BACILLUS CEREUS MCCB 101 AS BIOAUGMENTOR FOR DETRITUS DEGRADATION IN A SIMULATED ZERO WATER EXCHANGE SHRIMP GROW OUT SYSTEM

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by

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Certificate

This is to certify that the research work presented in the thesis entitled " ***Bacillus cereus*** **MCCB 101 as bioaugmentor for detritus degradation in a simulated zero water exchange shrimp grow out system** " is based on the original work done by Ms. Riya George (Reg.No.3570) under the guidance of **Dr. Robert H. Reed, Central Queensland University, Rockhampton, Queensland 4702, AUSTRALIA** and co-guidance of **Dr. I.S Bright Singh, Professor, School of Environmental Studies, Cochin University of Science and Technology, Kochi - 682022**, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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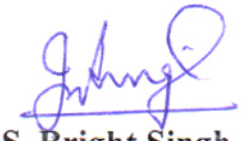

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Dedicated to

My beloved family

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UNRAVELING THE BIOREMEDIATION POTENTIAL OF AN AQUACULTURE BIOAUGMENTER *BACILLUS CEREUS* MCCB 101 IN THE DEGRADATION OF ORGANIC WASTE IN SHRIMP POND SEDIMENT 31-69

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Chapter 1

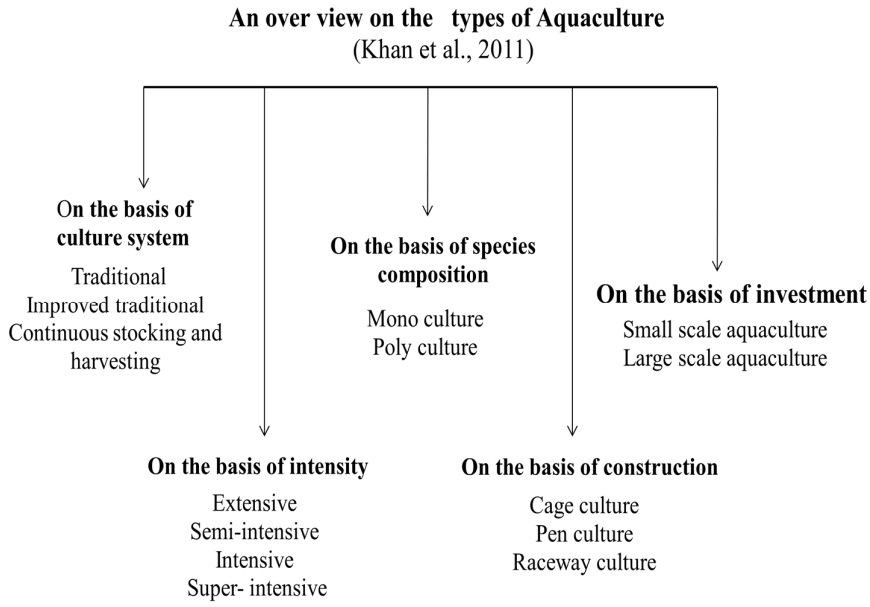
GENERAL INTRODUCTION

Aquaculture, "the Underwater Agriculture", is the farming of aquatic animals and plants namely finfish, shrimp, prawns, crabs, clams, oysters, mussels, seaweeds in water under controlled conditions. About 62% of all animals cultured are finfishes, 30% mollusks, and 8% crustaceans (Food and Agricultural Organization , 2007). The World aquaculture is categorized into inland culture, brackishwater culture and mariculture. Global fish production from inland culture (including brackishwater) and mariculture in 1980 was 2.35 million tonnes each. However, growth in inland culture outpaced mariculture with average annual growth rates of 9.2 and 7.6 percent, respectively. As a result, inland aquaculture steadily increased its contribution to total farmed food fish production from 50 percent in 1980 to 63 percent in 2012 .The aquaculture industry in India witnessed an enormous increase in the growth rate attaining second position in the total world fish production with annual fish production of 9.06 million metric tons 2012–2013 (FAO, 2014) in spite of declines in landings by capture from both inland waters as well as from sea. Moreover increasing human population coupled with plateauing of agricultural production and shrinking area available for agricultural or land based animal production, aquaculture industry has bright future for vast expansion to meet the demands for quality food.

In global perspective aquaculture is heavily dominated by Asia-Pacific region accounting for 89% production in terms of quantity and 77% in terms of value. Contribution by China to the global production is 67% in terms of quantity and 49% in terms of value (Eknath and Jena, 2008). India is reviewed as major maritime country, being home for more than 10% of global fish biodiversity (Ponnian and Sundaray, 2008).

Freshwater aquaculture demonstrated an overwhelming ten-fold growth from 0.37 million tonnes in 1980 to 4.03 million tonnes in 2010 with a mean annual growth rate of over 6 percent contributing to over 95 percent of the total aquaculture production. It comprises carps, catfishes, freshwater prawns, *Pangasius* and tilapia. In brackish water sector, focus is on shrimps (*Penaeus monodon* and *Penaeus vannamei*), and mussels and edible oysters undertaken in some coastal region of Kerala, to a limited extent. Carps in freshwater and shrimps in brackish water form bulk of the major aquaculture produce.

Precisely, aquaculture is economically more efficient and viable than land based animal farming systems in the sense that feed is efficiently converted to meat with more production of biomass per unit area. When the plant products are deficient of one or more of essential amino acids and essential fatty acids, fishes have well balanced amino acid and fatty acid profile and especially polyunsaturated fatty acids present in good quantity. Fish meat is highly digestible and considered as rich in several minerals and vitamins. Therefore, aquaculture sector as agro industry has the potential to meet the nutritional requirements of the ever increasing human population apart from generating employment opportunities and earn foreign exchange adding, to Indian economy.



1.1 Shrimp Farming

Shrimp continues to be the largest single commodity in terms of value, accounting for about 15 percent of the total fishery products internationally. In 2012, farmed crustaceans accounted for 9.7 percent (6.4 million tonnes) of food fish produced through aquaculture by volume, and 22.4 percent (US$30.9 billion) by value (FAO, 2014). Shrimp farming is reviewed as booming business compared to agriculture and animal husbandry (Kumar, 1997). As per 2014 reports of Food and Agriculture Organization of the United Nations, untapped areas suitable for shrimp farming still exists in India. Out of total 1.456 million hectare of brackish water area available in India, 0.902 million hectares are being utilized principally as shrimp farms. Majority of shrimp farms in India are export oriented. It earns foreign exchange and generate employment for large

Coastal contiguous population (Mishra *et al.*, 2008). Shrimp exports account for more than 70% (in terms of total earnings) of marine products (Antony et al., 2002). Shrimp is one of the largest single commodities accounted for nearly 17 % of total value of seafood products traded internationally and values more than US$ 14 billion. Approximately 70 % of produced shrimps are traded internationally, which makes it the most important and principal fishery commodity worldwide (FAO, 2009). This distinctly indicates the huge potential and global market of shrimp industry.

1.2 Cultured Shrimp species

All cultured and majority of captured shrimps across the globe belong to family Penaeidae of decapod crustaceans termed “Penaeids". The different shrimp species cultured globally are:

- Giant Tiger shrimp (*Penaeus monodon*)
- Western White shrimp (*Litopenaeus vannamei*)
- Indian white shrimp (*Penaeus indicus*)
- Western Blue Shrimp (*Penaeus stylirostris*)
- Chinese White shrimp (*Penaeus chinensis*, also known as *P. orientalis*)
- Japanese Kuruma shrimp (*Penaeus japonicus*)
- Banana shrimp (*Penaeus merguiensis*)
- Brown Tiger Shrimp (*Penaeus esculentus*)
- The Atlantic White Shrimp (*Penaeus setiferus*) (FAO, 2009)

In India, the tiger shrimp, *Peneaus monodon,* is the most commonly cultured species. Shrimp farming provides high returns in areas of production, processing and export and is regarded as a high pay-off economic activity (Krishnan *et al.*, 2000).

1.3 Practices and systems of shrimp culture

The shrimp farming in India are classified traditional, extensive, semi-intensive and intensive. The most commonly adopted shrimp farming practice in coastal areas are scientific extensive shrimp farming and traditional / improved traditional system. The categorization is on the basis of area, inputs used, stocking density, yield and water management.

1.3.1 Traditional aquaculture method

In this method, ponds consist of variety of fishes and a small proportion of shrimps. These systems are tide –fed with salinity variations depending on the monsoon regimes. Neither supplementary feeding nor fertilization is done. In this trap and culture method the entry of unwanted predators and undesirable species is the main disadvantage. The average production is low and ranges from 200 to 500 kg/ha/year of mixed species. The most well known of these systems are the Bheries of West Bengal and the paddy-cum aquaculture systems of Kerala, Goa and Karnataka (Hein, 2002).

1.3.2 Improved traditional systems

In improved traditional system, the entry of unwanted organisms is controlled; supplementary stocking is done with the desired species of shrimp seed with the adoption of improved environment friendly technology. The

production and productivity of the system can be increased with the yield levels varying between 1000 and 1500 kg/ha/season (Hein, 2002).

1.3.3 Extensive systems

In this method, square shaped ponds with excavated walls are used. Water enters the ponds through sluice gate. Wild seed enter through the sluice gate or purchased and stocked at rates of 2-5 per meter square with one or at the most two crops a year. There is very little complementary feeding, water or soil treatment (aeration, application of fertilizers etc.). In India extensive production systems of shrimp is found more profitable. In scientific extensive farming, for more effective integration and the use of land and water resources in the coastal areas, stocking with supplementary seed and application of pelleted feed are encouraged (Coastal Aquaculture Authority, 2006).

1.3.4 Modified extensive systems

In modified extensive system, the infrastructure remains the same as extensive systems, but pond preparation involves tilling, liming, and fertilization and stocking at higher density in the range of 5 to 10 per meter square. Feed consists of a combination of local feeds and locally produced or imported pellet feeds. One or two crops in the range of 600 to 1100 kg/ha can be produced. However, under extensive production system, the production cost is the lowest in India, which produces shrimps at US$ 1.07 per kilogram. In addition, labour cost remains 15% of the total production cost, the least compared to all other shrimp producing Nations. Precisely, extensive production system management is more profitable in India than any other systems (Leung and Engle, 2006).

1.3.5 Semi-intensive systems

This system is dependent upon reliable shrimp seed supply, preferably from hatcheries, well formulated shrimp feed in addition to the natural food. Ponds of 2-3 ha with 1 – 1.5 m depth are used and commonly stocked with hatchery-produced seed at the rate of 15 to 30 PL/m². Water exchange is regularly carried out by tide and supplemented by pumping. Natural food organism in the ponds are enhanced by applying organic manures such as cow dung, poultry droppings and inorganic fertilizers like super phosphate, urea etc. Extraneous materials such as water conditioners, probiotics etc, are used in this system to enhance the survival and growth. The duration of culture period is 4 - 5 months. Production yields range from 500 to 4000 kg/ha/yr. Semi intensive system is no longer recommended due to nutrient loading resulting in eutrophication of recipient water bodies, environment degradation and emergence of diseases.

1.3.6 Intensive systems

The ponds are 0.25-0.50 ha in size, with a square or rectangular shape, with four aerators per pond and a centralized drainage system to remove sludge and manage water flow.Stocking density is 25 to 100 PL/ m^2. The average production in India is about 4500 kg/ha/year. High stocking density and heavy feeding (4 -5 times a day) cause stress on the cultured stock accompanied by nutrient loading. These factors lead to serious environmental and health problems manifested through a host of diseases. The trend towards intensive shrimp aquaculture has been developed due to the anticipated high profit from farmed shrimp but high capital cost and operating costs make intensive shrimp farming a risky proposition. In intensive and semi intensive

production systems, inputs, especially cost of feed, constitutes major component per unit cost of shrimp produced; 35% of total production cost in India. Such systems are common in Thailand, Philippines, Malaysia, Taiwan and Australia. However, in India it is not frequently under implementation (James, 1999)

1.3.7 Zero water exchange shrimp production systems

The management of intensive aquaculture systems operated at very low water exchange rates (2% to 10% per day) lean on the methods developed to mitigate the inorganic ammonia–nitrogen buildup (NH^{4+} and NO_2^-) in respect to enrichment of pond water (Colt and Armstrong, 1981).In the production of marine shrimp in a zero-exchange system, it can be assumed that for every kg of feed at 35% protein, approximately 50.4 g of ammonia–nitrogen will be generated (Ebeling *et al* .,2006) .Unlike carbon dioxide which is released to the air by diffusion or forced aeration, there is no effective mechanism to release the nitrogenous metabolites out of the pond. Frequent exchange of pond water is not considerable due to environmental, economic and biosecurity reasons (Avnimelech, 1999).

There are several techniques which allow the reduction of this threat, maintaining at the same time the water quality within acceptable levels (reviewed by Crab *et al*., 2007). Most are designed to remove waste products from the culture but with added costs because of the need of additional space for waste removal in settling ponds (Van Rijn, 1996; Hargreaves, 2006) or through mechanical filters, generally followed by fine solid removal and foam fractionation, UV or ozone treatment and removal of dissolved organic waste in different types of biological filters (Greiner & Timmons, 1998; Malone &

Beecher, 2000; Gutiérrez-Wing & Malone, 2006; Timmons *et al*., 2006; Crab *et al*., 2007). Hence interest in closed /Zero water exchange aquaculture systems is increasing, due to its vested marketing advantages over associated problems like biosecurity and environmental deterioration in conventional faming systems (Menasveta, 2002; Ray, 2012). Zero water exchange production systems uses either a less intensive ecological approach or highly technical oriented biotechnical approaches (Kautsky *et al*., 2000).

The extensive zero-water exchange shrimp farming system in the periphery of Chilka lagoon (Orissa, India) is a typical representative of less intensive and sustainable farming systems. Five individual farms monitored over a complete production cycle were of acceptable levels of water and soil quality. With a mean Shrimp production of 145 kg/ hectare and net income of Rs. 63,250 per crop per hectare Chilka farms generated high benefit-cost ratio compared with shrimp farming system with regular water exchange in the same area, indicating high profitability and sustainability (Balasubramanian *et al*.,2004). As the biotechnological model involves great expenditure and specialized skills, the less intensive ecological approach is a viable alternative for resource-poor developing countries.

Zero water exchange technology was developed at an experimental scale through the 1980's and 1990's (Aquacop, 1985; Wyban and Sweeney, 1990; Hopkins *et al*., 1993; Sandifer and Hopkins, 1996). Research demonstrating low water exchange marine shrimp production systems have been conducted by Ebeling and LaFranchi, 1990; Santos and Ebeling, 1990. In the mid-90's a commercial shrimp farm, BAL in Belize, Central America, was designed and constructed using this technology (McIntosh *et al*., 1999).

BAL developed a zero water exchange and recycle strategy to reduce the effluents and sediments that would be released in to the environment by a typical intensive shrimp farm.

Meanwhile Zero water exchange systems managed by application of probiotics, and biofloc technology were made known with prime focus on biosecure, productive and sustainable farming .Zero-water exchange systems developed for large-scale pond production of marine shrimp traditionally was photoautotrophic algae based (Avnimelech *et al*., 1994; Hopkins *et al*., 1996). The ammonia buildup in these systems can be controlled by the manipulation of the carbon/nitrogen ratio in such a way as to promote the growth of heterotrophic bacteria (Avinimelech, 1999; McIntosh, 1999, 2001). As a result, the ammonia– nitrogen is removed from the system through assimilation into microbial biomass. It's a bonus for some aquaculture species (shrimp and tilapia) as this bacterial biomass produced in the intensive zero-exchange systems can be an important source of feed protein, reducing the cost of production and thus improving the overall economics (McIntosh, 1999; Moss, 2002).

Biofloc Technology (BFT) based on growth of microorganism in the culture medium, benefited by the minimum or zero water exchange has received alternate appellation such as ZEAH or Zero Exchange Autotrophic Heterotrophic System (Wasielesky *et al*., 2006) active-sludge or suspended bacterial-based system (Rakocy *et al*., 2004) single-cell protein production system (Avnimelech ,1989) suspended-growth systems (Hargreaves, 2006) or microbial floc systems (Avnimelech , 2007; Ballester *et al*., 2010). The microorganisms (biofloc) maintain water quality, by the uptake of nitrogen

compounds generating "in situ" microbial protein; control of bacterial community over autotrophic microorganisms is achieved using a high carbon to nitrogen ratio (C:N) (Emerenciano *et al*.,2009) and increase culture feasibility by reducing feed conversion ratio and a decrease of feed costs.. The carbon sources applied in BFT are often by-products derived from human and/or animal food industry, preferentially local available. Cheap sources of carbohydrates such as molasses (Samocha *et al*., 2007) glycerol (Crab *et al*., 2010) and plant meals (Hari *et al*.,2004 ; Emerenciano *et al*.,2012) can be applied before fry/post-larvae stocking and during grow-out phase, aiming to maintain a high C:N ratio (~15-20:1) and to control N compounds peaks. The carbon source serves as a substrate for operating BFT systems and production of microbial protein cells (Emerenciano *et al*.,2012). BFT under zero water exchange and limited discharge has been applied successfully in nursery phase of *L. vannamei* (Samocha *et al*., 2007) and *P. monodon* (Arnold *et al*., 2009) *Farfantepenaeus. paulensis* (Ballester *et al*., 2010). In *L. vannamei* nursery in under BFT conditions Cohen *et al*. 2005 reported survival rates ranging between 97% to 100%. Furthermore, Emerenciano *et al*. 2012 found that *F. brasiliensis* postlarvae grow with or without pelletized feed in biofloc conditions during 30-d of nursery phase, which was 40% more than conventional clear-water continuous exchange system.

Furthermore Suantika *et al*. (2012) reported increased survival rate of the giant freshwater prawn (*Macrobrachium rosenbergii*) by 10 -20% in a Zero Water exchange system administered with nitrifying bacteria and *Chlorella* . Synchronously the use of nitrifying bacteria and microalgae *Chaetoceros calcitrans* could sustain water quality, growth and FCR in super intensive white shrimp (*Litopenaeus vannamei*) zero water discharge (ZWD)

culture system broadcasting the new avenues by this modified green water technology in optimizing the nutrient (Suantika *et al*., 2015). In 2014 Joseph *et al*. evaluated field level performance of a zero water exchange shrimp farming protocol in terms of production of shrimp biomass and maintenance of water and sediment qualities in 10 earthen modified semi-intensive farms in different parts of Kerala, India which were managed through bioremediation and application of probiotics. A stable environment interms of water quality parameters except salinity and total hardness, sediment E*h* and pH were maintained without any significant variation during the culture period by the addition of Detrodigest^{TM}, an indigenous bioaugmentor compared to the control ponds that ensued mortality and culture failure. The average feed conversion ratio was close to the optimum and Cost benefit ratio indicated profitability in this study. Unlike the bio-flocks technique, bio augmentation does not require organic matter to control ammonia concentration (Hargreaves, 2006), which possibly makes the process of organic matter oxidation more efficient. Ebeling *et al*. (2006) stated that the heterotrophic bacteria have a significantly higher growth rate than the nitrifying bacteria.

In a ZWD system the decrement of dissolved oxygen budget due to the shrimp density and the accumulation of organic matter might reduce the oxygen budget of the system during the normal culture period can be streamlined by oxygen production of microalgae and constant aeration in ZWD system (Iba *et al*., 2014). A report on the growth of *Litopenaeus vannamei* by 46.6% accompanied by low biochemical oxygen demand than control by 70.2% in a superintensive zero water exchange system with weekly application of the bioaugmentation agent Comambio® (a commercial

product containing *Bacillus* spp.) is a radical surpass of this sustainable technology indicating the reduction in the fraction of organic matter (Salencia *et al.*,2016).Undoubtedly the balance between waste production and assimilative capacity in pond environment is of paramount importance for the success of closed systems.

1.4 Environmental impacts and challenges in shrimp farming

The tremendous growth in shrimp culture has lead to competition for water and land culminating in deforestation, eutrophication of receiving waters due to effluent discharge, modification of habitat of terrestrial and aquatic animals and impact on mangrove ecosystem (Thomas *et al*., 2010). There is dependence on formulated shrimp feed which has fish meal as the main protein ingredient for which there is overexploitation of trash fish stocks (Tacon, 2002 ; Sanchez – Martinez *et al*., 2007). The use of conventional chemotherapeutics has resulted in the increased drug resistance in pathogens and antibiotic residue in the produce resulting in consumer resistance (Verschuere *et al*., 2000). The potential impact of aquaculture effluent (Tacon, 2002) is in terms of discharge of organic matter (OM), nitrogen (N) and phosphorous (P) into the environment for each tonne of shrimp harvested, depending on the feed conversion ratio (FCR). Another major issue in shrimp farming in recent years has been the escalation in disease problems in many countries in Southeast Asia, Central and South America. Many of the outbreaks have been viral in origin and are exacerbated by poor water quality and high intensity of farms sharing intake and discharge waters (Moriarty,

1999; Kautsky *et al*., 2000) . He and Wu (2003) showed that only 13.9 % nitrogen and 25.4 % phosphorous in fish diets are utilized by aquatic animals, leaving the rest deposited in sediment. Nutrients such as nitrogen and phosphorus lead to eutrophication or algal bloom, excessive loss of oxygen resources, disease outbreak, low productivity, and undesirable changes in aquatic system (Jang *et al* .,2004 ; Cao *et al*., 2007). In addition, nitrogen compounds such as ammonium and nitrite can be toxic to aquatic animals at sufficiently high concentration, while nitrate may cause 'blue baby syndrome' potentially threatening public health (Nora'aini *et al*., 2005). The industry is, therefore, under increasing pressures from resource managers and non-government organizations to reduce nutrient and suspended solid discharge, while still remaining viable and profitable.

1.5 Importance of sediment and water quality parameters in the productivity of shrimp farms

Sediment and water quality plays an important role in increasing productivity of ponds. The changes in physico-chemical parameters such as temperature, pH, salinity, total suspended solids (TSS), dissolved gases and nutrients have been reviewed to influence the water quality and increased susceptibility to diseases of the organisms being cultured (Cheng *et al*., 2003). Salinity plays an important role on the physiological functions of the cultured organisms. The balance of salt and water in a tissue is very essential for maintaining the coordination in its physiological functions. Younger shrimps appear to tolerate wider fluctuations of salinity than the adults. Post-larvae of many penaeid species can tolerate wide salinity fluctuations having

little effect on their survival or growth. In pond condition, *P. monodon* can tolerate wide range of salinity from as low as 5 ppt to 40ppt. *P. merguiensis and P. indicus* prefer brackish water while *P. semisulcatus* and *P. japonicus* require more saline condition for growth (27–32 ppt).

The physical and chemical characteristics of pond water are very much influenced by the properties of bottom sediment. It provides food and shelter for the benthic organisms and also acts as the reservoir of nutrients for the growth of benthic algae which constitute food for aquatic organisms. The sediment also functions as buffer and governs the storage and release of nutrients into the water. It serves as biological filter through the adsorption of organic residues of food, excretory products and algal metabolites. The high bacterial load in the sediment helps in the decomposition and mineralization of organic deposits at the bottom.

Organic carbon is the most important factor determining fertility of soil. The range of organic carbon content was found to be between 2.2 to 2.5%. Burford and Williams (2001) in accordance with earlier reports of Banerjea (1967) pointed out that aquaculture production was found to be positively related with the soil organic carbon. According to him, pond soil with less than 0.5% organic carbon is low productive, 0.5 to 1.2% average productive, 1.5 to 2.5% high productive and greater than 2.5% less productive. Temperature influences photosynthesis, physiological response of cultured organisms and decomposition of organic matter and subsequent bio-chemical reactions. It is one of the most important physiological factors controlling growth and metabolism of shrimp (Das and Saksena, 2001;

Ramanathan *et al*., 2005). In the present study, the temperature was recorded between 17.4 to 29.8 ºC.

pH or the concentrations of hydrogen ions (H^+) present in pond water is a measure of acidity or alkalinity. pH 7 is a condition of neutrality and routine aquaculture occurs in the range 7.0 to 9.0 (optimum is 7.5 to 8.5). Exceedingly alkaline water (greater than pH 9) is dangerous as ammonia toxicity increases rapidly. It is an important chemical parameter to consider because it affects the metabolism and other physiological processes of cultured organisms. The growth of shrimps is retarded if pH falls below 5.0. Water with low pH can be corrected by adding lime to neutralize the acidity. Water of excessive alkalinity (pH values > 9.5) may also be harmful to shrimp growth and survival. In ponds which are excessively rich in phytoplankton, the pH of water usually exceeds 9.5 during late afternoon. However, at daybreak, the pH is usually lower. Excessive plankton growth can be corrected by water exchange.

Dissolved oxygen exerts tremendous effect on growth and production through its direct effect on feed consumption and metabolism and its indirect effect on environmental conditions. Oxygen affects solubility and availability of many nutrients. Low levels of dissolved oxygen can cause changes in oxidation state of substances from the oxidized to the reduced form. Lack of dissolved oxygen can be directly harmful to cultured organisms or cause substantial increase in the level of toxic metabolites. It is therefore important to continuously maintain dissolved oxygen at optimum levels of above 3.5 ppm (Li *et al*., 2006).

Availability of phosphorus determines productivity in culture ponds. According to Banerjea (1967) available phosphorus content of less than 30 ppm in pond sediments shows low production, 30-60ppm as average, and more than 60 ppm considered as highly productive.

The essential components of aquatic ecosystems are organic and inorganic form of potassium and calcium which influence organic productivity at the primary and secondary level in shallow coastal ponds traditionally used for shrimp and fish culture practices. The calcium and magnesium, along with their counter ion carbonate, comprise the basis for the measurement of 'hardness'. Optimum hardness for aquaculture is in the range of 40 to 400 ppm. Hard waters have the capability of buffering the effects of heavy metals such as copper or zinc which are in general toxic to fish. The hardness is a vital factor in maintaining good pond equilibrium. Reports on the acute toxicity of zinc, cadmium and copper on shrimps dates back to work of Ahsanullah *et al*. (1981). Severe structural damages, such as necrosis, loss of regular structure and infiltration of hemocytes in the gill tissues, as well as atrophy, necrosis and irregular tubular structure in the hepatopancreas of juvenile *Litopenaeus vannamei* exposed to five Cu concentrations ranging from 10 to 0.003% in a time and dose-dependent study (Frías-Espericueta *et al*., 2008) .

Alkalinity is the capacity of water to neutralize acids without an increase in pH. This parameter is a measure of the bases, bicarbonates, carbonates and, in rare instances, hydroxide. Total alkalinity is the sum of the carbonate and bicarbonate alkalinities. Some waters may contain only bicarbonate alkalinity and no carbonate alkalinity. The carbonate buffering

system is important regardless of the production method used. In pond production, where photosynthesis is the primary natural source of oxygen carbonates and bicarbonates are storage area for surplus carbon dioxide, hence never a limiting factor that could reduce photosynthesis, and in turn, reduce oxygen production. Also, by storing carbon dioxide, the buffering system prevents wide daily pH fluctuations. Without a buffering system, free carbon dioxide will form large amounts of a weak acid (carbonic acid) that may potentially decrease the night-time pH level to 4.5. During peak periods of photosynthesis, most of the free carbon dioxide will be consumed by the phytoplankton and as a result, drive the pH levels above 10 (Boyd, 1990; Shinde *et al*., 2011). As fish grow within a narrow range of pH values and either of the above extremes will be lethal to them.

Redox potential is an index indicating the status of oxidation or reduction. It is correlated with chemical substances, such as O_2, CO_2 and minerals composed of aerobic layer, whereas H_2S, CO_2, NH_3, H_2SO_4 and others comprise anaerobic layer. Microorganisms are correlated with the status of oxidation or reduction. With the degree of E*h*, it is indicative of one of the parameters that show the supporting ability of water and soil to the prawn biomass.

According to Alabaster and Lloyd (1980) maintenance of moderate to good shrimp farming is possible in water containing 25 to 80 mg/L suspended solid particles while TSS values of 80-100 mg/L and above do not support good fisheries. As per Jones *et al*. (2001a) shrimp ponds usually have very high loading of suspended solids and high densities of phytoplankton. High concentrations of inorganic nutrients in association with higher phytoplankton

density reflect a probable rich and productive environment supporting the notion that there was active mineralization of pond effluent (Boto and Wellington, 1988; Trott and Alongi, 2000).

Ammonia is present in two forms, ionized (NH_4^+) which is nontoxic, and, un-ionized (NH_3) the toxic form. Concentration of these depends on water temperature and pH. Higher the water temperature and pH, greater the concentration of the toxic form. Summation of both ionized and un-ionized forms is termed total ammonia nitrogen or TAN (Losordo *et al*., 1992; Sampaio *et al*., 2002).

Ammonia in ponds comes from feed and nutrients entering with the water other than it as the excretory product. If feed is uneaten, more ammonia will be present than if it is consumed by shrimp. For every kilogram of feed administered, about 30 grams ammonia will be excreted by shrimp. Un-ionized ammonia is very toxic to shrimp and causes gill damage and result in reduced growth even at low concentrations (Crab *et al*., 2007). The safe level reported is less than 0.02 - 0.3mg/L (Boyd and Tucker, 1998). Prevention of accumulation of toxic ammonia requires diligence in monitoring both ammonia and phytoplankton (which take up ammonia as nitrogen source) and respond quickly by reducing or stopping feeding, or fertilizing to stimulate more phytoplankton or resorting to exchange of water, since biological conversion of ammonia by ammonia oxidizing bacteria and nitrite oxidizing bacteria have several environmental constraints resulting in imbalanced nitrification.

Nitrite is another nitrogen compound that results from nitrification with well documented toxicity in shrimp. Nitrite is an intermediate product of the conversion of ammonia to nitrate by bacterial nitrification, which even at relatively low concentrations, 5 mg/L, is highly toxic (Boyd and Tucker, 1998) which can disrupt oxygen transport within cells and circulatory system (Lawson, 1995). Maintenance of healthy algal blooms encourages ammonia uptake which reduces the loading for bacteria to convert ammonia to nitrite to nitrate. In extreme nitrite concentrations, water should be exchanged.

1.6 Detritus in aquaculture

Detritus refers to non-living organic matter found in aquatic systems. It includes organic matter accumulated in sediment or the particulates and dissolved forms suspended in the water column (Moriarty and Pullin,1987). Detritus is a compound amorphous substance composed of the aggregates of living microorganisms together with the dead microbial fragments and their excreta such as fecal matter and other organic wastes (Yanagita, 1990). The wastes in hatcheries or aquaculture farms includes as:

(1) residual food and faecal matter;

(2) metabolic by-products;

(3) residues of biocides and biostats;

(4) fertilizer derived wastes;

(5) wastes produced during moulting and

(6) collapsing algal blooms (Sharma and Scheeno ,1999)

Accumulated sediments in shrimp ponds are highly reduced, enriched in organic matter and enriched in nitrogen and phosphorus (Hopkins *et al*., 1994). Characteristics of sludge vary depending on the type of culture system, pond management regime and inputs.

1.7 Bioremediation – an ecofriendly approach for disease control and sustainable aquaculture

The use of biological agents has gained popularity in aquaculture as an environment friendly approach to attain sustainability. It has become a reality that application of probiotics for maintaining health of animals and aquatic environmental quality is more sustainable and profitable (Wang *et al*., 2008; Moriarty and Decamp, 2009) than chemotherapeutics which are more costly, deleterious and often meet with consumer resistance (Sanders *et al*., 2003). In many countries, fish and shrimp farmers are being requested to meet stricter guidelines for product quality and effluent quality.

In this context microbial biotechnology not only assists in meeting regulatory requirements, but in fact improves profitability and sustainability of the industry as well (Moriarty, 1996, 1997). Bio-augmentation is a variant of bioremediation, where microbes applied modify the microbial communities in fish and shrimp ponds, in the intestinal tracts accompanied by decrease in waste output. Such microbial interventions manipulate microbial species composition to augment the rate of metabolic activity to carryout particular functions at faster rates than those occurring under existing conditions. The selected microbes should satisfy following criteria as stated by Moriarty and Decamp (2009).

- Microbes should be able to live and function under the environmental conditions of interest.
- Preferably be indigenous
- Non- pathogenic to humans
- The selected bacteria must not carry transmissible resistance genes against clinically important antibiotics.
- They must not produce toxins that affect humans, shrimp or fish.
- They should have appropriate functional properties for degrading the organic wastes, including the secretion of exo-enzymes for a wide range of organic polymers.

These biological agents address aquaculture challenges by improving water quality and reducing disease propensity caused by pathogenic bacteria (Fast and Menasveta 2000; Gomez-Gill *et al*., 2000; Jana and Jana, 2003; Hong *et al*., 2005). Beneficial bacteria incorporated in microbial feed is directed for protection from diseases (Gatesoupe, 1999), while Moriarty (1998) categorized them as amendments apart from oriented towards health improvement as the ones required for up-gradation of culture environment. A novel isolate, *Bacillus cereus* (NRRL 100132), was demonstrated for its outstanding capability in enhancing water quality and reducing *Aeromonas hydrophila* infection in *Cyprinus carpio* (Lalloo *et al*., 2007).

Potential mechanisms of action of biological agents include:

- competition for adhesion sites
- production of enzymes
- immune stimulation

- synthesis of antimicrobials
- competitive exclusion
- bioremediation
- competition for chemicals or for available energy
- intrinsic growth rate advantage

  (Verschuere *et al*., 2000; Holzapfel *et al*., 2001; Irianto and Austin, 2002; Hong *et al*., 2005).

1.8 The role of probiotics in aquaculture

"Pro" means favour, "Bios" means life. The term "probiotics" was introduced by Parker (1974). A widely accepted definition is taken from Fuller (1989), who considered that a probiotic is a cultured product or live microbial feed supplement, which beneficially affects the host by improving its intestinal microbial balance. There are variations in the actual understanding of the term probiotic. Gram *et al*., (1999) proposed that a probiotic is any live microbial supplement, which beneficially affects the host animal by improving its microbial balance. Salminen *et al*., (1999) considered a probiotic as any microbial (but not necessarily living) preparation or the components of microbial cells with beneficial effect on the health of the host. Aquatic animals have a much closer relationship with their environment. In fact, in seawater, pathogens proliferate independently of host and thereby opportunistic organisms reach a high density around aquatic animals (Moriarty, 1998). The bacteria present in aquatic environments are continuously ingested by the host which influences the composition of the gut microbiota (Chandrasekaran, 1985; Cahill, 1990.; Jorquera *et al*., 2001). The intensive interaction between the environment and the farmed aquatic animals

implies that the definition of probiotics has to be adapted for aquaculture. Based on this statement and the observation that organisms are capable of modifying the bacterial composition of water and sediments, Moriarty (1999) suggested that the definition of a probiotic in aquaculture should include addition of live naturally occurring bacteria to tanks and ponds in which animals live, as biological control agents, as discussed by Maeda *et al*. (1997).

Hence, to improve water quality and the immediate environment of fish and shrimp, probiotics are applied directly to the ponds. This type of biotechnology is equal to "bioremediation", the process which can be better stated as bioaugmentation involving manipulation of microorganisms in ponds to reduce pathogenic bacteria, enhance mineralization of organic matter and removal of undesirable waste compounds. However, Rengpipat *et al*. (2003) stated that bacteria added directly to pond water are not probiotics, and should not be compared with living microorganisms added to feed. But the effect of probiotic, *Bacillus coagulans* SC8168, as water additive on larvae shrimp (*Penaeus vannamei*) was significant based on the attainment of water quality, survival rate and digestive enzyme activity at different larval stages (Zhou *et al*., 2009). The presence of high levels of ammonia or nitrites not only pollutes the water but also blocks the appetite of the fish well before causing mortalities (Guillaume *et al*., 1999). Removal of ammonia can be carried out by addition of specialized nitrifying bacteria such as *Nitrobacter, Nitrosomonas* and denitrifying bacteria such as *Thiobacillus* and *Paracoccus*. Recent research shows that the use of commercial probiotics in *P. vannamei* ponds can reduce concentrations of nitrogen and phosphorus and increase

shrimp yield (Wang *et al*., 2005). The microbial species composition in hatchery tanks or large aquaculture ponds can be changed by adding selected bacterial species to displace deleterious normal bacteria (Moriarty, 1999). Douillett (1998) used a probiotic additive consisting of a blend of bacteria in a liquid suspension in intensive production systems. The probiotic blend improved water quality in fish and crustacean cultures by reducing the concentration of organic matter (OM) and ammonia. This procedure was accomplished by a series of enzymatic processes carried out in succession by various strains present in the probiotic blend. Addition of this blend to culture systems was found to reduce *Vibrio,* thereby minimizing vibriosis. Thus, amidst the controversies regarding the interchanging of terminology for addressing probiotic and bioaugmentor, these beneficial bacteria occupy their significant position in sustainable aquaculture under the green technology revolution.

1.9 *Bacillus* for bioremediation in aquaculture

Gram positive *Bacillus* species are attractive options as bacterial amendments in aquaculture as these organisms are found naturally in sediment, ingested by animals and unlikely to use genes of antibiotic resistance or virulence from Gram negative organisms such as *Aeromonas spp*. (Moriarty 1999). Literature reveals their dual role in disease control and in the improvement of environmental quality in aquaculture.

A commercial *Bacillus* spp. tested as probiotic on rainbow trout fry as feed additive gave significant survival in treatments higher than that of control (Bagheri *et al*., 2008). *Bacillus* spp. (*Bacillus subtilis* AB65, *Bacillus pumilus* AB58, *Bacillus licheniformis* AB69) isolated from local marine

environment as alternative to common antibiotics (oxytetracycline, chloramphenicol, gentamicin and bacitracin) used in Asian aquaculture were found to be ideal for bioremediation in shrimp hatcheries as well, especially to augment removal of total ammonia nitrogen (Banerjee *et al*., 2007).

Moriarty (1998) added *Bacillus* spp. as probiotic in penaeid shrimp ponds; results of the study showed increasing survival of animals and decreasing luminous *Vibrio* in pond water. In *P. monodon*, *Bacillus* used as probiotic was able to colonize both the culture environment and shrimp digestive tract and replace *Vibrio* spp. in gut of the shrimp, thereby increasing shrimp survival (Rengpipat *et al*., 1998). Meanwhile, Shariff *et al*. (2001) found that treatment of *P. monodon* with a commercial *Bacillus* probiotic preparation did not significantly increase survival.

Bacillus spp., are able to out-compete other bacteria for nutrients and space and can exclude them through production of antibiotics (Moriarty, 1998; Verschuere *et al*., 2000). Several antibiotics have been found produced naturally by a range of *Bacillus* spp., and it appears that other bacteria are unlikely to have resistance genes to all of them (Moriarty, 1998). Administration of *Bacillus* spp. also have been shown to increase shrimp survival by enhancing resistance to pathogens by activating both cellular and humoral immune responses (Rengpipat *et al*., 2000). *B. subtilis* was shown to produce a wide variety of antibacterial and antifungal compounds in culture media (Alexander, 1977; Katz and Demain, 1977; Korzybski *et al*.,1978), and novel antibiotics such as Difficidin and Oxydifficidin, that have activity against a wide spectrum of aerobic and anaerobic bacteria (Zimmerman *et al*., 1987), were found produced by them. In a study, Gram-negative bacteria

were found replaced with *Bacillus* probionts (Austin *et al*., 1995). Vaseeharan and Ramasamy (2003) reported antagonistic effect of *B. sublitis* BT23 on pathogenic. Vibrios in *P. monodon*, besides 90% reduction in cumulative mortality. This suggests that administration of *Bacillus* spp. as probiotics is an effective alternative to antibiotics for enhancing shrimp health.

Offset of culture production cycle is met with rapid increase in biomass, and water quality deterioration, mainly as a result of accumulation of metabolic wastes of cultured organisms, decomposition of unutilized feed, and decay of biotic materials. At this point of time, application of *Bacillus* spp. is reported useful for improving water quality and controlling pathogenic microorganisms (Prabhu *et al*., 1999; Irianto and Austin, 2002).

Species belonging to the genus *Bacillus*, are known to help in mineralization of organic matter and in reducing its accumulation (Shariff *et al*., 2001). Several different species of *Bacillus*, including *B. subtilis* and *B. licheniformis* produce oxygenases and thus are potentially important as candidates for large scale production for bioremediation of oil contaminated soil (da Cunha *et al*., 2006). Facultative anaerobes within the family Bacillaceae, can be used in consortia to enhance rate of methane production as well (Duran *et al*., 2006). *Bacillus* spp., which produces spores, grows aerobically and as facultative anaerobes, use nitrate or change to a fermentative metabolism when oxygen is absent. They possess an array of extracellular enzymes that can digest a wide range of polymeric organic substances (Priest, 1977) and are reported to provide substantial benefits to farmers (Ninawe and Selvin, 2009, Santos *et al*., 2009).

As part of bio-security measure, zero water exchange shrimp grow out systems have been developed by National Centre for Aquatic Animal Health, Cochin University of Science and Technology, incorporating bioremediation of detritus as the basic process. To accomplish this objective an effective detritus degrader, *Bacillus cereus* MCCB 101, could be isolated from shrimp culture system and developed it in to a product 'Detrodigest^{TM}', and had been widely used in aquaculture systems. The study described here was undertaken to know more about the organism and the precise mechanisms by which it accomplished bioremediation of detritus.

Objectives

1. Unraveling the bioremediation potential of an aquaculture bio-augmenter *Bacillus cereus* MCCB 101 in the degradation of organic waste in shrimp pond sediment.

2. Metagenomic approach to assess bacterial diversity in sediment of simulated zero water exchange shrimp culture system subjected to bioremediation of detritus

3. Bioaugmentation potential of *Bacillus cereus* MCCB101 in simulated zero water exchange shrimp grow out system of *Penaeus monodon* at high stocking density.

Chapter 2

UNRAVELING THE BIOREMEDIATION POTENTIAL OF AN AQUACULTURE BIO-AUGMENTER *BACILLUS CEREUS* MCCB 101 IN THE DEGRADATION OF ORGANIC WASTE IN SHRIMP POND SEDIMENT

2.1 Introduction

Developments in aquaculture sector are conceptualized to achieve eco-friendly practices for the well-being of aquatic environment (Avella *et al.,* 2010). Boyd (1995a) reported that the conditions of pond bottom strongly influence water quality via exchange of substances. Solids, semi solids and gaseous wastes generated from the residues of pond inputs such as uneaten feed and faecal matter get transformed into detritus creating anoxic conditions at the pond bottom leading to hydrogen sulfide production, pushing down sediment E*h* causing stress to the rearing stock. The sediment and water quality are of prime importance in an aquaculture environment, as the cultured species are in close contact with their surroundings. Hence, adopting bioremediation strategies sounds very much

relevant in maintaining healthy aquatic environment. Moriarty (1998) exemplified the term "water additives" for the probiotic bacteria applied directly into culture ponds or tanks to modify the microbial ecology of the water and sediment thereby reducing the load of pathogenic bacteria. The overall rearrangement of a niche amplifies the expression of specific catabolic traits and revert the metabolic competency in right direction at the right time necessary for the environmental clean up (Deonje *et al*., 2001). Ultimately an environment of low stability, subjected to stress is converted to a self purifying sustainable system. The sediment and water quality in *Penaeus .vannamei* grow out systems treated with commercial probiotics showed enhanced decomposition of organic matter, reduction in nitrogen and phosphorus concentrations and better production than control (Wang *et al*., 2005, 2008). Janeo *et al*. (2009) reported the effect of a bio-augmentor used in *Penaeus monodon* grow out system in lowering the ammonia and particulate organic matter. In this scenario, the present study was undertaken to evaluate the bioremediation potential and the mechanistic role of action of *Bacillus cereus* MCCB 101 (Gene Bank accession no. EF 062509) in bio-augmenting shrimp pond sediment microcosms for the effective degradation of organic matter substantiated in terms of extracellular enzymatic activities correlated with microbiological and physico-chemical parameters. This indigenous Gram positive bacterium isolated from shrimp pond sediment forms the sole component of a commercial product Detrodigest^{TM,} developed by National Centre for aquatic Animal Health (NCAAH) as an aquaculture pond probiotic for bioamelioration of detritus in aquaculture systems.

