CHAPTER 1

INTRODUCTION

1.1 PREAMBLE

Leukemia is a malignant cancer of the bone marrow and blood, characterized by an uncontrolled accumulation of abnormal blood cells leading to the inhibition of normal blood cell functions and in many instances, death. Leukemia causes more deaths than any other cancer among children aged around twenty (Kwan et al 2009). Among the antitumour drugs, bacterial enzyme L-asparaginase (L-asparagine amido hydrolase E.C.3.5.1.1), has been employed as the most effective chemotherapeutic agent in pediatric oncotherapy especially for acute lymphoblastic leukemia (ALL). L-asparagine is an essential amino acid for the growth of certain malignant cells whereas the normal cells are independent of its requirement (Savitri et al 2003). L-asparaginase catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia thereby selectively kills the leukemic cells. There is a great demand for new therapeutic L-asparaginase from a novel source. Actinobacteria are industrially the most important bacteria as they include the genus Streptomyces which produce large number of antibiotics, natural products and therapeutic enzymes. Marine Streptomyces have received great attention for anticancer agents in recent years. Marine Streptomyces are good source of salt tolerant L-asparaginase enzyme, which are suitable for therapeutic application (Gupta et al 2007, Dhavegi and Poorani 2008, Poorani et al 2009).
1.2 THE GENESIS OF THE THESIS

The search for new antitumour agents is still a priority goal for cancer therapy. Extensive screening has led to the discovery of several antitumour agents from microorganisms in particular bacteria. A great number of antitumour agents are natural products or their derivatives and antitumour enzymes. Antitumour L-asparaginases have been studied in several bacteria (Savitri et al 2003). L-asparaginases from Escherichia coli and Erwinia carotovora are currently used in therapeutic protocols for the treatment of ALL (Keating et al 1993). However, these L-asparaginases have attributed significant side effects mainly due to their contamination with glutaminase and endotoxins (Duval et al 2002).

Hence, a glutaminase free L-asparaginase with no toxicity from a new source will be more advantageous. In recent years, new actinobacteria with antitumour potential have been largely isolated from marine habitats. To accomplish this, the present study was undertaken. The aim of this investigation was to discover a novel marine actionobacteria which produces antitumour L-asparaginase. Further it was also proposed to characterize the enzyme with a view to knowing the possibility of its use as an alternative drug for cancer.

1.2.1 Marine Environment and its Importance

The marine biosphere is one of the earth’s richest habitats of microorganisms especially bacteria. Marine bacteria have a diverse range of enzymatic activity and are capable of catalyzing various biochemical reactions with their novel enzymes. Presently, marine bacteria are considered as potential source of therapeutic enzymes, metabolites and natural products, which possess novel properties. Salt tolerant enzymes from marine bacteria are interesting alternative for therapeutic purpose. There is an enormous scope
to investigate the probabilities of deriving therapeutic enzymes and new products of economic importance from potential marine bacteria for pharmaceutical purposes. Since marine environment is saline in nature and chemically closer to human blood plasma, it could provide bacterial therapeutic agents, mainly enzymes, that could be safer having no or less toxicity and side effects when used for human therapeutic application (Sabu 2003).

### 1.2.2 Marine Actinobacteria a Boundary Microorganism

Actinobacteria were first observed by Cohn in 1875 and named by Harz in 1877. Actinobacteria comprise a group of branching unicellular soil microorganisms. They have frequently been looked upon as a separate group of microorganisms occupying a mid position between the true fungi and the true bacteria (Das et al 2006). Actinobacteria hold excellent track record of metabolites production as they include the genus Streptomyces. A significant amount of effort has been focused on the successful isolation of actinobacteria from terrestrial sources for drug screening programs. The rate of discovery of new compounds from terrestrial actinobacteria has diminished in recent years, in contrast with marine actinobacteria (Liang et al 2009).

Marine actinobacteria have developed unique metabolic and physiological capabilities to ensure survival in extreme habitats. They produce several different antitumour agents and therapeutic enzymes which are not observed in terrestrial actinobacteria. The exploitation of marine actinobacteria as a source for therapeutic enzymes and other metabolites are still in their infancy (Bull and Stach 2007). Busti et al (2006) reviewed various novel metabolites of marine actinobacteria. They also suggested that the undiscovered new actinobacteria are greatly distributed in marine environment. Exploration of marine actinobacteria especially novel strains of the genus Streptomyces is imperative for pharmaceutical industries. Thus, it is
essential that new groups of actinobacteria from marine habitats are pursued as source of therapeutic enzymes and bioactive compounds.

