas stutzeri* strains from catfish pond, which were effective in lowering soluble N (NH_4, NO_2 and NO_3) levels in fishpond water from 10 mg/l to negligible amounts after 4 days. The nitrifying organisms are aerobic and have a high requirement for oxygen. Fernandes *et al*. (2010) have demonstrated that in high-density ponds, the aerators served to stimulate bacterial growth and activity which consequently maintained the quality of the water to match that of low-density ponds. They observed a marked increase in ammonium content in the non-aerated shrimp pond at the end of the culture period. This result is in agreement with those of Fernandes *et al*. (2010) who reported that the removal of ammonia was not significant due to lack of aeration and undetectable nitrifying bacteria (PCR -ve) in soil samples originally or less numbers of nitrifying bacteria causing poor biofilm formation. Fu *et al*. (2009) set up biological aerated filter bioaugmented with heterotrophic nitrifying bacterium *Lutimonas* sp. H10 for ammonia removal in the circulation water in a marine aquaculture, where the ammonia removal was not improved. This bioaugmentation failure was attributed to the poor biofilm forming ability of the inoculated strain.

Greenwater technology-An innovative bioagumentationCommon problem of aquaculture world-wide is vibriosis, predominantly in India creating economic losses (Raju 1994). In this particularly, *Vibrio harveyi* (Moriarty, 1999) is a luminous gram negative bacterium causing mortalities among *P. monodon* larvae, post larvae and cultured shrimp (Le Groumellec *et al.*, 1996). Even after the treatment of ponds with lime and chlorination couldn't be wipe out *V. harveyi* (Karunasagar *et al.,* 1996). *Vibrio* spp. are difficult to be controlled in aquaculture and related aquatic environment when the stocking density is high. Not all organisms are susceptible to infection, and both internal and surface-associated chemical defenses may account for the observed resistance of some species to microbial attack (Engel *et al.,* 2002; Lane and Kubanek, 2008). Probiotic technology provides a solution to these problems, wherein selected probiotic strains are added in the shrimp pond to displace deleterious normal bacteria. Ecologically realistic studies revealed that in the marine realm, numerous taxa have been suggested to use surface-associated defenses against competitors, foulers, and pathogens (Kelly *et al.,* 2003, Nylund *et al.*, 2005). Pan *et al.* (2008) and Fjellheim *et al.* (2010) have evaluated antagonistic activity of probiotic bacteria against pathogenic bacteria. The mechanism of probiotic bacteria is very difficult to understand. Controlling of pathogenic bacteria through a variety of mechanisms: competitive exclusion (Garriques and Arerals, 1995; Moriarty, 1999), improvement of water quality (Velmurugan, 2009), enhancement of immune response of host species (Andlid *et al.*, 1995), enhancement of nutrition of host species (Garriques and Arerals, 1995). Three types of probiotics such as soil probiotics, water probiotics and feed probiotics are used in aquaculture. Majority of these probiotics are normally applied for controlling abiotic stresses. However, there are few probiotics, which are used for the management of biotic stresses. There is always need to integrate bioaugmentation/supplement these organisms in the ponds for disease control. This will help in improving the environment of culturing ponds which leads to the higher production. The most widely studied microbial species from the coastal waters as a source of antibiotics has been the *Streptomyces* species (Blunt *et al.,* 2007). Reports on isolation, purification and structural elucidation of active compounds from pharmacologically promising marine organisms associated bacteria from Indian marine waters are scanty (Anand *et al.,* 2006; Krishnani 2010). There is a huge potential for venturing into the micro niche of exploring the chemical potential of the bacterial diversity associated with marine organisms as a source of novel biomolecules. Recent developments in molecular-biology-based techniques have led to rapid and accurate strategies for monitoring, discovery and identification of novel bacteria (Krishnani *et al.,* 2009a; 2010; Krishnani and Kathiravan 2010). The greenwater culture system is an innovative bioaugmentation technique, in which herbivores finfish mainly the grey mullet (*Mugil cephalus*) and milkfish (*Chanos chanos*) are propagated as bioremediators in fish cages in shrimp growing ponds. This method is proven most functional among all other (Baliao *et al.,* 1999; Baliao 2000) for coastal aquacultural management. Euryhaline fishes have broad diet spectrum and tolerance to poor water quality, which makes them ideal candidate species for zero-water exchange system. Bioaugmentation technology by integration of milkfish in pens in shrimp ponds has successfully been demonstrated (Krishnani *et al.,* 2012) (Fig. 3). Bioaugmentation technology can be used for controlling shrimp pathogenic bacteria with the result of higher shrimp production.

