1.0. Introduction

1.1. Marine microbes – a prolific producer of secondary metabolites

All life on earth is evolved from ocean (Newman and Cragg, 2007). Marine microorganisms are a crucial component of the Earth’s life-support system. With more than a billion microorganisms living in one cubic litre of seawater, the diversity of these mighty midgets is unparalleled. Marine environment serves not only as a source of untapped taxonomic diversity of microorganisms but also as source of novel secondary metabolites with unique chemical structure.

The secondary metabolites produced by the microorganisms are used to sense, adapt and respond to the external environment for their defense and survival. The molecular architecture of marine microorganisms are distinct from terrestrial counterparts in the physicochemical requirements, the biosynthetic pathways used and the elements employed in crafting the arsenal of the defensive molecules (Ramasamy et al., 2006). Small molecule screening programs over the past two decades revealed marine microorganisms to be a rich source of structurally diverse and highly bioactive secondary metabolites (Rath et al., 2005). For this reason scientists are avidly searching the marine environment for microbes that may lead to the isolation of new compounds with potential application in diverse fields.

1.2. Marine actinomycetes – a boundary microorganism

Amongst prokaryotes, the members of the phylum Actinobacteria are the dominant population and successful colonizers of all environments in the biosphere (Bull and Stach, 2007). Actinobacteria represents one of the largest taxonomic units of the 18 major lineages currently recognized within the domain bacteria, including 5 subclasses and 14 suborders (Zotchev, 2011). Actinobacteria belonging to the order Actinomycetales account for approximately 10,000 of the metabolites reported in the Dictionary of Natural Products, a biosynthetic capacity that remains without rival in the microbial world (Watve et al., 2001).
The name “Actinomycetes” was derived from Greek “atkis” (aray) and “mykes” (fungus), and has features of both bacteria and fungi. Although actinomycetes are considered as a pre-eminent source of bioactive natural products, in the hypothesis and process driven field of marine microbial ecology they are not often recognized as key species (Delong and Karl, 2005). Earlier, it was assumed that actinomycetes in marine environment are simply the consequence of terrestrial run-off and not part of indigenous microbiota (Bull et al., 2005). Furthermore, skepticism regarding the existence of indigenous populations of marine actinomycetes arises from the fact that the terrestrial bacteria produce resistant spores that are known to be transported from land to sea (Bull et al., 2000). Thus, it has been frequently assumed that actinomycetes isolated from marine samples are merely of terrestrial origin. The traditional perception of actinomycetes as authothonus terrestrial organisms has been sidelined in the recent past.

Ramesh and Mathivanan (2009) reported a tremendous diversity and novelty among marine actinomycetes. Culture-dependent and culture-independent methods have revealed immense actinobacterial diversity in different marine realms ranging from open ocean water to deep sea, from near-shore environment to inter-tidal environments, over the coral reef to invertebrates and plants (Zotchev, 2011). Furthermore, traditional and molecular approaches revealed the presence of actinomycetes in extreme environments that are previously considered hostile to life (Kristijanson et al., 2000), in association with hydrothermal vents (Kathiresan et al., 2005), below basalt aquifers (Chapelle et al., 2002), in the deep ocean subsurface (Parkes et al., 2014), inside Antarctic rocks (Hirsch et al., 2004) and in symbiotic association with invertebrates (Hill, 2004). Actinomycetes have evolved metabolic and morphological adaptations to enable their success in harsh environments. As marine environmental conditions are extremely different from terrestrial ones it is surmised that marine actinomycetes might produce unique bioactive compounds (Kijjoa and Sawangwong, 2004).
1.3. Actinomycetes - biotechnologically valuable prokaryotes

Since the discovery of actinomycin from the actinobacteria *Chainia purpurogena* in Salman Waksman’s laboratory at Rutgers University in 1940, followed by Streptomycin in 1943, actinomycetes served as a prolific producer of antibiotics and other secondary metabolites with biological activity. Actinomycetes are bacteria (Gram positive) with fungal morphology possessing high GC content in their DNA.

Actinomycetes are responsible for the production of about half of the discovered bioactive secondary metabolites (Berdy, 2005), notably antibiotics (Strohl, 2004), anti-tumor agents (Cragg and Newman, 2005), immunosuppressive agents (Mann, 2001) and enzymes (Oldfield et al., 1998). Due to the excellent track record on secondary metabolites production, immense efforts have been directed towards the successful isolation of novel actinomycetes from terrestrial sources for drug screening programs. Recently, the rate of discovery of new compounds from terrestrial actinomycetes has decreased, whereas the rate of re-isolation of known compounds has increased (Fenical et al., 1999). Therefore, bioprospecting terrestrial actinomycetes for secondary metabolite production is like screening ‘old friends’ for the costly rediscovery of known compounds (Jensen et al., 2005a).