1.2.3 Marine Streptomyces: A Potential Source for L-asparaginase

In general, biochemical and enzymatic properties of L-asparaginase vary in accordance to the bacteria and their growth conditions. Synthesis of L-asparaginase by wide variety of bacteria depicted that they it highly depend on the carbon and nitrogen source (Liu and Zajic 1973). Marine Streptomyces are good source for the production of L-asparaginase as they are able to metabolize various carbon and nitrogen sources in marine environment (Gupta et al 2007). Marine Streptomyces are nonpathogenic and are capable of producing high amount of L-asparaginase compared to E. coli L-asparaginase (Dhevagi and Poorani 2006). Currently, pharmaceutical industries are in great deal of exploring novel anticancer agents from undiscovered marine actinobacteria (Hakvag et al 2008). Marine Streptomyces are most prolific genus of actinobacteria as they are responsible for the production of antitumour compounds and therapeutic enzymes (Olano et al 2009). Marine actinobacteria are found to be a rich source for therapeutic L-asparaginase enzyme (Dhevagi and Poorani 2008).

1.2.4 L-asparaginase: A Potential Drug for Leukemia

L-asparaginase has been used as an important drug in acute lymphoblastic leukemia (ALL) for the past two decades. Data regarding the clinical trials of native L-asparaginases of Erwinia and E. coli origin have been in abundance and widely discussed in the last 30 years. L-asparaginase treatment for ALL is a major breakthrough in modern oncology as it induces complete remission in over 90% children within four weeks (Savitri et al 2003). Given the hypersensitivity associated with the native and modified preparations, the quest for new antitumour L-asparaginase with better
properties is expected for clinical applications (Narta et al 2007). Therefore, the search for marine actinobacterial L-asparaginase can be an alternative choice for the treatment of ALL without side effects.

1.3 LITERATURE REVIEW

Exploration of novel marine actinobacteria for therapeutic compounds has been notable in the past few years. A very limited literature is available on marine actinobacteria and its production of L-asparaginase. A detailed survey of the available literature on this study has been carried out and presented in this section.

1.3.1 Distribution of Marine Actinobacteria

The first report on marine actinobacteria was made by Nadson (1903) from salt mud. Occurrence and distribution of actinobacteria in marine environment was studied by Zobell and Upham (1944), Humm and Shephard (1946), Wood (1953) and Freitas and Bhat (1954). In India, distribution of marine actinobacteria was first reported by Baam et al (1966) from seawater collected in Bombay. An extensive survey of the distribution of actinobacteria in marine sediments of North sea and Atlantic ocean was carried out by Weyland (1969). This study suggests that marine actinobacteria are the best source for the unique bioactive compounds than the terrestrial counterpart. Systematic of marine actinobacteria was carried out by Walker and Colwell (1975). Prevalence of antagonistic marine Streptomyces in sediments of Portonovo (east coast region of India) was investigated by Lakshmanaperumalswamy (1978) and Vanajakumar et al (1981). Antibiotics potential of marine actinobacteria was reported by Okami and Hotta (1988). Chlorotetracycline and tetracycline producing S. aureofaciens was isolated from marine sediments (Yang and Ling 1989).
Actinobacteria from marine sediments were found to produce valuable therapeutic products (Goodfellow and Hayens 1984). Antibiotics producing actinobacteria were isolated from marine sediments by Okami (1986). Antimicrobial potential marine actinobacteria were isolated from sediments of east coast region of India (Ellaiah and Reddy 1987). Actinobacteria distribution in marine sediments was described by Jensen et al (1991). Occurrence of actinobacteria in mangrove sediments of Cochin was studied by Rathnakala and Chandrika (1993). Antimicrobial activity of marine actinobacteria from Chesapeake Bay was reported by Takizawa et al (1993). Indigenous Streptomyces population of marine actinobacteria in sediments of Georgia was reported by Moran et al (1995). Marine actinobacteria producing bioactive compounds were described by Davidson (1995). L-asparaginase potential Streptomyces plicatus and 16 strains of Streptomyces were isolated from marine environment by Dhevendran and Annie (1999). Marine actinobacteria are immense resource for therapeutic products (Chandrasekaran 1998). Systematics of marine Streptomyces in estuarine sediments of west coast region of India was reported by Dhevendran and Annie (1999). Marine actinobacteria from Taiwan strait, China was isolated by Zheng et al (2000).