Fig. 3. Greenwater technology in coastal shrimp aquaculture

Kathiravan and Krishnani (2014) isolated novel heterotrophic nitrifying and aerobic denitrifying bacteria from greenwater system of coastal aquaculture. Based on the 16S rRNA gene, FAME analysis and biochemical test, the isolates have been identified as *Pseudomonas aeruginosa* and *Achromobacter* sp. These have been named as *P. aeruginosa* strain DBT1BNH3 and *Achromobacter* sp. strain DBTN3. Denitrifying functional genes such as *nitrite reductase* (*nirS*), *nitric oxide reductase* (*qnorB*) and *nitrous oxide reductase* (*nosZ*) genes have been identified. Molecular techniques based on the functional gene revealed that these strains also found to be aerobic denitrifies indicating that they have an oxygen-tolerant denitrifcation system. These strains found to have a 27 kb plasmid coding for *nirS* and *nosZ*. The possibility of horizontal transfer of plasmid among Pseudomonadaceae and Alcaligenaceae families in coastal aquaculture has been explored. Combined nitrification and oxygen tolerant denitrification potential in the same isolates have been studied.

Bio-floc Based Bioremediation

Biofloc technology is a technique of enhancing water quality in aquaculture through balancing carbon and nitrogen in the system. This is considered as an resourceful alternative system since nutrients could be continuously recycled and reused. The technology has recently gained attention as a sustainable method to control water quality, with the added value of producing proteinaceous feed in situ (Crab *et al.,* 2012). The sustainable approach of such system is based on growth of microorganism in the culture medium, benefited by the minimum or zero water exchange. These microorganisms (biofloc) has two major roles: (i) maintenance of water quality, by the uptake of nitrogen compounds generating "in situ" microbial protein; and (ii) nutrition, increasing culture feasibility by reducing feed conversion ratio and a decrease of feed costs. The relative importance of this technique

depends on many factors, among them the daily feeding rate, suspended solids (biofloc) concentration, ammonia concentration, light intensity, and input carbon-to-nitrogen (C:N) ratio. The consumption of biofloc by shrimp or fish has demonstrated numerous benefits such as improvement of growth rate (Wasielesky *et al.*, 2010), decrease of FCR and associated costs in feed (Burford *et al.*, 2004b). Biofloc meal (also called "single-celled" protein), added to compounded feed is current focus of intensive research in nutrition fields (Kuhn *et al.*, 2009).

Biodegradation

The ultimate "sink" of the pesticides applied in agriculture and public health care is soil. Microorganism's plays major role in the degradation of pesticides into simpler non-toxic compounds. This process is known as "biodegradation". Hence, biodegradation is a process, by which microbial organisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment. Chemicals that show complete resistance to biodegradation are called "recalcitrant". Biodegradation of nitrogen and wastewater treatment are examples parameters in aquaculture. For successful biodegradation of pesticide in aquaculture soil, following aspects must be taken into consideration. i) Organisms must have necessary catabolic activity at fast rate to degrade the contaminant, ii) Congenial soil conditions for microbial growth and enzymatic activity (Soil moisture, temperature, pH and organic matter content) , iii) Cost effectiveness of bioremediation technology, Iv). Pesticide bioavailability (solubility).

Biotransformation

Biotransformation of heavy metals

Commonly found heavy metals in the environment include lead, mercury, arsenic, chromium, cadmium, nickel, zinc, and copper. Among these metals, chromium (Cr) is one of the most toxic pollutants generated by anthropogenic activities such as chromium electro-plating, leather tanning, wood preserving and manufacturing of steel and other alloys, bricks, dyes, pigments and fungicides, etc. The two significant oxidation states of chromium are hexavalence (Cr(VI)) and trivalence (Cr(III)) whereas the Cr(VI) is more hazardous than the Cr(III).The trivalent form of chromium, Cr(III), is about 1000 times less toxic than Cr(VI) and can be readily precipitated or adsorbed as $Cr(OH)_3$ by organic and inorganic substrates at neutral pH. Economically viable and eco-friendly products has been developed from bagasse and coconut husk for detoxification of toxic hexavalent chromium into least toxic trivalent chromium in coastal waters. Bagasse and coconut husk charred with acid have been found to be most effective for the detoxification of Cr(VI) into Cr(III) in acidic medium, due to their electron donating capacity, whereas the removal of Cr(VI) in the treatment with other products in alkaline medium was due to increase in native microbial community (Krishnani *et al.,* 2004: Parimala *et al.,* 2007). Conducting nano polymers have also been used in detoxification of Cr(VI) in to Cr(III). (Krishnani *et al.,* 2013).

