8.0. GENERAL DISCUSSION

Marine environment is unique embracing a plethora of microbial populations, which are very important due to their role in the degradation of organic matter, nutrient regeneration and of high value secondary metabolites. Bacteria play an important ecological role in the marine environment (Ravel et al., 1998). Actinomycetes from the marine and estuarine environment offer excellent potential for bioremediation of metal contaminated water and sediments (Ravel et al., 1998). Their activities and relative abundance might be controlled by the hydrobiological factors in an aquatic environment. In fact, the distribution of actinomycetes in the sea remains largely undescribed even today. The marine actinomycetes produce resistant spores, that are known to be transported from land into the sea where they can remain viable but dormant for many years (Bull et al., 2000; Felsenstein, 1993; Cross, 1981). It has persisted despite evidence that actinomycetes can be metabolically active and physiologically adapted to grow in seawater (Helmke and Weyland, 1984). There is evidence that, actinomycetes usually make up only a small proportion of the bacterial flora
in marine habitats, the actinomycetes distribution is much lower than in terrestrial habitats (Goodfellow and Haynes, 1984; Goodfellow, 1983).

Among the marine organisms, marine animals have been identified as a good source for drug development. Due to lack of cultivation technology and multiple years taken for the production of effective bioactive principles could not meet out the urgent demand of antimicrobial drugs. Marine invertebrates have developed highly specific relationship with numerous associated microorganisms and these associations are of recognized ecological and biological importance (Armstrong et al., 2001; Strahl et al., 2002). It has been reported that, the ratio of microorganisms with antimicrobial activity from invertebrates were higher than other sources (Ivanova et al., 1998; Burgess et al., 1999), which suggest that invertebrate associated microorganisms might play a chemical defence role for their host. This kind of microorganisms as a sustainable resource has high potential to biosynthesis more biologically active secondary metabolites with in a short span of time due to well developed cultivation technology. Bioactive natural products from sponges have been reported to have strong bioactivities including anticancer, antimicrobial and anti-inflammatory activities and are often applicable for medical use (Faulkner, 2002; Pettit et al., 2004). Unlike
other invertebrates, sponges harbour extraneous microorganisms on their surface in their canal system and in the intracellular matrix which constitute a large part of the body as much up to 40% of the total biomass (Santavy et al., 1990). Earlier investigations reveals that, many compounds found in sponges are biosynthesized through microorganisms associated with them or indeed produced by microorganisms (Bewley and Faulkner, 1998). To confirm this hypothesis, there has been a great deal of interest in isolating bioactive microorganisms from sponges (Mitova et al., 2003; Suzumura et al., 2003). With the aim of finding new bioactive compounds from marine microorganisms, the present study has initiated to isolate bacteria and actinomycetes showing potential antibacterial activity associated with variety of sponge species distributed along the coast of Palk Strait.

The present study has collected 36 sponge samples throughout the year at different seasons which were distinguished by colour and ornamentation. All the samples were critically subjected for the isolation of bacteria and actinomycetes by following standard procedures. It reveals that, all the sponge samples have reported to harbour either group of microorganisms. Marine invertebrates have developed highly specific relationships with numerous associated microorganisms and these
associations are of recognized ecological and biological importance (Sponga et al., 1999; Armstrong, 2001; Strahl et al., 2002). It is surprised to notice that, 22 sponge samples are not reported to harbour actinomycetes. It is quite obvious that, 0.1% of microbes from these sponge were amenable to culture using traditional techniques and therefore the vast majority of microorganisms associated with sponges particularly actinomycetes could not be identified using a culture based approach. The present study also observed that, the bacteria is found to be the dominant group of microorganisms associated with sponges than the actinomycetes.

**Relative composition of sponge associated bacteria and actinomycetes**

![Pie chart showing relative composition of bacteria and actinomycetes]

Burja and Hill (2001) reported that, 228 strains of bacteria, 25 fungal strains, 3 actinomycetes strains and 9 strains of cyanobacteria were isolated from 10 individuals of sponge samples from Australian Great Barrier Reef.
The results of the present study suggested that, the bacteria are the dominant group of microbes irrespective to the seasons and the collection sites. The present study also found that, the bacterial groups were found maximum during monsoon season (November-January) and this might be due to the higher nutrient derived from the fresh water runoff from the adjacent river which supports the maximum growth of bacteria during rainy season. The rain fall data from the local meteorological station reveals that, 9.75 mm highest rainfall (data not shown) has been reported during the study period when compared with the other seasons along Palk Strait. In contrary, the actinomycetes counts were found maximum during the summer season (May-July). This might be due to the availability of huge amounts of particulate nutrients introduced into the sea during monsoon by land runoff could deposit the nutrients during summer (Wilkinson and Garonne, 1980). Besides that, microbial sponge associates depends upon the transportation of waste products or active metabolites (Borowitzka et al., 1989), chemical defence (Unson et al., 1994) or contribution to mechanical structure (Wilkinson, 1978).

