General Introduction
1. GENERAL INTRODUCTION

The ever increasing human populace, emergence of new diseases and increasing incidence of bacterial resistance have necessitated the mankind to constantly look for new leads of drug resources. In recent years, new targets like AIDS, immunosuppression, anti-inflammation, Alzheimer disease, ageing processes and some tropical diseases, which are daunting the mankind, have been added to the inventory list (Kelecom, 1999).

The therapeutic natural products found in terrestrial plants and microorganisms have formed the basis of early drug development. Natural products are organic substances produced by organisms including microbes and plants that have long been exploited by people for a variety of purposes such as food, fragrances, pigments, insecticides and medicines. The chemical novelty associated with such natural products is higher than that of any other sources and hence, have long been recognized as an important source of therapeutically effective medicine. It includes a wide variety of chemical classes, such as terpenes, shikimates, polyketides, acetogenins, peptides and alkaloids of varying structures and a multitude of compounds of mixed biosynthesis (Wright, 1998).

Plants have long been the reliable source of medication for nearly 60% of the world’s population (Murugan, 2002). Of the 520 new drugs approved between 1983 and 1994, 39 % were natural products or derived from natural products. About 60 to 80% of the present day antibacterial and anticancer
drugs are derived from natural products (Harvey, 2000). A study using US based prescription data from 1993 demonstrated that natural products still play a major role in drug development and over 50% of the most prescribed drugs are the synthesis or design of the agent (Newman et al., 2000).

The world’s oceans, which cover almost 70% of the earth’s surface and over 90% of volume of its crust (Fenical, 1993; Whitehand, 1999), encompass a diverse array of fauna and flora, many of which have no terrestrial counterparts. The ocean life is represented by 34 of the 36 phyla in contrast to the 17 phyla of the terrestrial environment (Faulkner, 2002) and nearly 300,000 species of plants and animals have been described from the marine realm (Jimeno, 2002; Pomponi, 1999). But, it is presumed that this number is only a small percentage of the total number of species that are yet to be discovered and described (Winston, 1988; Malakoff, 1997). Marine ecosystem and the organisms living in there provide functions which are vital to human survival and well being.

The competition for space and food is intense in the marine environment and hence, marine organisms possess unique structures, metabolic pathways, reproductive systems, sensory and defense mechanisms as a result of adaptation to extreme environment ranging from cold polar seas to the great pressures of the ocean floor. Marine organisms especially sedentary forms like macro algae, sponges, ascidians, bryozoans and their associated microorganisms, which produce diverse bioactive substances to compete for space, to prevent the growth of fouling organisms on their surface
and to avoid predation, are the potential source of novel drug leads and have been much concentrated (Faulkner, 1995). These chemical substances play an important role in the interaction between living organisms and their environment and chemical signals in water are of vital importance for behaviour and reproduction of organisms especially settlement of larvae and spores. Hence, ocean is considered as an enormous resource pool (Cragg et al., 1997) and likely to continue to be a prolific source of new natural products for many years to come (Proksch et al., 2002).

Marine metabolites have been developed or currently being tested as antibiotics, analgesic and antiinflammatory agents, molecular probes, skin care products, sun screens, anticancer and antiviral agents (Fautin, 1998). But, the lack of an ethno-medical history, as far as marine environment is concerned, makes it difficult to identify marine organisms (Faulkner, 1992). The number of compounds isolated from various marine organisms has exceeded 10,000 (MarinLit, 2001) with hundreds of new compounds still being discovered every year (Faulkner, 2002). Since then, over 14,000 different natural products from marine organisms have been described (MarinLit, 2003) and hundreds of patents describing new bioactive marine natural products have been filed (Kerr and Kerr, 1999). Approximately, 10 to 15 different marine natural products are currently in clinical trials mostly in the areas of cancer, pain or inflammatory diseases (Proksch et al., 2002).