Balagurunathan (2004) proposed that marine sediments of Indian ocean are the suitable source for the isolation of antibiotic potential Streptomyces. Antibiotics producing Streptomyces griseobrunneus and 51 strains of Streptomyces were isolated from marine sediments of Parangipettai (Balagurunathan and Subramanian 2001). Sivakumar et al (2005) investigated the prevalence of marine actinobacteria in mangrove sediments of Pichavaram. This study suggested that mangroves are the rich source of Streptomyces and Micromonospora with bioactive compounds potential.

Widespread and persistent populations of marine actinobacteria in ocean sediments were reported by Mincer et al (2002). Marine actinobacteria
represent a valuable resource for anticancer drugs (Fenical 1993). Subsequent studies revealed that specific populations of marine adapted actinobacteria exist with significant new anticancer metabolite potential. Balagurunathan (2004) reviewed the literature on marine actinobacteria and suggested that they have promising future for drug industry. Antagonistic marine actinobacteria from sediments of Hainan Island was isolated by You et al (2005). The distribution of actinobacteria in the sea largely resides in sediments and is unexplored (Jensen et al 2005). Marine sediments are important resource for novel actinobacteria (Kim et al 2004) L-asparaginase activity of marine actinobacteria isolated from the sediments of Parangipettai was reported by Dhevagi and Poorani (2006). Marine actinobacteria are prolific source for natural products and new antitumour agents (Bull and Stach (2007) and Olano et al (2009)

Based on this conceptual frame of reference, the presence of indigenous marine actinobacteria in different marine environments is confirmed. On the same line, an extensive investigation on purification and characterization of a new antitumour L-asparaginase from a novel strain of marine actinobacteria was attempted in order to explore marine actinobacteria as a potential source for L-asparaginase for pharmaceutical industry.

1.3.2 Isolation of Marine Actinobacteria

Current progress in isolating marine actinobacteria from sea, especially from sediments has been notable in the past few years (Bull and Stach 2007). The isolation of marine actinobacteria from sediments requires enrichment of samples to improve the isolation and increase the counts of actinobacteria. Enrichment of samples in different selective media has led to the isolation of marine actinobacteria from sediments. Glucose asparagine medium, Grein and Meyer’s medium and Kuster’s medium are the best media for the isolation of actinobacteria (Jensen et al 1991). Starch casein medium is
an excellent medium for growth and maintenance of marine actinobacteria (Rathna Kala and Chandrika 1993).

Use of nalidixic acid (NA) in synthetic starch casein medium enhances isolation of actinobacteria from the marine sediments (Takizawa et al 1993). This protocol exploited the importance of ideal enrichment method for the isolation of marine actinobacteria and also the routes for the discovery of new actinobacteria with novel products. Considerable number of marine Streptomyces was isolated from sediments by using NA in different selective media (Dhevendaran and Annie 1999). Balagurunathan and Subramanian (2001) isolated marine Streptomyces from sediments of Parangipettai by using sterile seawater in the enrichment media. Dhevagi and Poorani (2006) used seawater and NA supplemented enrichment media for the isolation of marine actinobacteria. They suggested that starch casein medium is suitable not only to increase the counts of marine Streptomyces strains but also to maintain the diversity. Hakvag et al (2008) used cycloheximide and NA in yeast malt medium for isolation of actinobacteria from sea surface microlayer and sediments for antimicrobial metabolites. They also described that marine actinobacteria are a rich source of therapeutic agents and the success relies on the ability to isolate novel actinobacteria from the marine environments.

1.3.3 Novel Metabolites from Marine Actinobacteria

Although the exploitation of marine actinobacteria as a source for the discovery of secondary metabolites and enzymes are at an early stage, numerous novel metabolites, antibiotics, bioactive compounds, natural products and antitumour agents have been isolated in the past few years.

Lam (2006), reviewed various antimicrobial and anticancer metabolites produced by marine actinobacteria and their biological activities. He also opined that marine actinobacteria are prolific source for the discovery
of novel secondary metabolites. Olano et al (2009), reviewed various antitumour compounds and their antitumour properties, produced by marine actinobacteria. They expressed the view that recent development in molecular biology with 16S rRNA gene analysis leading to the identification of new marine actinobacteria with antitumour compounds potential.

Salinosporamide A (NPI-0052), a novel beta lactone gamma lactam produced by Salinospora tropica was investigated by Feling et al (2003). It is a proteasome inhibitor which induces apoptosis in multiple myeloma cells with mechanisms distinct from the commercial proteasome inhibitor anticancer drug Bortezomib (Jensen et al 2005).