Hentschel et al. (2001) reported that Aplysina sponges harbour large amount of bacteria which can amount 40% of biomass of the animal and
which exceed the bacterial concentration of the seawater by two order of the magnitude. Recently, Pabel et al. (2003) reported that, microbial community in sponges is still largely unknown, research for novel bacteria or those with novel activities is a worthwhile endeavour. The diverse wide assemblages of marine organisms in saline habitat thus encompasses a wide variety of chemical classes such as polyphenols, alkaloids, quinine, flavonoids, polyketites for their extreme survive. Still now a small number of plants, animals and microbes have reported to posse’s antimicrobial agents. Already 12,000 novel chemicals and hundreds of new compounds are being identified from marine sources (Fenical, 1993; Kobayashi and Ishidashi, 1993). Marine sponges are rich sources of structurally unique natural compounds, several of which have shown a wide variety of biological activities (Jacob Inbaneson and Ravikumar, 2011; Faulkner, 2002). Several studies proved that natural products from marine sponges are produced by microorganisms, which are associated commensally or symbiotically with marine invertebrates (Proksch et al., 2002). Isolation and cultivation of associated microorganisms with variety of bioactive compounds could help to solve the recognised problem of development of potential sponge derived drugs. To confirm this hypothesis, there has been a great deal of interest in isolating microorganisms with bioactivities from sponge and in recent years, a
number of novel compounds with biological activity have been discovered through cultivation of sponge associated microorganisms (Jacob Inbaneson and Ravikumar, 2011; Jayatilake, 1996; Mitova et al., 2003). With the aim of finding new bioactive compounds from marine microorganisms, the present study was initiated to identify the antibacterial activity of sponge associated microbes from Thondi coast of Palk Strait for the management of fish diseases.

Fishes are the first vertebrates with jaws, which are cold-blooded animals and can breathe through gills; there are about 36,000 species, which represent the 40% of the total vertebrate present. World fisheries and aquaculture production increased as 142 and 53 million in 2008 respectively and valued at around US$99 billion in 2008 (FAO, 2010b). Marine fisheries are conducted in all the oceans and seas of the world, including bays and estuaries. India is endowed with 2.02 million sq.km of Exclusive Economic Zone (EEZ) along with a coastline of 8129 km and 0.5 million sq. km continental shelf with a catchable annual fishery potential of 3.93 million tonnes occupying a very important strategic position in the Indian Ocean. The aquaculture resources in the country comprise 2.25 million hectares of ponds and tanks. Among the Asian countries, India ranks second in
aquaculture production and third in capture fish production and one of the
top leading exporters of sea foods (Kumar et al., 2003). In recent years,
marine fisheries affected by natural and human factors, before the industrial
revolution variation in climate occurred naturally. One among the reason is
disease caused in fishes. Marine sponges are an important component of
benthic communities throughout the world, regarding its biomass as well as
their potential to influence benthic or pelagic processes (Dayton et al., 1974;
Dayton, 1989; Gili and Coma, 1998; Maldonado et al., 2005). Marine
invertebrates have developed highly specific relationships with numerous
associated microorganisms and these associations are of recognized
ecological and biological importance (Sponga et al., 1999; Armstrong et al.,
2001; Strahl et al., 2002). It has been reported that, the ratio of
microorganisms with antimicrobial activity from invertebrates was higher
than from other sources (Ivanova et al., 1998; Burgess et al., 1999), which
suggested that, invertebrate associated microorganisms might play a
chemical defence role for their hosts.

The present study also made an attempt to find out the antibacterial
potential of sponge associated bacteria and actinomycetes against bacterial
fish pathogens. Initially an attempt has been made to isolate the bacterial fish
pathogens. A total of 5 different bacterial fish pathogens were isolated from
diseased fishes and the isolated fish pathogens showed disease symptoms
and mortality in *Mugil cephalus* and further confirms the pathogenic effect of
fish pathogens. Similar results are also identified with *Aeromonas bestiarum*
pathogenicity in carp fishes and the pathogenicity was classified as strongly
virulent, virulent and avirulent (Kozinska *et al.*, 2002).