A large number of new compounds with structures completely different from those isolated from terrestrial organisms were discovered from marine sources. Williams et al. (2007) reported that marine actinomycetes derived natural products possess unique structural features rarely or never found with the terrestrial sources. They also reported that the mode of action of secondary metabolites are specific, prompting them as a potential lead for drug development in cancer abatement and for other chronic diseases. Apart from therapeutic value, actinomycetes have a profound role in the marine environment. For example, degradation and turnover of various materials are mediated by the action of a variety of actinomycetes (Das et al., 2008a). The cellulolytic and chitinolytic activity of marine actinomycetes was reported by Pisano et al. (1992).
The increase or decrease in the number of a particular enzyme-producing microorganism indirectly indicates the concentration of substrate in the environment. Actinomycetes are also reported to contribute to the breakdown, recycling and mineralization of organic compounds (Goodfellow and Haynes, 1984), immobilization of mineral nutrients, fixation of nitrogen and other environmental protection measures (Goodfellow and Williams, 1983).

1.4. *Streptomyces*- a prolific source of bioactive compounds

*Streptomyces* is the largest genus of the actinomycetes under the family Streptomycetaceae, order Actinomycetales and suborder Streptomycineae (Anderson and Wellington, 2001). The term *Streptomyces* was first proposed by Waksman and Henrici (1943). Members of the genus *Streptomyces* differ greatly in their morphology, physiology and biochemical activities (Suneetha and Prathusha, 2011).

*Streptomyces* species are well known by a linear chromosome, complex morphological differentiation and by the ability to produce several bioactive secondary metabolites (Mervat, 2009). Marine environment harbors a wide range of distinct *Streptomyces* that are not present in the terrestrial ecosystem (Curtis et al., 2002). The immense diversity and the potential of synthesizing various bioactive compounds is the fundamental reason for attracting researchers towards marine *Streptomyces*.

Amongst actinomycetes, members of the genus *Streptomyces* remain a richest source of useful bioactive metabolites. Actinomycetes contribute to nearly 50% of the total microbial bioactive secondary metabolites produced, with 7600-8000 of these compounds (80%) are of *Streptomyces* origin (Berdy, 2005). Despite this astonishing productivity, it is predicted that only 10% of the total number of natural products synthesized by these organisms have been discovered (Watve et al., 2001). Bull and Stach (2007) reported that marine *Streptomyces* is an excellent reservoir for numerous chemical compounds with commercial application.
Maldonado et al. (2005) isolated 64 *Streptomyces* strains from the water samples collected from Andaman sea shore. Of the 64 isolates, 49 showed antibacterial and antifungal activity, the rest exhibited antagonistic activity against multidrug resistant pathogens. With increasing advancement in science and technology, there would be greater demands in future for new bioactive compounds synthesized by *Streptomyces* from various marine sources.

1.5. Probiotic properties of *Streptomyces*

Despite the source of several novel antibiotics, marine actinomycetes have been given less attention as probiotics. Probiotics are ‘live microorganisms which when administered in adequate amounts confer a health benefits on the host’ (FAO/WHO, 2002; Sanders, 2003). Probiotics inhibit the growth of pathogens and reduce the incidence of disease or lessen the severity of disease outbreaks (Gomez and Balcazar, 2008). Although there are sufficient reports to confer health benefits to humans and animals, the use of probiotic bacteria of non-human origin are still in controversy.

Among actinomycetes, *Streptomyces* exhibit excellent probiotic properties with no toxicity data published with respect to its consumption. Das et al. (2008b) enlists the prospects of using marine *Streptomyces* as potential probiotics. Although, it is generally admitted that probiotics exert beneficial effects, the knowledge on the mechanisms underlying is limited. Modes of action well accepted so far includes the production of inhibitory substances against pathogens, competition for essential nutrients and adhesion sites, supply of essential nutrients and enzymes, modulation of interactions with the environment and the development of beneficial immune responses (Verschuere et al., 2000; Balcazar et al., 2006). Further, in order to colonize the gastrointestinal tract, a potential probiotic microorganism should express high tolerance to acid and bile, ability to adhere to intestinal surfaces, persistence of gastrointestinal transportation, cholesterol-reducing and antimicrobial activities (Joborn et al., 1997; Wu et al., 2009).
1.6. Importance of iron

Iron (Fe) was once plentiful and valuable but now iron is dear and dangerous. Iron is an important bioactive element indispensable for the growth and metabolism of all living cells. Iron plays a key role in many biological processes like photosynthesis, nitrogen fixation, respiration, tri-carboxylic acid cycle, energy metabolism, electron transport, oxidation-reduction reactions and synthesis of DNA precursors (Touati, 2000). In addition, iron is required for the promotion of cell growth, proliferation and differentiation.