Abyssomicin C, a novel polycyclic polyketide antibiotic producing marine Verrucosispora strain was isolated by Riedlinger et al (2004). It is a potent inhibitor of Gram positive bacteria, particularly multiple drug resistant and vancomycin resistant pathogens. Diazepinomicin (ECO-4601) is a unique farnesylated dibenzodiazepinone produced by a Micromonospora strain Charan et al (2004). It possesses antibacterial, anti-inflammatory and antitumour properties. The preclinical development of ECO-4601 as an anticancer agent has been completed by Ecopia Biosciences Inc.

Enterocin and meroterpenoids are novel polyketides isolated from marine Streptomyces maritimus (Moore et al 2005). They exhibit broad antibiotic and anticancer activities. Currently, the gene manipulation approaches are under trial to generate new polyketides with therapeutic properties and to explore the biosynthetic pathways for drug discovery. The pre-eminence of natural products (Baker and Alvi 2004) and the success of marine natural products (Blunt et al 2004) have prompted the marine actinobacteria as a source of novel metabolite diversity for drug discovery (Ward and Bora 2006).
1.3.4 Bacterial Sources of L-asparaginase

The potential of antitumour L-asparaginase in cancer treatment was first proposed by Kidd (1953) by showing that guineapig sera could cause regression of transplanted lymphomas in mice and rats. Therapeutic potential of L-asparaginase from Guineapig serum was proved by Broome (1961). Large preparation of enzyme from guineapig sera was found to be tedious (Mashburn and Wriston 1964 and Yellin and Wriston 1966). Soon after, E. coli L-asparaginase antitumour activity was first demonstrated by Mashburn and Wriston (1964), L-asparaginase production using bacterial systems has received considerable attention. Bacterial L-asparaginases possess antitumour property and are a better source for the production of L-asparaginase, because they can be easily cultured (Roberts et al 1966). Further, the extraction and purification of L-asparaginase is also convenient, facilitating the large scale production (Liu and Zajic 1973). The search for a better source of this enzyme was led to a native form isolated from E. coli (Campbell and Mashburn 1969 and Whelan and Wriston 1969). This has prompted researchers to study wide variety of bacteria for their L-asparaginase activity. Assay procedure for the screening of microbial amidases was developed by Imada et al (1973). This quantitative assay facilitated subsequent screening of large number of bacteria for the selection of potential producer of L-asparaginase.

Distasio and Niederman (1976) characterized an antitumour L-asparaginase of Vibrio succinogenes which was immunologically distinct from the E. coli L-asparaginase. Similarly, Kitto et al (1979) characterized two functionally different asparaginases from Pseudomonas geniculata and only one form of L-asparaginase exhibited antitumour activity. L-asparaginase synthesis by Serratia marcescens was reported by Sukumaran et al (1979). Production of L-asparaginase by human clinical isolates of
Vibrio succinogens was described by Radcliffe et al (1979). Intracellular L-asparaginase from Streptomyces karnatakensis was reported by Mostafa (1982). L-asparaginase from Thermoactinomyces vulgaris was isolated by Mostafa and Ali (1983). L-asparaginase from Erwinia caratovora was purified by Lee et al (1989). Asparaginase gene from Erwinia chrysanthemi was cloned and expressed in E. coli by Harry et al (1986).

Corynebacterium glutamicum L-asparaginase was partially purified by Mesas et al (1990). Antitumour activity of E. caratovora L-asparaginase was reported by Maladkar et al (1993). The phenomenal behavior of cancerous cells such as programmed cell death through the inhibition of protein synthesis exploited to treat neoplasias using L-asparaginase (Story et al 1993 and Swain et al 1993). The L-asparaginase prepared from E. coli and E. caratovora has been used successfully to induce complete remissions of certain leukaemia and acute lymphoblastic leukemia (Muller and Boos 1998). The therapeutic responses of these asparaginase preparations have shown many unwanted side effects (Asselin et al 1993, Gallagher et al 1989, Duval et al 2002, Suzuki et al 2008, Uyttebroeck et al 2008 and Pasut et al 2007). These observations suggested the need to discover new L-asparaginase with therapeutic potential (Kotzia and Labrou 2007, Douer 2008 Veronese and Pasut 2009 and Kwan et al 2009).

