# ISOLATION, IDENTIFICATION AND LIPASE CHARACTERIZATION OF MARINE ACTINOBACTERIA OF GENUS STREPTOMYCES FROM ANDMAN COAST

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*By*


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## Certificate

This is to certify that the research work presented in this thesis entitled **"ISOLATION, IDENTIFICATION AND LIPASE CHARACTERIZATION OF MARINE ACTINOBACTERIA OF GENUS *STREPTOMYCES* FROM ANDAMAN COAST"** is based on the original work done by Ms. Sumitha Gopalakrishnan under our guidance at Department of Aquaculture and Fishery Microbiology, M. E. S. Ponnani College, Ponnani, Malappuram, Kerala, India 679 586, in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy and that no part of this work has previously formed the basis for award of any degree, diploma, associateship, fellowship or any other similar title or recognition.


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# Chapter 1
# General Introduction

## 1.1 Introduction

While contributing 97% of earth living habitat and covering approximately 71% of the surface of the planet earth, oceans are home for over 7 million species surviving in this world (Anon, 2017). It has been acknowledged as a promising source of food, a reservoir of various minerals, a major supplier of oxygen, and a regulator of climate (Pai, 2007). The vast diversity of oceanic environment is closely related to the plethora of habitats and microhabitats it supports. Since they are considered as one of the most productive regions of the world, they also support a diverse group of organism that is vital for human health and render various ecosystem services. The distribution and densities of marine fauna depend upon the habitat and trophic level they occupy. According to Coull and Bell (1979), the oxygen zone of the organically enriched mud site can support 20 times more biomass per volume of sediment than the oxic zone of the sand site. High production rates of benthic bacteria confirm the significant part they play in the organic matter degradation (Duyl and Kop, 1994) thus they perform significant roles in the benthic ecosystems (Kuwae and Hosokawa, 1999) by degrading organic matter within the sediment.

India is endowed with a coastline of about 7,500 km with an overall area of 2.02 million sq km of the exclusive economic zone. The Andaman and Nicobar Islands share 1962 km, which is about 25% of the country's entire coastline and comprises of 28% of the total Indian Exclusive Economic Zone with very rich biodiversity (Vijayakumar et al. 2007, Dam Roy et al. 2009). The coast of islands of Andaman is unique. There is an amalgamation of highly productive Mangrove-Coral –Rocky Sandy ecosystem. Thus this region has diverse marine environments and a variety of soil habitats. Among these ecosystems, mangroves and coral reef are found to be

most productive ecosystems and are ecologically as well as socio-economically important.

### 1.1.1. Mangrove ecosystems

Mangroves flourish in the intertidal zone and adapted to the ever-fluctuating environmental conditions of zero to high saline, often swamped, soft-bottomed anaerobic mud with high organic content (Khairnar et al. 2009). The mangrove microbial diversity is high, consists of Rhizobiales, Campylobacterales, Methylococcales, and Vibrionales, Actinomycetales, etc. (Gomes et al. 2010).These unique ecosystems are largely endowed with an unexploited source of novel microbial flora with a rich perspective to produce important bioactive metabolites (Jacob et al. 2005). Usha et al. (2014) studied the seasonalities in the distribution of actinobacterial distribution in the mangrove sediments of Nizampatnam and Coringa along the South East coast of Andhra Pradesh and isolated 69% of Streptomycete isolates which can produce commercially important enzymes and antimicrobial compounds. Bacterial diversity assessment of mangrove microbial community by Basak et al. (2016) from Dhulibhashani, Sundarbans revealed an abundance of Firmicutes, Cyanobacteria, Actinobacteria, Bacteroidetes, Acidobactreia, Nitrospirae, Planctomycetes, and Fusobacteria. Around 7% of actinobacteria isolated different depths of this mangrove area. Sethupathi et al. (2013) described the actinobacterial diversity in the Little Andaman islands where biotopes like dense mangrove coral ecosystem present. Meena et al. (2013) isolated 26 potential actinomycete strains from marine sediments of Minnie bay Mangrove area and *Streptomyces* spp. were reported abundant. Microbes occupy a key position in the ecological systems by maintaining sustainable natural productivity and also

constitute one among the major pool of potential metabolic pathways which have implications on future environmental and biotechnological research.

### 1.1.2. Coral ecosystems

The coral ecosystem is one of the largest biologically diverse, a complex ecosystem where chemical, biological, and physical factors interact and facilitate a symbiotic habitat of coral, algae and a complex consortium of bacteria, together demoted to as coral holobiont (Kelly et al. 2018). Coral reefs contribute to amazingly high biomass production rates in the coastal ecological systems but remain as some of the nutrient-depleted ecosystems on Earth (Kelly et al. 2018). Most of the microbial taxa inhabiting in the coral system are poorly categorised, due to limited representation by culturable isolates. Hodges et al. (2011) studied actinobacterial diversity in Anchialine cave systems and open coral reef system in the Bahamas, which showed domination of genus *Streptomyces* in open reef sediments and from marine cave sediment, samples a more diverse group of actinobacteria of genus *Solwaraspora* isolated. Mahmoud and Kaledar (2016) reported 19 different actinobacterial genera from coral reef system of the north Arabian Gulf. Nithyanand et al. (2011) isolated actinomycetes from the mucus of the coral *Acropora digitifera* and studied Phylogenetic characterization. The biodiversity of Indian coral reefs mostly depends on the two island ecosystems, Andaman and Nicobar as well as Lakshadweep islands. The diversity of corals from islands of Andaman and Nicobar include 424 hard coral species belonging to 86 genera and 19 families (Raghuraman et al. 2012). It was stated that the actinobacterial isolates from the coral sandy area were lesser than rocky coral area and areas of river discharge have shown higher actinobacterial density (Sethupathi et al. 2013).

### 1.1.3. Actinobacteria

Actinobacteria comprised of five subclasses, six orders and Fourteen suborders are one of the most prevalent taxa among Eighteen major lineages presently documented within the domain of bacteria (Ludwig et al. 2012). They are gram-positive microorganisms which acts like connecting link between fungi and bacteria (Barka et al. 2016). They are divided into two unequal subgroups viz. fermentative type, and a larger group called oxidative type (Lechevalier and Pine, 1977). These microorganisms have fragmented or non-fragmented branched mycelia (0.5-0.7 microns) which bear spores. Mycelium consists of aerial mycelium and substrate mycelium. Hopwood (1960) confirmed that the simple branching of substrate hyphae forms the aerial hyphae in *Streptomyces coelicolor*. Aerial hyphae give the entire colony a fluffy, velvety or floury covering. The aerial hyphae vary depending upon the species, nutritional and prevailing environmental conditions. The spores developed on the filaments of an aerial mycelium immediately after the appearance of the aerial mycelium and that some of them even began to germinate (Wildermuth, 1970). The genus *Streptomyces* is polysporous with long chains. Spores are covered with Sporangia, which is a sac-like structure. The morphology of sporangia differs in different species. There are different kinds of sporangia like a spiral or spindle-like sporangia, straight. Some Actinobacteria have densely packed spores form sporangia in the shape of the spheres and others they are stretched. Some are non-sporangia formers while sporangia are very short in some species. The common types are in the form of bristles, long straight, straight but not spiral, etc. (Dissel et al. 2014, Labeda, 1987).

Actinobacteria are present in common soil, freshwater, and marine condition. Terrestrial regions remain the most vital environment for Actinobacteria. According

to Xu et al. (1996), actinobacteria populations of the tropical and subtropical plateau were more complex than the cool temperate mountains and snowy mountain types. While thermophilic and psychrophilic actinomycetes were distributed even at 3,500 m above mean sea level. Actinomycetes were lower in the drier and poorer soil in the cooler climates. *Streptomyces* spp. are the most significant group in the actinobacteria, it represents approximately 90% of the total actinobacterial population of the soil (Nonomura and Ohara, 1960, Takizawa et al. 1993). Other genera like *Actinoplanes*, *Kineosporia*, and *Cryptosporangium* are generally inhabiting in the leaf-litter samples whereas strains of the suborder Streptosporangineae were isolated exclusively from soil environment (Nonomura and Ohara, 1960 a & b; Nonomura and Ohara, 1969 a & b; Nonumura and Ohara, 1971 a, b, c, & d; Pace et al.1986).

Marine Actinobacteria were once believed to be from terrestrial environment and washed into the aquatic environment (Goodfellow and Haynes, 1984). The first evidence for marine-derived actinobacteria that display a typical marine adaptation was given by Jensen et al. (1991) and the name *Salinospora* was given (Mincer et al. 2002). It was only recently established the existence of actinomycetes native to marine habitat and not purely a transcend contaminant from the shore and thus became an addition to the existing bacterial diversity within a variety of actinomycetes (Takizawa et al. 1993, Mincer et al. 2002, Stach et al. 2003 and Paul et al. 2005). The complete genome sequencing of the *Streptomyces coelicolor* A3 (2) was done by Bentley et al. (2002) was a good step forward for future research. Many studies on cultured and culture-independent marine actinomycetes in marine sediments revealed the vastness of its diversity and distribution in the marine environment (Mincer et al. 2005, Jensen and Mafnas, 2006).

Terrestrial Actinobacteria have various interesting antimicrobial potentials. They are the producers of most of the antibiotics (Okami, 1973, Waksman and Lechevalier, 1962). Marine actinomycetes are the producers of highly valuable natural products, the most significant source of new medicines. Till recently, studies on taxonomic nobility of marine-derived strains were less. Only a small group of taxa are involved in the considerable quantity of compounds already been utilized in the industry (Newman et al. 2003, Blunt et al. 2004, Keller and Zengler, 2004 and Paul et al. 2005). The genera *Micromonospora*, *Streptomyces*, and Nocardioforms screened from anoxic mangrove rhizosphere are fewer compared to terrestrial land which is found to be less because of the tidal influence (Tan, et al. 2009). Cytotoxic metabolites produced by certain mangrove inhabited *Nocardia* spp. found to inhibit gastric adenocarcinoma (Nicholson et al. 2006) strongly. In addition to their important influence on human health, actinobacteria have key ecological roles. Before focusing on antibiotic discovery, Nobel laureate Selman Waksman's work on soil bacteria and their impact on agricultural productivity were among the first to implicate Actinobacteria as important contributors to the process of plant biomass decomposition (Selman and Robert, 1931, Hopwood, 2007). More recently, Actinobacteria have been revealed as widespread symbionts of eukaryotes, helping herbivores gain access to plant biomass as nutritional mutualists and producing natural products as defensive mutualists (Coombs, 2004, Currie, 2006, Kaltenpoth, 2009, Book, 2016). Studies of Actinobacteria as defensive mutualists have led to the discovery of new antibiotics with potential pharmaceutical applications (Oh, 2009, Poulsen et al. 2011, Scott, 2008), renewing recognition of the value in understanding the ecology of Actinobacteria for drug discovery (Berenbaum, 2008).

Much like in drug discovery, the ecology and evolution of Actinobacteria also contribute to the development of a sustainable bioenergy industry. A number of aspects of the evolution, ecology, and physiology of Actinobacteria make them well suited for integration with cellulosic biofuel production. Industrial biofuel production requires enzymes that function under diverse conditions, depending on factors such as the biomass composition and pre-treatment protocol. The Actinobacteria that have evolved within particular niches can lead to the production of cellulases suitable for specific industrial usages. Thermostable enzymes were found in *Thermobifida fusca* and *Thermomonospora curvata* from heated composting environments and *Acidothermus cellulolyticus* from acidic hot springs (Stutzenberger, 1972, Tucker 1989, Wilson 2004, Ransom, 2007). Furthermore, given that actinobacteria freely secrete their enzymes and have evolved in some niches in close association with fungi, actinobacterial cellulases are promising candidates to complement fungal enzymes for plant biomass deconstruction. Actinobacteria are important for industrial use in various industrial processes (Demain, 2007). The ability to engineer strains that constitutively secrete cellulases at high levels (Book, 2016) and the resistance of their cell walls to processes, such as milling that may be important in sustainable cellulosic biofuel production (Paye et al. 2016).

The ecological roles of Actinobacteria in plant biomass degradation, in the soil, in compost, and in association with herbivorous animals have selected for the evolution of high cellulolytic abilities (Book, 2016). Indeed, a number of model cellulolytic bacteria occur in this phylum, such as *Cellulomonas* spp. and *Thermobifida fusca*, whose study has helped advance our understanding of the enzymology of cellulose degradation (Christopherson, 2013, Wilson, 2004). More broadly, the cellulolytic

abilities of this phylum are supported by genomic and proteomic analyses, which indicate that actinobacterial genomes have an amazing diversity of enzyme fold families and organizational architectures used to hydrolyze plant biomass compared to most other phyla of bacteria (Berlemont, 2013). In particular, of the genera, of *Streptomyces* and *Micromonospora*, some strains of *Mycobacterium*, and one strain of *Propionibacterium* are particularly enriched in carbohydrate-active enzyme (CAZy) genes.

Specifically, as actinobacteria are implicated as important decomposers of plant material in nature, their cellulolytic enzymes can be used to more efficiently break down plant biomass into simple sugars, which can then be used for the production of biofuels. In addition, the diverse biosynthetic capacity of actinobacteria, which evolved to mediate their environmental interactions, could be leveraged to produce a range of bio-products, including specialty biofuel compounds. This century, we have an urgent and critical need for bio-based approaches that help meet our energy requirements while reducing carbon emissions. There are many efforts underway to leverage the diverse ecology of actinobacteria to identify enzymes, pathways, and biosynthetic products that can contribute to biofuel and chemical production. Actinobacteria have the potential to further increase in prominence as they help us meet our global needs for renewable biofuels and other bio-products while mitigating the dangers of climate change.

Dessert condition is considered a harsh terrestrial environment hostile to the growth of plants and animals; only a certain species can survive. The diversity of actinobacteria showed a clear dominance of *Streptomyces* spp. at the subtropical dessert soil Thar, Rajasthan, other than these, species, such as *Nocardia* spp., *Nocardiopsis* spp. also reported by Cundell et al. (2016). Nithya et al. (2015)

isolated actinobacteria from different desert soil samples having antagonistic activity against various bacterial pathogens.

The actinobacteria contribute a significant part in the turnover of recalcitrant plant organic matter in the rhizosphere soil, and this region is considered as one of the best habitats for isolation of these microorganisms especially *Streptomyces* and *Nocardia*. According to Priyadharsini et al. (2016), paddy fields soil is one of the nutrient-enriched land and isolated *Streptomyces* spp., *Streptoverticillium* spp., *Actinomadura* spp., *Kitasatosporia* spp., *Nocardiopsis* spp., *Pseudonocardia* spp. and *Kibdelosporangium* spp. The *Streptomyces* spp. isolated from this area displayed the ability to hinder the growth of *Cyperus rotundus*.

Actinobacteria are considered to be indicators of the habitat, wide distribution of actinobacteria in aquatic habitats due to the washed in from the surrounding terrestrial habitats. Presence of *Thermoactinomyces* spp. and *Rhodococcus coprophilus* are known to be good indicators of the terrestrial environment (Rajani and Reshma, 2016). It is found that the spores of *Thermoactinomyces* are designed to withstand high temperature, but in the aquatic condition, there is no chance for such conditions.

Actinobacteria can be isolated from various environments, as they show adaptations to cop up with terrestrial as well as aquatic conditions. In *Actinoplanes* the spore vesicles have the ability to endure prolonged dryness, and they release the motile spores when rehydrated (Syker and Skinner, 1973). Freshwater habitat also show a rich diversity of Actinobacteria like *Actinoplanes*, *Micromonospora*, *Rhodococcus*, *Streptomyces* and the endospore-forming *Thermoactinomyces*, etc. Cross (1981). The extensive occurrence of *Micromonospora* in streams and other water bodies

explains their habitat versatility and considered them to be a vital part of the aquatic microflora (Anthony et al. 1993). Another interesting observation was done by Johnston and Cross (1976) that the occurrence of *Streptomyces* spp. found to be fewer in number in the deeper mud layers where micromonosporae were found to be predominant. In another study, a significant correlation between Micromonosporae and Thermoactinomycetes observed by AI-Diwany et al. (1978). It is believed from the adaptive features of both Micromonosporae and *Streptomyces*; they were washed into the aquatic system from the terrestrial environment. Micromonospora spores can exist dormant for several years until and unless reaching a favourable condition. In the case of *Streptomyces*, they are found very frequently from freshwater and aquatic environment. The *Streptomyces* shows wide tolerance of salinity and temperature, and they are found to be very active in very harsh conditions also. The freshwater environment comprises of species like *Actinomadura madurae*, *Mycobacterium kansasii*, *Arthrobacter* spp., *Corynebacterium* spp. and *Nocardia* spp. Thus it is proved that 2eappropriate substrates and conditions for growth. Actinobacteria are found to be native to such habitats where they hydrolyse cellulose, chitin, and lignin.

The marine environment is found to be very dynamic and versatile as the terrestrial and freshwater environment. Unlike terrestrial environment, the marine environment remains an unexplored source of novel actinobacteria diversity and thus of new metabolites. Marine actinobacteria survive in extreme conditions of high pressure, salinity, anaerobic condition, the 4ºC temperature to temperatures of over 40°C near hydrothermal vents at the mid-ocean ridges can produce different types of bioactive compounds compared with terrestrial ones. *Rhodococcus marinonascene* was the first actinomycete species to be characterized by the marine environment, which

supported the presence of innate marine actinobacteria. Members of the genera like *Dietzia*, *Rhodococcus, Streptomyces, Salinispora, Marinophilus, Solwaraspora, Salinibacterium, Aeromicrobium marinum, Williamsia maris,* and *Verrucosispora* have been designated as indigenous marine actinobacteria (Bull et al. 2005) and their antagonistic activity helps them to establish themselves and enrich the ecosystem by the degradation and mineralization of organic matter (Grossart et al. 2002, Rajani and Reshma, 2016). They form a vibrant living community in terrestrial and marine sediment. The Actinobacteria are involved in organic matter decomposition; biological nitrogen fixation - nitrification, de-nitrification; phosphate solubilisation; as well as cycles of major and minor nutrients and thus take part in a significant role in soil structure maintenance, disease control by producing antibiotics and plant growth promotion through secretion of hormones. This group of bacteria forms a major producer of antibiotics for various diseases, both bacterial as well as viral. Anti-cancerous property of the compounds isolated from these microbes is also promising. Research is also going on actinobacteria as producers of plant growth promoters. Diversity and resourcefulness of actinomycetes producing new metabolites is a need of new era since the microbial pathogens are gradually resilient to existing antibacterial compounds.The isolation of unexplored group and extraction of antimicrobial compounds is a critical element in drug discovery.

In India, studies were mainly concentrated on terrestrial, estuarine and mangrove actinomycetes (Batra et al. 1972, Kala and Chandrika, 1995, Vikineswary et al. 1997, Sivakumar, 2001, Dhanasekaran et al. 2005). An antibacterial methyl substituted β-lactam compound was isolated and characterized from *Streptomyces noursei* isolated from saltpan soil of Parangipettai. The role of antimetabolites especially from *Streptomyces* spp. have shown the highest larvicidal activity against

Anopheles mosquito larvae (Dhanasekaran et al. 2010). The studies on the marine actinomycetes were started only recently. Ellaiah and Reddy (1987) isolated actinomycetes from the marine sediments collected from Visakhapatnam coast. Taxonomy and antibiotic production studies on the actinomycetes from Indian shallow sea sediments were carried out by Balagurunathan and Subramanian (1993). Presently studies are mainly concentrated on the Isolation and characterization of antimicrobial compounds and extra-cellular enzymes producing marine actinomycetes from Indian EEZ (Patil et al. 2011, Sharma and Pant, 2001, Sujatha et al. 2005, Kathiresan et al. 2005, Sivakumar et al. 2005, Murugan et al. 2007, Ramesh et al. 2007, Gopalakrishnan and Philip, 2007, Sahu et al. 2007, Vijayakumar et al. 2007).

The group of Andaman Islands is an Indian archipelago in the Bay of Bengal. These islands are famous for its white sand beaches, mangroves, coral reefs which supports a vast diversity of marine life. Tourism is the source of major income for islanders. Tourism and related anthropogenic activities create lots of organic loads. In recent years due to overexploitation, island ecosystems are facing lots of changes. In the Andaman coast, the research on these organisms is still at the preliminary stage. Sivakumar et al. (2005) isolated Actinomycetes from the coral reef environment of Little Andaman Island. Sujatha et al. (2005) isolated 88 isolates of actinomycetes from 26 marine sediment samples near nine islands of the Andaman Coast. Of these isolates, 64 isolates were assigned to the genus *Streptomyces*, eight isolates to the genus *Micromonospora*, five to the genus *Nocardia*, seven to the family *Streptoverticilium* and four to the genus *Saccharopolyspora*. Among these isolates, three isolates showed very promising antagonistic activities against multi-drug resistant pathogens. Gopalakrishnan et al. (2013) studied the distribution of marine

Actinomycete species from the marine sediments along the South Andaman coast. There was no constructive effort has so far been made to investigate the ecological role of actinomycetes in the Andaman and Nicobar Islands. Under these circumstances, the present study on the habitat-related species diversity and distribution of common genera occurring in Andaman coast is very much relevant.

### 1.1.4. Actinobacteria as producers of enzyme

Chemical reactions in the living world are catalysed by highly specific proteins known as enzymes. Microorganisms are good producers of enzymes. According to the environmental cues, the microorganisms easily adjust themselves by producing various extracellular metabolites. In the phylum Actinobacteria, the resuscitating promoting factor (Rpf) protein (Puspita et al. 2013) are a good example. These capacities of microbes have made use for industries. These enzymes are active and stable than plant and animal enzymes (Anbu et al. 2013). Low nutrient requirements, rapid growth, ease to cultivate, vulnerable to genetic manipulation are making these microbes promising. At the beginning of the last century the German scientist Röhm (1915) used bovine pancreas extracts to remove stains from the cloth. It was he who introduced the use of biological extracts for industrial purpose. New microbial strains are always preferred by industries in order to harvest different enzymes to fulfill the present enzyme requirements. Mostly used industrial enzymes are hydrolytic enzymes like proteolytic, amylolytic, or lipolytic (Quax et al. 2006). The studies on the distribution of lipases (carboxylic acid ester hydrolases) in actinomycetes are a newly emerging field in the Biotechnology. Cholesterol esterase isolated from *Streptomyces lavendulae* displayed a lipolytic activity (Toshio et al. 1989).

Lipases of the EC 3.1.1.2 are important because they surely hydrolyze acylglycerol, oils, etc. (Qamsari et al. 2011)especially the Lipases isolated from *Rhodococcus erythropolis*. They are phthalate esters to a free phthalic acid and simple n-alcohols. These enzymes hydrolyse olive oil, and maximum tributyrin activity was shown at pH 8.6 and temperature 42˚C. The occurrence of a lipase (EC 3.1.1.3 group) reported from several *Streptomyces* strains by Chakrabarti et al. (1978).

There is a growing interest in lipases, particularly those which exhibit high stereospecificity which is useful for the resolution of racemic acids and alcohols applied as chiral substrates in organic synthesis (Cambou and Klibanov, 1984). There is a need for more attention to be paid to lipases production from Actinobacteria since these microorganisms are well known for their capacity to produce various secondary metabolites. A lipase-encoding gene from *Streptomyces cinnamomeus* was cloned and sequenced (Perez et al. 2002). As per literature, the extracellular Lipase from *Streptomyces exfoliatus* is the only lipase from the *Streptomyces* genus whose crystal structure has been recognised and the three-dimensional structure elucidated (Wei et al., 1998). From *S. exfoliatus* and *Streptomyces albus* two homologous lipases have been characterized, genetically there was no similarity to the two *Streptomyces* lipase sequences was found. This suggests a much higher variability of lipases than expected in this bacterial group (Sommer et al. 1997).

Cardenas et al. (2001) isolated lipases from *Streptomyces* spp which displayed comparatively improved activity than commercial lipase from Candida rugosa in the resolution of chiral secondary alcohols. The lipase from *S. halstedii* was exhibited very good activity in the synthesis of carbamates. Optimization is one of the most important criteria for developing new microbial process. Response surface analysis

is one of the vital tools to determine the optimal processing conditions. Gunalakshmi et al. (2008) optimised lipase production of *Streptomyces griseochromogenes* and found that maximum activity of the enzyme was at temperature 55°C, pH 7, 0.05% NaCl concentration. Vishnupriya et al. (2010) reported the lipase harvests of *Streptomyces griseus* were optimum at 72 h by using olive oil as a substrate and for sunflower oil and palm oil substrate, the maximum yields were obtained at 24h and 48hours of the incubation period. Thus optimization physiochemical parameters play a significant role in the production of enzymes.

Microorganisms like *Bacilli*, *Pseudomonas* and actinobacteria especially *Streptomyces* spp. are found to be good producers of hydrolytic enzymes like protease, amylase lipase, and cellulase. Most of the amylases produced for industries are from the fungal and bacterial origin (Pandey et al. 1999). Bacterial lipolytic enzymes are classified into eight families (families' I–VIII) based on the changes in their amino acid sequences and biological properties (Arpigny and Jaeger, 1999). Mostly the Lipase producers produce lipase enzyme of the family 1 and family 2 for, eg. *Streptomyces* spp., *Bacillus* spp., *Staphylococcus* spp. and *Pseudomonas* spp. (Arpigny and Jaeger, 1999). It was reported that lipase produced by *Streptomyces rimosus* and *S. scabies* are included in the family-II (Akoh et al. 2004). According to Ramnath et al. (2000) lipase enzymes from the microbes like *Pseudomonas* spp., *Aeromonas eutrophus*, *Moraxella* spp., *A. acidocaldarius*, *E. coli*, and *A. fulgidus, Moraxella* spp., *Psychrobacter immobilis*, mesophilic *Alcaligenes eutrophus*, and thermophilic *Bacillus acidocaldarius*, *Archeoglobus fulgidus* are coming in the Family of Lipases III-VIII.

### 1.1.5. Ecological significance of Lipases

The contamination of soil and water in the terrestrial, as well as the marine environment by urban wastes, industrial chemicals, and petroleum hydrocarbons, is a big problem for the developing country like India. The lack of proper planning and waste management as a result of sudden urbanization causes significant imbalance for the existing system. Hence with the advent of new microbe-based bioremediation tools removing contaminants has proven to be safe and economical. Bacterial activity is the primary process involved in the hydrolysis of organic pollutants. Extracellular enzyme activity is a crucial step in degradation and utilization of organic polymers (Karigar and Rao, 2011) as they break complex chemical bonds in the toxic molecules and reduce the toxic effect. This is the technique used to break down some of the recalcitrant compounds like organophosphate and carbamate insecticides. This mechanism is also effectively used for the biodegradation of oil spill. The ecological role of these important group of bacteria was also studied by screening for different extracellular enzymes like Lipase.

Most of the enzyme producing bacteria are isolated from the soil with high organic content. Aly et al. (2011) isolated *Streptomyces exfoliates* LP10 from oil-contaminated soil. Lipase activity of microbes are responsible for the drastic reduction total hydrocarbon from contaminated soil (Margesin et al. 1999, Riffald et al. 2006) and thus Lipase activity act as an indicator parameter for testing hydrocarbon degradation in soil. Microbial lipases are very versatile because of their potent application in industries. They can catalyze various reactions such as hydrolysis, interesterification, esterification, alcoholysis, and aminolysis (Prasad and Manjunath, 2011, Prasad et al. 2012). Lipases are ubiquitous enzymes that catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids (Sharma and

Sharma, 2011). Triglycerides are the main component of natural oil or fat. This can be further hydrolyzed to diacylglycerol, monoacylglycerol, glycerol, and fatty acids. Monoacylglycerols are used as an emulsifying agent in the food, cosmetic, and pharmaceutical industries.

Lipase is of much interest in the production of region-specific compounds which are employed in the pharmaceutical industry. Along with its diagnostic usage in bioremediation, lipase has many potential applications in food, chemical, detergent manufacturing, cosmetic, and papermaking industries, but the major bottleneck in its industrial adaptability is its production cost (Joseph et al. 2006).

### 1.1.6. Relevance of the study

Andaman and the Nicobar constitute a chain islands along the eastern Indian Ocean that is located southeast of the Indian subcontinent and separated by the Bay of Bengal. There was no major oil spill reported from Andaman other than a Japanese tanker collided with a small Indian vessel during 2006, spilling over 4,500 tonnes of oil into the Indian Ocean. In comparison with other coastlines of India, the coast of Andaman is considerably healthy, less polluted. The oil spillage, which is minimal, is mainly reported in the Haddo Jetty area, Mayabunder Port and aerial bay jetty. The present study indicates that the anthropogenic activities like tourism have initiated affecting the pristine Islands of Andaman slowly. Anthropogenic activities are one of the primary reasons for high organic content as well as eutrophication. Which leads to an increase in the presence of microbes.it is evident that where there are substrates, present naturally induce the production and activity enzymes.

In the present study, an attempt have been made to find out the actinobacterial distribution along the Andaman group of Islands in various habitats as well as

assessed the potential of the isolates from this pristine environment for their lipase activity.

## 1.2. Review of Literature

### 1.2.1. International Scenario

Nobel laureate Selman Waksman's work on soil bacteria and their impact on agricultural productivity were among the first to implicate actinobacteria as important contributors to the process of plant biomass decomposition (Waksman, 1931). Marine actinobacteria show an extensive distribution along all variety of marine sediments and habitats. Among actinomycetes, the genus *Streptomyces* was introduced by Waksman and Henrici in 1943, and it is the most predominant genera having more than 500 species. Antibiotic production by marine bacteria was first studied by Rosenfeld and Zobell (1947), and consequently, many others have studied the same (Hanefeld and Laatsch, 1991, Jensen and Fenical, 1994). Johnson and Rose (1968) collected the marine samples from the Indian Ocean, and isolated 127 actinomycetes and identified up to genus. Walker et al. (1975) observed that as the depth increases the number of *Streptomyces* decreased, Lechevalier and Pine (1977) divided actinobacteria into two unequal subgroups viz. fermentative type, and a larger group called oxidative type. The studies on fluctuations of environmental parameters which influence the abundance of microbial populations brought out much important information which help in understanding their distribution in various habitats ((Rheinheimer 1980, Wang and Han, 2014). Weyland et al. (1981) reported the *Streptomyces* was found to be decreased in number with increasing distance from shore and also observed that they were capable of growing in the absence of seawater, but better growth was observed in media containing

seawater. Although these bacteria represent a small component of the total bacterial population in marine sediments (Goodfellow et al. 1983), their role in the production of bioactive compounds makes them prominent. Thus they are defined as bacteria that can form branching hyphae at some stage of their development (Good fellow and Williams, 1983). Some indigenous marine actinomycetes like *Rhodococcus* and *Salinispora* spp. have been identified (Helmke and Weyland, 1984, Mincer et al. 2002, Maldonado et al. 2005, Pathom-aree et al. 2006). Goodfellow and Haynes (1984) reviewed the literature on the isolation of actinomycetes and suggested that only 10% of the actinomycetes are isolated from nature. According to Goodfellow and Haynes (1984), marine actinobacteria were once believed to be originated from dormant spores from the terrestrial system, and that washed into the aquatic system. On the basis of morphological characters like type and stability of mycelium, number and disposition of spores, formation of sclerotia, formation of flagellate elements; physical qualities like heat resistance and chemical composition of cell walls, whole cell composition, types of lipids, isoprenoid quinones, actinobacteria are divided into various groups (Goodfellow and Minnikin, 1985). The morphology of sporangia differs in different species. In some species, they are very short, straight, in the form of bristles, in others-long, straight but not spiral (Labeda, 1987, Dissel et al. 2014). Naganuma et al. (1990) studied vertical distribution marine bacteria from Suruga Bay and Japan Sea and found out that lipopolysaccharide (LPS) concentration was decreasing with increasing depth. Jensen et al. (1991) reported about the marine-derived actinomycetes, which exceptionally display marine adaptation. *Streptomyces* spp. among actinobacteria are considered to be dominant (Kampfer et al. 1991). The *Streptomyces* spp. shows wide tolerance to salinity. Some of the *Streptomyces* spp. isolated from marine sediment can even

tolerate zero salinity which justifies their terrestrial origin (Jensen et al. 1991). The research programs were conducted to upsurge the pool of actinomycete diversity (Takizawa et al. 1993, Mincer et al. 2002). According to Takizawa et al. (1993), marine Actinomycetes are distributed throughout the marine environment with streptomycetes predominating at shallow depths. Imada and Okami (1995) isolated marine actinomycete from a deep-sea sediment 1500m depth which can produce β-glucosidase inhibitor. *Bacillus, Pseudomonas,* and *Vibrio*, by the presence of their exoenzyme chitinase, can degrade biopolymer chitin (Kirchman, 1999). Bacterial densities and respiration rates in the sediment are found to be related (Field et al. 1997). Heterotrophic bacteria are the major contributors to the oceanic microbial population who help in the mineralisation of biological matter and the generation of carbon dioxide. According to Xu et al. (1996), Actinobacteria populations of the tropical and subtropical plateau were more complex than the cool temperate mountains and snowy mountain. Gooday (1990) and Stankiewicz (1997) have shown that *Clostridium pectinovorum* is responsible for the decomposition of pectin resulting in the end-products as pectic acid and methanol. The non-Streptomycetes are called rare actinomycetes (Vikineswari et al. 1997, Berdy, 2005). An interesting area of bacteriological research is the study of antagonistic properties towards human pathogens, they are (Multi-drug resistant) MDR strains or already resistant to earlier discovered antibiotics (Burgess et al. 1999). The biodiversity of marine microbes shows a pattern of distribution between different geographic regions and with time (Gaston, 2000). Curtis et al. (2002) estimated the diversity of these ecologically important prokaryotes and their limit. The microbial community of a deep sediment gas hydrate deposit was analysed by molecular genetic techniques by Marchesi et al. (2001). Mincer et al. (2002) described a major taxon of obligate

marine actinobacteria isolated from different depths. Actinomycetes distribution shows a low number in the drier and poorer soil in the cooler climates. Mincer et al. (2002) demonstrated that *Salinospora* spp. is an indigenous marine actinomycete. They are not merely a contaminant from the shore and added to the diversity of marine actinomycete taxa (Takizawa et al. 1993, Stach et al. 2003 and Paul et al. 2005). Miyake et al. (2003) described the distribution of antibiotic-resistant bacteria from different depth in Lake Biwa isolated from the shallow layer of sediment (0–1 cm), and by contrast, the isolates from the deep layer (9–10 cm) belonged to phylum Firmicutes. Stefanija et al. (2005) provided the first data on abundance, biomass, and biovolume of benthic bacteria along the eastern coast of the mid-Adriatic. Mincer et al. (2005) have conducted population studies of cultured and culture-independent marine Actinomycete taxa in ocean sediments and evaluated the diversity of marine actinomycete genus *Saliniospora*. Du et al. (2006) investigated the distribution of marine pigmented heterotrophic bacteria (PHB) and identified by sequencing. According to Kin (2006) demonstrated that indigenous marine actinomycetes exist in the oceans and are widely distributed in different marine ecosystems. Diversity and biogeography of marine actinobacteria reported by Ward and Bora (2006). Hopwood (2007) has opined that in addition to their important influence on human health, Actinobacteria have key ecological roles. In Mangrove rhizosphere soil, actinobacterial groups such as *Nocardioforms*, *Streptomyces*, *Micromonospora*, were found to be abundant, which is two times lesser than arid lands because of tidal effect (Tan, et al. 2009). More recently, actinobacteria have been revealed as widespread symbionts of eukaryotes, helping herbivores gain access to plant biomass as nutritional mutualists and producing natural products as defensive mutualists (Coombs, 2004, Currie, 2006, Kaltenpoth, 2009, Book, 2016). According

to Euzeby (2008), the genus *Streptomyces* of Actinomycetes group is the major component of the population. Gonzalez-Franco et al. (2009) reported that there is a clear reduction in Actinomycete diversity in the winter than other seasons like spring, it may be due to lower nutrients in rhizosphere soil. Yan Cai et al. (2009) isolated mesophilic and cryogenic actinomycetes from water, quagmire soil, lakeside soil and saline wasteland soil in the Qinghai Lake. Gontang et al. (2010) isolated 60 Actinobacteria representing 52 operational taxonomic units. Khan et al. (2010) isolated *Streptomyces tateyamensis* sp. nov., *Streptomyces haliclonae* sp. nov. and *Streptomyces marinus* sp. nov. was isolated from a marine sponge, *Haliclona* sp., collected from the coast of Tateyama City, Japan. Influence of Salinity on Bacterioplankton Communities from the Brazilian Rain Forest to the Coastal Atlantic Ocean was studied by Silveira et al. (2011). Mehravar et al. (2011) isolated 67 different actinomycete strain from Iran with antibacterial activity. Tamura et al. (2011) isolated eight actinomycete strains from Japan Phylogenetically; the isolates formed a single clade with the type strain of *Actinomycetospora chiangmaiensis*. Actinobacteria represent one of the largest taxonomy units with 18 major lineages including five subclasses, six orders and 14 suborders (Ludwig et al. 2012). According to De et al. (2014), marine bacteria provide a useful source for bioremediation because they can be used in extreme environmental conditions. Manivasagan et al. (2009) enlightened pharmaceutically active secondary metabolites of marine Actinobacteria. Innagi et al. (2015) isolated marine actinobacteria from off Hokkaido. Actinobacteria associated with reef coral Porites lutea documented by Kuang et al. (2015). Azman et al. (2015) reviewed the mangrove ecosystem is becoming a hot spot for studies of bioactivities and the discovery of natural products.

