CHAPTER- I
INTRODUCTION
1.1. Rationale of the research

'Therapeutic enzyme' is the term that has been known for almost 40 years. These have wide applications in the form of anticoagulants or thrombolytics, oncolytic agents and are also used as replacements for many of the metabolic deficiencies. These are required in very small amounts, but should have properties such as high purity and specificity. Some of the factors such as the size of the protein, properties of immunogenicity or eliciting immune response, half-life, etc. can influence the potentiality of these enzymes as therapeutic agents. Numerous enzymes have been in usage for the treatment of a wide variety of diseases from long time, in spite of their pharmacology being recognized only in the last few decades particularly in cancer therapy. Recent biotechnological advancements from past 10 years have enabled to produce cheaper, safer enzymes having high potency and specificity with a lower production cost [Michel Vellard, 2003]. Oncolytic enzymes are one such kind and are mainly categorized into two groups where one degrade small molecules which are required for tumor cells and the others are the ones which degrade macromolecules such as nucleic acids and membrane proteins. These enzymes have potential applications in the treatment of cancer and also play a very significant role in the diagnosis, curing and monitoring of many dreadful diseases. Enzymatic drugs differ from all other types of drugs in two key features namely [i] binding to targets with more specificity and affinity and [ii] they are catalytically active which can convert multiple target molecules into products. The above characters are reasons for developing them as potent drugs for treating a wide variety of disorders. Also, it is very important to fully understand the properties of enzyme and catalytic activities, in order to optimize its use and limit potential side effects [Kumar et al., 2009].
Cancer is defined as a disturbance of growth characterized primarily by excess proliferation of cells without apparent relation to the physiological demands of the organism involved. The disease still remains unconquered inspite of the fact that a very large number of studies are being carried out all over the world. Within the area of cancer treatment one exploits the knowledge of neither differences between normal and malignant cells, i.e. malignant cells lacking certain functions [Hyakuna et al., 2004].

Cancer drug therapy is undergoing a major transition from the pre-genomic era to the post genomic era. New technologies, particularly high throughput screening, combinational chemotherapy, gene expression micro array and computer aided drug designing are increasing the speed and efficiency of drug development [Michel Vellard, 2003]. Improved systemic drug therapy is particularly important for the treatment of leukaemia, where surgery and radiation can no longer be curative. The development of microbial enzymes for cancer therapy, has added to the choice of anti-leukaemic drugs which promises huge prospects for better treatment. The use of microbial enzymes in leukaemia therapy makes it important that the enzyme should be produced in large quantities and are usually required to be pure in their forms; therefore, their production costs are very high. If such a difficulty is circumvented, the potential use of enzymes in clinical medicine, particularly in general and cancer chemotherapy would be extended [Huisman et al., 2002].

Leukaemia is a form of cancer that is caused by alterations in proto-oncogenes or tumour suppressor genes or may be due to differences in microRNA genes related to hematopoietic stem cells or their related progenitors. These basic changes lead to alterations in the vital processes such as blocking the capacity of self-renewal of cells, cell differentiation and promote resistance to apoptosis [Pui et al., 2004; Frohling et al., 2008].
Leukaemia is a cancer of blood or bone marrow which is characterized by abnormal proliferation of blood forming tissues. It is divided into many large groups and major divisions include acute and chronic forms where acute leukaemia is most common forms of leukaemia and involves a rapid increase in the level of immature blood cells. Undifferentiated, immature state of blasts that are circulating which leads to rapid progression of disease and becomes fatal when untreated is commonly referred to as ‘acute’. Chronic leukaemia refers to a condition where the production of white blood cells increases abnormally. Chronic leukaemia differs from acute leukaemia and is divided into two types, namely myelogenous and lymphocytic. Another classification of leukaemia are also seen of which acute lymphoblastic leukaemia predominates in children, whereas acute myelogenous leukaemia is seen more in adults.

1.2 Acute Lymphoblastic Leukaemia (ALL)

Acute lymphoblastic leukaemia [ALL] is one of the forms of leukaemia, where malignant, immature white blood cells are multiplied and excessively produced in the bone marrow. It results in overcrowding of normal cells which also spreads to other organs such as liver, spleen and lymph nodes. The disease can be classified as low, average and high-risk forms and is mostly seen in between 2-4 years of age. The survival rate for childhood ALL was higher than 80% and about 50% cure in adults [Burger et al., 2003; Pui et al., 2004; Silverman et al., 2010], and has greatly improved from the last few decades [Smith et al., 1996; Weiss et al., 2006]. Due to the availability of chemotherapy and most advanced treatment protocols, potential cure rate of 60-70% for acute lymphoblastic leukaemia in children was achieved. The rationale of this therapy is to reduce the number of neoplastic cells that helps for prolonged survival by maintaining adequate quality of life [Michel Duval, 2002]. Improvements of more than 90% were
seen in childhood cancer, acute lymphoblastic leukaemia (ALL) due to the ongoing research on the enzymatic drug L-Asparaginase.