2.2. Materials and Methods

2.2.1 Determination of extra cellular enzymatic profile of the detritus degrading bacterium – *Bacillus cereus* MCCB 101 in vitro

The isolate, *Bacillus cereus* MCCB 101, was screened for the production of major extracellular hydrolytic enzymes such as amylase, cellulase, chitinase, lipase, protease and xylanase. For the qualitative analysis of extracellular hydrolases the culture was spot inoculated on to ZoBell's marine agar plates (15 g/L salinity) supplemented with different substrates. The medium supplemented with 1% soluble starch was used to detect amylase activity (Al Qodah *et al*., 2006). Gelatin and casein (1% each) incorporated ZoBell's marine agar plates were used for detecting proteases. For gelatinase production the plate was flooded with acidic mercuric chloride solution to visualize halo zone. Cellulose powder (Wills, 1983) and Purified colloidal chitin (1% each) (Khan *et al*., 2010)) were added in ZoBell's marine agar for detecting cellulase and chitinase activity, and the plates were incubated for a week. Lipase and xylanase activity were detected by incorporating 1% each tributyrin and oat spelt xylan in ZoBell's marine agar.

2.2.2 Optimization of cell count of *Bacillus* MCCB 101 to be supplied in bioassays

Top 2 cm layer pond sediment was freshly collected for the study from an active shrimp farm in Kodungalloor, Kerala, India, transported to lab, mixed thoroughly, and each of the experimental tanks of 25 L capacity (35cm length x 31cm breadth) was laid with 2 kg sediment. Sea water

having 15 g/L salinity was maintained at 2 inches above the sediment through out the experimental period. The system was left undisturbed for two days for settling prior to the experiment. A seed culture of *Bacillus* MCCB 101 prepared in ZoBell's broth was used in cell count optimization study.

2.2.3 Preparation of seed culture

Seed culture was generated in sterile ZoBell's broth prepared in seawater having 15 g/L salinity. A loopful of the culture from ZoBell's agar slant (prepared in seawater having 15 g/L salinity) was aseptically inoculated into the ZoBell's broth (100 mL) and incubated on a shaker at 100 rpm at room temperature (28±1 $^{\circ}$C) overnight. After incubation, the culture was Gram stained and checked for purity. Cell density of the culture was determined based on its absorbance at 600 nm from a standard graph generated based on absorbance versus cell count.

2.2.4 Application of seed culture

Based on cell count of the seed culture, as CFU, appropriate quantities of inoculate were added to each tank to attain final cell count of 10^4, 10^5, 10^6 and 10^7 CFU/mL of the overlying water. Dilution of the seed culture was accomplished using 0.1% peptone water and uniformly distributed in the tanks. The control tanks were maintained without addition of the seed culture. Experimental and control tanks were maintained in triplicates for each cell count, *Eh*, pH and total organic carbon of the sediment were measured at 0^{th} hour (before the addition of the bio-augmenter) 4^{th}, 7^{th} and 15^{th} day subsequent to the application of bio-augmenter.

2.2.5 Laboratory level study on the mode of action of *Bacillus cereus* MCCB 101 in shrimp pond sediment

2.2.5.1 Sampling and experimental setup

Bioassay systems developed in 25 L capacity fibre reinforced plastic (FRP) tanks (35cm length x 31cm breadth) with 2 inch thick sediment having 2cm overlying seawater having 15g/L salinity was inoculated with *Bacillus cereus* MCCB 101 to attain cell density of 10^5 CFU/mL, in triplicate. Control tanks were also maintained in triplicate without the inoculum. Impact of bio-augmentation was assessed by comparing the variations in the parameters such as extracellular enzymatic activity, total organic carbon, pH, E*h*, total plate count of bacteria, actinomycetes, fungi, total bacteria (direct count), concentration of total lipids, proteins and carbohydrates, in the sediment samples of bio-augmented (BT) and control tanks (CT) for a period of 2 weeks. Samplings were done on day 0 (before addition of the bioaugmenter), on 4^{th}, 7^{th}, 10^{th} and 15^{th} day after bio-augmentation. Surface sediment samples collected from fixed sites were pooled, mixed thoroughly and stored at -20^{o}C for all the physico-chemical analysis. For determining the direct bacterial counts, known quantity of sediment samples were transferred into sterile polypropylene tubes and fixed with known quantity of 2% (V/V) formalin prepared in seawater (Luna *et al*., 2002).

2.2.5.2 Estimation of soil pH, E*h*

To determine pH 1 part of sediment was homogenized with 2 parts of distilled water, stirred vigorously on a magnetic stirrer and pH determined using digital pH meter (Scientific Tech, India). The electrode of

ORP meter (Eutech Instruments, Japan) was directly inserted into the sediment in the tanks to around 1cm depth at 15 random sites in each of the tank and E*h* was recorded.

2.2.5.3 Determination of total organic carbon

For the determination of total organic carbon soil organic matter is oxidized under standard conditions with excess potassium dichromate in sulphuric acid, and the excess dichromate is determined by titration against standardized ferrous ammonium sulphate. From the value of dichromate consumed the organic carbon content can be calculated.

Weighed 0.2g finely divided ground soil in 500 mL conical flask. Pipetted out 10 mL 1 N potassium dichromate solution (24.52 g dry potassium dichromate dissolved in 500 mL distilled water) in to the flask. The flasks were swirled and mixed the dichromate with soil. Subsequently, 20 mL concentrated sulphuric acid was added and mixed by gentle rotation for 1 min to ensure complete contact. The flasks were incubated for 30 min, added 200 mL distilled water and 3 mL ferroin indicator. Titrated the contents of the flask against 0.25 N ferrous ammonium sulphate solution (98.0 g ammonium ferrous sulphate dissolved in 400 mL distilled water and 10 mL concentrated sulphuric acid made up to 500 mL). Color of the solution changed from blue to green and finally to greyish red at the end point (as the dichromate is reduced, the solution becomes greener and just before the end point clear bluish green color develops. As soon as there is slight excess of ferrous ion, color changes to greyish red which is being the reduced ferrous orthophenathrloine complex masked by the green chromium

ion). The distilled water blanks were also done in an identical manner using the same reagents.

Calculation

Total Organic carbon in soil (%) = (A-B) x normality of FAS x 0.003 x 100 / weight of soil taken

Where A = Volume of blank

B = Volume of titrant

FAS = Ferrous ammonium sulphate

2.2.5.4 Quantification of extracellular enzymatic activity in sediment using fluorogenic substrates

Potential extracellular enzyme activities in sediment samples were assayed using two classes of fluorogenic substrates viz., Methylcoumarinyl (MCA) and Methylumbelliferone (MUF) substrates (Sigma) as described in Hoppe *et al*. (1988). Stock solutions of following substrates

Sl. no	Substrates used	Acting enzymes
1	MUF-α-D-glucopyranoside	α-D-glucosidase (amylase)
2	MUF- β -D-glucopyranoside	β -D-glucosidase (cellulase)
3	MUF -N-Oleate	Lipase
4	MUF-N-acetylβ-D-glucosamine	Chitinase
5	MUF- β-D-Xyloside	Xylanase
6	Leucine-4-methylcoumarinyl-7-arnide	Aminopeptidase (exopeptidase)
7	Boc-Phe-Ser-Arg-MCA	Trypsin (endopeptidase)
8	Suc-Ala-Ala-Pro-Phe-MCA	Chymotrypsin (endopeptidase)

were prepared in parts of ethylene glycol monomethyl ether (methylcellosolve) and distilled water.

Except for Boc-Phe-Ser-Arg-MCA and Suc-Ala-Ala-Pro-Phe-MCA (0.5mM), stock solutions of all the substrates were prepared at 1mM concentration. Substrates were added into sediment samples at optimal concentrations ensuring maximum enzymatic activity as determined initially from a series of concentration of substrates until saturation was obtained.

Sediment samples diluted with sterile 15g/L salinity sea water at a ratio of 1:4 (w/v) were distributed at 0.5 mL aliquots in 2mL amber colored micro centrifuge tubes. Six replicates were maintained for each sample. Substrates were added to the slurries at concentrations slightly higher than their actual saturation concentrations viz., 150µM of 4-methylumbelliferyl-β-D-Xyloside, 100 µM of 4-methylumbelliferyl-N-acetyl β-D-glucosamine, 100 µM of 4-methylumbelliferyl-N-Oleate for lipase, 25 µM of MUF- β -D-glucopyranoside, 200 µM of MUF-α -D-glucopyranoside, 100 µM of L-Leucine-4-methylcoumarinyl-7-arnide (Leu-MCA), 150 µM of Boc-Phe-Ser MCA and Suc-Ala-Ala-Pro-Phe-MCA respectively. pH of the reaction mixture was adjusted using Phosphate buffer (pH 7.0) for MCA substrates and Tris HCl (pH 8.0) for MUF substrates. After incubation in the dark at room temperature for an hour, the reactions were terminated with Tris HCl of pH 8 for MCA substrates and sodium bicarbonate buffer of pH 10 for MUF, substrates respectively. Samples were vortexed and centrifuged at 2680*g* at 4ºC for 10min and the supernatant was used to measure the release of fluorogenic compounds in a spectrofluorometer (Hitachi F-2500) at an excitation and emission wavelength of 364/445nm for MUF and 380/440nm for MCA compounds respectively. Maximum fluorescence of MUF was at

pH 10.3 and MCA at 7.8 practically obtained by addition of 200µ1 of sodium bicarbonate buffer of pH 10.3 and Tris/HCl buffer of pH 7.8 added into Quartz cuvette prior to the addition of 1 mL of the sample. Blanks were generated using the same procedure on sediment and water samples with no substrate addition. Control samples were boiled for 30 min prior to the addition of substrates, and assayed using the same method as that described for the samples and was used to determine the non - enzymatically produced fluorescence. Hydrolysis rate of the substrates was determined after subtracting both the blank and control fluorescence intensity from the sample fluorescence. Increase in fluorescence after 1 hour was considered as a function of enzyme activity. Calibration curve for the fluorescence readings was constructed using standard solutions of methylumbelliferone and 7-amino-4-methylcoumarin in the concentrations between 0.1µM to 100µM. The maximum velocity of hydrolysis was expressed in terms of micromoles of fluorescein released per gram per hour of sediment samples analyzed and the data normalized to dry weight (60ºC, 24hr).

2.2.5.5 Estimation of total proteins in sediments

Protein content of sediment samples was determined following Hartree (1972) after modification by Rice (1982). This colorimetric method allows the reaction of proteins with rameic tartarate and the Folin-Ciocalteau reactive in basic environment (pH 10). The reaction provides stable blue coloration whose intensity is proportional to the protein concentrations in the sample. A quantity of 1 g sediment was added to 10 mL distilled water and vortexed for 1min and sonicated for 3 min with 30s interval between each min. An aliquot of 1mL sonicated sample was

transferred into tubes. To each tube was added 0.9 mL solution A and vortexed for a min and placed in a hot water bath at 50 °C for 10 min. Subsequently, 0.1 mL solution B was added and vortexed for a min and incubated at room temperature for 10 min. Finally 3 mL solution C was added and the tube was vigorously shaken with vortexing for 1 min. Tubes were placed in hot water bath at 50°C for 10 min. Samples were centrifuged at 800*g* for 15 min. After centrifugation, the supernatant was analyzed with the spectrophotometer (Shimadzu UV 1601) in an optical glass cuvette (1cm optical length) at absorbance of 650 nm against blank of reagent grade water. The sediment left after discarding the supernatant was dried at 60°C for 24 hours. A blank of reagents (1mL of reagent grade water) and at least three sediment replicates of samples previously calcinated at 550°C for 4 hours were processed in the same way.

Protein content of the samples was calculated from the calibration curve of standard solutions of BSA. Protein concentrations were normalized to dry weight and expressed as milligrams of albumin equivalents per gram of dry sediment.

2.2.5.6 Estimation of total carbohydrates in sediment

Concentrations of total carbohydrate were determined according to Dubois *et al.* (1956) and optimized for sediments following Gerchakov and Hatcher (1972) after minor modifications. This colorimetric assay is based on the reaction between sugars and phenols in presence of sulphuric acid. Sediment slurry was vortexed and sonicated for 3 min with 30s interval between each min of sonication. A quantity of 1 g sediment was added to 10 mL distilled water and vortexed for 1min and sonicated for 3 min with

30seconds interval between each min. Aliquoted 1mL sonicated sample was introduced into tubes and added 1mL of 5% phenol solution, and again vortexed for 1min. Tubes were left at room temperature for 10 min. To each tube was added 5 mL conc.H_2 $S0_4$ and mixed by vortexing for a min for developing visible colour change from light yellow to dark brown. Samples were centrifuged for 30 min at 800*g*. The supernatant was analyzed spectrophotometrically (Shimadzu UV 1601) at Abs_{485} and Abs_{600} against a blank of reagent grade water. Sediment samples previously calcinated at 550°C for 4 hours were processed in the same way. The sediment dry weight was assessed gravimetrically after desiccation at 60°C for 24 hours. Total carbohydrate content of the sample was estimated from a calibration curve of standard glucose solution.

2.2.5.7. Estimation of total lipids in sediments

The determination of total lipid concentration in marine sediment samples is generally carried out according to Bligh and Dyer (1956) and Marsh and Weinstein (1966), slightly modified to apply to the sediment matrix. Sediment slurry was vortexed and sonicated. A quantity of 1 g sediment was added to 10 mL distilled water, vortexed for 1min and sonicated for 3 min with 30seconds interval between each min. Aliquot of 1.25 mL chloroform and 2.5 mL methyl alcohol at room temperature were added to 1mL sonicated sample and vortexed for a minute, left at 4°C for 10 min and centrifuged at 800 *g* for 10 min. After centrifugation, the supernatant was withdrawn using Pasteur pipette, placed in test tubes, and 1.25 mL chloroform and 1.25 mL reagent grade water were added. The tubes were vigorously shaken by vortexing for 1 min in order to allow the

formation of water-methanol-chloroform emulsion. The tubes were centrifuged at 800 *g* for 5 min to separate the hydro alcoholic fraction (water-methanol) from the hydrophobic fraction (chloroform).The supernatant - hydro - alcoholic fraction was pipette out and the hydrophobic residual fraction (at the bottom of tube) was evaporated in a water bath at 100^0C for 10 min. Once at room temperature, added 2 mL conc.H_2SO_4 to each tube. The tubes were placed in water bath at 100°C for 30 min. During the reaction the acid turned to light yellow to brown. The tubes were then kept for cooling at room temperature for 5 min and transferred to ice for 5 min. To each tube, 3 mL reagent grade water was added and vigorously vortexed for 1 min. The supernatant was analyzed in a spectrophotometer (Shimadzu UV 1601) at an Abs of 375nm against blank of reagent grade water. A reagent blank (1mL of reagent grade water) and three sediment replicates of samples previously calcinated at 550°C for 4 hours were processed in the same way. After analysis, the remaining sediment after withdrawing the hydro alcoholic fraction was desiccated in the oven at 60^0C for 24 hours. Lipid concentration was calculated from calibration curves of standard solutions of triplamitine processed according to afore mentioned procedures.

2.2.5.8 Estimation of direct bacterial counts by epifluorescence microscopy (Danovaro, 2010)

To the sediment samples added tetra sodium pyrophosphate at a final concentration of 5mM and sonicated in ice for 3min with 30s interval between each minute at 40% amplitude and centrifuged at 371g for 1 min. An aliquot of 1mL supernatant was kept aside and the remaining discarded

after centrifugation at 800g for 1 min. Sediment samples were dried in an oven at 60^0C for 24-48 h, until constant weight was obtained.

The retrieved supernatant was subjected to serial dilution with 15 g/L salinity sterile seawater. From the highest dilution,1mL sample was stained with 40µl 0.025% (w/v) Acridine orange, incubated in dark at room temperature for 5 min, and then filtered through a syringe filter (0.2µm). The filter was washed with reagent grade autoclaved distilled water and air dried. The filter was examined under blue green light excitation (excitation at 450nm - 490nm) using an epifluorescence microscope (100X magnification). At least 25 optical fields were counted. A minimum of three replicates of each sample were analysed to minimize the count variance (Kirchmann *et al*., 1982).

Total bacterial counts were calculated using the following formula:
(Average cell number in each optical field) × [(Optical field coefficient × extraction coefficient × dilution factor of the sediment)/sediment dry weight)
Where Optical field coefficient = filtration area/counting area. Counting area (area of grid) =20.4mm^2. Extraction coefficient = 1.44

2.2.5.9 Estimation of total heterotrophic bacteria, actinomycetes and fungi of sediment through plate count

A quantity of 1.0 gm sediment was serially diluted in 15g/L salinity sea water aseptically and plated onto ZoBell's agar for heterotrophic bacterial counts as per standard procedures. Actinomycetes isolation agar (Hi-media) and Saboraud's dextrose agar (Hi-media) were used for

enumeration of actinomycetes and fungi respectively. Results were normalized to dry weight and expressed as CFU/gm of sediment.

2.2.5.10. Statistical analysis

Data expressed as mean ± standard deviation (S.D.). A one-way analysis of variance (ANOVA) was applied to compare bio-augmented groups and the controls at a significance level of $P < 0.05$, processed in MS - excel 2007 version.

Principal component analysis (PCA) was conducted on sediment data to detect trends of variation of parameters between the bioaugmented and control systems in the experiment. This analysis also uses an ordination plot to project the points of greater similarities closer together while samples more dissimilar are further apart. Unlike biological data, environmental data usually have mixed measurement scales, and similarity methods, such as normalised euclidean distance used in PCA, are more appropriate for environmental data (Clarke & Warwick 2001).In this study all the variables measured were included for the PCA using PRIMER 6+ software version .

2.3. Results

2.3.1 Qualitative analysis of extracellular hydrolytic enzymes

Bacillus MCCB 101 exhibited production of extra cellular enzymes such as amylase, protease and xylanase (halo zone ranging from 35 to 44 mm). It showed mild lipase activity (halo zone 15mm), whereas the culture was negative for cellulase and chitinase. Among the substrates used for detecting protease activity in the plate assay the zone of clearance was higher in gelatin plates (40mm) than that in casein (21mm**)** indicating higher gelatinase than caseinase activity.

2.3.2 Optimization of cell count of *Bacillus* MCCB 101 to be supplied for bioremediation in bioassay system

Determination of optimum cell count of *Bacillus* MCCB 101 was accomplished based on the response of sediment in the bioassay system in its pH, E*h* and total organic carbon on applying bio-augmenter. pH variations in sediment stood with in the range of 7.3 to 8.6 in both treated and control systems. *Eh* responded positively to the application of the bi-augmenter. An increment of 40 -50% in E*h* was recorded in tanks applied with 10^4,10^5,10^6 CFU of *Bacillus* MCCB 101/mL of the overlying water (2 inches depth). This status in *Eh* continued till 7 days, but got reduced subsequently. Notably, E*h* of the systems which received highest cell count (10^7cfu/mL of the over lying water) of the bio-augmenter did not show improvement and behaved as that of the control (Table 1).

Highest reduction of TOC was 36% in the systems supplied with cells to final CFU of 10^6 on 4^{th} day of application. This situation was maintained for 15 days reaching to a reduction of 29%. The tanks augmented with 10^4,10^5 and 10^7 cells experienced 33% decrease of TOC on the 4^{th} day which was also maintained till 15^{th} day. On 20^{th} day onwards substantial increase of TOC was noticed in all the systems (Table 1).

A highly significant negative correlation was found to exist between TOC and *Eh* on application of *Bacillus* MCCB 101 (Table 1). The correlation was highest in the system which was supplemented with 10^5 CFU/mL; accordingly it was selected as the optimum count suitable for further experiments. But in the systems provided with higher cell counts (10^6 and 10^7 cfu/mL) such a relationship was not noticed.

2.3.3 Physico - chemical quality of shrimp pond sediment on application of *Bacillus cereus* MCCB 101 at optimal cell count

2.3.3.1 Total organic carbon, pH and E*h*

In treated sediment in the bioassay system, total organic carbon (TOC) was found significantly ($P < 0.05$) reduced (50% from an initial value of 5.19 to 2.55) on 7th day of application. However, it was found getting increased subsequently over a period of 10 to 15 days (Fig.3). pH varied within narrow limits throughout the experimental period both in the control and treated systems (Fig.2). With an increment of 45% on 7^{th} day of application, E*h* was significantly high ($P < 0.05$) in the treated sediment (-173mV) while it remained very low (-291 mV) in the controls (Fig. 1).

2.3.3.2 Quantification of extracellular enzyme's activity in sediment

The enzymatic activities were found substantially enhanced in bio-augmented systems. Significant differences ($p < 0.05$) in the activity of xylanase, amylase, chitinase, cellulase, and protease were observed between the bio-augmented and control tanks. Notably, activity of these enzymes was maximum on 7th day of application of bio-augmenter with a mean value of 2.36 µM/gm/hr for xylanase (Fig.4), 1.16 µM/gm/hr for amylase (Fig.5), 6.49 µM/gm/hr for chitinase (Fig.6), 1.55 µM/gm/hr for cellulase (Fig.8) and 64 µM/gm/hr for proteases (Fig.9) and found to be higher than those recorded in controls. The activity of lipase increased significantly ($p < 0.05$) from 0.80 µM/gm/hr (0^{th} day) to 3.56 µM/gm/hr on 7^{th} day in treated tanks, while it increased from 0.35 (day 0) to 1.41 µM/gm/hr (day 7) only in control tanks (Fig.7). Activity of trypsin was significantly high ($p < 0.05$) in bio-augmented tanks (Fig. 10) on 4^{th} and 7^{th} day of application. Meanwhile, chymotrypsin activity was found only in bio-augmented tanks, especially on 4^{th}, 7^{th} and 10^{th} day of application having velocity of hydrolysis at the rate 38.46, 19.25 and 38.43 µM/gm/hr respectively (Fig.11).

2.3.3.3 Biochemical composition of organic matter

Total protein in the treated sediment samples was significantly depleted from that in controls from day 4 to 7 of application of the bio-augmenter. On 7^{th} day of application the reduction was significant ($p < 0.05$) by 45% compared to that of the control (Fig. 13). Total carbohydrate was also significantly lower in bio-augmented samples on all sampling days (Fig. 12). It got reduced by about 53% in the treated sediment on the 7^{th} day. Total lipids were significantly higher in the control systems compared to the bio-augmented tanks (Fig.14).

2.3.3.4 Measurement of microbial abundance

The direct bacterial counts in sediment as estimated by epifluorescence microscopy were significantly higher in bio-augmented systems ($p<0.0001$) than that in control systems. The bacterial cells increased from $2.47\times10^7 \pm 0.09$/gm to $6.14\times10^9 \pm 0.148$/ gm on day 4 recording $4.26\times10^9 \pm 0.112$ cells /gm in final sample (Fig 17). In untreated systems the trend in bacterial abundance was $3.19\times10^7 \pm 0.148$ cells/gm (day 0) to $3.15 \times10^7 \pm 0.07$ cells/gm (day 15). In parallel, average plate count of heterotrophic bacteria in bio-augmented sediment were also significantly higher ($p <0.05$) (Fig.15). Similar was the case of Actinomycete population, which was significantly higher ($p <0.05$) in bioaugmented sediments compared to control during the experiment (Fig.16). Fungal population also got augmented significantly on application of *Bacillus* MCCB 101 on 7th day ($1.06\times10^4 \pm 0.5$ CFU /gm) from the initial level of $6.04\times10^3 \pm 0.05$ CFU /gm; which however, went down to $1.8\times10^3 \pm 0.055$ CFU /gm on 15th day (Fig 18).

2.3.3.5 Principal component analysis

Principal Component Analysis (PCA) was performed on the normalized data set for the bioaugmented and control systems. The pattern of variation in sediment paramaters between bioaugmented and control systems was made clear in the PCA ordination (Fig.19). The analysis produced a total of 5 canonical axes. Principal axes 1 and 2 were found to be important and together they explained about 65% of the total variance between the sediment quality data sets of the two systems. Sediment E*h* , total organic carbon (TOC), enzymes viz xylanase , amylase ,Cellulase

along with total proteins , total carbohydrates (CHO) and direct bacterial counts (DBC) contributed significantly to the PC1, accounting for 53% of the variance in the data (Eigen value 9.55). PC2, which explained 11.9% of the total variance (eigen value 2.14), consisted primarily of the variables, viz pH, lipase, chitinase, protease, trypsin, Total heterotrophic bacterial counts (THB), Total actinomycetes counts (TAC) and total lipids (Table 2&3). An Eigenvalue gives a measure of the significance for the factor, which with highest Eigenvalue is the most significant. Eigenvalues of 1.0 or greater are considered significant.

The control system samples and day 0 sediment samples of bio-augmented systems (with high percentage of TOC and low E*h*) were ordinated on the left; while the remaining samples of bio-augmented systems that ordinates on right explain the efficiency of bio-augmentation strategy adopted in the progressive enhancement of sediment quality on application of bio-augmenter

2.4 Discussion

Shrimp production is characterized by high loads of organic matter in the form of pond inputs such as uneaten feed, faecal matters and organic fertilizers. The organic matter that accumulates at pond bottom forms sludge that may become toxic to cultured species (Tucker, 1985., Ayub *et al.*, 1993). Needless to say, in aquaculture, sediment and water quality is of prime concern. Boyd (1995) reported that the condition of pond bottom strongly influences water quality through exchange of substances across sediment water interface. A large accumulation of organic matter increases oxygen demand and creates/favours anaerobic conditions (Boyd, 2000). The

sediment used in the present study had exhibited initially E*h* of around -300 mV indicating high anaerobic state. Prevalence of anaerobic conditions in sediment produce undesirable gasses such as hydrogen sulfide and methane at the bottom causing acute stress to shrimp having negative impacts on appetite and feed conversion ratio. Redox potential is an indicator of the capability of a system for organic matter degradation. Looking at this angle, bio-augmentation significantly enhanced E*h* ($p<0.05$) by 45% on 7^{th} day (-173mV) of bio-augmenter application. This level of E*h* is better than the one reported (-200mV) in fish farm sediments (Pawar *et al*., 2002). Determining the efficacy of bio-augmentation through E*h* measurements is a novel approach adapted in this study. In the experimental systems during operations pH stood at optimum range (7.5 -8.0) suitable for aquaculture as stated by Boyd and Pippopinyo (1994).

On the other hand detritus in sediment to an extent (~ 2.0% TOC) is a nutritionally valuable food source important for the growth and survival of shrimps (Schroeder, 1987; Moriarty, 1997). Therefore, it was essential to determine the capability of *Bacillus* MCCB 101 to bring down organic matter during bio-augmentation. A single dosage of the bio-augmenter at an optimum cell count of 10^5 CFU/mL, *Bacillus* MCCB 101, could bring down the percentage of TOC in sediments to 2.5% on 7^{th} day of application from the initial value 5.19%. However, it was found increasing during subsequent days. Hence, it was concluded that weekly application of bio-augmenter could maintain the level of TOC in shrimp pond sediments at desirable limits. Banerjea (1 967) stated that the potential for fish production in ponds in India would be the highest where bottom soil would be

having 1.5 -2.5% organic carbon and it decreased both at lower and higher concentrations. Mean while, Hopkins *et al*. (1994) reported that during *Penaeus setiferus* culture the survival and production were very low in ponds with no moderate removal of sludge during culture period. Precisely, organic waste management during culture operations plays vital role in shrimp production. Therefore, a balanced removal rate of organic matter is preferred for ecological considerations. The results indicated that the optimum dosage of *Bacillus* MCCB 101 could decrease TOC and increase *Eh*. Whereas, higher cell counts applied (10^6 and 10^7 CFU/mL) reversed the process, probably due to the lack of sufficient dissolved oxygen required for higher population of heterotrophic bacteria for mineralization.

The chapter addresses efficiency of *Bacillus cereus* MCCB 101 in the bioremediation of organic wastes in shrimp pond sediment assayed in a simulated system. Variations in the patterns of extracellular enzymatic activity in the sediment of bio-augmented and control systems are coupled with relevant physico-chemical and microbiological parameters to understand the mechanistic action the bio-augmenter on application. Manipulation of environmental microbiota with probiotic microorganisms that enhance the growth of beneficial bacteria among detritivorous microbes in pond bottom have been reported by Lin (1995), Rengpipat *et al*. (1998) and Olafsen (2001).

Degradation of high molecular weight compounds to lower ones constitute the basis for heterotrophic metabolism, as compounds less than 600 Da can pass through pores in the cell wall. Microbial extra cellular hydrolytic enzymes are the major biological molecules for the

mineralization of sedimentary particulate organic carbon and nitrogen;, extracellular enzymes of heterotrophic bacteria are known to play a major role in the transformation of the organic matter (Azam *et al*., 1994) and in nutrient recycling of aquatic systems. Studies using artificial substrates to quantify these extracellular enzymatic activities in various aquatic environments are well documented in literature (Fabiano and Danovaro, 1998;Caruso and Zaccone, 2000; Sala *et al*., 2001; Caruso *et al*., 2003; Sebastián and Niell, 2004; Chrost and Siuda, 2006; Costa *et al*., 2007;Caruso, 2010). Methylumbelliferyl and Methylcoumaryl amide substrates have been introduced as useful and effective tools for the estimation of the extracellular enzyme activity and recognized that many extracellular enzymes are associated with bacterial cells (Hoppe *et al*.,1988, Meyer, 1986 ; Chrost, 1990 ; Hoppe, 1993 ; Poremba, 1995). These substrates (sigma) are analogous to major class of organic polymers encountered in aquaculture systems viz., proteins, starch, lipid, cellulose and chitin (Caruso, 2010). Their decomposition rates indicate the potential metabolic activity of the prokaryotic community in sediment.

In the present study xylanase, amylase, chitinase, cellulase and protease activities were highly significant ($P<0.05$) in bio-augmented tanks. Although not significant ($P>0.05$) the rate of lipase activity shifted from 0.80 µM/gm/hr (0^{th} day) to 3.56 µM/gm/hr (day-7) in treated tanks similar to the other enzymes. The total bacterial number (determined through epifluorescence microscopy), and viable heterotrophic bacterial and actinomycete counts were positively correlated with these enzymes and negatively correlated with the protein content of the sediment (r = -0.9, -0.7,

-0.8 respectively). Similar to these findings, Gallizia *et al*. (2005) reported bio-augmentation stimulated functional parameters such as β- Glucosidase and Leu- aminopeptidase (protease) activities in aquatic system. The V*max* (496.5 and 536.5 nM/mg/hr) observed of the above enzymes by Gallizia *et al*. (2005) was higher than that observed in the present study. However, the enzymatic activities were positively correlated with the bacterial population as observed here. Moreover, there are reports on wide distribution of β-Glucosidase and Leu - aminopeptidase (protease) in aquatic environments and have been considered as model ectoenzymes for studying bacterial degradation of natural polymers, carbohydrates and proteins (Chrost, 1992).

The total heterotrophic bacterial counts (THB) recorded in the sediment of simulated systems were significantly higher ($p<0.05$) than those of the controls. THB in bio-augmented sediment ranged between 2.82×10^5 to 3.02×10^6 CFU/g and in control tanks between 3.48×10^5 to 6.22×10^5 CFU/g. Similar findings were reported by Devaraja *et al*. (2002) where application of commercial probiotics in pond sediment significantly increased total bacterial plate counts of the treated sediments. THB in sediment samples reported by Nimrat *et al*. (2008) are comparable with the THB recorded in the present study. However, in tilapia pond bottom soil THB was much higher, $7.3\pm 1.1\times10^7$ CFU/g (Al-Harbi and Uddin, 2005).

Significant depletion of organic compounds was observed in bio-augmented tanks (Fig 13) having 45% reduction on 7th day from the initial value. Protein being the more labile fraction of organic matter the hydrolytic rates of protease, trypsin and chymotrypsin recorded the highest value among the quantified enzymes. Online with these findings organic matter

degradation in terms of the particulate carbohydrates and total lipids was also significantly higher in bio-augmented tanks with a mean value of 1553.61µg/gm and 13.08µg/gm accounting 53% and 25% reduction from the initial values respectively. The concentration of sedimentary organic matter followed a pattern lipid>CHO>protein in the present study. This finding is similar to that of Gallizia *et al*. (2005) who observed carbohydrates in harbor sediments greater in concentration than that of proteins. In contrast, Fabiano and Danovaro (1998) found proteins to be the major class of organic compound in marine sediment ranging from 232 - 4098 µg/gm sediment; the total CHO and the total lipids being the least represented.

Direct relationship between enzymatic activity and bacterial abundance has been reported (Romani and Sabater, 1999). Actinomycetes showed strong positive correlation with chitinase activity (r= 0.99) where as the activity of chymotrypsin was strongly correlated with total heterotrophic bacterial plate counts (r= 0.98). Actinomycetales, commonly termed as actinomycetes, cover the cultivable group of actinobacteria from diverse ecological niches. These Gram positive, filamentous bacteria are able to breakdown complex organic polymers (Das *et al*., 2008) as good sources of secondary metabolites. The contribution of fungal population on overall enzymatic activity was negligible except for chymotrypsin and xylanase (r values 0.9 and 0.4 respectively) on 7^{th} day of bio-augmenter application; however, it was negatively correlated with total bacterial counts. In terms of abundance, the population of bacteria exceeded fungi and hence there were possibilities of metabolic out competence. Antagonism between bacteria

and fungi is reported as an important controlling factor for microbial colonization and growth in aquatic plant litter (Mille-Lindblom and Tranvik, 2003). The significant increase ($p < 0.05$) in fungal plate counts (from 6.04 $\times 10^3$ CFU/gm to 1×10^4 CFU/gm) in bio-augmented tanks on the 7th day and its subsequent reduction to 5.2 $\times 10^3$ CFU/gm is negatively correlated ($r = -0.3$) with the total plate count of heterotrophic bacteria (1.3×10^6 CFU/gm) on 7th day of bio-augmenter application from a previous count (2.15×10^6 CFU/gm) on 4th day of application. The maximum cell count was attained (3.2×10^6 CFU/gm) on 15th day of application.

Overall increase in enzymatic activity in bio-augmented systems was negatively correlated with the concentration of total proteins, CHO, lipids and percentage of total organic carbon. In bio-augmented systems they were found depleted significantly ($p < 0.05$) by 45%, 54%, 26% and 50% respectively on 7th day of application of the bio-augmenter in contrast to the natural degradation of proteins, CHO and lipids (12%, 3%, 2.21% respectively) and not of total organic carbon in control systems. The data strongly suggest that *Bacillus cereus* MCCB 101 introduced into the system initiated a cascade of events to upgrade the sediment quality assessed in terms of functional parameters. Rapid degradation of accumulated organic wastes and overall improvement in the health and yield of cultured organisms have been recorded upon introducing active cells of selected microorganisms into ponds (Gatesoupe, 1999; Shishehchian, 2001; Devaraja, 2002). Application of probiotics in pond soil can accelerate decomposition of undesirable organics and other waste products

(Gatesoupe, 1999; Gross *et al*., 2003; Wang and Han, 2007) and perhaps even can increase ambient levels of oxygen (Boyd, 1995, 2000).

Vine *et al*. (2006) stated the need for selection of indigenous microbes as probiotics/bio-augmenters. The indigenous bacteria thus introduced are functionally linked to wastes present in sediments, and can contribute to the right metabolic competencies in the system, where the activities of other natural flora are limited, especially under organic matter loading at high rates at which ambient heterotrophic bacteria may not decompose and utilize or recycle the same effectively (Patel *et al*., 2002). The bio-augmenter used here is a benthic bacterial isolate obtained from shrimp pond sediment. Being indigenous, its application accelerates the entire microbial community to trap energy flux via high metabolic activities facilitated by enzymatic degradation. This drives the entire system to effective biodegradation compared to that in controls.

Bacillus MCCB 101 is a benthic isolate obtained from shrimp pond sediment. As shrimps are being fed with protein diet containing animal protein, the ponds happen to be rich in proteinaceous waste among which gelatin turns out to be the major component. In this context the higher gelatinase activity of *Bacillus* MCCB 101 has greater significance. Microbial extra cellular hydrolytic enzymes are the major biological molecules which mediate mineralization of sedimentary particulate organic carbon and nitrogen. The results suggested that, as *Bacillus* MCCB 101 produced variety of the hydrolytic enzymes for degrading organic matter to gain energy and carbon, it could be categorized to be an ideal organism for bioremediation in aquaculture.

The extracellular enzymatic profile of the isolate favours degradation of structural and associated polymers encountered in aquatic systems suggesting that, *Bacillus cereus* MCCB 101 can gain carbon and energy for growth from these compounds. Thus with less accumulation of organic matter, better penetration of oxygen into sediment can be achieved enhancing quality of sediment helping shrimps to dwell comfortably in the sediment water interface.

