CHAPTER 1

INTRODUCTION

1.1 ENZYMES

Enzymes are protein catalysts which catalyse lot of biochemical reactions both inside and outside the cell and life without enzymes are impossible. The distinguishable character of an enzyme is its ability to function in *invitro* systems with high specificity. Now a days, enzymes have become one of the important high value products due to their tremendous applications in various fields and have emerged as one of the major industry in the world. The major advantage of usage of enzymes in industry is due to their efficacy, specificity and eco-friendly nature.

Microbial enzymes are preferred over plant and animal enzymes due to their economic feasibility, high yields, consistency, ease of product modification, regular supply due to absence of seasonal fluctuations, rapid growth of microbes on inexpensive media, stability, and greater catalytic activity (Gurung et al. 2013). They are relatively more stable and provide greater diversity of catalytic activities than animal and plant source. Furthermore, the striking feature for replacement of animal and plant enzyme with microbial enzyme is that the possibility of enhanced enzyme yield by using genetically engineered microorganisms. The majority of enzymes currently used in industry are of microbial origin (Sabu et al. 2003; Sabu et al. 2005; Gurung et al. 2013). Enzymes are broadly classified as extracellular and intracellular enzymes. The intracellular enzymes are present within the cells
and catalyse the reactions within the cell. The extracellular enzymes are secreted outside the cell and are transported to different sites where the biochemical reactions are carried out. Often the extracellular enzymes are preferred over the intracellular enzymes due to the requirement of minimal downstream processing steps.

1.2 MICROBIAL THERAPEUTIC ENZYMES

Enzymes also have potential application as therapeutic agents. Therapeutic enzymes have a broad variety of specific applications such as oncolytics, thromolytics, anti-inflammatory agents etc (Table 1.1). Most of the therapeutic enzymes are also of microbial origin (Gurung et al. 2013).

Table 1.1 List of few important therapeutic enzymes of microbial origin

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Enzyme</th>
<th>Reaction</th>
<th>Source</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L-Glutaminase (Sabu et al. 2005)</td>
<td>L-Glutamine $\text{H}_2\text{O} \rightarrow$ L-glutamate + $\text{NH}_3$</td>
<td><em>Beauveria bassiana, Vibrio costicola, Zygossacharomyces rouxii</em></td>
<td>Antitumor</td>
</tr>
<tr>
<td>2</td>
<td>L-Asparginase (Sabu et al. 2005)</td>
<td>L-Asparagine $\text{H}_2\text{O} \rightarrow$ L-aspartate + $\text{NH}_3$</td>
<td><em>Pseudomonas acidovorans, Acinetrebacter sp.</em></td>
<td>Antitumor</td>
</tr>
<tr>
<td>3</td>
<td>β-Lactamase (Sabu et al. 2005)</td>
<td>β-Lactam ring hydrolysis</td>
<td><em>Citrobacter freundii, Serratia marcescens, Klebsiella pneumonia</em></td>
<td>Antibiotic resistance</td>
</tr>
<tr>
<td>4</td>
<td>Superoxide dismutase (Sabu et al. 2005)</td>
<td>$2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$</td>
<td><em>Mycobacterium sp., Nocadia sp.</em></td>
<td>Anti-oxidant, Anti-inflammatory</td>
</tr>
<tr>
<td>Sl. No</td>
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<tr>
<td>5</td>
<td>Ribonuclease (Gurung et al. 2013)</td>
<td>RNA hydrolysis</td>
<td><em>Yeast and Bacteriophages</em></td>
<td>Antiviral</td>
</tr>
<tr>
<td>6</td>
<td>Urokinase (Gurung et al. 2013)</td>
<td>Plasminogen-&gt; Plasmin</td>
<td><em>Bacillus subtilis</em></td>
<td>Blood clots</td>
</tr>
<tr>
<td>7</td>
<td>Uricase (Gurung et al. 2013)</td>
<td>Urate + O₂→ allantoin</td>
<td><em>Aspergillus flavus</em></td>
<td>Gout</td>
</tr>
<tr>
<td>8</td>
<td>Penicillin acylase (Gurung et al. 2013)</td>
<td>Binding the rings of (penicillin G) and (penicillin V)</td>
<td><em>Penicillium sp.</em></td>
<td>Penicillin / broad spectrum antibiotic production</td>
</tr>
</tbody>
</table>