1.3 Origin of the Disease ALL

Precise pathogenic events that cause ALL are still unknown, but in a few cases, the disease is inherited by disorders such as Ataxia telangiectasia, Bloom’s syndrome, Down’s syndrome, and Nijmegen breakage syndrome, or by exposing to some ionizing radiation or chemotherapeutic drugs [Hjalgrim et al., 2003]. Some of the reasons such as parental occupation, maternal reproductive history, parental tobacco or alcohol habits, prenatal vitamin use, maternal diet, exposure to solvents and pesticides, and power-frequency magnetic fields also lead to this disease [Ahlbom et al., 2000]. Some of the viruses are also the reasons for causing ALL that includes Epstein–Barr virus [EBV] and human immunodeficiency virus [HIV] [Lombardi et al., 1987; Li et al., 1993]. More cases of leukaemia have been seen after nuclear exposure or exposure to therapeutic radiotherapy [Brown et al., 1965]. ALL is sometimes found to be more because of higher socioeconomic status, that relates to better hygiene, less social contact in early infancy, and thus to a different exposure to infectious agents [Greaves et al., 1993]. A DNA virus [EBV] that causes infectious mononucleosis is also associated indirectly in causing leukaemia [Lombardi et al., 1987]. Recent studies clearly say that there is a link between onsets of seasons and is in turn linked with many infectious etiologies [Meltzer et al., 1989; Timonen et al., 1999].

1.4 Diagnosis of ALL

ALL can be diagnosed by physical examination of blood smears, immunotyping and cytogenetic studies. Disease aggressiveness can be known by DNA testing and invasions of the internal organs are verified by CT scanning and ultrasound.
1.5 Treatment with L-Asparaginase

Treatment mainly includes steroids, chemotherapy or radiation therapy. Chemotherapy is given in the initial treatment and it ends up in combination therapy [Bone marrow or stem cell transplantations]. The improvement rate of ALL in children during the early 60s due to combination chemotherapy was 4% and it has increased drastically to 80% in 1990s [Chen et al., 2004]. No surgical options can be done due to the wide distribution of the malignant cells throughout the body. Antileukemic effect of steroids potentiates L-Asparaginase activity and improves the outcome [Abshire et al., 2000; Moghrabi et al., 2007]. Thus, optimal use of L-Asparaginase is very important in the early therapeutic response and overall survival rate in children [Asselin et al., 1999].

Mainly three drugs are being used during the therapy viz. vincristine, steroidal drugs [Yang et al., 2008] and L-Asparaginase. Most of the conventional drugs used to treat acute leukaemia either directly target DNA or inhibit nucleic acid synthesis, some block protein synthesis by hydrolyzing an amino acid essential for leukemic cell growth or by interfering with the mitotic spindle apparatus. As these drugs are nonspecific, they have a narrow therapeutic index and sometimes produce adverse cytotoxic effects in various normal tissues. Meningeal Leukaemia can also be cured with L-Asparaginase [Oettgen et al., 1970].

L-Asparaginase [mono methoxy polyethylene glycol succinimidyl L-Asparaginase] is not employed as a single agent in the treatment protocols of Acute Lymphoblastic Leukaemia and lymphosarcoma. It is given in combination with drugs having definitive immunosuppressive effects. Strapinni et al [1984] reported that there is an enhancement in the polymerization rate of fibrin monomers or fibrin clot ability due to the treatment with L-Asparaginase. In treatment protocols, when L-Asparaginase in combination with methotrexate was given, synergistic anti-leukemic activity in a schedule
dependent fashion was observed. Also, due to the L-Asparaginase, apoptosis of leukaemic cells were also observed. It was reported that the enzyme reduces the intensity of pain in children without changes in the absorption rates of children [Ueno et al., 1997]. The enzyme was also found to be effective in patients with nasal type leukaemia and also attained success in patients after they have undergone bone marrow transplantation [Hyakuna et al., 2004; Yang et al., 2008].

Also, it was found that there is a reduction in levels of asparagine, glutamine and some other amino acids due to treatment with L-Asparaginase. L-Asparagine levels have been found to be strongly correlated with plasma L-Asparaginase activity, even at low enzyme activities of <50 U/L [Tsurusawa et al., 2004]. Correlation between the presence of anti-L-Asparaginase antibodies and L-Asparaginase activity has been observed by Zalewska, Beata and Bodalski. Immunologic cross-reaction between antibodies against various formulations of native Escherichia coli L-Asparaginase and PEG L-Asparaginase has been reported, but no such reaction has been found against Erwinia L-Asparaginase. The results of the Dana-Farber Cancer Institute ALL Consortium Protocol 95-01 for children with acute lymphoblastic leukaemia have shown that Erwinia is less toxic than Escherichia coli L-Asparaginase, but also less efficacious [Moghrabi et al., 2007]. Steiner has reported an undulating course of ammonia concentrations during L-Asparaginase containing induction treatment. It was concluded that ammonia levels may represent a suitable surrogate parameter of L-Asparaginase enzyme activity and may enable the monitoring of silent inactivation of L-Asparaginase due to the characteristic fluctuation profile.