Table 1: Effect of various cell counts on sediment E*h* and total organic carbon (TOC) in bio-augmented systems compared to that in controls (mean values of triplicates ± SD shown)

Days	10^4 cfu/ml		10^5 cfu/ml		10^6cfu/ml		10^7 cfu/ml		Control	
	E*h*	TOC	E*h*	TOC	E*h*	TOC	E*h*	TOC	E*h*	TOC
0	-106.4 ± 23.7	2.07 ± 0.33	-130 ± 31.15	1.925 ± 0.11	-119 ± 2.35	2.275 ± 0.18	-103.8 ± 5.97	1.975 ± 0.67	-115.46 ± 4.62	1.75 ± 0.24
4	-59.66 ± 11.4	1.4 ± 0.24	-73.46 ± 8.37	1.35 ± 0.45	-78.93 ± 9.29	1.45 ± 0.11	-134.46 ± 32.19	1.525 ± 0.30	-119.9 ± 4.61	1.625 ± 0.24
7	-46.2 ± 4.02	1.625 ± 0.17	-63.46 ± 15.4	1.55 ± 0.18	-64.53 ± 25.08	1.65 ± 0.15	-103.4 ± 4.10	1.675 ± 0 .38	-116.13 ± 2.94	1.675 ± 0.38
10	-90.13 ± 2.08	1.55 ± 0.18	-87.93 ± 10.47	1.6 ± 0.11	-106.8 ± 8.18	1.87 ± 0.25	-114.86 ± 14.87	1.7 ± 0.41	-114.66 ± 1.90	1.975 ± 0.24
15	-79.73 ± 12.64	1.85 ± 0.229	-82 ± 7.32	1.55 ± 0.18	-106.26 ± 7.13	1.6 ± 0.11	-107.33 ± 2.11	1.4 ± 0.26	-110.13 ± 5.08	1.5 ± 0.07
20	-104.44 ± 2.61	2.35 ± 0.30	-103.44 ± 5.93	2.125 ± 0.30	-102.44 ± 6.35	2.17 ± 0.27	-101.4 ± 3.78	2.57 ± 0.33	-100.44 ± 4.42	2.57 ± 0.04
Correlation(r)	-0.744		-0.76		-0.67		0.49		0.77	

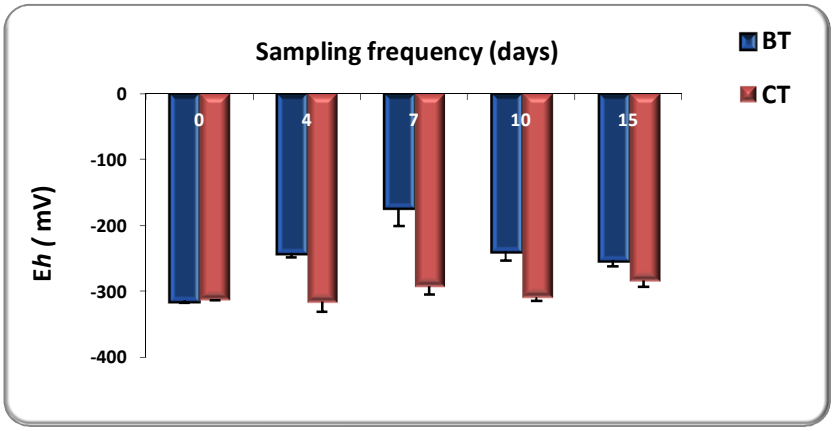


Fig 1: Variations in redox potential (*Eh*) of bio-augmented and control systems

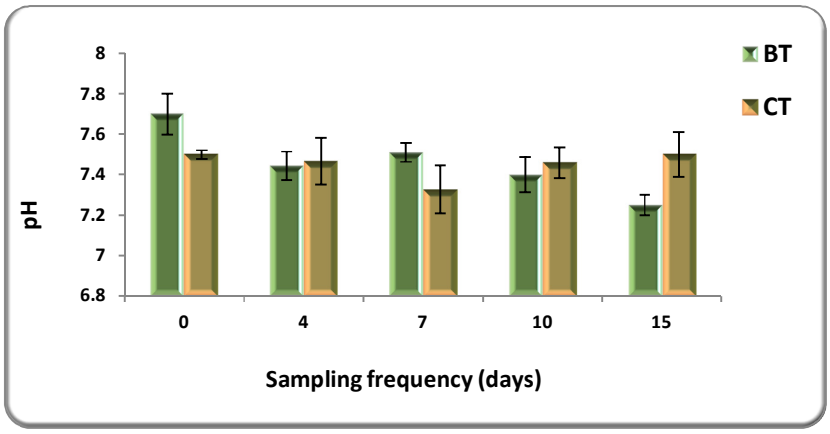


Fig 2: Variations in soil pH of bio-augmented and control systems

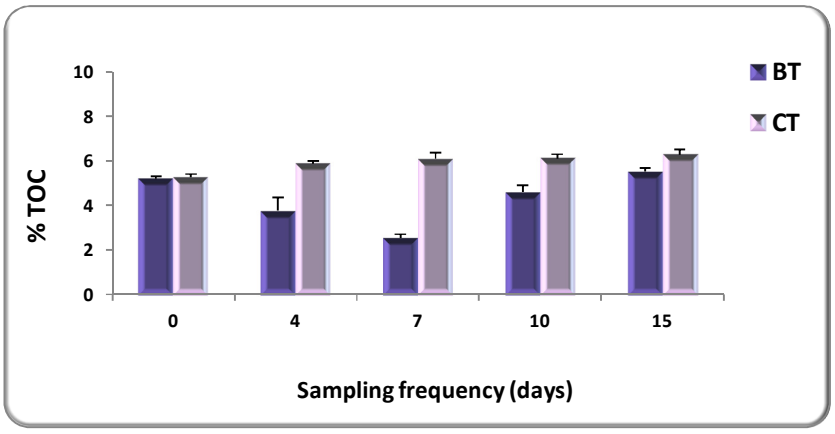


Fig 3: Variations in percentage of total organic carbon in bio-augmented and control systems

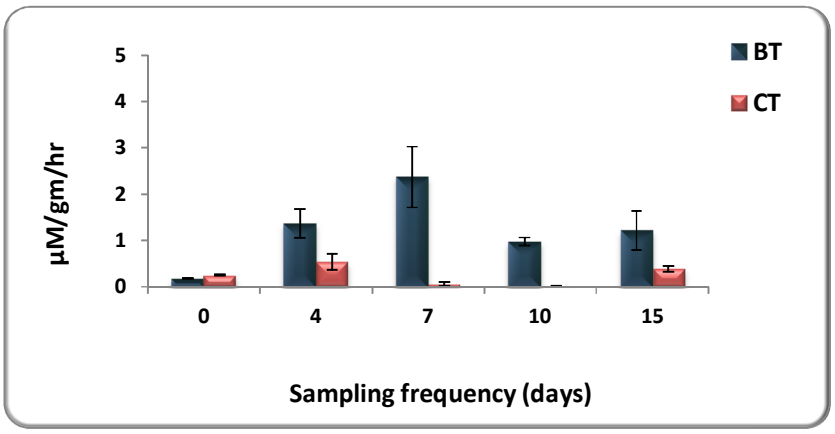


Fig 4: Pattern of xylanase activity in sediment samples of bio-augmented and control systems

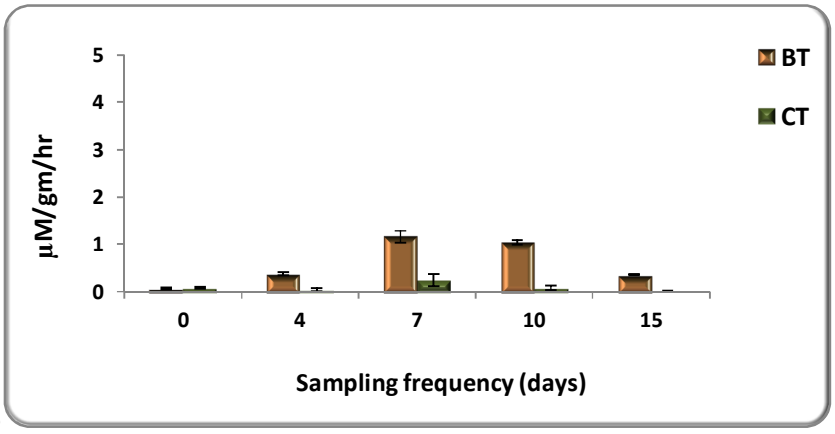


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Fig 5: Pattern of amylase activity in sediment samples of bio-augmented and control systems

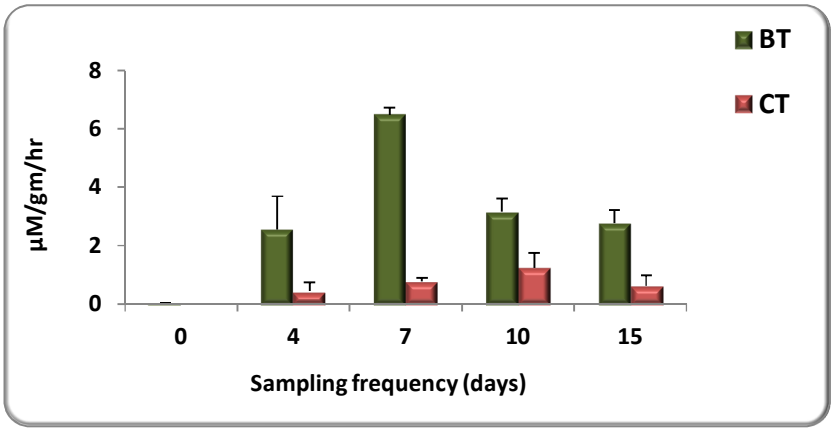


Fig 6: Pattern of chitinase activity in sediment samples of bio-augmented and control systems

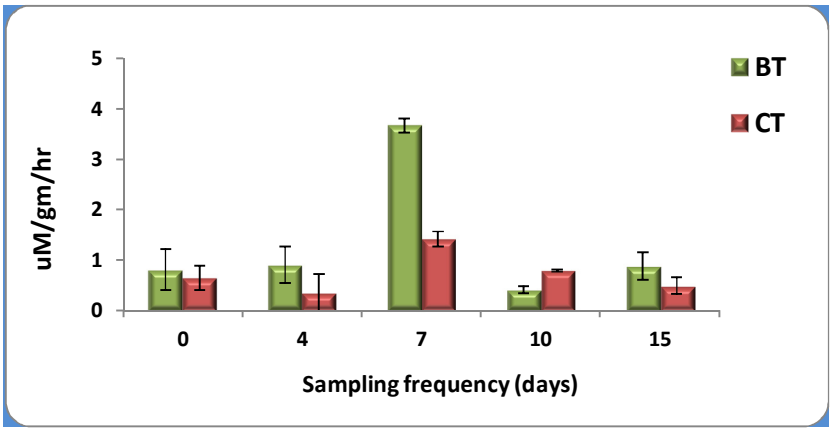


Fig 7: Variations in the activity of lipase in sediment samples of bio-augmented and control systems

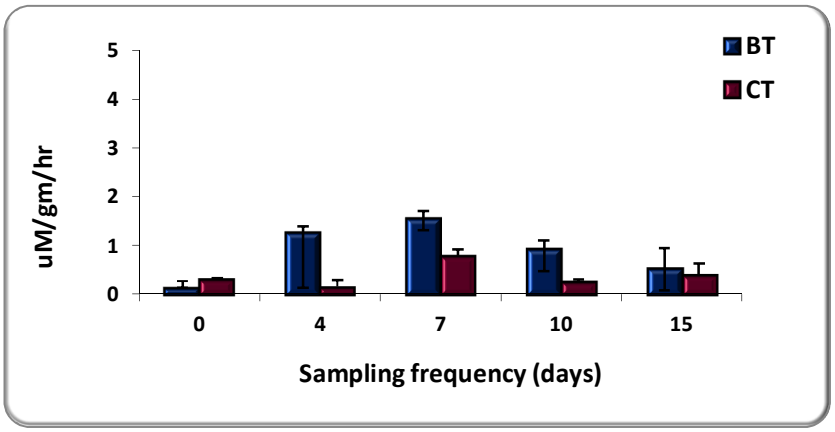


Fig 8: Variations in the activity of cellulase in sediment samples of bio-augmented and control systems

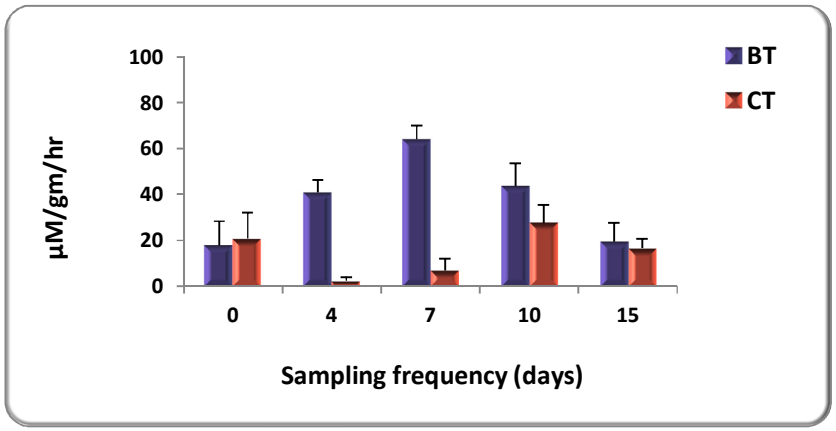


Fig 9: Variations in the activity of protease in sediment samples of bio-augmented and control systems

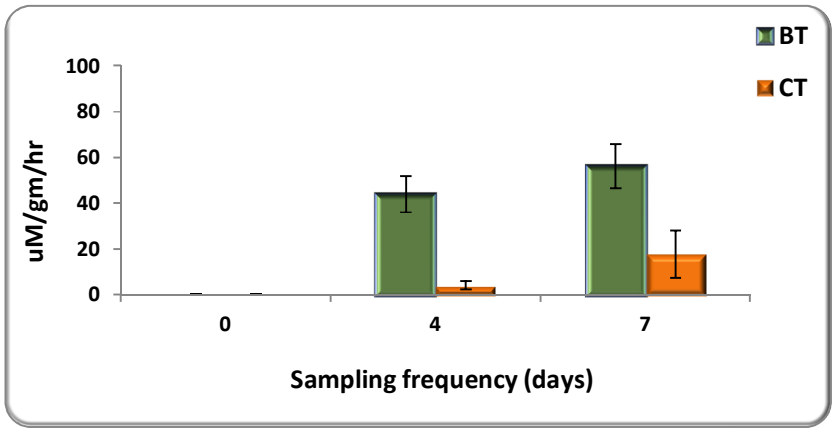


Fig 10: Variations in the activity of trypsin (endopeptidase) in sediment samples of bio-augmented and control systems

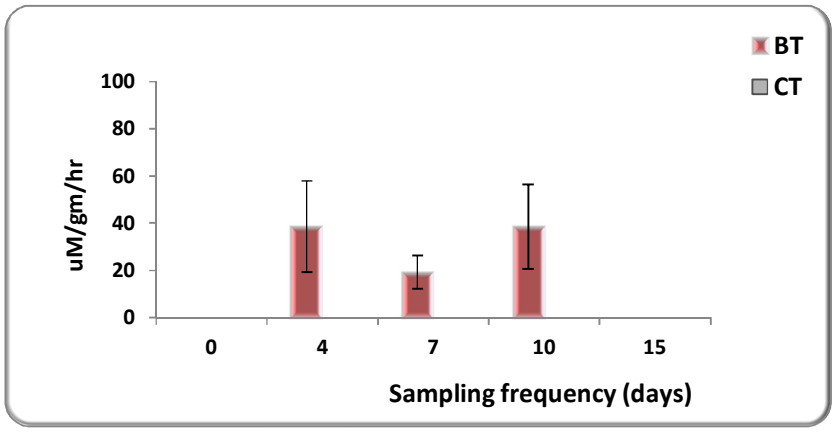


Fig 11: Variations in the activity of chymotrypsin (endopeptidase) in sediment samples of bio-augmented and control systems

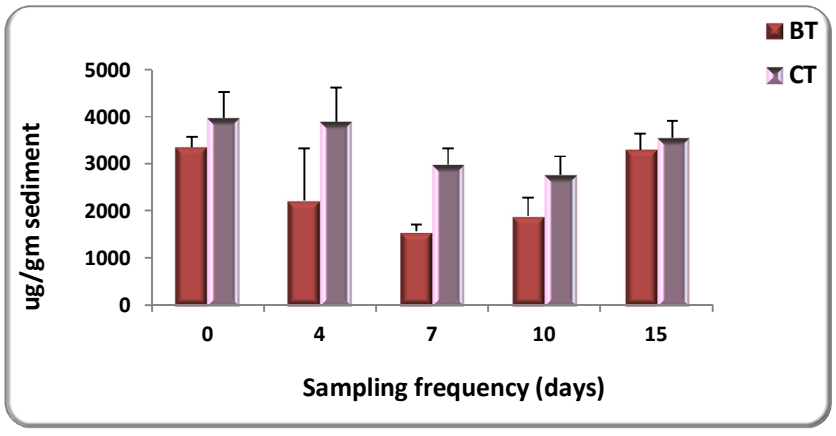


Fig 12: Impact of bio-augmentation in the concentration of total carbohydrates

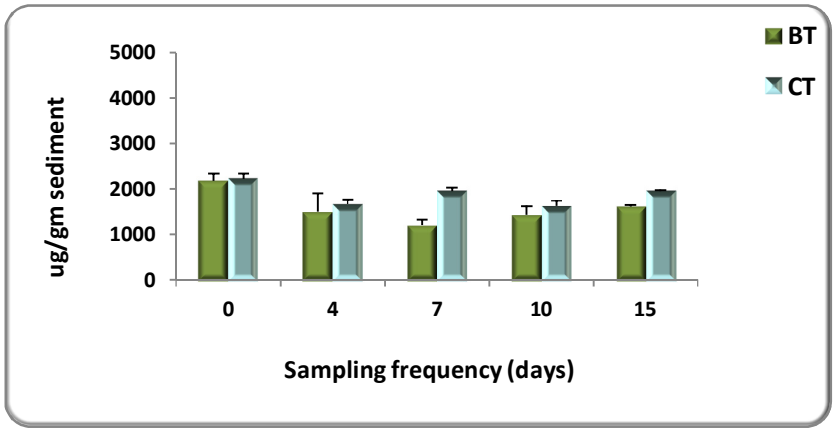


Fig 13: Impact of bio-augmentation in the concentration of total proteins

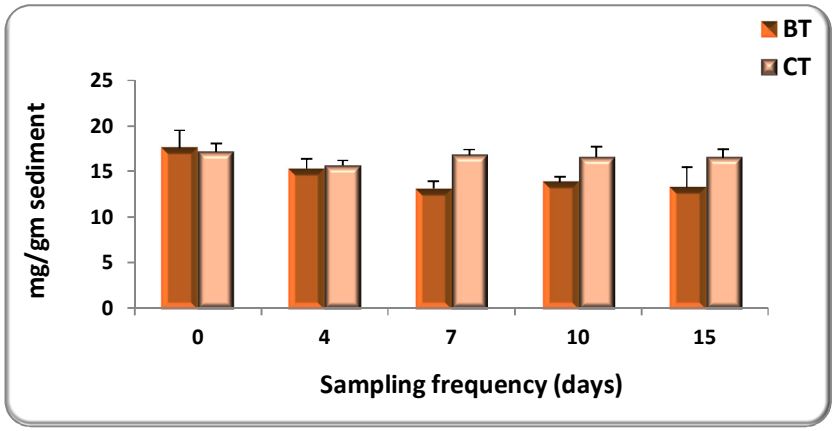


Fig 14: Impact of bio-augmentation in the concentration of total lipids

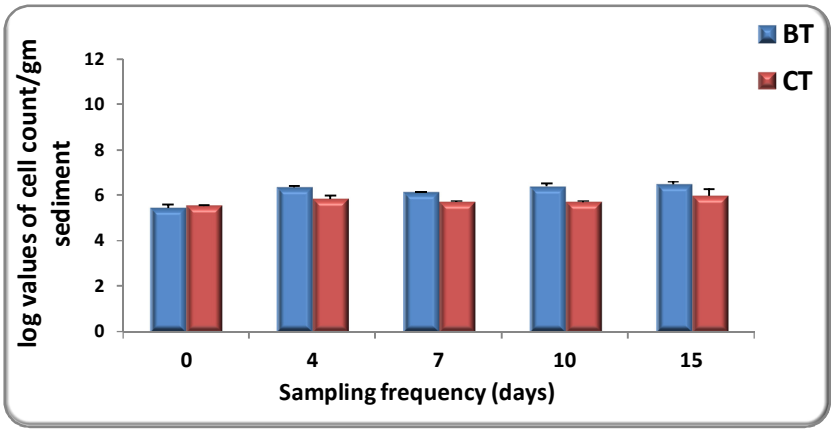


Fig 15 : Variations in total heterotrophic bacteria in bio-augmented and control systems

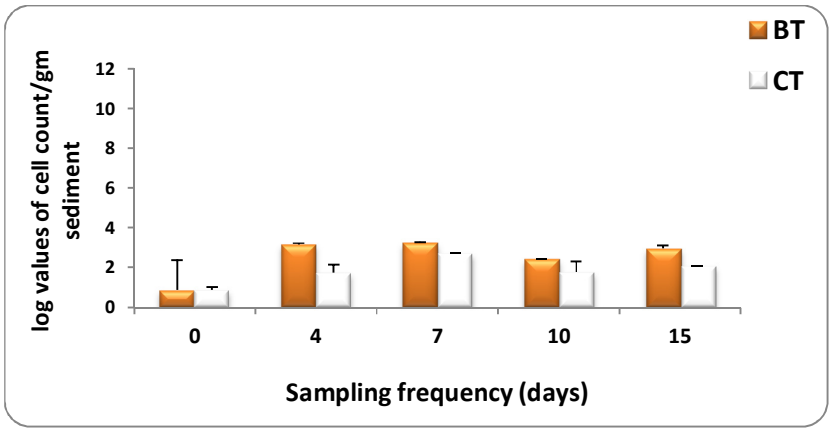


Fig 16 : Variations in total actinomycetes in bio-augmented and control systems

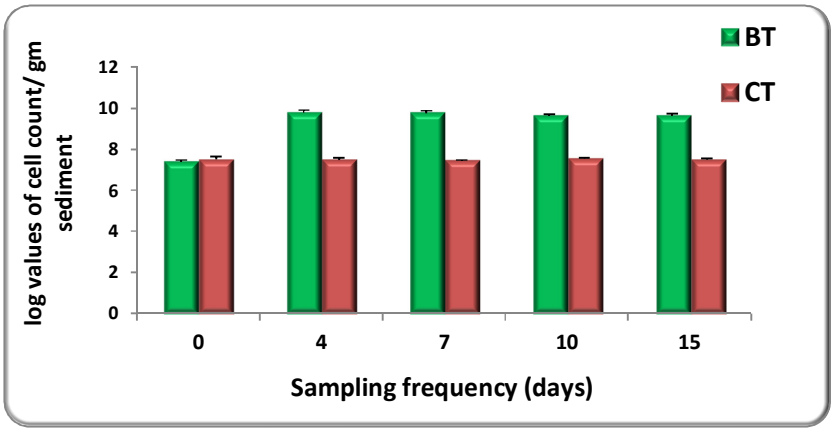


Fig 17: Pattern of total bacterial density in bio-augmented and control systems as estimated by epifluorescence microscopy

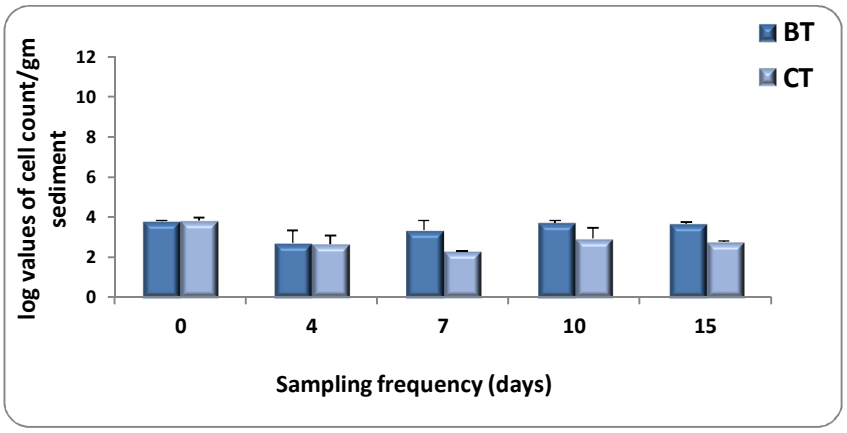


Fig 18: The pattern of fungal population in bio-augmented and control systems

Table 2: Results of Principal component analysis –Eigenvalues of principal components

PC	Eigenvalues	%Variation	Cum.%Variation
1	9.55	53	53
2	2.14	11.9	65
3	1.74	9.7	74.6
4	0.976	5.4	80.1
5	0.765	4.3	84.3

Table 3: Results of Principal component analysis –eigenvector values for principal components 1 & 2

Variable	PC1	PC2
TOC	-0.268	0.292
PH	-0.113	-0.478
Xylanase	0.288	0.027
Lipase	0.157	-0.294
Amylase	0.273	-0.102
Cellulase	0.278	-0.107
Chitinase	0.196	0.291
Protease	0.196	-0.25
Trypsin	0.2	-0.223
Chymotrypsin	0.275	-0.095
DBC	0.303	0.105
THB	0.247	0.339
TFC	0.026	-0.011
TAC	0.177	0.313
T-Prt	-0.265	-0.123
T-Cho	-0.247	0.236
T-Lip	-0.233	-0.264
E*h*	0.305	-0.078

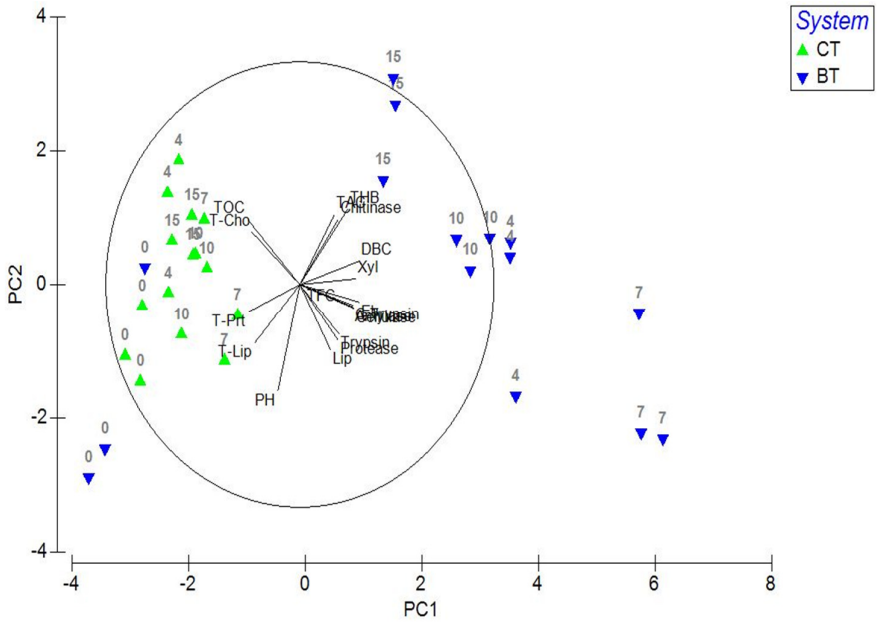


Fig 19: Principal component analysis of sediment parameters in Bioaugmented and Control Systems

Chapter 3

BIOAUGMENTATION POTENTIAL OF *BACILLUS CEREUS* MCCB 101 IN SIMULATED ZERO WATER EXCHANGE SHRIMP GROW OUT SYSTEM OF *PENAEUS MONODON* AT HIGH STOCKING DENSITY

3.1. Introduction

Among various aquaculture production systems, one of the fastest growing sectors is that of the penaeid shrimp. Black tiger shrimp *Penaeus monodon* is the prominent species of *Penaeus* being cultured commercially in many countries because of its high economic value in the international market. In India, the export oriented shrimp based coastal aquaculture is mainly dominated by the giant tiger prawn (*Penaeus monodon*), can grow-up to a large size (40 - 60g) followed by the Indian white prawn (*Fenneropenaeus indicus*) (FAO, 2011).

Aquaculture intensification practices emerged for productivity enhancement to reconcile the inadequacy of land and water resources

(Brune *et al*., 2003; Delgado *et al*., 2003; Piedrahita, 2003; Avnimelech *et al*., 2008). Intensive aquaculture production systems avoids unplanned wasteful use of water per kg biomass produced in contrast to normal production systems that requires upto 45 m^3 water per kg biomass produced in ponds (Verdegem *et al*., 2006) .However Such farming practices avenues the risk of disease outbreaks too (Kautsky *et al*.,2000; Guan *et al*., 2003). To tranquilize the environmental impacts of effluent discharge due to rapid expansion and to knock off disease contamination risks from externally polluted water supply, shrimp culture in recent years has evolved from open system with frequent water discharge to closed system with limited or zero water discharge (Arnold *et al*., 2009).

Zero water exchange systems furnish an environmental friendly green water technology of shrimp production with guaranteed biosecurity, defined as "the sum of all procedures in place to protect living organisms from contracting, carrying, and spreading diseases and other non-desirable health conditions" (Moss *et al*., 1998). Significant reduction in released nutrient-rich water allows the system to be operated with native or exotic species with minimal risk of escapement into receiving waters while simultaneously decreasing the probability of disease introduction from water exchange (Moss, 2002).

The major problem associated with closed system is the rapid eutrophication of culture ponds, resulting from increasing concentrations of nutrients (ammonia and nitrite) and organic matters over the culture period (Thakur and Lin, 2003). Likewise, Tacon *et al*., (2002) revealed that closed zero-water exchange culture systems can only biologically support a certain

level of nutrient input and shrimp biomass without the system 'crashing' and compromising shrimp growth and survival. Obviously, the balance between waste production and assimilation capacity in pond environment is of paramount importance for the success of closed system. Waste management of zero water exchange aquaculture production systems by application of viable heterotrophic bacteria is a sensible approach to appraise growth of culture organisms, mortality and the overall expansion of total biomass in the system.

The utilization of probiotic applications to RAS has become recognized as a beneficial practice to shrimp aquaculture (Ninawe and Selvin, 2009). These bacteria once established aid in the maintenance of water quality in general, most often with respect to nitrification (Kuhn *et al*., 2010). Further advantages to bacterial supplementation include microbial floc as natural food to improve growth (Burford *et al*., 2004; El-Haroun et *al*., 2006), prevention of pathogen proliferation through competitive exclusion (Verschuere *et al*., 2000), and immunostimulation of the immune system of target organisms (Ganguly *et al*., 2010). Thus sufficient literature concerning the benefits of probiotic application in aquaculture is available but a scientific substantiation on bioaugmentation mechanism of a probiotic is scarce. Therefore, in this context, an investigation was undertaken to observe the impact of an indigenous bio-augmenter, *Bacillus cereus* MCCB101 on stabilizing the ecological and environmental parameters to sustain growth of *P. monodon* at high stocking density in a simulated zero water exchange shrimp grow out system.

3.2 Materials and Methods

3.2.1 Bioassay system

Fiber reinforced plastic (FRP) tanks of 25litre capacity (half meter square) were used as bioassay systems. A set of tanks for bio-augmentation (here after referred to as 'bio-augmented systems' abbreviated as BT in figures) and a set of control tanks (here after referred to as 'control systems' abbreviated as CT in figures) were maintained in triplicate. Bottom of the tanks were laid with fresh pond sediment collected from an active shrimp farm in Kumbhalanghi, kochi after harvest of reared stock. Top layer of sediment collected from random sites of the farm were pooled, mixed and layered in 3 inches thickness in both the sets of tanks meant for bio-augmentation and as control.

3.2.2 Pre-conditioning of sediment

Sediment in the set of tanks meant for bio-augmentation was alone pre-conditioned prior to adding seawater while the control systems were left undisturbed. The absorbance of overnight grown *Bacillus* MCCB 101 in 15 g/L salinity ZoBell's broth was adjusted to a cell count of 10^5 CFU/mL. This seed culture was re-suspended to a final volume of 150 mL peptone water and applied in systems meant for bio -augmentation. The sediment samples were collected at random for periodic estimation of E*h*, pH and total organic carbon. The application of bio-augmenter was continued once in a week till the total organic carbon of sediment had dropped to 2% and E*h* rose to around -150 mV.

3.2.3 Stocking and management of the system

When sediment E*h* and total organic carbon in the bio – augmented systems were in desirable limits for shrimp culture, both bio –augmented and control systems were filled with sea water 20 L at 15 g/L salinity, and left undisturbed for 2 days. They were stocked with *Penaeus monodon* juveniles tested nested PCR negative for WSSV at a stocking density of (10 nos per tank) 20 nos/m^2.The total biomass per tank ranged between 25- 36 mg provided with an aeration rate of 10L min^{-1} . Average weight of juveniles stocked ranged between 2.5 to 3 gms and were fed with 6% of their body weight twice a day 4 g of shrimp feed / tank daily. Frequency of bio-augmentation was weekly once during day 1 - 30^{th}, on alternate days from day 30 - 60^{th} and daily from day 60 - 90^{th} decided as per the conditions prevalent in the systems. Water and sediment samples were analyzed periodically for the following parameters.

3.2.4 Analysis of water quality parameters

3.2.4.1 pH, temperature, dissolved oxygen and salinity

pH was measured using digital pH meter, temperature using maximum–minimum thermometer, salinity using refractometer and dissolved oxygen using DO meter.

3.2.4.2 Estimation of ammonia (Solorzano, 1969)

Ammonia reacts in moderately alkaline solution with hypochlorite to monochloramine, which, in the presence of phenol, catalytic amounts of nitroprusside ions and excess hypochlorite, gives indo-phenol blue. The formation of monochloramine requires a pH between 8 and 11.5. At a pH

higher than 9.6, precipitation of Mg and Ca ions as hydroxides and carbonates occurs in seawater. However, these ions can be held in solution by complexing them with citrate.

An aliquot of 10 mL sample was taken in a test tube, added 0.4 mL phenol solution (20 g of crystalline phenol dissolved in 95% V/V ethyl alcohol), 0.4 ml sodium nitroprusside (1 g dissolved in 200 mL distilled water) and 1.0 mL oxidizing solution [alkaline reagent (100 g sodium citrate and 5 g sodium hydroxide dissolved in 500 mL distilled water) and sodium hypochlorite 4: 1 ratio]. Absorbance was measured at 640 nm after 1 hr incubation at room temperature. A series of standards (4.714 mg ammonium chloride dissolved in 100 mL double distilled water gave 10µg/mL ammonia - nitrogen) were prepared and the factor value was calculated.

Ammonia - nitrogen in mg/L = Factor value x Absorbance of the sample

Factor Value = Concentration of standards/ absorbance.

3.2.4.3 Estimation of nitrite and nitrate (Bendschneider and Robinson, 1952)

Under acidic condition (pH 2.0 to 2.5) nitrite ion (NO_2^-) as nitrous acid (HNO_3) react with sulphanilamide to form a diazonium salt, which combines with N- (1-naphthyl)-ethylene diamine dihydrochloride (NED dihydrochloride) to form a bright coloured pinkish red azo dye. The colour produced is directly proportional to the amount of nitrite present in the sample.

Nitrate is determined by converting nitrate to nitrite using a mixture of phenol – sodium hydroxide and copper sulphate - hydrazine sulphate.

Reagents are added and incubated at dark for 18 hours, added acetone and complexed with sulphanilamide and NED.

3.2.4.4 Nitrite analysis

An aliquot of 10 mL sample was taken in a test tube, added 0.2 mL sulphanilamide (5 g dissolved in a mixture of 50 mL concentrated hydrochloric acid and 450 mL distilled water) and 0.2 mL NED (0.5 g dissolved in 500 mL distilled water). The absorbance was taken after 8 minutes at 543 nm. Series of standards (4.925 mg sodium nitrite dissolved in 100 mL gave 10µg/mL) were prepared and calculated the factor value.

3.2.4.5 Nitrate analysis

To the 10 mL sample added 0.4 ml phenol – sodium hydroxide solution [This solution was prepared by the mixing phenol solution (46 gm dissolved in 1 L distilled water) and sodium hydroxide (30 g dissolved in 2 L distilled water) at 1:1 ratio] and 0.2 mL hydrazine sulfate – copper sulphate solution [this solution prepared by the mixing hydrazine sulphate (14.5 g hydrazine sulphate dissolved in 1 L distilled water) and copper sulphate (0.1 g copper sulfate dissolved in 1 L distilled water) at 1:1 ratio], incubated in dark for 18 - 24 hours. After incubation 0.4 mL acetone, 0.2 mL sulphanilamide and 0.2 mL NED were added. Absorbance was measured after 8 minutes at 543 nm. A series of standards (6.0707 mg sodium nitrate dissolved in 100 mL gave 10 µg/mL nitrate - nitrogen) were prepared and calculated the factor value.

Calculation

Concentration of nitrate in sample in mg/L = [(x-y) x 100/ efficiency]

Where x = Absorbance of nitrate x Factor value of nitrate

Y = Corresponding concentration of nitrite

Efficiency = (A/B) x 100

Where A = Observed concentration of standard (absorbance x factor value of nitrite)

B = Original concentration of standard prepared.

Efficiency measures the percentage of nitrate converted into nitrite

3.2.4.6 Estimation of inorganic phosphate (Ascorbic acid method) (Strickland and Parson, 1972)

Ammonium molybdate and potassium antimonyl tartarate react in acid medium with orthophosphate to form a heteropoly acid - phosphomolybdic acid- that is reduced to intensely coloured molybdenum blue by ascorbic acid. Intensity of the colour can be measured photometrically and this is related to the concentration of phosphate by Beer-Lambert's law.

An aliquot of 10 mL sample was taken in a test tube, 1.6 mL combined reagent [mixed 5 mL 5 N sulphuric acid (14 mL concentrated sulphuric acid diluted to 100 mL distilled water), 0.5 ml potassium antimonyl tartarate (0.686 g dissolved in 250 mL distilled water), 1.5 mL ammonium molybdate (4 g dissolved in 100 mL distilled water) and 3 mL ascorbic acid (1.76 g dissolved in 100 mL distilled water)] were added. Absorbance was measured at 880 nm after 10 min incubation. A series of standards (54.87 mg anhydrous potassium dihydrogen phosphate dissolved in 250 mL distilled water gave 50 µg PO_4^{3-}/ mL) were prepared and calculated the factor value.

Concentration of phosphate ions in sample in mg/L

= factor value x absorbance.

3.2.4.7 Estimation of alkalinity

Alkalinity of the water samples were measured by titrating with standard hydrochloric acid (APHA, 1995). Titration to pH 8.3 or decolorisation of phenolphthalein indicator indicate complete neutralization of OH^- and half of the CO_3^-, while pH 4.5 or sharp change from yellow to pink of methyl orange indicator, indicate total alkalinity (complete neutralization of OH^-, CO_3^- and HCO_3^-). Alkalinity is commonly expressed in mg $CaCO_3$/ L.

An aliquot of 100 mL sample was taken in a conical flask and added 3 drops of phenolphthalein indicator. If there has been no colour produced, the phenolphthalein alkalinity turns out to be zero. If a pink colour gets developed it is titrated against 0.1N hydrochloric acid (8.3 mL of concentrated hydrochloric acid in 1L distilled water) till the colour disappears or pH 8.3 is attained. Subsequently, an aliquot of 3 drops of methyl orange was added to the same flask, and continued titration till pH dropped to 4.5 or the yellow colouration changed to orange. The volume of hydrochloric acid used was noted. In case the pink colourations did not appear after addition of phenolphthalein, the estimation of methyl orange alkalinity was continued.

Calculation

Phenolphthalein alkalinity (mg/L $CaCO_3$) = (A x 1000) / mL sample

A = Pink to colorless end point

Total alkalinity of sample in mg $CaCO_3$ /L

= (Normality of HCl x volume of HCl consumed/volume of sample taken) x 50 x 1000

3.2.4.8 Estimation of hardness

EDTA forms a chelated soluble complex when added to a solution of certain metal cations (APHA, 1995). Metal cations in the presence of Eriochrome Black T at a pH of around 10.0 become wine red. When EDTA is added as a titrant, the calcium and magnesium will be complexed, and when complexation is complete, the solution turns from wine red to blue, marking the endpoint of titration.

An aliquot of 50 mL sample was taken in a conical flask, added 2 mL buffer solution (1.179 g disodium salt of EDTA, 780 mg magnesium sulphate in 50 mL and 16.9 g ammonium chloride dissolved in 100 mL distilled water and added with 143 mL concentrated ammonium hydroxide, diluted to 250 mL) and a pinch of Eriochrome Black T indicator (Ground 100 mg of the Eriochrome Black T indicator with 100 g of sodium sulphate). Titrated against 0.01 M EDTA (18.1612 g EDTA dissolved in 1L distilled water) till wine red colour changed to blue, and calculated the hardness as follows:

Total hardness (mg $CaCO_3$/L) = Equivalent hardness x 100

Equivalent hardness of water sample (mg/L)

= Volume of titrant x molarity of titrant x 1000 / volume of sample taken.

3.2.4.9 Estimation of calcium

Calcium in water samples was measured by EDTA titrimetric method (APHA, 1995). Calcium can be determined directly, with EDTA, when the pH was made sufficiently high that the magnesium is largely precipitated as the hydroxide and an indicator is used that combines with calcium only. Several indicators give a color change when all of the calcium has been complexed by EDTA at a pH of 12 to 13. Magnesium is estimated as the difference between hardness and calcium.

An aliquot of 50 mL water sample was taken in a conical flask, 1 mL 1N sodium hydroxide (40 g NaOH dissolved in 1 L distilled water) and pinch of murexide indicator were added. Titrated immediately against 0.05 M EDTA solution (18.612 g dissolved in 1 L distilled water) till pink colour changed to purple and calcium and magnesium were calculated as follows:

Calcium (mg Ca/L) = A x normality of EDTA x 40 x 1000/ vol.of sample
Where A = titer value for calcium
Magnesium = Total hardness - calcium

3.2.5 Sediment quality parameters

3.2.5.1 Estimation of E*h*, pH and total organic carbon

Methodology same as described in Chapter 2, Sections 2.2.5.2. and 2.2.5.3.

3.2.5.2 Estimation of ammonia, nitrite, nitrate and inorganic phosphate

The concentrations of these soluble ions were determined using filtrates of sediment samples. Sediment water ratios used in the procedure is

1:2 W/V, incubated on a mechanical shaker for 1hr and then filtered through Whatman No.42 filter paper. The filtrates were subjected to the same analytical procedures mentioned for water samples in sections 3.2.4.2. to 3.2.4.5. The concentration of the ions was expressed in mg/g sediment.

3.2.5.3 Estimation of bio-available forms of organic matter

The concentration of total proteins, carbohydrates and lipids in sediment samples were estimated as described in Chapter -2, Sections 2.2.5.5 to 2.2.5.7**.**

3.2.5.4 Quantification of extracellular enzymatic activity in sediment and water samples

Estimation of potential activity of extracellular enzymes in sediment samples were done as described in Chapter 2, Section 2.2.5.4. Six fluorogenic substrates were used in the study as shown below:

Sl.No	Substrates used	Acting enzymes
1	MUF-α-D-glucopyranoside (4-methylumbelliferyl - α –D-glucoside)	α-D-glucosidase (amylase)
2	MUF- β -D-glucopyranoside (4-methylumbelliferyl - β –D-glucoside)	β -D-glucosidase (cellulase)
3	4-methylumbelliferyl-N-Oleate	Lipase
4	4-methylumbelliferyl-N-acetyl β-D-glucosamine	Chitinase
5	4-methylumbelliferyl- β-D- xyloside	Xylanase
6	L-Leucine-4-methylcoumarinyl-7-arnide (Leu-MCA)	Aminopeptidase (exopeptidase)

The substrates were added to sediment samples at their optimum concentrations. They were 100µM of 4-methylumbelliferyl- β-D-xyloside, 4 -MUF-N-acetyl β-D-glucosamine,, 4-methylumbelliferyl-N-Oleate, MUF-α-D- glucopyranoside, 200 µM of MUF- β -D- glucopyranoside, and 50 µM of Leucine - MCA.

For water samples, the optimum concentration of substrates added was 10 µM for quantifying proteases, xylanase and amylase, 32 µM for cellulase and 64 µM for chitinase.

For assaying water samples, substrates were added at their final concentrations as mentioned above. The final volume of each mixture was 1 ml. The mixtures of water sample and substrate solution were incubated at 25^0C or at the in situ temperature for 1h, before and after which the fluorescence of hydrolytic product, 7-amino-4- methylcoumarin (AMC) and methyl Umbelliferyl (MUF) was measured with a spectrofluorometer (Hitachi F-2500) at an excitation/emission wavelength of 380/460 nm and 364/445 nm respectively on termination of the reaction. The fluorescence was calibrated using the slope of a calibration curve obtained from several concentrations of AMC and MUF. Controls were prepared with autoclaved filtered seawater collected from the same sample. The controls were assayed using the same method in order to define the non–enzymatically produced AMC and MUF. The hydrolysis rate of substrate was determined after subtracting both the blank fluorescence intensity of seawater and concentration of non–enzymatically produced AMC from the concentration of AMC in the reaction. The hydrolyzed rate of substrate, namely the

enzymatic activity, was calculated for nanomoles of substrate per hour per liter of water sample (nM L^{-1} h^{-1}).

3.2.5.5 Estimation of variations in microbial abundance

The population of total heterotrophic bacteria, *Vibrio*, actinomycetes and fungi in sediment and water samples of bio-augmented and control systems were estimated by standard plate count method. The samples were serially diluted and plated on to ZoBell's agar, TCBS agar, Actinomycetes isolation agar and Sabouraud's dextrose agar respectively and incubated at room temperature for 24hrs for heterotrophic bacteria and *Vibrio*, 72 hrs for fungus and 10 days for actinomycete population.

3.2.5.6 Direct bacterial counts by epifluorescence microscopy

The sediment and water samples were stained using acridine orange prior to microscopic observations. The analytical procedure for sediment and water samples was as described in Chapter -2, Section 2.2.5.8. Unlike sediment samples the water samples were processed directly following serial dilutions.

3.2.6 Statistical analysis

Data were expressed as mean ± standard deviation (S.D.). A one-way analysis of variance (ANOVA) was applied to compare bio-augmented groups and the controls at a significance level of $P < 0.05$, processed in MS - excel 2007 version.

Principal Component Analysis (PCA) was performed on the log-transformed and normalized environmental data set of the bioaugmented

and control systems – treating the sediment and water quality parameters separately.

3.3 Results

3.3.1 Sediment pretreatment in bio-augmentation systems

A single dosage of *Bacillus cereus* MCCB 101 in the systems meant for bio-augmentation could precondition the sediment by significantly reducing the percentage of total organic carbon from 4.11± 0.32 % to 2.80 ± 0.42 % on the 7^{th} day (Fig. 2). A second administration on 7^{th} day significantly reduced TOC to 1.59 ± 0.042 % as observed on 15^{th} day in bio-augmented tanks .The control systems recorded a mean value of 3.89 ± 0.27 during the same period. Similar was the case with redox potential prevalent in the bio-augmented systems (-153.6 ± 6.36 mV) significantly higher to that of control (-241± 9.1 mV) (Fig. 3). In the bio-augmented system pH dropped to 7.4 ± 0.09 while in the control system it remained at 8.4 ± 0.35 (Fig. 1).

3.3.2 Water quality analysis

The mean temperature range until 21 days was 21.56 ± 0.9 ^{0}C in both the category of systems. A rise in temperature to 28.43 ± 0.2 ^{0}C was recorded in the systems towards the end of the experiment (Fig. 5). In the bio-augmented systems pH was around 7.64 ± 0.14 which never exceeded 8.33 ± 0.03 (Fig. 4). However, in the control systems it had gone up to 9.2 ± 0.13 on 21^{st} day of the experiment (Fig. 4). Alkalinity remained at 265 ± 21.2 mg/L on 90^{th} day in the bio-augmented systems, while in the control systems it had gone up to 691.60 ± 114.43 on 21^{st} day itself (Fig. 6). Salinity in both the systems ranged between 15 – 22 g/L. Calcium showed an

increasing trend in the bio-augmented systems (Fig. 8) in contrast to that of magnesium which fluctuated greatly during the experimental period (Fig. 7). Notably, both these parameters showed a declining trend in the control systems.

TAN levels were significantly high ($p<0.05$) in control systems. On day 15 and 21 it went up to 17.58 ± 4.35 mg/L and 20.8 ± 0.55 mg/L respectively. During the corresponding period in the bio–augmented system it was significantly ($p < 0.5$) low (2.44 ± 0.9 and 1.39 ± 0.57 mg/L respectively) (Fig. 9). Even on the 60th day of experiment TAN in the bio – augmented system had gone to 3.34 ± 1.14 mg/L only, by the time which the shrimps in the control systems had suffered heavy mortality. The mean values of NO_2^- – N in bio – augmented systems were 0.05 ± 0.01 mg/L, 0.67 ±0.33 mg/L, 1.39 ± 0.27 mg/L on day 0, 15 and 21 and in control systems it was 0.85 ± 0.26 mg/L, 2.44 ± 0.37 mg/L and 2 ± 0.51 mg/L respectively (Fig .10). Notably, only in the bio-augmented systems NO_3 – N could be recorded during the period of 30 - 90 days (Fig. 11) suggesting establishment of nitrification.