Stack et al. (2003) designed new primers for Actinobacteria. Ghai et al. 2013 described a deep-branching lineage of marine Actinobacteria with very low GC content. Chen et al. (2015) tried Molecular Phylogenetic Identification of Actinobacteria. Mizuno et al. (2015) described the genomes of four novel marine Actinobacteria derived from the Mediterranean deep. A high diversity of cultivable Actinobacteria (10 genera) was retrieved from fjord environments by Undabarrena (2016) using five different isolation media. The study indicates that the Comau fjord is a promising source of novel actinobacteria Chen et al. (2016) isolated A total of 5 actinobacterial classes, 17 orders, 28 families, and 52 genera were detected by pyrosequencing from the Deep-Sea Sediments along the Southwest Indian Ridge. Many actinobacteria have a mycelial lifestyle and undergo complex morphological differentiation. (Barka et al. 2016). Spatial and vertical distribution of bacterial communities in the eastern Indian Ocean detailed characterization of community structures by pyrosequencing suggested by Wang et al. (2016). Natural Product Potential of the Genus *Nocardiopsis* produces a wide variety of secondary metabolites accounting for its wide range of biological activities. (Ibrahim et al. 2018). Phylogenetic diversity and investigation of plant growth-promoting traits of actinobacteria in coastal salt marsh plant rhizospheres from Jiangsu, China were identified by Gong et al. (2018). Reza et al. (2018) described the first whole genome sequencing (WGS) study on the microbial community of the Ofunato Bay in Iwate Prefecture, Japan).

Waksman and Lechevalier (1962) have observed that the terrestrial Actinobacteria are the producers of most of the antibiotics and the same was supported by Okami, 1973. Thermostable enzymes were found in Thermobifida fusca and Thermomonospora curvata from heated composting environments and in

Acidothermus cellulolyticus from acidic hot springs (Stutzenberger, 1972, Tucker, 1989, Wilson, 2004, Ransom 2007). Lytic enzymes can degrade a wide diversity of recalcitrant compounds such as lignocelluloses (Crawford, 1978). *Streptomyces* spp. are responsible for the production of 80% of antibiotics in the world. Indeed, a number of model cellulolytic bacteria occur in this phylum, such as Cellulomonas spp. and *Thermobifida fusca*, whose study has helped advance our understanding of the enzymology of cellulose degradation (Christopherson, 2013). Actinobacteria are good producers of lytic enzymes, especially *Streptomyces* spp. Because of their metabolic diversity, actinomycetes are a great source of lytic enzymes, antibiotics and a great deal of other bioactive metabolites (Alderson et al. 1993, Sanglier et al. 1993). Marine actinomycetes are the producers of highly valuable natural products, the most important source of new medicines. Although only very few natural products studies have assessed the taxonomic nobility of marine-derived strains, it has yielded exciting new chemistry. Most of these secondary metabolites are potent antibiotics like neomycin, chloramphenicol (Kieser et al. 2000). A complete genome sequencing of the model Actinomycete *Streptomyces coelicolor* A3 (2) was done by Bentley et al. (2002). Grossart et al. (2002) have demonstrated that actinobacteria colonise in marine organic aggregates which affect the degradation and mineralization of organic matter, and their antagonistic activity is found to be significant. Bacteria belonging to the order Actinomycetes (commonly called actinomycetes) account for approximately 7000 compounds ((Newman et al. 2003, Blunt et al. 2004 and Paul et al. 2005). Only a small group of taxa are accounted for the vast majority of compounds already discovered. For example, of the 53 known bacterial phyla, only five reported to produce anti-infective agents (Keller and Zengler, 2004) and among these five classes, Actinobacteria more specifically.

Oskay et al. (2004) isolated Actinobacteria that had the capability of producing novel antibiotics with high antibacterial activity. Exploitation of marine environments as a pool of new actinomycetes for bioactive compound discovery is a recent trend since the rediscovery of the already existing compound was a problem (Kin, 2005). Jensen et al. (1991) isolated five new actinomycetes from marine sediments of the island of Guam. Among actinomycetes, it is estimated that approximately 7,600 compounds are produced by *Streptomyces* sp. (Berdy, 2005). Similarly, Nocardia isolated from mangrove soil produced new cytotoxic metabolites that strongly inhibited human cell lines, such as gastric adenocarcinoma (Nicholson et al. 2006). Actinobacteria are important for industrial use in various industrial processes (Demain, 2007). Studies of Actinobacteria as defensive mutualists have led to the discovery of new antibiotics with potential pharmaceutical applications (Scott, 2008, Oh, 2009, Poulsen et al. 2011), renewing recognition of the value in understanding the ecology of Actinobacteria for drug discovery (Berenbaum, 2008, Smanski 2015). The ability to engineer strains that constitutively secrete cellulases at high levels (Book 2016) and the resistance of their cell walls to processes, such as milling that may be important in sustainable cellulosic biofuel production (Paye et al. 2016). Marine environments are a largely untapped source for the isolation of new microorganisms with the potential to produce active secondary metabolites (Solecka et al. 2012). Actinobacteria constitute one of the largest bacterial phyla which are universally distributed in both aquatic and terrestrial ecosystems (Ludwig et al. 2012). Macroecological patterns of marine bacteria studied by Amend et al. (2012). Research is carried out in this area to disclose newer preventive and potential therapeutic strategies shows some bioactive compounds with their source and mechanism of action (Wang and Han,

2014) with modifications. The ecological roles of Actinobacteria in plant biomass degradation, in the soil, in compost, and in association with herbivorous animals have selected for the evolution of high cellulolytic abilities (Book, 2016).

The genus *Streptomyces* appears to be the most important candidate; it represents 90% of all soils. Motile actinomycetes of the general, Kineosporia, Actinoplanes and Cryptosporangium were isolated from mangrove leaf-litter samples. Actinobacteria isolated from the samples of the deepest abyss, the Challenger Deep off the Marianas (Stach et al. 2004).Thus they distributed in the wide range of environments. Marine actinomycetes turn out to be important contributors of bioactive compounds, but their ecological role in the marine ecosystem is largely neglected. Bull and Stach (2007) reviewed the importance of actinobacteria as producers of the natural product. Frequency and dominance of *Streptomyces* in various sources have been reported by Vijay Kumar et al. 2007.

### 1.2.2. Indian Scenario

Exploration of actinobacteria diversity in Indian coastal waters was initiated by Baam et al. (1966) who isolated two antagonistic actinobacteria from the Bombay coastal waters. Lakshmanaperumalsamy (1978) has isolated 518 strains from Parangipettai coastal sediments. Vanajakumari (1979) brought out information on actinobacteria diversity in marine molluscs in and around the Parangipettai. The relationship between the nutrients and sediments and bacterial population of the Andaman Sea was studied by Dhevendaran et al. (1987), who has found that clayey sediment can retain nutrients and which can be available for microorganisms than sandy sediment, so they support the more actinobacterial population. The studies from India were mainly concentrated on terrestrial, estuarine and mangrove

actinomycetes (Batra et al. 1972, Alavandi et al. 1995, Kala and Chandrika, 1995, Vikineswary et al. 1997, Sivakumar, 2001). Ellaiah and Reddy (1987) reported actinomycetes from the marine sediments collected from Visakhapatnam coast. Taxonomy and antibiotic production studies on the actinomycetes from Indian shallow sea sediments were carried out by Balagurunathan (1992) and Balagurunathan and Subramanian (1993). Kerkar et al. 1994 isolated an antibiotic-producing marine *Streptomyces* sp. from an intertidal zone of the Andaman Islands. Presently studies are mainly concentrated on the isolation and characterization of antimicrobial compounds and extra-cellular enzymes producing marine actinomycetes from Indian EEZ (Patil et al. 2011, Sharma and Pant, 2001, Sujatha et al. 2005, Kathiresan et al. 2005, Sivakumar et al. 2005, Murugan et al. 2007, Ramesh et al. 2007, Gopalakrishnan and Philip, 2007, Sahu et al. 2007, Vijaya kumar et al. 2007). It was found that halophilic microorganisms produce hydrolytic enzymes which can lead to precipitation of proteins. Moreover, sea water is closer to human blood plasma, and so these microorganisms are expected to produce products including enzymes which are less toxic and have low or zero side effect when a therapeutic application to humans are performed (Sabu et al. 2003). Dhanasekaran et al. (2005) isolated actinobacteria from soil samples belonging to the saltpan regions of Cuddalore, Parangipettai and observed dominance of *Streptomyces* spp. Vijaya kumar et al. (2007) have recorded 192 actinomycetes colonies from 18 marine sediment samples from the Palk Strait region of Bay of Bengal where *Streptomyces* show a clear dominance. Rajasegar et al. 2008 studied the distribution of sediment nutrients in the Vellar estuary and reported higher total organic carbon during monsoon season. Shirodhkar et al. (2009) and Sundaramanikam (2008) investigated and correlated the seasonality and nutrient influx on streptomycete in intertidal

regions and described the importance of Monsoon season. Actinomycetes can be isolated from soil and marine sediments. Meena et al. 2013 isolated 26 actinobacterial strains consist of potential *Streptomyces* spp. with very good enzyme activity especially lipase activity from the marine sediments collected from various sites of Port Blair. According to Gnanam et al. 2013, factors like sediment structure, nutrient availability, Total organic carbon, and total nitrogen have a high influence on the distribution of actinobacteria. Nithya et al. (2015) has isolated actinobacteria from different desert soil samples and morphologically identified. Rajani and Reshma, (2016) isolated actinobacteria from various aquatic habitats and interpreted that these might have been washed in from surrounding terrestrial habitats. Garbeva et al. (2004) and Gonzalez-Franco et al. (2009) reported that actinomycete counts were higher in rhizosphere soils and also described clayey soil had a stronger effect over the microbial structure than sandy soils. At the subtropical dessert soil Thar, Rajasthan, *Streptomyces* spp. were dominant (Cundell et al. 2016). Priyadharsini et al. (2016) isolated *Streptomyces* from Paddy fields. Low temperature, high salinity, the absence and high pressure are characteristic the marine environment, and heterotrophic bacteria are well adapted to them.

Research on actinobacteria is very few from Andaman and Nicobar Islands. Chandramohan and Nair (1992) isolated 69 strains of potential Streptomycetes from the sediments of the Andaman and Nicobar Islands and studied their ecological distribution. Chinnaraj and Untawale (1992) isolated 9 actinobacteria from the submerged part of mangroves from the Andamans. The total organic carbon and nitrogenous nutrients significantly influence actinobacterial population (Ghanem et al. 2000, Adinarayana et al. 2007, Vijaya Kumar et al. 2007, Manivasaganm, 2009 and Gnanam et al. 2013). The mangrove ecosystem is an extensively unexplored

source for actinomycetes with the potential to produce secondary metabolites of biological importance. In Andaman and Nicobar Islands Sivakumar et al. (2005) isolated actinomycetes from the coral reef environment of Little Andaman Island. According to the various studies conducted earlier by Sujatha et al. (2005), Ramesh et al. (2009), Suthindhiran et al. (2010) in the Andaman Islands showed *Streptomyces* as dominant genera and their occurrence in the Bay of Bengal. The population density of actinobacteria from the islands of the Little Andaman assessed by Sahu et al. (2007), Swarnakumar et al. (2008). The ecology and a population density of marine actinobacteria from twelve stations in the Little Andaman Island were reported by Sethupathi et al. (2013). Gopalakrishnan et al. (2013a) have studied the microbial status of the coastal habitats of the Havelock Island, the Andamans. Human impact and presence of bacterial pathogens on the limestone cave of the Baratang Island, the Andamans was reported by Gopalakrishnan et al. (2013b). Samples collected from stations Yerratta mangrove area, Austin Creek and Aerial bay jetty were found to be rich sources of actinobacteria compared with the sandy areas like Kalipur 2, Carbyn's cove (Gopalakrishnan et al. 2013a).

The present study indicates that the anthropogenic activities like tourism started effecting pristine Islands of Andaman. Anthropogenic activities are one of the major reasons for high organic content as well as eutrophication, which lead to an increase in the presence of microbes. It is evident that where there are substrates, present naturally induce the production and activity enzymes.

## 1.3. Objectives of the Study

1. Distribution of Actinobacteria with special reference to *Streptomyces* spp. of Andaman Coast
2. Screening and Media Optimisation for Lipase producing *Streptomyces* spp.
3. Partial Characterization of Lipase enzyme produced from *Streptomyces albus* ST04

## 1.4. Organization of Thesis

The thesis consists of five chapters. Chapter 1 discuss the General insight about marine Actinobacteria, their distribution, and Bio potentiality. Chapter 2 gives an account Distribution Streptomycetes of marine actinomycetes in the coast of Andaman. Screening and Media Optimisation for Lipase producing *Streptomyces* spp. described in Chapter 3. Chapter 4 explores the partial Characterization of Lipase enzyme produced from *Streptomyces albus*. Chapter 5 is dedicated to Summary and Conclusion and followed by References and Appendices.

# Chapter 2

# Distribution of Streptomyces in the Coastal Sediments of Andaman

## 2.1. Introduction

Actinobacteria, the inhabitants of terrestrial as well as marine habitats, are well known for their production of secondary metabolites which forms the basis for many antibiotics. It has been emphasized in many studies that only approximately 10% of the total available actinomycetes could be isolated from nature (Goodfellow and Haynes, 1984). Although they have been prolifically abundant in terrestrial soil, marine ecosystems have now been considered a goldmine of their untapped source of novel microorganisms with the potential for the production of important secondary metabolites (Solecka et al. 2012). Actinobacteria among such microorganisms are of exceptional interest, as well as known hyper-producers of diverse compounds having immense biological activities (Manivasagan et al. 2009; Solecka et al. 2012).

It is estimated that about 7,600 different bioactive compounds have their source from *Streptomyces* species of actinomycetes (Berdy 2005). Being potent producers of antibiotics, like neomycin and chloramphenicol, most of them have the capacity to synthesize wide range of biologically active compounds that are either antitumor/ anticancer/ antimalarial/ anti-inflammatory as well as immunosuppressive agents and lytic enzymes (Alderson et al. 1993, Sanglier et al 1993, Kieser et al. 2000). These along with other categories of extracellular metabolites assist them to survive in the environment they inhabit. Due to these capabilities, *Streptomyces* can survive in all types of environments and thus forms the largest genus within the family Streptomycetaceae (Kampfer et al. 1991).

The pioneering works on actinobacterial diversity from Indian waters were of Baam et al. (1966), who isolated and characterized antagonistic actinobacteria from the Bombay coast, Lakshmanaperumalsamy (1978) studied 518 strains of Streptomyces

from the coastal sediments of Parangipettai. A similar study by Vanajakumari (1979) unearthed the actinobacterial diversity in marine molluscs from Parangipettai.

Streptomycetes are interesting microbes, distributed in all possible habitats and are found to exist and flourish in these environments through suitable adaptations. They inhabit mostly in the soil as well as decaying vegetation and are characterized by supporting complex secondary metabolism. The specific characteristics include the formation of spores and production of a volatile metabolite, geosmin which is responsible for the distinct "earthy" odour. Garbeva et al. (2004) and Gonzalez-Franco et al. (2009) reported the highest abundance of actinomycete in rhizosphere soils in comparison with bulk soils irrespective of seasons.

The combination of nutrients in each habitat influences Actinobacteria especially *Streptomyces* spp. to produce different types of extracellular metabolites for their subsistence. *Streptomyces* spp. are responsible for the production of 80% of antibiotics in the world because they can decompose a variety of recalcitrant compounds including lignocelluloses, other biopolymers and xenobiotic compounds which occur in soil and litter (Crawford 1978; Goodfellow and Simpson 1987; Warren 1996; Schrijver and Mot 1999). Meena et al. (2013) isolated 26 actinobacterial strains of potential *Streptomyces* spp. with excellent enzyme activity especially lipase activity from the marine sediments of the coastal regions of Port Blair.

Studies have shown that various habitats differ in the composition as well as an abundance of actinobacteria. The combination of multiple species in each habitat is defined by the capabilities of them to coexist with other fauna and flora as well as the prevailing environmental parameters. The parameters like depth, light

penetration, salinity, pH, pressure, dissolved oxygen, micro, and macronutrients, etc. influence the distribution of Actinobacteria. The distribution of microbes is also affected by structure and type of sediments. Similarly, the nutrient content in the sediment especially, of a higher percentage of small particles like clay, clay-silt or clay Silt showed more abundance and diversity of microbes (Garbeva et al. 2004). Gonzalez-Franco et al. (2009) reported that the winter season showed lesser Actinomycete diversity than other seasons like spring, it may be due to lower nutrients in rhizosphere soil.

Aquatic microbes are generally influenced by salinity rather than other environmental parameters. The *Streptomycete* spp. shows wide tolerance to salinity. Some of the *Streptomyces* spp. isolated from marine sediment can even tolerate zero salinity which justifies their terrestrial origin (Jensen et al. 1991). The *Streptomyces* spp. were found mainly distributed in the nearshore waters, and it is presumed that these actinobacteria are terrestrial forms, washed off to coastal waters. Those forms which are living in the intertidal region are generally tolerant to fluctuations in various environmental conditions. In tropical areas, seasons have great influence on the diversity of terrestrial as well as aquatic fauna and flora including microbes. In India, the monsoon, South West Monson as well as North East Monsoon influence the variations in the environmental parameters including nutrients distribution, especially in the nearshore waters.

The Andaman and Nicobar islands are situated between Lat. 6 $^{0}$ 45 N-13 $^{0}$ 41 E and Long. 92 $^{0}$ 57-93 $^{0}$ 57 E in the southeast of Bay of Bengal (Kar et al. 2011). The archipelago consists of a group of fragile ecosystems like coral, rocky or/and sandy intertidal regions as well as fringed with rich mangrove habitats and considered to be one among the most pristine diversity hotspots in the world. They comprise more

than 572 scattered islands, comprising the Andaman Islands with a total area of approximately 6408 $km^2$ and the Nicobar Islands with 1814 $km^2$. Both groups of islands are hilly type terrain except Car Nicobar (Dam Roy et al. 2009). The Andaman and Nicobar Islands endowed with extensive mangrove ecosystems in Andaman (929 $km^2$) and Nicobar Islands (37 $km^2$) (Balakrishnan 1989; Andrews and Sankaran 2002; Ragavan et al. 2014). Coastal regions of Andaman and Nicobar Islands are endowed with clean beaches as well as clear and less polluted waters compared to other coastal regions of India. The region includes mangrove area with highly productive soil, sandy beaches, rocky coastal areas and coral reefs with less anthropogenic activities as well as fish landing centres, harbours, residential areas, market areas and agricultural lands where anthropogenic activities are high. The most extensive coastal landforms in the Islands are different types of beaches (Garzanti et al. 2013) along with mangroves, coral and rocky shores which support a unique diversity of flora and fauna. Due to the highest and unique species diversity, these island groups are treated as a distinct ecoregion and are classified as one of the 12 biogeographical zones of India (Rogers and Panwar, 1988).

Even though many studies on bacterial diversity and distribution from Andaman and Nicobar region are available (Swarnakumar et al. 2008; Gopalakrishnan et al. 2013a), the information on Actinobacteria are scarce. The few studies comprised the work on the ecological distribution of *Streptomyces* spp. from marine sediments, submerged part of mangroves from various parts of Andaman including Little Andaman Island (Chandramohan and Nair 1992; Chinnaraj and Untawale 1992; Sahu et al. 2007; Sujatha et al. 2005; Ramesh et al. 2009; Suthindhiran et al. 2010; Gopalakrishnan et al. 2013 a&b). It was reported that the frequency and dominance of *Streptomyces* spp. were found to be varied among different substrate sources in

Andamans (Vijay Kumar et al. 2007). The type of sediments, nutrients especially calcium, magnesium, fluoride, inorganic phosphate, nitrite, phosphorus, potassium, TOC concentrations and presence of heavy metals influence bacterial abundance in the Andaman Sea (Noronha et al. 1981; Sethupathi et al. 2013).

The present chapter gives a detailed account on the depth-wise occurrence of marine Actinobacteria along the coastal sediments of Andaman Islands as well as the effect of environmental parameters and their seasonal fluctuations on its abundance in various habitats.

## 2.2. Materials and methods

### 2.2.1. Study Area

The present study was carried out along the coastal waters of Andaman group of islands (Fig. 2.1.). The sampling was conducted from three major zones viz. South (Port Blair to Baratang), Middle (Baratang to Billiground) and North Andaman (Between Billiground to Diglipur). South Andaman stations were Burmanallah, Carbyn's Beach, Chattam, Chidiyatappu, Dignabad, Junglighat, Kodiyaghat, Marina Park, Science Center, Minni Bay, Sippighat, Collinpur Beach and Wandoor. Middle Andaman stations were Billiground, Betapur, Yeratta, Bakultala, Rangat, Nimbutala, Kadamtala, Karmatang, Austin Creek and Mayabunder. North Andaman stations were Kalighat, Aerial Bay Jetty, Durgapur, Kalipur, Shyam Nagar, Kalighat 2, Kalipur 2, Durgapur 2 and Shibpur (Plate 2.1.-2.4.).

**Figure 2.1.A. The Andaman Islands showing three zones – North, Middle, and South Andaman**

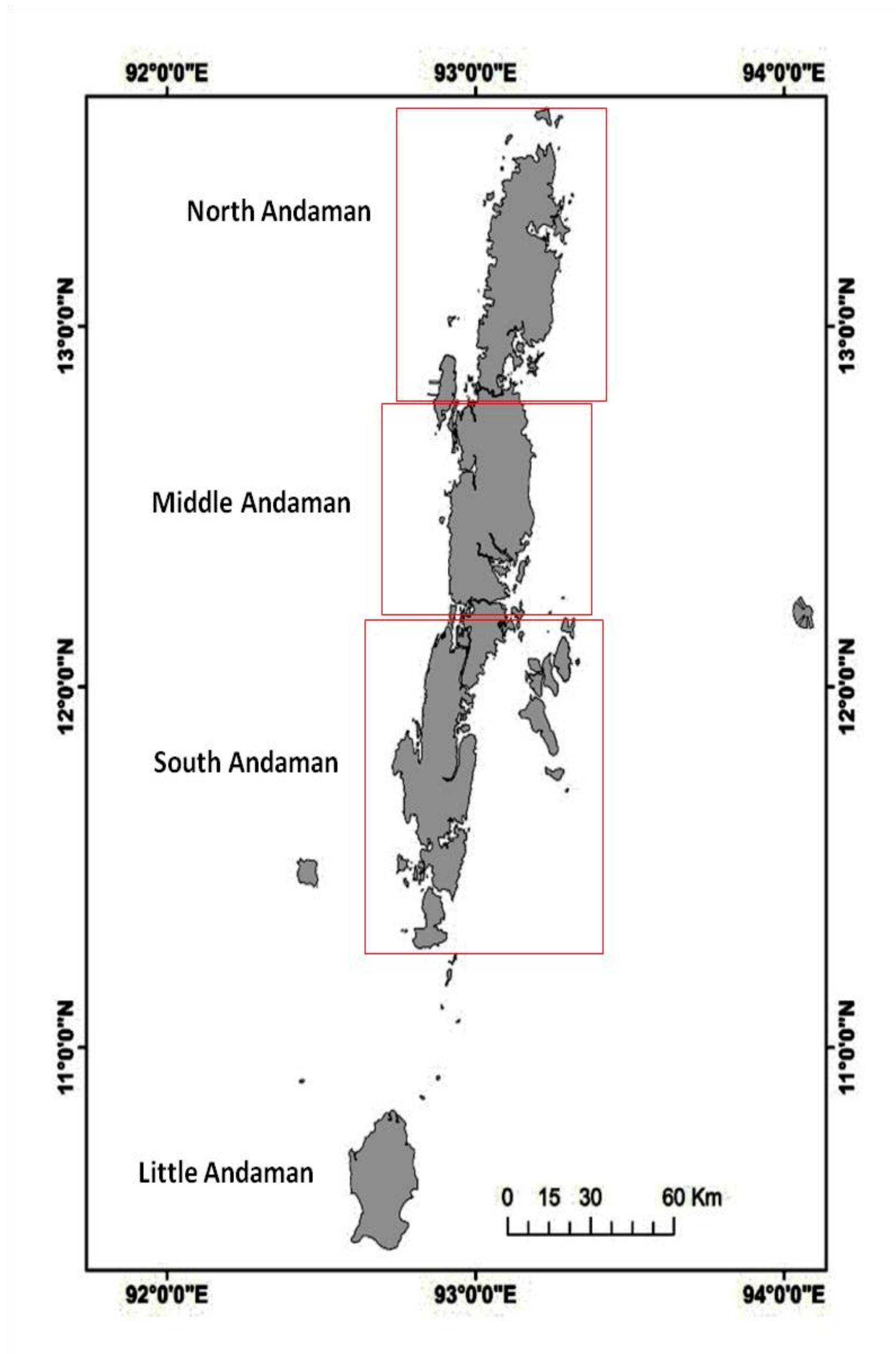


**Figure 2.1.B. South Andaman**

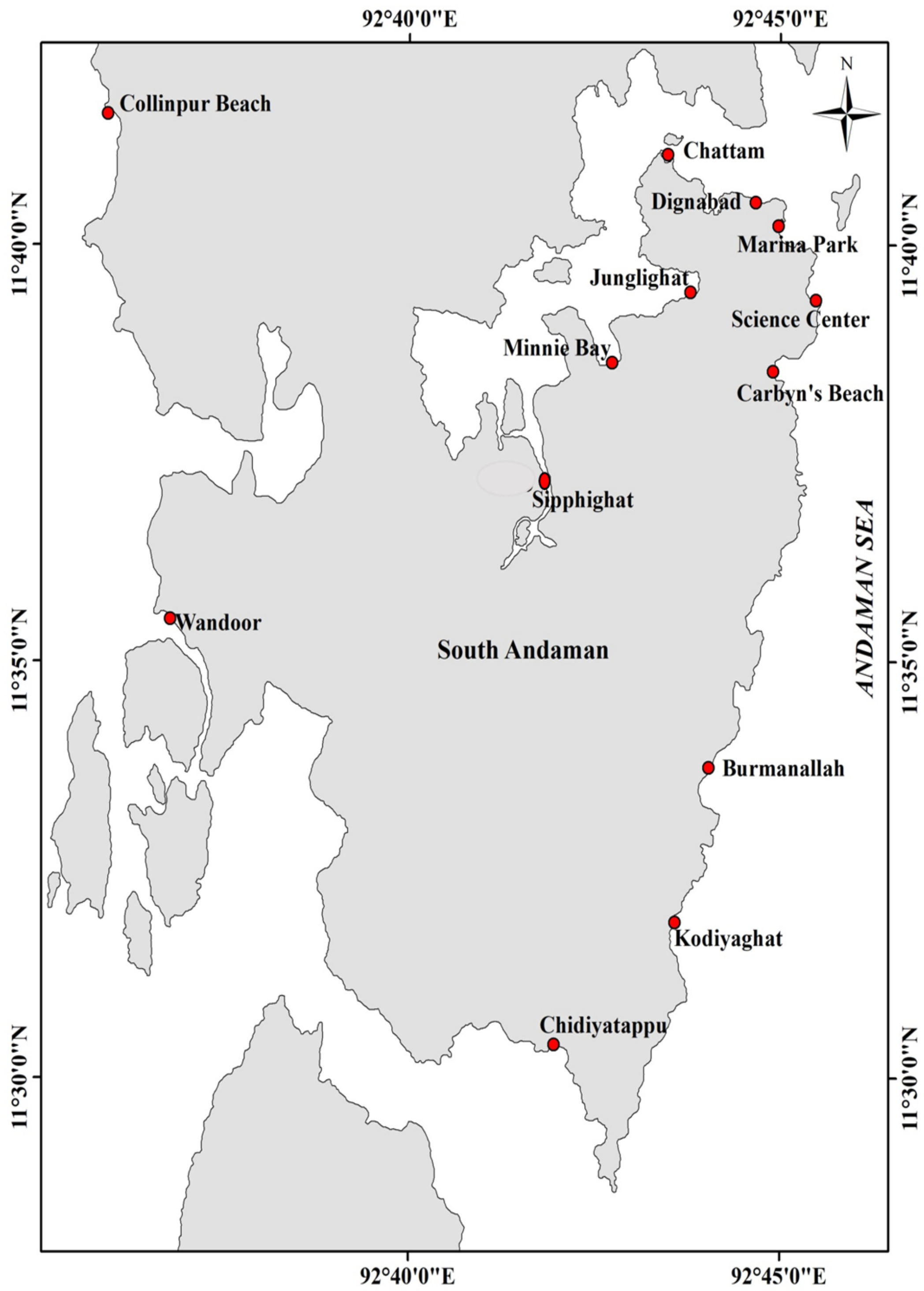


**Figure 2.1.C. Middle Andaman**

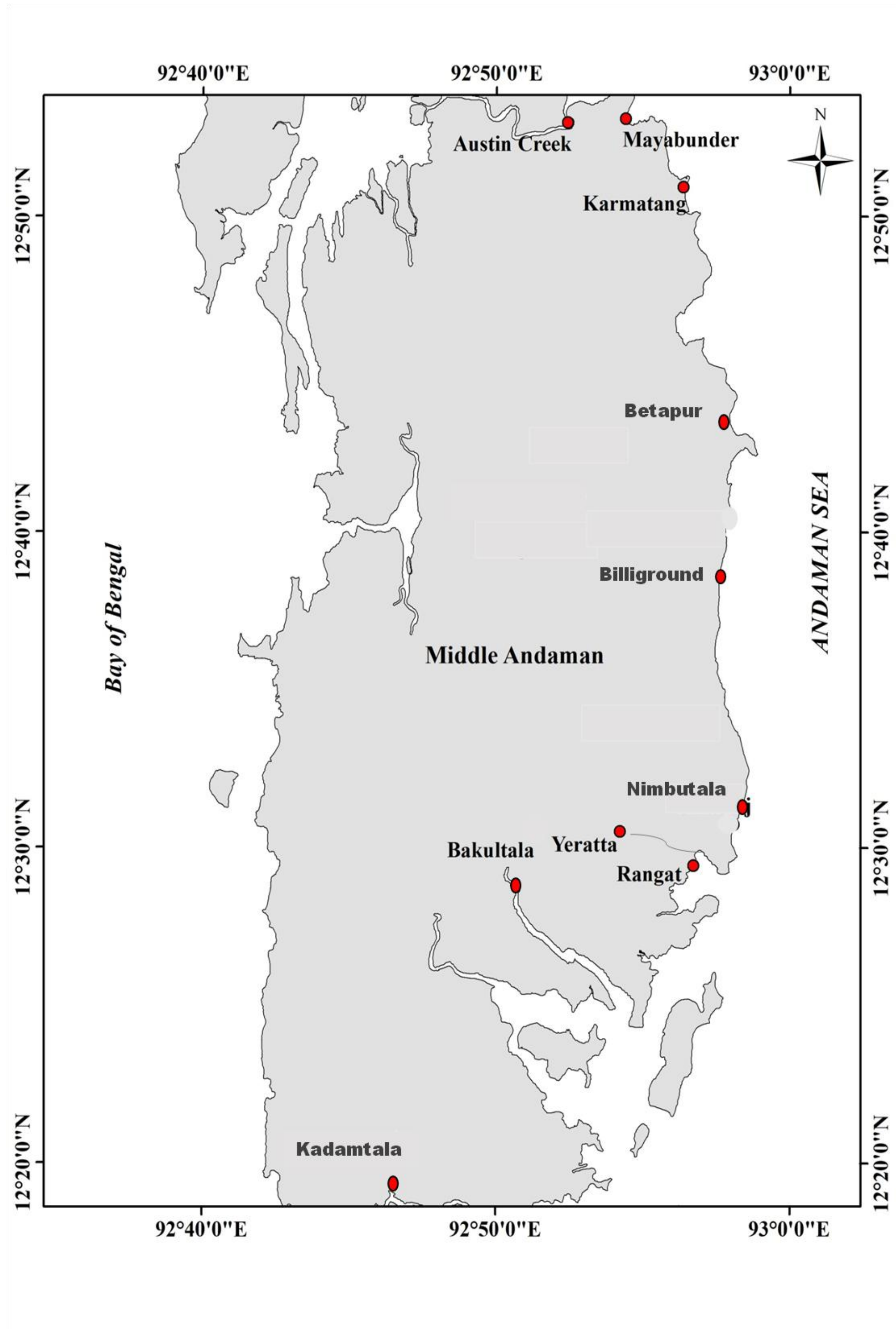
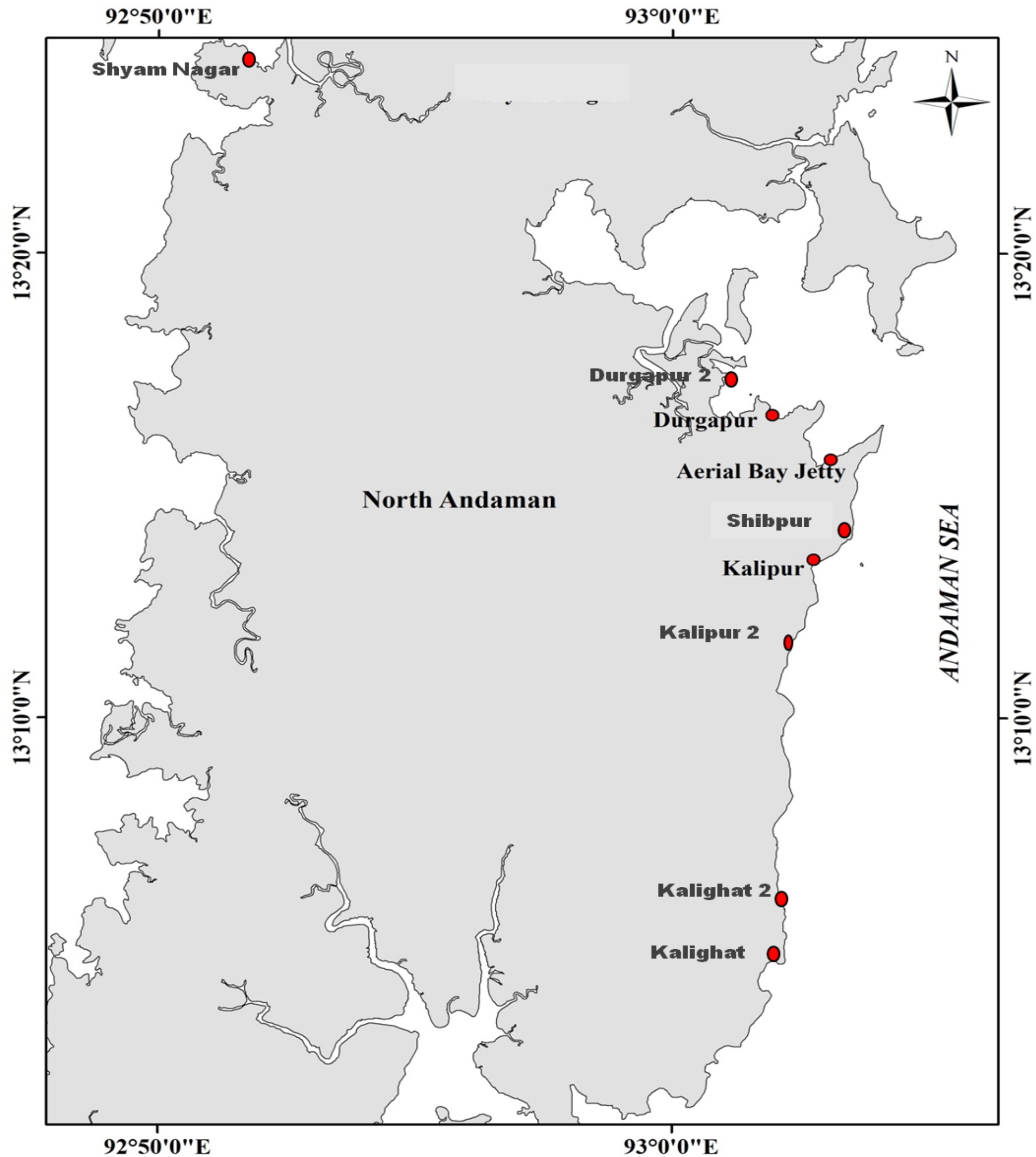


**Figure 2.1.D. North Andaman**




Mangrove – coral, mangrove – rocky shore, mangrove, coral – rocky shore interactions were chosen while fixing the stations for adequate representation of various types of ecological systems and their combinations. The areas where anthropogenic interactions like tourism, fishing activities, and waste disposal are experienced were also considered while fixing the stations (Table 2.1.).