### 1.3 AMIDASES IN THE TREATMENT OF LEUKEMIA

The potential application of therapeutic enzymes is in the treatment of cancer. Cancer, especially leukemia, is a malignant cancer of bone marrow and blood. It is characterized by the accumulation of abnormal blood cells which inhibits the function of normal blood cells and in some cases it may also lead to death. Among the pediatric cancer in developed countries, Acute Lymphoblastic Leukemia (ALL) constitutes the major part with affecting 30-45 per 1,000,000 children each year (Kwan et al. 2009; El-Ghonemy 2014). Several types of treatments such as chemotherapy, radiation therapy are available; enzyme therapy is also equally effective. Microbial L-asparaginase and L-glutaminase are amidases, which have earned attention due to their anti-tumor properties (Robert et al. 1970; Bauer et al. 1971; Abell & Uren...
Certain tumor cells require non-essential amino acids such as L-asparagine and L-glutamine in higher concentration than the normal cells for their rapid growth. The continued growth of the tumor cells could be checked by restricting the availability of such amino acids. Amidases including L-asparaginase and L-glutaminase were found to be promising in reducing the concentration of these amino acids by converting L-asparagine and L-glutamine into aspartate and glutamate respectively. The usage of amidases brings about rapid depletion of these amino acids in the blood supply and selectively inhibits the growth of tumor cells. Thus, application of amidases offers an attractive method to target tumor cells (Noura et al. 2014).

The Food and Drug Administration (FDA) and World Health Organization (WHO) have approved L-asparaginase for the effective treatment of (ALL) and lymphsarcoma (Egler et al. 2016). L-asparaginase, from the terrestrial bacteria such as *Escherichia coli*, *Erwinia carotovora* and *Erwinia chrysanthemi*, are currently used for the treatment of leukemia. ELSPAR, ONSPAR, ERWINASE and KIDROLASE are the brand names of L-asparaginase drug available for the treatment of ALL (Noura et al. 2014; El-Ghonemy 2014). But the clinical application of L-asparaginase is often limited by majorly three factors. First, the side effects associated with L-asparaginase administration, including immunosuppression and pancreatitis (Wang et al. 2003). Second, about 10% of successfully treated patients suffer a relapse with the appearance of tumors that are resistant to further L-asparaginase therapy (Woo et al. 2000). Lastly, prolonged treatment with L-asparaginase improves the growth of resistant tumors and increases the metastatic activity (Asselin et al. 1999). Hence, there is a need for alternative enzyme drug that is more compatible to human blood and include less or no side effects in
patients. A parallel interest in L-glutaminase has arisen from the demonstration that microbial L-glutaminase also exhibits anti-tumor activity against ALL. A glutaminase-asparginase enzyme from *Achromobacter* has shown anti-leukemic effect in patients with ALL in a preliminary clinical study (Spiers et al. 1979).

1.4 **L-GLUTAMINASE**

L-glutaminase (L-glutamine amidohydrolase E.C. 3.5.1.2) catalyses the hydrolysis of L-glutamine to glutamic acid and ammonia (Nandakumar et al. 2003; Iyer & Singhal 2009). L-glutaminase has attained much importance due to their potential application as a therapeutic enzyme.

Cancer cells, especially ALL cells cannot synthesis L-glutamine, as they lack properly functioning glutamine biosynthetic machinery and therefore require large amount of L-glutamine for their rapid growth. These cells depend on the exogenous supply of L-glutamine for their survival and rapid cell division. Hence, the use of L-glutaminase deprives the tumor cells from L-glutamine and causes selective death of L-glutamine dependant tumor cells. Thus, it can act as a possible candidate for enzyme therapy (Tanaka et al. 1988; Nandhakumar et al. 2003; Hensley et al. 2013; El-Ghonemy 2014). In recent years, L-glutaminase in combination with or as an alternative to L-aspararginase could be used as in enzyme therapy for cancer particularly leukemia (Sabu et al. 2003).

1.4.1 **L-GLUTAMINASE from Marine Bacteria**

The marine biosphere is the richest habitat of microorganism especially bacteria. The organic and inorganic wastes expelled by human and industries are dumped into oceans. Biodegradation of these complex materials are eventually brought about by the microorganisms through the action of
their enzymes. Thus, the marine microorganisms serve as potent source of several enzymes with novel and improved properties (Kumar & Chandrasekaran 2001).

The marine environment, particularly sea water, which is saline in nature and chemically closer to human blood plasma, could provide microbial enzymes that are safer with no or less side effects when administered for human therapeutic application. The major restriction in the application of terrestrial L-asparginase in the treatment of ALL is the development of allergic reactions in the patients and ultimately leading to inactivation of the drug (Woo et al. 2000). This could be effectively suppressed by the application of L-glutaminase from marine source (Sabu et al. 2000). L-glutaminase from marine bacteria have been reported to have very high affinity for substrate and pH optima in the range 7.4, which is an indication of better therapeutic regime over the other terrestrial bacterial glutaminase whose pH optima ranges from pH 5-7. Therefore, L-glutaminase produced from marine bacteria may hold more potential for treating ALL rather than L-asparaginase from terrestrial bacteria.