Biotransformation of potent green house gases

Nitrous oxide is a potent greenhouse gas, contributor to ozone layer destruction and its released from fixed N. This process is entirely controlled by microbial activities. Mitigation of N_2O emissions to the atmosphere has been attributed exclusively to denitrifiers possessing NosZ, the enzyme system catalyzing N_2O to N_2 reduction. ANAMMOX is the only process, which prevent greenhouse gases produced during denitrification. Non-denitrifying populations with a broad range of metabolisms and habitats are potentially significant contributors to N_2O consumption. These diverse microbial taxa possess divergent nos clusters with genes that are related yet evolutionarily distinct from the typical nos genes of denitirifers (Sanford *et al.*, 2012).

Biosorption

Water contaminated with heavy metals are generally cleaned by chemical precipitation, adsorption using activated carbon, evaporation, electrochemical treatment and the use of ion-exchange resins. However, their high cost restricts large-scale use for the abatement of heavy metal pollution. In recent years the search for low-cost materials has grown. The use of lignocellulosic agrowastes is environmentally viable for effective removal of heavy metals such as Pb^{2+}, Cd^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+} (Krishnani and Ayyappan 2006). Complete removal mechanisms of heavy metals using biosorbents developed from paddy straw and bagasse have been ascertained through various mechanistic approaches such as batch isothermal, experimental breakthrough curves, Langmuir isotherm, Scatchard plot, scanning electron microscopy, X-ray photo electron spectroscopy, Fourier transform infrared spectroscopy and potentiometric analysis (Krishnani *et al.,* 2008; 2009). Chemical modification applied to the native agro-wastes led to improvements of the cation exchange and adsorption capacities. This is due to increase in surface area, facilitation of transport of metal ions to the functional binding sites in the form of multiple strong and weak acidities and ligands on biosorbent. Development of biosorbent could provide cheap alternative of costly conventional techniques because of its higher fixation capacity, effectiveness in a wide range of pH such as alkaline, slightly alkaline, neutral and even slightly acidic waters (Krishnani *et al.*, 2008).

Nanoremediation

New nanoparticles and its role in environmental remediation is the subject of extensive research. Nano-biotechnology has appeared as new and innovative weapon to counteract multiple problems related to abiotic and biotic stress in fishery. Application of nanostructured materials can be alternative of physical, chemical and biotechnological measures to remediate waste water. Nanotechnologies that perform pollutant degradation are particularly attractive for organic contaminants. Krishnani *et al.* (2012) have synthesized silver-ion-exchanged-zeolite, which has successfully been demonstrated for nanobioremediation of ammonia and shrimp pathogenic bacteria. The use of conducting nanopolymers and PEG based

amphiphilic nanopolymers have also been successfully demonstrated for detoxification of Cr(VI) and bactericidal activity (Krishnani *et al.,* 2014; 2015). Zeolites are three-dimensional, microporous and crystalline solids, which have increasingly been used in water treatment because of their ion exchange properties and thermal stability (Fig. 4). Zeolites have cation exchange capacity for various applications in industries, agriculture and aquaculture because of its cage-like structure consisting of SiO_4 and AlO_4 tetrahedra joined by shared oxygen atoms. Nano(bio)remediation to detoxify these contaminants could be an efficient, economical approach.

Fig. 4. Zeolite materials

Enzymatic Bioremediation (Recombinant DNA technology)

Expression cloning is one of the most basic techniques of molecular biology to study protein function, wherein, DNA coding for a protein of interest is cloned into a expression vector, which may has special promoter elements to drive production of the protein of interest (Alberts *et al.,* 2002). Gene transfer among bacteria (gene bioaugmentation) has the potential to become a powerful tool in environmental management (Pepper *et al.,* 2002). This is a process of obtaining enhanced activity after gene transfer from an introduced donor organism into a member of the indigenous soil population.

Nutri-bioremediation

In the last decade, extensive research has been done on nutritional remediation on environmental contaminant especially pesticide. Among all form of pesticide, the organochlorine are most dangerous in term of persistence. The endosulfan is more persistent in aquaculture soil, with an average field half-life of 50 days, but if it adheres to clay particles it will persist for many years in soil and water. Kumar *et al.* (2011, 2012, 2014a, 2014b) have developed a nutritional remediation system which protect the aquatic organism especially fish to develop immunity against pesticide. The nutritional components viz. methyl donors (Muttappa *et al.*, 2014), pyridoxine (Akhtar *et al.*, 2010), yeast RNA, omega 3 fatty acid (Jha *et al.,* 2007), microbial leven (Gupta *et al.,* 2013) have very important nutritional component which have role in remediation against environmental contamination.