Sponges, corals, ascidians, bryozoans, polychaetes, molluscs, starfish, plankton, sea weeds, fungi and bacteria are the major marine groups
exhibiting bioactivity. Porifera is the most studied phylum followed closely by the Cnidaria, Chromophycota, Rhodophycota, Mollusca, Chordata and Echinodermata. Out of thousands of natural products isolated to date from marine organisms, 25% are from algae, 33% from sponges, 18% from coelenterates and 24% from representatives of other invertebrate phyla such as ascidians, opisthobranch molluscs, echinoderms and bryozoans (De Varries and Beards, 1995; Rinehart et al., 1988; Yamada et al., 2000; Oliveira et al., 1985; Potts and Faulkner, 1992).

The first notable discovery of biologically active compounds from marine resources is the serendipitous isolation of the C-nucleosides, Spongouridine and Spongothymidine from the Caribbean sponge Cryptotheca crypta in the early 1950s (Bergman and Feeney, 1951). But the systematic investigation of the marine environment as a source of novel biologically active substances began in the mid 1970s. During the decade from 1977 to 1987, 2500 new metabolites from a variety of marine organisms have been reported (Cragg et al., 1999).

Tunicates have been an interesting source of more than 130 chemically unique bioactive natural products, which were previously unknown, over the past 10 years. The marine alkaloid Ecteinascidin 743 (ET 743), an anticancer drug isolated from Ecteinascidia turbinata of Caribbean Sea, now entered into market in the name of Trabectedin (Yondelis) and has been approved in the Europe as a second line treatment for advanced soft tissue sarcoma (Wan, 2007). Bryostatins, are the most important unusual
metabolites isolated from a purple brown bryozoan *Bugula neritina* (Pettit, 1991) and *Amathia convoluta* (Hale et al., 2002). Conotoxins, the extremely potent venoms of predatory cone snails *Conus* sp., have yielded complex mixtures of small peptides (6-40 amino acids) that have provided models for novel painkillers like Ziconotide (Cragg et al., 1999; Olivera, 2000).

The sea hare, *Dolabella auricularia*, collected from the Indian ocean has been the source of 15 cytotoxic peptides Dolastatins, which has remarkable in vitro cytotoxicity and impressive in vivo anti-tumour activity at low doses (Smith et al., 2001). A most potent amphotericin B, which showed potent cytotoxic activity against L1210 murine leukemia cells and antifungal activity, was isolated from the egg mass of Spanish dancer nudibranch *Hexabranchus sanguineus* (Kerman et al., 1988)

Though, many potent compounds have been isolated from marine invertebrates, the continuous production of the same has posed great challenges due to practical difficulties with the availability and cultivability of the source organisms. The supply of raw material as to the ratio of biomass to yield is not favorable. The concentrations of many highly active compounds in marine invertebrates are often very low. For example, about one metric ton of wet weight of the tunicate *Ecteinascidia turbinata* is required to obtain approximately 1 g of the promising anti-cancer agent ET-743 (Mendola, 2000). Similarly, one metric ton of the sponge *Lissodendoryx* sp. is needed to get as little as 300 mg of a mixture of two halichondrin analogues, the powerful cytostastic polyketides (Hart et al., 2000). So, the harvest of the
large amounts of biomass of the source organisms from nature has the risk of extinction of the concerned species.

Mariculture of the target species is an option and considerable progress has been made with the culture of the bryozoan *Bugula neritina* (Mutter and Wills, 2000) and the tunicate *Ecteinascidia turbinata* (Mendola, 2000). However, not only the culture production is far from that will be needed once these compounds enter the drug market, but also is subjected to uncertain natural phenomenon (Proksch et al., 2002). Many factors including seasonal factors, like cycles or reproductive rates, diet, physical and chemical conditions, distribution by phylogenetic affiliation, geographic location, water depth or associations with symbiotic microorganisms influence the production of bioactive substances in marine organisms.

Many of the compounds isolated from marine environment are very large and complex molecules which require elaborate biochemical processes and so, the total synthesis of these complex natural products posed great challenges. The possibility of determining the essential features of the molecule necessary for activity have led, in some instances, to the synthesis of simpler analogues with similar or better activity of anticancer drugs like Bryostatin-1 and Ecteinascidin-743, synthetic analogues of marine organism metabolites. Manipulating biosynthetic pathways is another route to generate compounds with enhanced bioactivities.