In many ecological niches iron is present as insoluble iron oxides and makes its acquisition by microorganisms (indeed of higher organisms) a considerable challenge (Dave and Dube, 2000). Thus, iron on which life is so dependent becomes scarce and growth limiting. Chelation and reduction are the main approaches that have been adopted by bacteria to close the ‘concentration gap’ between ferric iron solubility and iron requirement. Although iron is crucial for life, an excess of iron is toxic. This is due to the deleterious effects of reactive oxygen species (ROS), such as the hydroxyl radical (OH+) which is produced via the Fenton reaction (Figure 1).

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\begin{align*}
\text{Fe}^{3+} + \text{O}_2^- & \rightarrow \text{Fe}^{3+} + \text{O}_2 & (1) \\
\text{Fe}^{3+} + \text{H}_2\text{O}_2^- & \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- & (2) \\
\text{O}_2^- + \text{H}_2\text{O}_2 & \text{Fe} \rightarrow \text{O}_2 + \text{OH}^- + \text{OH}^- & (3)
\end{align*}
\]

**Figure 1. Iron-catalyzed redox reactions of biological importance.**

- equation 1. shows the reduction of Fe (III) by the superoxide radical (O$_2$).
- equation 2. Fenton reaction that results in the production of the hydroxyl radical (OH\(^+\)) and the hydroxide anion (OH\(^-\)) from hydrogen peroxide(H$_2$O$_2$).
- equation 3. shows the iron-catalyzed Haber-Weiss reaction.

The highly reactive OH\(^+\) radical is able to induce cell death through initiating a series of chemical reactions with different biomolecules, resulting in DNA oxidation, mitochondrial damage and peroxidation of membrane lipids (Bergeron et al., 2003). In addition, excess free iron can also react with unsaturated lipids to produce alkoxy and peroxy radicals (Lieu et al., 2001).
Richardson and Ponka (1997) reported that rapidly growing cancer cells require more iron for their growth and metabolism than do resting cells. Iron accumulation in many organs has been shown to correlate with the process of carcinogenesis (Richardson et al., 2009). In the absence of iron, cells cannot proceed from the G1-to S-phase of the cell cycle. Therefore, establishing iron deprivation by chelation could potentially contribute to the prevention and management of cancer. To chelate iron and to establish equilibrium constant, microorganisms produce multidentate organic ligands known as siderophores.

1.7. **Siderophores - the iron chelators**

Siderophores are low molecular weight (<1000 daltons) iron chelators. Lankford coined the term siderophore in 1973 from a Greek term meaning “iron carrier”. These low molecular weight ligands were selectively evolved to sequester the transition metal iron in a soluble form, via specific membrane receptors (Hider and Kong, 2010).

The study of siderophores and their function in microorganism might seem like a recondite pursuit, but the importance of iron transport against natural odds places it in a more prominent perspective. Iron chelators in seawater are predominantly of bacterial origin. The production of siderophores and the proteins required for their uptake needs to be tightly regulated in order to avoid toxic oxygen radical formation (Neilands, 1984). Therefore, the rationale for siderophore synthesis is not only to overcome the insolubility of available iron but also to regulate and control its uptake, as at higher concentration it becomes toxic (Guerinot, 1994).

Research in the field of siderophores started six decades ago when Neilands discovered fungal ferrichrome in 1952. Currently, there are almost 500 compounds that have been identified as siderophore. However, knowledge on siderophores produced by marine actinomycetes is relatively recent and only a few reports are available. Raymond et al. (2004) reported that siderophores differ widely in their overall structure from one species to another.
1.8. Nature and classification of siderophores

Depending on the functional groups that coordinate iron chelation, microbial siderophores are classified as hydroxamate, catecholate and carboxylate (Neilands, 1990; Sandy and Butler, 2009).