1.3.5 L-asparaginase Activity of Actinobacteria

Asparaginase from Gram positive bacteria has received little attention since very few of them were known to produce L-asparaginase. Among the Gram positive bacteria, actinobacteria have gained special importance as they include the genus Streptomyces which are responsible for bioactive compounds. Unfortunately, few reports are available about the L-asparaginase production from actinobacteria. Among actinobacteria, notably the genus Streptomyces produce L-asparaginase (Wade et al 1971).
Synthesis of L-asparaginase by Streptomyces griseus was reported by De Jong (1972). L-asparaginase activity of Streptomyces in soils of Kuwait was reported by Mostafa and Salama (1979). Intracellular L-asparaginase from Thermoactinomyces vulgaris was reported by Mostafa and Ali (1983).

L-asparaginase activity of marine Streptomyces in sediments of Kerala was studied by Dhevendaran and Annie (1999). Similarly, L-asparaginase activity of marine Streptomyces in sediments of Parangipettai was reported by Dhevagi and Poorani (2006). These studies suggest that marine adapted Streptomyces are the prolific source for therapeutic L-asparaginase. This was supported by Gupta et al (2007). L-asparaginase activity of marine actinobacteria was studied by Sahu et al (2007), suggesting that marine actinobacteria can be a new source for antitumour L-asparaginase.

Since isolation and identification of marine actinobacteria are tedious, and time-consuming, very limited reports pertaining to the L-asparaginase activity of marine Streptomyces are available to date.

1.4 OBJECTIVES

Significant progress has been made recently in discovering new marine actinobacteria with biopharmaceutical potential from marine sediments. Marine actinobacteria have shown to produce antitumour compounds. It is important to screen the marine actinobacteria for selecting antitumour compound producers. The selected potential strains can be further identified for the characterization of the compound. Pharmaceutical industry is being recognized as an important consumer for commercial therapeutic enzymes. Enzymes that are antitumour compounds are in great demand for use as therapeutic agents against cancer in modern oncology. L-asparaginase has proved to be a promising agent for the treatment of acute lymphoblastic leukemia (Keating et al 1993). The recent pharmacological reports have
shown that existing L-asparaginases are attributed to hypersensitivity and new L-asparaginase is imperative. This prompted the search for a new source of antitumour L-asparaginase with high purity and less adverse effects for cancer therapy. The study of L-asparaginase activity of marine actinobacteria has been reported in detail. Yet the purification and antitumour activity of marine actinobacteria L-asparaginase has not been reported so far. Therefore a systematic study on marine actinobacteria for new antitumour L-asparaginase was aimed and carried out. The following sections brief about the main objectives of the present investigation followed by organization of the thesis.

The main objectives of the present investigation are as follows:

1. Isolation of marine actinobacteria from the marine sediments of Parangipettai, East coast region of India.
2. Screening of the marine actinobacteria for L-asparaginase activity, and for the selection a high potential strain.
3. Identification of the potential strain by combination approach.
4. Purification of L-asparaginase from the potential strain.
5. Characterization and evaluation of antitumour activity of the enzyme.
6. Studies on fermentation kinetics of the enzyme production.

Based on the above objectives, this investigation was carried out and the results obtained are discussed in the following chapters.

1.5 ORGANIZATION OF THE THESIS

This dissertation focuses on the isolation of marine actinobacteria for the selection of an L-asparaginase potential strain, its authentic identification, purification of L-asparaginase, characterization and
fermentation kinetics of the enzyme production. A novel antitumour L-asparaginase from marine Streptomyces sp strain EPD 27 was investigated with a series of studies as organized under the following headings:

1. Identification of marine Streptomyces sp strain EPD 27.
2. Purification of L-asparaginase from strain EPD 27.
4. Fermentation kinetics of L-asparaginase production from strain EPD 27.

This dissertation is divided into five chapters including the introduction.

Chapter 1 introduces the subject and the objectives of the present investigation.

Chapter 2 describes the isolation of marine actinobacteria from the sediments of Parangipettai, East coast of India, and screening of the isolates for their L-asparaginase activity. It also touches upon the selection of a high L-asparaginase potential strain and its authentic identification.

Chapter 3 deals with the purification of L-asparaginase from the strain EPD 27. A modified procedure was developed to simplify the purification of L-asparaginase to the maximum with enzyme yield sufficient to evaluate the antitumour activity was described.

Chapter 4 explains the characterization and antitumour activity of the purified marine L-asparaginase on related cancer cell lines.

Chapter 5 outlines the effect of various culture condition on growth and yield of enzyme in batch fermentation.