MULTI-OMIC APPROACHES

Multi-omics techniques help in making rapid and accurate strategies for monitoring, detection and characterization of novel bacteria and their functional genes implicated in detoxification processes. A better understanding of genetic diversity of these extremophiles is of keen interest because of its relevance to bioremediation of priority pollutants.

Genomics

16S rRNA gene and functional gene based techniques

Molecular identification by 16S rRNA sequence is a valuable tool for identifying and characterizing bacterial diversity, which can be helpful in understanding the marine and freshwater bacterial interaction. One classical molecular approach to assess and monitor contaminated sites is targeting the ribosomal RNA gene. The exploration of microbial world has been revolutionized by 16S rRNA gene based studies, but such studies mainly decipher microbial diversity and phylogenetic description of community members, providing little insight into the genetics, physiology and biochemistry of the members. A major limitation of the 16S rRNA gene approach lies in, biasness towards dominant bacterial groups, intra-genomic heterogeneity of the 16S rRNA gene itself and presence of mosaicism in gene which ultimately leads to the selective analysis of phylogenetically diverse bacteria. Identifying genes directly involved in the chemical process of interest could complement 16S rRNA gene based approaches. Molecular approaches based on functional genes allow a greater resolution for the study of genetic differences in natural populations. Molecular methods detect the presence of the genes responsible for the degradation capability, when the molecular pathway is known. A better knowledge of these functional genes is required to develop molecular approaches for environmental studies on the ecology of microbial population. These molecular techniques are proved to be most effective in detection of low copy numbers of target genes. The real time PCR technique has been used to assess quantitatively specific functional genes at very low concentrations in complex community in a variety of environmental samples.

Nitrogen is the most vital element in our atmosphere and a component of many biomolecules, is essential for growth and development of all organisms (Christoph *et al.,* 2007). As atmospheric nitrogen cannot be directly utilized, it must be reduced to ammonia by nitrogen-fixing bacteria present in the environment for the availability of living systems. The gene encoding *nifH* is largely unique to nitrogen fixing bacteria, which convert atmospheric nitrogen into ammonia by producing dinitrogenase reductase enzyme.

Ammonia oxidizing bacteria (AOB's) are extremely slow growing organisms and resist culture. This makes them difficult to be detected in coastal environments by cultivation-dependent traditional methods,resulting in under estimation of AOB's by several orders of magnitude. This under estimation of AOB's in culture based methods is due to their slow growth rates, long incubation period, the small size of the colonies and co-contamination with fast growing heterotrophic bacteria (Underhill, 1999; Torsvik *et al.*, 1990). Alternatively,

culture independent molecular techniques are therefore used to monitor bacterial populations in order to recover the nearly entire diversity. Molecular detection systems based on the functional genes, which do not rely on traditional cultivation methods appear promising in determining microbial populations in aquaculture systems (Krishnani and Kathiravan 2010; Krishnani *et al.*, 2009b). Real time PCR has shown much higher sensitivity for the detection of nitrifying bacteria (Geets *et al.,* 2007; Wallenstein and Vilgalyas 2005). A quantitative real-time PCR assay targeting *amoA* was developed to estimate AOB population size in coastal soil, greenwater system of coastal aquaculture and commercially available bioaugmentors. Krishnani *et al.* (2010; 2012), for the first time, have successfully demonstrated bagasse as a biostimulator to control ammonia and to achieve higher shrimp production in coastal aquaculture with major emphasis on the quantification of indigenous ammonia oxidizing bacteria onto bagasse biofilm using molecular technique targeting *amoA* genes. Nitrification is carried out by two distinct autotrophic bacterial groups: ammonium-oxidizing bacteria (AOB), and nitrite oxidizing bacteria (NOB) (Hollocher *et al.,* 1981; Hooper *et al.,* 1997).

$$NH_3 + O_2 + 2e^- + 2H^+ \xrightarrow{AOB} NH_2OH + H_2O \xrightarrow{NOB} NO_2 + 5H^+ + 4e^- \longrightarrow NO_3$$