Concentration of inorganic phosphorus in bio-augmented systems was significantly lower than that of the control ($p <0.05$, day 0 – day 21). The highest level was observed during the 3rd week in control systems and 4th week in the bio –augmented systems (Fig .12).

3.3.3 Microbiological analysis of water samples

The heterotrophic bacterial plate counts and the direct bacterial counts showed significant differences ($p<0.05$) between the bio-augmented and control systems. The heterotrophic bacterial plate count ranged between $2.48 \times 10^6 \pm 0.04$ to $6.3 \times 10^8 \pm 0.04$ CFU/mL in the bio-augmented systems

and $5.6 \times 10^5 \pm 0.01$ and $8.1 \times 10^6 \pm 0.1$ CFU/mL in the controls (Fig .19). The epifluorescence count (total bacterial number) was significantly high in bio-augmented systems with the highest density on day 75 and 90 having $7.68 \times 10^{10} \pm 0.06$ and $4.48 \times 10^{10} \pm 0.01$ cells respectively (Fig . 21). The total *Vibrio* counts were higher ($p>0.05$) in the water column of bio-augmented systems than that of the controls. (Fig.23). However, no significant differences ($p>0.05$) existed in the fungal population between the bio-augmented and control systems (Fig. 22).

3.3.4 Extracellular enzymatic activity in water samples

The xylanase activity exhibited similar pattern in both the systems but were higher in bio-augmented ones. The maximum activity was observed on day 15 (8646.61 nM/L/hr and 6243.43 nM/L/hr) in bio-augmented and control systems respectively (Fig. 13). While the lipase activity recorded was high in control systems (1032 ± 177.19 nM/L/hr) on 15th day, and it dropped to 557.80 ± 157.6 nM/L/hr on 21st day (Fig .16). The hydrolytic activity of amylase was significantly high ($p < 0.05$) in bio-augmented systems on 21st day accounting for about 7829 ± 732.05 nM/L/hr. The maximum activity recorded in the control systems was 5636.7 ± 895.5 nM/L/hr on 15th day, much lower to that of the bio-augmented systems (Fig. 14). A decline in potential activity of amylase happened on 30th, 45th and 60th day and increased on subsequent days (Fig .14). The activity of cellulase progressed to maximum of 1249.78 ± 290.76 nM/L/hr commencing from 45th day onwards in the bio-augmented systems. However, in the control systems the highest activity observed was on 15th

day (1129.85 ± 197.9 nM/L/hr) which declined to 276.55 ± 26.15 nM/L/hr on 21st day (Fig. 17).

The rate of chitinase activity peaked on 45th and 90th day in bio-augmented systems recording 4535.86 and 5036.82 nM/L/hr respectively, whereas, it was comparatively less in the control systems with a mean value of 1295.48 ± 122.3 nM/L/hr on day 0 – day 21 (Fig 15). Apart from variations in the pattern, protease activity was significantly higher ($p < 0.05$) in bio-augmented systems (Fig 18). The highest rate of activity of 6013.41 nM/L/hr was attained on 75th day of the experiment in bio – augmented systems. Meanwhile, in the control systems, , the maximum activity was 1140.94 ± 306.1 nM/L/hr on 15th day, significantly lower than that of bio-augmented samples which showed potential activity of 2550.32 ± 174.75 nM/L/hr on15th day.

3.3.5 Analysis of physico-chemical parameters in sediment

Sediment quality parameters such as total organic carbon, E*h*, pH, TAN and inorganic phosphorus showed significant differences between bio-augmented and control systems. Even though an increase in TOC content was noticed in two sampling points, the level could be maintained at 2.56 ± 0.1 % in the bio-augmented systems. In control systems, on the contrary,, initial value of 4.02 ± 0.3 increased to 6.24 ± 0.2 % on 21st day of the experiment (Fig .26). Similar trend was observed with respect to redox potential and sediment pH. Redox status of -248.66 ± 9.5mV observed in the control systems on 21st day was significantly lower compared to that in bio-augmented systems (-174.33 ± 2.3mV) (Fig 24). pH ranged between 8.1

and 9.5 in controls, whereas the mean pH during the experiment was 7.95 ± 0.15 in the bio-augmented sediment (Fig.25).

TAN recorded 0.057 ± 0.004 mg/gm on 21st day in the sediment of control systems, significantly higher from that of bio-augmented ones (0.008 ± 0.0007 mg/gm) (Fig 36). Application of bio-augmenter on the 30th and 60th day was helpful to maintain the level of TAN at 0.009 ± 0.0007 mg/gm and 0.017 ± 0.005 mg/gm on 45th and 90th day respectively. The levels of NO_2^- – N was significantly higher in control systems ranging between 0.068 ± 0.006 mg/gm on day 0 to 0.24 ± 0.02 mg/gm on day 21 (Fig 37). Meanwhile, in bio-augmented systems, a mean value of 0.034 ± 0.016 mg/gm of sediment could be maintained until 21st day of the experiment. Nitrification was highly efficient in bio-augmented sediment as NO_3 – N was highest on day 21 (9.80 ± 1.5 mg/gm), and could sustain the level (4.85 ± 0.02 mg/gm) until completion of the experiment (Fig.38). During progression of the experiment, oxidation of NO_2- N to NO_3 – N declined in sediment samples of control systems and more toxic NO_2- N started getting accumulated. Concentration of inorganic phosphorus was significantly lower in the bio-augmented systems compared to that of the controls (Fig.43).

3.3.6 Microbiological analysis of sediment samples

Microbial community showed significant differences between bio-augmented and control systems. The heterotrophic bacterial plate counts ranged between 1.76 × 10^6 ± 0.18 CFU/gm (day 0) to 5.39 × 10^7 ± 0.06 CFU/gm (day-90) in the bio-augmented systems, and 7.36 × 10^5 ± 0.07 CFU/gm (day 0) to 8.1× 10^6 ± 0.081 CFU/gm (day 21) in the controls

(Fig.40). The total *Vibrio* counts were significantly lower ($p < 0.05$) in the bio-augmented sediment (day 0 to day 21) than that of the control (Fig 44). The daily application of bio-augmenter was found to decrease *Vibrio* count in the bio-augmented sediment towards the end of the experiment. Meanwhile, significant increment in total actinomycetes and total bacteria was observed in the bio-augmented sediment ($p < 0.05$). Bio-augmentation was found associated with higher fungal population, which, however, showed decline in the control systems.

3.3.7 Impact of bio-augmentation on total proteins, carbohydrates and lipids.

The total protein increased substantially from 246.01 ± 31.79 (day 0) to 1085.73 µg/mg (day 21) in control systems. The concentration was significantly low in bio-augmented systems (21.18 ± 6.62 and 392.25 ± 24.38 µg/mg) on day 0 and 21 respectively (Fig. 34). Similarly, significant reduction ($p<0.05$) of total lipids was observed in the bio-augmented systems compared to that in the controls (Fig.35). Total CHO was significantly high in control systems ranging between 153 ± 10.24 µg/mg on day 0 to 650.99 ± 21.66 µg/mg of sediment on day 21. The corresponding activity observed in bio-augmented systems was 90 ± 7.85 µg/mg and 355.19 ±12.24 µg/mg of sediment respectively (Fig. 33). In spite of the increment in the total CHO and lipid concentrations in bio-augmented sediment on 75th day it gradually decreased in subsequent samples (Fig. 35). From 30th day onwards regular application of the bio-augmenter could be correlated with low levels of total proteins in the bio-augmented systems.

3.3.8 Extracellular enzymatic activity in sediment

During the course of the experiment activity of xylanase significantly increased ($p<0.05$) to 156.62 ± 30.7 nM /gm/hr on 15th day in control systems from an initial velocity of 27.56 ± 5.56 nM /gm/hr and declined to 23.34 ± 5.06 nM /gm/hr on 21st day (Fig. 27). However, the maximum velocity of hydrolysis recorded for xylanase in bio-augmented sediments was 40.76 ± 2.31 nM /gm/hr on 15th day. Similar trend was observed with chitinase also, where the course of enzymatic activity was significantly higher in the control sediment with the highest activity on 15th day, which got reduced on 21st day (Fig. 29). The frequency of bio-augmentation could maintain the level of xylanase and chitinase activities in the bio-augmented systems from 30th day untill the end of the experiment.

The mean value of amylase activity was 33.85 ± 4.36 nM /gm/hr in control systems. The activity recorded on day 75 and 90 in bio-augmented systems was comparatively higher with the observed values, 167 ± 46.97 nM /gm/hr and 186.06 ± 17.2 nM /gm/hr respectively (Fig. 28). Comparing the mean values for 21 days, lipase activity was higher in the sediment of bio–augmented systems recording 19.5 ± 4.74 nM /gm/hr for a corresponding activity of 11.38 ± 2.07 nM /gm/hr in the control sediment samples. Subsequent dosages of the bio-augmenter peaked the lipase activity in bio-augmented sediment to 39.04 ± 3.17 nM /gm/hr on 90th day (Fig. 30).

Activity of cellulase in bio-augmented systems ranged between 49.03 ± 6.48 nM /gm/hr (day 0) and 179.77 ± 3.73 nM /gm/hr (day -90) with the lowest rate of activity, 14± 6.38 nM /gm/hr, on 21st day of the

experiment. The rate of cellulase activity was significantly low ($p < 0.05$) in control tank sediment measuring 15.19 ± 3.34 nM /gm/hr, 24.62 ± 2.51 nM /gm/hr, 16.35± 3.39 nM /gm/hr on day 0, 15 and 21 respectively (Fig. 31).

The protease activity was significantly high ($p<0.05$) in bio-augmented sediment (Fig.32). The rate of enzymatic activity got shifted from 110.52 ± 6.1 nM /gm/hr (day0) to 472.53 ± 45.61 nM/gm/hr (day 90) during the experimental period which peaked on day 75 with a recorded velocity of 692.91 ± 44.6 nM /gm/hr of sediment. In contrast, the estimated protease activity in the control sediment was reduced to 89.74 ± 7.26 nM /gm/hr on 21st day from an initial rate of 111.08 ± 4.06 nM /gm/hr.

3.3.9. Principle Component Analysis of sediment and water quality parameters

Principal Component Analysis (PCA) was performed on the log-transformed and normalized environmental data set of the bioaugmented and control systems – treating the sediment and water quality parameters separately (Fig.47 & 48). The analysis produced a total of 5 canonical axes in both sediment and water compartments. Principal axes 1 and 2 were found to be most appropriate in explaining the observed variations among samples, and together they explained about 61.9% and 56.9% of the total variance respectively in the sediment and water quality data.

Among the sediment quality parameters, total organic carbon (TOC), pH, hydrolytic enzymes like lipase, amylase, cellulase, protease along with direct bacterial counts (DBC), total actinomycetes count, total heterotrophic fungal counts (TFC) contributed significantly to the PC1, accounting for

34.7% of the variance in the data (Eigenvalue 7.29), while PC2, which explained 27.2% of the total variance (eigenvalue 5.74), consisted primarily of the variables like, total Vibrios, heterotrophic bacterial plate counts, total lipids, proteins, carbohydrates, chitinase total organic carbon, total ammonia nitrogen (TAN), pH and sediment phosphate (Table 1&2). Majority of sedimentary parameters vector ordinated towards the top left and bottom left position in PCA plot, towards bioaugmented system, which was suggestive of effective biaoaugmentation. Total ammonia nitrogen (TAN) and TOC loaded on top right and bottom position, towards control systems, explaining the existence of high TOC, low E*h* and high alkalinity in control systems.

Among the water quality parameters, temperature, calcium hardness, nitrate, enzymes chitinase and protease, direct bacterial counts and heterotrophic bacterial plate counts accounting for 35.2% of the variance in the data (Eigenvalue 7.05) contributed significantly for PC1 axis. The PC2 that explains 21.7% of variance primarily consists of contribution from pH, alkalinity, magnesium hardness, total ammonia nitrogen (TAN), lipase and phosphate (Eigenvalue 4.33) (Table 3&4).Among the water quality parameters, control systems shows principally high TAN and nitrite accumulation, and thus loaded onto the top right of PCA plot . An eventual improvement in water quality parameters is observed in bioaugmented system with high density of microbial populations, nitrification potential and enzyme activities (except for lipase) beyond day 30, with these samples ordinating to top left of PCA plot (Fig.48).

3.3.10. Survival rate of shrimps

Shrimps in the control systems suffered 100% mortality after 21 days of stocking. Meanwhile, the average survival was 80% in bio-augmented system on completion of the experimental period of 21 days. An average biomass input of 30.3 gm/tank yielded 96.66gm/tank on completion of the study period (90 days) attaining average weight of 18.12 ± 0.5g per animal. There was 55.55 % survival at the end of the study in bio-augmented systems in contrast to 100% mortality in the control systems on 21st day of the experiment (Fig .46).

3.4 Discussion

Zero water exchange technology as part of farm management strategies ensures bio-secured, closed shrimp production systems to prevent pathogen entry and subsequent disease outbreak. Such a strategy has been adapted in a few countries as part of bio-security measure, and to safeguard the surrounding environment from impairment due to discharge of nutrients and suspended solids (Funge-Smith and Briggs, 1998). Although high-intensity production of *P. monodon* is limited, studies over recent years have demonstrated improvements in culturing this species at high densities. In the present study, potential of *Bacillus cereus* MCCB 101 in bio-augmenting zero water exchange shrimp grow out system of *P.monodon* of high stocking density was investigated in a simulated system.

In intensive and semi-intensive culture systems nitrogen loading rates from feed happens to be high. Out of the total nitrogen 90% enters the pond as formulated feed, 5% of total nitrogen enters through the intake water of which 22% alone is converted to harvested shrimp, 14% remains in

sediment, most of the remainder (57%) gets discharged to the environment and 3% goes unaccounted and assumed to be lost into the atmosphere by volatilization of ammonia or denitrifcation. (Jackson *et al*., 2003). Accumulation of feed and other organic matter in a culture system in relation to higher biomass leads to enhancement of total organic matter, total soil nitrogen and ammonia – nitrogen (Millamena, 1990). This leads to rapid eutrophication with zero water exchange shrimp culture systems imposing tremendous stress to the cultured stock reducing the systems' carrying capacity (Lin, 1995).

One of the most important aftermaths with the intensification of shrimp culture is the build up of toxic NH_3 - N (Lightner, 1993). Ammonia is a major end product of protein metabolism and is excreted as unionized ammonia across the gills of aquatic organisms. It is also formed by microbial mineralization of organic matter (Moriarty, 1997). In water, ammonia exists in two forms: un-ionized ammonia (NH_3) and ionized ammonium, (NH_4^+). Sum of the two forms (NH_4^+, NH_3) is referred to as total ammonia – nitrogen (TAN) or simply ammonia. The relative concentration of each of these forms is primarily a function of pH, temperature, and salinity (Anthonisen *et al*., 1976).

Nitrogen in the form of ammonia (NH_3) and nitrite (NO_2^-) are toxic to shrimp; however, the toxicity depends on various factors including species tolerance, water characteristics (pH, temperature, salinity and dissolved oxygen) and exposure duration (Hargreaves, 1998; Barajas *et al.,* 2006). A 'safe' concentration of TAN and nitrite for culturing juvenile *P. monodon* is reported to be 3.7 mg/L and 3.8 mg/L respectively (Chen and

Lei, 1990). The TAN concentrations in the present study peaked to 20.85 ± 0.55 mg/L in control tanks. It was reported earlier that in *P. monodon* grow out system, even with frequent water exchange ammonia might increase up to 6.5 mg/L (Chen and Tu, 1991). The NH_3-N exceeding 1.0 ppm has been reported in Indian shrimp culture ponds (Prabhu *et al*., 1999; Rao *et al*., 2000).

TAN levels in bio-augmented systems during initial 21 days were significantly lower than that of the control systems. It indicated that apart from nitrification, NH_3 – N was removed from the system via nitrogen assimilation into bacterial biomass. Significant difference ($p<0.05$) in relative bacterial densities during 21 days in bio-augmented and control tanks supports the above statement. Consequently accumulation of NO_2 -N was significantly lower in bio-augmented systems. Nitrite accumulation has significant effects on shrimp performance and is inversely correlated with shrimp growth rate (Lin and Chen, 2003; Vinatea *et al*., 2010). Nitrite is reported to cause methemoglobinemia that creates hypoxia leading to nitirite toxicosis (Tomasso, 1994). Nitrite peaked up to 2.4mg/L in control tanks. Banerjee *et al*. (2010) reported that the use of indigenous *Bacillus pumilus* in shrimp PL rearing tanks lowered the concentration of TAN and NO_2 -N compared to those of the control tanks. Lalloo *et al*. (2010) demonstrated reduction in concentrations of waste ions, *in vitro* and *in vivo*, in ornamental fish aquaculture using *Bacillus* sp. Devaraja *et al*. (2013) observed that the use of indigenous *Bacillus* spp. improved water quality in *P. monodon* culture systems. Reports of non performing bioremediators (Timmermans and Gerard, 1990; Shariff *et al*., 2001) have also been noticed in literature.

During organic matter decomposition by heterotrophic bacteria CO_2 is released as the end product. This can augment chemoautotrophs for oxidation of inorganic compounds such as ammonia. The nitrate formation chemistry in sediment and water samples of bio-augmented systems in the present study indicated such a possibility where *Bacillus cereus* MCCB 101 augmented chemoautotrophic bacteria for efficient nitrification.

Higher nitrification rates in sediment samples are indicative of active sediment microbial community that ranged between 10^5 to 10^7 CFU/g sediment. The observed THB in the present study was higher than those reported by Devaraja *et al.* (2002) and Tendencia *et al*. (2006) viz., 3.75×10^5 CFU/g and 6.8×10^5 CFU/g respectively in pond bottom soil, but in agreement with modified extensive brakishwater shrimp pond sediment THB according to Abraham *et al*. (2009). Addition of probiotic *Bacillus* sp. to the rearing waters was found to enhance bacterial counts to 1x 10^6 CFU/mL in bio-augmented systems compared to $1x10^4$ CFU/mL in control tanks as reported by Nimrat *et al*. (2012). Significant increase ($p < 0.05$) in THB in the water column of bio-augmented systems observed in the present study adds to this information.

The increase in nitrate nitrogen in bio-augmented systems from day 45 points to the necessity of increasing the frequency of application of the bio-augmenter progressive to crop age to enhance nitrogen cycling in closed systems. This argument holds good for bio-augmented sediment samples when alternative (30^{th} – 60^{th} day) and daily (60^{th} – 90^{th} day) bio-augmentation increased the nitrification rates. Nitrate, unlike ammonia and nitrite, is less toxic to shrimp; however, high concentration (100 mg/L) was reported to be lethal (Rijin *et al.,* 2006). This corroborate with the findings

of Devaraja *et al*. (2013) on the necessity of additional doses of *Bacillus* sp. during bioremediation for restoring cell density to attain the desired results. Janeo *et al*. (2009) demonstrated that application of bio-augmenter four times a week was highly beneficial and ideal for shrimp culture.

The reduction in nitrate formation in bio-augmented sediment on day 30 and 60 might be due to increase in organic matter. Hargreaves and Tucker (1996, 2003) reported that in intensive culture systems nitrogen loading rates from feed is higher, and even minor changes in process rates could result in accumulation of potentially toxic intermediates. Subsequently, NO_2^- _ N concentration in bio-augmented sediment was found maximized on 60^{th} day in the present study.

The NO_3^- N build up in the water column of bio-augmented systems is reflective of the slowly establishing nitrifying bacterial community in a microenvironment satisfying fastidious chemoautotrophic growth require ments by the heterotrophic bacterium applied, generating inorganic carbon during breakdown of organic matter. Significantly low alkalinity in bio-augmented systems is indicative of autotrophic consumption of inorganic carbon as CaCO3. In control systems alkalinity was significantly higher. Alkalinity in estuarine waters varied from 10 – 400 mg/L $CaCO_3$ (Campos *et al*., 2009) and for aquaculture it should not be less than 20 mg/L $CaCO_3$, below which it can affect phosphate solubility (Wurtz, 2002). The minimum alkalinity recorded in the present study was 65 mg/L $CaCO_3$.

Decomposition of organic matter in control systems at slow rates accumulated organic input pushing down E*h* to -248 mV creating anoxic conditions in the sediment. Oxidation – reduction potential (Redox

potential) of sediment has been identified as an appropriate indicator of organic accumulation in sediment (Pearson and Black, 2000). The maximum acceptable range of redox potential in a cage farm of salmon was -150 mV (Shakouri, 2004). The redox potential of mud, at any stage, should not be lower than – 200 mV (Muralidhar *et al*., 2001). Bottom soil quality has long been recognized as a factor influencing water quality and aquatic animal production (Boyd, 1990, 1992; Munsiri *et al*., 1996). In-spite of the high stocking density and feeding rates, higher level of dissolved oxygen could improve E*h* in the bio-augmented systems throughout the experiment, a factor which could be attributed to the beneficial effects of bio-augmenter.

Dissolved oxygen in bio-augmented systems was well above the minimum requirement of 3.5 ppm for shrimp farming (Boyd and Arlow, 1992); whereas in the control systems it had reduced to < 3 mg/L. Cheng and Liu (2001) showed that dissolved oxygen of 4–6 mg/ L provided favorable conditions for nitrifying bacteria.

Although the optimum salinity suitable for shrimp culture is about 15-25 g/L (Boyd, 1995) the recent studies indicated that *P. monodon* could be cultivated in low saline conditions also (Abraham and Sasmal, 2009). No significant variations in salinity were observed in the present study and it ranged between 15 – 22 g/L in both the systems. pH recorded in bio-augmented systems was within the optimum range recommended 7.5 –8.0 for sediment (Boyd and Pippopinyo, 1994) and 7.5-8.5 for water (Chiu, 1987); however, was found to cross desirable limits in the control systems. The average temperature was found to be lower than the optimum range (25-30^{0}C) recommended by Boyd and Arlow (1992), till 21st day of

operation in both the systems, however, bio-augmented systems attained optimal temperature along with the progress of the experiment.

Besides breaking down organic matter, heterotrophs are efficient competitors for phosphate as well (Drakare, 2002) and perhaps maintain optimal levels of this nutrient. The low levels of phosphate in the water column of bio –augmented systems in the present study could be indicative of enhanced heterotrophic phosphate uptake by bacterial community. Low phosphate concentrations in treated tanks is in agreement with the findings of Wang *et al*. (2005), that ponds applied with commercial probiotics had significantly lower total inorganic phosphorus than the control ponds.

There was significant reduction of presumptive *Vibrio* in the bio-augmented sediment. Several authors have reported reduction in *Vibrio* load in ponds applied with probiotics (Prabhu *et al.,*1999 ; Ravichandran and Jallaluddin, 2000; Wang *et al*., 2005). Similar observations were made by Devaraja *et al*. (2002) in probiotic treated shrimp pond sediment and water. On the contrary, *Vibrio* counts of water samples recorded in the present study was higher in the bio-augmented systems than that of the controls. *Vibrio* species are endemic to marine environment, and turn out to be opportunistic pathogens when the natural defence mechanism of the animals are suppressed (Lightner, 1993) as a result of exposure to stressful environmental conditions. It implies that better environmental quality would diminish the possible emergence of Vibriosis in spite of higher load of the pathogen.

Significantly high counts of fungal and actinomycetes population in bio-augmented sediment reflected prevalence of better environmental quality for chemoheterotrophs imparted by bio-augmentation to act upon organic matter. Fungi and actinomycetes are capable of heterotrophic nitrification as well (\Odu and Adeoye, 1970) but related studies in aquaculture are unseen in literature in contrast to ample reports on bacteria involved in nutrient cycling in shrimp culture systems (Abraham *et al*., 2004; Wang *et al*., 2005). Generally, low to moderate nutrient concentrations stimulate fungal activity (Gulis and Suberkropp, 2003; Gulis *et al*., 2006) but eutrophication accompanied by low levels of oxygen reduces the decomposition by aquatic fungi (Medeiros *et al*., 2009).

The rate-limiting step in the degradation of organic matter is the hydrolysis of macromolecules by extracellular enzymes (Meyer–Reil, 1987). Studies regarding microbial extracellular enzymes in aquatic environments are mostly focused on protease and glucosidases, as within the bulk of organic matter proteins and carbohydrates have been identified by several authors as the most available food source for microbial metabolism (Danovaro *et al*., 1999; Mayer *et al*., 1995). Nevertheless, such an approach is lacking in aquaculture research even though probiotic application is reported to significantly improve the sediment and water quality.

Bacillus cereus MCCB 101 used in this study secreted extracelluar enzymes susch as protease, amylase, and lipase which initiated the process of organic matter degradation in aquatic ecosystems (Boyd and Gross, 1998). Apart from that, the increased rate of enzymatic activities seen in

bio-augmented systems is the overall contribution of microbial community that was stimulated by bio-augmentation. Significantly abundant ($p < 0.5$) prokaryotic community in bio-augmented systems estimated in terms of total bacterial number and heterotrophic plate counts showed positive correlation ($r => 0.5$) with protease, amylase, chitinase, cellulase activities of sediment and water. Direct relation between ecto-enzymatic activity and bacterial abundance is seen in literature (Romani and Sabater, 1999). Similar to the findings in the present study, Gallizia *et al*. (2005) reported that bio-augmentation strongly stimulated β – glucosidase activity in harbor sediments with a maximum rate of 496.5 nM/mg/h, and was positively correlated to total bacterial number.

Low levels of amylase activity in the sediment could be attributed to the fact that organic matter reaching sediment would be low in easily degradable compounds such as starch leading to a decreased requirement for amylase activity whereas less degradable compounds like cellulose are more likely to reach sediment (Arnosti,1998; Boeitus,1995).

Published values of β – glucosidase activity in sediment of marine ecosystems (King, 1986 ; Fabiano and Danovaro,1998) are in agreement with the observed activity here, except for those determined in deep water sediments (Poremba,1995) which are lower than those obtained in this study.

Leucine aminopeptidase activity measured using Leu- MCA fluorogenic substrate is used as a model proteolytic activity (Hoppe 1983). In-spite of their variations, the measured protease activity in water samples

of bio-augmented and control systems was lower than reported by Gallizia *et al.* (2005) and higher than documented by Obayashi and Suzuki (2005). In contrast, the α – glucosidase, β –glucosidase activities of water samples were higher in comparison with the study of Gallizia *et al.* (2005) Such differences result from microbial diversity among ecosystems which differ in the nature of organic matter reaching the sediment and probably in the structure of bacterial communities.

Vrba *et al.* (2004) noted a significant but weak correlation between α - glucosidase, β- glucosidase, chitinase activities and bacterial numbers suggesting that euakaryotes including fungi should be considered potentially important enzyme producers in aquatic environments as estimated by their size fractionation experiments. In the present experiment, the relative contribution of fungi to enzyme activites in bio-augmented water samples was restricted to xylanase alone ($r = 0.515$) probably due to its significantly low counts than control ($p < 0.5$). However, in sediment samples the fungal community was positively correlated with protease, cellulase and chitinase activities. A significant positive correlation was seen between the actinomycete population and protease, cellulase, amylase and lipase activities in sediment samples ($r = > 0.5$).

As an overall impact, the resultant organic matter depletion in 21 days were significantly higher in bio-augmented systems in terms of total protein, CHO and lipids as estimated, and maintained the TOC and E*h* in desirable limits. Their concentrations determined in the present study is in agreement with the findings of Danovaro *et al.* (2001) who reported total protein concentrations of 1422 µg/gm, total CHO of about 2210.1 µg/gm and total lipids of 775.2 µg/gm in deep sea sediment. Daily application of

bio-augmenter in treated tanks from 60th day had pronounced impact on enzymatic activities, which regardless of increase in day of culture regulated the organic matter accumulation and thereby upgraded the redox status in bio-augmented sediment through mineralization (Moriarty, 1997).

Optimum range of organic carbon in the pond sediments is between 1-3% (Banerjea 1967; Wudtisin and Boyd, 2006). According to Boyd (1995) bottom organic carbon concentration seldom exceeds 5%. Lower concentration is unfavorable for the growth of benthic organisms that are important food for many species and higher concentrations favor anaerobic conditions at the sediment water interface. Accordingly, range of organic carbon in control tanks were exceedingly high than recommended. High stocking density and inadequate treatment in control tanks resulted in accumulation of wastes at exceedingly high rates that natural bacterial community could degrade. The overall enzymatic profile was less influenced by natural process in control systems, except for chitinase and xylanase activities in sediment ($p<0.05$) and lipase and cellulase activites in water ($p = ns$) at the rates higher than that in bio-augmented systems and thus resulted in eutrophic systems with high level of nutrients. Moreover environmental factors such as concentration of mineral elements, dissolved oxygen, temperature, pH could also modify enzyme activities (Chrost, 1990; Richardot *et al.,* 2000).

Thus bio-augmentation using *Bacillus* cereus MCCB 101 maintained a balance between waste accumulation and assimilation in the culture system which was of paramount importance for the success of closed systems. Krantz and Norris (1975) stated that survival rates of 60 to 80% are to be expected for *P. monodon* under suitable rearing conditions. Average

survival of 70-80% is quite possible if ideal conditions are maintained for *P. monodon* . The shrimps in control systems suffered 100% mortality after 21 days of stocking. Poor growth and low survival in the control ponds could be due to the deterioration of sediment quality which leads to high redox value and low oxygen level in the pond bottom. Dissolved oxygen concentrations also would be dropped to levels that might have impacted negatively on the shrimp. The average survival rate of shrimp was 80% in bio-augmented systems at the end of 21 days of culture in the present study.

On completion of the study period (90 days) the shrimps of average size 18.12 ± 0.5g were harvested from bio-augmented systems with thrice the output of the biomass introduced per tank. In the end there was 55.55 % survival in the bio-augmented systems. In general, reduced growth and/or survival of juvenile shrimp cultured at high densities were thought to result from a combination of factors, which included decrease in the availability of space and natural food (Maguire and Leedow, 1983 ; Peterson and Griffith, 1999) increase in adverse shrimp behavior such as cannibalism (Abdussamad and Thampy, 1994) degradation of water quality and the generation of anaerobic sediment (Arnold *et al* ., 2005 ; 2006a) although the latter two were insignificant in the present study with respect to bio-augmented systems.

High-intensive production of juvenile shrimp has been most successful with *L. vannamei*, where high survival (97.5%) and fast growth (1.12 g shrimp in 50 d) at stocking densities of 3300 m^{-3} produced biomass yields of 4.25 kg m^{-3} (Cohen *et al*., 2005). The present experiment in the simulated system was meant to verify the feasibility and to study the

scientific basis of bioremediation of detritus in a closed system using an indigenous bio-augmenter. Therefore, it is technically difficult to have a comparative analysis with yields from out door full-scale cultures reported in literature. Nevertheless on the basis of the results, it could be suggested that shrimps would grow successfully in a closed culture system provided appropriate bio-augmentation technology was adopted, preferably using indigenous bio-augmenters. *Bacillus cereus* MCCB 101 stands out as an appropriate bio-augmenter for detritus degradation in Indian conditions for shrimp culture.

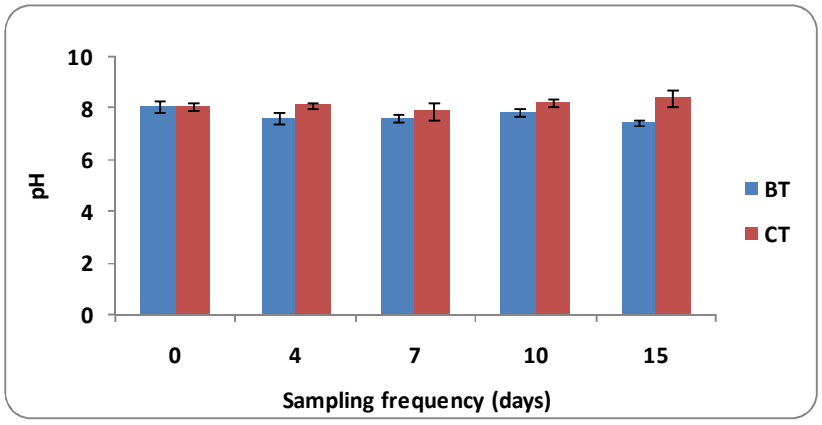


Fig 1: Variations in pH of sediment during pre – conditioning

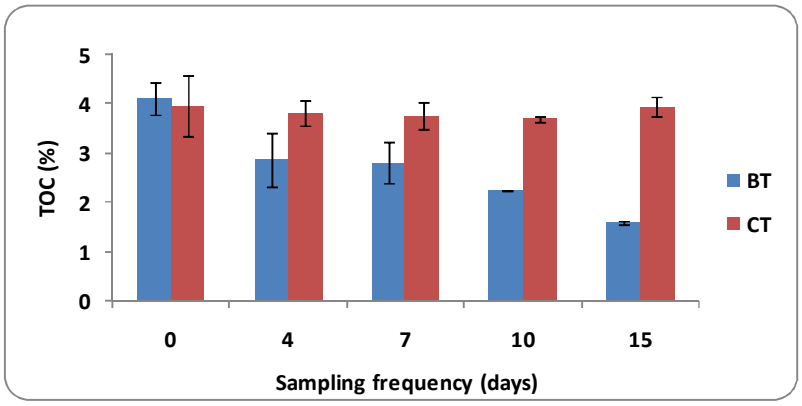


Fig 2: Variations in percentage of TOC in sediment during pre- conditioning

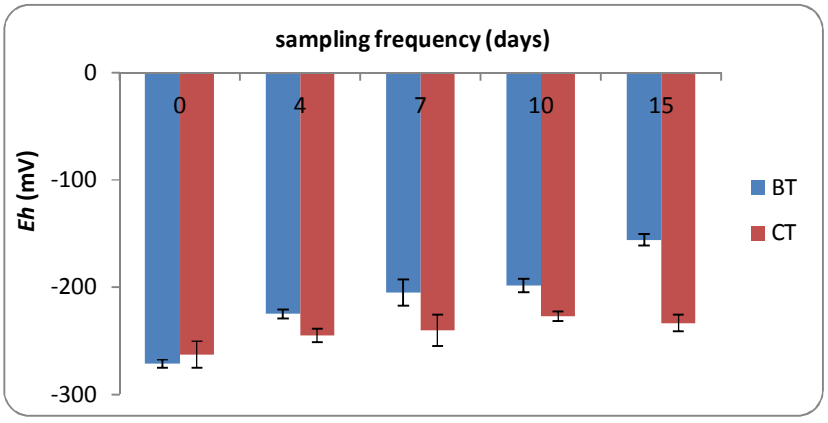


Fig 3: Variations in *Eh* of sediment during pre- conditioning

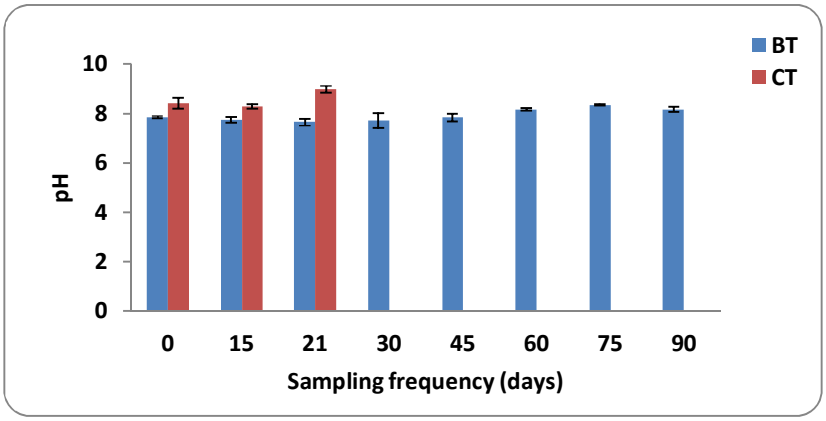


Fig 4: Variations in pH of water column in bio-augmented and control systems

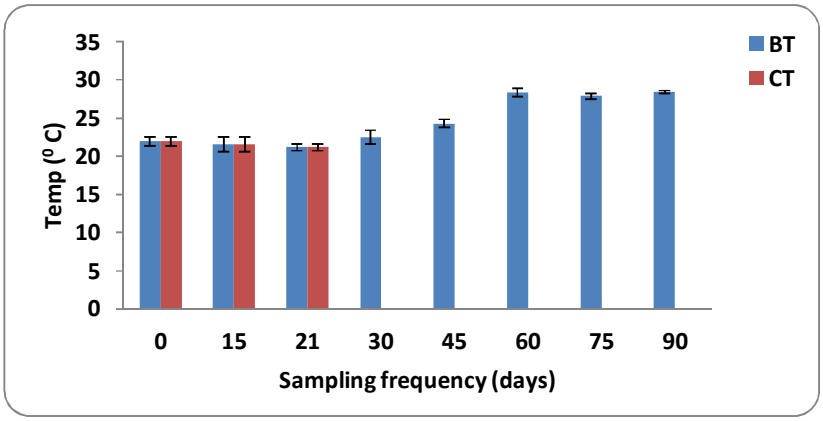


Fig 5: Variations in temperature of water column in bio-augmented and control systems

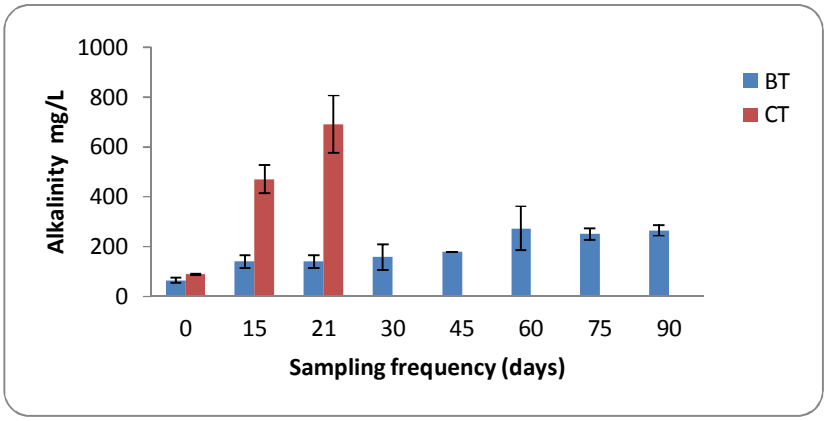


Fig 6: Variations in alkalinity of water in bio-augmented and control systems

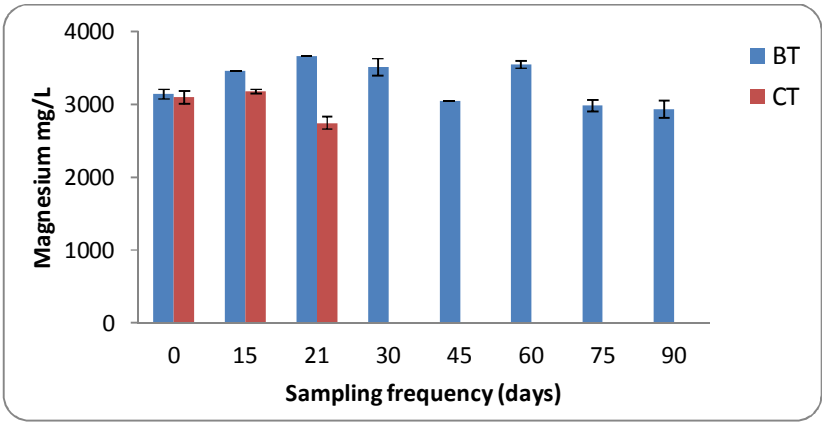


Fig 7: Variations in magnesium content of water in bio-augmented and control systems

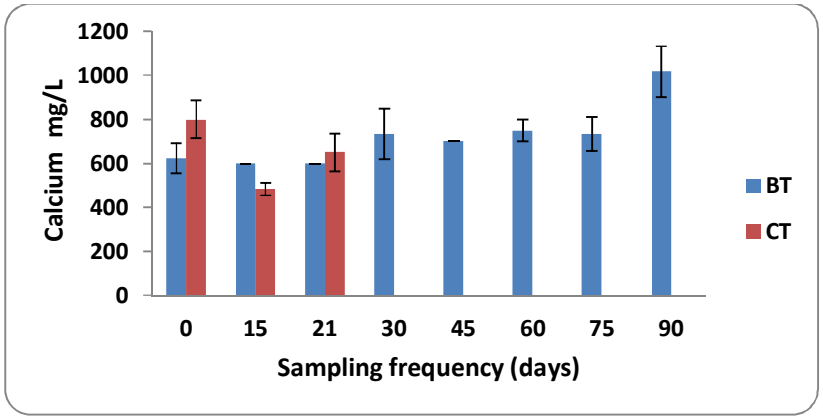


Fig 8: Variations in calcium content of water in bio-augmented and control systems

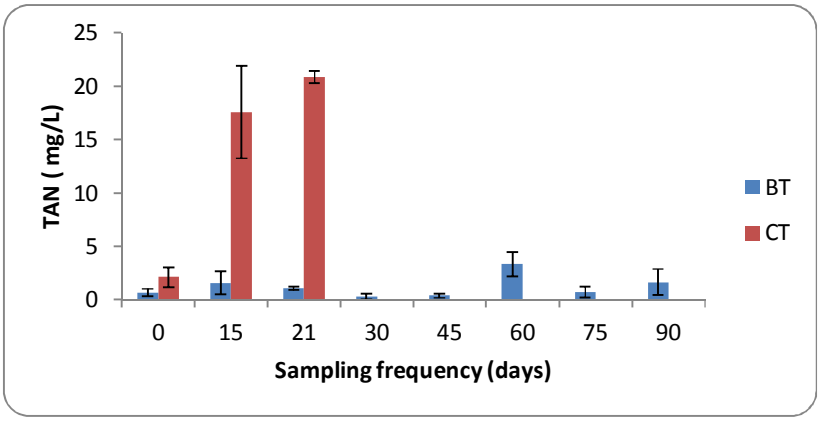


Fig 9: Variations in total ammonia nitrogen in water of bio-augmented and control systems