The present study was also made an attempt to identify the
antibacterial potential of the isolated sponge associated microbes against the
fish pathogens and the results suggest that, the maximum level of
antibacterial activity was identified with THB-131 and ACT-21 strains.
Krishnakumar (2005) reported that, the isolated actinomycetes from
Kanyakumari coast were tested against antibiotic sensitive human pathogens
to choose the potential strains of antagonistic actinomycetes. Out of 63
actinomycetes isolates were tested against ten antibiotic sensitive human
pathogens, 33 strains showed antagonistic activity against atleast one
pathogen, 31 actinomycetes isolates showed antagonistic activity against
atleast one eye pathogens and 43 actinomycetes strains showed antagonistic
activity against atleast one human antibiotic resistant pathogens.
Similarly, Santos et al. (2010) reported 12 sponges associated bacterial isolates with antibacterial activities. Chelossi et al. (2004) reported that, isolates of more than 8.8% of all subculture colonies were active against several pathogens. Premanand et al. (2011) also reported 4 out of 75 bacterial strains from sponges showed antibacterial activity against Vibrio harveyii, Vibrio paraheamolyticus and Aeromonas hydrophila.

Based on the preliminary screening, the present study has made an attempt to findout the dose dependant antibacterial activity through MIC and MBC experiments. It shows that, the MIC and MBC values of the most promising strains of total heterotrophic bacteria (THB) and actinomycetes (THB-131 and ACT-21) and the results showed that, the THB-131 and ACT-21 showed concentration dependent MIC values between the ranges of 125-1500 µg.ml⁻¹. The minimum bactericidal concentration (MBC) test was also carried out by the present study and it shows that, THB-131 and ACT-21 showed concentration dependent MBC values ranged from 500 -1500 µg.ml⁻¹. Gandhimathi et al. (2008) reported that, the minimum inhibitory concentration and minimum bactericidal concentration of Nocardiopsis dassonvillei showed active against tested pathogens at 300-600 µg.ml⁻¹. Bhosale et al. (2002) and Bernard and Pesando (1989) reported that, the
extracts from *Cymodacea roundata* showed sensitivity against several *Bacillus* sp. In addition, Ravikumar *et al.* (2011b) reported that, the ethanolic extracts of seagrass *Syringodium isoetifolium* root extracts exhibited MIC and MBC value of 1000 μg. μl⁻¹ against 3 bacterial fish pathogens *viz.*, *Aeromonas hydrophila*, *Bacillus subtilis* and *Serratia* sp. The results confirmed that invertebrate associated microorganisms are potential resources of bioactive natural products due to their competition for nutrition and light (Inovova *et al.*, 1998; Burgees *et al.*, 1999; Armstrong *et al.*, 2001).

The results of the phylogenetic analysis of 16S rRNA gene sequences of fish pathogens revealed that, the FPAU01, FPAU02, FPAU03, FPAU04 and FPAU05 strains are belongs to the genus *Bacillus* sp., RPAUOCAS1 (JF899538), *Bacillus* sp. RPAUOCAS2 (JF899539), *Bacillus cereus* RPAUOCAS3 (JF899540), *Bacillus cereus* RPAUOCAS4 (JF899541) and *Bacillus* sp. RPAUOCAS5 (JF899542) respectively. Similar reports on the phylogenetic analysis of fish pathogens *Bacillus* sp. were identified by Ha *et al.* (2002) and Parvathi *et al.* (2009). Ampofo and Clerk (2010) reported that, the *Bacillus* is the predominant pathogen in diseased fishes. Contrastingly, Sowunmi *et al.* (2008) reported that, most of the fish pathogens are gram positive and gram negative in *C.gariepinus* and *T. Zilli*. Eventhough these bacterial strains are
belonging to the same genus, the stem and loop in the RNA secondary structure showed variation among the *Bacillus* sp. Saravanakumar *et al.* (2011) reported the dominance of *Bacillus* sp. (45.87%) in sponge associates along Gulf of Mannar region. Moreover, these isolates differed in the energy threshold, cluster factor, conserved factor, compensated factor, conservatively part of sequences, greedy parameter and treated sequences as indicated by Genebee software (http://www.genebe.nsu.su). Ravikumar *et al.* (2012) identified variations among the 5 species of same *Streptomyces* genus by using RNA secondary structure.