Ammonia monooxygenase (*amoA)*, that catalyzes the oxidation of ammonia to hydroxylamine, and *nitrite oxido-reductase* (*norB*) that catalyzes oxidation of nitrite to nitrate are frequently used to study genetic diversity of ammonia and nitrite oxidizing nitrifying bacteria (McTavish *et al.,* 1993; Rotthauwe *et al.,* 1997). Due to their higher diversity compared with 16S rRNA genes, functional genes allow a greater resolution for the study of genetic differences in natural populations of AOB and NOB (Purkhold *et al.,* 2000). Furthermore, horizontal transfer of *amoA* and *norB* genes within autotrophic AOB and NOB appears to be minimal or absent, which allows an inference of the evolutionary history of the organism based on these genes (Treusch *et al.,* 2005). The gene encoding NxrA is unique to NOB, which oxidizes nitrite into nitrate by producing nitrite-oxidoreductase enzyme.

Denitrification is a dissimilatory biological process for waste treatment in nitrogen recovery, in which oxidized nitrogen compounds such as nitrates and nitrites are used as alternative electron acceptors for energy production (Tamegai *et al.,* 2002; 2007). In contrast to nitrifying bacteria, denitrifying ability is widespread among bacteria due to lateral gene transfer. Denitrifying bacteria are phylogenetically diverse facultative anaerobes, which can switch from oxygen to nitrogen oxides as terminal electron acceptors under anoxy conditions. Denitrification consists of four reaction steps by which nitrate is reduced into dinitrogen gas by the metaloenzymes *nitrate reductase (Nar)*, *nitrite reductase (Nir)*, *nitric oxide reductase (Nor)* and *nitrous oxide reductase (Nos)* (Philippot 2002). *Nitrite reductase* (*nirS*) and *nitrous oxide reductase* (*nosZ*) genes are markers to detect denitrifiers.

$$NO_3 \xrightarrow{Nar} NO_2 \xrightarrow{Nir} NO \xrightarrow{Nor} N_2O \xrightarrow{Nos} N_2$$

Metagenomics

Knowledge and monitoring of microbial ecology is required to analyze the fate of toxicants, which helps to predict and optimize bioremediation performance. An understanding of microbial populations in the system is very important since biodiversity may play a major role in enhancing indigenous bioaugmentation predictability and reliability. Soils are estimated to harbor up to 10^{10} bacteria of about 10^4 different ribotypes per gram, of which more than 99% can not be cultured by present methods. Unculturable microorganisms represent the majority of uneplored ecological microcosms. Microbial biotechnology has undergone paradigm shift with emergence of unseen majority of unculturable microbes and their potential for applications in industries remains untapped. Metagenomics offers a powerful tool for examining the diversity of both cultivable as well as the uncultivable members in a population (Krishnani 2010). Application of real-time PCR and DGGE facility in the same also provides the facility to quanfity the diversity within the said (Meena *et al.*, 2015). Moreover, the discovery of highly conserved regions, especially within 16S rDNA has proved a robust tool for the high-throughput assesment of the diversity of both culturable and unculturable microbial communities (Roger and McClure, 2003; Watanabe and Baker, 2000; Amann *et al.*, 1995). This approach boosted the studies on bioremediation because of its robustness in exploration of the contaminated environment-associated microbiota (Fig. 5). Metagenomic analysis involves isolating DNA from an environmental sample, cloning the DNA into a suitable vector, transferring the clones in to a host bacterium and screening the resulting transformants (Fig. 6). Recent advancement in molecular technology including high throughput sequencing, phenotypic/genotypic microarrays offer excellent opportunity to exploit unculturable microbes for biotechnological processes.

