1.0 INTRODUCTION

L-asparaginase (EC 3.5.1.1) is an anti neoplastic agent, generally used for treatment of a type of cancer i.e. Acute Lymphoblastic Leukemia (ALL) and non Hodgkin’s Lymphoma (NHL), which are prevalent in children aged up to 10 years and some adults. Anti neoplastic activity of this enzyme was initially suggested by Broom (1961) and proved by Mashburn and Wriston (1964). It was found that guinea pig serum suppressed the growth of lymphosarcomas in mice. It’s presence in guinea pig serum was first reported by Broome (1961). L-asparaginase was later shown to be the active factor. L-asparaginase is an enzyme that destroys L-asparagine external to the cell. Normal cells are able to make all the asparagine they need internally whereas tumor cells become depleted rapidly and die. Cancer cells differentiate themselves from normal cells in diminished expression of L-asparagine. (Mannan et al., 1995 and Swain et al., 1993). Due to lack of asparagine, these cells depend on uptake of asparagine from surrounding cells and tissues. Many investigators have reported that L-asparaginase inhibits tumor growth in the mouse, rat, dog, and human, through the inhibition or deletion of tumor-specific cells. Its chemotherapeutic potential in treating acute lymphoblastic leukemia and lymphosarcoma has been one of the most eminent discoveries of modern times (Broome et al., 1961, and Mashburn et al., 1964). Cells in ALL patients are unable to synthesize L-asparagine due to deficiency or absence of asparagine synthetase enzyme (Keating et al.1993). Hence, they are not capable of producing L-asparagine and mainly depend on the L-asparagine from circulating plasma pools (Swain et al., 1993). In this condition, when the L-asparaginase is injected intravenously, decreases the blood concentration of L-asparagine by hydrolysis of L-asparagine into L-aspartate and ammonia (Wristen et al., 1973 and Capizzi et al., 1984). Inability of ALL cells to uptake L-asparagine
results in inhibition of protein synthesis and their ultimate death. Clinical trials indicate that this enzyme is also a promising agent in treating some forms of neoplastic cell disease in man (Oettgen et al., 1967). It catalyses the conversion of L-asparagine to L-aspartate and ammonia, and this catalytic reaction is essentially irreversible under physiological conditions (Prakasham et al., 2007). This clinical action of this enzyme is attributed to the reduction of L-asparagine, since tumour cells are unable to synthesize this amino acids are selectively killed by L-asparagine deprivation (Prista et al., 2001).

L-asparaginase have been isolated, purified and experimentally used as an anticancer agent in human patients (Clavell et al., 1986 and Story et al., 1993). This enzyme is routinely screened in laboratory using Nessler’s reagent (Imada et al., 1973). Although this enzyme is produced by various microorganisms including prokaryotes and eukaryotes, bacterial L-asparaginase can cause hypersensitivity in the long term use due to allergic reactions and anaphylaxis (Reynolds et al., 1993). Several scientific groups have studied L-asparaginase production and purification in attempt to minimize impurities that produce allergic reactions (Campbell et al., 1967, Boss et al., 1997 and Gallagher et al., 1999). It has been observed that eukaryotic microorganisms like yeast and filamentous fungi have a potential for L-asparaginase production (Wade et al., 1971, Wiame et al., 1985 and Pinheiro et al., 2001). Some researchers also purified L-asparaginase from plants (Bano et al., 1980). In the view of toxic effect of bacterial L-asparaginase, the present study was designed for screening novel L-asparaginase producing microorganisms viz. bacteria, actinomycetes and fungi. This source may provide better L-asparaginase producing microbial strains with lesser toxicity.
1.1 Enzyme reaction

L-asparaginase belongs to an amidase group that produces aspartic acid and ammonia by asparagine hydrolysis.

\[
\begin{align*}
\text{L-asparagine} & \quad \text{L-asparatate} \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{H}_2\text{N} & \quad \text{NH}_3^+ \\
\text{O} & \quad \text{O} \\
\text{NH}_3^+ & \quad \text{Ammonia}
\end{align*}
\]

1.2 L-asparaginase as an antitumor drug

Many enzymes have been used as drugs like wise L-asparaginase attracted much attention because of its use as effective therapeutic agent against lymphocytic leukemia and other kinds of cancer in man (Ekert et al., 1982, Ali et al., 1989 and Krasotkina et al., 2004). L-asparaginase in the treatment of leukemia and other lymphoproliferative disorders has expanded immensely. For these reasons L-asparaginase has established itself to be an indispensable component.