**Table 2.1. General features of the stations selected for sampling**

| Station | Mean S‰ | Mean S.S.T. °C | Mean Water pH | Sediment Properties (Mean Values) | | | Seabed Characteristics* | Isolates Mean cfu/g (%) |
|---|---|---|---|---|---|---|---|---|
| | | | | pH | TOC % | Texture | | |
| **South Andaman** | | | | | | | | |
| Burmanallah | 32 | 26.7 | 7.9 | 5.8 | 1.2 | Silty Clay | R, M | 8 |
| Carbyn's Beach | 31 | 26.7 | 8.1 | 6.7 | 0.6 | Sandy | M, R, S | 2 |
| Chattam | 33 | 26.2 | 8.0 | 7.3 | 1.1 | Sandy Muddy | S | 3 |
| Chidiyatappu | 33 | 26.2 | 8.0 | 5.9 | 0.9 | Clayey Silt | M, C | 4 |
| Dignabad | 32 | 27.0 | 8.1 | 5.2 | 0.6 | Sandy Clay | S | 4 |
| Junglighat | 31 | 26.7 | 7.8 | 5.9 | 0.8 | Muddy Sandy | S | 4 |
| Kodiyaghat | 33 | 27.2 | 7.9 | 7.4 | 0.7 | Sandy Silt | M, R | 2 |
| Marina Park | 32 | 27.0 | 8.3 | 6.2 | 0.5 | Sandy Clay and Silt | S, R | 6 |
| Science Center | 34 | 27.7 | 8.1 | 6.3 | 0.5 | Sandy Clay Silt | R, S | 2 |
| Minnie Bay | 33 | 26.8 | 8.2 | 7.8 | 0.5 | Clayey Silt | M | 1 |
| Sippighat | 31 | 27.3 | 8.0 | 6.5 | 0.6 | Clayey Silt | M | 3 |
| Collinpur Beach | 32 | 27.2 | 7.9 | 6.7 | 0.5 | Sandy Silt | M, S | 1 |
| Wandoor | 32 | 27.0 | 7.9 | 7.1 | 0.6 | Sandy Silt | M, S | 2 |
| **Middle Andaman** | | | | | | | | |
| Billiground | 30 | 27.2 | 7.5 | 6.4 | 0.5 | Sandy | S, with Shells | 2 |
| Betapur | 29 | 26.8 | 7.5 | 6.8 | 0.5 | Sandy Silty | S | 4 |
| Yeratta | 32 | 26.0 | 7.9 | 5.6 | 2.0 | Clay Silty | M | 6 |
| Bakultala | 32 | 26.7 | 8.0 | 6.9 | 1.1 | Clay sandy | M | 3 |
| Rangat | 32 | 26.8 | 7.3 | 6.8 | 1.3 | Clay Silty | M | 3 |
| Nimbutala | 31 | 27.0 | 7.9 | 6.7 | 1.2 | Silty clay | M | 3 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Kadamtala | 32 | 26.7 | 7.9 | 6.9 | 0.9 | Clay Silty Sandy | M | 2 |
| Karmatang | 33 | 27.3 | 7.9 | 6.7 | 1.7 | Sandy Slay | M, R | 3 |
| Austin Creek | 33 | 27.7 | 7.8 | 7.1 | 0.6 | Sandy | M | 5 |
| Mayabunder | 32 | 26.5 | 8.0 | 6.7 | 1.0 | Coarse Sandy | M, R | 6 |
| **North Andaman** | | | | | | | | |
| Kalighat | 30 | 27.0 | 8.0 | 6.2 | 0.8 | Gravel Sandy | S, R | 3 |
| Aerial Bay Jetty | 31 | 27.3 | 7.3 | 5.9 | 0.8 | Muddy Sandy | M, S | 4 |
| Durgapur | 32 | 27.5 | 8.0 | 6.6 | 0.7 | Sandy | S | 1 |
| Kalipur | 32 | 28.0 | 8.0 | 7.2 | 0.6 | Sandy clay | S | 3 |
| Shyam Nagar | 33 | 26.7 | 7.8 | 7.0 | 1.1 | Clay Silty Sandy | S, M | 2 |
| Kalighat 2 | 33 | 26.7 | 7.9 | 6.6 | 0.8 | Silty Sandy | S | 2 |
| Kalipur 2 | 33 | 26.8 | 7.9 | 6.9 | 0.7 | Silty Sandy | S | 3 |
| Durgapur 2 | 34 | 27.2 | 7.9 | 6.5 | 1.1 | Silty sandy | S | 3 |
| Shibpur | 34 | 27.2 | 7.9 | 6.3 | 0.5 | Coarse Sandy | S | 2 |

***S = Sandy; R = Rocky; M = Mangrove; C = Coral**

### 2.2.2. Sampling procedure

Sediment samples were collected from 32 stations at 0-1m, 5-6m and 10-11m depths during 2013-14 and 2014-15. Sampling was conducted three times in a year (three seasons) during Pre-Monsoon or Dry season (February to May second week), South West Monsoon (May Third week to September) and North West Monsoon (October to January).

Air and water temperature measurement using a mercury thermometer, pH estimation using pH meter (Model – ESICO 1010) and Salinity measurement using hand-held refractometer (Model- ERMA) were carried out at each station at the time

of sampling. Total Organic Carbon estimation was carried out following Schumacher et al. (2002). The Nitrite, Phosphate, and Silicate were analyzed following Strickland and Parsons (1972).

At each depth, triplicate samples of sediment and water were collected following aseptic microbiological sampling methods (Sivakumar et al. 2005, 2007). So, altogether nine samples of sediment and water were collected from each station during a season. These samples were analysed separately for the marine actinobacterial load (Sivakumar et al. 2007). The samples from 0-1m were collected from the shore directly. While the samples from 5-6m and 10-11m were collected by skin or scuba diving (Plate 2.5., Fig. 33-34), water samples (50ml) were collected using sterilized sampling bottles without any air bubbles to avoid contamination. Separate 1000ml samples were also collected for analysis for assessing environmental parameters. Sediment samples (100g) from the first 5cm surface were collected using a sterilized spatula and was transferred to a sterile polyethylene zip bag, and the samples were taken to the laboratory inside an ice box with ice packs for further analyses.

### 2.2.3. Pre-treatment of samples

As it is assumed that other bacteria outnumber the actinobacterial population in the habitat, many selective isolation methods have been employed to eliminate undesirable bacteria. Thus the soil samples were subjected to a series of pre-treatment methods such as:

1. **Heat treatment method** (Tsao et al. 1960)

The sediment samples were inoculated at room temperature as well as after heat treatment at different temperatures viz. 40, 50, 70, 100 and 120$^{o}$C for 1 hour in a hot

air oven. The treated samples were plated in Starch Casein Agar media to isolate Actinomycetes (Sivakumar et al. 2005).

2. **Phenol treatment 1.5%** (Lawrence, 1956)

Sediment samples were air dried and inoculated in Starch Casein Agar media containing Nalidixic acid, phenol (1.5%) and cycloheximide (50ug) for avoiding fungal contamination. The colonies with powdery, leathery appearance oppressed to media were selected for further purification. Morphological characters like types of substrate mycelia and aerial mycelia, temperature tolerance at 27 - 65$^{o}$C, the colour of spores, aerial and substrate mycelia were used for identification of isolated colonies.

3. **Calcium Carbonate Enrichment** (Jensen et al. 1991)

Calcium Carbonate enrichment was done with the treatment of 2% $CaCO_3$ on air-dried sediment samples. The inoculated plates were incubated at 37°C for 10-15 days. The purified isolates were stored in -80 $^{o}$C for further analysis.

The Actinobacteria (1083 Nos.), isolated and maintained in the Microbiology Laboratory of MES College, Ponnani from nearshore sediments of Andaman and Nicobar Islands were used for the present study.

### 2.2.4. Purification of Isolates

The purification of Actinomycetes was done by streaking on nutrient agar and stocked in soft nutrient agar vials with overlaid sterile liquid paraffin (Jensen et al. 1991). Nutrient Agar slants used for maintaining working cultures were preserved under refrigeration at 4°C.

### 2.2.5. Morphological and Cultural Characterization

The features of Morphology, physiology, and biochemistry of the strains were recorded as per International Streptomyces Project (Shirling and Gottlieb, 1966) and Bergey's Manual of Systematic Bacteriology (Williams et al. 1989).

The isolates were streaked on to different media as follows:

1) Starch casein agar (Starch 10.0 g; Casein 1.0 g; $K_2HPO_4$ 0.7 g; $KH_2PO_4$ 0.3 g; $MgSO_4.7H_2O$ 0.5 g; $FeSO_4.7H_2O$ 0.01 g; $ZnSO_4$ 0.001 g; Agar 20.0 g; seawater 1L; pH 7.2)

2) Yeast extract-malt extract agar (ISP 2) (Yeast extract 4.0 g; Malt extract 10.0 g; Dextrose 4.0 g; Agar 20.0 g; seawater 1L; pH 7.2)

3) Glycerol asparagine agar (ISP 5) (L- Asparagine 1.0 g; Glycerol 10.0 g; $K_2HPO_4$ 1 g; Agar 20.0 g; seawater 1 L; pH 7.2) and

4) Nutrient agar (Peptone 0.5 g; Beef extract 0.3 g; Agar 2 g; seawater 100 ml)

#### 1. Morphology

The colony characteristics like mature sporulating aerial mycelium colour, the structure of substrate mycelium, macromorphology, diffusible pigment, colony reverse colour, colony texture, etc. were recorded after observing the plates under a research microscope (Olympus CX21i) following Tresner and Backus (1963).

#### 2. Coverslip Culture technique

The filamentous actinomycetes morphology was studied by employing coverslip culture. The inoculation of isolates was carried out in marine Actinomycete broth media and was incubated for 1-2 days at 28°C. Plates were prepared in Casein starch peptone yeast malt extract agar medium (Casein 3.0 g; Maize starch 10.0 g; Peptone 1.0 g; Yeast extract 1.0 g; Malt extract 10.0 g; $K_2HPO_4$ 0.5 g; agar 20 g; sea water 1 L; pH 7.4). Sterile coverslips (3-4) were prepared by autoclaving and were inserted

into the agar medium at an angle of 45°. The inoculation was done by dispensing a loop full of Actinomycete spore suspension at the point where the medium and coverslip joints. The incubation of plates was carried out for 4-8 days at 28°C, and the coverslips were removed after incubation and were observed under microscopes with high power oil immersion objectives (100X). Morphology of aerial as well as substrate mycelia, sporogenous hyphal arrangement and morphology (straight, flexuous, spiral-shaped) were recorded following ISP (Shirling and Gottlieb, 1966; Nonomura 1974).

**3. Biochemical characterization**

The physiological and biochemical tests for characterization was carried out following Berd (1973).

**4. Decomposition of organic substrates**

a) Tyrosine: Tyrosine (0.5 g) was mixed with 10 ml of distilled water and autoclaved. This suspension was poured into 100 ml sterile nutrient agar media (agar 20 g; beef extract 3.0 g; seawater 1 L; peptone 5.0 g; pH 7.2) at 50°C, thoroughly mixed and plated. After spot inoculation of isolates, plates were incubated at 28°C for 5-7 days. The positive result was taken as the clearing zone around the actinomycete colony.

b) Hypoxanthine: Autoclaved hypoxanthine (0.5 g) was mixed with 10ml of distilled water and was mixed with 100 ml of sterile nutrient agar at 50°C. The media was further mixed thoroughly and poured into petri plates. After spot inoculation of isolates, plates were incubated at 28°C for 5-7 days. The positive result was taken as the clearing zone around the actinomycete colony.

c) Xanthine: Ten millilitre of distilled water was mixed with 0.4 g Xanthine and autoclaved. This suspension was mixed with sterile nutrient agar media (100 ml) and

poured into petriplates. After spot inoculation of isolates, plates were incubated at 28°C for 5-7 days. Clearing zone around the Actinobacterial colony was recorded as positive.

d) Casein: Decomposition of casein was done in skim milk agar. 10 ml of skim milk was autoclaved with 100 ml distilled water. To 100 ml of seawater, 2g of agar was added and autoclaved separately. Two components were mixed thoroughly and poured into petri plates. After spot inoculation of isolates, plates were incubated at 28°C for 5-7 days. Clearing zone around the Actinobacterial colony was recorded as positive.

e) Esculin: When Esculetin reacts with ferric ammonium citrate, a dark brown to the black complex will be produced in the medium. Slants were prepared with esculin (0.1%) agar media (agar 20 g; esculin 1 g; peptone 10 g; seawater 1 L; ferric ammonium citrate 0.5 g; pH 7.2). Inoculation was done with loopful of actinomycete spores in these slants. The control slants were prepared with the same agar base without esculin were also inoculated. The incubation for the slants was carried out for 1-2 weeks at 28°C. Blackening of the slants was observed as positive.

**5. Lysozyme resistance**

Sterile glycerol broth (peptone 5 g; glycerol 70 ml; sea water 1000 ml; pH 7.2) and 5 ml of lysozyme solution (100 mg of lysozyme [Himedia] in 100 ml of 0.01 N hydrochloric acid sterilized by seitz filtration) were mixed and was poured in test tubes. One drop of culture suspension using Pasteur pipette was inoculated to these tubes as well as controls containing glycerol broth without lysozyme. Weekly readings were done for two weeks. Good growth in both tubes was taken as positive and negative if growth was good in the control tube but poor or absent in other tubes with lysozyme.

**6. Urea hydrolysis**

Production of Urea was tested using Christensen's urea agar medium (yeast extract 0.1 g; Agar 20 g; NaCl 20 g; $KH_2PO_4$ 9.1 g; Phenol red 4 ml of 0.25% solution; $Na_2HPO_4$ 9.5 g; Urea 20 g; distilled water 1000 ml; pH 6.8). Dissolved all ingredients other than urea in 950 ml of distilled sea water and sterilized in an autoclave at 15 lbs for 15 minutes. Sterilized urea using diethyl ether was dissolved sterile distilled water (50 ml). This urea suspension mixed with basal medium was used for preparing slants (3 ml each). Pink colour formation in the medium after incubation indicated urea hydrolysis by the colony.

**7. Nitrate reduction**

Nitrate broth supplemented with potassium nitrate (0.1%) was used for nitrate reduction test. An inverted Durham's tube was introduced into the basal medium (Peptone 5.0 g; Potassium nitrate 1.0 g; Beef extract 3.0 g; seawater 1L; pH 7.2). The medium was inoculated with a spore suspension of isolates and incubated for one week with un-inoculated tubes as a negative control. Equal volumes of reagents A (α-naphthyl amine 0.5 g; acetic acid (5 N) 100 ml) and B (sulfanilic acid 0.8 g; acetic acid (5 N) 100 ml) added to the inoculated tubes to detect Nitrate reduction. The red colour formation due to azo dye indicated nitrate reduction positive. If colourlessness observed in the suspension after the addition of reagents, a small pinch of zinc dust was added to the medium to find if the medium remained colourless for a positive result. Turning medium into pink after the addition of zinc powder was considered the negative result.

**8. Hydrogen sulfide production**

Sterile lead acetate strips (5%) were inserted through the mouth of the test tube when the nitrate reduction test is carried out to test Hydrogen sulphide production. A positive result is the blackening of the strips.

**9. Citrate utilization**

Simmon's citrate agar medium (HiMedia) ($(NH_4)_2HPO_4$ 1.0 g; Sodium citrate 0.2 g; $K_2HPO_4$ 1.0 g; $MgSO_4.7H_2O$ 0.02 g; NaCl 15.0 g; bromothymol blue 0.02 g; Agar 20 g; seawater 1 L; pH 6.9) was used for testing citrate utilization. The colour change from green to Prussian blue of the medium was recorded as a positive result.

**10. Melanin production**

Peptone yeast extract iron agar (ISP6) (Peptone 15 g; Ferric ammonium citrate 0.5 g; Sodium thiosulphate 0.08 g; Dipotassium phosphate 1 g; Protease peptone 5 g; Yeast extract 1.0 g; Agar 20 g; pH 7.2; sea water 1L) and Tyrosine agar (ISP 7) (Glycerol 15.0 g; $MgSO_4.7H_2O$ 0.5 g; L-Asparagine 0.5 g; $K_2HPO_4$ 0.5 g; L-Tyrosine 0.5 g; $FeSO_4.7H_2O$ 0.01 g; seawater 1 L; Agar 20 g; pH 7.2) was used to test melanin production ability. The slants were inoculated with loopful of spores and incubated for 5-7 days at 28°C. The positive test was determined by the formation of brown to back diffusible pigment in the medium.

**11. Carbohydrate utilization**

The utilization of carbohydrate was tested following the International Streptomyces Project (Shirling and Gottlieb, 1966). Carbohydrate utilization kit (HiMedia) was used for testing utilization of carbon sources like glucose, trehalose, mannitol, sucrose, lactose, arabinose, inositol, sorbitol, ribose, and xylose. Change in colour of the carbohydrate medium from purple to yellow due to acid reaction indicated a positive result.

### 12. Biogranulation property

The ability to produce biogranules (self-immobilization) was tested by inoculation of spores from a single colony into nutrient broth (50 ml) prepared in sea water. The inoculum was incubated on an incubator shaker (150 rpm) for seven days at 28°C. The formation of biogranules of each strain was observed regularly, and the diameter of the biogranules was measured using pipette analysis (Jayaraj et al. 2012). Based on the diameter, the granules were classified into small (0.5-2mm), medium (2-4mm) and large (>4mm).

### 13. Hydrolytic enzyme production

The isolates were screened for production of various hydrolytic enzymes - Protease, Amylase, Lipase, Chitinase, Arylsulfatase, Phosphatase, Ligninase, DNase, Pectinase and Cellulase.

#### a. Amylase activity

Amylase activity was tested using starch (1%) supplemented nutrient agar medium (Agar 2 g; Beef extract 0.3 g; Peptone 0.5 g; sea water 100 ml; pH 7.0). Spot inoculation of the culture plates was followed for testing the activity of enzymes. The inoculated culture plates were incubated for 24 to 48 hours. Grams Iodine reagent (1g Iodine; 2g potassium iodide; 300ml distilled water) was flooded on the plate. The absence of blue color around the culture spot or clearing zone around the culture spot is a positive result for amylase activity.

#### b. Protease activity

Nutrient Gelatin Agar (Peptone – 5gm, Gelatin – 120gm, Beef extract – 3gm, pH 6.8) was used for testing protease enzyme activity. Spot inoculation was done with a loopful of culture along with the spores and incubated for 24 to 48hr. Then the plates

were flooded with mercuric chloride solution (15%) which consequently liquefy gelatin to amino acids to produce a clear zone around culture spot indicated a positive result.

**c. Lipase activity**

Lipase activity of the isolates was determined by inoculating loop full of cultures in Tributyrin Agar (Himedia, Mumbai) incubated for 3 to 5 days. The clearance zone around the colonies was observed as a positive result, and the diameter was recorded.

**d. Pectinase activity**

Production of pectinase was tested using pectin Agar (Pectin 0.5g; $CaCl_2.2H_2O$ 0.02g; NaCl 2g; $FeCl_3.6H_2O$ 0.001g; Agar 2g; yeast extract 0.1g; seawater 100ml; pH 7.0). Spot inoculation was carried out, and the plates were incubated for 5 to 7 days at room temperature (28 ± 2°C). The plates were flooded with 1% Cetavlon (Cetyl trimethyl ammonium bromide), after incubation and were allowed to stand for 20-30 minutes. Clearance zone was observed as positive.

**e. Cellulase activity**

Testing cellulase production was carried out using cellulose agar (Casein hydrolysate 0.05 g Agar 2 g; Cellulose powder 0.5 g Yeast extract 0.05 g; $NaNO_3$ 0.01 g; seawater 100 ml; pH 7.0) medium. Spot inoculated plates were incubated at room temperature for 7 to 10 days at 28 ± 2°C. A positive result was the zone of clearance around the colonies.

**f. DNase activity**

Spot inoculation of isolates was done on DNase agar (Agar 2 g; DNA 0.2 g; Tryptone 3 g; sea water 100 ml; pH 7.0) and incubated for five days at 28 ± 2°C.

The zone of clearance around the colonies after flooding plates with 1 N HCl was recorded as positive.

### g. Arylsulfatase activity

The production of arylsulfatase was tested using nutrient agar (Peptone 0.5 g; Agar 2 g, Yeast extract 0.1 g; Ferric phosphate 0.002 g; seawater 100 ml, pH 7.0) supplemented with Tripotassium phenolphthalein disulfate (PDS). Spot inoculated plates were incubated for 5-7 days at room temperature (28 ± 2°C). The formation of pink colour around the colonies due to the release of phenolphthalein from PDS when the agar plates were exposed to ammonia vapour after incubation was recorded as positive.

### h. Phosphatase activity

The isolates were spot inoculated on nutrient agar basal media supplemented with 0.01% phenolphthalein diphosphate and Incubated for 5-7 days at room temperature (28 ± 2°C). The agar plates were exposed to ammonia vapour after incubation. The development of pink colour around the colonies due to the release of phenolphthalein from phenolphthalein diphosphate was recorded as a positive result.

### i. Ligninase activity

Lignin degradation test was carried out using Crawford's agar (Glucose 0.1 g; Agar 2 g; Yeast extract 0.15 g; $CaCl_2$ 0.05 g; $KH_2PO_4$ 0.1 g; $MgSO_4$ 0.002 g; $Na_2HPO_4$ 0.45 g; sea water 100 ml; pH 7.0) as the basal medium. Supplement of 0.02% methylene blue to the basal medium was done before plating. The spot inoculated plates were incubated for 5 to 14 days at room temperature (28 ± 2°C). Halo zone formation or decolourisation or of methylene blue was considered as a positive result.

### j. Chitinase activity

Spot inoculation on chitin agar media prepared with 5% colloidal chitin in mineral basal media was used for chitinase activity test. The composition of the media is as follows: Colloidal chitin 5g; Agar 2g; $K_2HPO_4$ 0.07g; $MgSO_4.7H_2O$ 0.05g; $MnCl_2.4H_2O$ 0.0001g; $KH_2PO_4$ 0.03g; $FeSO_4.7H_2O$ 0.001g; $ZnSO_4.4H_2O$ 0.0001g; seawater 100 ml; pH 7.0). Zone of clearance after incubation of the plates at 28°C for 7-14 days was recorded as a positive result.

Based on the utilization of carbohydrate viz. D-glucose, L-Rhamnose, D-Fructose, Xylose, D-Galactose, Salicin, Lactose, Raffinose, Sucrose, L-Arabinose, Cellulose and Inositol, similarity tree (NTSYS v 2.02 software) was plotted for preliminary genus identification and grouping.

The cell wall composition was determined by thin layer chromatography. The samples were pelletized and treated with 6N HCl to detect the presence of meso Diaminopimelic acid. Vacuum evaporation method was used for evaporation of HCL present in the sample before spotting on the TLC Plates (Merck). A solvent system comprising of methanol: distilled water: 6N HCl: Pyridine (80:26:4:10) was used for running the plates (5-6 hour). The plates were then sprayed with 0.2% Ninhydrin in acetone after air dried. The development of plates was carried out in a hot air oven at $100^{o}C$ for 3 minutes, and the presence of maroon coloured spots (Sherling and Gotilleb 1968, Jenson et al. 1984) was observed. Statistical analysis was carried out following SPSS Statistics v20 (IBM Corp.) for comparison of means and multiple regression analysis, while Primer v6 (Plymouth Marine Laboratory) was used to study the diversity parameters and Principal Component Analysis was conducted to understand the effect of environmental parameters on the distribution of these actinomycetes.

The genus Streptomyces was identified based on the ISP standards as given in table 2.2. (Williams et al. 1989).

**Table 2.2. Cultural characteristics of Genus *Streptomyces***

| Sl. No. | Characters | Media used | Result |
|---|---|---|---|
| 1 | Colour of aerial mycelium | ISP2 (Yeast extract-Malt extract Agar) | Grey, White, Red, Green, Blue, Violet |
| 2 | Melanin production | ISP 7 (Tyrosine Agar) | Brown, Blackish brown, Greenish brown |
| 3 | Production of Soluble pigment | ISP 2, ISP 5 (Glycerol-asparagine Agar), ISP 7 | Violet, Blue, Yellow, Green, Orange, Red |
| 4 | Morphology of Spore chain | Starch Casein Agar | Recti-Flexibiles, |
| 5 | Utilization of nine different sugars | Himedia HiCarbo $^{TM}$Kit | Arabinose, Zylose, Inositol, Manitol, Fructose, Rhamnose, Sucrose, Raffinose |

## 2.3. Results

### 2.3.1. Composition of Actinobacteria and general distribution

The present study shows that the isolates from the sediments along the Andaman coastal waters included *Streptomyces* spp., *Nocardia* spp., *Actinomadura* spp., *Streptosporangium* spp., *Actinoplanes* spp., *Bifidobacterium* spp., *Streptoverticillium* spp. and *Micromonospora* spp. (Table 2.3.). The results of the morphological and biochemical analysis are shown in Plates 2.5-2.7. (Fig. 35-51).

**Table 2.3. Biochemical characterization of Actinomycetes isolated from Andaman coast**

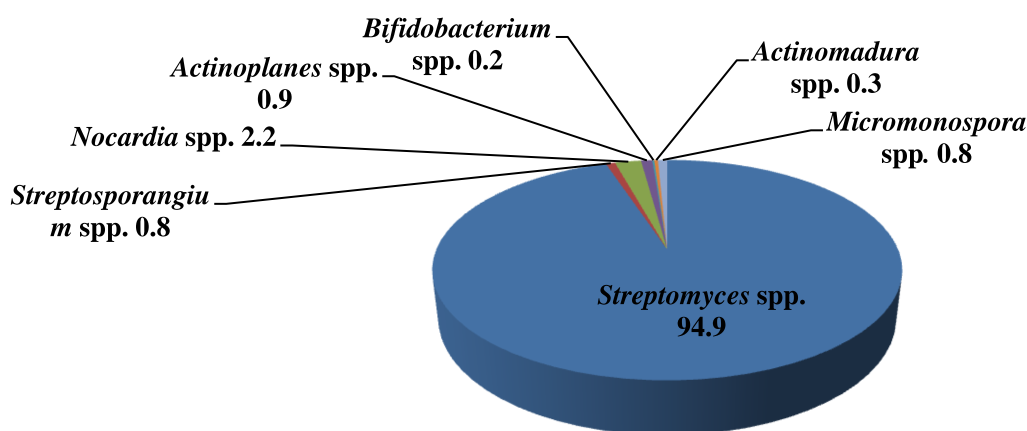
| Characteristics | Representative Colonies that showed a positive result | Possible Species |
|---|---|---|
| **Aerial mass colour** | | |
| White | 128 | SM, AM, NO, AP, MM |
| White cream/yellow | 136 | SM, AM, NO, SP |
| White changing to grey | 58 | SM |
| White change to red | 42 | SM |
| Grey | 145 | SM |
| Red/Orange/Pink | 27 | SM, MM, NO, SP |
| **Substrate Mycelium** | | |
| Substrate Mycelium | 594 | MM, AP, AM, SP, SM |
| Fragmented Substrate Mycelium | 14 | NO |
| **Sporophore morphology** | | |
| Sporangia Formation | 578 | AP, AM, SP, SM |
| Straight | 145 | SM |
| Spiral | 230 | SM |
| Flexous | 111 | SM |
| Retinaculum apertum | 50 | SM |
| Conidia Formation | 13 | AM, AP |
| **Pigment production** | | |
| Melanin | 129 | SM |
| Reverse colour | 115 | SM |
| Soluble colour | 134 | SM |
| Isolates showing pigmentation | 158 | SM |
| **Carbohydrates utilised by the isolates** | | |
| D-glucose | 640 | SM, SP, AP, NO, MM, AM |
| D-Fructose | 632 | SM, SP, AP, NO, MM, AM |
| L-Rhamnose | 450 | SM, SP, AP, AM |
| D-Galactose | 148 | SM, SP, MM, AM |
| Lactose | 219 | SM |
| Sucrose | 354 | SM, SP, MM |
| L-Arabinose | 630 | SM, SP, AP, NO, AM |
| Raffinose | 604 | SM, SP, MM |
| Xylose | 630 | SM, SP, NO, MM |
| Salicin | 405 | SM, SP, AP, MM, AM |
| Cellulose | 309 | SM, SP, MM |
| Inositol | 617 | SM, SP, MM |
| Oxidase | 386 | SM, SP |
| Tyrosine | 14 | NO |
| Xanthine | 14 | NO |
| Hypoxanthine | 16 | NO, AP |

| **Enzyme Activity** | | |
|---|---|---|
| Catalase | 514 | SM |
| Urease | 386 | NO, SM |
| Amylase | 568 | SM, MM, NO, AP, AM |
| Protease | 571 | SM, MM |
| Lipase | 549 | MM, NO, SM |
| Chitinase | 12 | SM, MM |
| Cellulase | 18 | SM, MM, NO |
| Lysozyme | 14 | NO |
| Caesin | 452 | SM, MM, AP, AM |
| **Presence of Di Amino Pimelic Acid** | 549 | SM |

SM – *Streptomyces*; MM – *Micromonospora* spp.; AP – *Actinoplanes* spp.; AM – *Actinomadhura* spp.; NO – *Nocardia* spp.; SP – *Streptosporangium* spp.

The most dominant group encountered in the present study was *Streptomyces* spp. (94.9%) of all the culturable species of Actinobacteria (Fig. 2.2.). They were mostly distributed in the 0-1 m depth of South, Middle, and North Andaman Islands, and their predominance in depths 5-6m and 10-11m were also perceptible.

**Figure 2.2. Percentage composition of Actinobacteria from Andaman coast**




The Streptomyces was found to be distributed in all the depth zones, in all habitats and during all seasons. The distribution of Actinomyces other than *Streptomyces* spp. is shown in Table 2.3.

**Table 2.3. Depth wise profile of Actinomycetes, other than *Streptomyces* spp. isolated from Andaman**

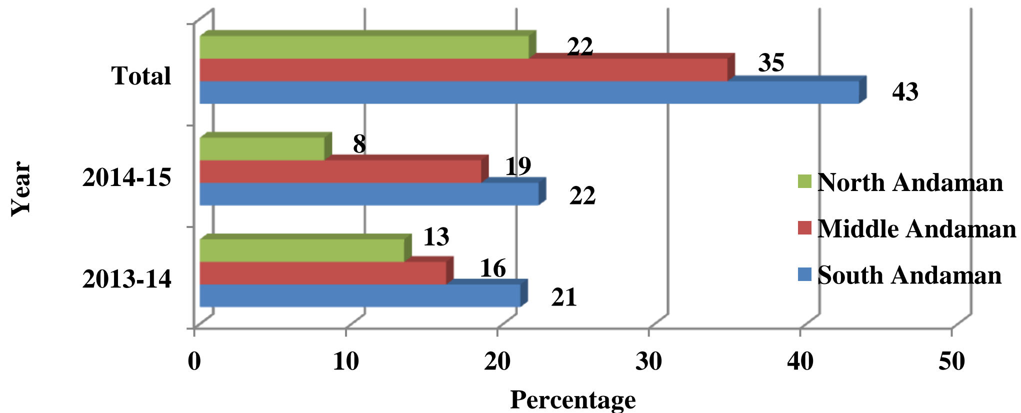
| Zone | Station | 0-1 m | 5-6 m | 10-11 m | Total |
|---|---|---|---|---|---|
| ***Streptosporangia* spp.** | | | | | |
| **South Andaman** | Chattam | 1 | 0 | 0 | 1 |
| | Junglighat | 0 | 1 | 0 | 1 |
| | Marina Park | 0 | 0 | 1 | 1 |
| | Wandoor | 0 | 0 | 1 | 1 |
| | **Total** | **1** | **1** | **2** | **4** |
| **Middle Andaman** | Billiground | **0** | **1** | **0** | 1 |
| | Yeratta | **0** | **0** | **1** | 1 |
| | **Total** | **0** | **1** | **1** | **2** |
| **North Andaman** | Kalipur 2 | 0 | 1 | 0 | 1 |
| | Kalipur | 0 | 1 | 1 | 2 |
| | **Total** | **0** | **2** | **1** | **3** |
| | **Grand Total** | **1** | **3** | **3** | **9** |
| ***Nocardia* spp.** | | | | | |
| **South Andaman** | Burmanallah | 0 | 1 | 0 | 1 |
| | Carbyn's Beach | 1 | 1 | 2 | 4 |
| | Junglighat | 0 | 2 | 1 | 3 |
| | Marina Park | 0 | 1 | 0 | 1 |
| | Sippighat | 0 | 1 | 1 | 2 |
| | **Total** | **1** | **6** | **4** | **11** |
| **Middle Andaman** | Bakultala | 0 | 1 | 1 | 2 |
| | Nimbutala | 0 | 1 | 0 | 1 |
| | Kadamtala | 0 | 0 | 1 | 1 |
| | Austin Creek | 0 | 1 | 0 | 1 |
| | **Total** | **0** | **3** | **2** | **5** |
| **North Andaman** | Kalighat | 1 | 1 | 1 | 3 |
| | Shyam Nagar | 0 | 1 | 1 | 2 |
| | Kalipur 2 | 0 | 2 | 0 | 2 |
| | Durgapur 2 | 0 | 1 | 1 | 2 |
| | **Total** | **1** | **5** | **3** | **9** |
| | **Grand Total** | **2** | **14** | **9** | **25** |
| ***Actinoplanes* spp.** | | | | | |
| South Andaman | Sippighat | 0 | 1 | 0 | 1 |
| | Collinpur Beach | | | 1 | 1 |
| | **Total** | **0** | **1** | **1** | **2** |
| Middle Andaman | Billiground | 0 | 0 | 1 | 1 |
| | Betapur | 0 | 1 | 0 | 1 |
| | Bakultala | 0 | 1 | 0 | 1 |
| | Karmatang | 0 | 1 | 1 | 2 |
| | **Total** | **0** | **3** | **2** | **5** |
| North Andaman | Kalipur | 0 | 2 | 1 | 3 |
| | **Total** | **0** | **2** | **1** | **3** |
| | **Grand Total** | **0** | **6** | **4** | **10** |

| | | | | | |
|---|---|---|---|---|---|
| ***Actinomadura* spp.** | | | | | |
| Middle Andaman | Karmatang<br>Mayabunder | 0<br>0 | 1<br>0 | 0<br>1 | 1<br>1 |
| | **Total** | **0** | **1** | **1** | **2** |
| North Andaman | Kalipur | 0 | 0 | 1 | **1** |
| | **Total** | **0** | **0** | **1** | **1** |
| | **Grand Total** | **0** | **1** | **2** | **3** |
| ***Micromonospora* spp.** | | | | | |
| South Andaman | Chattam<br>Sippighat | 0<br>0 | 2<br>1 | 0<br>1 | 2<br>2 |
| | **Total** | **0** | **3** | **1** | **4** |
| Middle Andaman | Nimbutala<br>Kadamtala | 0<br>0 | 0<br>1 | 1<br>0 | 1<br>1 |
| | **Total** | **0** | **1** | **1** | **2** |
| North Andaman | Aerial Bay Jetty<br>Kalipur 2 | 0<br>0 | 1<br>1 | 1<br>0 | 2<br>1 |
| | **Total** | **0** | **2** | **1** | **3** |
| | **Grand Total** | **0** | **6** | **3** | **9** |
| ***Bifidobacterium* spp.** | | | | | |
| Middle Andaman | Billiground<br>**Total** | 0<br>**0** | 1<br>**1** | 0<br>**0** | 1<br>**1** |
| North Andaman | Durgapur 2<br>**Total** | 0<br>**0** | 1<br>**1** | 0<br>**0** | 1<br>**1** |
| | **Grand Total** | **0** | **2** | **0** | **2** |

### 2.3.2. Zone wise distribution of *Streptomyces* spp.

The zone wise spatial distribution analysis has shown that culturable *Streptomyces* spp. was highest (43%) in South Andaman (Fig. 2.3.) followed by Middle Andaman (35%) and least in North Andaman (22%). The same trend followed during both the years of study.