The most promising isolates of sponge associated microbes (ACT-21 and THB-131) which showed maximum antibacterial activity were also subjected for the species level identification through 16S rRNA sequencing. 16S rRNA sequencing analysis has been widely applied for the study of the diversity of microbial community and for strain identification and the results suggested that, the ACT-21 and THB-131 were showed maximum similarity with *Streptomyces* sp., and *Bacillus* sp. and named as *Streptomyces* sp. RPAUACT-21 (JF899543) and *Bacillus* sp. RPAUTHB-131 (JN083779) which belongs to the family of *Streptomycetaceae* and *Bacillaceae* respectively. Zhang *et al.* (2006) reported that, the phylogenetic analysis using 16S rRNA
gene sequences of the isolates belonged to seven genera of culturable actinobacteria including Actinoalloteichus, Micromonospora, Nocardia, Nocardipsis, Pseudonocardia, Rhodococcus and Streptomyces. The dominant genus was Streptomyces, which represented 74% of the isolates. Jiang et al. (2008) find out that, the phylogenetic analysis based on 16S rRNA gene sequencing from marine sponge Iotrochota sp. isolates belonging to the genera Streptomyces. The majority of the strains belonging to the genus Streptomyces. Jiang et al. (2007) reported that, the phylogenetic analysis of culturable actinobacteria isolated from the marine sponge Haliclona sp. belonged to the genera Streptomyces, Nocardiopsis, Micromonospora and Verrucosispora by using 16S rRNA gene sequencing. Hence, Streptomyces sp., is widely distributed in nature and found to be the good source of commercial enzymes and therapeutically useful bioactive molecules (Stach and Bull, 2005; Selvakumar, 1979). Joseph Selvin et al. (2004) reported that, among the sponge associated microbes the Streptomyces sp. occupies 6%. Earlier investigation reveals that, Streptomyces sp. strains isolated from marine environment have many novel bioactive compounds with several unique structures (Lee et al., 1998; Itoh et al., 2003). Jiang et al. (2007) isolated 54 numbers of actinomycetes from marine sponge Haliclona sp., of these Streptomyces group is the dominant form than the other species.
In intensive aquaculture farms, fish populations are continuously challenged by both natural (like temperature, crowding etc.) and artificial (like pollutants) stress factors. Such stressors often interfere with function of innate (nonspecific) and adaptive arms of the immune system, which results in immunosuppression. Such compromise in the immunological status of the fish results in increased susceptibility to a wide variety of biological stressors, such as bacteria, viruses and parasites which could potentially lead to population reductions (Chakrabarty, 1998) and were reported for sporadic episode of mass mortality. The accelerating pace at which man made changes are occurring in the aquatic environment seems to have channeled substantial interest for immediate and efficacious solutions (Chakrabarty, 1998). Problems with present antibiotics, drugs and chemical treatment to prevent disease in fish set the stage for the new concept in disease prevention-immunostimulants.

Recently the use of immunostimulants was introduced as a prophylactic measure (Anderson, 1992; Muliani et al., 1998). An immunostimulant is a substance that elevates non-specific defence mechanisms and specific immune response if the treatment is followed by vaccination or infection (Anderson, 1992). So far, a number of
immunostimulants that include a very heterogeneous group of substances like levamisole, lipopolysaccharides, glucans, peptidoglycans and muramyl dipeptide have been tested in a variety of fish species (Anderson, 1992; Sakai, 1999).

Many substances from different sources (bacterial component, chemical agents, animal or plant extracts etc.,) have been studied as prospective immunostimulants in fish (Sakai, 1999; Anderson, 1992 and Cooper, 1995) and some have been reported to give a significant degree of protection against several diseases commonly found in farmed fish, such as *Vibrio harveyi* (Villegeas *et al.*, 2004 and Joseph Selvin *et al.*, 2004). Bioactive substances from natural resources such as jellyfish is considered friendly to our environment (Joseph Selvin *et al.*, 2004). Keeping this view in mind, the present study has been undertaken to find out the immunomodulatory effect of crude extracts from sponge associated *Bacillus* sp. and *Streptomyces* sp. It reveals that, there is increasing level of ALP in the fish infected with pathogen alone but the level of ALP does not showed any marked difference in the fish injected with pathogen alongwith the crude extract from sponge associated microorganisms. Gaafar *et al.*, (2010) reported that, the fish exposed with the pesticide showed marked increase in the level of AST and
ALP at the beginning of the experiment due to hepatic toxicity which leads to extensive liberation of the enzymes suffered from a significant decrease in their levels. The decrease in activity of AST, ALP and ALT in fish exposed to pesticides was also reported by different authors (Elboushy, 1994 and Begum, 2004). It is very clear from the present findings that, there is no hepatic toxicity and hence the level of ALP is within the normal level.