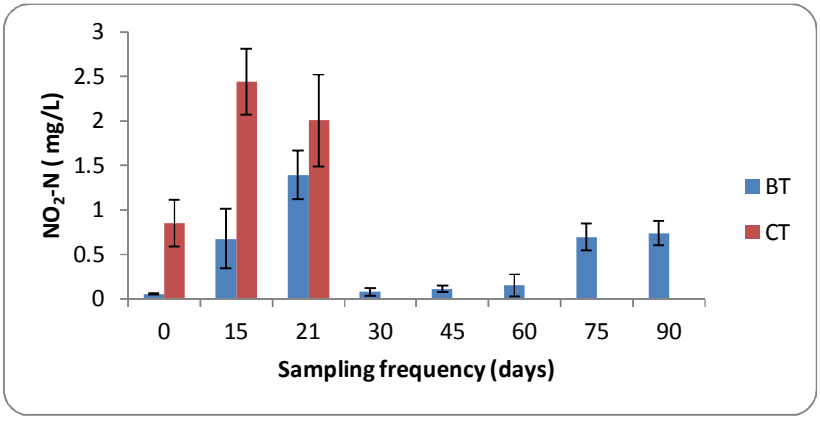


Fig 10: Variations in NO_2^--N of water in bio-augmented and control systems

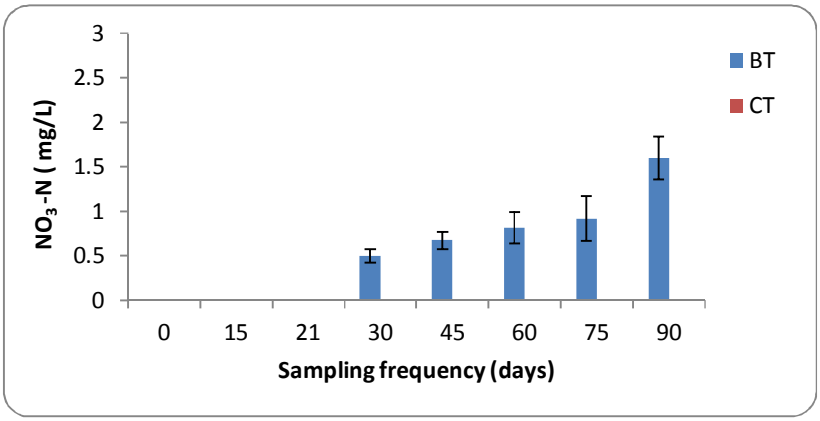


Fig 11: Variations in NO_3-N of water in bio-augmented and control systems

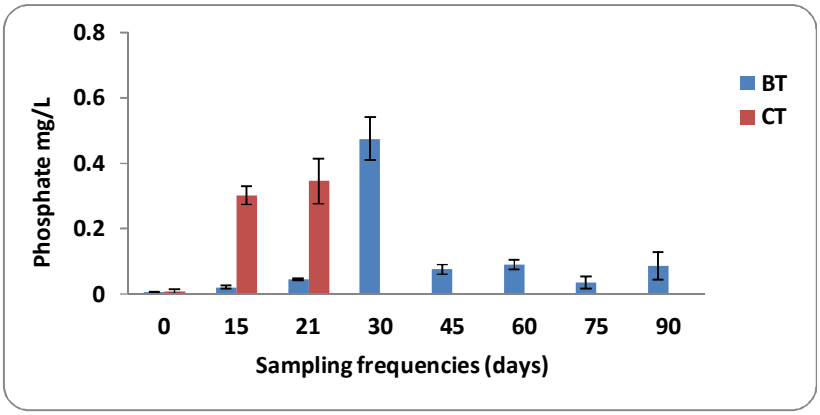


Fig 12: Variations in PO_4 - P of water in bio-augmented and control systems

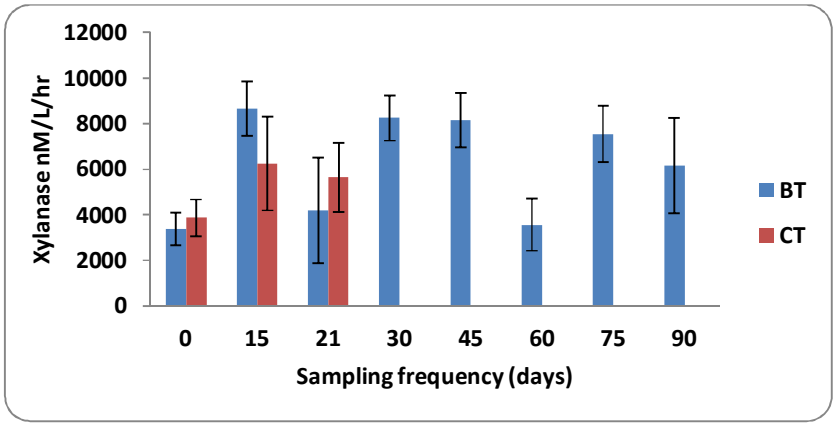


Fig 13: Pattern of xylanase activity in water column of bio-augmented and control systems

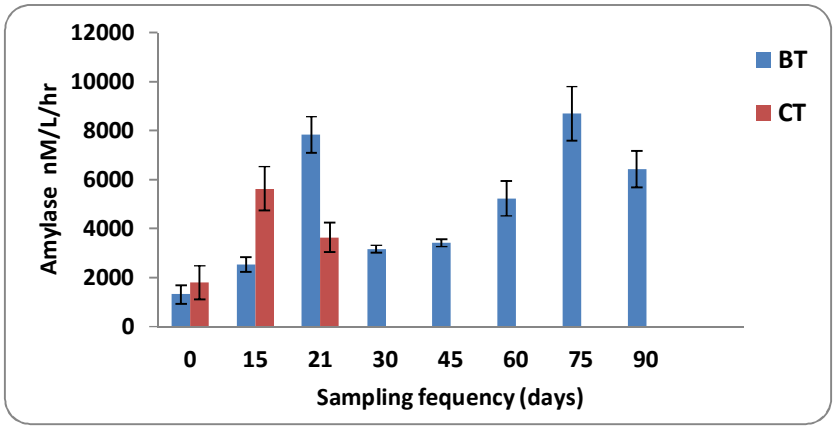


Fig 14: Pattern of amylase activity in water column of bio-augmented and control systems

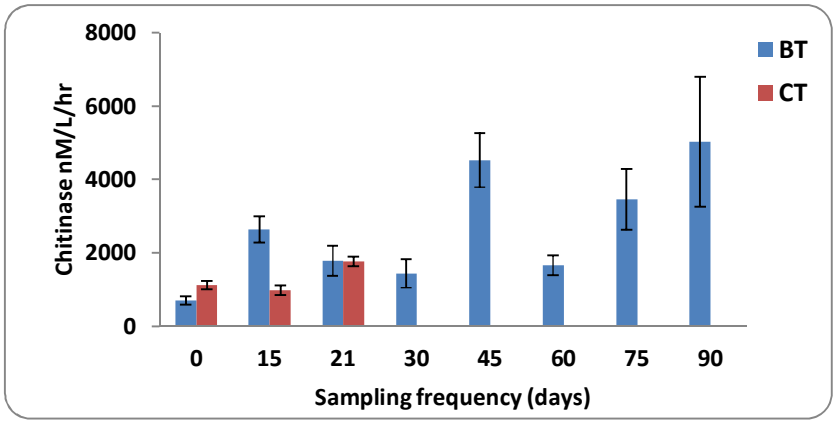


Fig 15: Pattern of chitinase activity in water column of bio-augmented and control systems

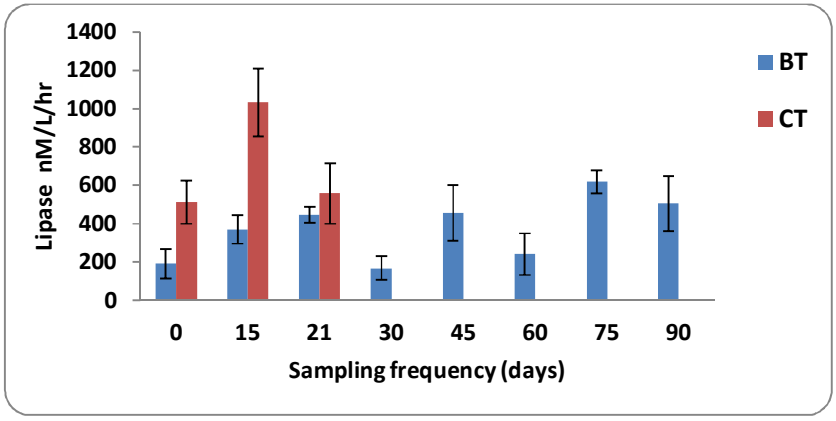


Fig 16: Variations in lipase activity of water in bio-augmented and control systems

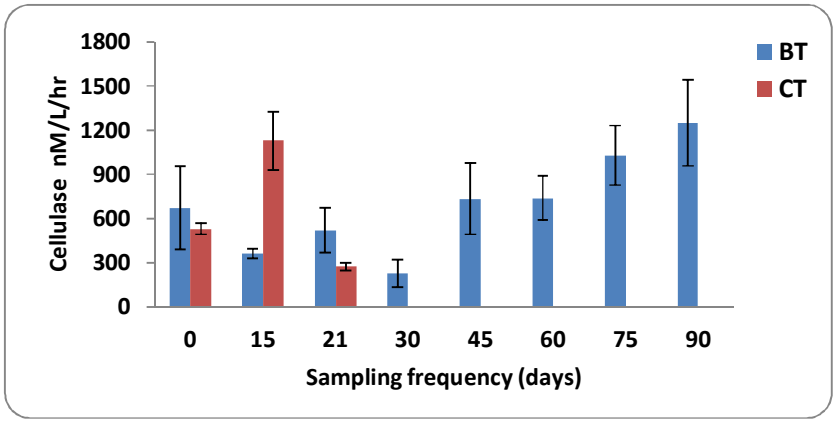


Fig 17: Variations in cellulase activity in water column of bio-augmented and control systems

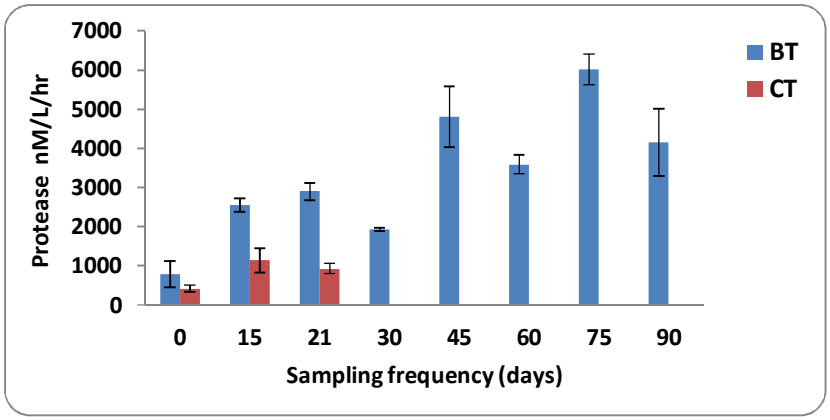


Fig 18: Variations in protease activity in water column of bio-augmented and control systems

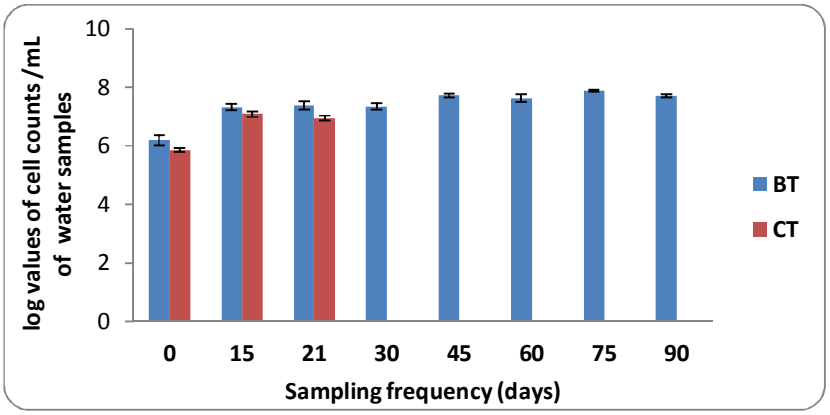


Fig 19: Variations in total heterotrophic bacterial counts in water column of bio-augmented and control systems

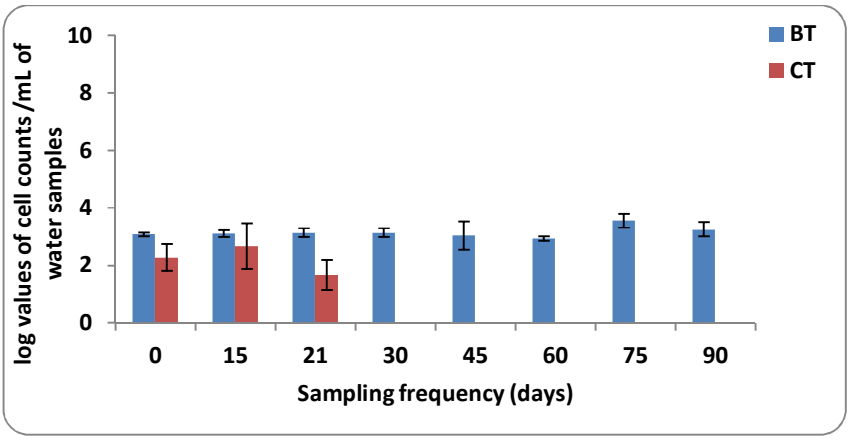


Fig 20: Variations in total actinomycetes plate counts in water column of bio-augmented and control systems

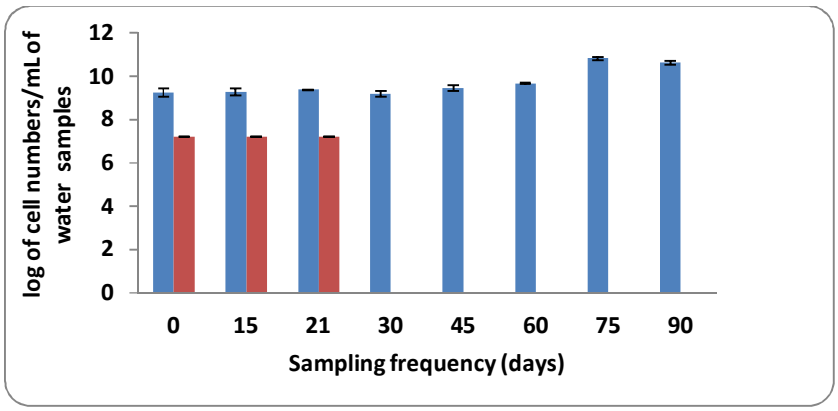


Fig 21: Variations in total bacterial abundance in water column of bio-augmented and control systems as estimated by epifluorescence microscopy

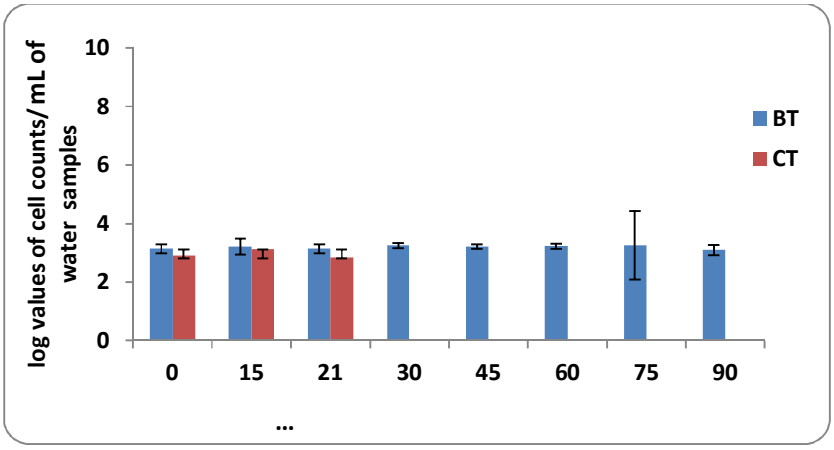


Fig 22: Variations in fungal population in water column of bio-augmented and control systems

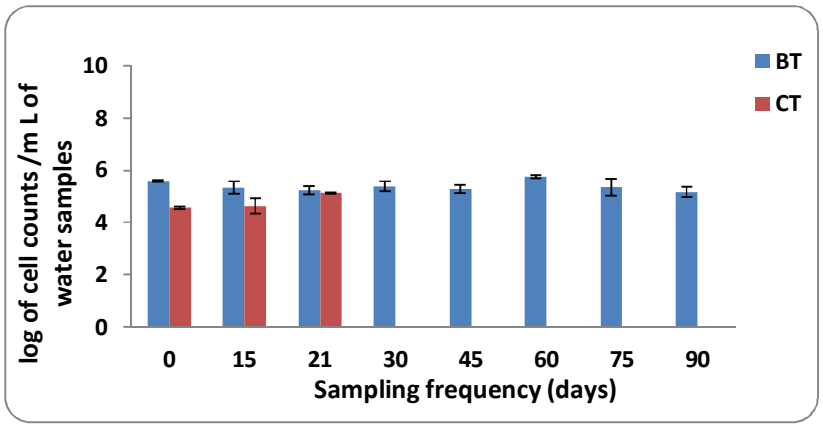


Fig 23: Total *Vibrio* counts in the water column of bio-augmented and control systems

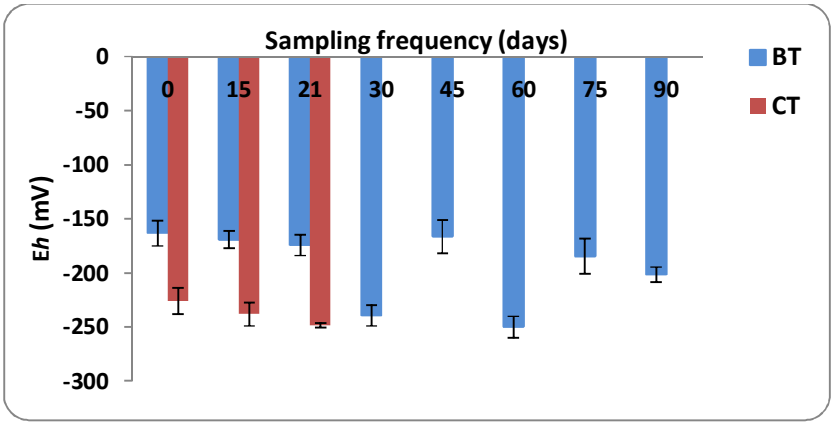


Fig 24: Variations in redox potential (*Eh*) of sediment in bio-augmented and control systems

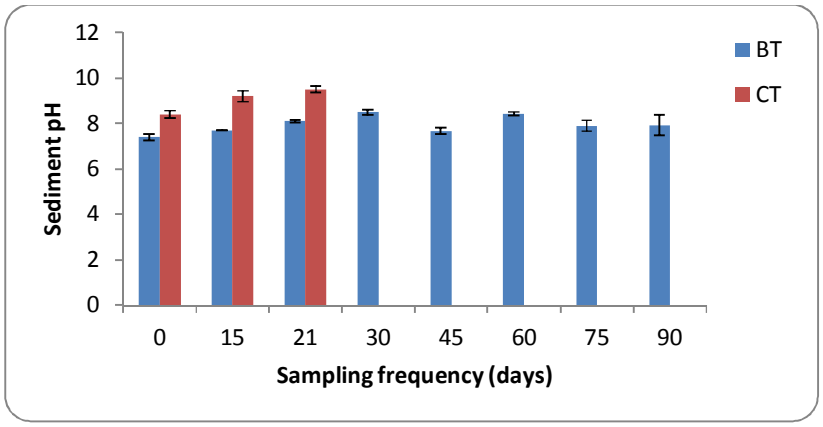


Fig 25: Variations in pH of sediment of bio-augmented and control systems

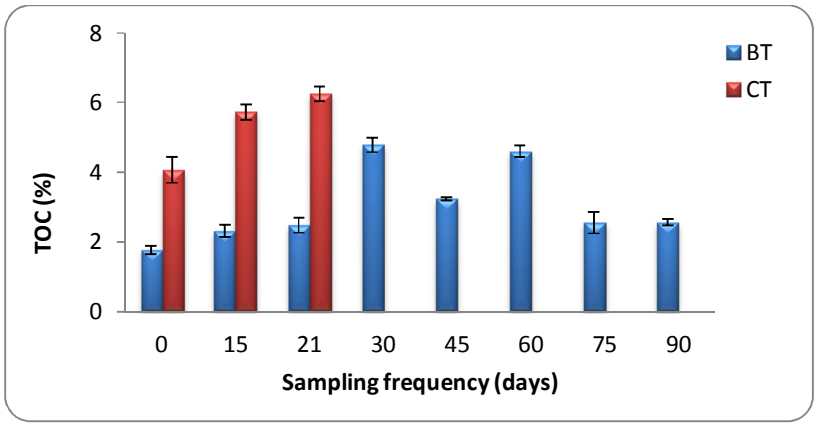


Fig 26: Variations in the percentage of total organic carbon in sediment of bio-augmented and control systems

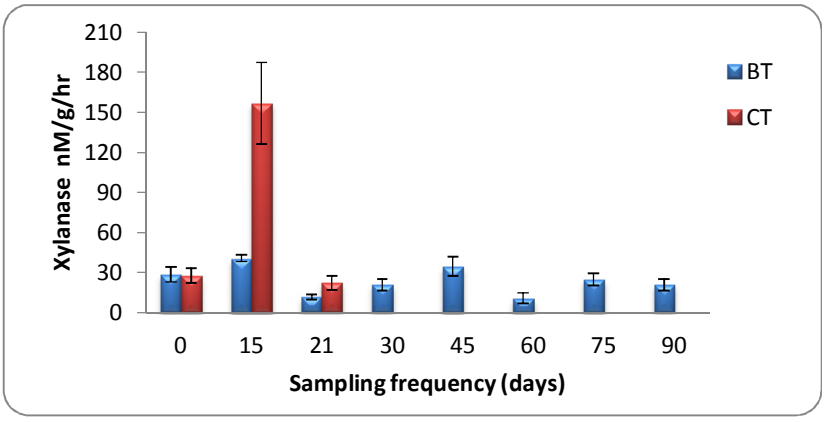


Fig 27: Pattern of xylanase activity in sediment of bi-augmented and control systems

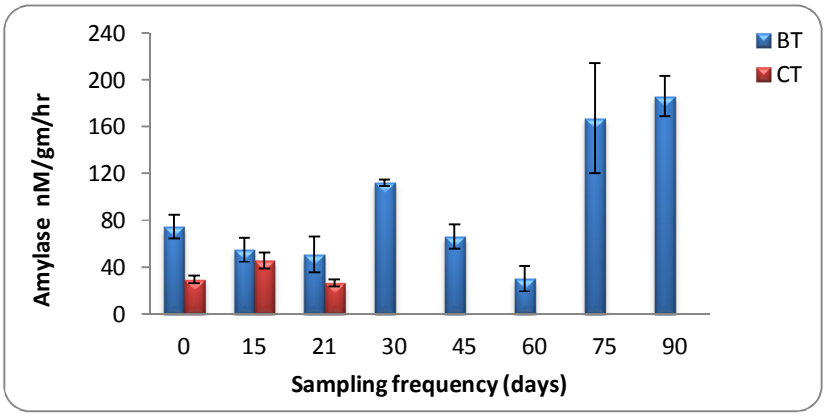


Fig 28: Pattern of amylase activity in sediment of bio-augmented and control systems

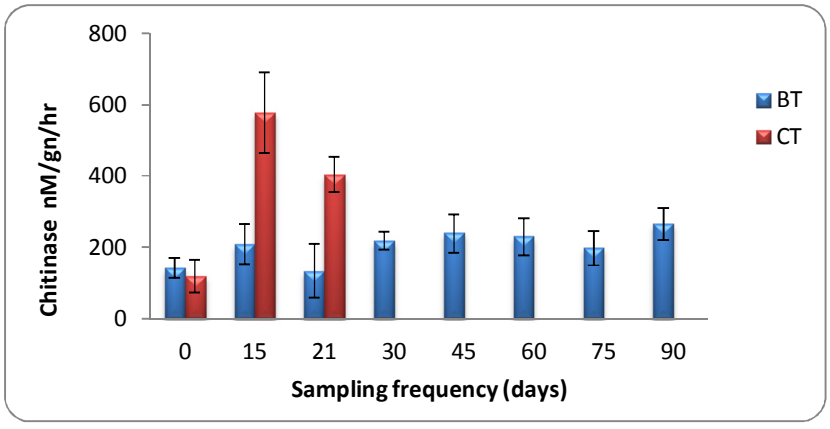


Fig 29: Pattern of chitinase activity in sediment of bio-augmented and control systems

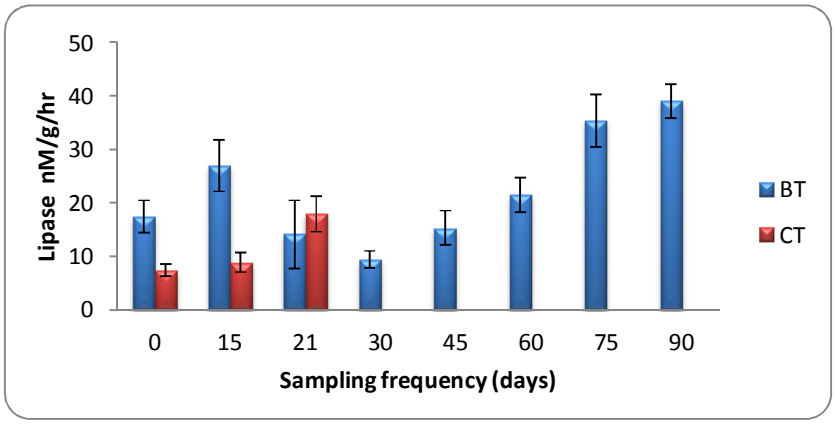


Fig 30: Pattern of lipase activity in sediment of bio-augmented and control systems

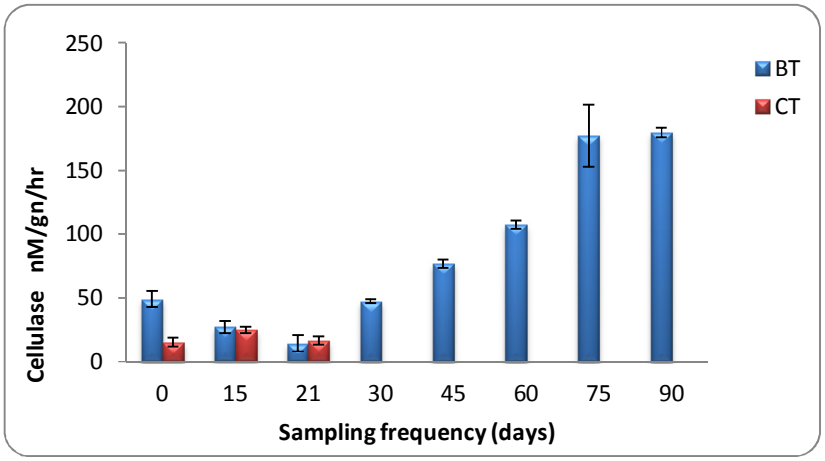


Fig 31: Pattern of cellulase activity in sediment of bio-augmented and control systems

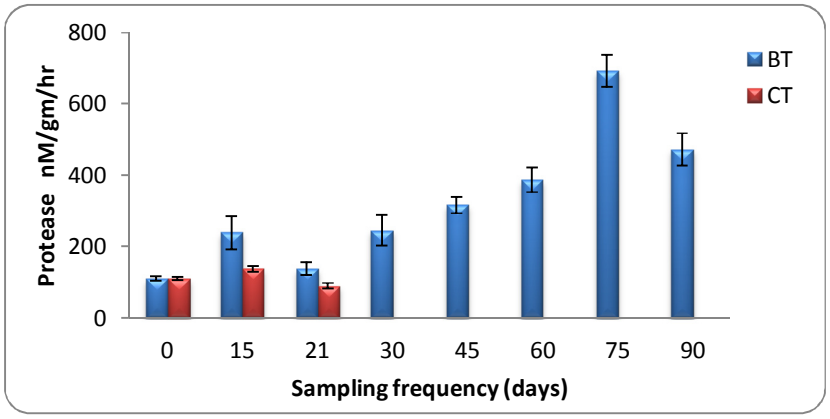


Fig 32: Pattern of protease activity in sediment of bio-augmented and control systems

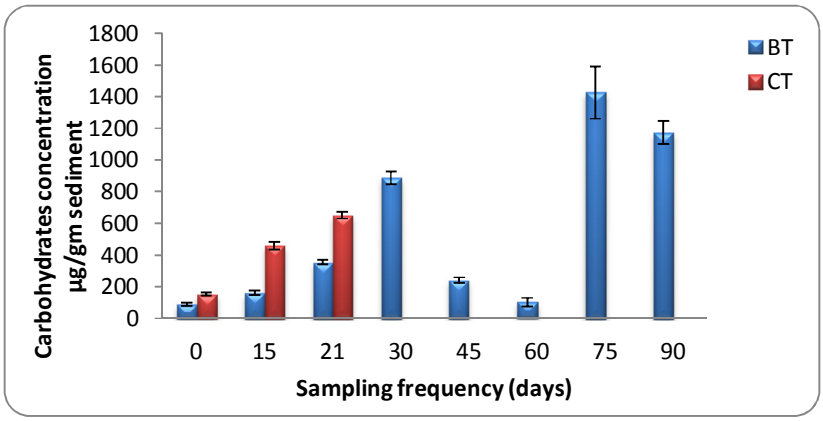


Fig 33: Variations in total carbohydrates in sediment of bio-augmented and control systems

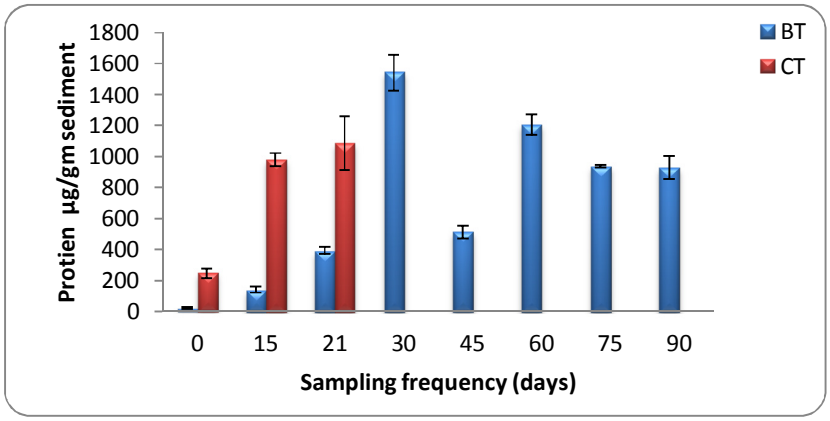


Fig 34: Variations in total protein in sediment of bio-augmented and control systems

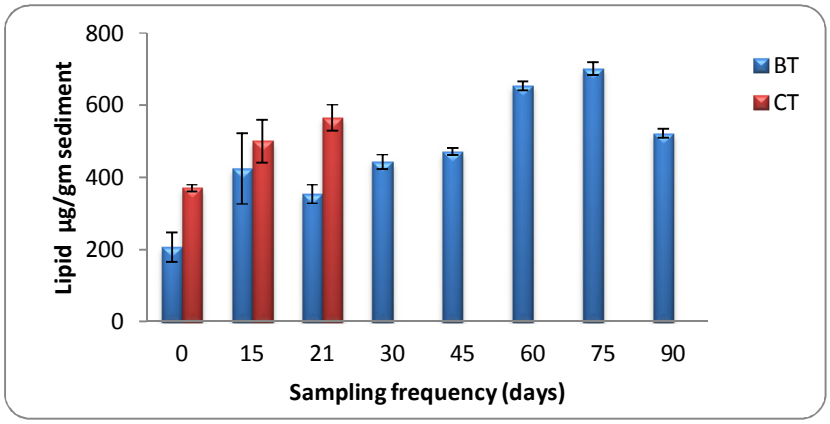


Fig 35: Variations in total lipids in sediment of bio-augmented and control systems

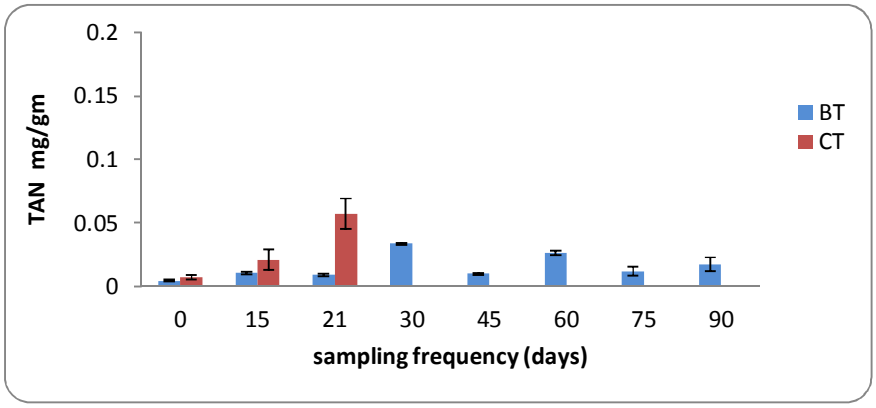


Fig 36: Variations in total ammonia nitrogen (TAN) in sediment of bio-augmented and control systems

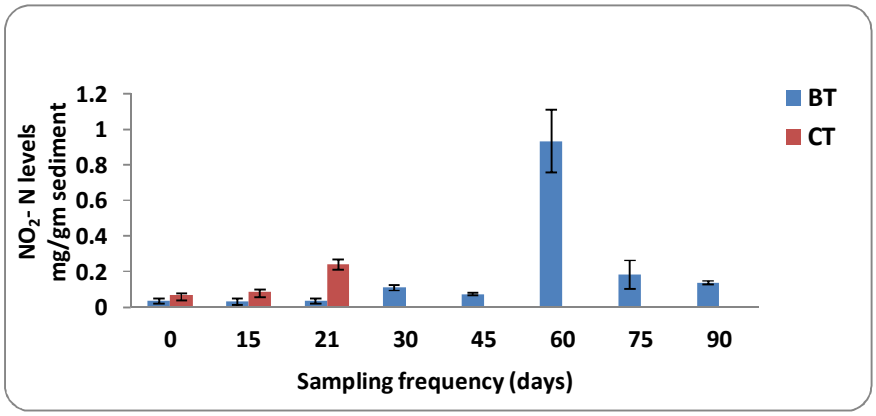


Fig 37: Variations in NO_2^--N in sediment of bio-augmented & control systems

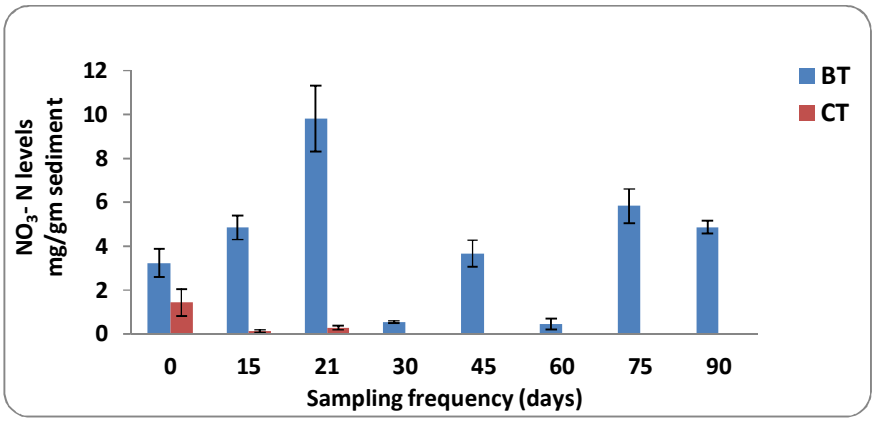


Fig 38: Variations in NO_3- N in sediment of bio-augmented & control systems

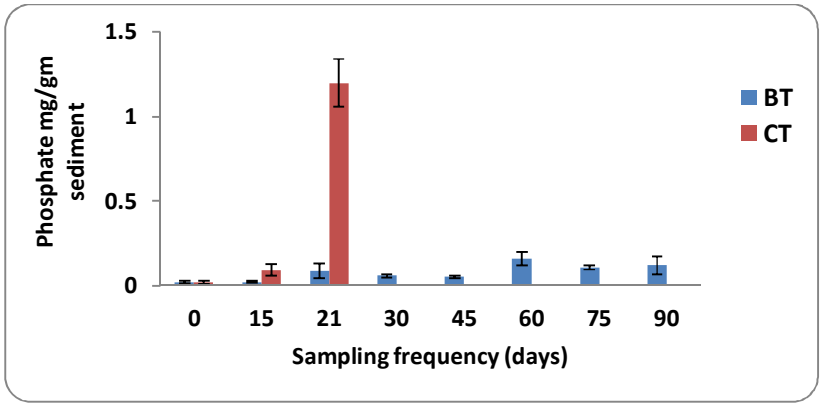


Fig 39: Variations in PO_4 –P in sediment of bio-augmented and control systems

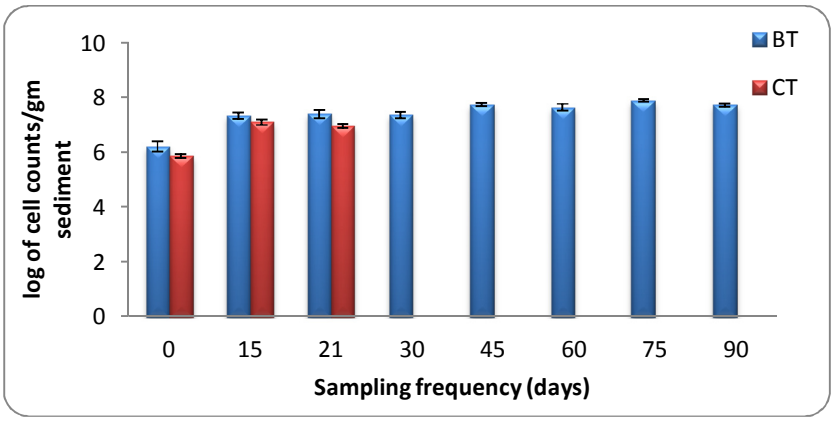


Fig 40: Variations in total heterotrophic bacteria as CFU in bio-augmented and control systems

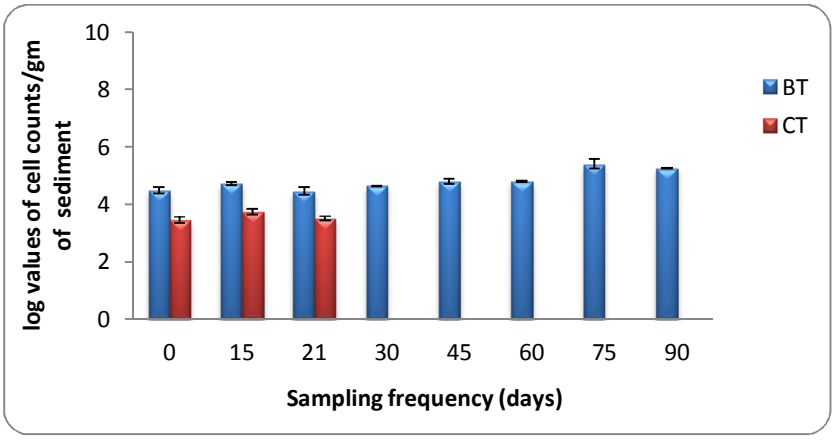


Fig 41: Variations in total actinomycetes as CFU in bio-augmented and control systems

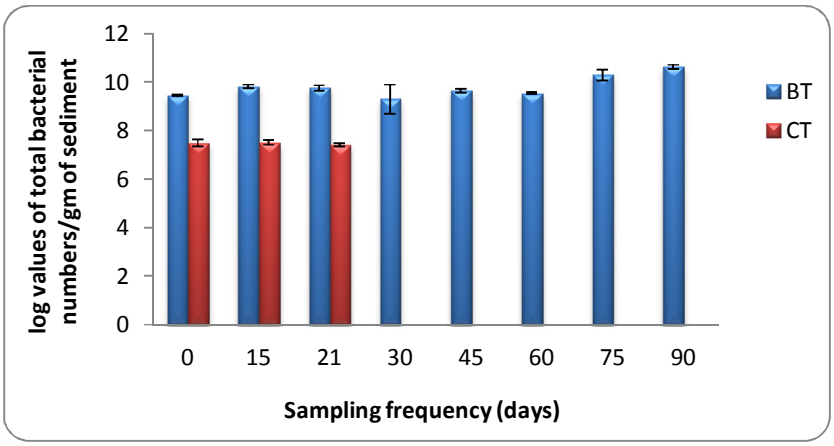


Fig 42: Total bacteria determined through Epifluorescence microscopy in sediment of bio-augmented and control systems

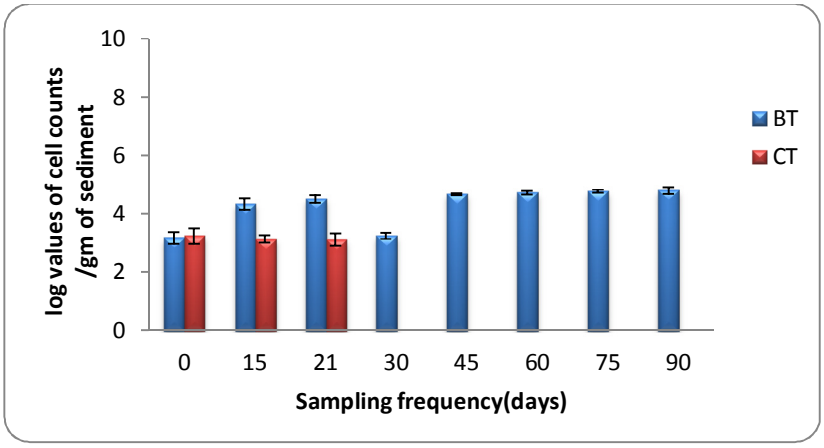


Fig 43: Pattern of fungal population in sediment of bio-augmented and control systems

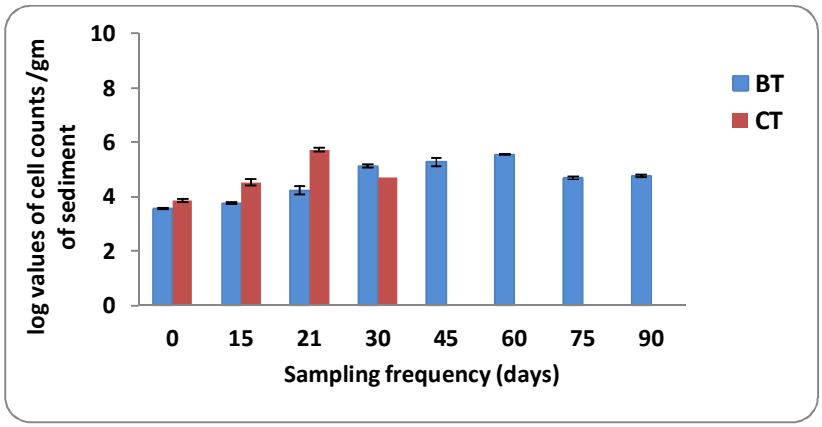


Fig 44: Pattern of total *Vibrio* in sediment in bio-augmented and control systems