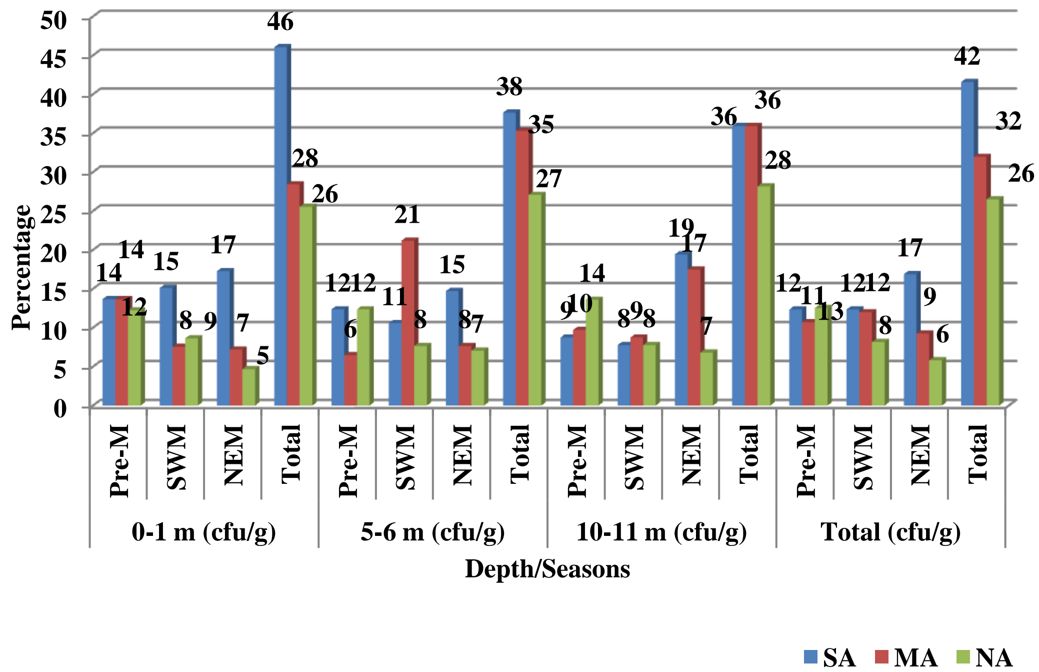
**Figure 2.3. Zone wise distribution of *Streptomyces* spp.**




South Andaman dominated in the number of *Streptomyces* spp. isolates (42%) followed by Middle (32%) and North (26%) (Fig.2.4.). Except for SWM, South Andaman had contributed the highest number of *Streptomyces* spp. in all seasons, while both South and Middle Andaman contributed equally (12% each). South Andaman recorded the highest number of *Streptomyces* spp. isolates from all seasons at 0-1m depth (46%) except for Pre-Monsoon season where South and Middle Andaman shared dominance (14% each).

Even though the contribution of South Andaman was highest in 5-6m depth (38%), Pre-Monsoon have shown a shared dominance between South Andaman and North Andaman (12% each), the predominance of Middle Andaman during SWM (21%) and dominance of South Andaman in NEM (15%). The dominance of South Andaman was discernible in all depth zones except for 10-11m depth zone, where SA, as well as Middle Andaman, have recorded an equal number of isolates (36% each). North Andaman dominated during Pre-Monsoon season (14%), while Middle Andaman dominated during South West Monsoon season (9%). Whereas, South Andaman recorded the highest number of isolates during North East Monsoon (19%).

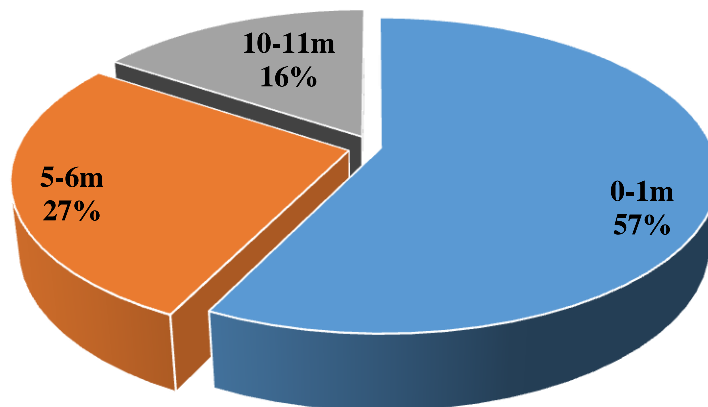
**Figure 2.4. Zone wise distribution of *Streptomyces* spp. in various depths**




## 2.3.3. Depth wise distribution of *Streptomyces* spp.

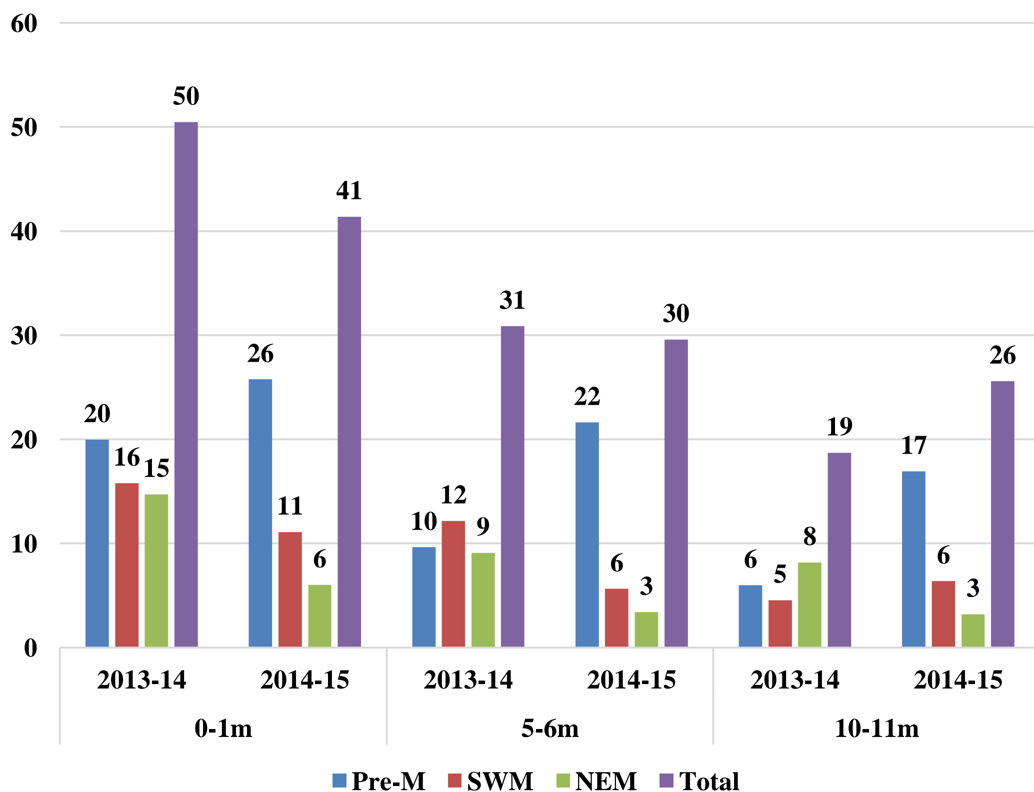
The results of bathymetric distribution studies have shown a high abundance of *Streptomyces* spp. in the nearshore region than the deeper waters (Fig. 2.5.). About 57% of the isolated *Streptomyces* spp. were from 0-1 m depth, 27% of Streptomyces spp. from 5-6 m while 16% were isolated from 10-11 m.

**Figure 2.5. Depth wise distribution of *Streptomyces* spp. from Andaman Coast**



The results indicate that there is perceptible variation in the abundance of *Streptomyces* spp. based on depth in the Andaman waters. The preponderance of a high number of *Streptomyces* spp. within the 0-1m depth zone (50 & 41% first and second year respectively) which further declines with depth are an indication that this genus has an affinity towards shallow depths. Fig. 2.6. depicts the variations in the abundance of *Streptomyces* spp. with depth during the three seasons in 2013-14 and 2014-15.

**Figure 2.6. Depth wise distribution of *Streptomyces* spp. at various seasons**



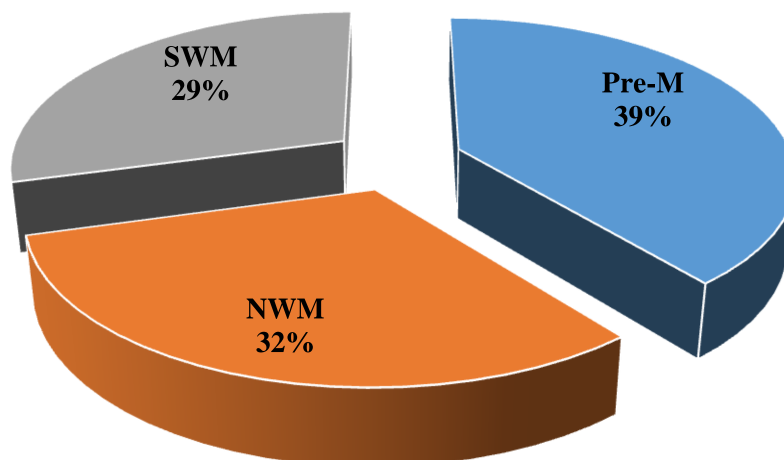

In the year 2013-14 a higher percentage of *Streptomyces* spp. was recorded during pre-monsoon season (20%) from the 0-1m depth zone followed by SWM and NEM (Fig. 7) and a similar trend was seen during 2014-15. At 5-6m depth, a higher

number of isolates were recorded during SWM in 2013-14 (12%), while it was higher in Pre-Monsoon during 2014-15 (22%). More number of isolates were recorded during 2014-15 in the depth 10-11m. In this depth, a higher number of isolates were recorded in NEM during 2013-14, while it was highest in pre-monsoon season during 2014-15.

### 2.3.4. Season-wise distribution of *Streptomyces* spp.

Season-wise distribution of Streptomyces has shown variation between wet season (rainy) and dry season. Pre-monsoon (dry season) recorded greater occurrence (39%) of Streptomyces (Fig. 2.7.) compared to monsoon (wet season viz., Southwest and Northeast monsoon), which was recorded to the tune of 32% and 29% respectively.

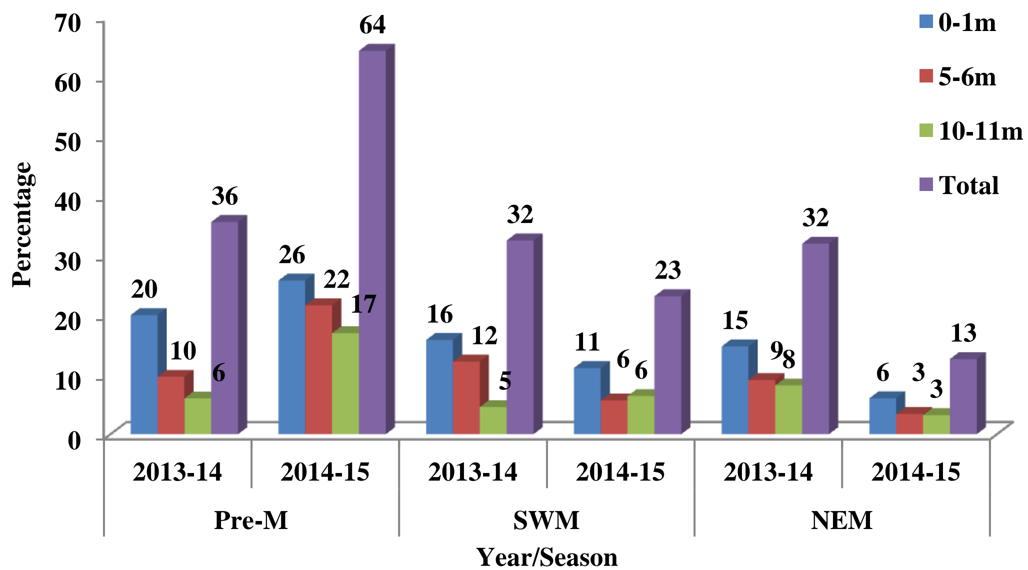
**Figure 2.7. Season-wise distribution of *Streptomyces* spp. from Andaman Coast**




Higher cfu/g of *Streptomyces* spp. were recorded during Pre-Monsoon followed by South West Monsoon and North East Monsoon during first as well as the second year of study (Fig. 2.8.). During the pre-monsoon season of 2013-2014, a higher

percentage of *Streptomyces* spp. (20%) were isolated from 0-1m depth followed by 10% from the 5-6m depth and 6% from 10-11m depth. While in the year 2014-2015, 26% *Streptomyces* spp. were isolated from the 0-1m depth, 22% from the 5-6m depth and 17% from 10-11m depth.

**Figure 2.8. Season-wise distribution of *Streptomyces* spp. at various depths**



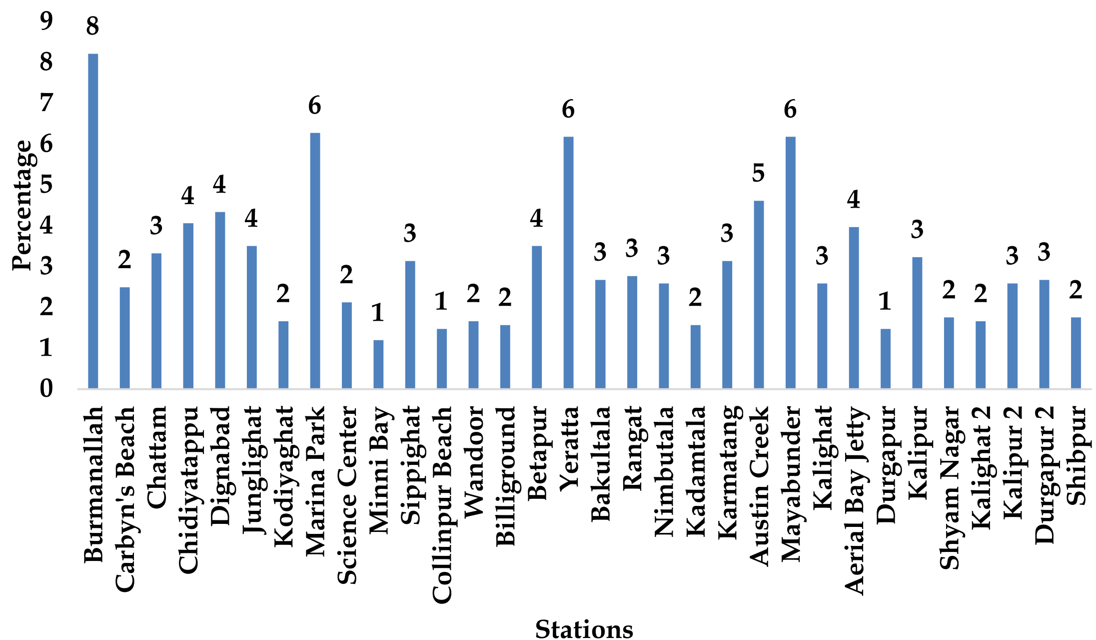

In the South West monsoon season, domination of *Streptomyces* spp. isolates were higher at 0-1m depth during 2013-14 (16%) followed by 12% from 5-6m and 5% from 10-11m depth. Whereas during the same season during 2014-2015, 11% were isolated from 0-1m depth, 6% from the 5-6m depth and 6% from 10-11m depth. Meanwhile, during the North East Monsoon of 2013-2014, 15% *Streptomyces* spp. were isolated from 0-1m followed by 9% from 5-6 m and 8% from 10-11 m depth. In the succeeding year, however, 6% of Streptomyces spp. were isolated from the 0-1m depth and 3% each from 5-6m and 10-11m depth.

### 2.3.5. Station-wise distribution of *Streptomyces* spp.

#### 2.3.5.1. Overall distribution

The station-wise distribution of *Streptomyces* spp. in the coast of Andaman islands is shown in Figure 2.9. The results indicated that the highest occurrence of *Streptomyces* spp. was encountered from Burmanallah, (8%) followed by Marina Park, Yeratta and Mayabunder (6% each).

**Figure 2.9. Station-wise distribution of *Streptomyces* spp. from Andaman Coast**



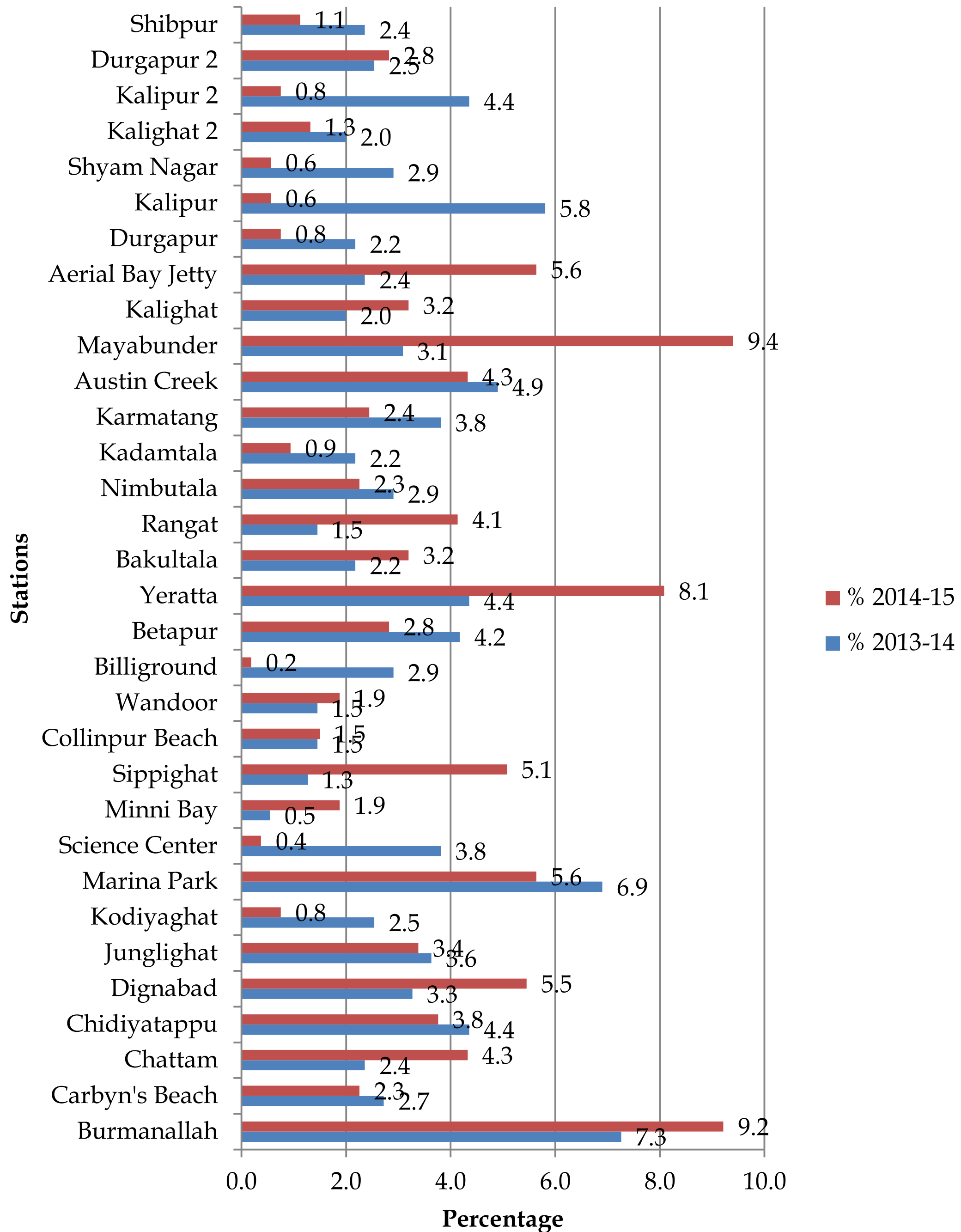

All other stations from the South Andaman, Middle Andaman and North Andaman were within the range of 1% to 5%. The results indicate that there was a rather uniform pattern of distribution of *Streptomyces* spp. from all the stations sampled.

#### 2.3.5.2. Year wise distribution

The year wise distribution has shown variation in abundance of *Streptomyces* spp. in most of the stations (Fig. 2.10.) During 2013-14, Burmanallah has shown highest

percentage contribution (7.3%) followed by Marina Park (6.9), Kalipur (5.8), Austin Creek (4.9) and the least was from Minnie Bay (0.5%).

**Figure 2.10. Distribution of *Streptomyces* spp. during 2013-14 & 2014-15**



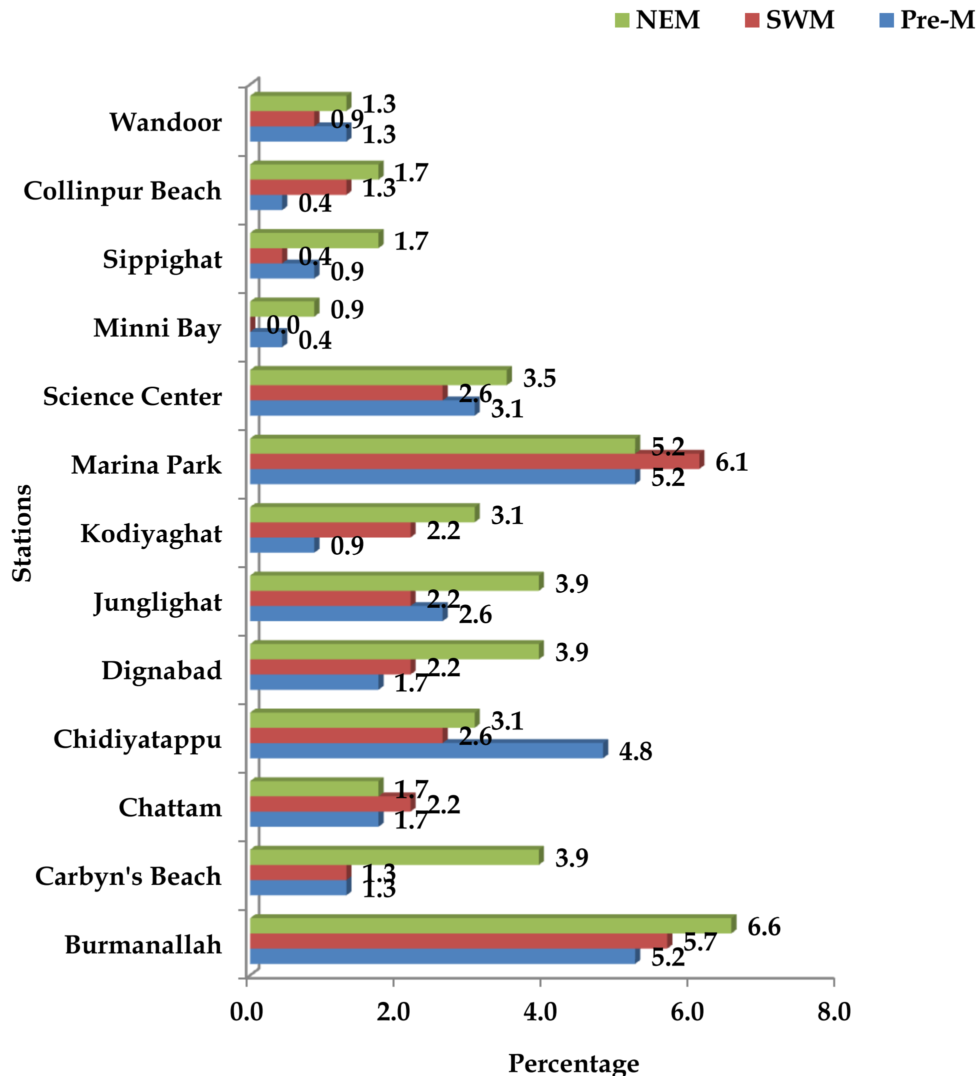

While during 2014-15, Mayabunder contributed highest (9.6%) percentage of Streptomyces followed by Burmanallah (9.2), Yeratta (8.1), Arial Bay Jetty (5.6),

Marina Park (5.6), Dignabad (5.5), Sippighat (5.1) and the least was recorded from Billiground (0.2%).

### 2.3.5.3. Season-wise Distribution

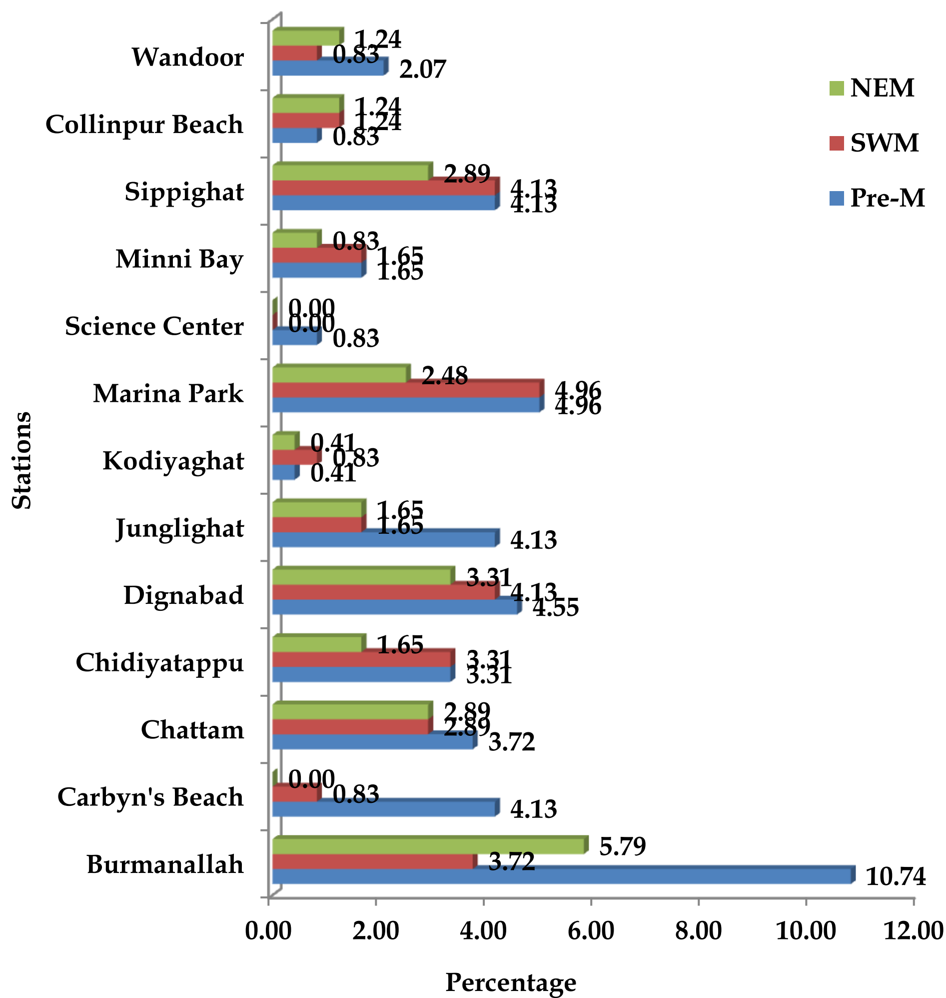
During the NEM of 2013-14, Streptomyces dominated in most of the stations except Chidiyatappu, Chattam and Marina Park (2.11.). During Pre-Monsoon, the highest percentage was recorded from Chidiyatappu (4.8%) while during SWM it was from Chattam (2.2%) and Marina Park (6.1%).

**Figure 2.11. Season-wise distribution of *Streptomyces* spp. in South Andaman during 2013-14**



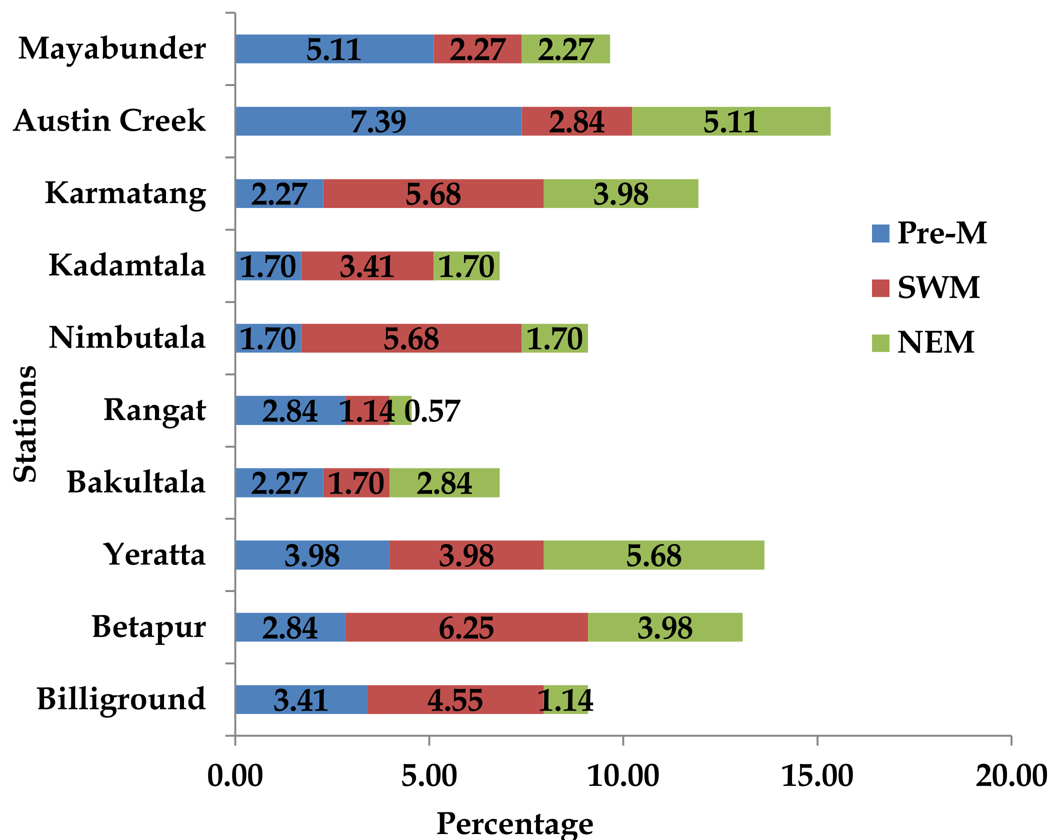
During 2014-15, Pre-Monsoon has shown dominance in all the stations with the highest in Burmanallah (10.74%) followed by Marina Park (4.96%), Dignabad (4.55%) (Fig. 2.12.). Pre-Monsoon contributed the highest percentage in Chidiyatappu (4.8%) while SWM in Chattam (2.2%) and Marina Park (6.1%). During South West Monsoon, Marina Park has shown the highest percentage of cfu/g (4.96%) followed by Sippighat (4.13%), Dignabad (4.13%), Burmanallah (3.72%) and no isolate could be recorded from Science Centre.

**Figure 2.12. Season-wise distribution of *Streptomyces* spp. in South Andaman during 2014-15**



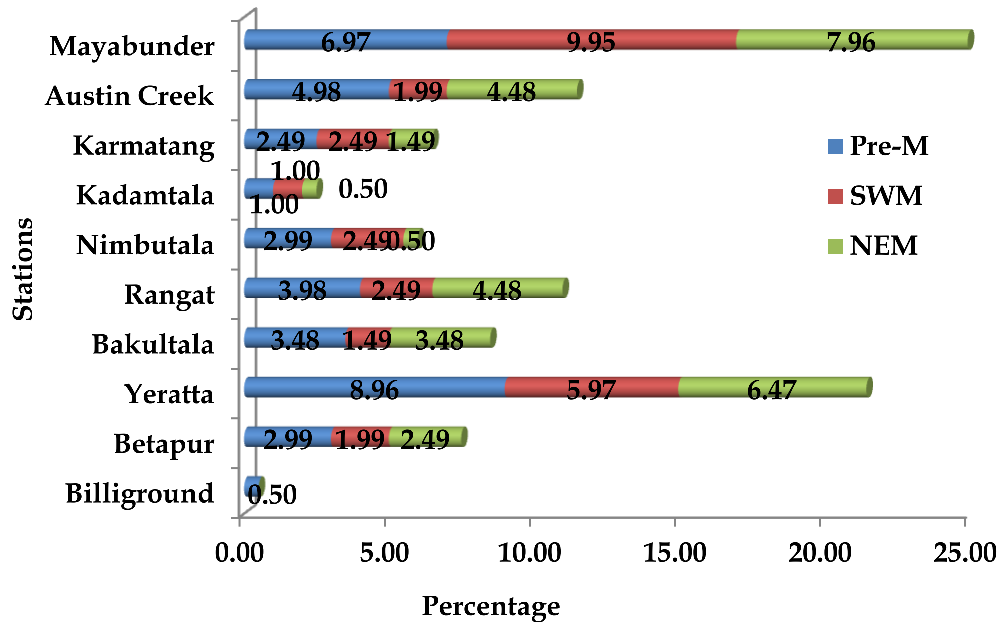
The season wise distribution of *Streptomyces* spp. have shown that the highest percentage cfu/g was recorded from Austin Creek (7.39%) during Pre-Monsoon of the year 2013-14 followed by Betapur (6.25%), Nimbutla (5.68%), Karmatang (5.68%) during SWM and Yeratta (5.68%) during NEM. The least was Rangat during NEM (0.57%). The highest percentage of isolates were recorded from SWM and NEM during this year.