The present study also observed that, there is no marked change in the level of transaminases and serum proteins which are the good sign of immunomodulatory effect of crude extracts injected to the fish. Saeed (1983) reported that, the level of transaminases and the level of protein were decreased due to the hepatic necrosis of fish exposed to pesticides. Also the severe hepatic necrosis leads to lack of cells from which the enzymes are produced. The rapid decrease in total protein in some treatments without the addition of crude bioactive compounds is associated with active degradation of proteins under stress. This fact is correlated to the development of resistance toward to toxic stress. Proteins being involved in the architecture and physiology of the cell, they seem to occupy a key role in cell metabolism. Catabolism of proteins makes a major contribution to the total energy production in fishes. Under stress situations may constitute a physiological
mechanism with an important role in providing energy to cope with the stress situations. Therefore, depletion of total protein content might also be attributed to the destruction or necrosis of cellular function and consequent impairment in protein synthetic machinery (David et al., 2004). When an animal is under toxic stress, diversification of energy occurs to accomplish the impending energy demands and hence the protein level is depleted (Neff, 1985). The depletion of total protein content may be due to breakdown of protein into free amino acid under the effect of mercury chloride at the lower exposure period (Shakoori et al., 1996). Reduction in protein content in liver of exposed fish might be due to either arrested metabolism in the liver or to use it to build up new cells or enzymes to reduce the stress (Sakr and Al lail, 2005).

It is also observed from the present study that, the level of RBC is decreased in all the other treatments except the fish injected with pathogen and the extract from Streptomyces sp. This reveals that, there is no change in the level of hemoglobin and also the respiratory functions. The decreased level of RBC due to the chronic toxicity was observed in other treatments. Gaafar et al., (2010) reported that the hematological parameters showed a significant decrease in Hb, RBC’s count and PCV, this reveals the prominent
anemic effect of pesticides which is confirmed by the results of the blood indices. Which in turns revealed the hemolytic effect of pesticide and may explain the chocolate discoloration of parachymatus organs as hemoglobin may be converted into methemoglobin with resultant hemolysis and reduced blood oxygen carrying capacity which accumulates with the irritant effect of edifenphos pesticides causing respiratory distress to the fish. The severity of anemia also is magnified by the hypoproteinemic effect showed by the pesticide. The marked decrease in the RBC’s count was in significant with those reported by other workers. Rani et al., (1987) and Venkateshwarthlu et al. (1990) proved these changes in blood parameters in catfish intoxicated with the organophosphate pesticide dichlorvos.

The present finding also reveals that, the level of WBC has marked changes in the treated fish in all other treatments except the fish injected with pathogen and the crude extract from *Streptomyces* sp. Changes in the white blood cell and the differential counts, neutrophils, lymphocytes and monocytes indicated a stress condition in *T. guineensis*. The increased percentage of neutrophils and monocytes and decrease percentage of lymphocytes in the circulating blood of *T. guineensis* agrees with the report of Johansson-Sjöbeck et al., (1978) in *Anguilla anguilla* exposed to handling
stress, but contrary to the findings of Salonius and Iwama (1993) who reported increased in lymphocytes percentage in Atlantic salmon subjected to handling stress. This disparity may be due to duration of the stress. The salmons were only exposed for a period of four hours and the *T. guineensis* for seven days.

Glucose is a carbohydrate that has a major role in the bioenergetics of animals, being transformed to chemical energy (ATP), which in turn can be expressed as mechanical energy (Lucas, 1996). In suboptimum or stressful conditions (internal or external), the chromatin cells release catecholamine hormones, adrenaline and noradrenaline toward blood circulation (Reid *et al.*, 1998). Those stress hormones in conjunction with cortisol mobilize and elevate glucose production in fish through glucogenesis and glycogenolysis pathways (Iwama *et al.*, 1999) to cope with the energy demand produced by the stressor for the fight of flight reaction (Wedemeyer *et al.*, 1990). Glucose is then released (from muscle and liver) toward blood circulation and enters into cells through the insulin action (Nelson and Cox, 2005). But in the present study, the level of glucose is increased in all other treatments except the fish injected with pathogen and crude extracts from sponge associated microorganisms *Bacillus sps.* and *Streptomyces sps.* and hence the fish have
enough energy from the food source rather than the other degenerative metabolic pathways.

In conclusion, this study has provided information for fish biologist on the usefulness of the crude extract from sponge associated Bacillus sps and Streptomyces sps. to be used as a effective immunostimulants so as to enable to maintain the fish health through improved innate and humoral immune responses.