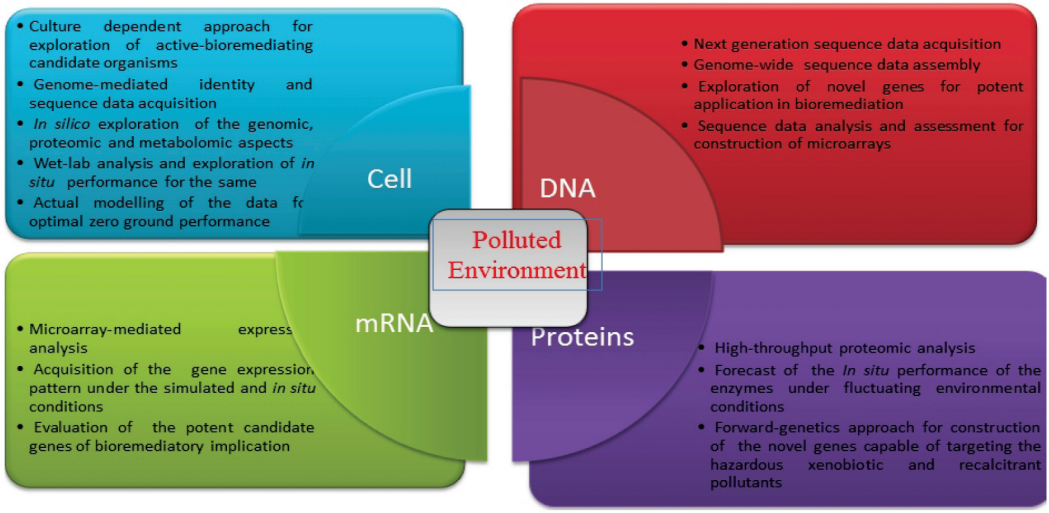


Fig. 5. Combined application of omic-approaches for the screening of potent candidates and developement of a most effective bioremediation model for a contaminated ecological nich.

The culture dependent approach consent the user direct analysis of working microbial system under a given environment, moreover it also provides the opportunity for acquiring the detailed expression profiles under various physico-chemical conditions and the typical pollutants as metabolic substrates. The metagenomic studies make available the exact picture of the diversity in attendance and thought to contribute the bioremediation process. The direct analysis of *in situ* RNA and proteins clears the gene expression pattern therein, thus the cumulative data extracted considering all the aspects presents a powerfull tool for design and developement of models with improved function and processivity under the conditions.

Although metagenomics has revealed very interesting and useful information about biological systems, there are many barriers to extracting the full range of information that is locked in the genomes of uncultured microbial world. Metagenomic analysis mainly depends on technical innovations to provide improved method of DNA extraction from wider range of organisms facilitating aggressive bacterial cell lysis. Adoption of nucleic acid methods based on the sequencing of metagenomic clone libraries has provided an insight into the diversity of microbial populations in terms of sequence and the phylogenetic information of an individual clone. This has led to the realization that microbial populations are much more diverse than previously thought using traditional culture methods. The potential for application of metagenomics to biotechnology seems endless. Krishnani *et al.* (2009b; 2010; 2013; 2014) have successfully demonstrated the applications of metagenomics for examining uncultivable microbial diversity implicated in biotransformation of nitrogenous, sulfurous toxicants and potent greenhouse gases in coastal aquaculture.

Diversity of nitrifying, denitrifying and sulfur oxidizing bacteria have been examined in coastal aquaculture using metagenomics. Metagenomic clonal libraries have been created for various functional genes implicated in metabolism of nitrogen, sulphur and methanogenesis viz.*Ammonia monooxygenase*(*amoA), nitrite oxido-reductase (norB), nitrate reductase (napA and narG), nitrite reductase* (*nirS*), *nitric oxide reductase (qnorB and cnorB), nitrous oxide reductase*(*nosZ), dinitrogenasereductase* (*nifH), sulfate thioesterase/ sulfate thiohydrolase (soxB),* and *methane monooxygenase* (*pmoA*) have been studied in Indian coastal aquaculture. Nucleic acid methods based on sequencing of clone libraries provided sequence and the phylogenetic information of an individual clone. The abundance of these functional genes reveals the presence of nitrifying and denitrifying organisms in coastal aquaculture (Krishnani 2010; Krishnani *et al.,* 2009; 2010; 2012).

A huge diversity of extremophiles exists in nature and they have been studied by both culturing and culture independent methods. These extremophiles are bestowed with a variety of stress tolerance mechanisms to withstand the high salt concentration, desiccation, low pH, high concentration of heavy metals, cold and freezing stress and other abiotic stresses known to exist in aquaculture ponds. This ability of extremophiles to thrive in stressed conditions have been acquired by evolving unique genes and cellular processes and are known to harbour a wealth of genes/cellular pathways which can be implicated in bioremediation and stress mitigation. Metagenomic approaches are largely used to decipher the genes involved in stress tolerance in these unculturable tolerant microbes in

aquaculture ecosystem. The existing mechanism of stress tolerance in these unculturable microbes is likely to be quite distinctive to the stress tolerance mechanism known to exist in their culturable counterparts. To date, a number of novel potential genes/ORFs/pathways well implicated in stress tolerance viz. salt tolerance (Kapardar *et al.,* 2010, Culligan *et al.,* 2012, Gao *et al.,* 2013), heavy metal tolerance (Mirete *et al.*, 2007, Morgante *et al.,* 2014) and hydrocarbon degradation pathway (Sierra-García *et al.,* 2014) are retrieved using functional metagenomics approaches. These potential candidate genes can be exploited to impart resistence to benefiacial microbial members in aquaculture ecosystems.