Hydroxamate group-bearing siderophores are synthesized mainly by Gram-positive filamentous bacteria (actinomycetes) and fungi. In 1979, Armstrong and Van Baalen isolated the first hydroxamate-type siderophore from Agmenellum quadruplicatum. Hydroxamate siderophores include ferrioxamines, ferrichromes and coprogens.

Catecholate siderophores are also referred as phenolates. Catecholates are produced only by bacteria. Catecholates are the second most common siderophore produced by bacterial species. In 1970, enterobactin (also known as enterochelin), the first tricatechol siderophore, was isolated from culture fluids of Escherichia coli, Aerobacter aerogenes and Salmonella typhimurium. Enterobactins, bacillibactins, vibriobactin, yersiniabactin, salmochelin etc., are the examples of catechol-type siderophore (Raymond and Dertz, 2004).

Carboxylates are earlier called as complexones. Carboxylate are produced exclusively by fungi belonging to Zygomycota (mucorals) and few bacteria (Rhizobium and Staphylococcus species) (Drechsel and Winkleman, 1997). Carboxylate type siderophores was first isolated from Rhizobium meliloti (Smith et al., 1985).

1.9. Iron chelators – therapeutic application

Resistance of pathogenic bacteria to different antibiotics is wobbling the medical field all over the world. Iron chelation therapy is being considered as a suitable, viable alternative treatment for various infectious diseases (Mollmann et al., 2009). Siderophores are less preferably exploited in therapeutic applications. Development of new compounds with anticancer potential has been garnering considerable attention in the recent times. In this contest, siderophores will serve as a viable alternative to the present day drugs.
In addition to anticancer potential, chelation of iron is reported to inhibit the growth of microbial pathogens (Murugappan et al., 2012). Removal of iron has shown to improve the clinical outcome in a number of infectious diseases (You et al., 2005). By binding redox-active iron, chelators play a beneficial role in the treatment of pathological conditions like thalassemia, ischemia reperfusion injury, neurodegenerative diseases and inflammation.

Marine actinomycete genera have been reported to produce novel compounds with various biological activities (Jensen et al., 2005b). The mode of action of actinomycetes derived compounds are unique, prompting their investigation as potential leads for the development of drugs in the treatment of cancer, as well as other chronic diseases. Desferrioxamine (DFO), a siderophore produced by Streptomyces pilosus is has been used for the treatment of cancer for more than three decades (Wong et al, 2004). However, siderophores are less preferably exploited in therapeutic applications.

1.10. Optimization – response surface methodology

Optimization of the process involved in the synthesis of valuable products has received much attention in the recent past. The purpose of optimization is to improve the performance of the system and to increase the yield without increasing the cost. It is inevitable to optimize the medium constituents as they contribute for the high cost of production. Optimization studies can be conducted either by adopting conventional method of changing one factor at a time or by varying several factors and examining their effects and interaction at a time using simulation studies.

Optimizing all the essential parameters at a time can eliminate the limitations of one factor at a time optimization process which is laborious and time consuming. Statistical experimental design such as response surface methodology (RSM) depicts the interactive effects of the components (Uma and Sathyanarayana, 2007). Response surface methodology was developed by Box and Wilson in the year 1951. RSM is a collection of mathematical and statistical techniques widely used to determine the effects of several variables.
The statistical design of experiment using RSM is an organized approach that yields more reliable information per experiment than unplanned approaches (Nikel et al., 2005). Statistical data analysis allows visualization of the interactions among several experimental variables, leading to the prediction of data in areas not directly covered by experimentation (Papaneophytou and Kyriakidis, 2012). RSM is a tool used to construct the model, design and test the trials, seek and optimize the significant factors, identify the optimum conditions and establish the numerical correlation for the target responses (Bas and Boyaci, 2007). RSM is useful for developing, improving and optimizing processes in which a response of interest is influenced by several variables (Mohamad et al., 2011). RSM is used not only for optimization but also for determination of kinetic constants and investigation of enzyme stability kinetics.

1.1.1. Cancer abatement

For the last several decades, the search for agents that may treat or ameliorate the scourge of cancer have involved all aspects of chemistry and pharmacology. Natural products (NPs) have played an extremely important role in cancer abatement. Initially, as the major source of drugs used for direct treatment and currently as modulators of specific cellular pathways in the tumor cells. Development of naturally derived anticancer drugs is crucial and isolation of novel compounds has become an important part of cancer research.