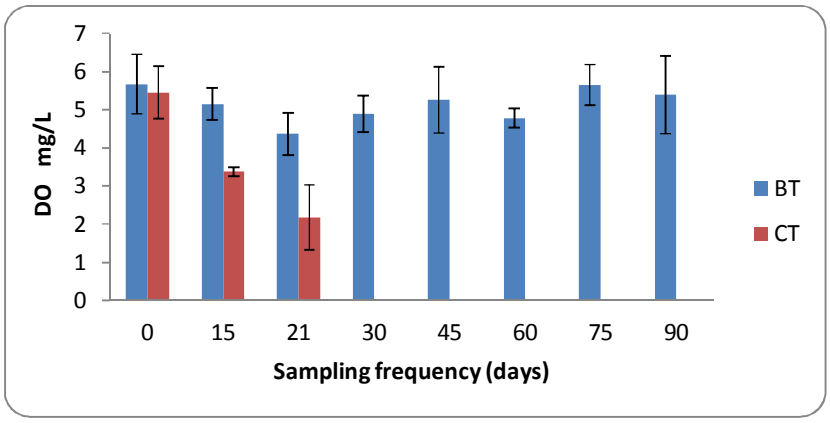
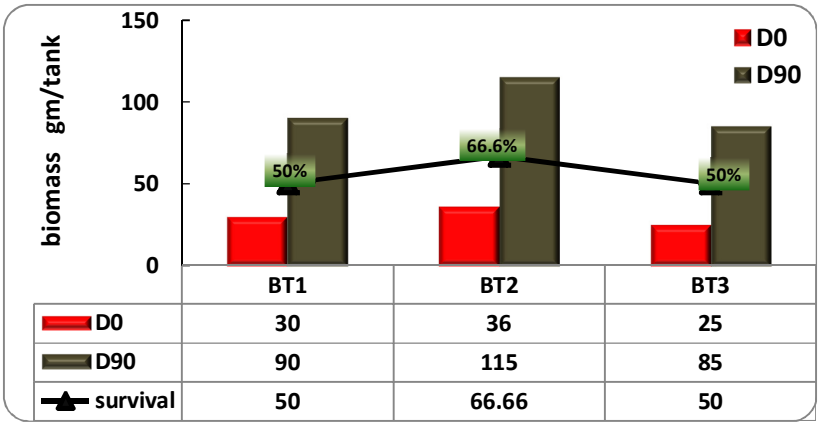


Fig 45: Pattern of dissolved oxygen in bio-augmented and control systems



	BT1	BT2	BT3
D0	30	36	25
D90	90	115	85
survival	50	66.66	50

Fig 46: Percentage survival of shrimp and total biomass yield in bio-augmented systems BT1, BT2 & BT3 represents replicates of bio-augmented tanks

Table 1: Results of Principal component analysis of sediment samples – Eigenvalues of principal components

PC	Eigenvalues	%Variation	Cum.%Variation
1	7.29	34.7	34.7
2	5.71	27.2	61.9
3	2.69	12.8	74.7
4	1.59	7.6	82.3
5	1.08	5.1	87.4

Table 2: Results of Principal component analysis sediment samples – eigenvector values for principal components 1 & 2

Variable	PC1	PC2
S-TOC	-0.245	0.26
S-pH	-0.27	0.253
S-TAN	-0.164	0.3
S-NO_2	0.144	0.084
S-NO_3	0.071	-0.114
S-PO_4	-0.172	0.259
S-Xylanase	-0.143	-0.008
S-Lipase	0.256	0.128
S-Amylase	0.261	-0.003
S-Cellulase	0.287	0.147
S-Chitinase	-0.096	0.273
S-Protease	0.316	0.161
S-DBC	0.355	-0.051
S-THB	0.25	0.24
S-TVC	-0.002	0.359
S-TAC	0.36	0.02
S-TFC	0.308	0.074
S-Total Protein	0.088	0.259
S-Total CHO	0.088	0.259
S-Total Lipid	0.073	0.368
S-*Eh*	0.071	-0.297

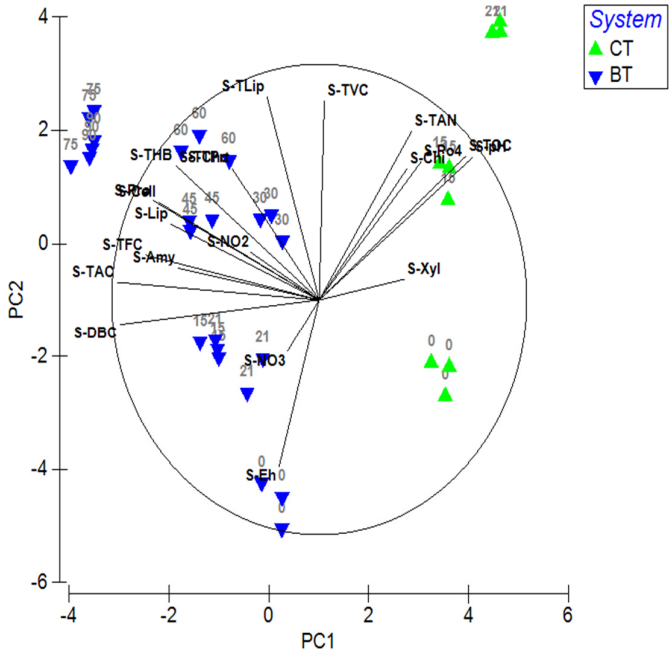


Fig 47 : Principal component analysis of sediment parameters in Bioaugmented and Control Systems

Table 3: Results of Principal component analysis of water samples – Eigenvalues of principal components

PC	Eigenvalues	%Variation	Cum.%Variation
1	7.05	35.2	35.2
2	4.33	21.7	56.9
3	1.78	8.9	65.8
4	1.72	8.6	74.4
5	1.12	5.6	80

Table 4: Results of Principal component analysis water samples – Eigenvector values for principal components 1 & 2

Variable	PC1	PC2
W-pH	0.048	0.369
W-Temp	-0.306	0.057
W-Alk	-0.147	0.388
W-Ca	-0.321	0.012
W-Mg	-0.052	-0.279
W-TAN	0.183	0.33
W-NO_2	0.044	0.115
W-NO_3	-0.315	0.059
W-PO_4	0.019	0.281
W-Xylanase	-0.096	0.093
W-Lipase	0.02	0.389
W-Amylase	-0.234	0.253
W-Cellulase	-0.189	0.214
W-Chitinase	-0.295	0.14
W-Protease	-0.353	0.056
W-DBC	-0.34	-0.148
W-THB	-0.324	0.017
W-TVC	-0.196	-0.206
W-TAC	-0.195	-0.212
W-TFC	-0.182	-0.162

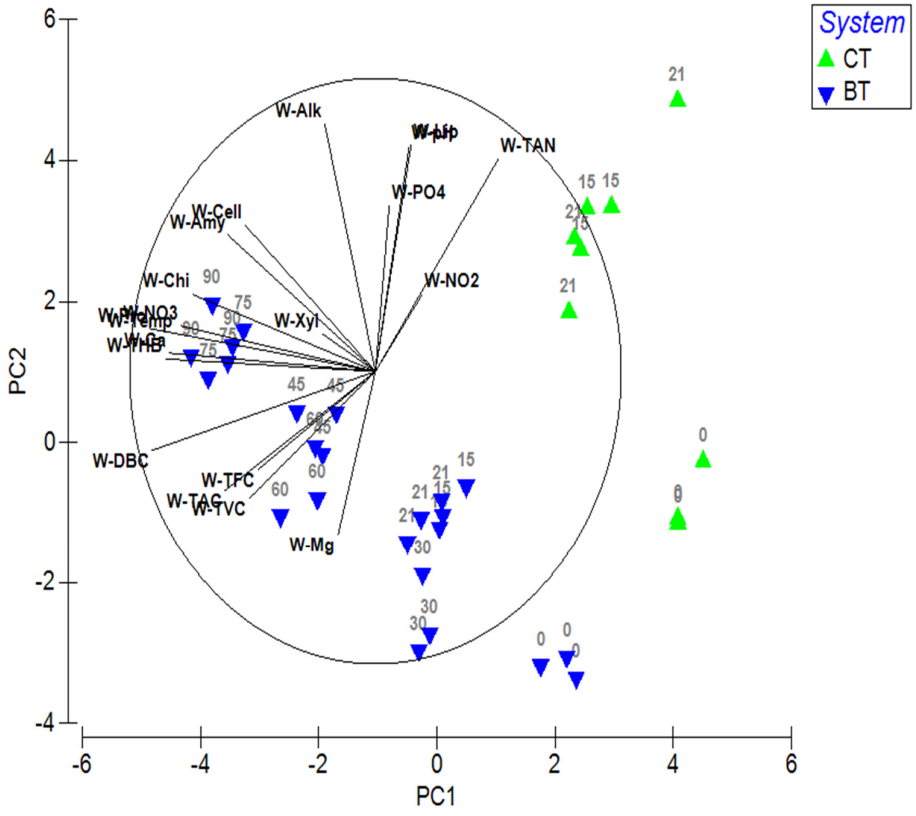


Fig 48 : Principal component analysis of water quality parameters in Bioaugmented and Control Systems

Chapter 4

METAGENOMIC APPROACH TO ASSESS BACTERIAL DIVERSITY IN SEDIMENT OF SIMULATED ZERO WATER EXCHANGE SHRIMP CULTURE SYSTEM SUBJECTED TO BIOREMEDIATION OF DETRITUS

4.1 Introduction

Sediment and soil represent some of the most complex microbial habitats on Earth. There may be several thousand species of bacteria in 1 g soil (Torsvik *et al*., 1990).Since only a small percentile (<1%) of existing bacteria are cultivable by standard microbiological methods,>99% of the microorganisms in any environment observed through a microscope remain largely unexplored (Hugenholtz, 2002).With recent advances in genomics and sequencing technologies, microbial community analyses using culture-independent molecular techniques have initiated a new era of microbial ecology.

Prokaryotic diversity analysis based on 16S rRNA gene sequence analysis provides means for understanding ecologically complex microbial community structure through identification of otherwise obscure (un-cultivable) population. The sequence-based phylogenetic techniques provide a less biased picture of the community composition than would any cultivation technique (Amann *et al*., 1995).Molecular analyses of environmental communities strengthened the findings that the cultivable fraction represented<1% of the total prokaryotic species present in any given system.Methods developed for community analysis based on (the amplification of the 16S rRNA gene include Temperature Gradient Gel Electrophoresis (TGGE) and Denaturing Gradient Gel Electrophoresis (DGGE) , single strand conformation poly- morphism (SSCP), amplified ribosomal DNA restriction analysis (ARDRA) and terminal restriction fragment length polymorphism (T-RFLP) (Tiedje *et al*., 1999).Comparative analysis of 16S rRNA sequence of microorganisms has been universally applied for the understandings of microbial community structures in nature infer relationships among organisms due to the ubiquity of ribosomal RNA molecules in all microorganisms, to (Pederson *et al*., 1996; Wise *et al*., 1999; Lee *et al*., 2000).The rRNA molecules are comprised of highly conserved sequence domains, interspersed with more variable regions. In general, the essential rRNA domains are conserved across all the phylogenetic domains, thus universal tracts of sequences can be identified (Olsen *et al*., 1986).

ARDRA is based on the principle that the restriction sites in the RNA genes are phylogenetically conserved (Massol-Deya *et al*., 1995).Since ARDRA uses universal 16S rRNA gene primers, it is expected

to be applicable to the identification of most bacterial species from any kind of environmental sample.The DNA sequence variations present in PCR-amplified 16S rRNA genes from environmental DNA is generally digested with tetracutter restriction endonucleases and restricted fragments are resolved on agarose or polyacrylamide gels and is analysed in amplified ribosomal DNA restriction analysis (ARDRA) (Smit *et al*., 1997). It is useful for rapid monitoring of microbial communities or to compare microbial diversity in response to changing environmental conditions.

ARDRA has been used to characterize the genetic diversity and community changes of microbial populations in environmental samples such as soils (Porteous *et al*., 1994; Smit *et al*., 2006), reactors (Massol-Deya *et al*., 1995; Massol-Deya *et al*., 1997;Fernandez *et al*., 1999) wastewater (Becker *et al*., 1998) and in the analysis of mixed bacterial populations from different environments (Moyer *et al*.,1994; Martínez-Murcia *et al*.,1995; Acinas *et al*.,1997).ARDRA approach has been successfully tested to detect differences in activated sludge bacterial communities fed on domestic or industrial wastewater, and subject to different operational conditions (Gich *et al*., 2000). ARDRA also detects interspecies, interstrain as well as interoperon variability and enables a relatively fast multiple strain analysis (Heyndrickx *et al*., 1996). Thus technique is appropriate to obtain indicative phylogenetic and taxonomic information.

Although ARDRA gives little or no information about the type of microorganisms present in the sample, it can be used for a quick assessment

of genotypic changes in the community over time, or to compare communities subject to different environmental conditions. ARDRA is also used for identifying the unique clones and estimating OTUs in environmental clone libraries based on restriction profiles of clones (Smit *et al.,* 1997).Therefore, ARDRA can be designated as a common methodology for a rapid molecular characterization based on the generation of so called "genetic fingerprints". One of the major limitations of ARDRA is that restriction profiles generated from complex microbial communities are sometimes too difficult to resolve by agarose/Poly acrylamide gel electrophoresis (PAGE).

In the present study 16S rRNA gene clone libraries have been made to investigate the genetic diversity of microbial communities in sediment in bio-augmented and control systems. To assess the diversity of an environmental sample, modern molecular tools based on the PCR amplification of 16S rRNA gene, the phylogenetic anchor, are employed (Handelsman, 2004).Amplified ribosomal DNA restriction analysis (ARDRA) which is based on DNA sequence variations present in PCR-amplified 16S rRNA genes also serve as an effective tool in phylogenetic analysis (Smit *et al*.,2006). Clonal types were initially grouped on the basis of amplified rRNA gene restriction analysis (ARDRA). The 16S rRNA gene from representatives of different clusters were sequenced and compared with those available from the DNA databases.

4.2 Materials and methods

4.2.1 Sample collection

Sediment samples for metagenomic analysis were collected on the 7th day of stocking in bioassay experiment mentioned in Chapter - 3. Bio-augmented sediment samples were collected from the systems along with those from controls in triplicates. Upper layer of sediment was scooped out using spatula randomly from various sites, pooled and stored at -80^{0}C until DNA extraction.

4.2.2 DNA extraction from sediment

Metagenomic DNA having good yield and purity was obtained from sediment sample employing Powersoil DNA isolation Kit (MOBIO Laboratories Inc., USA). Accordingly, 0.5 g sediment was added to the power bead tubes provided andgently vortexed. An aliquot of 60µl solution C1 was added and vortexed at maximum speed for 10 min. Solution C1 contains SDS and other disruption agents required for complete cell lysis. The tubes were centrifuged at 10,000x*g* for 30 seconds and supernatants were transferred to 2 mL collection tubes provided. To this 250 µl solution C2 was added and incubated at 4^0 C for 5 min. The tubes were centrifuged at 10,000 x *g* for 1 min at room temperature and aliquots of 600 µl of the supernatant were transferred to 2 ml collection tubes. Aliquots of 200 µl solution C3 was added, incubated at 4^0 C for 5 min and centrifuged at 10,000x *g* for 1 min. Solution C2 and C3 is patented Inhibitor Removal Technologies® (IRT). It contains a reagent to precipitate non-DNA organic and inorganic material including humic substances, cell debris, and proteins.

Aliquot of 750 µl supernatant was transferred to clean 2 ml collection tubes. To the supernatant 1200 µl solution C4 a high concentration salt solution was added and vortexed for 5 seconds. Approximately 675 µl supernatant was loaded on to spin filter and centrifuged at 10,000x *g* for 1 min at room temperature. The flow through was discarded and additional 675 µl supernatant was added to the spin filter, centrifuged at 10,000x g for 1 min at room temperature. The remaining supernatant was also added, centrifuged and the flow through was discarded. To the spin filter 500 µl C5 solution an ethanol based wash solution was added and centrifuged at 10,000x *g* for 30 seconds at room temperature. The flow through was discarded and tubes containing spin filter was carefully placed in clean 2 ml collection tubes provided and 100 µl of sterile elution buffer solution C6 was added to the center of white filter membrane. The tubes were centrifuged at room temperature at 10,000 x *g* for 30 seconds and the spin filter was discarded.

4.2.3 Estimation of DNA yield

The metagenomic DNA extracted was quantified by spectrophotometric analysis. The concentration of DNA was determined by measuring the absorbance of 50 times diluted sample at 260nm in a 1-cm light –path length quartz cuvette. The ratio of absorbance at 260 nm and 280 nm was used as measure for contamination of the DNA with RNA and protein. The ratio of absorbance at 260 nm and 230 nm was used as a measure of humic acid contamination. Visual comparison was done under UV light in a gel documentation system (Biorad, USA) after electrophoresis of 8 µl DNA solution on 0.8% agarose gel with 1X TAE buffer (Tris base acetic acid, 0.5 M EDTA, pH 8.0) and ethidium bromide staining.

4.2.4 Amplification of the universal bacterial 16S rRNA gene fragment

Amplification of 1500bp bacterial rRNAgene fragment was performed using universal primers, 16S1 and 16S2 (Table 2.1), which were complementary to the conserved region at the 5'and 3' ends of the rRNA gene corresponding to the 9-27 and 1477 -1498 of the *E.coli* 16S rRNA gene.

The bacterial rRNA gene was amplified using Emerald PCR reaction mix (Takara, BIO Inc. Japan) in a total volume of 25 µl. To thereaction mix universal primers at 0.2µM concentration each and 1µl template DNA (75/ng/µl) were added. The amplification was carried out in a thermal cycler (Epperndorf, Germany)programmed forinitial denaturation at 95^0C for 3 min, followed by 24 cycles of denaturation at 98^0C for 10seconds, annealing at 58^0C for 40 seconds and extension at 72^0C for 1' 30 seconds followed by final extension at 68 ^{0}C for 10 min.

Primers for the amplification of universal bacterial rRNA gene fragment

Primer	Position	Primer Sequence (5'- 3')
16S1	9-27	5' GAGTTTGATCCTGGCTCA 3'
16S2	1477- 1498	5' ACGGCTACCTTGTTACGACTT 3'

The amplicon obtained from the above amplification was observed in 1% agarose gel with 1XTAE buffer and size of the product was determined by comparing with molecular weight marker (1Kb, New

England Biolabs) and the results were documented using Gel documentation system (Biorad, USA)

4.2.5 Cloning and transformation of the amplified universal bacterial 16S rRNA gene fragment

PCR products from three 25 µl reactions of universal 16S rRNA genes were cloned in to pGEM-T Easy vector (promega, USA). The ligation mix (10 µl) consisted of 5 µl ligation buffer (2X), 0.5 µl vector (50 ng/ µl), 3 µl PCR product and 1 µl T4 DNA ligase (3U/ µl). The ligation mix was incubated at 4^0C overnight. The entire ligation mix was used to transform *Escherichia coli* DH5 α competent cells prepared using $CaCl_2$. The mix was briefly centrifuged and added to 10 mL glass tubes previously placed on ice to which 100 µl competent cells were added and incubated on ice for 20 min. A heat shock at 42^0 C was given for 90 seconds, immediately the tubes were placed on ice for 2 min and 600 µl of Super Optimal broth with Catabolite repression (SOC) medium was added and incubated for 2 hours at 37^0 C with shaking at 250 rpm. The transformation mixture (100 -200 µl) was spread on Luria Bertani (LB) agar plates supplemented with ampicillin (100 µg/ml), IPTG (100mM) and X-gal (180 µg/ml) in triplicate. The plates were incubated at 37^0C overnight. The clones were selected by blue/white screening. The white colonies were selected and streaked to purity on LB-Amp+X-gal + IPTG plates and incubated at 37^0C overnight.

To confirm the insert, PCR of white colonies was carried out using the vector primers T7 (5'TAATACGACTCACTATAGGG3') and SP6 (5'GATTTAGGTGACACTATAG3'). White colonies (template) picked from the plates were dispensed into the PCR mix (25 µl) containing 2.5 µl

(10X) PCR buffer, 2.5 µ1 (2.5 mM) dNTPs, 1 µl 10 pmol/ µl T7 and SP6 primers, 0.5U *Taq* polymerase and the remaining volume was made up with MilliQ. The *Taq* polymerase was added after initial denaturation. The thermal cycling conditions were as follows: initial denaturation at 39^0C for 5 minutes, held at 80^0C for addition of *Taq* polymerase, 35cycles of 94^0C for 15 seconds, 57^0C for 20 seconds, 72^0C for 60 seconds, and final extension at 72^0C for 10 minutes and held at 4^0C. The amplicons were observed in 1% agarose gel and size of the product was determined by comparing with molecular weight marker (1Kb) and documented in a gel documentation system.

4.2.6 Plasmid extraction

Plasmids from positive colonies were extracted using a modified protocol of plasmid mini preparation kit (Sigma Aldrich, USA). Aliquot of 4mL overnight grown recombinant *E. coli* culture was centrifuged. To the pellet added 200µl of re-suspension buffer (25mM Tris, 10Mm EDTA, 50Mm glucose and 100µg/mL RNAase), 200µl lysis buffer (.2N NaOH, 1% SDS) and neutralization buffer (3M potassium acetate and glacial acetic acid). Centrifuged at 12,000 *g* for 10 min. The supernatant was carefully withdrawn and added equal volume of ice cold isopropanol and kept in ice for 10 min. Centrifuged again and pellet washed with 70% ethanol. The pellet was air dried and dissolved in 25µl Milli-Q water. Glycerol stocks of the clones were maintained in LB – ampicillin medium at -80^0 C.

4.2.7 Amplified ribosomal DNA restriction analysis (ARDRA)

Amplified rDNA restriction analysis (ARDRA) was performed to analyze the diversity among 146 clones (73 clones each from both systems) in shrimp pond sediment. The extracted plasmids were diluted 20 times and the inserts re-amplified using the universal primers, 16S1 (GAG TTT GAT GGC TCA) and 16S2 (ACG GCT ACC TTG TTA CGA CTT) at same PCR conditions for amplification of 16S rRNA gene. The amplicons obtained were observed in 1% agarose gel and product size was determined by comparing with molecular weight marker (1Kb) and documented in a gel documentation system.

Each PCR product was digested with *Alu 1,Hae III*and *Taq* I (New England Biolabs, USA) in separate reactions. The digestion was performed for 3.5 hours in 20 µl reaction volume containing 5 µl PCR product, 2 µl buffer (50mM NaCl, 10mM, Tris – HCl, 10mM $MgCl_2$, 1nM dithiothreitol), 12.8 µl milliQ water, and 0.2 µl (10U/ µl) each restriction enzyme. Restricted products were run on 3% agarose gel in 1X TAE buffer for 3 hours at 100V. The band pattern was scored for the absence / presence (0/1) of individual loci using Quantity one software (Biorad, USA) and analyzed using the software NTSYSpc (Version 2.02i, Applied Biostatistics Inc., USA). The data matrix prepared in MS Excel spreadsheets was converted to proprietary matrix file by the programme NT edit and rectangular datamatrix generated was analyzed by the programme NTSYSpc. Similarities were calculated by simple matching coefficient using the statistical module, sequential agglomerative hierarchical nested cluster method (SAHN) and clustering was achieved by un-weighted pair group method with arithmetic mean (UPGMA).

4.2.8 Phylogenetic analysis

Selected clones based on ARDRA were sequenced using ABI Prism 3700 Big Dye sequencer at SciGenom, Cochin. The vector regions in the sequences were analysed using VecScreen analysis tool of NCBI database. The vector region cleaned sequences were matched with the GenBank database using Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al*., 1990). The non-chimeric sequences were aligned with the published sequences from the NCBI database using ClustalW. Distance matrices and phylogenetic tree were constructed from the aligned data sets of the bio-augmented and control system clone library using Mega 6.06 software (Tamura *et al*., 2013). The 16S rRNA phylogenetic tree was constructed using UPGMA statistical method and Kimura 2-parameter substitution model with 1000 bootstrap replications. The bacterial community structure of bio-augmented and control systems sediment were analyzed using Geneious (Ondov *et al*., 2011) and VITCOMIC (Mori *et al*., 2010) 16SrRNA biodiversity analysis tools. Indices like Jaccard index, Lenon index and Yue and Theta index (Chao *et al*., 2006) were calculated using the clustering results of VITCOMIC for the statistical comparison of taxonomic composition between the bio-augmented and control systems.

4.3 Results

4.3.1 DNA extraction and 16S rRNA gene amplification

Sediment samples were analyzed for bacterial diversity. The Genomic DNA extracted from sediment samples were predominantly >10 Kb in size (Fig.1) and on amplification using universal bacterial specific16S rRNA gene PCR primers 1500bp product could be amplified from

metagenomic DNA extracted from sediment of bio-augmented and control systems (Fig.2). The PCR product was cloned to produce 16S rRNA gene library representing each sample.

4.3.2 ARDRA

To differentiate the clones for sequencing, the plasmids extracted (Fig. 5) from PCR positive clones (Figs. 3&4) were re-amplified (Fig.6) and subjected to ARDRA using tetracutter restriction enzymes *Alu 1, Hae III* and *Taq* I (Figs.7-12). The band pattern types developed using each restriction enzyme was variable and distinct. Each ARDRA-type was composed by 2 to 5 distinct bands with molecular weights (MW) ranging from 100 to 1000-bp. Hence a combination of the ARDRA pattern generated independently from each restriction digestion enzyme was used in this study followed by plotting a dendrogram to differentiate the clones based on the ARDRA patterns generated as shown in Figs.13 &14. ARDRA pattern of 73 clones from control sediment gave dendrogram of 34 clusters / OTUs (Fig. 14). However, the digestion of equal number of clones from bio-augmented sediment generated 42 clusters/OTUs (Fig.13) exhibiting different pattern. Representatives of each cluster were sequenced. The dendrogram of the clones from bio-augmented sediment showed distance coefficient of 0.74 to 0.98 while in control sediment it was 0.71 to 0.94.

4.3.3 Phylogenetic analysis

On comparing the 16S rRNA gene sequences from the bio-augmented and control sediment with GenBank database using BLAST algorithm, major hits were with uncultured proteobacteria. The phylogenetic

tree constructed with the aligned sequences of the bio-augmented sample and 99% similar sequences from GenBank database showed a major clustering of sequences within the taxonomic composition of the bio-augmented sediment. A few of the clones formed a cluster with uncultured bacteria isolated from various marine, river and polluted sediment. Few of the clones were related and branched off from the uncultured delta proteobacteria, uncultured spirochaetes, uncultured acidobacteria, *Maritimibacter*, *Rugeria pomeroyi*, uncultured alpha proteobacteria, uncultured beta proteobacteria, uncultured nitrospirae, uncultured chloroflexi, uncultured bacterioidetes and *Muricauda* sp (Fig.15). In case of phylogenetic analysis of the sequences from control sediment with 99% similar sequences from GenBank database showed a major clustering of sequences within the clones and few of the clones clustered and branched off from uncultured bacteria, uncultured soil bacteria, uncultured sludge bacteria and uncultured delta proteobacteria (Fig. 17).

The phylogenetic tree constructed with the aligned sequences from bio-augmented sediment consisted of six clusters (Fig.16) while the same from control sediment had only five (Fig. 18). Using the diversity estimation of MEGA 6.0, the mean inter-populational diversity obtained for the bio-augmented sediment was 5.167 ±1.009, whereas in control sediment it was only 3.188 ±0.686.

An overall taxonomic composition of bacteria from control and bio-augmented sediments were visualized using VITCOMIC (Figs.19 & 20). In the VITCOMIC map (Fig. 21) the different font colour of each species name corresponded to its phylum name. Large circle indicated

boundaries of BLAST average similarities (inner most circle starting at 80% followed by 85, 90, 95 and 100% similarity of the database sequence). The size of the dots indicated the relative abundance of the sequences in the samples. The red dots represent taxonomic group of bio-augmented sediment, blue of control sediment, grey the common taxonomic group found both in bio-augmented and control sediment. The VITCOMIC map showed that most of the taxonomic communities of both the samples belonged to Proteobacteria. The comparative taxonomic composition generated by VITCOMIC map revealed the relative abundance and species composition found in the bio-augmented and control sediment. The map showed relative abundance of >10% of *Geobacter* sp. and *Gramella* sp. followed by <5% relative abundance of *Thermodesulphovibrio* sp., *Desulfococcus* sp., *Pelobacter* sp. and *Flavobacterium* sp. in bio-augmented sediment. In control sediment, a relative abundance of <10% of *Frankia* sp. was observed followed by < 5% *Pelobacter carbinolicus*, *Pelotomaculum thermopropionicum*, *Syntrophobacter fumaroxidans*, *Syntrophus aciditrophicus* and *Thioalkalivibrio* sp. Information on the nearest phylogenetic groups derived for clones of bioaugmented and control sediments from VITCOMIC map is shown in tables 1 and 2 .Groups that are unique to and common among clones of bioaugmented and control sediments is represented in table 3. Comparative analysis showed a difference between both the communities as indicated by Jacard index, Lenon index and Yue and Clayton Theta of 0.109, 0.2 and 0.0723 respectively.

The obtained 16S rRNA amplicon data was also submitted through the Geneious bioinformatics platform to a distributed cloud compute resource.

The data were then analyzed using the Ribosomal Database Project Database (RDP) Classifier. The RDP Classifier assigns sequences derived from bacterial gene to the corresponding taxonomy model using a 'Naïve Bayesian Classifier' for rapid assignment of rRNA sequences. The Geneious 16S Biodiversity Tool accurately thus aligned a taxonomy (in the range of domain to family) along with a confidence-estimate for each sequence by comparing them to the RDP database. The output was then displayed in a web browser using Krona chart, which produced an interactive HTML5 hierarchical graph of the bacterial diversity in the sample. Krona displayed abundance and hierarchy simultaneously using a radial space-filling display. The Krona chart features a red-green color gradient signifying average e-values of BLAST hits within each taxon, with red being the highest observed e-value (least significant) and green being the lowest (most significant).

As depicted in krona chart generated using geneious software (Fig.22) 16SrRNA amplicons of bioaugmented sediment clones metagenome belong to the taxa Proteobacteria, Bacteroidetes, Acidobacteria, Planctomycetes, Nitrospirinae and Chloroflexi. Overall Proteobacteria contributes to 41% in terms of abundance and richness. This phylogenetic group includes δ proteobacteria, followed by α, β and γ proteobacteria. δ proteobacteria includes 12% Desulfobacteriacea , 5% Soranginiiaeae, 2% Syntrophobacteriaceae and 2% Nanocystineae . 7% Rhodobacteriacecae, 5% Rhodospirillaceae and 2% Hyphomicrobiaceae adds to 14% abundance of the class α proteobacteria. β and γ, proteobacteria are composed of 2% each of like Thiollkalispiraceae , Burk holderiales incertaesedis and Alcaligenaceae of the bacterial community.

Bacteroidetes homes 21% of the clones in which 17% of the abundance is linked to family Saprospiraceae , 2% of Prolixibacteraceae and 2% of Flavobacteriaceae. Acidobacteria family of GP9, GP 10, GP11 forms minor group representatives of Phylum Acidobacteria. Similar pattern of abundance was seen with Chloroflexi wherein the phylum is represented by 5% Cladilineaeceae, 2% Dehalococcideae and 2% Anaerolineaceae. Phylum Nitrospirinae is comprised of a single family 7% Nitrospirinaceae. A 5% of Tepidisphaeraceae and 2% Candidatus Brocadiaceae makes up Planctomycetes. BRC1 a unique and least represented group of 2% could also be observed in the heirarchial layout of biaougmented sediment clones.

The krona chart on 16SrRNA biodiversity of control system showed that clones were distributed among the Proteobacteria as the major phylum occupying about 61% of the population, followed by 11% Chloroflexi and Actinobacteria Bacteroidetes and Acidobacteria represents 6% and 8 % of the clones,whereas Planctomycetes, Firmicutes and Gemmatimonadetes are evenly distributed minor phyla accosting for about 3% of the community (Fig.23). Among the Proteobacteria α and γ proteobacteria computes 21% each and is relatively abundant than β group that holds 19% representatives of the phyla. However the class δ proteobacteria signifies only 3% of the clones.

More specifically 16S rRNA amplicon data of control system categorized 13% clones in among family Rhodobacteriaceae , 10% teams with Sinobacteriaceae ,8% in Desulfotobacteriaceae and Anaerolinaceae .Rhodospirillaceae , Ectothiorhodospiraceae, Syntrophobacteriaceae, Actinomicrobales and GP21 of Acidobacteria comprehends 5% each . Remaining clones tranquilly reckons 3% each among 15 families as shown in figure 23.

4.4 Discussion

4.4.1 ARDRA based bio-augmented shrimp pond sediment bacterial community analysis

The metagenomic approach of analyzing diversity of microbial community with respect to bio-augmentation of shrimp pond sediment has not so far been reported, and present study is first of its kind. The basic strategy of bio-augmentation is the overall re-arrangement of organisms in a niche so that specific catabolic traits are expressed in increasing the metabolic competency in the right direction at the right time necessary for environmental cleanup (Deonje *et al*., 2001). In this study, it has been attempted to unravel the bacterial diversity when the shrimp pond sediment is bio-augmented with *Bacillus cereus* MCCB101.The microbial community has been analysed using culture independent molecular techniques and diversity determined based on phylogenetic analysis. The concept of metagenomic approach using 16S rRNA gene sequence to understand the microbial interactions and community analyses is useful in monitoring changes in microbial diversity upon bio-augmentation.

Primarily, 16S rRNA gene PCR products were generated from metagenomic DNA of bio-augmented and control samples and were cloned into pGEM-T-Easy vector system. The positive clones obtained were re-amplified with 16S rRNA primers, and the PCR products were subjected to ARDRA. The ARDRA performed with *Alu*-1, *Hae*-III and *Taq*-I enabled determination of variations in the restriction band pattern among the clones. These tetracutter restriction enzymes have been shown to be useful tools for screening environmental clone libraries (Liu *et al*., 1997; Ravenschlag *et al*.,

1999) and the common restriction fragment patterns, the so-called operational taxonomic units (OTUs), obtained from such analysis assisted in distinguishing the taxonomic groups. The variations in restricted band pattern and the distance among the clones viewed in the dendrogram conferred the initial identification of diversity. Based on the dendrogram obtained using NTSYS software, bio-augmented shrimp pond sediment generated 42 clusters/ OTUs from 73 clones that were analysed while control sediment provided 34 clusters/ OTUs. Similar kind of ARDRA based studies were used for comparative analysis of bacterial community structure in contaminated and non-contaminated river reservoir sediment (Wu *et al*., 2012) and black sea coastal sediment (Todorova *et al*., 2014). ARDRA based approach was used for assessing the phylogenetic diversity of mangrove sediments of Cochin backwaters (Vincent *et al*., 2013). In another study of microbial diversity in marine sediments from Japan, ARDRA was used for investigation of the microbial community structure (Urakawa *et al.*, 1991). Thus the ARDRA based investigation of microbial community structure with regard to bio-augmented and control sediments are substantially supportive in understanding diversity of the microbial community.

Based on cluster analysis of ARDRA, clones were selected and sequenced for confirmation of variations in the microbial community structure. The 16S rRNA gene sequences obtained from bio-augmented and control sediment samples were matched with NCBI GenBank database for identification of microbial phylotypes. The phylogenetic analysis was carried out following two approaches (1) phylogenetic tree construction

using MEGA 6.06, and (2) VIsualization tool for Taxonomic COmpositions of MIcrobial Community (VITCOMIC) based on 16S rRNA gene sequences. In the phylogenetic tree analysis of sequences obtained from bio-augmented and control sediment samples with GenBank database, it was observed that a major clustering of sequences was within the taxonomic group. In bio-augmented sediment samples, few sequences formed clusters with uncultured bacteria, while others branched off from uncultured alpha proteobacteria, beta proteobacteria, delta proteobacteria, uncultured nitrospirae, uncultured bacteroidetes, uncultured spirochaetes, uncultured acidobacteria, *Maritimibacter*, *Rugeria pomeroyi*, uncultured chloroflexi, and *Muricauda* sp. The phylogenetic analysis of control sediment samplesexhibited clustering with uncultured delta proteobacteria, uncultured bacteria from soil and sludge. The phylogenetic analysis of bacteria of bio-augmented samples showed major difference from the control sediment samples in having more diversity, and clustering with uncultured alpha proteobacteria, beta proteobacteria, uncultured bacteroidetes, uncultured nitrospirae, uncultured spirochaetes and uncultured acidobacteria. The presence of uncultured beta proteobacteria revealed the presence of ammonia oxidizing bacteria (AOB) as they were composed of members of the beta subdivision of the class Proteobacteria (Teske *et al*., 1994). Uncultured bacteroidetes revealed the presence of anaerobic bacteria, and uncultured nitrospirae the presence of nitrogen fixing bacteria. The diversity estimation of the bio-augmented and control sediment showed more diversity of 5.167 ±1.009 in bio-augmented samples. Results of the phylogenetic analyses revealed changes in the microbial community of bio-

augmented shrimp pond sediment using *Bacillus cereus* MCCB101 compared with that of the control sediment.

VIsualization tool for Taxonomic COmpositions of MIcrobial Community (VITCOMIC) based on 16S rRNA gene sequences is another approach to visualize and identify the bacterial taxonomic representation.VITCOMIC can analyze millions of bacterial 16S rRNA gene sequences and calculate the overall taxonomic composition for a microbial community. The 16S rRNA gene sequences of genome-sequenced strains are used as references to identify the nearest relative of each sample sequence. With this information, VITCOMIC plots all sequences in a single figure and indicates relative evolutionary distances.

The VITCOMIC map showed relative abundance of >10% Geobacter spp. and *Gramella* spp. in the bio-augmented shrimp pond sediment. The presence of *Geobacter* spp. in the bioaugmented sample was predominant which implied the anaerobic degradation of organic matter in the sediment. *Geobacter* spp. are the microorganisms in a variety of subsurface environments in which Fe (III) reduction is important (Sneyenbos *et al*., 2000; Holmes *et al*., 2002; Anderson *et al*., 2003). *Geobacter* species has the ability to couple the oxidation of acetate to the reduction of Fe(III) (Lovley *et al*., 1987; Lovley and Phillips, 1988). The ability to oxidize acetate is important because acetate is the central intermediate in the anaerobic degradation of organic matter in sedimentary environments (Lovley and Chapelle, 1995). Most of the growth of *Geobacter* spp. during bioremediation can be attributed to Fe(III) reduction (Finneran *et al*., 2002).FamilyGeobacteraceae is the largest group of Fe(III)-

reducing bacteria, within the 'Deltaproteobacteria'(Lovley *et al*., 2004). The relative abundance of *Gramella* spp., member of Bacteroidetes, indicated representation of organisms responsible for degradation of high molecular weight organic matter, a predicted preference for degradation of polymeric carbon compounds (Bauer *et al*., 2006). In the control sediment samples <10% relative abundance of actinomycete *Frankia* spp. was seen, and most of the representatives belonged to the uncultured delta Proteobacteria. A representative of *Geobacter* spp. could also be seen, however, the relative abundance in comparison to that of the bio-augmented sample was very less. The indices calculated in VITCOMIC for comparative analysis showed significant differences between both the communities.

Geneious is a flexible software application framework for the organization and analysis of biological data, with a focus on molecular sequences and related data types (Kearse *et al*., 2012). Geneious software has been employed in diversity studies of various habitats (Masters *et al*., 2011; Sere *et al*, 2012). In the present study by employing this software the bioaugmented and control sediment derived clones were mainly grouped within phylum Proteobacteria. This might be due to Proteobacteria comprising the largest and most phenotypically diverse division of prokaryotes (Gupta 2000). However the abundance and the proportions of *Gamma Proteobacteria* varied greatly between the samples which reflect different ecological functions that they could perform in the system.

Sulphur cycling in aquaculture systems is important as un-ionised dissolved hydrogen sulphide (H_2S) is extremely toxic to many aquatic organisms, even at natural levels (Knezovich *et al*., 1996). It is particularly

relevant for sediment-based aquaculture systems as sulphate reduction can account for over 50% of organic matter degradation in marine sediments, leading to the production of considerable quantities of H_2S (Muyzer & Stams, 2008). In shrimp aquaculture, phototrophic purple and green sulphur bacteria that performed anoxygenic photosynthesis at low light intensities under anaerobic conditions were frequently mass cultured and applied to ponds to bioremediate H_2S (Antony & Philip , 2006).In the present study the control and bioaugmented clone libraries contained purple sulfur bacteria of families Chromatiaceae, Ectothiorhodospiraceae and sulphur oxidising family Thiotrichales of gammaproteobacteria cladre, Sulphate reducing Desulfobacteraceae and Syntrophobacteraceae of deltaproteobacteria, class alphaproteobacteria represented by aerobic photo- and chemoheterotrophic purple non-sulfur bacteria Rhodobacteraceae which perform photosynthesis in anaerobic environments and are deeply involved in sulfur and carbon biogeochemical cycling and also Rhodospirillaceae that can grow photoheterotrophically and chemoheterotrophically and the green nonsulfur bacteria phylum Chloroflexi known to comprise numerous chemotrophic bacteria of diverse ecophysiology and phylogeny (Overmann,2008). Redox potential can principally drive shifts in bacterial community composition and predicted functional capacity. The variations in sediment *Eh* will have differentially affected relative distribution and abundance of bacterial community of bioaugmented and control systems .Within redox-stratified sediments bacteria with very different metabolic capacities maybe distributed according to redox potential and the spatial distribution of electron acceptors. Hence the relatively high abundance of phylum Proteobacteria among control sediment clones dictates the presence of

sulphur rich environment. Rhodobacteraceae is an ecologically diverse group involves members that are mainly engaged in sulphur geochemical cycling (Garrity *et al.*, 2005).

A successful bioremediation should involve the combined actions of a diverse consortium of heterotrophic, chemolithotrophic and phototrophic bacteria which could be seen among bioaugmented clones library. These clones exhibited the presence of taxa with heterotrophic metabolism, including aerobic and anaerobic respiration and fermentation; chemolithotrophic; methylotrophic and phototrophic metabolisms, performing both oxygenic and anoxygenic photosynthesis. In contrast control sediment clones exhibited limited functional diversity, chemoorganotrophic (heterotrophic) bacteria being restricted 6% Bacteroidetes. A broad spectrum of heterotrophic bacteria with a good enzymatic capacity and ability to multiply rapidly is essential to maximise rates of carbon mineralization to carbon dioxide for effective bioremediation with minimum sludge accumulation, hence the heterotrophic community in the bioaugmented sediment is presumed to utilise the full spectrum of carbon oxidation pathways including aerobic, anaerobic and fermentative respiration. The phyla Bacteroidetes and Planctomycetes, are classified as taxa with aerobic metabolisms capable of oxidising a range of complex polymeric carbon compounds including sugars, alcohols, organic acids, amino acids and carbohydrates (Schloss *et al* .,2009) the relative abundance of these groups were high among bioaugmented clones underscoring the enhanced capacity for organic matter degradation in this sediment.

Phylum Bacteroidetes represent second most abundant group among bioaugmented sediment clones. The family Saprospiraceae *seems to the* prominent group sharing 17% of the Phylum. Saprospiraceae family isolated from aquatic environments is well known for its ability to hydrolyse and utilise complex carbon sources. The family Saprospiraceae consists of the genera *Aureispira*, *Haliscomenobacter*, *Lewinella*, and *Saprospira* and is represented by five type strains found in various habitats like freshwater lakes (Schauer *et al*., 2005) in hypersaline mats (Ley *et al* ., 2006), marine sponges and algae from the southern coastline of Thailand (Hosoya *et al* ., 2006) from activated sludge (Van Veen *et al*., 1973), and also present in a methanol-fed denitrifying sequencing batch reactor (Ginige *et al*., 2005) and are associated with the predation of algae and other bacteria in their environment. Members of Bacteroidetes are generally associated with the degradation of complex organic materials (Cottrell and Kirchman, 2000 ; Riemann *et al*., 2000). Therefore, in sediments this may be involved in the hydrolysis of polysaccharides and utilize the hydrolysates as energy and carbon sources for growth. Interestingly, this group is unique to bioaugmented sediment clone library in the present study. Flavobacteriales that can utilize high-molecular-weight DOM such as proteins or chitin (Cottrell and Kirchman, 2000) were evenly distributed in the clone library of both systems. Lui *et al*. (2013) reported that Proteobacteria and Bacteroidetes were the dominant groups in the crab pond sediment .Apart from Proteobacteria and Bacteroidetes Fan *et al*. (2017) found Chloroflexi were especially abundant in tilapia pond sediment. Similarly Chloroflexi accounting for 11% and 9% respectively among the control and bioaugmented sediment libraries were observed. Zhang *et al*.