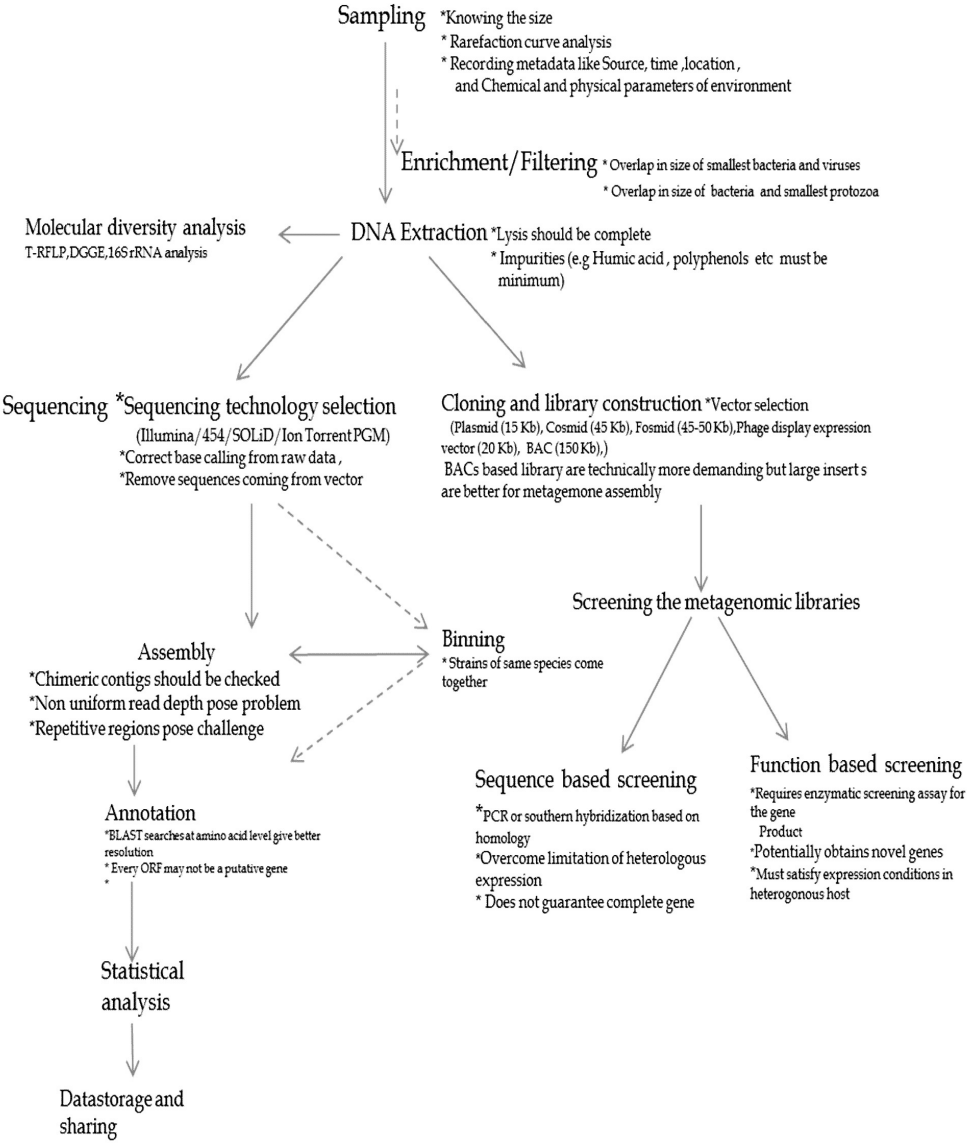


Fig. 6. Work flow diagram of core steps of a typical metagenomic study (Dotted lines represent optional steps)

Metabolomics (Metabolic profiling of microorganisms implicated in bioremediation)