1.4.2 Production of L-Glutaminase

In view of the potential application of L-glutaminase in the treatment of leukemia, there exists a pressing need to develop viable bioprocess technologies for the commercial production of the enzyme. Their commercial production using marine bacteria could make possible its wide application in cancer chemotherapy. Conventionally, large scale production of microbial enzymes are carried out by submerged fermentation (SmF) which favours low contamination problem, low cost of production and better process control. Inspite of all these advantages of SmF, there is an increasing interest in solid state fermentation (SSF) for large scale production of microbial enzymes. SSF is described as the fermentation process which occurs in the
absence or near absence of free water by employing a natural substrate or inert substrate as solid support (Bhargav et al. 2008). SSF offers certain advantages over SmF like high productivity, easy recovery and lower capital and recurring costs due to the feasibility of using various agro-industrial residues such as wheat bran, rice bran, oil cakes etc.

The success of SSF largely depends on the type of substrate and the microorganism used. The marine bacteria apart from producing extracellular enzymes, they also possess the ability to colonize on barren surfaces. The unique property of marine bacteria to adsorb onto solid particles and their ability to survive in extreme conditions makes them ideal for their use in SSF (Chandrasekaran 1996). Agro-industrial residues such as wheat bran, rice bran, maize bran, wheat straw, rice straw, rice husk etc are generally regarded as the best substrate for SSF due to their low cost and easy availability (Pandey et al. 1999). Wheat bran however holds the key, and most commonly been used, in various processes.

Keeping in view the anti-cancer potential of L-glutaminase, and since only very few reports are available on the production of L-glutaminase from marine environment, the primary objective of the present study was to isolate a potent L-glutaminase producing marine bacterial strain and to maximize the yield of the enzyme.

The following section brief about the objective of the present study and the organization of the thesis.

1.5 OBJECTIVE OF THE PRESENT STUDY

A great demand for commercial therapeutic enzymes always exists in pharmaceutical industries. Enzymes especially that are anti-tumor in nature are preferably used against cancer in modern oncology. L-glutaminase has
proved to be a promising agent for the treatment of ALL (Tanaka et al. 1988). From the reports, it is evident that L-asparaginase, especially from the terrestrial bacterial source, which are currently used for the treatment of leukemia, are attributed to hypersensitivity and thus need for an alternative enzyme is imperative. This prompted the search for new source of L-glutaminase enzyme. Therefore, a research was carried out with the objective of selective isolation, molecular identification of potentially useful L-glutaminase producing bacterial strain and to maximize the L-glutaminase yields under SmF and SSF. This was followed by purification and characterisation of L-glutaminase and determination of its anti-oxidant and anti-tumor potential.

The main objectives of the present study are as follows

- Selective isolation of L-glutaminase producing bacterial isolate from the marine sediments collected from the different sea shores of South India.
- Biochemical and molecular identification of the potent bacterial isolate.
- Production of L-glutaminase under submerged fermentation (SmF).
- Increase the yield of L-glutaminase under SmF with the help of statistical methods.
- Production of L-glutaminase under solid state fermentation (SSF).
- Maximizing the L-glutaminase production under SSF using statistical methods.
• Purification and characterisation of the produced L-glutaminase enzyme.

• Study on the anti-oxidant and anti-tumor potential of L-glutaminase.

This thesis is divided into six chapters.

Chapter 1 describes the introduction and main objectives of the present study.

Chapter 2 outlines the literature review of L-glutaminase.

Chapter 3 focuses on the selective isolation of the L-glutaminase producing potential bacterial strain from the sediments collected from the different sea shores of South India. It also discusses about the biochemical and molecular identification of the potential bacterial strain.

Chapter 4 deals with the analysis of production pattern of the enzyme by the potent isolate under SmF. The L-glutaminase production was maximized using statistical techniques such as Plackett and Burman Design (PBD) method and Response Surface Methodology (RSM).

Chapter 5 describes the production pattern of L-glutaminase under SSF. Statistical techniques were employed to maximize the enzyme production.

Chapter 6 outlines the various techniques adopted for the purification of the enzyme followed by characterization and the evaluation of the anti-oxidant and anti-tumor potential of L-glutaminase.

Chapter 7 discusses the summary and conclusion of the entire study.