Iron chelation therapy has been considered as a possible treatment of various diseases, including cancer (Krishnaraj et al., 2014). Iron, the metal is used in catalysis of DNA synthesis and for a variety of enzymes concerned in electron transport and energy metabolism. Due to the essential role which iron plays in metabolic processes of mammalian systems, it seemed probable that siderophores would also prove useful in controlling the growth of neoplastic cells. Siderophores, preferentially inhibits DNA synthesis, resulting in interference with the growth of cancer cells (Kanoh et al., 2006). Earlier data demonstrated that desferrioxamines (iron-free siderophores) can significantly reduce the growth of aggressive tumors in patients suffering from neuroblastoma (NB) or leukemia (Buss et al., 2003; Lovejoy and Richardson, 2003; Richardson, 2005).
1.12. Application of siderophores

The importance of siderophore extends beyond their role in iron acquisition and iron chelation therapy. Siderophores and their derivatives have a lot of applications like heavy metal biosorption, antibiosis, plant growth promotion, quorum sensing etc.

Heavy metal biosorption

Siderophores are generally viewed as biological iron uptake agents, recent evidence has shown that siderophores play a significant role in the biogeochemical cycling and biological uptake of metals like Al, Cd, Cu, Mn, Pb, Zn, etc., and trace levels of the radionuclide’s gallium-67 and indium (Kiss and Farkas, 1998; Neubauer et al., 2000). Therefore, siderophore-mediated sequestering system is reported to moderate heavy metal toxicity in different environments (Clarke et al., 1987). Recently, siderophores have been used in phytoremediation process (Braud et al., 2009). Dimkpa et al. (2009) reported siderophore mediated metal tolerance in plants. Microbial siderophores play an important role in complexing toxic metals and in increasing their mobility in soils (Yan et al., 2011).

Antagonistic potential

Apart from iron chelation, siderophore production is considered as one of the virulence factors in bacteria. Fgaier and Eberl (2011) reported that siderophore production in microorganisms possesses a competitive advantage over other species which lack the ability. In recent years, new and novel antibiotics have been designed to accommodate iron binding functional group (Watanabe et al., 1987). Antimicrobial efficiency of siderophore is in accordance with many of the commercially available antibiotics. Most of the siderophore are reported to inhibit the protein biosynthetic mechanism of the target organisms (Murugappan et al., 2012). Siderophore from Klebsiella pneumoniae has been used as an anti-malarial agent and in cosmetics as deodorants (Johnson et al., 2008). Mabeza et al. (1999) and Stirrett et al. (2008) reported the utilization of hydroxamate siderophores in treatment of malaria, tuberculosis, leprosy and cholera.
Plant growth promotion

Predominant group of plant growth promoting rhizobacteria (PGPR) produces siderophores (Crowley, 2006). Siderophore-producing rhizobacteria improve plant health by inhibiting the growth of pathogens by limiting the iron available. Siderophore-producing bacteria are released to improve the iron nutrition at the same time results in lowering the chemical inputs such as pesticides and fertilizers (Murugappan et al., 2013). Disease prevention and growth promotion by siderophore producing soil bacteria is a promising technology in plant growth development. Iron is likely to be crucial for nitrogen-fixing organisms because the enzymes involved need large amounts of iron (Geider, 1999).

Quorum sensing

Evidence suggests that siderophore plays a significant role as signaling molecules. “quorum sensing” is the term used to describe the cell density-dependent regulation of bacterial physiology, including gene expression (Waters and Bassler, 2005). Quorum sensing bacteria excrete low molecular weight chemical “messenger” molecules into the environment, which when a critical concentration is reached, trigger a signal transduction cascade. This signal cascade results in the alteration of gene expression. Siderophore production and other iron transport genes are reported to be under “quorum sensing” control (Guan and Kamino, 2001). Recent reports suggest that siderophores themselves can have a cell signaling function in addition to their iron uptake and transport roles (Lamont et al., 2002).

The bioprospecting strategy which is the subject of this work is based upon the premise to meet the demand for new pharmaceutical compounds and to combat the antibiotic resistant pathogens. Researchers in the recent past have been forced to look for novel microorganisms in marine environment for secondary metabolites. Of the marine organisms, actinomycetes are prolific but underexploited source for the discovery of novel secondary metabolites. Relatively, Gulf of Mannar, the nose of Indian Ocean has rarely been explored for microbial diversity and microbial metabolites. Therefore, in the present study an attempt has been made to isolate marine actinomycetes from different locations of Gulf of Mannar for the discovery of novel bioactive compounds for siderophore production, probiotic properties and anticancer activity. It is also intended to investigate the influence of various nutrients on siderophore production by optimization process.