L-asparaginase is the first enzyme with antitumor activity to be intensively studied in human beings. It is an enzyme drug of choice for acute lymphoblastic leukemia in children used in combination therapy (Schemer et al., 1981). The discovery of L-asparaginase, a medicinal agent for the treatment of malignant tumors, was made in 1922. Clementi (1922) showed that guinea pig serum contained a high activity of L-asparaginase, whereas other mammals were found to be devoid of this enzyme. A component exclusively present in guinea pig serum was proposed to be responsible for tumoricidal effects (Kidd et al., 1953). The growth of cell line derived from Walker carcinosarcoma was shown to be dependent on L-asparagine (Neuman et al., 1956). Similar results were obtained with mouse leukemia cell line. It was further shown that growth of normal cells was not dependent on L-asparagine, making L-
asparaginase a potential tumour specific drug. L-asparaginase purified from guinea pig serum was a tumourcidal agent (Broome et al., 1961) against implanted 6C3HED cells (serum from newborn guinea pigs lacking L-asparaginase was devoid of antitumour activity).

L-asparaginase for acute lymphoblastic leukemia was a major breakthrough in modern oncology as it induced complete remissions in over 90% children within four weeks as reported by Gallagher et al., 1999.

1.3 Sources of L-asparaginase

L-asparaginases are distributed throughout the animal, plant and microbial kingdoms. Considerable research has been undertaken for the production of L-asparaginase (both extracellular and intracellular) by variety of microorganisms. Among all these system, L-asparaginase derived from bacterial and fungal sources have dominant application in pharmaceutical sector. Attempts have been made to specify the cultural conditions and selection of superior strains of the bacteria for large scale production.

The advantages of using microorganisms for the production of L-asparaginase include:

- Bulk production capacity
- Economical
- Microbes are easy to manipulate to obtain enzymes with desired characteristics.

L-asparaginase production using different bacterial sources have been reported by various scientists. The major bacterial species that produce this enzyme include Escherichia coli (Khushoo et al., 2005) Erwinia cartovora (Aghaiypour et al., 2001), Serratia marcescens, Pseudomonas acidovoras, Pseudomonas aeruginosa
(Bessoumy et al., 2004), *Erwinia chrysanthemi* (Kotzia and La brou, 2007), *Enterobacter aerogenes* (Mukherjee et al., 2000), *Candida utilis* (Kil et al., 1995), *Thermus thermophilus* (Prista and Kyriakidis, 2001). Certain L-asparaginase producing fungal species have also been isolated and studied viz. *Aspergillus tamari, A. terrus, A. penicillium, Hypomyces solani, Nectria haematococca* (Sarquis et al., 2004), and *Fusarium* species like *Fusarium roseum, F. saloni*. L-asparaginase production is also reported by a yeast species *Saccharomyces cerevisae* (Sarquis et al., 2004) and an algal species named *Chlamydomonas microalgae* (Paul, 1982).

L-asparaginase has used as processing aid in food industry to reduce the formation of acrylamide during frying of in starchy foods. The demand for this enzyme will increase several fold in coming years due to its potential application in food processing in addition to its clinical applications (Wei et al., 1998 and Lingappa et al., 2005).

A wide range of microorganisms such as bacteria, fungi, yeast, actinomycetes, algae, animals and plants have proven to be proficient sources of this enzyme. It has been reported that eukaryotic microorganisms like yeast and fungi also have the potential for L-asparaginase production. (Wade et al., 1971 and Pinheiro et al., 2001). For example, the mitosporic fungi genera such as *Aspergillus, Penicillium, and Fusarium*, have been commonly reported in scientific literature to produce asparaginase (De-Angeli et al., 1979 and Imada et al., 1973).

1.4 Treatment with L-asparaginase

Current treatment protocols of ALL and lymhosarcoma do not employ L-asparaginase as a single agent. In fact, it is always a part of multiple agent regimens and combined with drugs having definitive immunosuppressive effects.
Comparison of L-asparaginase isolated from Erwinia *caratovora* and *E.coli* has been made (Cavana *et al.*, 1976). Enhanced polymerization rate of fibrin monomers or fibrin clottability by L-asparaginase treatment has been reported by Strappinni *et al.*, (1984). The binding of *E.coli* L-asparaginase to the plasma membrane of normal human mononuclear cells was demonstrated by Mercado *et al.*, (1999). L-asparaginase in combination with methotrexate has shown synergic anti-leukemic activity in a schedule dependent fashion (Aguayo *et al.*, 1999).

In humans, acute lymphoblastic leukemia cell lines have been markedly inhibited by asparaginases. Cell cycle arrest in G1 phases, which results in apoptosis of leukemia cells, is induced by L-asparaginase (Ueno *et a.*, 1997). Kelo *et al.*, (2002) have reported L-asparaginase action on peptides and their effects on metabolism in the human body. L-asparaginase has been found to be effective in nasal type leukemia treatment as well (Yong *et al.*, 2003). Hyakuna *et al.*, (2004) have reported successful asparaginase treatment followed by bone marrow transplantation in leukemia patients. Deamination of glutamine may enhance the anti-leukemic effect of L-asparaginase (Panosyan *et al.*, 2004). Correlation between the presence of anti-asparaginase antibodies and L-asparaginase activity has been observed by Zalewska *et al.*, (2004). Immunologic cross-reaction between antibodies against various formulations of native *E.coli* L-asparaginase and PEG L-asparaginase has been reported but no such reaction has been found against *Erwinia* L-asparaginase (Avramis and Panosyan, 2005). A result of the Dana-Farber Cancer Institute ALL Consortium Protocol 95-01 for children with acute lymphoblastic leukemia has shown that *Erwinia* is less toxic than *E.coli* asparaginase, but also less efficacious (Moghrabi *et al.*, 2006).
1.5 Side Effects of administration of bacterial L-asparaginase