The process of chemotherapy mainly consists of three different phases which include remission–induction phase, intensification or consolidation phase and continuation phase. Major drugs like azacytidine, decitabine, lonafarnib, temozolomide,
prednisone, dexamethasone, daunorubicin, doxorubicin, anthracyclines, idarubicin, flavopiridol, glucocorticoids, vincristine, tipifarnib are used in the treatment protocols along with L-Asparaginase. More than 99% tumour cells are destroyed and normal functions of the cells are not affected in the remission induction phase of chemotherapy. Drugs such as L-Asparaginase, vincristine and glucocorticoids are used in this phase and more than four drugs are used in combination in cases of high risk conditions. This phase is generally used to reduce the effects of toxicity [Pui et al., 2006]. In remission-induction phase, small increase in concentrations of L-Asparaginase leads to toxic effects leading to death [Avramis et al., 2002; Douer et al., 2007; Wetzler et al., 2007]. In intermediate risk ALL conditions, double re-induction is enhanced where vincristine and prednisone did not prove to be beneficial, but improvements were observed due to increased concentrations of L-Asparaginase. Drugs such as anthracyclins, cyclophosphamide, cytarabine and methotrexate showed improvement in post remission treatments [Avramis et al., 2002; Kantarjian et al., 2004]. Long-term continuation treatment is used in children with high risk ALL to prevent or forestall relapse which has also found to enhance the survival rate in intensification and consolidation phases [Clavell et al., 1980; Grahm et al., 1998; Muller et al., 1998; Amylon et al., 1999]. Number of clinical trials were carried out with respect to the drug L-Asparaginase, and the beneficial impact of it during the induction phase of randomized trials was first demonstrated by Jones and colleagues [Jones et al., 1977]. Sallan and colleagues (1993) carried out further studies and explained about the importance of Escherichia coli L-Asparaginase. Anti-leukemic effect of steroids potentiates L-Asparaginase activity which improves the outcome. Thus, optimal use of enzymatic drug L-Asparaginase is very important in the early therapy for overall survival of children with ALL [Asselin et al., 1999; Moghrabi et al., 2007].
1.6 Enzyme market

Efficacy studies were performed for L-Asparaginase sourced from guinea pig serum, but production of huge quantities of the enzyme was difficult to obtain. Even though enzyme L-Asparaginase was found to be present in wide sources like plants and animals, microorganisms are selected due to easy extraction procedures. Microbes were proved to be more efficient and cheaper sources to obtain the enzyme. Microorganisms such as algae, fungi, bacteria, actinomycetes and yeast are very efficient producers of the enzyme but they differ in enzymatic properties. Among all the sources of enzyme, *Escherichia coli* and *Erwinia chrysanthemi* were found to be used for large scale production as L-Asparaginase from these two sources have been reported to have low toxicity [Duval et al., 2002]. Clinically, today L-Asparaginase is available under various trade names like Elspar obtained from *Escherichia coli* [marketed commercially by Merck and Co.], Oncaspar [Pegasparagase, modified version], Crisantaspase, Kidrolase and Erwinase. Crisantaspase is obtained from *Erwinia chrysanthemi* [available as *Erwinia* L-Asparaginase from Ogden BioServices Pharmaceutical Repository in the United States] and is used mostly in combination with other anti-cancer drugs or in patients allergic to *E. coli* preparations. Erwinase is also used as a part of therapy along with chemotherapy or radiotherapy. Along with these, other form of enzyme is PEG–L-Asparaginase in which native *Escherichia coli* L-Asparaginase has been covalently conjugated to monomethoxypolyethylene glycol [PEG]. Peg-L-Asparaginase [available commercially from Rhone-Poulenc Rorer as Oncaspar] is approved by the Food and Drug Administration for use in combination chemotherapy for the treatment of patients with Acute Lymphoblastic Leukaemia who are hypersensitive to native [unmodified] forms of *Escherichia coli* L-Asparaginase [Silverman et al, 2010] and also it has the advantages of good plasma clearance property which helps in avoiding frequent medication [Ettinger et
al., 1993; Roberts et al., 2002] enhanced plasma half-life, less toxicity, increased drug toxicity, increased drug stability and solubility [Schellekens et al., 2002]. *Escherichia coli* were found to be a source for isolating different isozymes of L-Asparaginase [Irion et al., 1979].

By considering the above facts in view, the present work is aimed to obtain an enzymatic drug L-Asparaginase for better treatment of acute lymphoblastic leukaemia. The present work is therefore taken with the following objectives.

1. *In silico* characterization of L-Asparaginase for understanding the conservation of amino acids, domains and motifs in the perspective of glutaminase side activity.
2. *In silico* engineering of L-Asparaginase to have reduced glutaminase activity
3. Molecular dynamics studies for analyzing the stability of modeled enzyme
4. Optimization of fermentation conditions to produce glutaminase free L-Asparaginase from *Pectobacterium carotovorum*
5. Cloning and heterologous production of L-Asparaginase in *E.coli* BL21 [DE3].
6. Site directed mutagenesis and analysis of L-Asparaginase variant for its reduced glutaminase activity