(2016) reported Proteobacteria, Firmicutes, Acidobacteria, Chloroflexi and Bacteroidetes were the bacterial phyla with more than 10% abundance in sediment samples in *Litopenaeus vannamei* culture ponds.

Bioremediation of nitrogenous compounds relies on maximising chemolithotrophic processes that remove potentially toxic compounds (ammonia and nitrite) via nitrification, which is mediated in a step-wise process by ammonia-oxidising bacteria (AOB) and nitrite oxidising bacteria (NOB). The presence of Nitrospinaceae confirms the functional guild of nitrite oxidizing bacteria in bioaugmented sediment unlike that in control sediment. Relatively high percentage of uncultured Nitrospira and Nitrospinaceae is reported to provide high activity in transforming nitrite to nitrate in pearl mussel ponds (Zheng *et al*., 2016)

In general, results of ARDRA followed by phylogenetic analysis and application of VITCOMIC and geneious bioinformatics tools could depict the re-arrangement happened in microbial diversity and taxonomic composition on application of *Bacillus cereus* MCCB 101 for bio-augmentation of shrimp pond sediment.

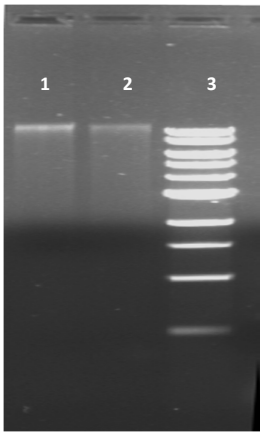


Fig 1: Metagenomic DNA extracted using Power soil DNA isolation Kit

Lane 1: **Bioaugmented sediment**
Lane 2: **Control sediment**
Lane 3: **1500 bp marker**

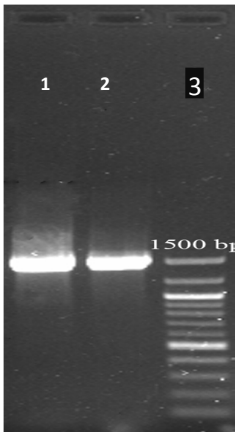


Fig 2: Amplification of 16S rRNA gene by universal primers

Lane 1: **Bioaugmented sediment**
Lane 2: **Control sediment**
Lane 3: **1500 bp marker**

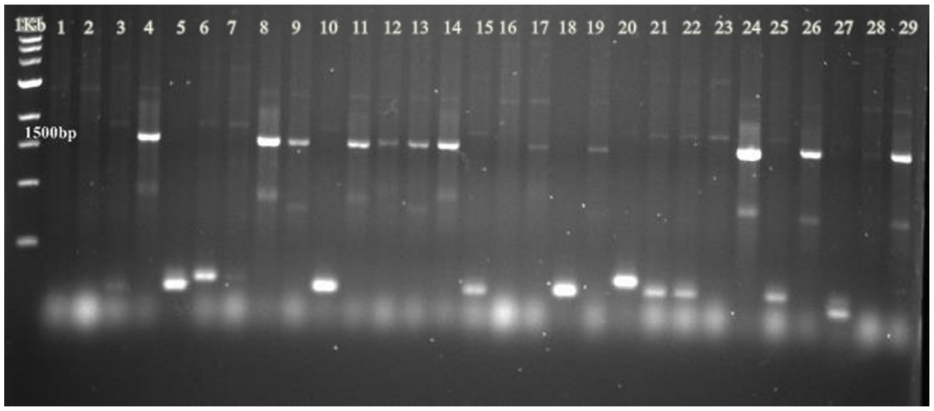


Fig 3: showing colony PCR result of bio-augmented sediment clones

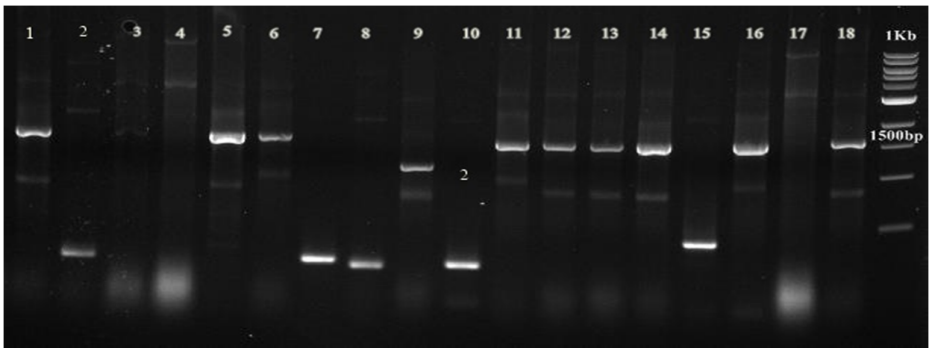


Fig 4: showing colony PCR result of control sediment clones

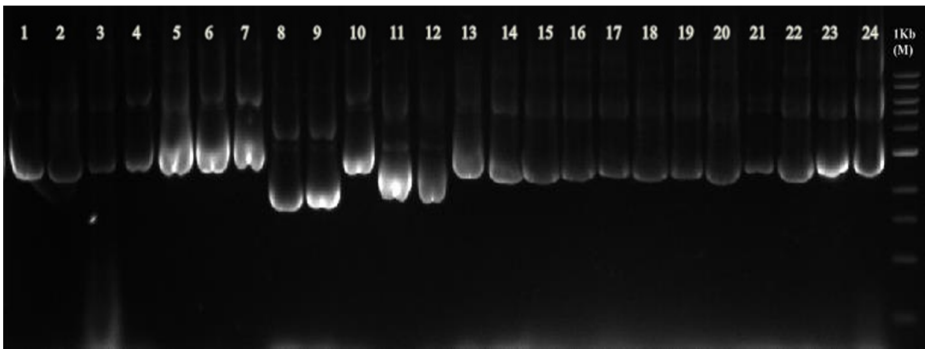


Fig 5: Plasmids extracted from colony PCR positive clones

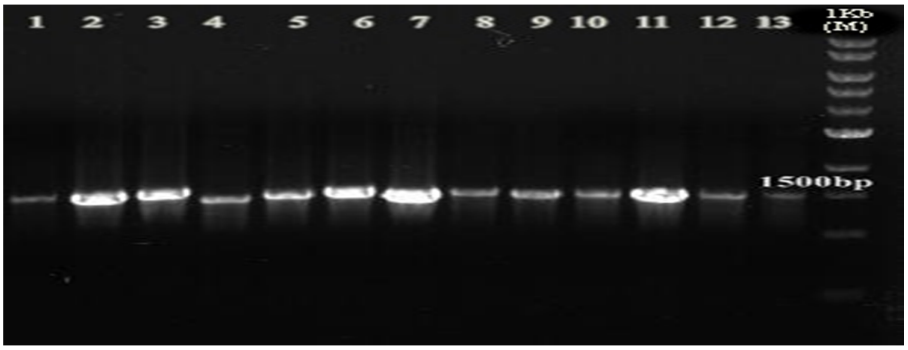


Fig 6: 16SrRNA gene re-amplification from extracted plasmids

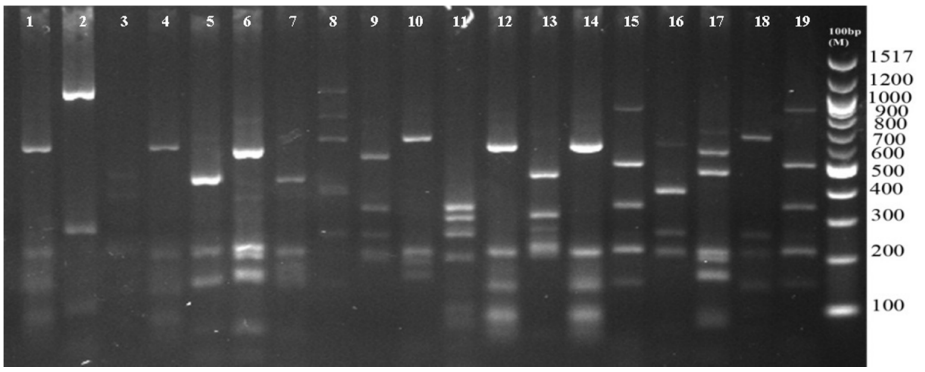


Fig 7: ARDRA patterns of *AluI*
restriction digestion products from bioaugmented sediments

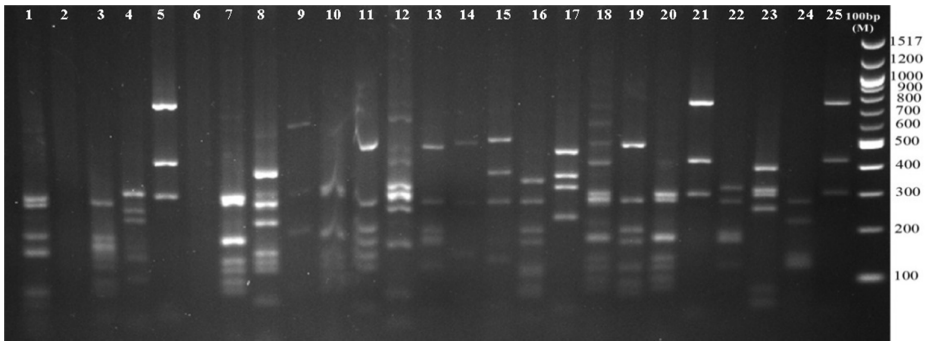


Fig 8 : ARDRA patterns of *HaeIII*
restriction digestion products from bioaugmented sediments

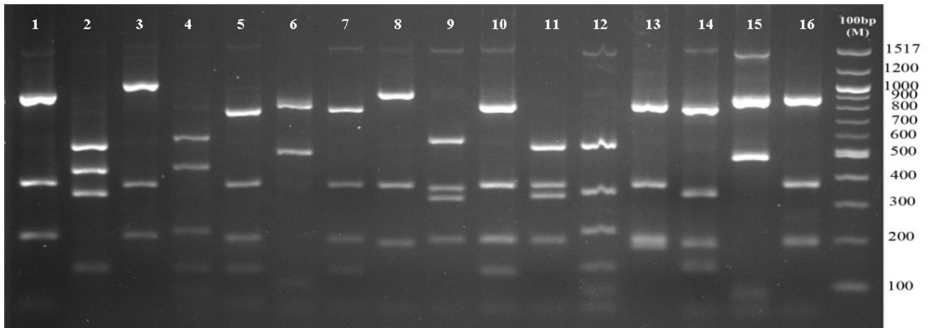


Fig 9: ARDRA patterns of *Taq I*
restriction digestion products from bioaugmented sediments

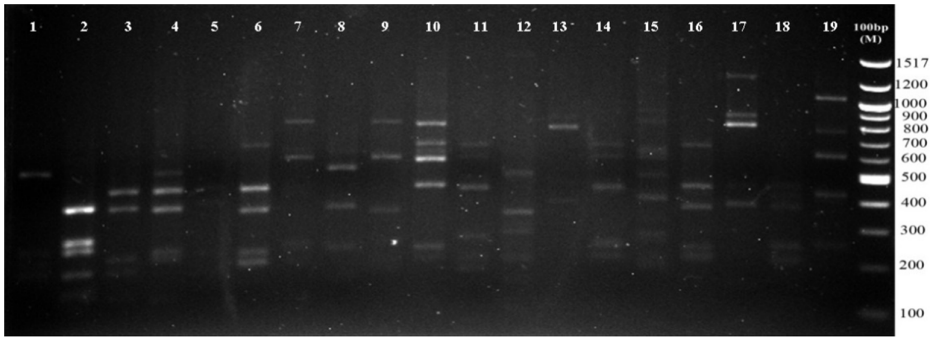


Fig 10: ARDRA patterns of *AluI*
restriction digestion products from control sediments

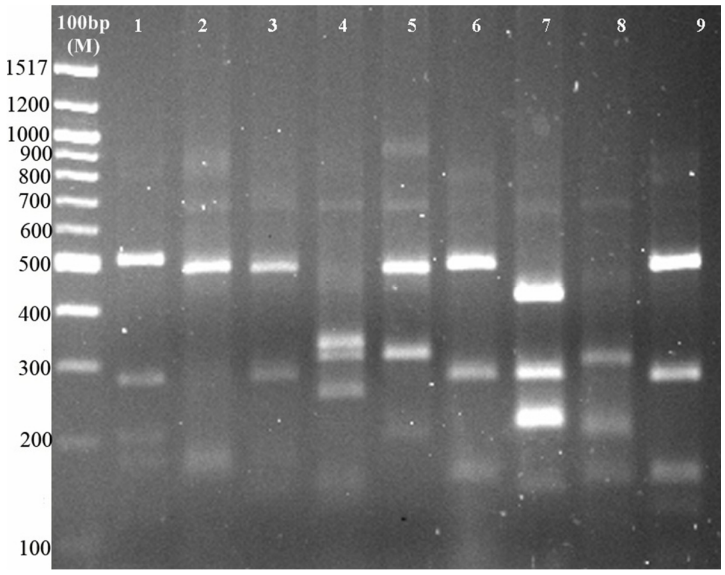


Fig 11: ARDRA patterns of *HaeIII*
restriction digestion products from control sediments

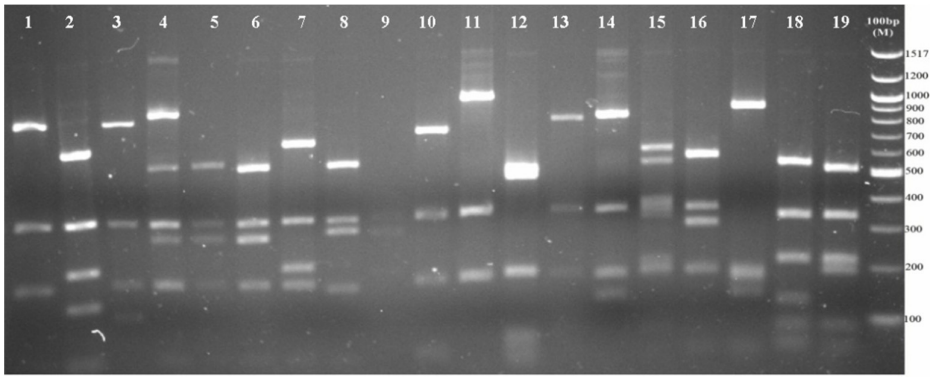


Fig 12: ARDRA patterns of *Taq I*
restriction digestion products from control sediments

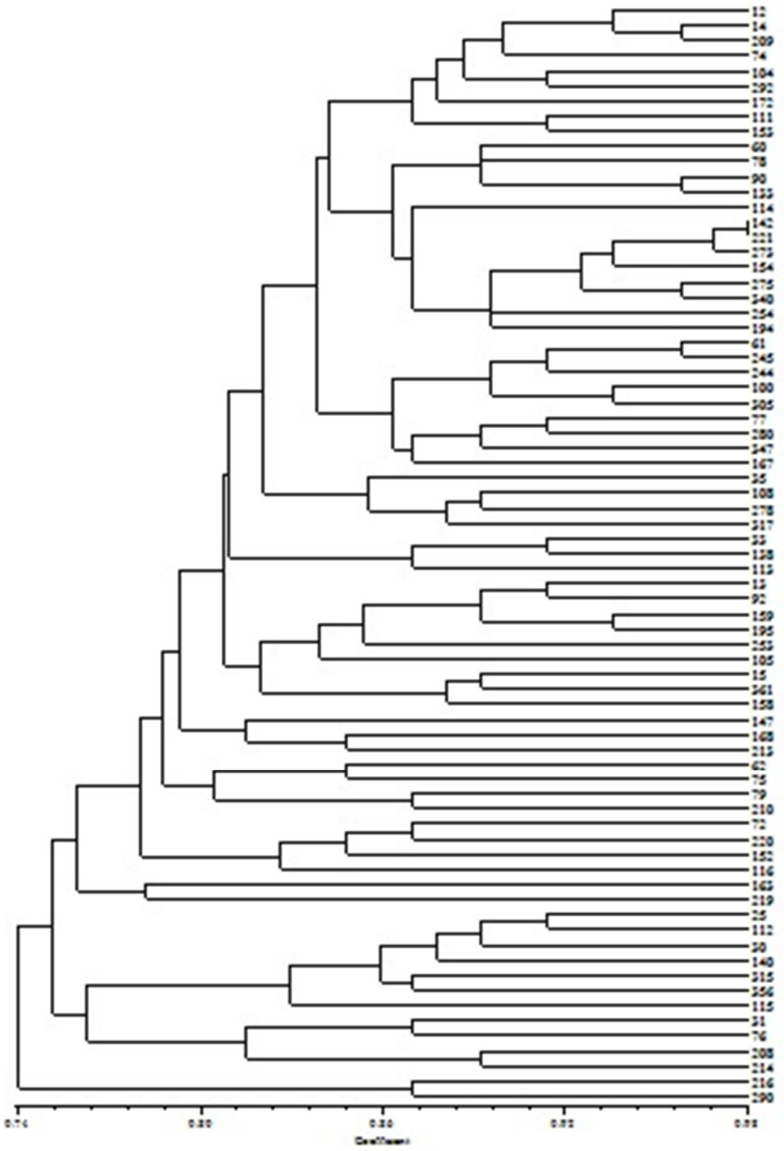


Fig 13 : Dendrogram constructed based on ARDRA pattern of bioaugmented sediment

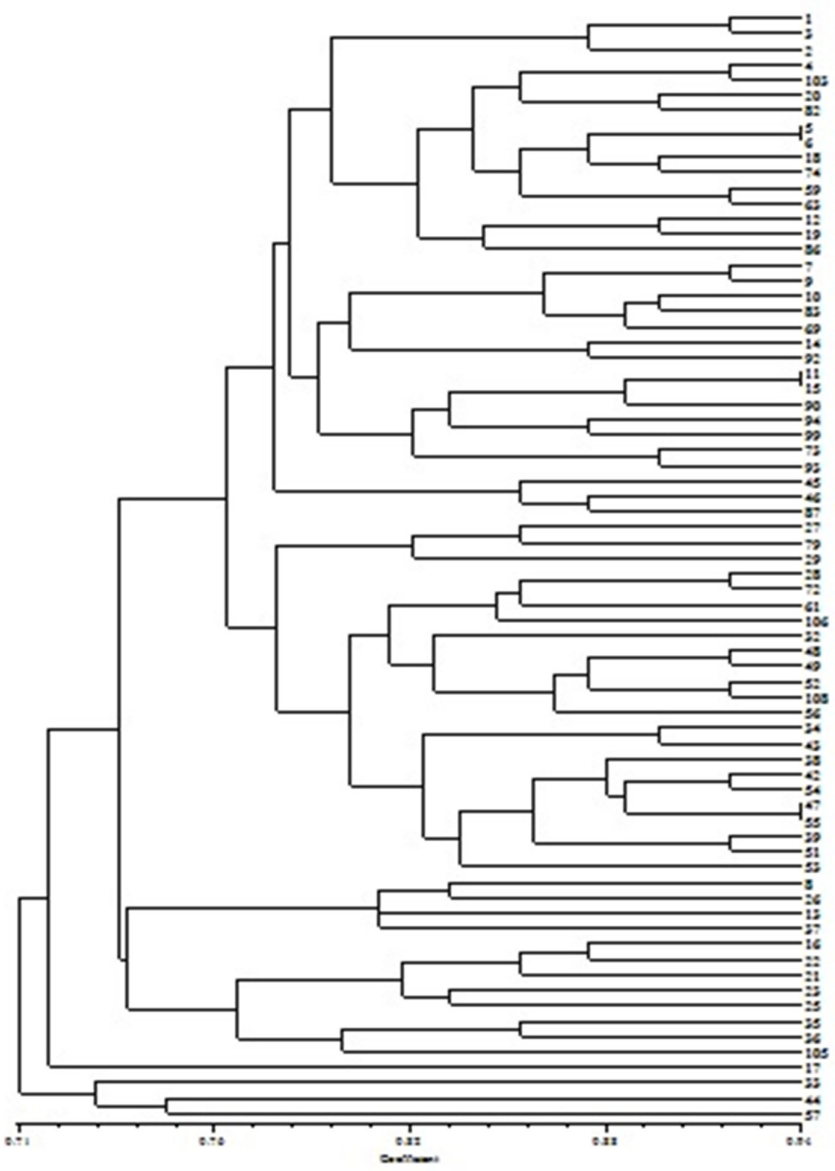


Fig 14: Dendrogram constructed based on ARDRA pattern of control sediment

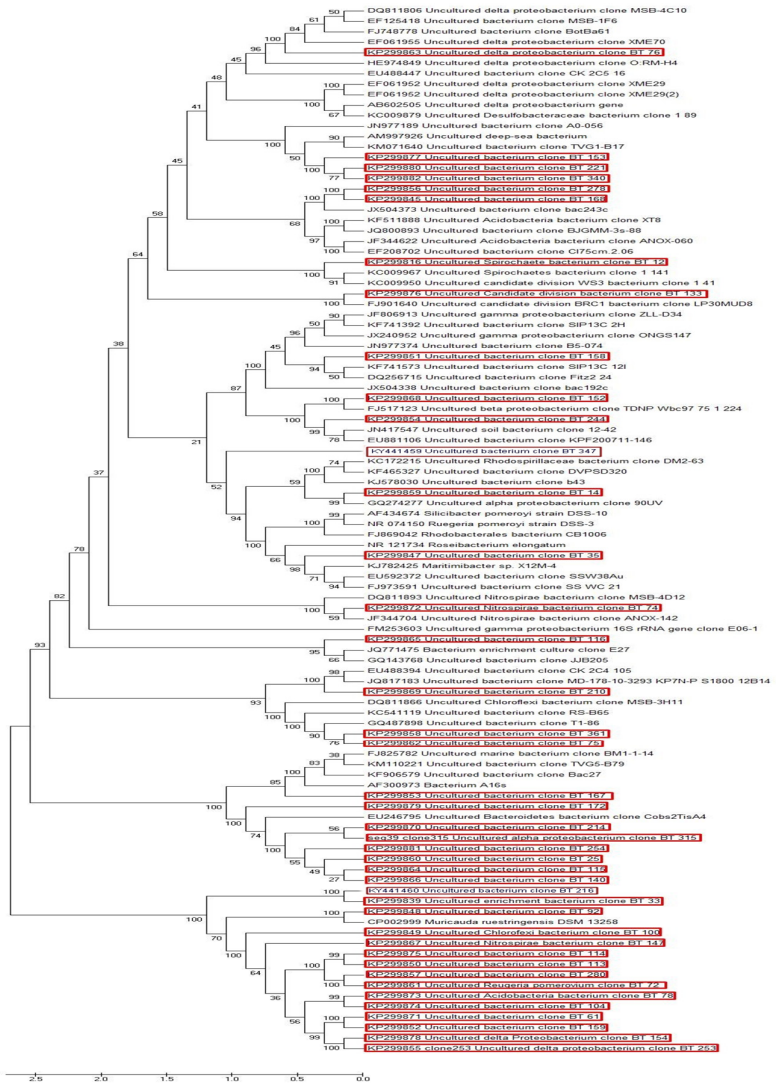


Fig 15

Phylogenetic analysis of representative 16S rRNA gene sequences obtained from bio-augmented sediment samples (indicated using red highlighted box) with 99% similar GenBank sequences. The UPGMA tree constructed were computed using Kimura-2 parameter substitution model with 1000 bootstrap replications. The bar represents 0.5% sequence divergence

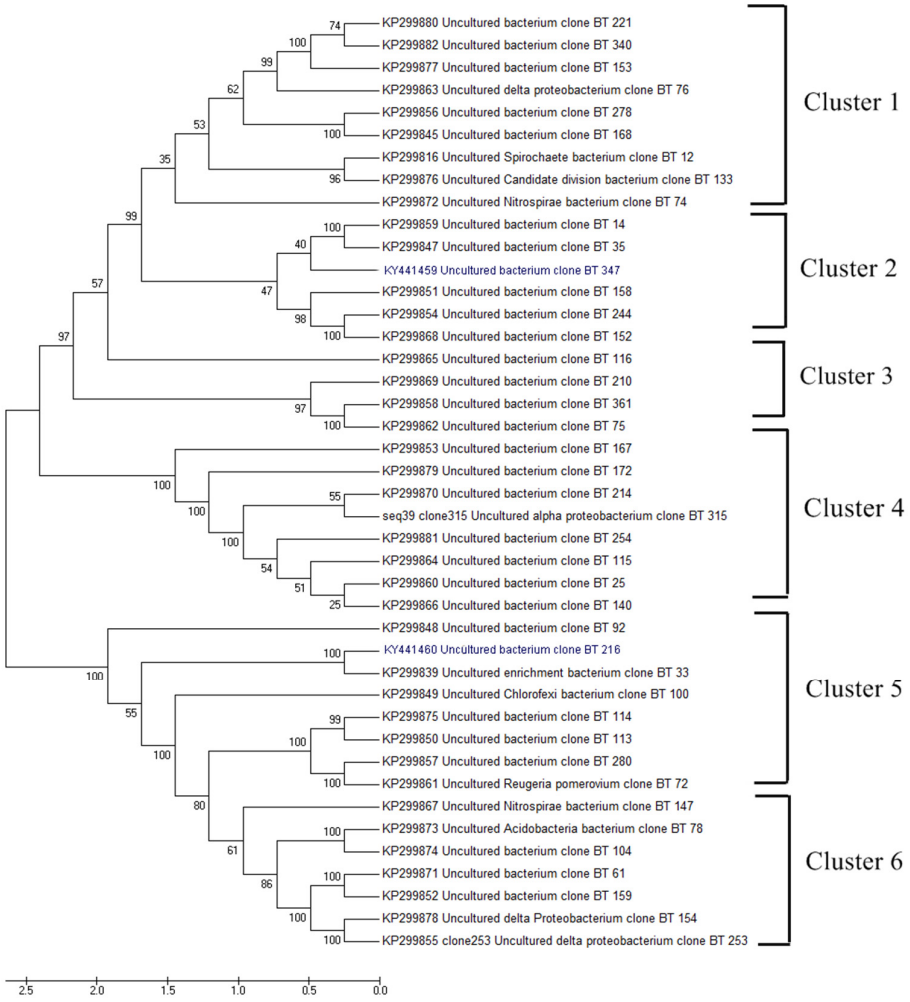


Fig 16

Phylogenetic analysis of representative 16S rRNA gene sequences obtained from bio-augmented sediment samples. The UPGMA tree constructed were computed using Kimura-2 parameter substitution model with 1000 bootstrap replications. The bar represents 0.5% sequence divergence.

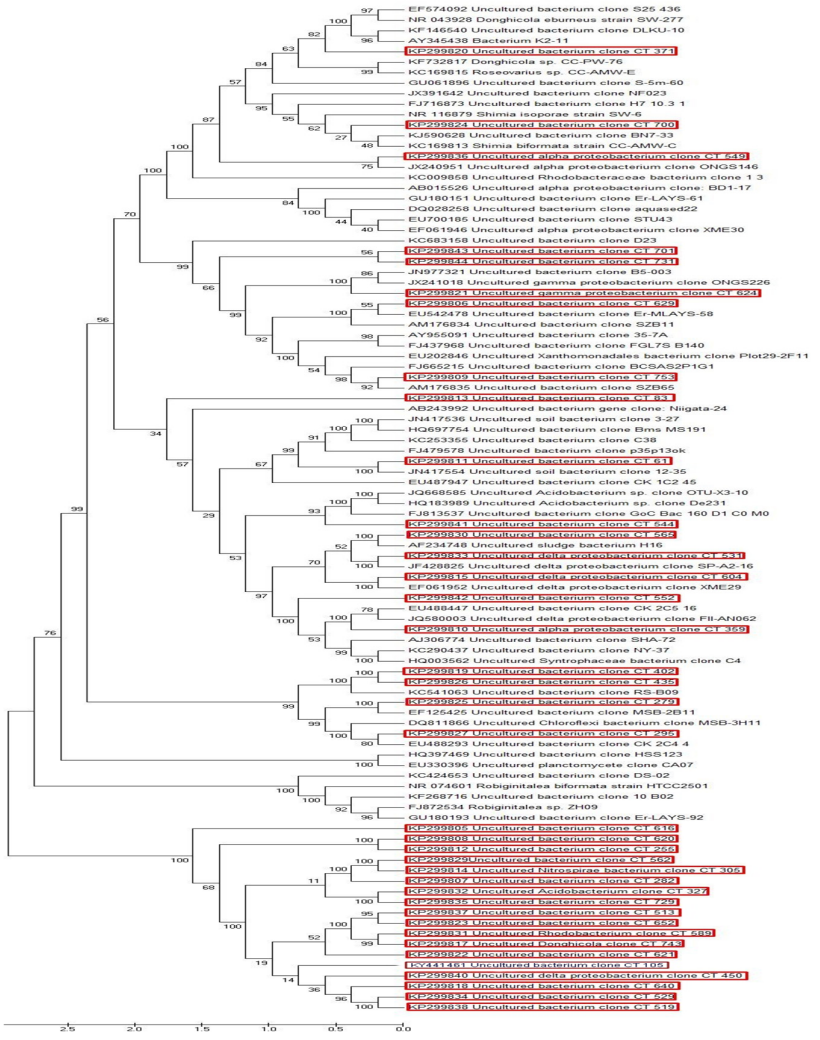


Fig 17

Phylogenetic analysis of representative 16S rRNA gene sequences obtained from control sediment samples (indicated using red highlighted box) with 99% similar GenBank sequences. The UPGMA tree constructed were computed using Kimura-2 parameter substitution model with 1000 bootstrap replications. The bar represents 0.5% sequence divergence.

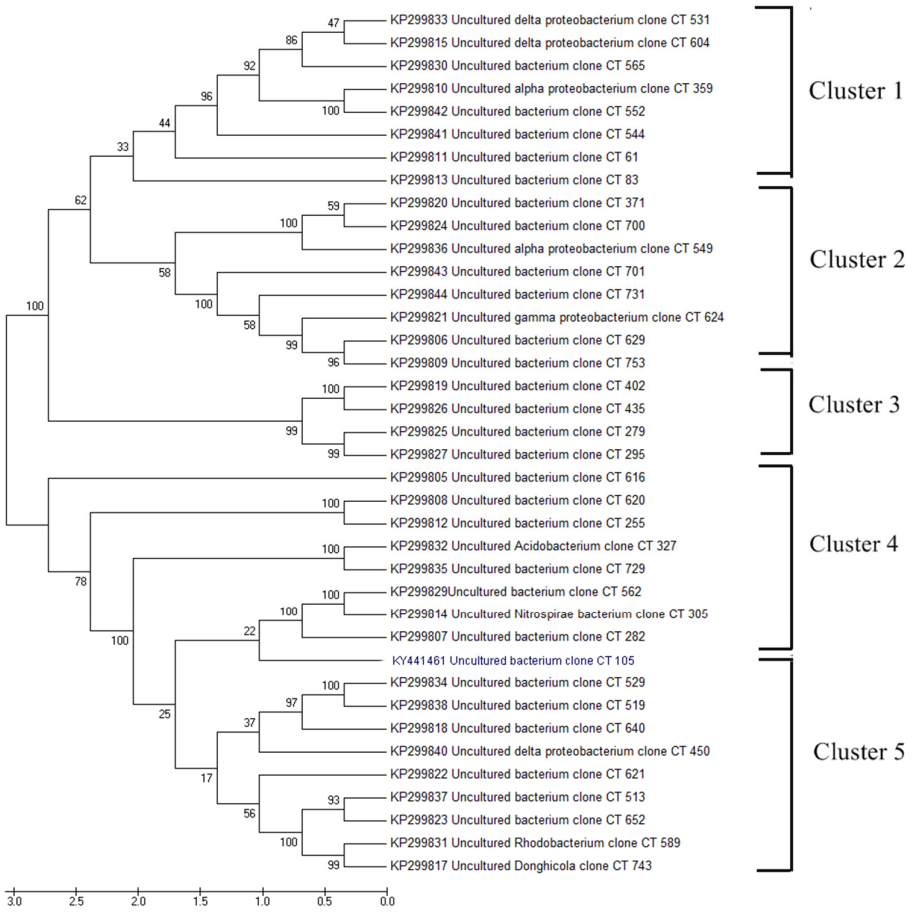


Fig 18

Phylogenetic analysis of representative 16S rRNA gene sequences obtained from control sediment samples. The UPGMA tree constructed were computed using Kimura-2 parameter substitution model with 1000 bootstrap replications. The bar represents 0.5% sequence divergence.

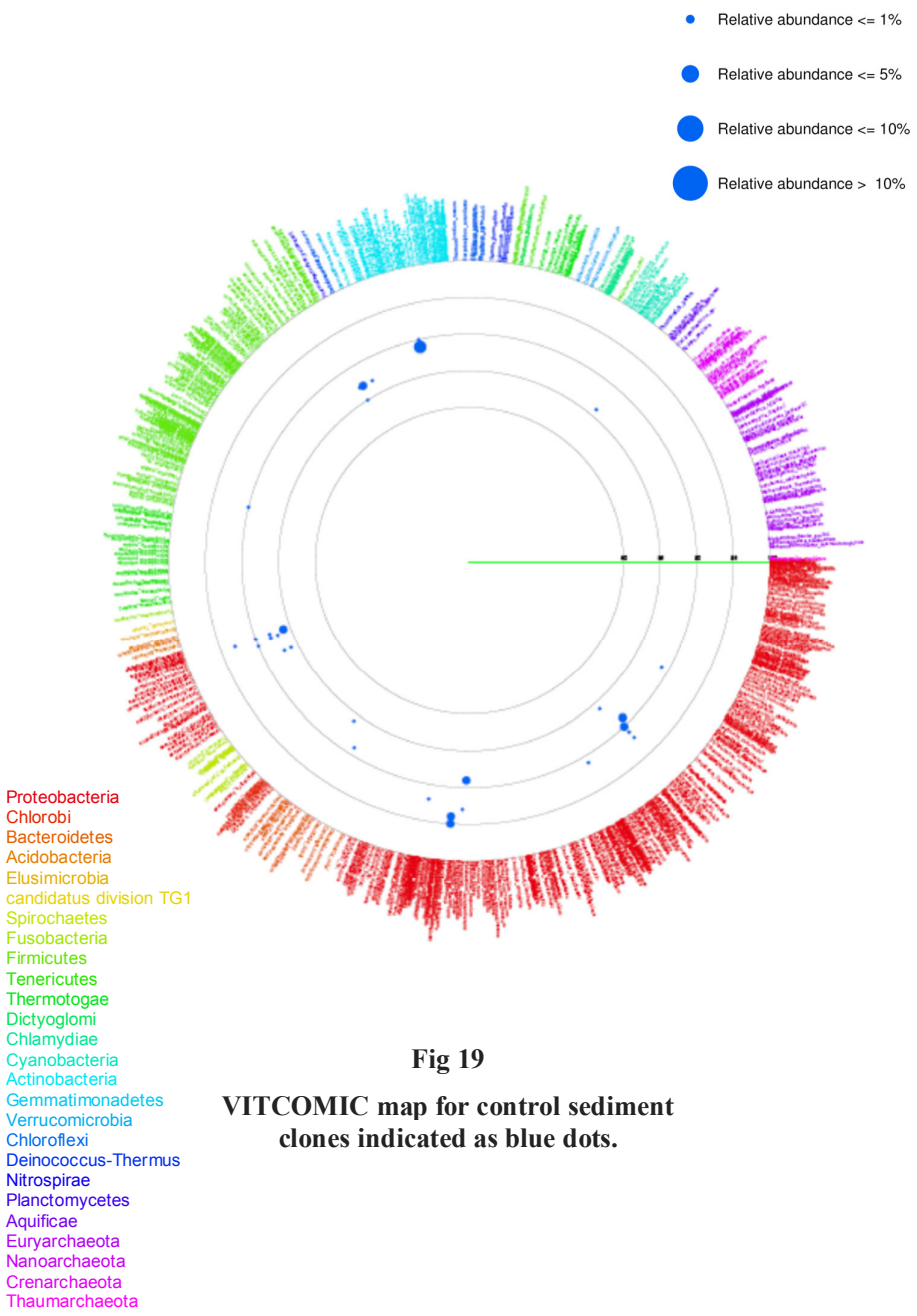


Fig 19

VITCOMIC map for control sediment clones indicated as blue dots.

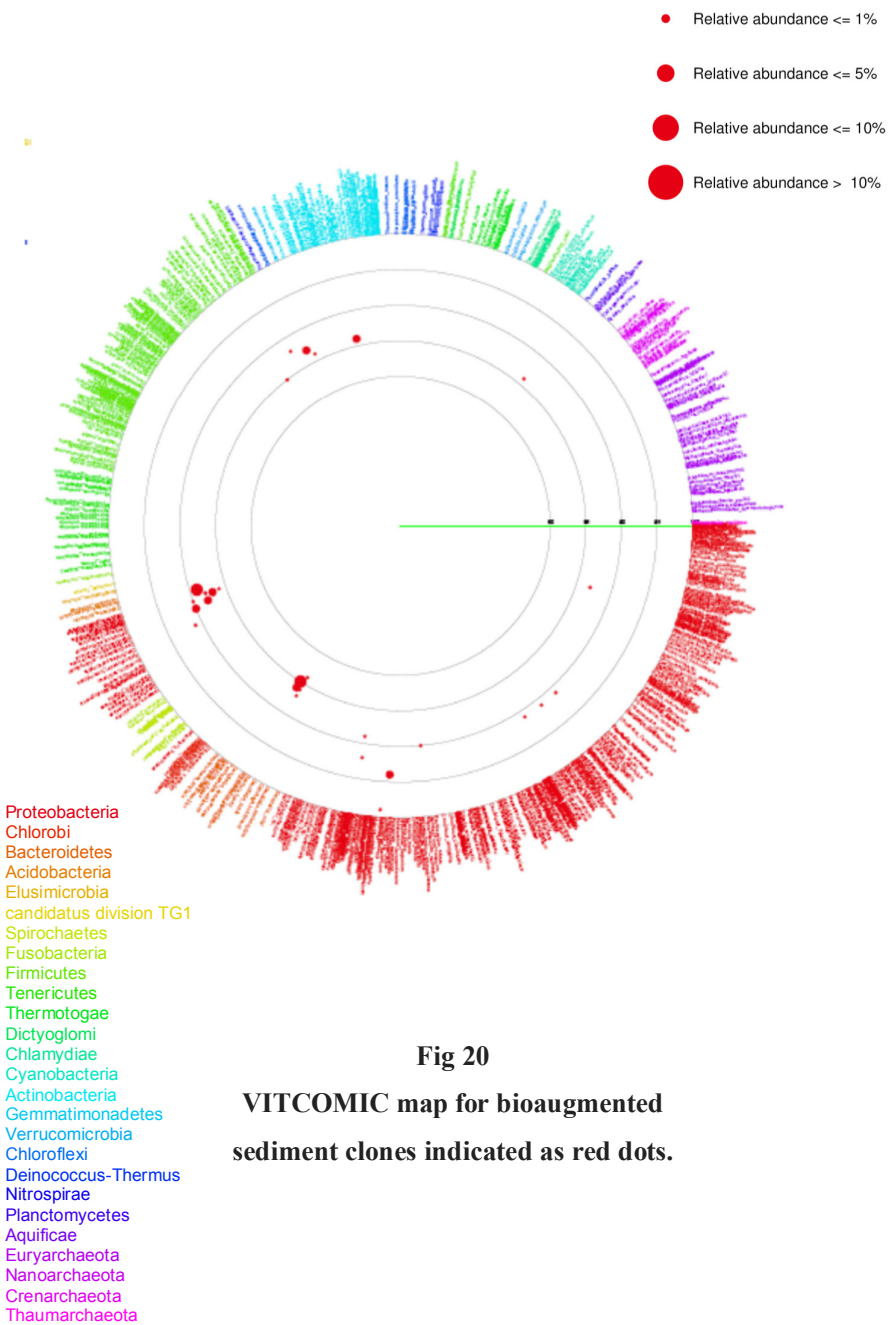


Fig 20

VITCOMIC map for bioaugmented sediment clones indicated as red dots.

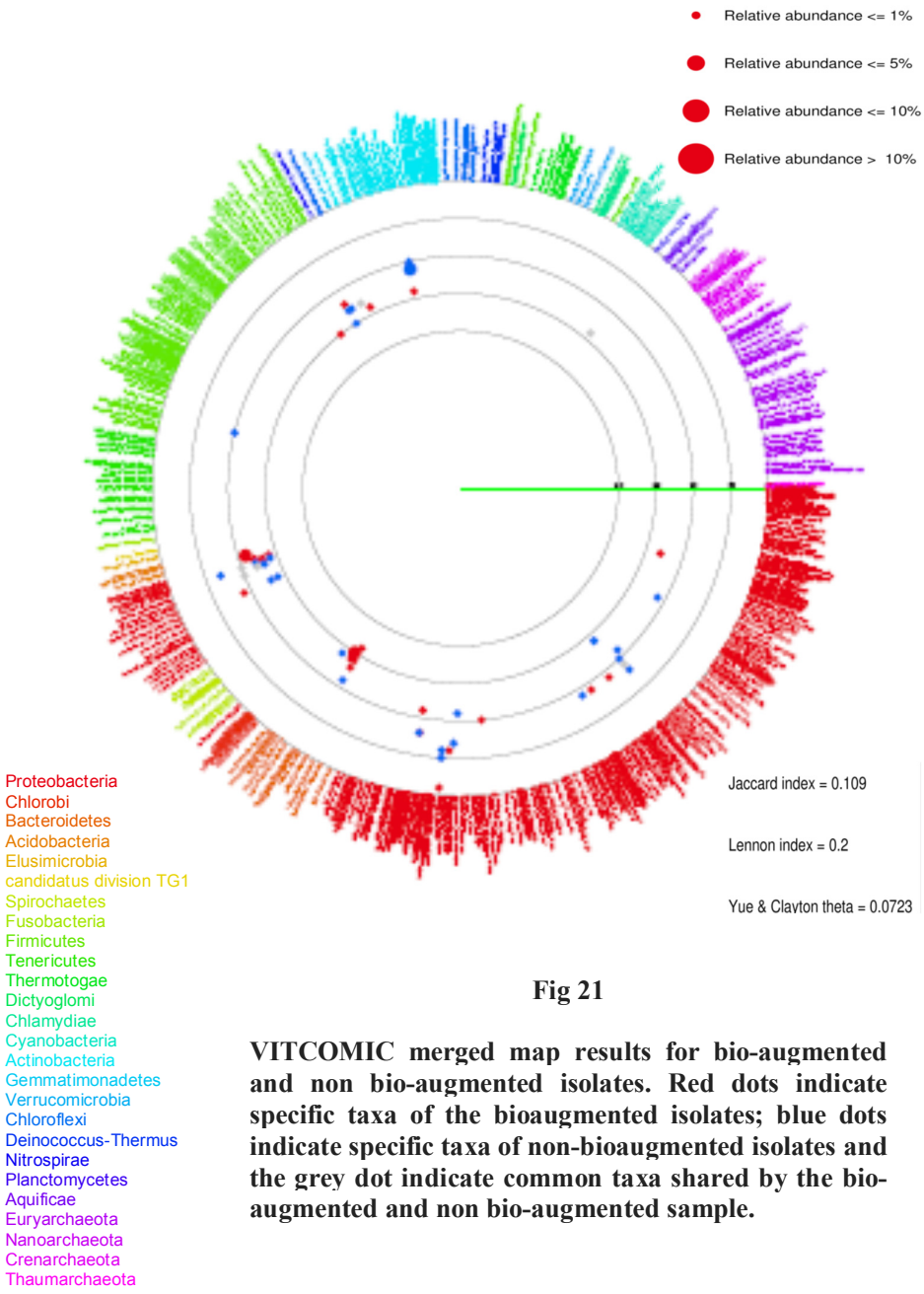


Fig 21

VITCOMIC merged map results for bio-augmented and non bio-augmented isolates. Red dots indicate specific taxa of the bioaugmented isolates; blue dots indicate specific taxa of non-bioaugmented isolates and the grey dot indicate common taxa shared by the bio-augmented and non bio-augmented sample.