Metabolomics is aimed at detection and quantitation of endo-cellular and extracellular metabolites in biological samples. All the genomic approaches discussed above aid the understanding regarding the physiological capabilities of the candidate microbes, but the functioning of the individual microbes in a complex ecological nich can be better understood through the models capable to deal with the number of reactions concurrently occuring inside the cell at the given moment under the conditions, such possibilities can be easily probed out using the *in silico* models for microbial metabolism (Covert *et al.*, 2001; Paramanik and Keasling, 1998). Metabolomics is mainly concerned with identification and quantification of small molecule metabolites of size <1500 Da in the metabolome of an organism (Fig. 5). It enables understanding of the mechanisms of biological and biochemical processes in composite arrangements. An array of metabolites are elaborated by microorganisms under varying physio-chemical environments which need to be explored in details. This generates probability of finding some novel metabolites of microbial origine; capable of performing an important role for removal of various pollutants from contaminated environments. Such kind of explorations (Fig. 5) can be easily carried out utilizing the high-throughput techniques like LC-MSMS/ GC-MSMS, ICPMS, AAS, etc., for high-throughput analysis of proteins, 2D gel and MALDI-TOF are preferred ones. The metabolites like siderophores (400-1500 Da) have been demonstrated for their role in bioremediation of heavy metals like Hg and Pb. The said have also been demonstrated for thier role in precipitation of uranium from low grade ores. Metabolomics has been applied in various research areas including abiotic and biological-stress studies, biomarker discovery, functional genomics and integrative systems biology.

Bioinformatics and proteomics in bioremediation

The bioinformatics combines biological sciences with information technology, it deals with the *in silico* strategy for data analysis. The data generated from the above strategies is tremendous and is quite difficult to maintain and analyse and store. Thus the use of robust computational tools for the proper mentainance and and analysis of the said are of prime importance. Moreover, various computational tools are also available to monitor the *in situ* bio-physico-chemical aspects at fine level. This generates scope for the fine-level assessment of a model and its actual implementation probability. Today, the next generation sequencing technology geterates millions of base sequences within days, this huge amount of data needs to be assembled *in silico* and the probability of presence of partially complete / complete countigs can also be tested; using which the novel genes capable of contributing a large share in bioremediation processes may be explored. Similar is the fact with proteins where various domains of proteins can be manipulated *in silico* followed by wetlab assessment for improved processivity. Addition / deletion of specific domains can significantly alter the functionality of proteins, thus it is important to identify and manupulate such domains using cutting-age computational tools for protein analysis and visualization.

Today, number of micorganisms capable of utilizing environmental pollutants for their growth have been demonstrated. Moreover, significant additions to the count are being regularly made. The computational approach will prove most convenient for the acquisition of the knowledge regarding functional biodegradation systems within these microorganisms for the cleavage of various xenobiotic and recalcitrant pollutants. Along with this, the toxicological evaluation of such compounds is necessary (Ekins *et al.*, 2007). The Environmental Protection Agency has developed hirarchy of toxicity values of various compounds. These and similar kind of evaluations can be done using the available databases and / tools. Khan *et al.* (2013) have made a comprehensive listing of useful tools and databases in this regard. The international bioinformatics centres including NCBI, EMBL, CBS, Munich, EBI and ExPASy have developed a number of bioinformatics tools and made them publically available. These include sequence alignment tools, primer design tools, motif finders, sequence similarity search tools, protein modelling tools, gene identifiers etc. The University of Michigan Medical School have made good assortment of such tools at the online web-page of department of computational medicine and bioinformatics. (http://www.ccmb.med.umich.edu/bioinf-core/tools?field_bioinfotool_category_tid=All).

These alongwith similar tools and databases have accumulated variety of data related to individual gene and proteins, thus serving as hubs of information for system biologists (Jeong *et al.*, 2001; Fraser *et al.*, 2002; Rison and Thornton, 2002). Today it has therefore became quite easier task to evaluate the detrimental potential of a given pollutant to the environment and its possible fate therein, particularly from the perspective of microbial degradation.

CONCLUSION

Conclusively, bioremediation has tremendous potential to alleviate the existing pollution, toxicity and abiotic stresses in aquatic ecosystems in a sustainable and ecofriendly manner. It is perceived to benefit the aquatic communities, ultimately increasing the productivity of aquatic ecosystems. The adoption of molecular techniques developed originally for other areas of biotechnology and their improvisation and extrapolation to gain a comprehensive understanding of microbial community dynamics has proved very effective in understanding the molecular basis of bioremediation. The knowledge so created has provided us the sound foundation and experimental framework, on which novel stretegies of bioremediation can be devised. Current bioremediation stretegies have brought a noticeable advantage of minimizing consumption and release of water, recycling organic matter reduced pathogens introduction improved biosecurity and enhanced ecosystem productivity. Bioremediation stands to benefit greatly and advance even more rapidly with the adoption of molecular techniques developed originally for other areas of biotechnology. These techniques promise to provide a better understanding and better control of environmental biotechnology processes, thus enabling more cost effective and efficient bioremediation of toxic waste and contaminated environments.

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