**Figure 2.14. Season-wise distribution of *Streptomyces* spp. in Middle Andaman during 2013-14**



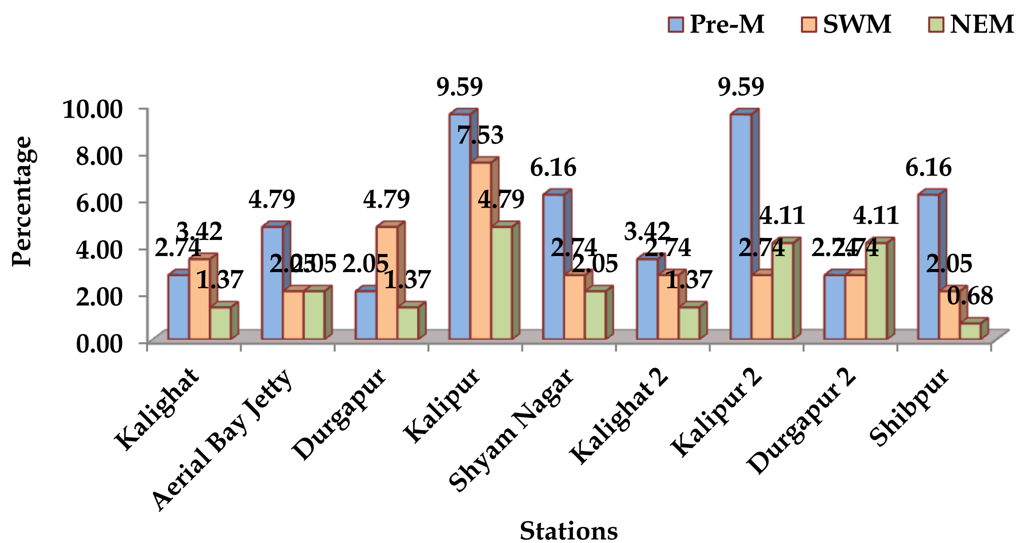

In North Andaman, Mayabundar contributed highest during 2014-15 SWM (9.95%) as well as NEM (7.96%) (Fig. 2.15.) followed by Yeratta, contributing 8.96% during Pre-Monsoon, 6.47% during NEM and 5.97% during SWM. Billiground recorded 0.5% during Pre-Monsoon, and *Streptomyces* spp. were not isolated during other seasons.

**Figure 2.15. Season-wise distribution of *Streptomyces* spp. in Middle Andaman during 2014-15**




The highest number of isolates was recorded at Kalipur in all the seasons during 2013-14 from North Andaman (Fig. 2.16.). Pre-Monsoon contributed highest in all stations except for Kalighat, Durgapur and Durgapur 2.

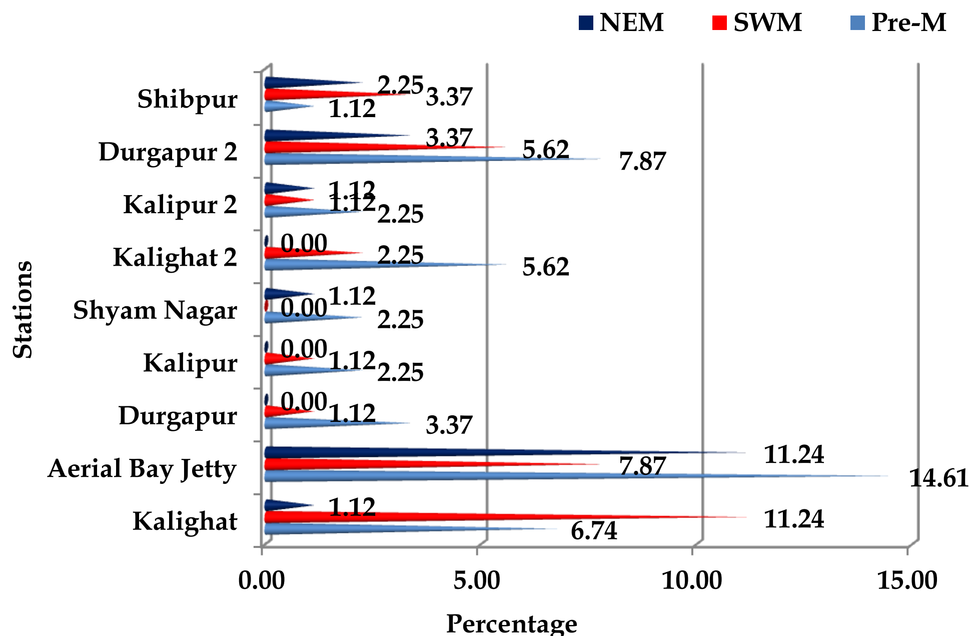
**Figure 2.16. Season-wise distribution of *Streptomyces* spp. in North Andaman during 2013-14**



The percentage cfu/g was highest at Kalipur and Kalipur 2 during Pre-Monsoon (9.59% each) followed by Shibpur (6.16%), Shyam Nagar (6.16%), Aerial Bay Jetty (4.79%) and the least was from Durgapur (2.05%). During SWM, the highest number of isolates were recorded from Kalipur (7.53%) followed by Durgapur (4.79%) and the least was from Shibpur (2.05%). While less number of isolates were recorded during NEM with a highest of 4.79% at Kalipur and the least 0.68% at Shibpur.

During 2014-15, Pre-Monsoon contributed to the highest in all stations except Kalighat and Shibpur (Fig. 2.17). Highest percentage cfu/g was isolated from Aerial Bay Jetty (14.61%) during Pre-Monsoon season followed by Durgapur 2 (7.87%), Kalighat (6.74%), Kalighat 2 (5.62%) and the least was from Shibpur (1.12%).

**Figure 2.17. Season-wise distribution of *Streptomyces* spp. in North Andaman during 2014-15**



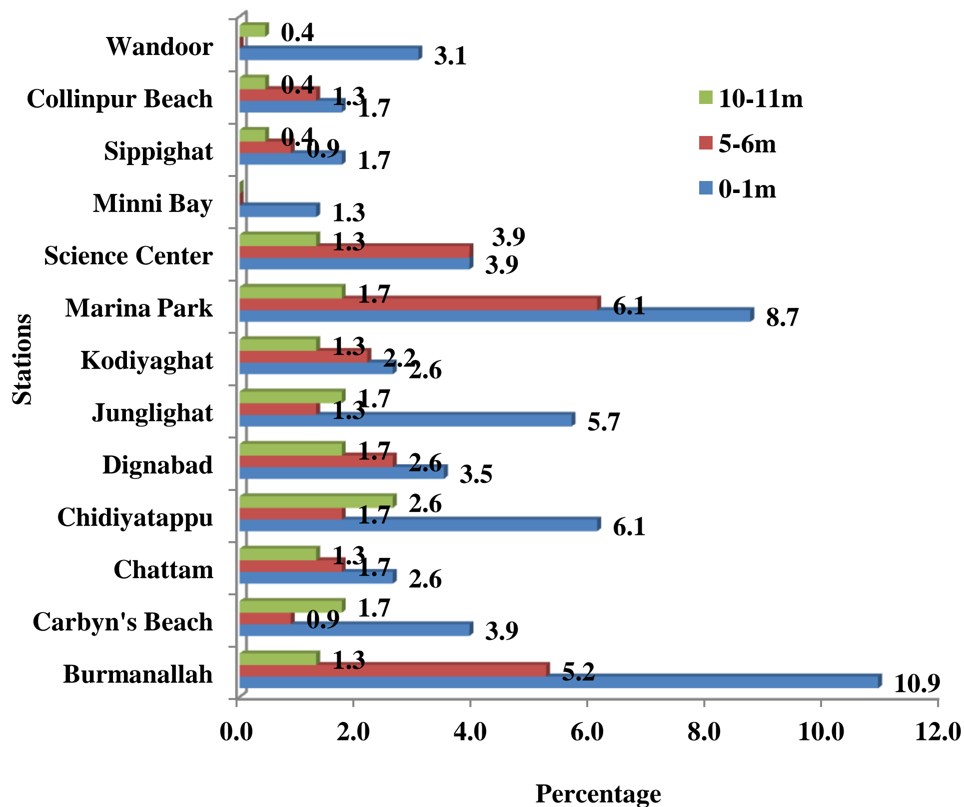

During SWM, the highest number of isolates were recorded from Kalighat (11.24%) followed by Aerial Bay Jetty (7.87%, Durgapur 2 (5.62%) while no isolates were

recorded from Shyam Nagar. NEM has shown a very low abundance of *Streptomyces* spp. even though a higher number was recorded at Aerial Bay Jetty (11.24%). The samples from Kalighat 2, Kalipur and Durgapur, recorded no *Streptomyces* spp. during this year.

### 2.3.5.4. Depth wise distribution

*Streptomyces* spp. isolates were found to be distributed mostly in the 0-1m depth range in all the stations except Science centre (Fig. 2.18.) during 2013-14, where 0-1m, as well as 5-6m, has shown an even distribution of isolates (3.9%). Few stations like Carbyn's Beach (1.7%), Chidiyatappu (2.6%), Junglighat (1.7%) and Wandoor (0.4%) have shown higher abundance in the 10-11m depth than 5-6m.

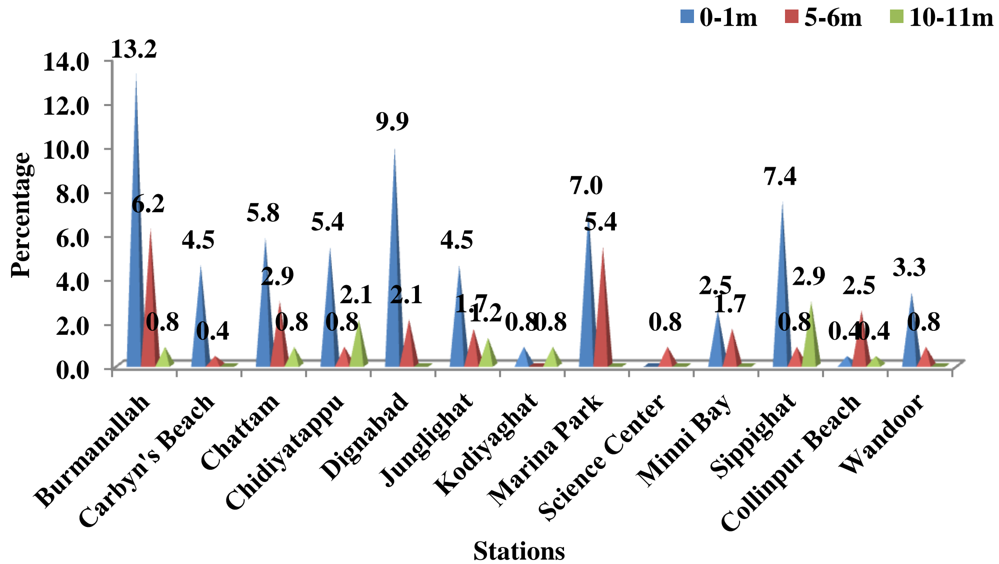
**Figure 2.18. Depth wise distribution of *Streptomyces* spp. in South Andaman during 2013-14**




Burmanallah recorded the highest percentage of isolates (13.2%) in 2014-15 in South Andaman at 0-1m depth zone (Fig. 2.19.), and a similar trend was found in all the stations. Dignabad (9.9%) followed by Sippighat (7.4%), Marina Park (7.0%),

Chattam (5.8%) were the other significant contributors. Other depth zones contributed much less.

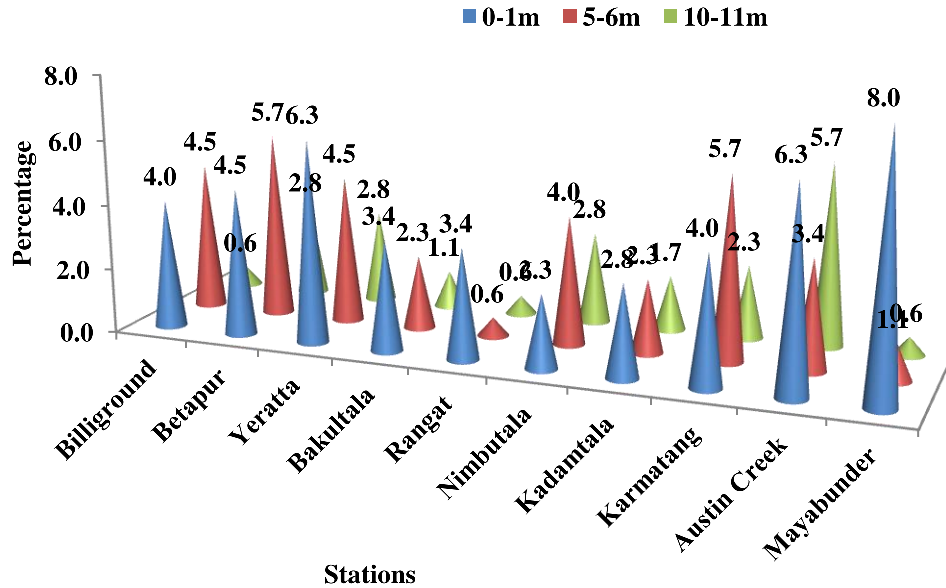
**Figure 2.19. Depth wise distribution of *Streptomyces* spp. in South Andaman during 2014-15**




The highest recorded number of isolates from the 5-6m depth zone was Burmanallah (6.2%) followed by Marina Park (5.4%), and no isolates were recorded from Kodiyaghat at this depth zone. While its very less occurrence recorded from 10-11m depth zone with a highest from Sippighat (2.9%) with no records from Carbyn's Beach, Dignabad, Marina Park, Science Centre, Minnie Bay, and Wandoor.

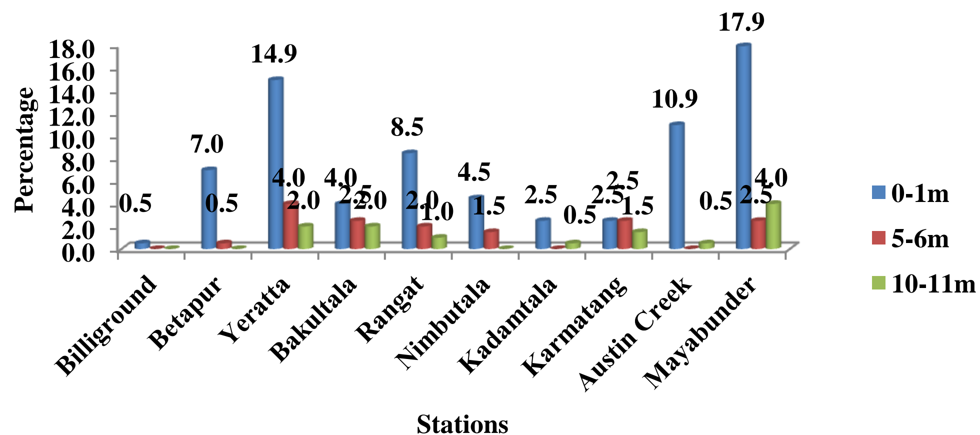
During 2013-14, Mayabunder contributed highest cfu/g (8.0%) at 0-1m depth from Middle Andaman (Fig. 2.20.). Whereas 5-6m depth contributed a higher number of isolates from Karmatang (5.7%), Nmbutala (4.0%), Betapur (5.7%) and Billiground 4.5%) during this year. The least was recorded at 10-11m at Billiground, Rangat, and Mayabunder (0.6% each) and 5-6m at Rangat (0.6%).

**Figure 2.20. Depth wise distribution of *Streptomyces* spp. in Middle Andaman during 2013-14**



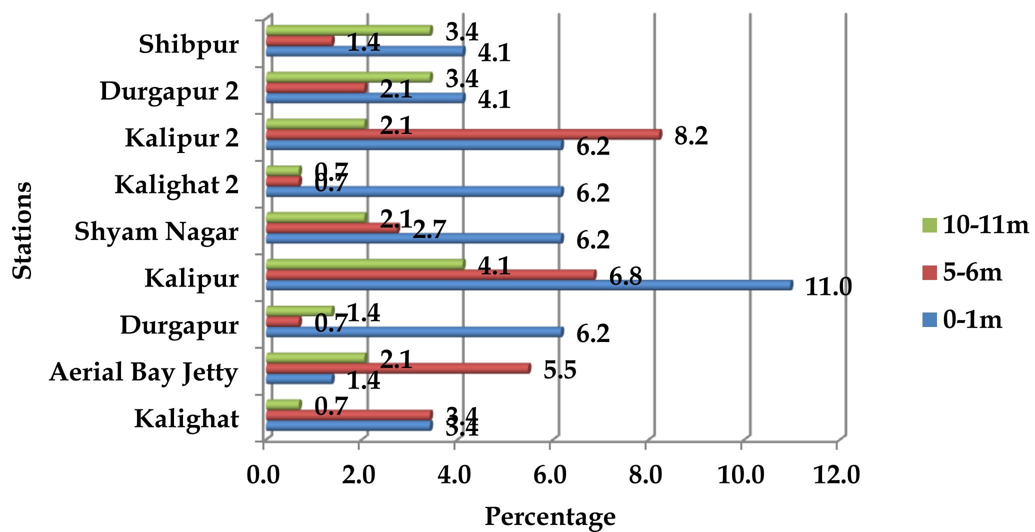

During the year 2014-15 also Mayabunder (17.9%) has contributed the highest number of isolates (Fig. 2.21.) at 0-1m depth, and other stations followed the same trend. This was followed by Yeratta (14.9%), Austin Creek (10.9%), Rangat (8.5%), Betapur (7.0%) and the least was recorded from Billiground (0.5%). While at 5-6m depth, the highest number of cfu/g was recorded from Yeratta (4.0%), whereas no isolates from Billiground, Kadamtala and Austin Creek.

**Figure 2.21. Depth wise distribution of *Streptomyces* spp. in Middle Andaman during 2014-15**



In North Andaman, 0-1m depth zone dominated in all stations except Culber Bay, Aerial Bay Jetty and Kalighat. Kalipur (11.0%) contributed the most number of isolates from 0-1m depth zone during 2013-14 followed by Durgapur, Shyam Nagar, Kalighat 2 and Kalipur 2 (6.2% each).

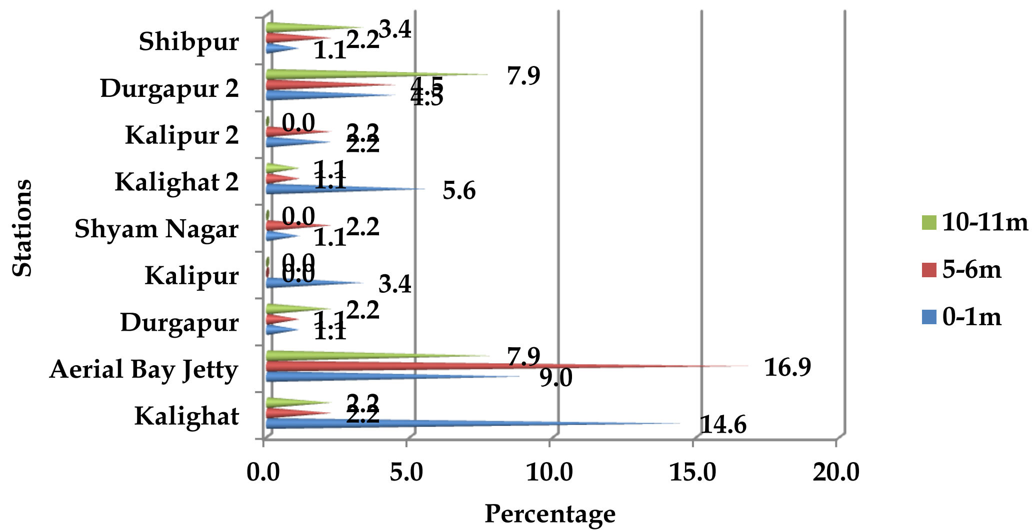
**Figure 2.22. Depth wise distribution of *Streptomyces* spp. in North Andaman during 2013-14**




Whereas Kalipur 2 contributed the highest (8.2%) from 5-6m depth zone followed by Kalipur (6.8%), Aerial Bay Jetty (5.5%) and the least was from Durgapur and Kalighat 2 (0.7% each). Kalipur recorded the highest number of isolates (4.1%) from 10-11m depth followed by Durgapur 2 and Shibpur (3.4% each) and the least from Kalighat and Kalighat 2 (0.7% each). During 2014-15, the highest cfu/g was observed from Aerial Bay Jetty (16.9%) at 5-6m depth. Kalighat recorded 14.6% cfu/g of *Streptomyces* spp. isolates at 0-1m depth followed by Aerial Bay Jetty (9.0%), Kalighat 2 (5.6%) and the least from Durgapur and Shibpur (1.2% each). While apart from Aerial Bay Jetty, all other stations have shown comparatively less number of isolates and Kalipur recorded no isolates during this year from 5-6m

depth. Aerial Bay Jetty and Durgapur 2 recorded the highest number of isolates at 11-12m depth zone (7.9% each) followed and Kalipur with no records.

**Figure 2.23. Depth wise distribution of *Streptomyces* spp. in North Andaman during 2014-15**




While apart from Aerial Bay Jetty, all other stations have shown comparatively less number of isolates and Kalipur recorded no isolates during this year from 5-6m depth. Aerial Bay Jetty and Durgapur 2 recorded the highest number of isolates at 11-12m depth zone (7.9% each) followed and Kalipur with no records.

### 2.3.6. Effect of Environmental parameters on the distribution of *Streptomyces*

During the present study, Salinity, pH, Total Organic Carbon, Nitrite and Phosphate were found to be influencing the distribution and abundance of *Streptomyces* spp. in Andaman waters (Fig. 2.24.). The variation of environmental variables with respect of seasonality depicted the variations in the pre-monsoon samplings during the southwest and northeast monsoon seasons.

**Figure 2.24. Effect of Environmental parameters on the distribution of *Streptomyces* spp.**

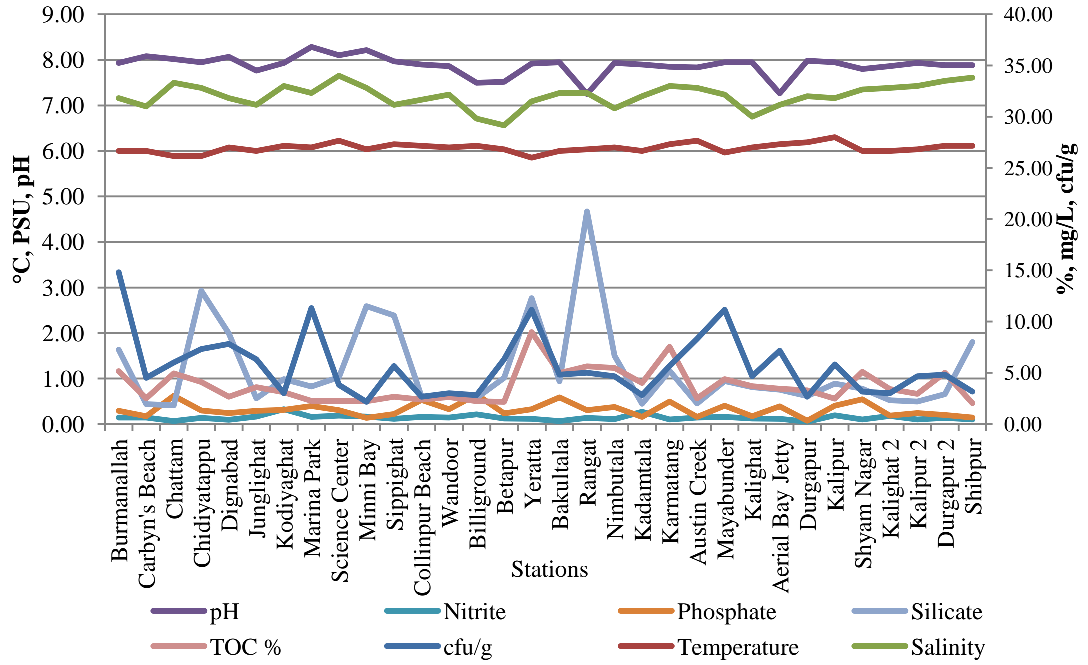
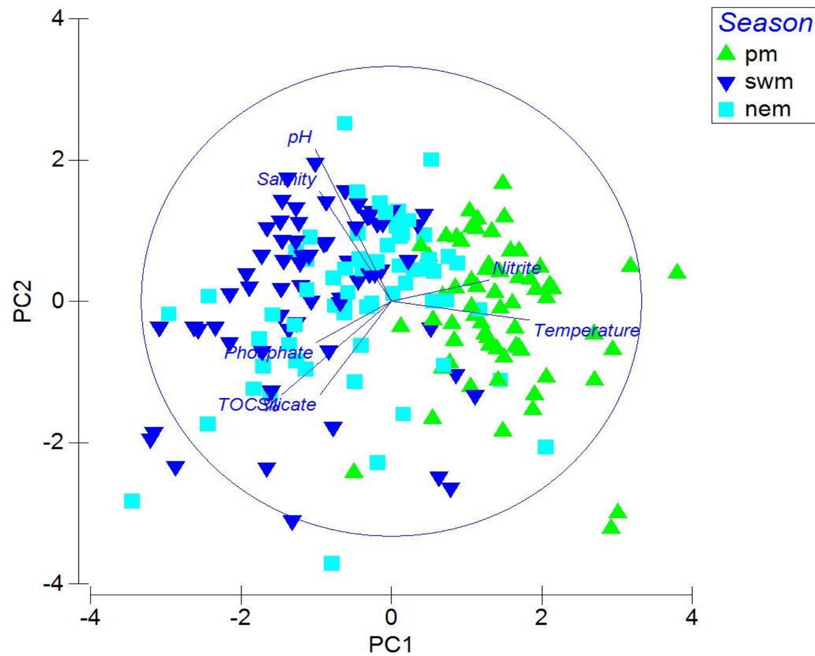



**Figure 2.25. Principal Component Analysis**



Further analysis showed that the five principal components could explain 83.3% of variation with two significance axes (PC1 and PC2) explaining about 45.8% of the total variation among the seasonal samplings (Fig. 2.25.). The PC1 axis (eigenvalue 1.98) and PC2 (eigenvalue 1.23) axes contributed a total variation of 28.2% and 17.6% respectively. PC1 axis explained variation regarding temperature, nitrite, and phosphate while PC2 axis explained variation for salinity, silicate, and TOC%. The pre-monsoonal period samplings were influenced with high temperature and nitrite concentration. The rest of the environmental variables were the influencing factors for the southwest monsoon and northeast monsoon season samplings. Coefficients in the linear combinations of variables making up PC's are given in Table 2.4.

**Table 2.4. Coefficients in the linear combinations of variables making up PC's**

| Variable | PC1 | PC2 | PC3 | PC4 | PC5 |
|---|---|---|---|---|---|
| **Temperature** | 0.554 | -0.081 | -0.069 | 0.206 | 0.268 |
| **Salinity** | -0.287 | 0.467 | -0.462 | 0.379 | 0.306 |
| **pH** | -0.305 | 0.648 | 0.092 | -0.093 | -0.276 |
| **Nitrite** | 0.393 | 0.09 | -0.255 | 0.497 | -0.699 |
| **Phosphate** | -0.301 | -0.176 | 0.501 | 0.743 | 0.17 |
| **Silicate** | -0.285 | -0.398 | -0.676 | 0.07 | 0.102 |
| **TOC %** | -0.438 | -0.397 | 0.019 | -0.034 | -0.48 |

BIOENV analysis was carried out using the seven measured environmental variables (temperature, salinity, pH, nitrite, silicate, phosphate, TOC%) along with the number of *Streptomyces* spp. isolates (cfu/g). The results revealed that temperature, pH, Nitrite and TOC % were the best subset that could explain the variation in the distribution of *Streptomyces* spp. with a Spearman correlation coefficient, ρ of 0.026.

**Table 2.5. Linear correlation (Pearson)**

| | Temperature | Salinity | pH | Nitrite | Phosphate | Silicate | TOC % | cfu/g |
|---|---|---|---|---|---|---|---|---|
| **Temperature** | | 0.0084 | 0.0001 | 0.0001 | 0.0109 | 0.0135 | 0.0001 | 0.0817 |
| **Salinity** | -0.1896 | | 0.0002 | 0.2947 | 0.3880 | 0.1095 | 0.6500 | 0.0294 |
| **pH** | -0.3204 | 0.2604 | | 0.1611 | 0.5125 | 0.3526 | 0.7791 | 0.8169 |
| **Nitrite** | 0.3073 | -0.0760 | -0.1015 | | 0.0508 | 0.1087 | 0.0067 | 0.7039 |
| **Phosphate** | -0.1833 | 0.0626 | 0.0475 | -0.1411 | | 0.4380 | 0.0049 | 0.7918 |
| **Silicate** | -0.1779 | 0.1158 | -0.0674 | -0.1161 | 0.0562 | | 0.0007 | 0.3247 |
| **TOC %** | -0.3693 | 0.0329 | 0.0203 | -0.1947 | 0.2019 | 0.2416 | | 0.0086 |
| **cfu/g** | 0.1259 | -0.1571 | 0.0168 | -0.0276 | 0.0191 | 0.0714 | 0.1890 | |

Statistically linear (Pearson) correlation was performed to correlate the environmental variable and the *Streptomyces* spp (Table 2.4.). TOC % showed significant positive correlation (r=0.18, p=<0.05) while salinity showed significant negative correlation (r=-0.15, p= <0.05). Temperature, pH, phosphate, and silicate showed weak positive correlation while nitrite showed a weak negative correlation.

## 2.4. Discussion

In the present study distribution of the actinobacteria belonging to the genus *Streptomyces* from Andaman coast is described. Actinobacteria were isolated from the sediment samples collected from coastal waters of Andaman from three different depths during three seasons for two years. *Streptomyces* spp. predominated most of the isolates irrespective of depths and seasons. The other genera like *Actinomadura*, *Actinoplanes*, *Bifidobacterium*, *Micromonospora, Nocardia*, *Streptosporangium*, and *Streptoverticillium* were meagrely represented. Sujatha et al. (2005) had described a similar pattern of distribution of actinobacteria isolated from eight sampling stations from Andaman & Nicobar Islands. It was reported that in mangrove rhizosphere, Actinobacterial genera such as *Streptomyces,*

*Micromonospora*, and *Nocardioform* are abundant (Tan et al. 2009). According to Euzeby (2008), the genus *Streptomyces* of Actinomycetes group is the major component of the population. So the distribution of this major genus along the coast of Andaman is very significant since the Andaman Islands are home to a widespread mangrove stands with great diversity. In a similar study, Meena et al. (2013) isolated 26 Actinobacterial strains collected from various sites of Port Blair south Andaman which comprised of a higher representation of *Streptomyces* spp.

Zone-wise distribution pattern has shown that South Andaman harbour more Streptomycetes population. South Andaman was the most populated, experiencing more human activities than the other two zones Middle and North Andaman (Bandopadhyay and Carter, 2017). According to Gnanam et al. (2013), factors like sediment structure, nutrient availability, total organic carbon, and total nitrogen have a significant influence on the distribution of actinobacteria. It was found from the present study that clay type of sediment was a good source of Actinobacteria population than sandy sediments. Samples collected from stations Yerratta mangrove area, Austin Creek, and Aerial bay jetty were found to be rich sources of actinobacteria compared with the sandy areas like Kalipur 2, Carbyn’s cove. The results are in consonance to the earlier reports (Narayani et al. 2018) from these regions. As clayey sediments can retain more nutrients which can be available for microorganisms than sandy sediments, and thus they support more actinobacteria population (Dhevendaran et al. 1987).

Depth-wise analysis has shown that *Streptomyces* spp. has a higher percentage distribution in the nearshore area than offshore region. It was assumed that marine actinobacteria were washed into the ocean from the terrestrial environment through continuous run-offs as well as other process and got adapted to marine conditions

(Jensen et al. 1991). Predomination of streptomycetes at shallow depths was reported by Takizawa et al. (1993) from the Chesapeake Bay. Miyake et al. (2003) isolated Actinobacteria from the shallow layer of sediment (0–1 cm) and found that the isolates from the deep layer (9–10 cm) mostly belonged to phylum Firmicutes. Walker et al. (1975) observed that 98% of the streptomycetes observed were from water less than 3m deep and also the abundance decreased quickly with an increase in water depth. However, during the present study some stations like Durgapur 2 and Shibpur, a similar trend was not seen. This may be due to the island-specific conditions as well as more or less similar salinity distribution in these stations. Weyland et al. (1981) reported that the abundance of *Streptomyces* spp. decrease with increase in distance from shore and also observed that the isolated streptomycetes were capable of growing in freshwater, but better growth was found in media containing seawater. The present study also reported a similar pattern, and it was interesting to find that the nearshore Streptomycete isolates showed more tolerance towards salinity and temperature than offshore isolates.

Seasons play a significant role in the distribution of streptomycetes. Shirodhkar et al. (2009) and Sundaramanikam (2008) correlated the seasonality and variations in nutrients on Streptomycete in intertidal regions and described the importance of monsoon season in its distribution. Gnanam et al. (2013) and Ghanem et al. (2000) observed total organic carbon and nitrogenous nutrients significantly influenced the actinobacterial population. Similar results were reported from the sediments of Bay of Bengal by Adinarayana et al. (2007), Vijaya Kumar et al. (2007) and Manivasagan (2009). Results emerging from the present study shows the season-wise distribution of Streptomycetes from the coast of Andaman has high variation between the seasons. The Pre-monsoon season or non-rainy season has shown a

higher abundance of Streptomycetes than North West monsoon and South West monsoon. The Andaman and Nicobar region experiences heavy rainfall for almost nine months in a year with three months of actual dry season. So it may be inferred that the distribution pattern of marine actinobacteria in Andaman has less effect during rainy seasons.

The present study has shown that the distribution of *Streptomyces* spp. along the coast of Andaman is mainly influenced by Total organic carbon and Salinity and was statistically confirmed by PCA. It is evident from the present results that when salinity increases, or when the distance from shore increases, the number of isolates decreases. The present results support the argument that these microbes were initially terrestrial forms but washed into the ocean through continuous flushing out due to run-off as well as other ecosystem processes and adapted in the marine environment (Jensen et al. 1991). The pre-monsoon season affects the distribution of Actinomycetes in Andaman waters since the remaining months during southwest monsoon as well as northeast monsoon the physicochemical parameters remain more or less similar.

The actinobacteria from marine sediments of islands of Andaman is less documented and characterized. These bacteria represent a small component of the total bacterial population in marine sediments (Goodfellow et al. 1985), and so their role in the marine environment is complex and difficult to assess. During the present study also, the isolation and characterization of these microbes were difficult since they grow slowly and relatively low numbers could be found in relation to many common unicellular bacteria. Actinobacteria especially *Streptomyces* spp. are a good source for lytic enzymes. Their metabolic diversity makes them the major producers of antibiotics along with a great deal of other bioactive metabolites (Alderson et al.