Under these circumstances, the striking similarities between invertebrate metabolites and the known microbial metabolites has raised a
question on the real origin of invertebrate metabolites. Bacterial associations, quite common among marine invertebrates, have previously been recorded. Epibiotic bacteria on the larvae of some crustaceans protect them from fungal infection through the production of simple antimicrobial compounds (Gil-Turnes et al., 1989). Marine epiphytic bacteria associated with nutrient rich algal surfaces and invertebrates, have also been shown to produce antibacterial secondary metabolites which inhibit the settlement of potential competitors (Jensen and Fenical, 1994; Bernan et al., 1997). Chemoautotrophic bacterial association with deep-sea hydrothermal vents of vestimentiferean tubeworms, which help the tubeworms to thrive in sulfidic marine environment, are an excellent example of symbiosis, wherein the bacteria produce metabolic energy from the oxidation of hydrogen sulfide and provide organic compounds to the worm and in return, the worm provides the bacteria with CO₂, O₂, H₂S, NH₃ and minerals (Vrijenhoek et al., 2007).

Some bioactive compounds isolated from invertebrates originate from symbiotic microorganisms, like Tetrodotoxin, Saxitoxin, Okadaic acid, Surugatoxins, 2,4–diacetylphloroglucinol etc. (Schmitz et al., 1981; Nakamura et al., 1989; Isnansetyo et al., 2003). The direct contribution of associated bacteria (endosymbionts) to natural product biosynthesis in many sponges has been established. The antifungal cyclic peptide theopalauamide and the cytotoxic macrolide swinholide A isolated from the sponge Theonella swinhoei has been linked to the endosymbiotic filamentous and unicellular bacteria in the same sponge (Bewley et al., 1996; Bewley and Faulknar, 1998).
The cyclodepsipeptide jaspaklinolide (syn. jaspamide), isolated from marine *Jaspis* sponges and used as a molecular tool in cell biology as a cell permeable f-actin probe (Bubb et al., 1994), showed a close structural relation to chondramide D known from the myxobacterium *Chondromyces crocatus* (Jensen et al., 1996). The structural features of ET-743 from the tunicate *Ecteinascidia turbinata* reveals striking similarities to safracin B, a metabolite of *Pseudomonas flourescens* (Ikeda et al., 1983). This chemical similarity is in fact so pronounced that biotechnologically available cyanosafracin B provides a commercially feasible precursor for the partial synthesis of ET-743 (Cuevas et al., 2000). Symplostatin 1, a close structural analogue of dolastatin 10 isolated from the marine mollusc *Dolabella auricularia*, currently in phase II clinical trials, was found to be a metabolite of the blue-green alga *Symploca hydnoides* (Harrigan et al., 1998; Luesch et al., 2001 and 2002).

The marine environment harbours microbes, typically ranging from $10^3$ to $10^6$ per milliliter with as many as $10^9$ per milliliter in marine sediments (Austin, 1988), capable of exhibiting bacteriolytic and antibiotic activity. Marine microorganisms encompass a complex and diverse assemblage of microscopic life and less than 5 per cent have so far been cultured and identified. Bacteria help regulate rates of organic matter mineralization, nutrient cycling and energy transfer in aquatic environments (Azam and Worden, 2004). They produce a variety of metabolites, some of which can be used for drug development (Fenical, 1993; Davidson, 1995; Nair et al., 1992; Grossart et al., 2004).
Bacteria inhabit the surface and internal spaces of marine organisms. Interaction between epibiotic marine bacteria and their host organisms are known to play a significant role in marine ecosystem. They often form symbiotic association with the host organisms. Symbiosis between bacteria and primitive organisms were an absolute necessity for the diversity and evolution of multicelled eukaryotic organisms (Margulis, 1993). The metabolic and physiological capabilities of marine microorganisms that allow them to survive in their unique habitats also provide great potential for the production of metabolites not found in terrestrial environments (Colwell, 1993).