CT identity	Nearest Bacterial taxonomic Group identified	Percentage of similarity (%)	CT identity	Nearest Bacterial taxonomic Group identified	Percentage of similarity (%)
BT 12	*Geobacter sp.*	88	BT244	*Aromatoleum aromaticum*	91
BT 14	*Mesorhizobium loti*	89	BT253	*Desulfococcus oleovorans*	90
BT 61	*Syntrophus aciditrophicus*	88	BT278	*Geobacter bemidjiensis*	87
BT 74	*Thermodesulfovibrio yellowstonii*	87	BT280	*Dinoroseobacter shibae*	94
BT 78	*Geobacter uraniireducens*	88	BT361	*Acidothermus cellulolyticus*	86
BT 104	*Pelobacter carbinolicus*	87	BT216	*Natranaerobius thermophilus*	85
BT 114	*Magnetospirillum magneticum*	90	BT25	*Flavobacterium psychrophilum*	85
BT 133	*Shewanella loihica*	87	BT72	*Ruegeria pomeroyi*	99
BT 153	*Geobacter sulfurreducens*	89	BT75	*Acidothermus cellulolyticus*	86
BT 154	*Desulfococcus oleovorans*	90	BT76	*Syntrophobacter fumaroxidans*	91
BT 172	*Gramella forsetii*	84	BT115	*Flavobacterium psychrophilum*	85
BT 221	*Geobacter sulfurreducens*	89	BT116	*Rhodopirellula baltica*	86
BT 254	*Flavobacterium psychrophilum*	85	BT140	*Flavobacterium psychrophilum*	85
BT 340	*Geobacter sulfurreducens*	89	BT147	*Thermodesulfovibrio yellowstonii*	87
BT 35	*Dinoroseobacter shibae*	94	BT152	*Methylibium petroleiphilum*	91
BT 92	*Gramella forsetii*	86	BT210	*Dehalococcoides sp.*	86
BT 100	*Pelotomaculum thermopropionicum*	88	BT214	*Flavobacterium psychrophilum*	86
BT 113	*Mesorhizobium sp.*	92	BT315	*Flavobacterium psychrophilum*	86
BT 158	*Thioalkalivibrio sp.*	91	BT33	*Geobacter sp.*	86
BT 159	*Syntrophus aciditrophicus*	88	BT68	*Geobacter bemidjiensis*	87
BT 167	*Gramella forsetii*	87	BT 347	*Pelobacter carbinolicus*	90

Table 1: Nearest phylogenetic groups derived for clones of bioaugmented sediments from VITCOMIC map.

Clone Identity	Nearest Bacterial taxonomic Group identified	Percentage of similarity (%)	Clone identity	Nearest Bacterial taxonomic Group identified	Percentage of similarity (%)
CT 1	*Parvibaculum lavamentivorans*	89	**CT 44**	*Rhodopirellula baltica*	86
CT 2	*Frankia sp.*	90	**CT 45**	*Thioalkalivibrio sp.*	90
CT 9	*Pelobacter.carbinolicus*	90	**CT 46**	*Frankia alni*	89
CT 12	*Paracoccus denitrificans*	93	**CT 52**	*Gramella forsetii*	89
CT 13	*Syntrophobacter fumaroxidans*	86	**CT 53**	*Nitrosococcus oceani*	89
CT 14	*Pelobacter carbinolicus*	93	**CT 56**	*Syntrophus aciditrophicus*	87
CT 16	*Nitrosococcus oceani*	89	**CT 61**	*Frankia alni*	89
CT 17	*Syntrophobacter fumaroxidans*	87	**CT 63**	*Parabacteroides distasonis*	86
CT 18	*Ruegeria pomeroyi*	94	**CT 83**	*Thermodesulfovibrio yellowstonii*	87
CT23	*Burkholderia xenovorans*	91	**CT 86**	*Frankia alni*	89
CT27	*Candidatus desulforudis*	87	**CT 93**	*Desulfococcus oleovorans*	90
CT28	*Ruegeria pomeroyi*	94	**CT 105**	*Pelobacter carbinolicus*	86
CT29	*Thioalkalivibrio sp.*	91	**CT 106**	*Ruegeria pomeroyi*	95
CT32	*Alkaliphilus oremlandii*	90	**CT1**	*Parvibaculum lavamentivorans*	89
CT34	*Mesorhizobium loti*	92	**CT5**	*Thioalkalivibrio sp.*	90
CT35	*Ruegeria pomeroyi*	95	**CT48**	*Syntrophus aciditrophicus*	88
CT38	*Pelotomaculum thermopropionicum*	85	**CT51**	*Pelobacter carbinolicus*	86
CT39	*Thioalkalivibrio sp.*	92	**CT90**	*Pelobacter carbinolicus*	88
CT42	*Candidatus desulforudis*	87	**CT99**	*Coxiella burnetii*	86
CT82	*Pelotomaculum thermopropionicum*	87	**CT103**	*Saccharophagus degradans*	89

Table 2: Nearest phylogenetic groups derived for clones of control sediments from VITCOMIC map.

Phylogenetic groups unique to Bioaugmented sediments	Phylogenetic groups unique to Control sediments	Phylogenetic groups common in Bioaugmented and control sediments
Acidothermus cellulolyticus	***Alkaliphilus oremlandii***	*Desulfococcus oleovorans*
Aromatoleum aromaticum	***Burkholderia xenovorans***	*Gramella forsetii*
Dehalococcoides sp.	***Candidatus desulforudis***	*Mesorhizobium sp.*
Dinoroseobacter shibae	***Coxiella burnetii***	*Pelobacter carbinolicus*
Flavobacterium psychrophilum	***Frankia sp.***	*Pelotomaculum thermopropionicum*
Geobacter spp	***Nitrosococcus oceani***	*Ruegeria pomeroyi*
Magnetospirillum magneticum	***Parabacteroides distasonis***	*Syntrophobacter fumaroxidans*
Methylibium petroleiphilum	***Paracoccus denitrificans***	*Syntrophus aciditrophicus*
Natranaerobius thermophilus	***Parvibaculum lavamentivorans***	*Thermodesulfovibrio yellowstonii*
Rhodopirellula baltica	***Saccharophagus degradans***	*Thioalkalivibrio sp.*
Shewanella loihica		

Table 3: Phylogenetic groups unique and common to bioaugmented and control sediments .

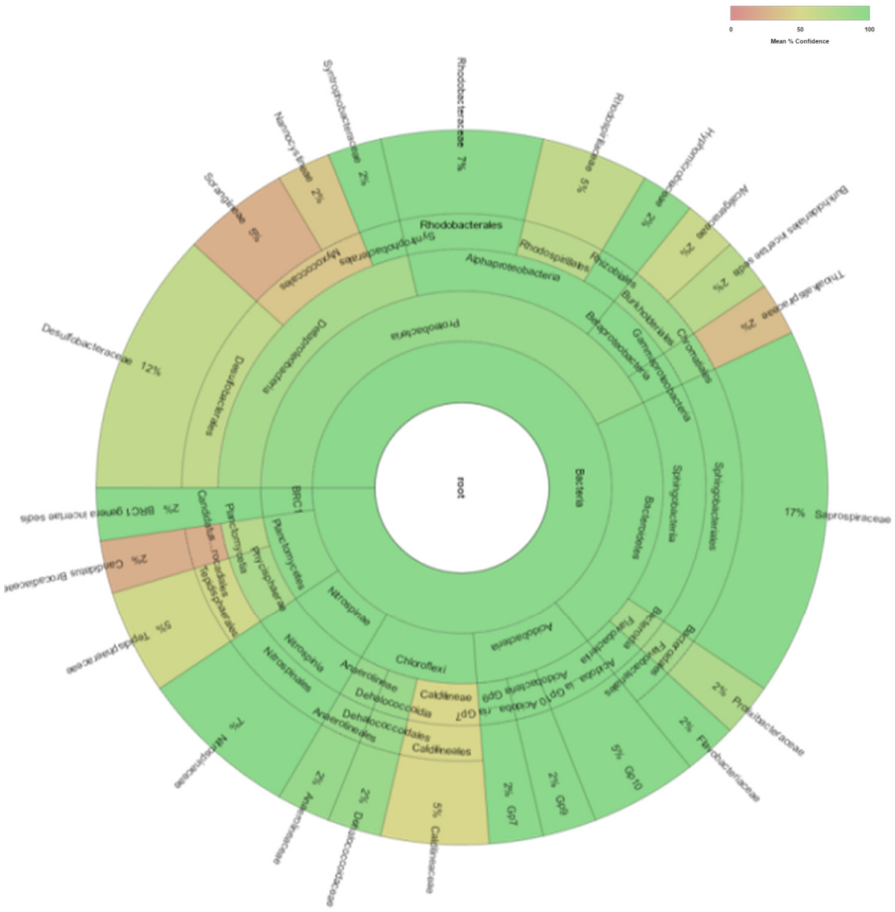


Fig 22: Geneious map on the distribution pattern of phylogenetic groups within clones of bioaugmented sediments

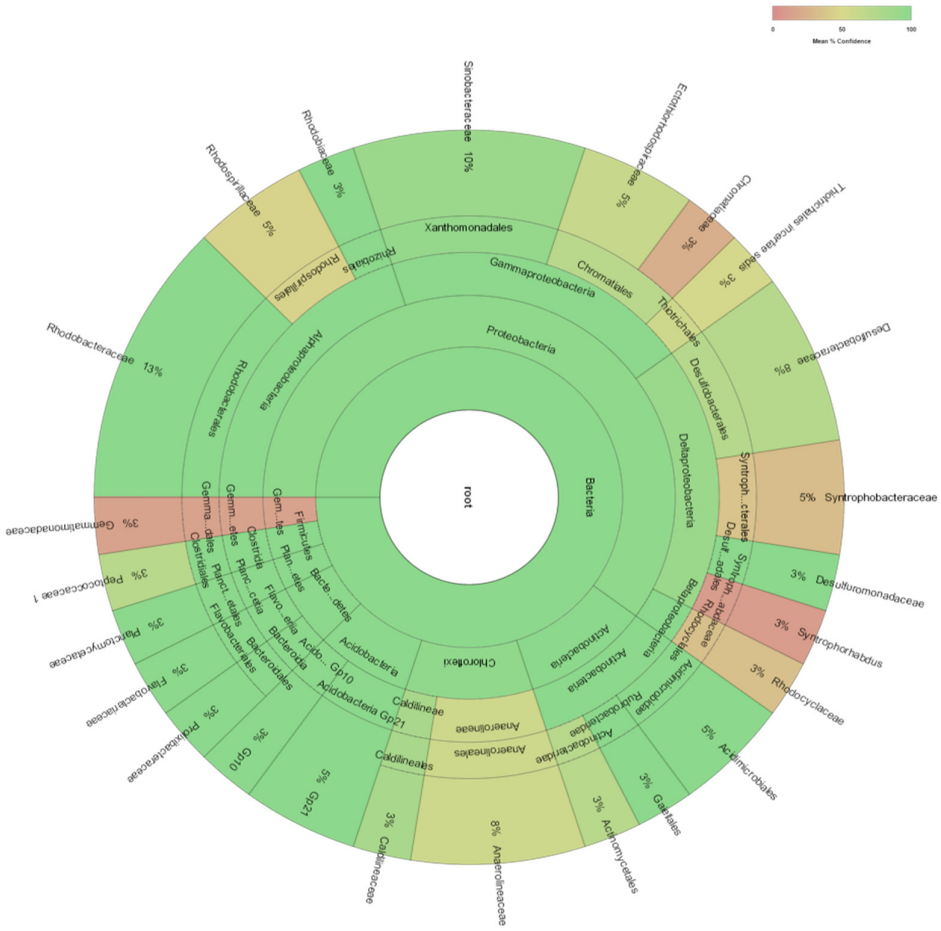


Fig 23: Geneious map on the distribution pattern of phylogenetic groups within clones of control sediments

Chapter 5

SUMMARY AND CONCLUSIONS

Aquaculture is one of the fastest growing food sectors in the world. In aquaculture, shrimp culture has experienced rapid expansion globally because of its faster growth rate, short culture period, high export value and demand in the International market. The culture potential of black tiger shrimp *P. monodon* is well known due to its production performance and profitability. The innovative technologies developed in the industry to improve the production and to maximize the returns per unit area has been accompanied by serious environmental impacts. Eutrophication of nearby water bodies which receive substantial amount of effluent, discharged from farms result in environmental impairment and subsequent disease outbreak.

Extensive, modified extensive and semi intensive cultures have been differentiated based on the management strategies adopted in terms of pond size, stocking density, feeding rate and the water exchange. Under open culture, the systems are prone to epizootics due to pathogen introduction through the incoming water, brought by vectors, reservoir hosts, infected tissue debris and free pathogens themselves. In this scenario, to prevent entry of pathogen and to reduce the consequences of effluent discharge in to the surrounding environment an alternative strategy has become a necessity.

Zero water exchange shrimp culture system is one of the alternatives to address the problem. But, these systems are also characterized by buildup of nutrients and unfavaourable environmental conditions in the absence of effective management measures such as bioremediation. Alternations in water NH_4^+-N, NO_2^--N, H_2S toxicity, in soil redox potential less than -150 mV, Vibriosis and subsequent mortality are commonly met with zero water exchange culture systems as crop progresses. Hence, bioremediation and its related technologies are envisaged to maintain the overall environmental quality and beneficial pond ecology to attain sustainability and economic viability.

The present work was designed to gain scientific background on the role of *Bacillus cereus* MCCB101 in degrading organic matter in shrimp grow out systems. This could be addressed through bioremediation in shrimp pond sediment under simulated high stocking density zero water exchange grow out system of *P.monodon*. Further, possible shifts in bacterial community with respect to relative species abundance of ecological significance in bio-augmented and control sediment could be assessed by ARDRA and VITCOMIC.

Salient findings of this study are as follows:

- Microbial extra cellular hydrolytic enzymes are the major biological molecules for the mineralization of sedimentary particulate organic carbon and nitrogen. The hydrolytic enzyme profile of *Bacillus cereus* MCCB101 included amylases, proteases and xylanases.

- Among the proteases, gelatinase production was higher than caseinase. Shrimps being immensely fed with protein containing feed, the ponds are rich in proteinaceous wastes.

- *Bacillus cereus* MCCB101 being a benthic isolate obtained from shrimp pond sediment, its familiarity with proteinaceous substrate, accounts for the higher gelatinase activity.

- On bio-augmentation with *Bacillus cereus* MCCB101 the redox status of treated sediment could be improved by 45% where as in the control sets the sediment exhibited highly reduced state of -291mV.

- A single dosage of *Bacillus* could bring down the percentage of TOC in the sediments to 2.5% on day 7 from the initial value 5.19%, which was found to go up subsequently. Hence, it is concluded that weekly application of bio-augmenter can sustain the level of TOC in shrimp pond sediment with in desirable limits.

- The enzymatic activities quantified using fluorogenic substrates revealed high xylanase, amylase, chitinase, cellulose, protease and lipase in bio-augmented sediment.

- Hydrolytic rates of protease, trypsin and chymotrypsin recorded the highest value among the quantified enzymes.

- The total lipid, CHO and protein concentrations in bioaugmented sediment underwent a net depletion of 25%, 53% and 45% respectively.

- The total heterotrophic bacterial plate counts (THB), direct bacterial counts, fungal plate count and actinomycetes count were found significantly increased in bio-augmented sediment.

- While bio-augmentation, reshuffling of microbial community was found to have taken place mediated by *Bacillus cereus* MCCB 101, resulting in a cascade of events upgrading the quality of sediment.

- Hence, implementation of bio-augmentation technology to precondition pond sediments using *Bacillus cereus* MCCB 101 prior to starting a culture cycle provide a cleaner pond bottom with less accumulated organic matter and fine quality sediment habitat for shrimps.

- Zero water exchange culture systems ensure bio-secured, closed shrimp production system to prevent viral entry into the culture system avoiding subsequent disease outbreaks.

- Farming at high density aims economic profitability with limited space in short span.

- Rapid eutrophication is a major problem associated with zero water exchange shrimp culture system due to the accumulation of uneaten feed and metabolic wastes in relation to higher biomass.

- Bio-augmenting *P.monodon* simulated grow out system having high stocking density using *Bacillus cereus* MCCB 101 provided promising results for translating in to field conditions.

- Total amonia nitrogen (TAN) concentration in the water column of control systems peaked to 20.85 ± 0.55 mg/L.

- However, TAN in the bio – augmented system was within acceptable limits during the initial 21 days and was significantly lower than the control systems.

- Apart from nitrification, NH_3 – N was found to have removed from bio-augmented systems via nitrogen assimilation into bacterial biomass as well, with less accumulation of nitrite in the water column.

- Comparative bacterial density assessed during 21 days of the experiment significantly differed between bio-augmented and control systems.

- The nitrate formation in sediment and water column of bio-augmented systems indicated enhancement of nitrification by chemoautotrophic bacteria.

- The study substantiates the necessity of increasing the frequency of bio-augmentation, progressive to crop age to enhance nitrogen cycling in closed systems assessed from the increased nitrification rates in accordance with increase in the frequency of bio-augmenter application.

- Alkalinity was significantly low in bio-augmented systems.

- Accumulated organic matter created anoxic conditions in the control systems with E*h* of -248mV.

- In spite of high stocking density and feeding rates, the higher levels of dissolved oxygen improved E*h* in the bio-augmented systems throughout the experimental period as the positive impact of application of bio-augmenter.

- Dissolved oxygen in bio-augmented systems were well above the minimum requirement of 3.5 ppm for shrimp farming whereas in the control systems it got reduced < 3 mg/L.

- No significant variation in salinity was observed between the bio-augmented and control systems and it ranged between 15 – 22ppt.

- The pH recorded in treated tanks was within the optimum range recommended for sediment (7.5 –8.0) and water (7.5-8.5), however, was found to cross the desirable limits in control systems.

- Low levels of inorganic phosphorus was seen in bio-augmented systems indicative of enhanced heterotrophic phosphate uptake by bacterial community.

- Bio-augmentation leads to enhancement of microbial population in sediment water systems compared to control systems.

- Increased rate of enzyme activity in bio-augmented systems is the overall contribution of microbial community stimulated by *Bacillus cereus* MCCB 101.

- Direct bacterial counts and heterotrophic plate counts were positively correlated with protease, amylase, chitinase and cellulase activities of sediment and water samples.

- Fungal community was positively correlated with protease, cellulase, chitinase and xylanase activities.

- Actinomycete population showed significant positive correlation with protease, cellulase, amylase and lipase activity in sediment samples.

- As an overall impact the resultant organic matter depletion in 21 days was significantly higher in bio-augmented systems in terms of total protein, CHO and lipids, and TOC and E*h* that could be maintained in desirable limits.

- Mineralization was enhanced by daily application of the bio-augmenter from 60^{th} day. Low percentage of TOC, regardless of day of culture indicated that the organic matter accumulation could be regulated with increased rate of enzyme activity and improvised redox status in bio-augmented systems.

- High stocking density and inadequate treatment in control systems resulted in accumulation of wastes at exceedingly high rates that natural bacterial community could degrade.

- Overall enzymatic profile was less influenced by the natural degradative processes in control systems, resulting in nutrient accumulation.

- The shrimps in control systems met with 100% mortality after 21 days of stocking influenced by multiple factors involved in deterioration of sediment and water quality.

- Prevalence of anoxic conditions as observed with low redox status, low dissolved oxygen concentration, toxicity imparted by high levels of ammonia and nitrite might have lead to the mortality.

- Average survival of shrimp was 80% in bio-augmented tanks after 21 days of stocking related to better environmental conditions prevalent in the bio-augmented systems.

- On completion of the study (90 days) shrimps of average size, 18.12 ± 0.5g with 55.55 % survival could be resulted from the bio-augmented systems.

- Metagenomic DNA of good quality could be extracted from bio-augmented and control sediment using Power Soil DNA isolation kit.

- From each set of sediment, 73 clones positive for 1500bp 16S rRNA gene inserts were proceeded for ARDRA using tetrameric restriction enzymes *Alu 1, Hae III* and *Taq* I.

- The dendrogram constructed based on ARDRA of bio-augmented sediment clones showed a distance coefficient coverage of 0.74 to 0 .98 while in control sediment it was 0 .71 to 0 .94

- Of the 73 clones subjected for ARDRA in control sediment, 34 clusters could be identified, and of the clones from bio-augmented sediment, 42 clusters could be resulted. Representative of each cluster was sequenced.

- On comparing the 16S rRNA gene sequence from bio-augmented and control sediment with GenBank database using BLAST algorithm, major hits were with uncultured proteobacteria.

- The phylogenetic tree constructed with the aligned sequences of the bio-augmented sample and 99% similar sequences from GenBank database showed a major clustering of sequences within the taxonomic composition of the bio-augmented sample.

- A few of the clones formed cluster with uncultured bacteria isolated from various marine, river and polluted sediments.

- Few of the clones were related and branched off from the uncultured delta proteobacteria, uncultured spirochaetes, uncultured

acidobacteria, *Maritimibacter*, *Rugeria pomeroyi*, uncultured alpha proteobacteria, uncultured beta proteobacteria, uncultured nitrospirae, uncultured chloroflexi, uncultured bacterioidetes and *Muricauda* spp.

- Phylogenetic analysis of the control sediment bacterial sequences with 99% similar sequences from GenBank database showed a major clustering of sequences within the clones and few of the clones were clustered and branched off from uncultured bacteria, uncultured soil bacteria, uncultured sludge bacteria, and uncultured delta proteobacteria.

- The phylogenetic tree constructed with the aligned 16S rRNA sequences of the bio-augmented samples alone consisted of six clusters while that constructed out of the sequences from control sediment had only five.

- Using the diversity estimation employing MEGA 6.0, the mean inter population diversity obtained for the bio-augmented sediment was 5.167 ±1.009, whereas in comparison the control had only 3.188 ±0.686.

- The VITCOMIC map revealed the relative abundance and species composition found in the bio-augmented and control sediment, and most of the taxonomic communities of both the systems belonged to Proteobacteria.

- The red dots represented taxonomic group of bio-augmented sediment, blue of control sediment and grey the common taxonomic group found both in bio-augmented and control sediment samples.

- Size of the dots indicated relative abundance of the sequences in the samples.

- The map showed relative abundance of >10% of *Geobacter* spp. and *Gramella* spp. followed by <5% relative abundance of *Thermodesulphovibrio* sp., *Desulfococcus* sp., *Pelobacter* spp. and *Flavobacterium* spp. in bio-augmented sample.

- In control samples, a relative abundance of <10% of *Frankia* spp. was observed followed by < 5% of *Pelobacter carbinolicus*, *Pelotomaculum thermopropionicum*, *Syntrophobacter fumaroxidans*, *Syntrophus aciditrophicus* and *Thioalkalivibrio* spp.

- The comparative taxonomic analysis generated by VITCOMIC map showed differences between both the communities as indicated by Jacard index, Lenon index and Yue and Clayton Theta of 0.109, 0.2 and 0.0723 respectively.

- The heirarchial layout of bacterial phyla generated using geneious software grouped bioaugmented sediment clones metagenome to the taxa Proteobacteria, Bacteroidetes, Acidobacteria, Planctomycetes, Nitrospirinae and Chloroflexi. Overall Proteobacteria contributes to 41% in terms of abundance and richness.

- Among the 21% Bacteroidetes 17% of the abundance was linked to family Saprospiraceae. The relative abundance of this group in bioaugmented clone library underscores the enhanced capacity for organic matter degradation in this sediment.

- Phylum Nitrospirinae was seen only among bioaugmented sediment clones library. The presence of Nitrospinaceae confirms the functional guild of nitrite oxidizing bacteria in bioaugmented sediment unlike that in control sediment is suggestive of a population capable to bioremediate nitrogenous compounds

- 16SrRNA biodiversity of control system portraits Proteobacteria as the major phylum occupying about 61% of the population, followed by 11% Chloroflexi and Actinobacteria. Bacteroidetes and Acidobacteria represents 6% and 8 % of the clones, whereas Planctomycetes, Firmicutes and Gemmatimonadetes are evenly distributed minor phyla accosting for about 3% of the community

- The relatively high abundance of phylum Proteobacteria among control sediment clones dictates the presence of sulphur rich environment.

Bio-augmentation brings about overall reshuffling of microorganisms in a niche for the expression of specific catabolic traits enhancing mineralization and less accumulation of organic wastes.

Ultimately an environment of low stability, subjected to stress is converted to self purifying sustainable system.

Based on the results generated in this study, it was concluded that shrimps could be grown successfully in a closed culture system under zero water exchange mode adopting bio-augmentation technology using the indigenous bio-augmenter *Bacillus cereus* MCCB 101.

Scope of further research

- This study demonstrates that bioaugmenting sediment microcosms with *Bacillus* MCCB 101 has tremendous influence in boosting sediment *Eh* .Hence a better understanding on sediment chemistry with respect to *Eh* in detail could further substantiate the spatial distribution of electron acceptors in the redox stratified sediments on applying the bioaugmenter.

- Immobilization of bioaugmenter on an inert substance and its fine tuning for better mastery in sediment is yet another promising perspective of our future research work.

- Our investigation on one time shift of bacterial diversity in bioaugmented and control system sediments were very much informative on the hierarchy of bacterial lineage. Therefore further study on the shift of bacterial diversity associated with sediment,

water and shrimp with time in a Zero water exchange high stocking density shrimp production system should be attempted.

- The next stage could be to up-scale the treatment systems to determine cost-effective production and expand application of the systems described.

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APPENDICES

Appendix 1: Standard graphs of MUF substrate.

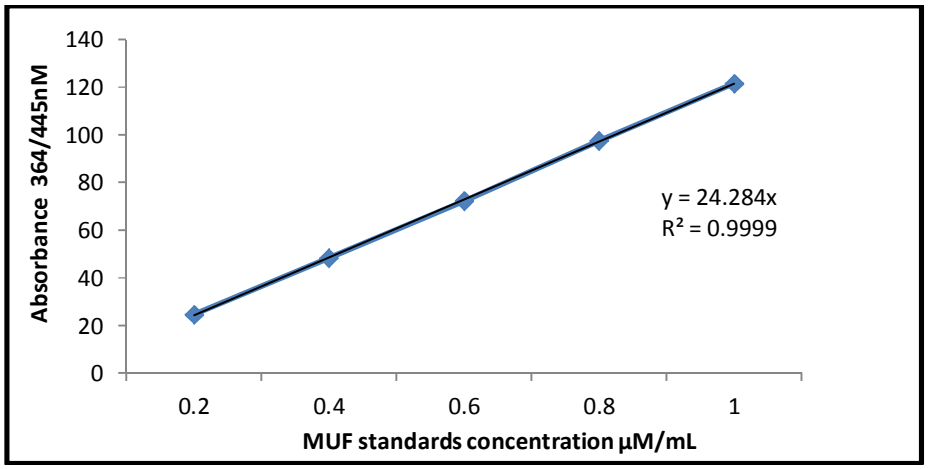

Fig A1: 1

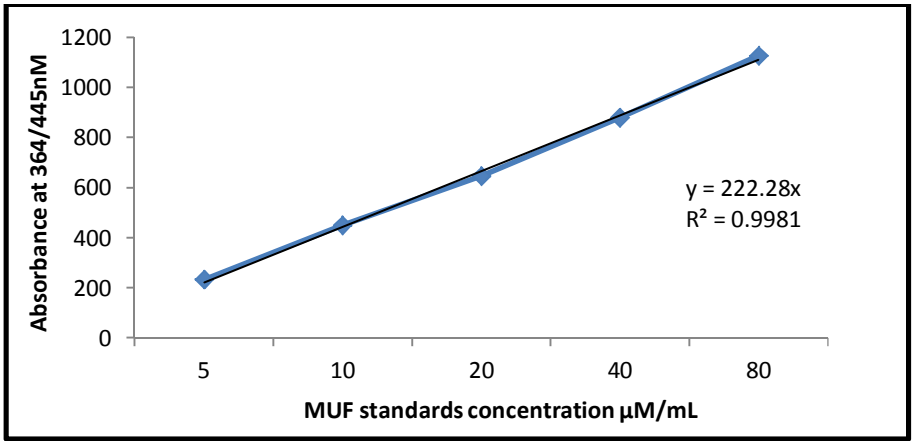

Fig A1: 2

Appendix 2: Standard graphs of MCA substrate.

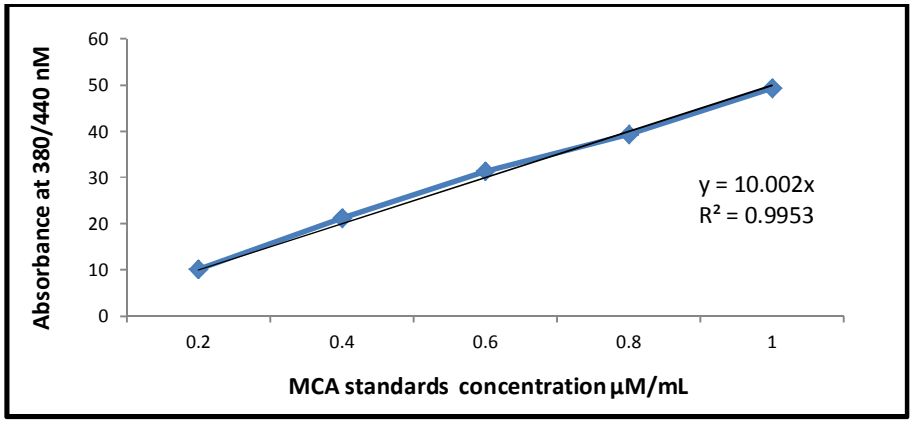

Fig A2: 1

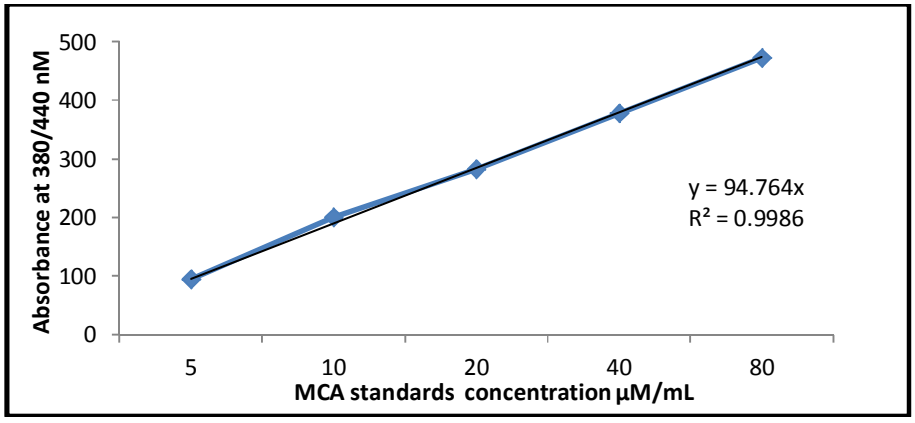

Fig A2: 2

Appendix 3: Standard graph of Bovine serum albumin (BSA) for estimation of total proteins

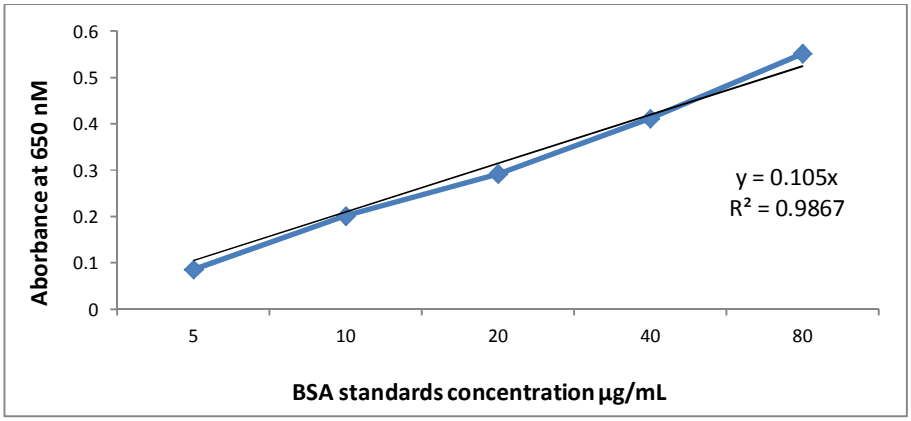

Fig A3: 1

Appendix 4: Standard graph of Glucose for estimation of total carbohydrates

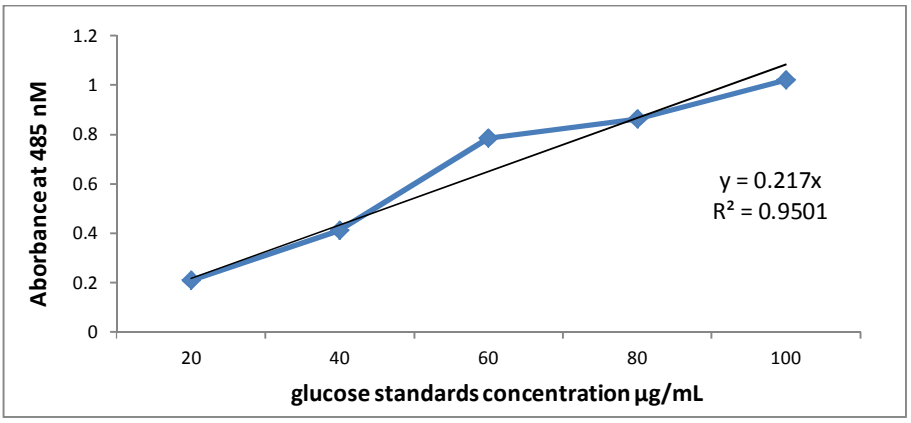

Fig A4: 1

Appendix 5: Standard graph of tripalmitine for estimation of total lipids

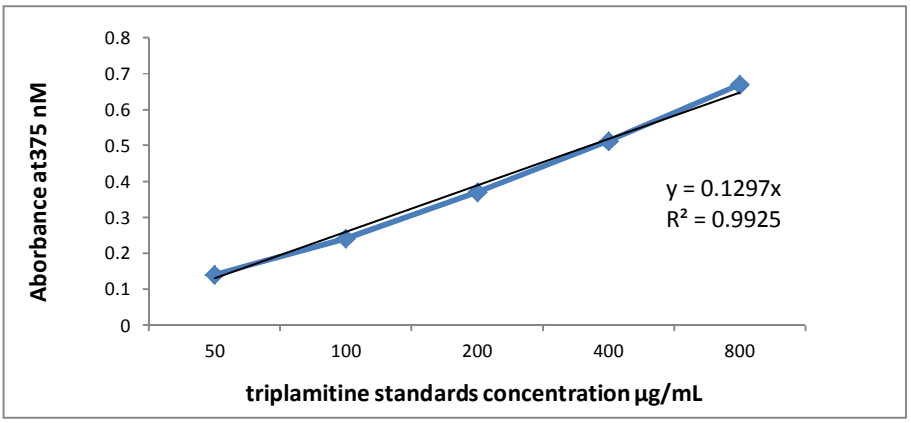

Fig A5: 1

Appendix 6: Accession no. of clones obtained from Bioaugmented system

Sl.No	Clone Identity	Accession Number	Definition
1	Clone BT 168	KP299845	16S ribosomal RNA gene,partial Sequence
2	Clone BT 42	KP299846	16S ribosomal RNA gene,partial Sequence
3	Clone BT 35	KP299847	16S ribosomal RNA gene,partial Sequence
4	Clone BT 92	KP299848	16S ribosomal RNA gene,partial Sequence
5	Clone BT 100	KP299849	16S ribosomal RNA gene,partial Sequence

6	Clone BT 113	KP299850	16S ribosomal RNA gene,partial Sequence
7	Clone BT 158	KP299851	16S ribosomal RNA gene,partial Sequence
8	Clone BT 159	KP299852	16S ribosomal RNA gene,partial Sequence
9	Clone BT 167	KP299853	16S ribosomal RNA gene,partial Sequence
10	Clone BT 244	KP299854	16S ribosomal RNA gene,partial Sequence
11	Clone BT 253	KP299855	16S ribosomal RNA gene,partial Sequence
12	Clone BT 278	KP299856	16S ribosomal RNA gene,partial Sequence
13	Clone BT 280	KP299857	16S ribosomal RNA gene,partial Sequence
14	Clone BT 361	KP299858	16S ribosomal RNA gene,partial Sequence
15	Clone BT 14	KP299859	16S ribosomal RNA gene,partial Sequence
16	Clone BT 25	KP299860	16S ribosomal RNA gene,partial Sequence
17	Clone BT 72	KP299861	16S ribosomal RNA gene,partial Sequence
18	Clone BT 58	KP299862	16S ribosomal RNA gene,partial Sequence
19	Clone BT 76	KP299863	16S ribosomal RNA gene,partial Sequence
20	Clone BT 115	KP299864	16S ribosomal RNA gene,partial Sequence
21	Clone BT 116	KP299865	16S ribosomal RNA gene,partial Sequence
22	Clone BT 140	KP299866	16S ribosomal RNA gene,partial Sequence

23	Clone BT 147	KP299867	16S ribosomal RNA gene,partial Sequence
24	Clone BT 152	KP299868	16S ribosomal RNA gene,partial Sequence
25	Clone BT 210	KP299869	16S ribosomal RNA gene,partial Sequence
26	Clone BT 214	KP299870	16S ribosomal RNA gene,partial Sequence
27	Clone BT 67	KP299871	16S ribosomal RNA gene,partial Sequence
28	Clone BT 74	KP299872	16S ribosomal RNA gene,partial Sequence
29	Clone BT 78	KP299873	16S ribosomal RNA gene,partial Sequence
30	Clone BT 104	KP299874	16S ribosomal RNA gene,partial Sequence
31	Clone BT 114	KP299875	16S ribosomal RNA gene,partial Sequence
32	Clone BT 133	KP299876	16S ribosomal RNA gene,partial Sequence
33	Clone BT 153	KP299877	16S ribosomal RNA gene,partial Sequence
34	Clone BT 154	KP299878	16S ribosomal RNA gene,partial Sequence
35	Clone BT 172	KP299879	16S ribosomal RNA gene,partial Sequence
36	Clone BT 221	KP299880	16S ribosomal RNA gene,partial Sequence
37	Clone BT 254	KP299881	16S ribosomal RNA gene,partial Sequence
38	Clone BT 340	KP299882	16S ribosomal RNA gene,partial Sequence
39	Clone BT 33	KP299839	16S ribosomal RNA gene,partial Sequence

40	Clone BT 12	KP299816	16S ribosomal RNA gene,partial Sequence
41	Clone BT 347	KY441459	16S ribosomal RNA gene,partial Sequence
42	Clone BT 216	KY441460	16S ribosomal RNA gene,partial Sequence

Appendix 7: Accession no. of clones obtained from Control system

Sl.No	Clone Identity	Accession Number	Definition
1	Clone CT 616	KP299805	16S ribosomal RNA gene,partial Sequence
2	Clone CT 629	KP299806	16S ribosomal RNA gene,partial Sequence
3	Clone CT 282	KP299807	16S ribosomal RNA gene,partial Sequence
4	Clone CT 620	KP299808	16S ribosomal RNA gene,partial Sequence
5	Clone CT 753	KP299809	16S ribosomal RNA gene,partial Sequence
6	Clone CT 359	KP299810	16S ribosomal RNA gene,partial Sequence
7	Clone CT 61	KP299811	16S ribosomal RNA gene,partial Sequence
8	Clone CT 255	KP299812	16S ribosomal RNA gene,partial Sequence
9	Clone CT 83	KP299813	16S ribosomal RNA gene,partial Sequence

10	Clone CT 305	KP299814	16S ribosomal RNA gene,partial Sequence
11	Clone CT 604	KP299815	16S ribosomal RNA gene,partial Sequence
12	Clone CT 743	KP299817	16S ribosomal RNA gene,partial Sequence
13	Clone CT 640	KP299818	16S ribosomal RNA gene,partial Sequence
14	Clone CT 402	KP299819	16S ribosomal RNA gene,partial Sequence
15	Clone CT 371	KP299820	16S ribosomal RNA gene,partial Sequence
16	Clone CT 624	KP299821	16S ribosomal RNA gene,partial Sequence
17	Clone CT 621	KP299822	16S ribosomal RNA gene,partial Sequence
18	Clone CT 652	KP299823	16S ribosomal RNA gene,partial Sequence
19	Clone CT 700	KP299824	16S ribosomal RNA gene,partial Sequence
20	Clone CT 279	KP299825	16S ribosomal RNA gene,partial Sequence
21	Clone CT 435	KP299826	16S ribosomal RNA gene,partial Sequence
22	Clone CT 295	KP299827	16S ribosomal RNA gene,partial Sequence
23	Clone CT 573	KP299828	16S ribosomal RNA gene,partial Sequence
24	Clone CT 562	KP299829	16S ribosomal RNA gene,partial Sequence
25	Clone CT 565	KP299830	16S ribosomal RNA gene,partial Sequence
26	Clone CT 589	KP299831	16S ribosomal RNA gene,partial Sequence

27	Clone CT 327	KP299832	16S ribosomal RNA gene,partial Sequence
28	Clone CT 531	KP299833	16S ribosomal RNA gene,partial Sequence
29	Clone CT 529	KP299834	16S ribosomal RNA gene,partial Sequence
30	Clone CT 729	KP299835	16S ribosomal RNA gene,partial Sequence
31	Clone CT 549	KP299836	16S ribosomal RNA gene,partial Sequence
32	Clone CT 513	KP299837	16S ribosomal RNA gene,partial Sequence
33	Clone CT 519	KP299838	16S ribosomal RNA gene,partial Sequence
34	Clone CT 450	KP299840	16S ribosomal RNA gene,partial Sequence
36	Clone CT 544	KP299841	16S ribosomal RNA gene,partial Sequence
37	Clone CT 552	KP299842	16S ribosomal RNA gene,partial Sequence
38	Clone CT 701	KP299843	16S ribosomal RNA gene,partial Sequence
39	Clone CT 731	KP299844	16S ribosomal RNA gene,partial Sequence
40	Clone CT 105	KY44146	16S ribosomal RNA gene,partial Sequence

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