1993, Sanglier et al. 1993). They can utilize and decompose a wide diversity of compounds including polymers and xenobiotic compounds (Crawford, 1978, (Goodfellow and Simpson, 1987, Warren, 1996, Schrijver and Mot, 1999). The results of the present study are significant as the depthwise and season wise distribution and abundance of various *Streptomyces* spp. isolates are described for the first time from this remote islands the Indian EEZ. These actinobacteria are found to be mostly distributed in the coastal waters and inhabited less in deeper environments. The study also has confirmed that the clayey nutrient-rich sediments support more actinobacteria than the other course sediments. The *Streptomyces* spp. are known for their bioactive potential throughout the world, and many workers have stressed the importance of characterizing marine *Streptomyces* spp. So the present study reveals that there is the untapped potential of Actinobacteria available in the sediments of this pristine islands which can be further effectively be utilized for future biotechnological as well as medical industries.

## Plate 2.1.

1. Burmanallah

2. Carbyn's Beach

3. Chattam

4. Chidiyatappu

5. Dignabad

6. Junglighat

7. Kodiyaghat

8. Marina Park

Plate 2.2.

9. Science Centre

10. Minnie Bay

11. Sippighat

12. Collinpur Beach

13. Wandoor

14. Billiground

15. Betapur

16. Yerrata

## Plate 2.3.

17. Bakultala

18. Rangat

19. Nimbutala

20. Kadamtala

21. Karmatang

22. Austin Creek

23. Mayabunder

24. Kalighat

## Plate 2.4.

25. Arial Bay Jetty

26. Durgapur

27. Kalipur

28. Shyam Nagar

29. Kalighat 2

30. Kalipur 2

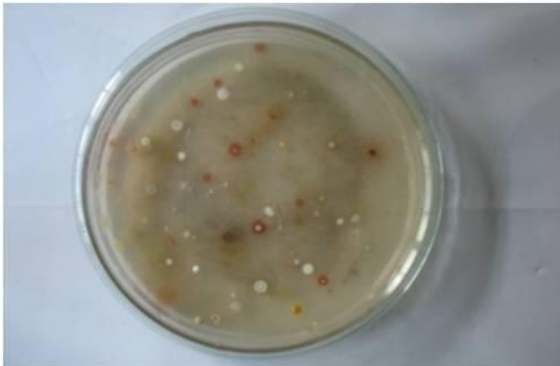
31. Durgapur 2

32. Shibpur

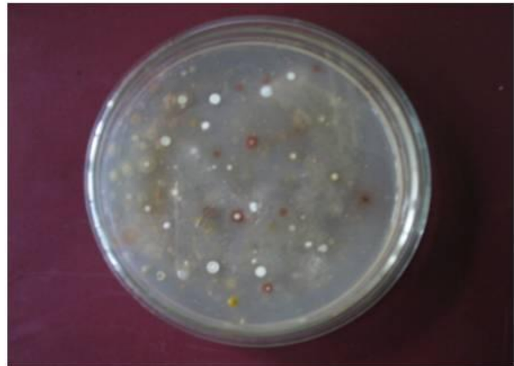
# Plate 2.5.

33. Sampling by skin diving

34. Sampling using SCUBA



35. Culture Photo A



36. Culture Photo B

37. Culture Photo C

38. Culture Photo D

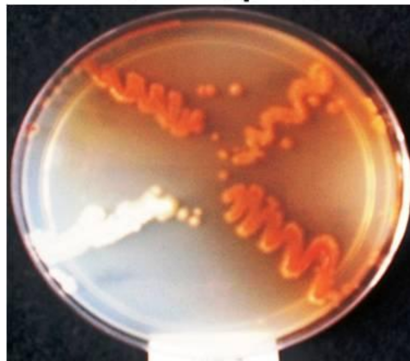
39. ISP 1 Top view

40. ISP 1 Bottom view

## Plate 2.6.

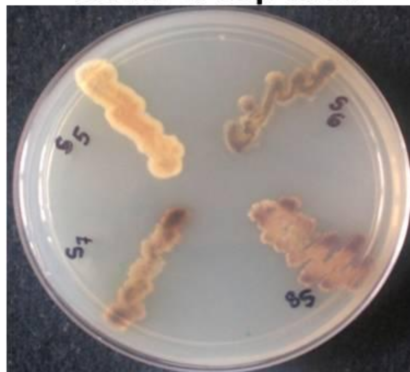
41. ISP 2 Top view

42. ISP 2 Bottom view



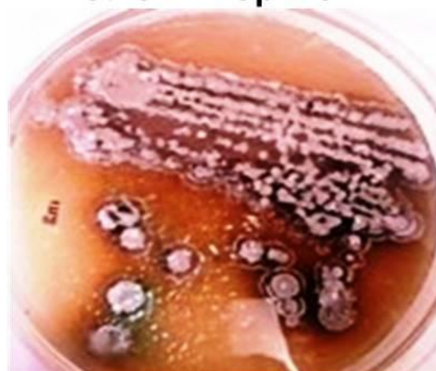
43. ISP 5 Top view

44. ISP 5 Bottom view



45. ISP 7 Top view

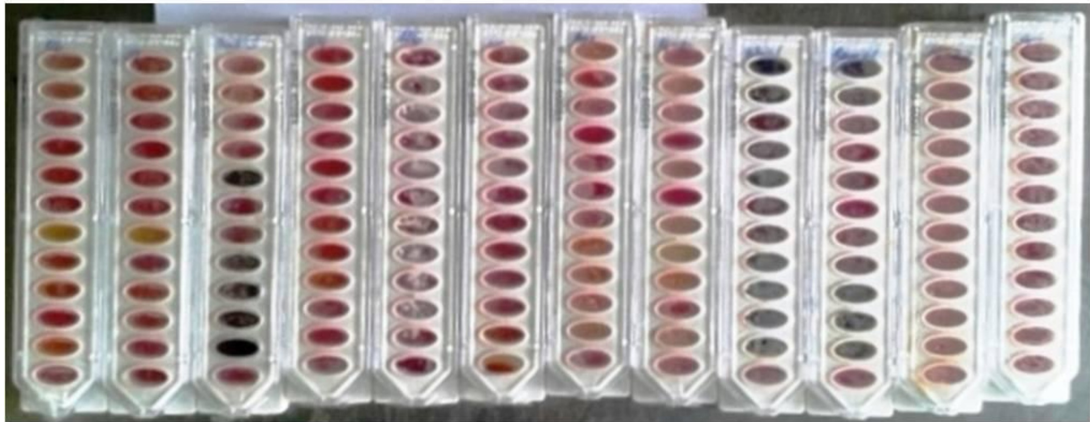
46. ISP 7 Bottom view



47. *Streptomyces* sp.

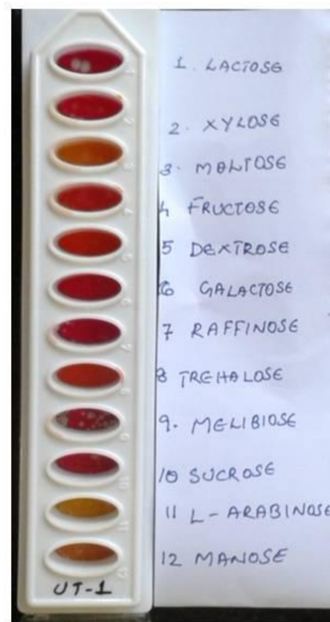
48. *Streptomyces* sp. Microscopic view

# Plate 2.7.



49. Carbohydrate utilization tests A

50. Carbohydrate utilization tests B




51. Carbohydrate utilization tests C

# Chapter 3

# Media Optimization for Lipase Production by *Streptomyces albus ST 04*

## 3.1. Introduction

Actinobacteria, especially genera from Streptomycetaceae are important producers of various compounds of biological importance especially for human usage, like antibiotics. Marine actinomycetes are noble microbes, producers of highly valuable natural products and considered to be an important source of new medicines. Although only very few studies on natural products have assessed the taxonomic nobility of strains from marine habitats, it has generated new understanding. It is interesting to note that only a small number of taxa are accounted for the bulk of the already discovered bioactive compounds till date and the most important group being Actinomycetales (actinomycetes) (Newman et al. 2003, Blunt et al. 2004, Keller and Zengler, 2004 and Paul et al. 2005).

Enzymes are proteins and are also known as biological catalysts. Isolation and purification of enzymes are complicated processes which include several steps to isolate a single type of protein from a cluster of the protein complex. Even though the enzymes can be isolated from tissues or microbe samples, the quality and quantity of enzyme from a particular sample is very important. Preliminary screening and quantification have to be performed before going through the isolation and purification process.

Actinobacteria, especially *Streptomyces* spp. are very good producers of extracellular enzymes. Industrially important enzymes like Protease, Lipase, Amylase, Chitinase, etc. are very important and one among the best source of novel and quality enzymes are *Streptomyces* spp. from various wild habitats. In this study, streptomycetes isolated from sandy, muddy, coral, rocky and mangrove habitats were isolated, characterized and screened for lipase activity.

Lipases, which are hydrolases, play an essential role in Lipid metabolism. Chemically this group of enzymes is known as Triacylglycerol hydrolases (EC 3.1.1.3) (Aloulou et al. 2006). These enzymes catalyse the hydrolysis ester bonds of glyceride which are present in fat and lipids. Microorganisms such as bacteria and fungi are good producers of extracellular lipases which shows a broad substrate specificity to break down the insoluble lipid into soluble polar components and thus facilitate absorption (Lie et al. 1991). The major character of lipases is that they are more active with insoluble ester substrates as against esterases that act only on soluble ester substrates (Sarda and Desnuelle, 1958, Entressangles and Desnuelle, 1968).

The lipases show a wide range of substrate specificity, adaptable molecular structure and stable in most of the organic solvents (Jaeger et al. 1999, Elibol and Ozer, 2000). These enzymes were isolated from many genera, *Achromobacter,* Acinetobacter, *Alcaligenes, Arthrobacter, Burkholderia*, *Chromobacterium, Staphylococcus*, *Streptococcus*, *Streptomyces* etc. as well as from the strains *Serratia marescens*, *Pseudomonas aeruginosa*, *Bacillus* sp. etc. (Abramic et al. 1999, Hasan et al. 2006, Selva Mohan, 2008, Dutta and Ray, 2009, Sangeetha et al. 2008). The increased demand for these enzymes in various industries supports the increased quest for lipases from these microorganisms as well as better purification and characterization (Sommer et al. 1997, Dartois et al. 1992, Cruz et al. 1994). Lipase enzymes of microbial origin play a significant role in oleochemical industries to catalyse a number of useful reactions including esterification, transesterification and leather industries. They are used in industries like organic chemical processing, detergent manufacturing, biosurfactant synthesis, dairy, agrochemistry, paper, nutrition, cosmetics and medicine (Haki et al. 2003), etc. Even though

Streptomycetes were recognized for their excellent enzyme activities, earlier works were not much concentrated on the lipases (Jaeger et al. 1999) except for few studies (Aly et al. 2012).

Isolation and characterization of genetically homologous lipases from *Streptomyces exfoliatus* and *S. albus* (Perez et al. 1993); cloning and sequencing of extracellular lipase-encoding gene from *S. cinnamomeus* (Sommer et al 1997); three dimensional structure elucidation of lipase enzyme from *S. exfoliatus* (Wei et al. 1998) were some of the earlier works related to lipase production from *Streptomyces* spp. Recent works include the isolation of *S. exofoliatus* from oil-contaminated area and screening for lipase production (Aly et al. 2012), comparison of inducer substrates Tween and triglycerides (vegetable oils) of different types in the medium and evaluation of Lipase enzyme activity from *S. halstedii* (Srilekha et al. 2014) etc.

Media optimization is an important step, which helps to quantify the composition of an ideal medium as in industry, 30 to 40 % costs account for growth medium (Joo et al. 2002, Laxman et al. 2005, Hajji et al. 2008). This will enable industry to formulate best nutritional as well as physic-chemical conditions so that the microorganism utilize components efficiently to obtain a cost-effective metabolite yield at the end of each production cycle (Hajji et al. 2008, Oskouie et al. 2008, Bhunia et al. 2011, Bayoumi and Bahobil, 2011).

Statistical methods are effective tools while optimizing various components as these experimental designs create a platform for better understanding of conditions affecting the stability of media (Montogomery, 2003, Myers and Montogomery, 2002). The statistical approach such as fractional experimental design, RSM and

Analysis of variance (ANOVA) has been successfully experimented and verified (Ghanem et al. 2000, Senthilkumar et al. 2005, Ghafari et al. 2009).

RSM is used to design experiments, to construct empirical models, evaluate the impacts of component factors and search for the optimum conditions in the model. The standard RSM mainly consists of two parts. First, it conducts an experiment to screen the important factors. Mostly it is based on a first-order design such as the $2^{n-k}$ fractional factorial designs (FFDs) or designs such as Plackett Burman design for screening (Breyfogle, 1992, Deming and Morgan, 1993, Onsekizoglu et al. 2010, Harry et al. 2001). This is suitable for identifying the most significant components in the medium formula. Second, it conducts a more intensive study of fewer factors and over a smaller region based on a second-order design such as the CCD (Box and Wilson, 1951). RSM is a helpful tool to study the effects of components as well as interaction among components. The experimental designs, the actual values are changed to a scale from 1 to 1 for screening and optimizing variables. This will eliminate the effects of variable ranges to facilitate the data analysis and obtain inferences (Mills et al. 2010). RSM has facilitated modelling and optimization of bioprocesses like fermentations (Sen, 1997), enzymatic reactions (Ferreira-Dias et al. 1998), product recovery (Annadurai et al. 1996) and techniques for enzyme immobilization (Chang et al. 2007). Widely used designs in RSM are the second-order models such as Central Composite Box-Behnken and Doehlert as they engage a wide range of functional forms (Dean and Voss, 2006). This flexibility in the approach permits for a close approximation of the true response surface (Adinarayana and Ellaiah, 2002, Doddapaneni et al. 2007, Li et al. 2007). According to The experimental designs and RSM applied successfully in the fermentation process and achieved improved product yields with reduced development time,

process variability and overall costs. In Biological Sciences, statistical experimental designs were not prevalently used except in biodegradation and bioremediation, biomaterials and bioprocessing technologies, genetic engineering, etc (Kwang-Min and David, 2005).

The concentrations of lipid carbon, nitrogen, pH, temperature, DO, etc. affects lipase enzyme production (Elibol and Ozer, 2001, Farshid et al. 2016) and is essential for obtaining a high lipase yield. In an important finding, cloned lipase was produced from the metagenome of *S. lividans*, and it has shown a maximal activity (4287 U/mg) at pH 8.5 and 60°C towards p-nitrophenyl butyrate (Cote and Shareck, 2010).

Pristine island ecosystem of Andaman and Nicobar Islands is a biodiversity hotspot, and the actinobacteria isolated from the coast of Andaman showed a clear dominance of *Streptomycetes* spp. (Refer to Chapter 2). These actinobacteria are important antibiotic as well as anticancer compound producing agents and also an important source of extracellular enzymes like Lipases. Most of the extracellular compounds were found to identify from the terrestrial area, but the marine environment is an emerging area for exploitation. So the present chapter discusses about the optimization of physical parameters and nutrient factors influencing culture conditions of lipase-producing marine *Streptomyces* spp. by RSM to maximize lipase enzyme productivity, isolated from coastal waters around the Andaman Islands.

## 3.2. Materials and Methods

### 3.2.1. Screening for Lipase

The pure cultures were screened for lipase activity by spot inoculation in tributyrin agar plates (Himedia) and were incubated for 3 to 5 days. The formation of the clearance zone around the colonies was noted as a positive result (Plate 3.1-3.2, Fig. 1-10), and the diameter of the zone was recorded. Among the 1083 streptomycete isolates, five isolates were found to have good lipase activity and were preserved at -80°C at glycerol stock.

### 3.2.2. Calculation of Enzyme Activity

Further screening of lipase activity of these selected cultures was done based on the titration method with olive oil hydrolysis (unit enzyme activity) (Selvamohan et al. 2008). Culture sample (200ul) was added to the substrate (2ml homogenized olive oil in 1% gum acacia, 1 ml of Tris-HCl buffer pH 8.0) and incubated at room temperature for 30 minutes on a magnetic stirrer. Ethanol: acetone mixture (1:1) was added (4ml) and titrated against NaOH (0.05N) after adding 2- 3 drops of Phenolphthalein indicator until light pink colour appears.

Lipase activity was calculated as micromoles of free fatty acids formed from olive oil per mL of crude lipase enzyme as per equation:

$$\mathbf{Activity} = \frac{\mathbf{(Vs - Vb)\ x\ N\ x\ 1000}}{\mathbf{S}}$$

Where,

$V_S$ is the volume of 0.05M NaOH solution consumed by the enzyme-substrate cocktail (ml);

$V_B$ is the volume of 0.05M NaOH solution consumed in the titration by the substrate (Control) cocktail (ml).

N is the molar strength of the NaOH solution used for titration (0.05M)

S is the volume of substrate cocktail solution (10ml).

One unit (U) of lipase enzyme is defined as the amount of enzyme required to liberate 1μmol of fatty acids from triglycerides.

### 3.2.3. Estimation of protein

Protein content was estimated following Lowry et al. (1951) at different stages of purification using BSA as standard. Suitable aliquots of samples with 10-100ug of protein, added 1ml and 5ml of alkaline copper reagent (50 ml of 2 % $Na_2CO_3$ in 0.1 N NaOH mixed with 1ml of 0.5% CUSO4 in 1% sodium potassium tartrate) and mixed thoroughly and incubated for 10 min at room temperature. To this, Folin reagent (0.5 ml of 1 N) was added and vortexed. After 30 minutes of incubation in the dark, the blue colour developed and OD was measured at 660 nm UV-Visible spectrophotometer (Systronic-112).

The *Streptomyces* sp. isolated from Yerratta mangrove region showed good lipase activity and was identified using 16s r DNA.

### 3.2.4. Optimization of Media for lipase production by Response Surface Methodology

Media optimization of *Streptomyces* spp. for lipase production was carried out using Placket Burman design (Harry et al. 2001). Composition of basal media consisted of peptone (10 g/L); $KH_2PO_4$ (1.5 g/L); inducer (0.5g/L); MgCl2 (0.5g/L); NaCl (0.3g/L%) ; Yeast extract (10g/L) and Incubation time (96 hrs).

### 3.2.4.1. Design of experiment

#### a. Plackett - Burman Design

Various components of the medium and culture characteristics of *Streptomyces* spp. was assessed for screening by Placket Burman Design. Each factor was tested by design at two levels: -1 (low level) and +1 (high level).

The first order model base for 2 Level Factorial experimental design is as follows:

$$Y = \beta_0 + \Sigma\beta_i x_i$$

Where Y is the enzyme activity, $\beta_0$ is the model intercept, and $\beta_1$ is the linear coefficient and *xi* is the level of the independent variable. The screening was done for 11 assigned variables in sixteen experimental designs.

The purified *Streptomyces* cultures were inoculated in culture flasks aseptically and incubated at various operational conditions. According to the operational conditions, the extent of lipase activity was evaluated. Optimization of media components was done to improve the lipase activity of *Streptomyces* ST04. Initially this was carried out by considering eleven variables viz. pH (4-10), inoculum volume (0.5-1ml); temperature (20-40°C), peptone (2-10g/L); $KH_2PO_4$ (0.5-1.5g/L) inducer (0.1-0.5g/L), $MgCl_2$ (0.5-1.5g/L), NaCl (0.1-0.3g/L%), Yeast extract (2-10g/L), Agitation (100-200rpm) and Incubation time (48-96hrs) as depicted in table 3.1.

The Box-Behnken design was employed for further optimization of levels after 2-level Factorial design to find out the most influential variables for the improved lipase production. According to the suggestions of the model, multiple trials were conducted, and the data were analysed by ANOVA. In order to omit trial errors, the experiments were carried out in triplicate and also tested for lack of fit of the data by using the second-degree polynomial model.

**Table 3.1 Variables selected for analysis by 2 Level Factorial design and their values at high and low levels**

| Sl. No | Nutrient code | Nutrient | Low value coded as (-1) (g/L) | High value coded as (1) (g/L) |
|---|---|---|---|---|
| 1 | A | Temperature | 20.0 | 40.0 |
| 2 | B | pH | 4.0 | 10.0 |
| 3 | C | Inducer | 0.1 | 0.5 |
| 4 | D | Inoculum volume | 0.5 | 1.0 |
| 5 | E | Yeast extract | 2.0 | 10.0 |
| 6 | F | Peptone | 2.0 | 10.0 |
| 7 | G | $MgCl_2$ | 0.5 | 1.5 |
| 8 | H | Agitation | 100.0 | 200.0 |
| 9 | J | Incubation | 48.0 | 96.0 |
| 10 | K | NaCl | 0.1 | 0.3 |
| 11 | L | $KH_2PO_4$ | 0.5 | 1.5 |

**b. Box-Behnken Design**

The variables selected by using the 2 Level Factorial experimental design were further fine-tuned by using Box-Behnken design of Response Surface Methodology (RSM) (Box and Behnken, 1960, Guo-qing et al. 2004). Each factor was studied at three different levels (-1, 0, +1) in this design as given in table 3.2., yielding a set of 17 experiments (Table 3.3.).

**Table 3.2. Variables included in the Box-Behnken design and their values at high and low levels.**

| Parameters | Units | Level of variable (%) coded as | | |
|---|---|---|---|---|
| | | (-1) | (0) | (+1) |
| pH | | 4.0 | 7.0 | 10.0 |
| Inducer | (g/L) | 0.1 | 0.3 | 0.5 |
| NaCl | (g/L) | 0.1 | 0.2 | 0.3 |

**Table 3.3. Box-Behnken design matrix showing the Lipase production using *Streptomyces ST04***

| Runs | pH | Inducer (g/L) | NaCl (g/L) |
|---|---|---|---|
| 1 | 0 | -1 | -1 |
| 2 | 0 | 0 | 0 |
| 3 | -1 | 0 | -1 |
| 4 | 1 | 0 | -1 |
| 5 | 0 | 0 | 0 |
| 6 | 0 | 0 | 0 |
| 7 | -1 | 1 | 0 |
| 8 | 1 | -1 | 0 |
| 9 | 1 | 0 | 1 |
| 10 | 0 | 0 | 0 |
| 11 | 0 | -1 | 1 |
| 12 | -1 | 0 | 1 |
| 13 | 0 | 0 | 0 |
| 14 | 0 | 1 | 1 |
| 15 | -1 | -1 | 0 |
| 16 | 1 | 1 | 0 |
| 17 | 0 | 1 | -1 |

The experimental design with respect to the values of the parameters in the actual and coded form was summarized in tables 3.2. and 3.3. The whole set of experiments was carried out in triplicates, and average values were taken as a response. A second order polynomial equation was then fitted to the data by using the Design-Expert software.

**Final Equation in Terms of Coded Factors:**

$$\text{Lipase Activity} = +61.80 + 0.9375A + 1.88\,B + 0.5625\,C - 0.2500\,AB + 0.1250\,AC - 0.5000\,BC - 1.96\,A^2 - 3.09B^2 - 1.21\,C^2$$

where A - pH; B- Inducer and C- NaCl

### 3.2.4.2. Design-Expert software

Design-Expert software version 11 (Stat-Ease Inc., Minneapolis, MN 55413) was used for designing the 2 Level Factorial and Box-Behnken models and data analysis.

### 3.2.5. Molecular identification of *Streptomyces* ST04

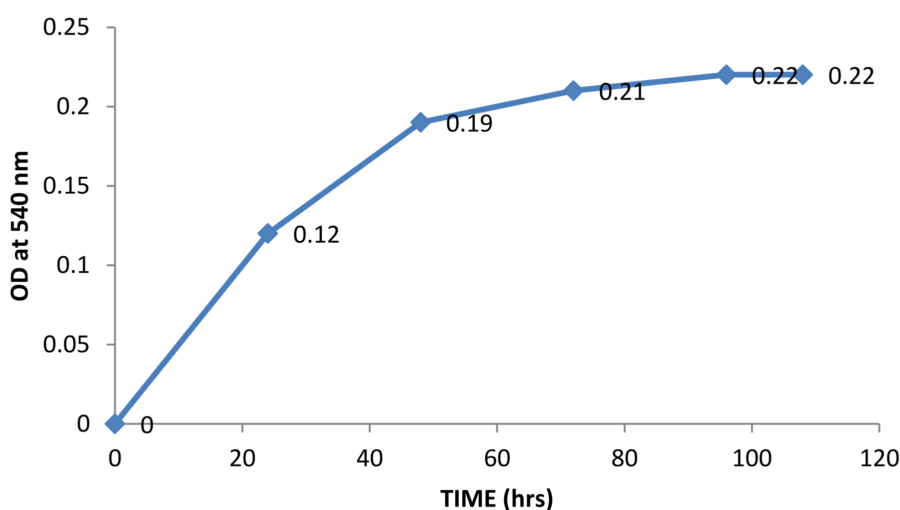
Total genomic DNA from the selected *Streptomyces* strain ST04 was isolated as per standard protocols (Sambrook et al. 1989, Sivakumar et al. 2005). The PCR amplification was done using the primers 8f (5'AGAGTTTGATCCTGGCTCAG 3'), and 1492r (5'GGTTACCTTGTTACGACTT 3') and Sanger sequence were generated using ABI PRISM 3730xl DNA Analyzer.

## 3.3. Results

### 3.3.1. Growth curve determination of ST04

The nutrient broth was used to study the growth pattern of the selected *Streptomyces* sp. ST04. Figure 3.1. shows the growth curve obtained from the measurement of optical density at 540nm. It is understood from the growth curve that the cells attain its maximum growth after 96 hours of incubation and the stationary phase continued up to 108 hours. The maximum lipase production was reported at 96 hours of incubation consistent to the change or concentration of media components.

**Figure 3.1. Growth curve of strain ST04**



### 3.3.2. Optimization of Lipase production using 2 Level Factorial Design

Twelve different runs were performed for optimization of Lipase production using ST04 and the results obtained was enlisted in table 3.4.

**Table 3.4. The yield of Lipase at various runs of 2 Level Factorial design**

| Run | A | B | C | D | E | F | G | H | J | K | L | Lipase production (U/ml) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | -1 | 1 | -1 | -1 | 1 | -1 | 1 | 1 | - 1 | 1 | 11.5 |
| 2 | 1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | - 1 | 1 | 1 | 14.5 |
| 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 15 |
| 4 | 1 | - 1 | -1 | -1 | 1 | -1 | 1 | 1 | - 1 | -1 | -1 | 8.5 |
| 5 | -1 | -1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | 1 | -1 | 13 |
| 6 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | - 1 | 1 | -1 | 12 |
| 7 | -1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | 1 | -1 | 1 | 11 |
| 8 | -1 | 1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | -1 | 14 |
| 9 | 1 | - 1 | 1 | 1 | -1 | -1 | 1 | -1 | -1 | -1 | 1 | 11 |
| 10 | - 1 | 1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | -1 | -1 | 12.5 |
| 11 | -1 | -1 | -1 | 1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | 11 |
| 12 | -1 | -1 | 1 | -1 | 1 | 1 | 1 | -1 | -1 | 1 | -1 | 13 |
| 13 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | -1 | -1 | 9 |
| 14 | -1 | -1 | 1 | -1 | -1 | -1 | -1 | -1 | 1 | 1 | 1 | 10 |
| 15 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | 12 |
| 16 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | 1 | 12 |

Highest lipase activity observed was 14.5U/ml at the $2^{nd}$ run. The results were fitted into a factorial model using 2 Level Factorial Experimental Design. pH, Inducer, and NaCl were identified as significant variables contributing to the lipase production. The result is depicted using a Pareto Chart (Figure 3.2.) and the 3D (Figure 3.3. A-C). Table 3.5. depicts the ANOVA results of the 2 Level Factorial Design.

**Figure 3.2. Pareto Chart showing the significant variables obtained in 2 level Factorial Design experiment**

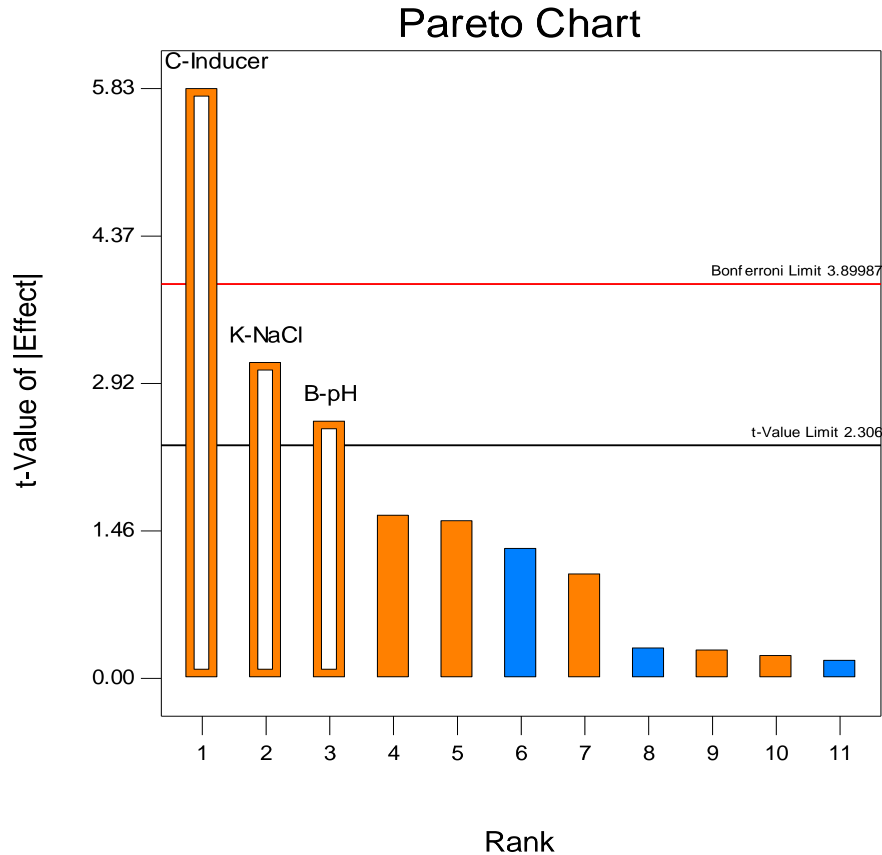



**Figure 3.3. The interactive effect of significant variables in the lipase production using ST04 (A-C).**

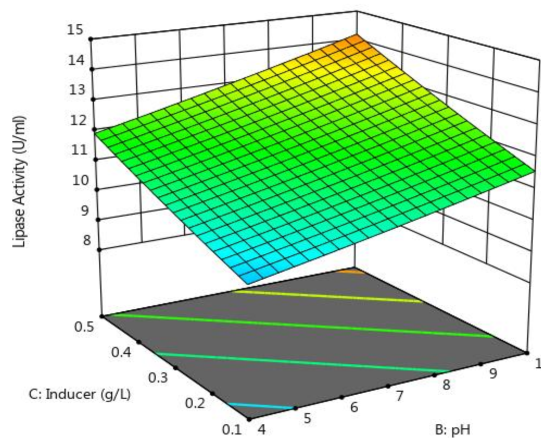
**A. Effect of Inducer and pH**

**Design-Expert® Software**
Factor Coding: Actual
Original Scale

**Lipase Activity (U/ml)**
8.5 15

X1 = B: pH
X2 = C: Inducer

**Actual Factors**
A: Temparature = 30
D: Inculum volume = 0.75
E: Yeast extract = 6
F: Peptone = 6
G: $MgCl_2$ = 1
H: Agitation = 150
J: Incubation = 72
K: NaCl = 0.2
L: $KH_2PO_4$ = 1



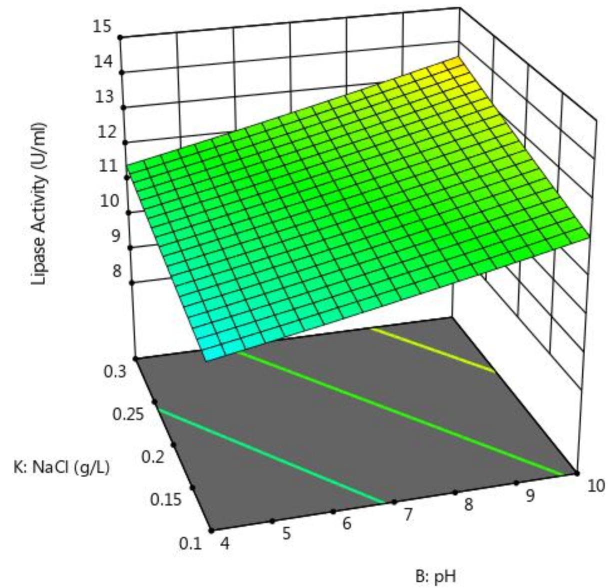
## B. Effect of NaCl level and pH

**Design-Expert® Software**
Factor Coding: Actual
Original Scale

**Lipase Activity (U/ml)**

X1 = B: pH
X2 = K: NaCl

**Actual Factors**
A: Temparature = 30
C: Inducer = 0.3
D: Inculum volume = 0.75
E: Yeast extract = 6
F: Peptone = 6
G: MgCl2 = 1
H: Agitation = 150
J: Incubation = 72
L: KH2PO4 = 1



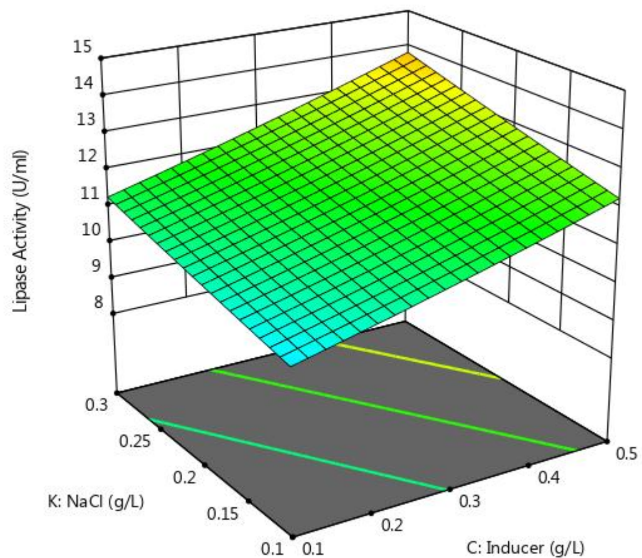

## C Effect of NaCl and Inducer

**Design-Expert® Software**
Factor Coding: Actual
Original Scale

**Lipase Activity (U/ml)**

X1 = C: Inducer
X2 = K: NaCl

**Actual Factors**
A: Temparature = 30
B: pH = 7
D: Inculum volume = 0.75
E: Yeast extract = 6
F: Peptone = 6
G: MgCl2 = 1
H: Agitation = 150
J: Incubation = 72
L: KH2PO4 = 1



At high pH with supplementation of inducer in the medium, an increased lipase production was observed. Also, a high concentration of NaCl and inducer showed similar results. The model was fitted into a linear equation represented as follows:

| **Final Equation in terms of coded factors:** |
|---|
| Ln (Lipase Activity) = +2.46 +0.870x B +0.1017× C+0.0598xK |

**Table 3.5. Analysis of Variance (ANOVA) of 2 Level Factorial Design**

| **ANOVA for selected factorial model** | | | | | | |
|---|---|---|---|---|---|---|
| **Analysis of variance table [Partial sum of squares – Type III]** | | | | | | |
| Source | Sum of Squares | df | Mean Square | F Value | p-Value | |
| | | | | | Prob>F | |
| Model | 0.3437 | 3 | 0.1146 | 43.13 | < 0.0001 | significant |
| B-pH | 0.1210 | 1 | 0.1210 | 45.57 | < 0.0001 | |
| C-Inducer | 0.1654 | 1 | 0.1654 | 62.27 | < 0.0001 | |
| K-NaCl | 0.0573 | 1 | 0.0573 | 21.56 | 0.0006 | |
| Residual | 0.0319 | 12 | 0.0027 | | | |
| Cor Total | 0.3756 | 15 | | | | |

**Table 3.5.1**

| | | | |
|---|---|---|---|
| Standard Deviation | 0.051 | R-Squared | 0.9151 |
| Mean | 2.46 | Adj R-Squared | 0.8939 |
| C. V. % | 2.09 | Pred R-Squared | 0.8491 |
| PRESS | 0.0567 | Adeq Precision | 19.2851 |
| -2 Log Likelihood | -54.09 | BIC | -43.00 |
| | | AICc | -42.45 |

The model is significant as per the Model F-value of 43.13. Present results indicated that pH, Inducer and NaCl are significant.