Antibacterial activity among marine bacteria is a well-known phenomenon and has been demonstrated in a number of studies (Isnansetyo et al., 2003; Uzair et al., 2006). Several bioactive compounds isolated from marine bacteria in the last decade formed new resources for the development of medically useful compounds (Donia and Haman, 2003; Anand et al., 2006). However, their ecological role and degrees of adaptation to the marine environment is largely unknown (Bush, 2004). Over 120 of the most important medicines (penicillins, cyclosporin A, adriamycine, etc.) in use today are obtained from terrestrial microorganisms (Alanis, 2005).

Penicillin represents the first antibiotic in the history of natural products from microorganisms (Bentley, 2000). Since the discovery of penicillin (penicillin G) in 1928 (Fleming, 1929), intensive research on soil derived bacteria and fungi, have shown that microorganisms are a rich source of structurally unique substances (Fenical, 1993). The isolation of cephalosporin (cephalosporin C) in 1948 by Giuseppe Brotzu from the fungus
Cephalosporium acremonium was considered as one of the early isolations of secondary metabolites from marine sources. Later on, Burkholder and his co-workers have isolated the highly brominated pyrrole antibiotic pentabromopseudiline from the bacterium Pseudomonas bromoutilis (Burkholder et al., 1966a). Till the mid eighties, many groups of important bacterial metabolites like the antibacterial cephalosporin C, streptomycin, tetracyclines, erythromycin, vancomycin, the antifungal amphotericin B, imidazoles, griseofulvin, strobilurins and many other compounds that play a role in therapeutics and agriculture were discovered (Grafe, 2000).

A number of surface associated marine bacteria have been found to produce antibiotics. The bacterium Pelagiobacter variabilis isolated from the seaweed Pocockiella variegata produced phenazine antibiotics, pelagiomicins (Imamura et al., 1997). Two bicyclic depsipeptides, salinamide A and B from Streptomyces sp. isolated from the surface of the jellyfish Cassiopeia xamachana, though exhibited moderate antibiotic activity, showed potent topical anti-inflammatory activity in chemically induced mouse ear edema assays (Trischman et al., 1994; Moore et al., 1999). Novel anticancer compounds, chandrananimycins A, B and C have been isolated from Actinomadura sp. (Maskey et al., 2003). A bacterial antibiotic, MC21-A (3, 3', 5, 5'-tetrabromo -2,2'-biphenyldiol), a brominated anti-MRSA substance was isolated from Pseudoalteromonas phenolica (Ishnansetyo and Kamei, 2003).

The highly cytotoxic proteasome inhibitor, salinosporamide A, isolated from Salinospora sp., a new genus proposed by Fenical and his colleagues in
2002 to a group of rare obligate marine actinomycetes from the ocean sediments (Mincer et al., 2002), displayed a potent *in vitro* cyclotoxic activity against human colon carcinoma (Feling et al., 2003). Mechercharmeycin A, a cyclic-peptide isolated the bacterium *Thermoactinomyces* sp. showed cytotoxic activity against human lung carcinoma and human leukaemia (Kanoh et al., 2005).

Bioactive agents were isolated extensively from *Streptomyces*, *Altermonas/Pseudoalteromonas*, *Bacillus*, *Vibrio*, *Pseudomonas* and *Cytophaga* associated with seawater, sediments, algae and marine invertebrates. They are able to produce quinones, polyenes, macrolides, alkaloids, peptides and to a lesser extent terpenoids. In recent years, the extremophiles like acidophiles, alkalophilis, halophiles, baro, thermophiles and psycrophiles have attracted greater interest among researchers.

The wealth of microbial diversity in the world's oceans as indicated by the preliminary studies make this as a promising frontier for the discovery of new medicines, although their taxonomy are not well defined (Blunt et al., 2005). So, the objectives of the study were to isolate the potential bioactive associated bacterial strains from the marine sources, culture them by optimizing their growth and production of bioactive metabolites, to screen against human bacterial pathogens, to identify the potent strains through molecular characterization, to assess pharmacological property and to isolate and elucidate the structure of the active metabolite.