### 3.3.3. Box-Behnken Design

The Box-Behnken design indicated that lipase production was high at a medium concentration of NaCl, at an Inducer concentration of 4g/L and pH 8. During the optimization, a maximum lipase activity of 17U/ml could be achieved (Table 3.6.). The $R^2$ value of the predicted model was found to be significant. Figure 3.4. A-C shows the results of response surface analyses of significant variables in Lipase production. ANOVA results of the design are shown in Table 3.7.

**Table 3.6. Box-Behnken design matrix showing the Lipase production using ST04**

| Runs | pH | Inducer g/L | NaCl g/L | Lipase Production (U/ml) |
|---|---|---|---|---|
| 1 | 0 | -1 | -1 | 9.5 |
| 2 | 0 | 0 | 0 | 17 |
| 3 | -1 | 0 | -1 | 12 |
| 4 | 1 | 0 | -1 | 14 |
| 5 | 0 | 0 | 0 | 16.5 |
| 6 | 0 | 0 | 0 | 17 |
| 7 | -1 | 1 | 0 | 13 |
| 8 | 1 | -1 | 0 | 11 |
| 9 | 1 | 0 | 1 | 15.5 |
| 10 | 0 | 0 | 0 | 16.5 |
| 11 | 0 | -1 | 1 | 11.5 |
| 12 | -1 | 0 | 1 | 13 |
| 13 | 0 | 0 | 0 | 17 |
| 14 | 0 | 1 | 1 | 14.5 |
| 15 | -1 | -1 | 0 | 9 |
| 16 | 1 | 1 | 0 | 14 |
| 17 | 0 | 1 | -1 | 14.5 |

**Figure 3.4. The Response Surface Plot of the combined effects of significant variables on the lipase production using ST04 (A-C)**

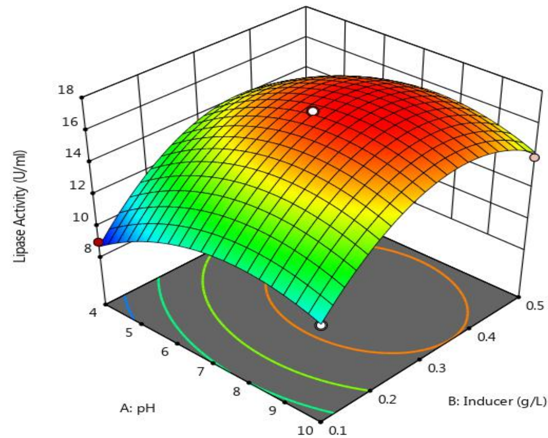
## A Effect of pH and Inducer

Design-Expert® Software
Factor Coding: Actual

Lipase Activity (U/ml)
Design points above predicted value
Design points below predicted value
9 17

X1 = A: pH
X2 = B: Inducer

Actual Factor
C: NaCl = 0.2



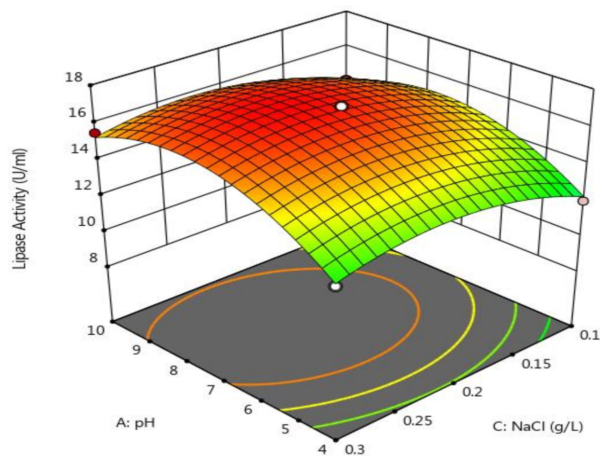

## B Effect of pH and NaCl

Design-Expert® Software
Factor Coding: Actual

Lipase Activity (U/ml)
Design points above predicted value
Design points below predicted value
9 17

X1 = A: pH
X2 = C: NaCl

Actual Factor
B: Inducer = 0.3




## C Effect of Inducer and NaCl

Design-Expert® Software
Factor Coding: Actual

Lipase Activity (U/ml)
Design points above predicted value
Design points below predicted value
9 17

X1 = B: Inducer
X2 = C: NaCl

Actual Factor
A: pH = 7

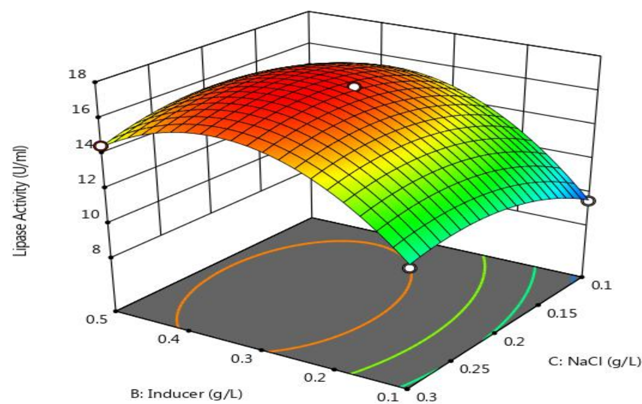


**Table 3.7. Analysis of variance (ANOVA) for the response surface quadratic model**

| Analysis of variance table [Partial sum of squares – Type III] | | | | | | |
|---|---|---|---|---|---|---|
| **Source** | **Sum of Squares** | **df** | **Mean Square** | **F Value** | **p-value Prob > F** | |
| Model | 107.64 | 9 | 11.96 | 113.52 | < 0.0001 | Significant |
| A-pH | 7.03 | 1 | 7.03 | 66.74 | < 0.0001 | |
| B-Inducer | 28.12 | 1 | 28.12 | 266.95 | < 0.0001 | |
| C-NaCl | 2.53 | 1 | 2.53 | 24.03 | 0.0017 | |
| AB | 0.2500 | 1 | 0.2500 | 2.37 | 0.1674 | |
| AC | 0.0625 | 1 | 0.0625 | 0.5932 | 0.4664 | |
| BC | 1.00 | 1 | 1.00 | 9.49 | 0.0178 | |
| $A^2$ | 16.22 | 1 | 16.22 | 153.92 | < 0.0001 | |
| $B^2$ | 40.14 | 1 | 40.14 | 380.97 | < 0.0001 | |
| $C^2$ | 6.19 | 1 | 6.19 | 58.75 | 0.0001 | |
| Residual | 0.7375 | 7 | 0.1054 | | | |
| Lack of Fit | 0.4375 | 3 | 0.1458 | 1.94 | 0.2643 | Not Significant |
| Pure Error | 0.3000 | 4 | 0.0750 | | | |
| Cor Total | 108.38 | 16 | | | | |

**Table 3.7.1**

| Statistical optimization | Variables | Lipase activity (U/ml) |
|---|---|---|
| 2 Level Factorial | pH, Inducer, NaCl | **14.5** |
| Box-Behnken Design | pH, Inducer, NaCl | **17** |

The ANOVA for the response surface quadratic model F-value of113.52 implies the model is significant. The model was significant at 95% confidence level with a Prob>F of < 0.0001. The P-value less than 0.05 indicated that the model terms indicated that model terms are significant. Here A, B, C, BC, $A^2$, $B^2$ $C^2$ are

significant model terms. It was found that the Lack of Fit F-value (1.94) is not significant in relation to the pure error.

### 3.3.4. Molecular characterization of Streptomyces ST04

Five *Streptomyces* spp. which showed good Lipase activity was identified up to species level and gene sequences were submitted to NCBI Genebank and got Accession Number ACC. NO:- KC95427 (*Streptomyces carpaticus*), KC818233 (*S. thermocarboxydus*), KF002486 (*S. albus*), KF002487 (*S. diastaticus*), KF002488 (*S. violaceorubidus*). *Streptomyces* ST04 have shown 96% similarity to *Streptomyces albu*s.

## 3.4. Discussion

Lipolytic enzymes are a group of enzymes that catalyses the hydrolysis of Triacylglycerols into Monoacylglycerol, and fatty acids and glycerol (Bomscheuer, 2002, Casas-Godoy, et al. 2012, Fan et al., 2012, Davender et al. 2012, Ramnath et al. 2000). Lipases and esterases form a diverse group of hydrolytic enzymes that catalyses the lipids like triglycerides (Gandhimathi et al. 2009, Aly et al. 2011). Lipases have potential to be used in the processing of oils and fat, cosmetics, diagnosis and detergents (Ninawe et al. 2006).

The lipolytic enzyme activity is mostly expressed by microbes in habitats of oil contamination. The Andaman Islands are bestowed with pristine beaches, less disturbed mangroves, coral and rocky environment. These habitats provide excellent environments for the bacterial population with natural enzyme activities. During the present study, *Streptomyces* spp. isolated from the coast of Andaman have shown good production of protease, amylase, and lipase. Isolates from mangrove are good producers of amylase enzyme. Kafilzadeh et al. (2015) isolated very good amylase producing actinobacteria from mangrove forest of southern Iran.

A number of Actinomycetes strains have the ability to hydrolyse oils and fats (Gunalakshmi et al. 2008). The present study has found that the isolates from mangrove area shown good lipase activity. The demand for the industrial enzymes of microbial origin is increasing day by day, and there has been a number of works has been conducted in *Streptomyces* spp. especially Amylase and cellulase activity of *Streptomyces* spp. (Chakraborty et al. 2012). Lipase activity of *Streptomyces* spp. was found to be less, compared with the studies conducted in other enzyme activities. Lipases are a group of an important enzyme, as they process ecological importance, which quickens the degradation of fat particles. Takamoto et al. (2001) described that these enzymes possess the ability to degrade polyurethane in an organic solvent. The above work mainly concentrated on the screening and isolation of Lipase enzyme from streptomycetes species. Vishnupriya et al. (2010) observed *S. griseus* strain has good Lipase activity in olive oil, sunflower oil, and palm oil. Rajinikanth et al. (2016) observed extracellular lipase production by *S. fungicidicus* RPBS-A4 isolated from the Bay of Bengal. In the present study isolation and screening of lipase-producing *Streptomyces* sp. were done to find out the most promising lipase producing candidate. It is the preliminary step for understanding the fermentation process in Actinobacteria.

Media optimization plays a significant role to identify the ideal condition for the production of the maximum enzyme. The nutritional requirements as well as the growth of every organism is distinctive and is the same for enzyme production also. So it is essential that proper components in correct combination are to be determined for the culture medium along with optimized culture characteristics (Laxman et al. 2005, Hajji et al. 2008, Rai and Mukherjee, 2010, Pillai et al. 2011). The optimization of culture media through the Plackett–Burman and the Box–Behnken

design has shown that pH, salinity as well as inducer concentration are the significant variables for enzyme production of *Streptomyces* sp. ST04. Gholamhossein et al. (2013) also reported that pH is one among the most important variable affecting the enzyme production. Lang et al. (2002 suggested that proper nutrition and environmental parameters are essential to enhance the enzyme production. While Tanyildizi et al. (2005) indicated that the same parameters could not be suitable for all microorganism and most of the sugars in the media repress the production of lipase. However, Devraj et al. (2018) found out that when compared with other carbon and nitrogen sources, the addition of 5% yeast extract and 3% sucrose in the medium has enhanced lipase production. Thus microorganisms maintain uniqueness in its nutritional requirements and there is no defined medium microbial metabolite production (Tari et al. 2006, Beg et al. 2003). Limited number of experiments and less time consumption are found to be an add-on advantage. The experimental designs like factorial design used to plan experiments to get finest responses such as product yield and productivity (Wang and Lu, 2004).

According to the design, eleven parameters were examined to attain maximum lipase production. The factors including environmental factors (pH, NaCl, temperature), carbon sources (olive oil), nitrogen sources (yeast extract), and divalent cations ($MgCl_2$, $KH_2PO_4$) were used in the defined medium for six days. Olive oil used as the substrate for production of lipase. Among natural oils, olive oil has been mentioned as one of the best inductors and substrate for lipase production (Bornscheuer, 2002). In the present study along with Inducer olive oil concentration, pH and Sodium Chloride concentration enhanced the production of the enzyme. The optimum pH was found to be 8, which indicate that these *Streptomyces* ST04 produces the highest quantity of lipase at alkaline pH. Whereas other works have

shown that maximum lipase activity was recorded between 7-7.5 (Kulkarni et al. 2002, Sekhon et al. 2008). In the present study, the temperature did not show any significant role in Lipase production. Even though other factors also important for the growth and lipase production, it is not necessary that all these factors will become limiting factors for the species.

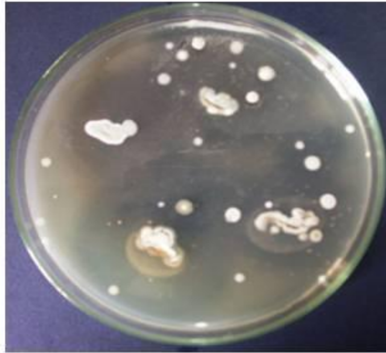
Isolates were identified by conventional methods like morphological and biochemical characteristics (Nonomura, 1974, Shirling and Gottlieb, 1966) and promising isolates were identified by molecular method (O'Donnell et al. 1993, Sambrook et al. 1989), and sequences were submitted to NCBI. Molecular identification is found to be a backbone of the characterization based on 16S rDNA gene sequence analysis.

The isolates showed lipase activity were identified as KC95427 (*Streptomyces carpaticus*), KF002486 (*S. albus*), KC818233 (*S. thermocarboxydus*), KF002487 (*S. diastaticus*), KF002488 (*S. violaceorubidus*). *Streptomyces* sp. isolate ST04 was isolated from the station Yerratta of northern Andaman Mangrove area where the sediment was clay type. The isolate showed 96% similarity to *S. albus* (Acc. No. KF002486). Sommer et al. (1997) described two *Streptomyces* lipase sequences, the 28-kDa lipase of *Streptomyces* sp. strain M11 and the similar-sized lipase of *S. albus*. Aly et al. (2012) explained two homologous lipases characterized from *S. exfoliatus* and *S. albus.* Sathya Priya et al. (2012) found out lipase activity of *S. albus* isolated from sediments of Tiruchendhur coastal areas of Tamil Nadu was at pH 10.0 and 50°C.

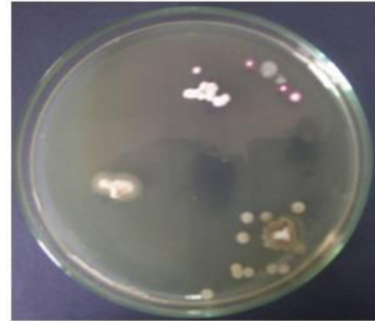
In order to formulate proper media, it is very important to optimize components (Singh and Satyanarayana, 2006) and parameters. The present study has optimized

media and other conditions to maximize the production of lipase enzyme produced by *S. albus* ST04 with minimal supplementation of organic nitrogen sources such as yeast extracts, peptone for economical production of the enzyme.

# Plate 3.1.



1. Lipase Activity 1

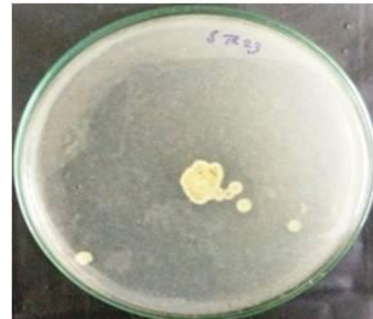


2. Lipase Activity 2

3. *Streptomyces carpaticus* 1

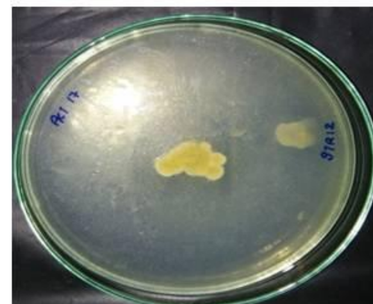
4. *Streptomyces carpaticus* 2

5. *Streptomyces diastiticus*



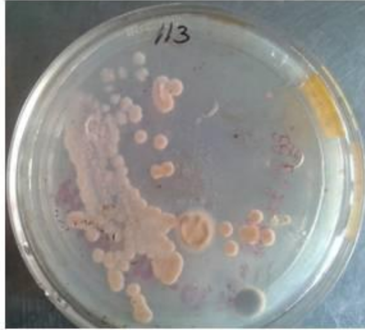
6. *Streptomyces diastiticus* 2

7. *Streptomyces thermocarboxidus* 1

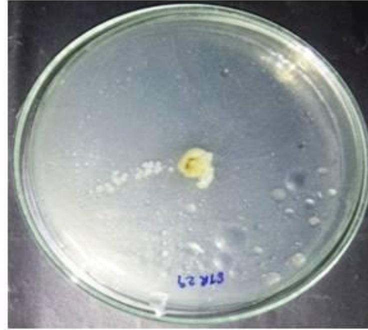


8. *Streptomyces thermocarboxidus* 2

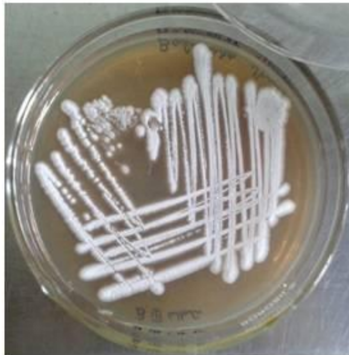
# Plate 3.2.




**7. *Streptomyces violaceorubidus* 1**



**8. *Streptomyces violaceorubidus* 2**



**9. *Streptomyces albus* 1**

**10. *Streptomyces albus* 2**

# Chapter 4

# Purification and Characterization of Lipase from *Streptomyces albus* ST 04

### 4.1. Introduction

Microbial enzymes have vivid uses in industries and medicine and are more active and stable than plant and animal enzymes. In addition, the easiness of production of enzymes from microbial sources by fermentation leads to applications in biochemical diversity and susceptibility to genic engineering. Industries are eager to produce new microbial strains which will be able to overcome current requirement and more. The fermentation technology and its introduction in microbial production with the aid of new strain or selected strains helps large-scale production of industrially certain products and process, for example, detergent, starch, and textile industries. The recombinant DNA technology has an unavoidable role in the introduction of the improved and modified production process. It helps to produce enzymes with more potential applications. Furthermore, the developments and advances in microbial biotechnology provide different kinds of enzymes displaying new activities, adaptability to new conditions leading to their increased use in industrial purposes via protein engineering and mutagenesis.

Due to the wide range nature of reactions, enzymes are being classified according to their enzyme catalysing reaction. Among them lipase has got pertinent role in the industry due to its potential applications in different industrial sectors and microbial biotechnology such as detergent manufacturing, food ingredient production, wastewater treatment, paper processing, pharmaceutical production, fine chemical synthesis and manufacture of pesticides, cosmetics and single cell protein (Jaeger et al. 1999). They can be used as a catalyst for the synthesis of esters and trans-esterification of the oil for the production of biodiesel (Gunstone, 1999, Poonam et al. 1995, Gulati et al. 2005). Lipases, (triacylglycerol acyl hydrolases, EC3.1.1.3), are natural catalysts of the hydrolysis of triacylglycerols into di- and

monoacylglycerols, fatty acids, and glycerol at an oil-water interface, a phenomenon known as interfacial activation (Schmidt et al. 1998). Currently, lipases become an automatic choice as a biologically active enzyme because they can be applied to chemo, regio and enantio selective hydrolyses and also in the syntheses of a broad and vivid range of compounds.

If we consider the vividness of the application, the availability of the lipase enzymes with characteristic features is still a limiting factor. This is why researchers are fond of doing there works on various strains having versatile characteristic properties in improved and modified productions. There are a number of lipase-producing bacteria, but only a few are commercially exploited as wild or recombinant strains. Among these, the important ones are *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium*, *Enterococcus*, *Corynebacterium, Streptomyces,* and *Pseudomonas*. In a variety of biotechnological applications lipases from *Pseudomonas* are widely used. Lipase producing bacteria have been found in various habitats such as soil contaminated with oil, dairy waste, industrial wastes, oil seeds, decaying food, compost heaps, coal tips and hot springs. According to the biological properties of enzymes and conserved sequence motifs, 53 sequences of bacterial lipases and esterase's were compared and classified (Arpigny andJaeger, 1999). Only about 2.0 percent of the world's microorganisms have been tested as enzyme sources. Among them, *Streptomyces* are the most important industrial microorganisms because of their capability to produce numerous bioactive molecules having industrial relevance, particularly antibiotics and enzymes (Lam, 2006).

*Streptomyces* bacteria are widely distributed in nature, especially in the soil. From currently known 364 species of *Streptomyces*, most of them are important in industry for producing antibiotics, some secondary metabolites having antibacterial, antifungal, antiviral, and antitumor nature, as well as immunosuppressants, antihypercholesterolemic activities. Due to diverse metabolism of *Streptomyces* spp. for degrading the insoluble present in other organisms, like lignocelluloses and chitin, these strains are important in soil environment. This is why the *Streptomyces* strain grown in coastal water of Andaman Islands was used for current study. The strain was subjected to amplification of 16S rRNA for determining the genetic relatedness using DNA from the strain, and the sequence analysis was carried out. The sequences of *Streptomyces* species having good lipase activity were identified as *S. carpaticus*, *S. thermocarboxydus, S. albus*, *S. diastaticus*, *S. violaceorubidus*. Among them isolated strain got more genetic relatedness with *Streptomyces albus, i.e.* 96%, and so it was named as *Streptomyces albus* ST 04. Considering the biotechnological importance of bacteria in enzyme modification, the present research reported the optimization of media and process parameters by a classical method in solid state fermentation for maximum production of lipase under optimum conditions.

## 4.2. Material and methods

### 4.2.1. Mass production of Lipase from *S. albus* ST 04

The isolate ST 04 was mass cultured in basal medium incorporated with optimized nutrients (in g $L^{-1}$ distilled water) peptone (10 g $L^{-1}$); $KH_2PO_4$ (1.5 g $L^{-1}$); inducer (0.5 g $L^{-1}$); $MgCl_2$ (0.5 g $L^{-1}$); NaCl (0.3 g $L^{-1}$%); Yeast extract (10g $L^{-1}$). Enzyme production of the bacterial cultures was carried out using submerged state fermentation under optimized conditions (25$^{o}$C pH 7.0+/-0.05 rpm and supplied with

sterile air at the rate 2.51 $min^{-1}$). For enzyme extraction, the culture was centrifuged at 10000 rpm for 10 min at 4°C, and the supernatant was stored as 200 ml aliquots at -20°C and used for further purification and characterization.

### 4.2.2. Enzyme purification

#### 4.2.2.1. Ammonium Sulphate precipitation of *S. albus* ST 04

Extraction of the enzyme was performed by Ammonium Sulphate precipitation (20%, 40%, 60%, and 80%) and partial purification of lipase following the method of Englard and Seifter (1990). The filtrate broth (crude) was collected and centrifuged at 10000 rpm for 15 min at 4°C to obtain a cell-free filtrate. After performing a test for sterility, 200 ml of the cell-free filtrate containing lipase was collected and determined their lipase activity. About 200 ml of the crude enzyme extract were first brought to 20% (w/v) saturation with solid Ammonium Sulphate (enzyme grade). The precipitated proteins were regimented by centrifugation for 15 min at 10000 rpm. The resulted pellet was dissolved in 25mM Sodium Phosphate buffer at pH 7.4. The left supernatant was precipitated again with Ammonium Sulphate to achieve 40%, 60%, and 80% (w/v) saturation. Thus crystal Ammonium Sulphate (Sigma Aldrich, Bengaluru) required to precipitate lipase was optimized by its addition, at 80% saturation to the crude extracts precipitated well. The addition of Ammonium Sulphate was done under constant stirring at 4°C for 30 min and kept overnight at 4°C with stirring. Precipitated protein was collected by centrifugation at 10000 rpm for 15 min at 4ºC. The precipitate was resuspended in 25 mM Na-phosphate buffer (pH-7.4).

#### 4.2.2.2. Dialysis

The precipitate obtained after Ammonium Sulphate precipitation was further dialyzed against (Sigma Aldrich, Bengaluru) 25 mM Na-phosphate buffer (pH-7.4). Dialysis was carried out by solute exchange and to enhance the speed of exchange the buffer should be gently stirred. Dialysis tube (Sigma-Aldrich) was first treated to remove the humectants and protectants like Glycerin and Sulphur compounds present in it and to make the pores of the tube more clear. The pretreatment involved washing of the tube in running water for 3-4 hours, dipping in 0.3 % (w/v) sodium sulfide at 80ºC for 1 min, further washing with hot water (60ºC) for 2 min followed by acid wash in 0.2 % (v/v) Sulphuric acid. Finally, the tube was rinsed with distilled water. The precipitated protein was dialyzed in the pretreated dialysis tube overnight for 48 hours at 4ºC with several changes of buffer to increase the efficiency of the dialysis and assayed for lipase activity, protein content, and specific activity. After dialysis, the sample was collected and stored under -20°C.

#### 4.2.2.3. Gel Filtration chromatography

Purification of lipase of *S. albus* ST 04 was carried out by the Sephadex G-100 (Sigma Aldrich, Bangalore) column chromatography. The lipase active fractions were pooled and separated in Sephadex G-100 column (2×20 cm). The lipase was eluted with the same buffer at a flow rate of 0.5 ml/min. The fractions (3ml) were collected and analyzed for lipase activity and protein content. The fractions showing the highest lipase activity were pooled, dialyzed and stored at 4°C for SDS-PAGE and other enzyme characterization.

### 4.2.3. Enzyme assay

#### 4.2.3.1. Estimation of Lipase activity of *S. albus* ST 04

Lipase activity was measured by a titrimetric method using olive oil as substrate at pH 7.0. The reaction cocktail contains by 5% (w/v) olive oil in 100 mM sodium phosphate buffer, pH 7. Nearly 0.1ml of purified lipase enzyme was added to the reaction cocktail of 10 ml separately and incubated for 15 min at 37°C at 100 rpm in a shaker incubator. After 15 min, 1ml of Acetone: Ethanol solution (1:1) was added for quenching the reaction and fatty acids were extracted by adding and swirling the contents swiftly. Phenolphthalein indicator of 2-3 drops was then added to each of the reaction mixtures with respect to different crude lipase enzymes of isolates and the control. The contents of each reaction mixture were titrated with 0.05M sodium hydroxide solution and the endpoint being the pink color at pH 10.0. Lipase activity was calculated as micromoles of free fatty acids formed from olive oil per mL of crude lipase enzyme as per equation:

$$\text{Activity} = \frac{(\text{Vs} - \text{Vb}) \text{ x N x } 1000}{\text{S}}$$

Where,

'$V_S$' is the volume of 0.05M sodium hydroxide solution consumed during the blank titration against the reaction mixture in ml.

'$V_B$' is the volume of 0.05M sodium hydroxide solution consumed during the titration against the reaction mixture in ml.

'N' is the molar strength of the sodium hydroxide solution used for titration and say it as 0.05M.

'S' is the volume of substrate cocktail solution is to be taken and say as 10ml.

Lipase activity can be measured in the unit, and one unit (1U) of lipase enzyme is defined as the amount of enzyme required to liberate 1µmol of fatty acids from triglycerides.

### 4.2.3.2. Protein estimation of *S. albus* ST 04

Protein content was estimated according to the method of Lowry *et al*., 1951 using bovine serum albumin (BSA) as the standard. To estimate the protein, an aliquot of 100 µL of the sample was made up to 1 mL with distilled water and added to 5 mL of freshly prepared working reagent (0.5 % Cupric sulphate and 1.0 % Sodium potassium tartrate in distilled water), mixed thoroughly, and incubated for 10 min. 0.5 mL of Folin and Ciocalteau's phenol reagent was added and incubated for 30 min. The absorbance of the blue color developed was measured at 660 nm in a UV-Visible spectrophotometer (Systronics Model 118, Ahmedabad).

### 4.2.3.3. Calculation of yield of protein, the yield of enzyme activity, a fold of purification of *S. albus* ST 04

The yield of protein and enzyme activity of each fraction obtained during purification is the percentage activity obtained by dividing the total protein content or activity of that fraction with the total protein content or activity of the crude extract was measured based on following equations.

$$\text{Yield of Protein} = \left(\frac{\text{Total protein content of the fraction}}{\text{Total protein content of the crude extract}}\right) \text{x } 100$$

$$\text{Yield of Activity} = \left(\frac{\text{Total activity of the fraction}}{\text{Total activity of the crude extract}}\right) \text{x } 100$$

$$\text{Fold of purification} = \left(\frac{\text{Specific activity of the faction}}{\text{specific activity of the crude extract}}\right) \text{x } 100$$

$$\text{Specific Activity} = \frac{\text{Enzyme activity (U/mL)}}{\text{mg of protein/mL}}$$

### 4.2.3.4. SDS-PAGE analysis of purified lipase of *S. albus* ST 04

The purity of the fractions, showing lipase activity, was checked by SDS-PAGE by the method of (Laemmli, 1970). The molecular weight of lipase was determined by comparison with standard molecular marker proteins (Promega Corporation, USA).

### 4.2.3.5. Procedure for SDS-PAGE Analysis of *S. albus* ST 04

The purified samples were then subjected to SDS- PAGE (using 12% resolving gel, 4% stacking gel) at a constant current of 12mA.

**A. Preparation of separating gel**

The desired solution was pipetted out into the sandwich template. 1cm of water was added on the top of the separating gel solution. The gel margin was kept uniform and allowed it to polymerize for 30 minutes.

**B. Preparation of stacking gel**

The stacking gel solution was poured onto separating gel using a micropipette until the solution reaches the top of the plate and carefully inserted a comb into the gel sandwich without trapping air bubbles. For the completion of the polymerization, the gel was kept for 30 minutes. After stacking gel has polymerized, the comb and basal spacer were carefully removed and fixed the gel setup into the electrophoresis apparatus.

**C. Sample preparation for SDS-PAGE**

To the purified enzyme, equal volume of sample buffer (0.05% bromophenol blue, 5% β-mercaptoethanol, 10% glycerol, and 2% SDS in 0.25M Tris–HCl buffer; pH 6.8) was added and then boiled at 100°C for 2 min, cooled to room temperature, and 30 µl sample and 5 µl low molecular weight markers were loaded onto the gel.

**D. Running SDS-gel**

The electrode plugs were attached to appropriate electrodes. The power supply was turned on to 60V until the sample entered into the separating gel and continued at 100V till the end of separating gel. After the dye front migrated to 1cm from the bottom of the gel in 120 minutes, turned off the power supply and spacers from the gel plate were carefully removed and proceeded for staining.

**E. Commassie brilliant blue staining of proteins**

The gel was agitated in staining solution for 5 hours on a slow rotary shaker. The container was covered with a lid during staining and destaining to prevent evaporation. The stain was poured out and rinsed the gel with distilled water. Destain solution was added and destained the gel for 3 hours. The gel was visualised on a transilluminator.

**4.2.3.6. Protein structure prediction of *S. albus* strain**

For understanding the nature of the lipase protein, the structure of Streptomyces *albus* sequence of protein in UniProt (D6B385) was developed by homology modelling method with the aid of structure of lipases/ esterase( PDB ID: 4v2i) obtained from protein data bank in the Swiss modelling tool (Water house et.al,2018). For getting the more insight about the sequence, the physicochemical analysis of the sequence was carried out in PROTPARAM tool for determining the molecular weight, theoretical pI, amino acid composition, instability index, aliphatic index and grand average of hydropathicity (GRAVY). For identifying domains of the predicted structure obtained PDB ID was submitted in PDB SUM from Procheck (Laskowski, 2009). The molecular structure of the obtained protein was visualized using PyMOL programme (Delano, 2002).

### 4.2.4. Enzyme characterization of *S. albus* ST 04

#### 4.2.4.1. Effect of various substrates on lipase activity

The substrate specificity of the enzyme to various substrates, pH, temperature, and various metal ion concentrations was evaluated.

##### 4.2.4.1.1. Effect of pH on lipase enzyme activity of *S. albus* ST 04

The effect of pH on lipase enzyme was monitored in the range of 3-11 using the 25 mM Na-phosphate buffer for 60 min at 28$^{o}$C. The standard lipase assay conditions were used for measuring relative activity of the enzyme.

##### 4.2.4.1.2. Effect of temperature on lipase enzyme activity of *S. albus* ST 04

The effect of temperature on lipase enzyme was monitored in the range 35$^{o}$C to 50$^{o}$C in 25 mM Na-phosphate buffer (pH-7.4). The enzyme was incubated at the temperature range for 1hr, and the assay was carried out.

##### 4.2.4.1.3. Effect of substrate concentration on lipase enzyme activity of *S. albus* ST 04

The effect of substrate concentration on enzyme was determined 25 mM Na-phosphate buffer (pH-7.4) with levels of *p*-nitrophenyl palmitate) as substrate ranging from 1% to 10%.

##### 4.2.4.1.4. Effect of metal ions on lipase enzyme activity of *Streptomyces albus* ST 04

The effect of various metal ions including $CaCl_2$, $Na_2CO_3$, $MgCl_2$, $FeSO_4$, $KNO_3$, $ZnSO_4$, and $MnSO_4$ on lipase activity in 25 mM Na-phosphate buffer (pH-7.4) was determined at a concentration of 1 mM.

##### 4.2.4.1.5. Enzyme kinetics of lipase produced by *Streptomyces albus* ST 04

The Kinetics of Lipase enzyme, the constants $K_m$ and $V_{max}$ were determined by carrying out the enzyme reaction at a various substrate concentration of lipase say 1

to 10 % in sodium- phosphate buffer (pH9.0) at 45°C (Segel, 1975). The enzyme activities were calculated and expressed in (U/mL). Using the software Graphpad prism 8.0 Michaelis-Menten plot was constructed and Vmax and Kmax were values were determined. Using Lineweaver–Burk plot initial velocity was plotted against the concentration of the substrate by means of linear transformation of the Michaelis-Menten equation.

## 4.3. Results

### 4.3.1. Production and purification of lipase enzyme from *S. albus* ST 04

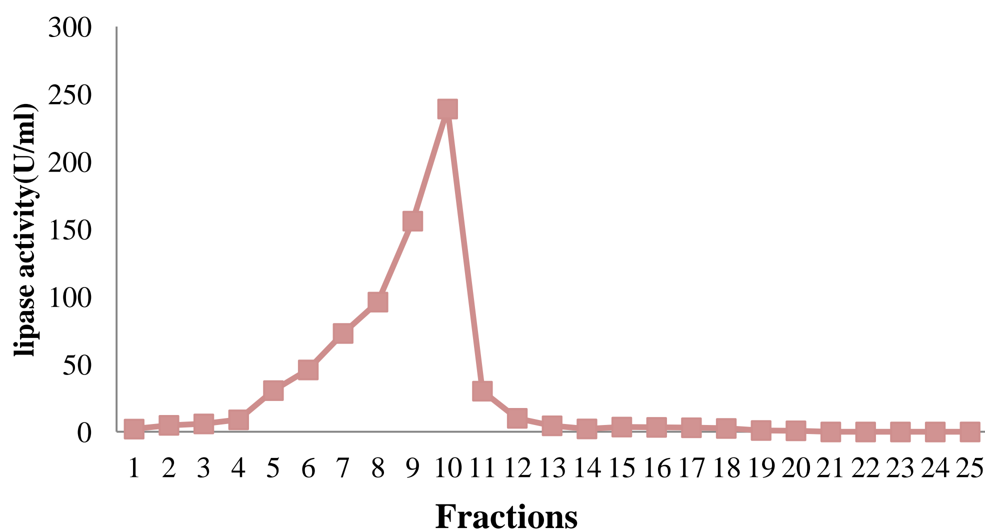
In the present study, mass culture of *Streptomyces albus* ST 04 was carried out using optimized media. Lipase purification was done to get a protein of interest and to remove unnecessary substances. The purification process of lipase occurs in a sequential manner. The enzyme produced over 108 hours of culture was purified by ammonium sulphate precipitation for salting out the protein. For increased enzymatic activity desalting was performed to remove the traces of salt. According to Pabai *et al.* increased lipase activity depends on the concentration of ammonium sulphate. The crude enzyme (500ml) produced have shown a specific lipase activity of 2.81 U/mg. The enzyme was purified as per the steps are summarized in Table 4.1. Ammonium Sulphate precipitation up to 70% showed lipase activity. The reduction in activity was observed beyond this point and may be due to the drop in pH, which affects the enzyme activity. The desalting process could achieve 16.8 fold purification of the enzyme with 81.9% yield and specific activity of 47.19 U/mg of protein. The final purification was done using gel-filtration chromatography using a Sephadex G-100 column. The different eluents were pooled and dialyzed for concentrating (Fig 4.1). After concentration, it shows an increase in specific activity from 47.19 to 238.89 U/mg which confers the removal of most of the contaminated

proteins. The final purified enzymes have exhibited 84.9 fold purification and 41.9% yield of the enzyme.

**Table 4.1. Purification steps of lipase from *S. albus* ST 04**

| Purification Steps | Total Protein (mg/ml) | Total Enzyme Activity (U) | Specific Activity (U/mg of protein) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 18.23 | 51.3 | 2.81 | 1.0 | 100.0 |
| Ammonium sulphate, post dialysis | 0.89 | 42 | 47.19 | 16.8 | 81.9 |
| Sephadex G-100 | 0.09 | 21.5 | 238.89 | 84.9 | 41.9 |

**Figure 4.1. Elution profile of different fractions from *Streptomyces albus* ST 04 on Sephadex G-100 column chromatography**



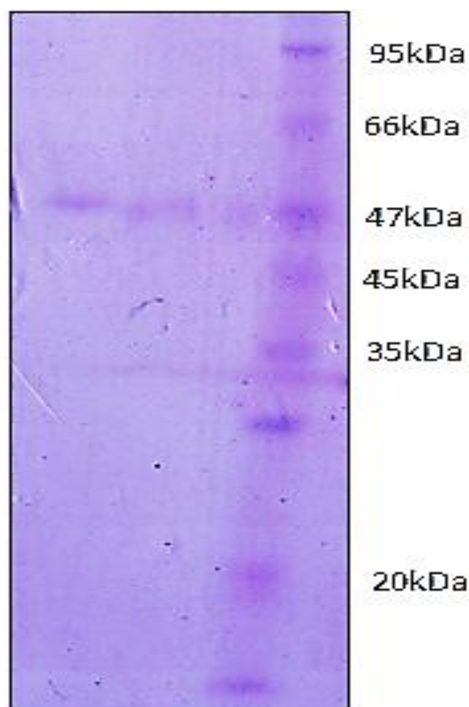

### 4.3.2. Characterization of Lipase enzyme

#### 4.3.2.1. Determination of the molecular weight of purified Lipase enzyme

The purification of lipase enzyme was confirmed by analysis with SDS PAGE, which showed a single band on staining with Coomassie Brilliant blue G250

indicating the homogeneity of the preparation (Fig 4.2). SDS PAGE analysis of purified enzyme yielded a single band at 47 KDa.

**Figure 4.2 Analysis of *S. albus* ST 04 lipase in SDS-PAGE**




### 4.3.2.2. Protein structure prediction of *Streptomyces albus* strain

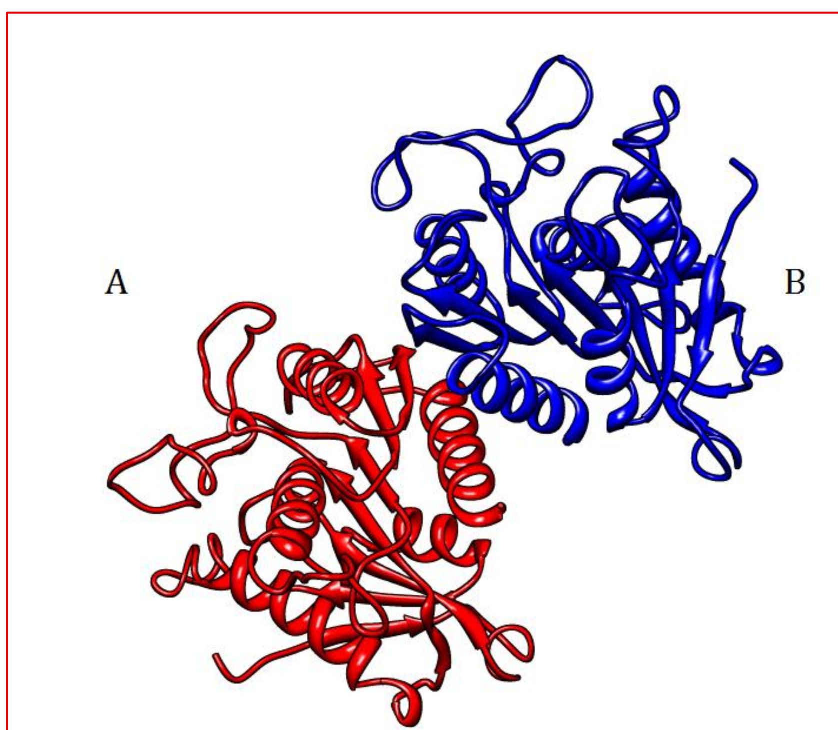
For getting more insights on lipase enzyme of the *Streptomyces albus* ST 04 strain, the protein structure was deduced from the amino acid sequence of *Streptomyces. albus* present in the uniprot database (uni prot id D6B385) by means of swiss modeling. The physicochemical properties of the lipase enzyme were established with the aid of the bioinformatics tool program. Table 4.2 provides the physiochemical values of the lipase enzyme of *Streptomyces albus.* In the tool, the parameters like molecular weight (MW), negatively charged residues (NCR), positively charged residues (PCR), Theoretical PI (TP), Aliphatic index (AI), Instability Index (II) and Grand average of hydropathy (GRAVY) were examined. The *Streptomyces albus* shows a theoretical pI of 6.41, the molecular weight of 31.983 kDa having an almost equal number of positive and negative charged

residues with an instability index of 36.09, Aliphatic index 75.16 and GRAVY value of -0.170. Based on the parameters, the tertiary model was built using 4vzi as the template. The modeled structure was shown in fig 4.3 and has two chains A &B and exists as a dimer (Laskowski, 2009).

**Table 4.2. Physicochemical properties of *Streptomyces albus***

| Parameters | Lipase of *S. Albus* |
|---|---|
| **MW** | 31983 Da |
| **NCR** | 26 |
| **PCR** | 25 |
| **TP** | 6.41 |
| **II** | 36.09 |
| **AI** | 75.16 |
| **GRAVY** | -0.170 |

**Figure 4.3 Predicted tertiary structure of *Streptomyces albus***

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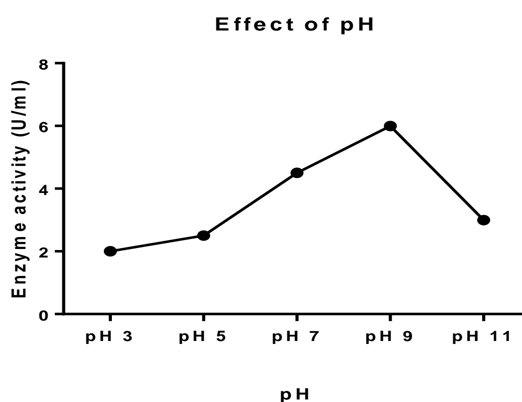
### 4.3.2.3. Characterization of lipase from *Streptomyces albus* ST 04

#### 4.3.2.3.1.. Effect of pH on enzyme activity

The Lipase activity was studied at a pH range 3-11 at 28°C. The percentage of lipase activities at different pH was shown in Fig 4.3. The optimum pH for lipase enzyme activity of *S. albus* ST 04 was found to be 9.0.and least activity at the pH 3.0.

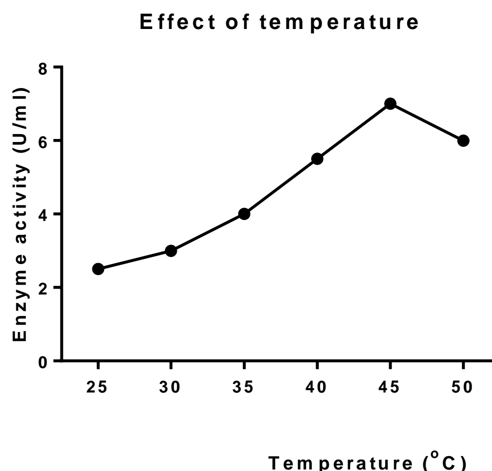
**Figure 4.3 Effect of pH on the lipase enzyme of *Streptomyces albus* ST 04**




#### 4.3.2.3.2. Effect of temperature on enzyme activity

The activity for purified lipase enzyme was checked at different temperatures ranging from 25-50°C. The enzyme was found to exhibit the highest activity at an optimum temperature of 45°C. There was a significant reduction of activity at 50°C (Fig 4.4).

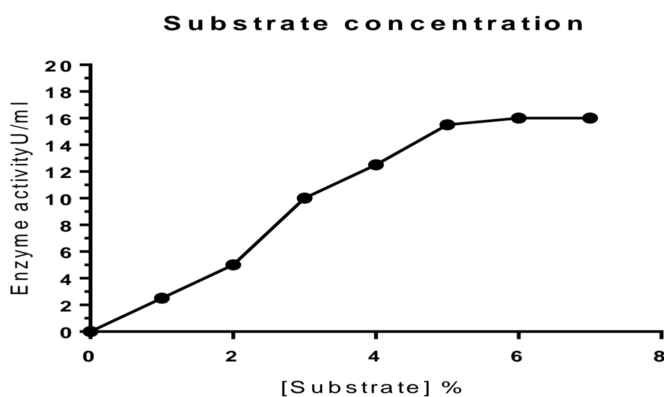
**Figure 4.4 Effect of temperature on the lipase enzyme of *Streptomyces albus* ST 04**



### 4.3.2.3.3. Effect of substrate concentration on enzyme activity

The substrate concentration is a very significant part of enzyme activity. Substrate concentration 0-8% was added and checked for the enzyme activity. It was found that at substrate concentration 6% showed stable activity curve which is showed Fig 4.5.

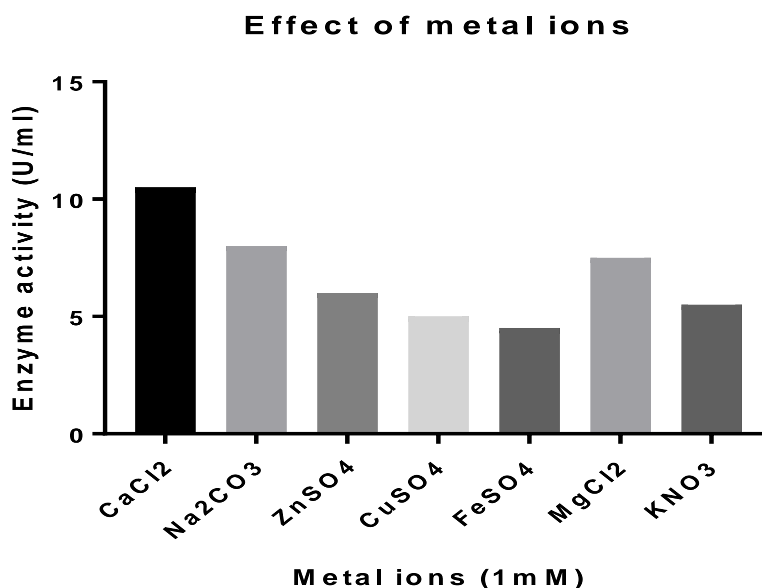
**Figure 4.5 Effect of substrate concentration on the lipase enzyme of *Streptomyces albus* ST 04**




### 4.3.2.3.4. Effect of metal ions on enzyme activity

The effect of various metal ions on purified lipase enzyme activity was studied by using chloride, sulphate and nitrate ions of metal ions.

**Figure 4.6 Effect of Metal ions on the lipase enzyme of *Streptomyces albus* ST 04**

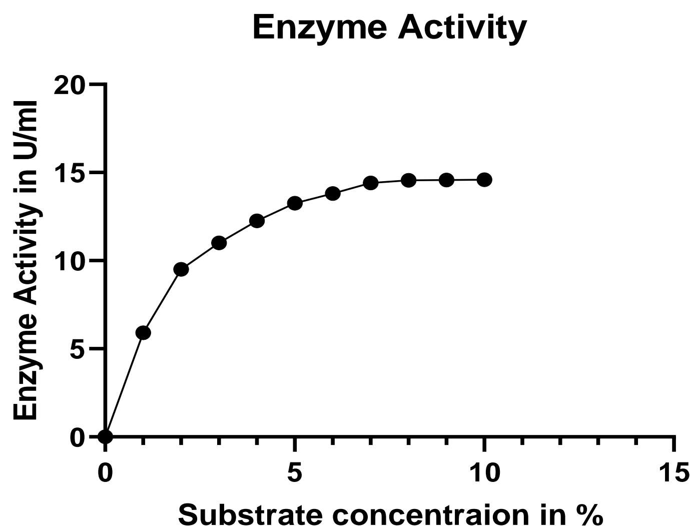


The addition of Ca $^{2+}$, Na $^{2+}$, Mg $^{2+}$ result in activation of lipase enzyme. The addition of $Ca^{2+}$ showed most enhanced enzyme activity of 10U/ml followed by $Mg^{2+}$, $Na^{2+}$, $Zn^{2+}$, $K^{2+}$ and the least was with $Fe^{2+}$.

### 4.3.2.4. Kinetics of lipase derived from *Streptomyces albus* ST 04

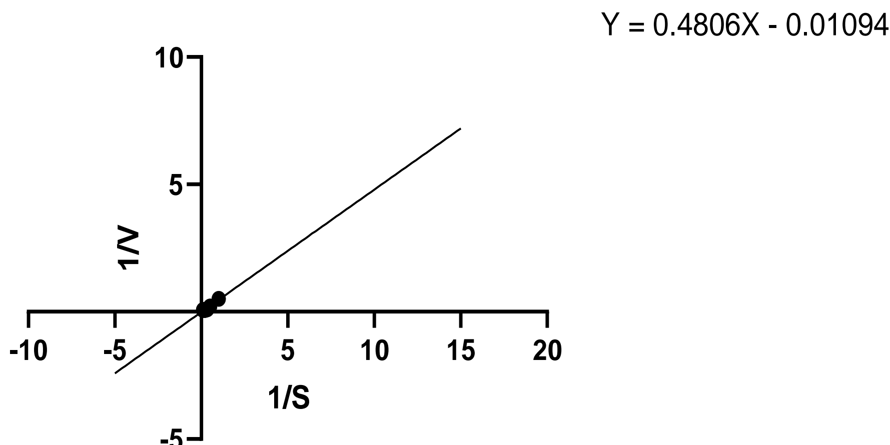
By using the various substrate concentrations at optimized conditions the Kinetic parameters $K_m$ and $V_{max}$ of lipase enzyme were estimated (Figure 4.7). As the function of the concentration of substrate $K_m$ and $V_{max}$ were estimated by plotting the initial velocity data.

**Figure 4.7 Michaelis Menten plot for the lipase activity at various concentration of substrate**




Lineweaver-Burk plot was used obtaining the kinetic parameters (Figure 4.8) by solving the Michaelis-Menten equation using Prism 8.0 software (GraphPad Software, Inc. CA, USA).

**Figure 4.8: Lineweaver–Burk plot of lipase produced by *Streptomyces albus* ST 04**




The lipase enzyme exhibited a $K_m$ value of 4.56% of substrate concentration with a $V_{max}$ value of 23.6 U/mL. The kinetic value of lipase enzyme shows significant with an $R^2$ of 0.9385. Lineweaver-Burk plot was constructed by transformed Michelis-Menten Data, and linear regression was calculated from the perfect line of the graph as Y = 0.4806X - 0.01094.

## 4.4. Discussion

Bacterial lipases are greatly influenced by nutritional and physicochemical factors, such as temperature, pH, carbon and nitrogen sources, inorganic salts, agitation and dissolved oxygen concentration (Aires-Barros et al. 1994, Gupta et al. 2004). Temperature changes the physical properties of the cell membrane, thus influences extracellular enzyme secretion (Pagu et al. 2013). Some unique properties of lipase, such as stability in organic solvents, being active without the aid of cofactors, broad substrate specificity, high enantioselectivity, temperature, and pH dependence are the reasons for the enormous biotechnological potential of microbial lipases (Jaeger and Reetz, 1998). So as a part of the process of development, the generation of kinetic data becomes very necessary.

Generally, enzymes are concentrated by means of salt precipitation of solvent precipitation. These techniques do not require sophisticated tools and are rapid, simple and economical. However, the recovery yield of enzymatic activity due to irreversible denaturation of protein is sometimes meager. The precipitate formed at 70% saturation of $(NH_4)_2SO_4$, showed a 16.8 fold increase in specific activity compared to the crude sample and was used for further purification employing different chromatographic methods. Lipases are reported to be monomeric proteins having molecular weights in the range of 16-670 kDa. In the present study, the molecular weight of purified lipase from *S. albus* ST 04was approximately 47kDa. From the phylogenetic analysis, it was clear that the *Streptomyces* Albus. ST 04 got more similarity with *Streptomyces. Albus* strain and so the physicochemical properties of the available *Streptomyces. Albus.* was carried out. The physicochemical parameters it was evident that the neutral nature of theoretical pI (6.41) was due to the similarity in the numbers of negative chain residues and positive chain residues. Moreover, the GRAVY value indicated the solubility of the protein and was computed as negative. The instability value will tell the nature of the protein, and the obtained II value 36.09 directly indicates that the protein was stable, since the stable protein will produce an II value less than 40 (Laskowski, 2009). By considering the stable nature of the protein, the tertiary structure was modeled using PDB ID 4vzi as a template; the developed structure has got two chains A and B and existed as a dimer. Moreover, in 2015, Todosiichuk et al. conducted a study that *S. Albus* strains were subjected to mutagenesis associated with the multistage selection. In the study, he observed a characteristic variation in bactereolytic enzyme activity and antibiotic activity due to induced mutation. The result was obvious and gives limelight in the characterization of a lipase gene from

*S.albus* ST 04 since it has only 96% similarity with the *S. albus* Strain. Moreover, just a few works were carried out in the characterization of lipase from *Streptomyces albus.* For understanding possible structure-stability relationship among different *Streptomyces* lipases, the various *Streptomyces* sp. was examined. It was noted that the presence of amino acids in different lipases was varied from 234-306. This variation is reflected in the stability of the lipase protein, due to disulfide bridges produced by cysteine. In *S. albus* strain it was observed at cysteine in 242 and 258. It was already reported that the presence of disulphide bridge bond between cysteines in 242 and 252 amino acid residues, the thermostability of putative lipase *S. coelicolor*. The lipase protein structure of *S. albus* was precisely similar to that putative lipase *S. coelicolor* (Vujaklija et al. 2003). Thermostability is an essential requirement for industrial applications of enzymes. By considering these factors of *Streptomyces* strain, the protein size will come only below 30000 Da. The Protein Databank of the crystal structure of extracellular lipase from *Streptomyces rimosus* (PDB ID 5MAL) also reported that it has two chains, A & B, and has a size of 235 amino acids .i.e., approximately 24000 Da and so it may appear as a dimer at 48000 Da. The extracellular lipases extracted from bacteria were purified by ammonium sulphate precipitation & dialysis, Sephadex G-100 column chromatography. The enzyme activity of lipase was examined for further purification and molecular weight determination by SDS-PAGE. The molecular weight of the enzyme was estimated to be 47 kDa. This may be due to dimer formation in the isolated enzyme, and it will be further confirmed by implementing molecular tools. The dimer formation and the stability of lipase will not entirely agree with earlier reports as lipase exists as a monomer. Many authors have a similar report with other bacterial strains. The work of Sangeetha et al. in 2008 in molecular characterization of a

proteolysis-resistant lipase from *Bacillus pumilus* SG2 also supports our data that instead of obtaining 20 kDa band they got 40 kDa band. They also mentioned the possibility of a dimer, and it will increase the thermostability of the lipase obtained. The lower molecular size of 50 kDa lipase was purified from the culture filtrate of *Streptomyces cinnamomeus* (Sommer et al. 1997). From the literature, the molecular weight of lipase for *Pseudomonas putida* were between 29 to 45 kDa (Chartrain el al. 1993). All these data's supports the derived dimer structure of *Streptomyces albus* and variation of the protein size with of *Streptomyces albus* (expected size of the dimer is 63 kDa) may due to 4% variation. So for characterizing lipase isolated from *Streptomyces albus* ST 04 strain, more molecular studies will be carried out.

Lipases are stable over a wide range of pH from pH 4 to 11 and temperature in the range from 10 to 96 oC (Patil et al. 2011). Lipase from *Pseudomonas fluorescens* SIK W has an acidic pH optimum of 4.8, whereas lipases from *Bacillus stearothermophilus* SB-1, *Bacillus atrophaeus* SB-2 and *Bacillus licheniformis* SB-3 show activity over a broad pH (3-12) (Nagar et al. 2013). Mostly bacterial lipases prefer pH 7.0 for optimum growth and lipase activity (Gupta et al. 2004). The studies in streptomycetes lipases reported that It was stable in a wide range of pH from 4.0 -10.0 and for *Streptomyces variabilis it was observed in between 9.0-9.5* (Selvam et al. 2013). Generally, we can say that the lipase obtained from *Streptomyces* spp. strains have alkaline pH stable in nature. Reports on the thermostability of streptomycetes lipases have reported them to be fully stable; stability is between 15°C and 65°C, and stable between 55°C and 65°C. Aly et al. (2011) observed that the lipase production was maximum in between 35°C and 40°C for *Streptomyces exfoliates* while Sirisha et al. (2010) also reported the same *Staphylococcus*. However, many of the earlier researchers reported that it was in

between 30°C to 35°C Selvam et al. (2013). However, certain reports are available for thermophilic organisms, especially *Streptomyces,* isolated from marine sediments; the optimum temperature may go up to 55°C Sathyapriya et al. (2012). This observation will support our data as our optimum temperature is 45°C and confirms that the strain isolated from marine sediments is *Streptomyces* spp.

Metal ions like $ZnSO_4$, $MnCl_2$, $FeSO_4$, $MgSO_4 \cdot 7H_2O$, and $CaCl_2$ were also found to interfere with the lipase activity. Metals ions can either stimulate or inhibit lipase activity. Metal cations play important roles in the structure and function of enzymes particularly Ca2+, and some of the lipases are strictly calcium-dependent (Nagar et al. 2013). Magnesium chloride (0.6 mM) in combination with calcium chloride (0.4 mM) tremendously increased lipase activity up to 3 fold (60.92 U/ml) in Burkholderiacepacia RGP-10 (Rathi et al. 2001). *Bacillus* sp. A 30-1 (ATCC 53841) lipase required a complex medium containing $Ca^{2+}$, $Mg^{2+}$ , $Na^{+}$, $Co^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $K^{+}$, $Mn^{2+}$, $Mo^{2+}$, and $Zn^{2+}$ (Wang et al., 1995). Calcium ions increased lipase activity in *Microbacterium* sp. by 50%, $Na^{+}$ and $Ba^{+}$ salts showed no effect on lipase activity, while $Zn^{2+}$, $Mg^{2+}$, $K^{+}$, and $Li^{+}$ ions decreased lipase activity by 35, 14, 23 and 41 % respectively (Tripathi et al. 2014). In the data, the increase in activity due to $Ca^{2+}$ influence will support the above data.

The effect of substrate concentration for maximum lipase activity was calculated and was observed at 6 %. The $K_m$ (for lipase) and $V_{max}$ values of this enzyme were calculated by Lineweaver-Burk plot method (Segel, 1975). By comprising all the data, we say that after purifying the 108 hr cultured *Streptomyces albus* ST 04 was able to produce at an average lipase activity of 23.6 U/ml in th purified stage. The optimum condition for the lipase activity for the lipase obtain from the strain was finalized as pH 9.0, temperature 45°C and a substrate concentration of 6%. Based on

the substrate specificity microbial lipases may be divided into three categories: namely nonspecific, regiospecific and fatty acid-specific. Nonspecific lipases result in the complete breakdown of triacylglyceride to fatty acid and glycerol by acting randomly on the triacylglyceride molecule (Jaeger et al. 1999, Gupta et al. 2004). Region-specific lipases hydrolyze only primary ester bonds (i.e., ester bonds at atoms C1 and C3 of glycerol) of triacylglyceride to give free fatty acids. The third group exhibits a clear fatty acid preference and comprise fatty acid specific lipases (Jaeger et al. 1999, Gupta et al. 2004). The 14.5g/m lipase activity shows the specificity of the substrate in the reaction.

The present chapter, reports the purification of lipase from soil isolate, *Streptomyces albus* ST 04. The purification studies, SDS-PAGE and zymography analysis showed that the enzyme is having a molecular mass of 47kDa. Enzyme activity is highly stable at a pH of 9 and active up to a pH of 9.5. The enzyme is most stable at a temperature of 45°C and temperature stability is continued up to 50°C. The enzyme activity and substrate specificity have a relevant role in lipase production. More of the region specificity of the enzyme will increase its demand. A few works only carried out in the field of *Streptomyces* species also supports the lipase production in *Streptomyces albus* ST 04. Moreover, the molecular work on the characterisation of lipase from *Streptomyces strain* is very few, and the present work gives an insight for establishing the region-selectivity of the lipase in the various substrate by means of prokaryotic as a well eukaryotic expression of the protein.

# Chapter 5

# Summary and Conclusion

Oceans are the dynamic system that remains our yet to be explored natural resource. The biological diversity of these ecosystems offers scope for the study of marine living organisms. A variety of offensive and defensive mechanisms have evolved to allow organisms to gain selective advantage and to cope with competitors. To thrive in this competitive niche, these marine organisms produce bioactive metabolites. Almost every class of marine organisms from smallest microbes onwards produces a variety of bioactive compounds with unique characteristics which may be entirely distinct from those of the terrestrial environment. The bioactive compounds are excellent candidates for a variety of applications in the pharmaceutical, agricultural and food industries. Of all the marine microbes, the actinobacteria have special consideration in view of the proven biosynthetic capabilities of numerous isolates from the soil. Marine Actinobacteria can exist in various ecological niches and to adjust with various environmental stress they produce secondary metabolites as well as extracellular enzymes like Amylase, Lipase, Cellulose, Chitinase, especially genus *Streptomyces*. They are valued as producers of therapeutically important compounds. Most of the natural products are derived from these marine microbes. These valuable prokaryotes also show a universal distribution.

In this scenario, distribution of the actinobacteria especially its important candidate genus *Streptomyces* were studied from the Islands of Andaman, an ecosystem which has not been explored much especially after the post tsunami situation. These isolates were characterized up to generic level based on biochemical characteristics as well as microscopic observation. These isolates were screened for hydrolytic enzyme lipase, and characterization of lipase enzyme was done.

During the present study samples were collected zone wise, i.e., South (Port Blair to Baratang) comprising 14 stations, Middle (Baratang to Billiground) with 10 stations

and North Andaman (between Billiground to Diglipur) from 9 stations. From each station, depth-wise sampling was conducted from 0-1 m, 5-6 m and 10-11m depths. Samples were collected aseptically by skin diving during 2013-14 and 2014-15. Collected sediment samples were pre-treated and were serially diluted after reaching the laboratory for further processing.

The isolation and characterization of these microbes were difficult since they grow slowly, relatively low numbers in relation to common unicellular bacteria. Heat treatment method and Phenol treatment (1.5%) method were employed in order to avoid all possible interfering bacteria (especially gram negative) and isolated all possible culturable Actinobacteria.

A total of 1083 actinobacteria isolates of genus *Streptomyces* were isolated during the study. The *Streptomyces* spp. (94.9%) was the most predominant actinobacterial isolates encountered during the study. The zone wise spatial distribution analysis showed that culturable *Streptomyces* spp. was more (43%) in South Andaman followed by Middle Andaman (35%) and North Andaman (22%). High abundance of *Streptomyces* spp. was recorded in the nearshore region than the deeper waters, i.e., 57% of the isolated *Streptomyces* spp. were from 0-1 m depth, 27% from 5-6 m and 16% from 10-11 m.

Season-wise distribution of *Streptomyces* showed variation between wet season (rainy) and dry season. During pre-monsoon (dry season), 39% *Streptomyces* were isolated followed by 32% during North West Monsoon and 29% in Southwest Monsoon (wet season). Results of BIOENV analysis revealed that temperature; pH, Nitrite and TOC % were the best subset that could explain the variation in the distribution of *Streptomyces* spp. with a Spearman correlation coefficient, ρ of 0.026. Pearson correlation was performed to correlate the environmental variable

and the *Streptomyces* spp. TOC % showed significant positive correlation (r=0.18, p=<0.05) while salinity showed significant negative correlation (r=-0.15, p= <0.05). Temperature, pH, phosphate, and silicate showed weak positive correlation while nitrite showed a weak negative correlation.

It is evident from the present study that when salinity increases, or when the distance from shore increases, the number of isolates decreases. It supports the argument that these microbes were initially terrestrial forms and whether the predominance of these group post tsunami needs further investigation. The pre-monsoon season affected the distribution of actinomycetes in Andaman waters since during southwest monsoon and northeast monsoon the physicochemical parameters remained more or less similar.

Present study depicts Actinobacteria especially *Streptomyces* spp. as a good source for lytic enzymes. Five isolates which showed good lipase activity were selected for further studies. Optimization of lipase production media for *Streptomyces* ST04 was done by using Plackett - Burman and Box - Benhken Analysis. Significant variables contributing to the lipase production were identified as pH, inducer, and NaCl. Using 2- factorial design a 14.5 U/ml enzyme activity observed. Further lipase production optimisation was done by the Box-Behnken design, indicating that lipase production was high at medium NaCl concentration, 4 g/L of inducer concentration (olive oil) and pH of 8, following which maximum lipase activity of 17 U/ml was acheived.

The isolates were identified through barcoding using 16S rDNA and the NCBI Genebank accession numbers KC954275 (*Streptomyces carpaticus*), KC818233 (*Streptomyces thermocarboxydus*), KF002488 (*S. albus*), KF002486 (*S. diastaticus*),

KF017203 (*S. violaceorubidus*) were received. *Streptomyces* sp. ST 04 showed 96% similarity to *Streptomyces albus.*

Enzyme production of the bacterial cultures was carried out using submerged state fermentation under optimized conditions 25$^o$C pH 7.0+/-0.05 rpm and supplied with sterile air at the rate 2.5 1/min. For enzyme extraction, the culture was centrifuged at 10000 rpm for 10 min 4$^o$C and the supernatant stored in 200 ml aliquots at -20$^o$C and used for further purification and characterization. The crude extract showed a total enzyme activity of 51.3 U and specific activity is 2.81 U/mg.

Ammonium Sulphate precipitation was done under constant stirring at 4°C for 30 min and then stirring was continued for overnight at 4°C. Precipitated protein was collected by centrifugation at 10000 rpm for 15 min at 4ºC. The precipitate was resuspended in 25 mM Na-phosphate buffer (pH-7.4). Crude enzyme precipitated at 80% saturation of Ammonium Sulphate.

Following this, dialysis was conducted overnight and the Na-phosphate buffer (pH-7.4). The buffer was changed several times to increase the efficiency of the dialysis. The precipitated protein was dialyzed in the pre-treated dialysis tube for 48 hours at 4ºC and assayed for lipase activity, protein content, and specific activity. After dialysis, the sample was collected and stored under -20°C. After salting out and dialysis, total protein present was 0.89 mg/ml. Total enzyme activity was found to be 42 U, and specific enzyme activity was 47.19 U/mg.

The lipase active fractions were pooled and separated using a Sephadex G-100 column (2×20 cm). The lipase was eluted with the same buffer at a flow rate of 0.5 ml/min. The fractions (3ml) were collected and analysed for lipase activity and protein content. After Sephadex column purification, each fraction was assayed,

total protein was found to be 0.09 mg. Total enzyme activity was 21.5 U. Specific activity of the fraction was 238.89 U/mg.

The purified lipase sample on analysis with SDS PAGE showed a single band on staining with Coomassie Brilliant blue G250 indicating the homogeneity of the preparation. SDS PAGE analysis of purified enzyme yielded a single band of 47 kDa. The activity of the enzyme was further characterized and lipase activity was found to be maximum at pH 9.0 and least at the pH 3.0. The enzyme was found to exhibit optimum activity at 45$^{o}$C. There was a significant reduction of activity at 50$^{o}$C. The substrate concentration was a very significant part of enzyme activity. It was also found that at substrate concentration 6% showed stable activity.

The present study gives an account of the distribution of the genus *Streptomyces* along the vast coastline of islands of Andaman. Only a few works were conducted in islands of Andaman especially against a post-tsunami scenario. A detailed account of the distribution of *Streptomyces* spp., both depth-wise as well as season wise was depicted showing abundance towards the coastal area at depths 0-1 m. Deep zones isolates was described for the first time from these remote islands the Indian EEZ. The study also has confirmed that the clayey nutrient-rich sediments support more actinobacteria than the other course sediment. Habitat-wise mangrove area was found to be a rich source of *Streptomyces* than the coarse sandy area/coral area. Seasonality also plays a significant role in the distribution of streptomycetes. Season-wise distribution of *Streptomyces* from the coast of Andaman has high variation between the seasons. The Pre-monsoon season or non-rainy season has shown a higher abundance of *Streptomyces* than North West monsoon and South West monsoon. The Andaman and Nicobar region experiences heavy rainfall for almost nine months in a year with three months of actual dry season. So it may be

inferred that the distribution pattern of marine actinobacteria in Andaman has less effect during rainy seasons. Genus *Streptomyces* of Actinobacteria was found to be a notable producer of various bioactive compounds. *Streptomyces* spp. showed good production of lytic enzymes like Protease, Lipase, Amylase, Chitinase, Cellulase, etc. Most of the enzyme concentrations were achieved by precipitation with salts or solvents. The precipitate formed at 70% saturation of $(NH_4)_2SO_4$, showed a 16.8 fold increase in specific activity compared to the crude sample and was used for further purification employing different chromatographic methods. The final purified enzymes exhibited 84.9 fold purity and 41.9% yield of the enzyme. The enzyme lipase purified from *Streptomyces albus* ST04 is most stable at a temperature of 45°C and temperature stability continued up to 50°C and showed an optimum activity in the pH 9. In the present study, the molecular weight of purified lipase from *S. albus* ST04 was approximately 47 kDa. The effect of substrate concentration for maximum lipase activity was calculated and was observed at 6%. The enzyme activity and substrate specificity have a significant role in lipase production. More the regional specificity of the enzyme, the demand will increase. Very few works have been carried out on *Streptomyces* spp. as producers of lipase enzyme, especially in *Streptomyces albus*. So the present work is very important in the recent scenario of the ever-growing requirement of these bioactive compounds and enzymes for biotechnology or bio-prospecting industry.

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