Bacterial L-asparaginase could cause an allergic reaction like skin rash, difficulty in breathing, decreased blood pressure, sweating or loss of consciousness. It may interfere with blood clotting, raise blood sugar levels, raise liver enzyme blood tests, and cause liver disease in some patients. The other common side effect of this medication is vomiting (Ali et al., 1994). Sarquis et al., 2004 in their studies report that L-asparaginase produced by fungal strain was not toxic and appeared to have myelosuppressive and immunosuppressive activity. The various fungi viz. Aspergillus nidulans (Shaffer et al., 1988) Aspergillus, penicillum and Fusarium spp (Sarquis et al., 2004), Aspergillus niger (Mishra et al., 2006), A. terreus (Baskar et al., 2011) and Bipolaris spp.(Lapmak et al., 2010) were reported to produce L-asparaginase.

Besides minor side effects such as an allergic reaction and vomiting, L-asparaginase therapy of ALL has some serious side effects. Onset of venous thrombosis in children undergoing histopathologic disease due to ALL therapy has been reported by Sahoo and Hart (2003). L-asparaginase associated hyperlipidemia with hyperviscosity syndrome in a patient with T-cell lymphoblast lymphoma has been reported by Meyer et al. (2003). Neutropenic entercolitis (NE) has been observed as an unusual acute complication of neutropenia, associated with leukemia and lymphoma (Radulovic et al., 2004). Hypersensitivity reactions to chemotherapeutic actineoplastic agents such as L-asparaginase (Rossi et al., 2004) have been reported. Ikarashi et al., (2004) reported tubular and glomerular dysfunction due to ALL chemotherapy. Chen et al., (2004) observed that urethral obstruction was due to L-asparaginase induced pancreatitis during treatment of ALL. Myocardial ischemia has been observed in a patient with acute lymphoblastic leukaemia (Saviola et al., 2004) due to L-asparaginase therapy.
An outburst of acute pancreatitis, called “drug induced pancreatitis” (DIP), has been reported by Trivedi and Pitchumoni (2005). A cerebral thrombotic complication in adolescent leukemia patients (Imamura et al., 2005) has been attributed to L-asparaginase treatment. Acute hepatic dysfunction (Aoki et al., 2005) and immunodeficiency in children with ALL (Brodtman et al., 2005) have been other major side effects. Ocular complications arise due to L-asparaginase treatment (Foroozan, 2005), but symptoms ease out by discontinuing treatment and carrying on treatment of heparine. Growth hormone deficiency in children receiving chemotherapy for acute lymphoblastic leukemia has been reported by Haddy et al., (2006). Growth impairment after cranial radiation (CR) can result in diminished adult height. A study on the consequences of L-asparaginase on antithrombin levels in plasma from acute lymphoblastic leukemia patients, HepG2 cells, and plasma and livers from mice treated with this drug has been carried out by Hernandez-Espinosa et al., (2006). They reported that L-asparaginase treatment induced severe, acquired, and transient type I deficiency of antithrombin (and 1-antitrypsin) with intracellular accumulation of the nascent molecule, increasing the risk of thrombosis.

1.6 Resistance to L-asparaginase

A cell line resistant to L-asparaginase expressed high levels of asparagine synthetase activity as reported by Andrulis et al., (1990). This was due to increased expression but without amplification of the genes encoding asparagine synthetase. Holleman et al., (2003) reported that drug resistance in ALL is associated with impaired ability of cells to induce apoptosis. Also, PARP and Procaspace-2 expression is related to drug resistance in childhood ALL. Resistance to L-asparaginase results in a decreased ability of tumor cells to undergo apoptosis (Savitsky et al., 2003). The differential
expression of genes has been studied in drug sensitive and drug resistant ALL and it is associated with drug resistance and response to treatment (Holleman et al., 2004). Resistance to L-asparaginase has been associated with the over expression of asparagine synthetase (AS). ALL patients have been found resistant to chemotherapeutic agents such as antracyclines, vincristine and asparagine. An in vitro chemosensitivity assay is a good indicator of cellular response to chemotherapy (Arrifin et al., 2005). In rat serum cells and in ARJ cells, L-asparaginase treatment depletes cellular asparagine; also, cellular glutamine levels have been found to be severely reduced along with a marked decrease in the activity of glutamine synthetase (GS). The inhibition of GS in the presence of L-asparaginase triggers apoptosis (Rotoli et al., 2005). GS may thus form a target for the suppression of a L-asparaginase resistant phenotype. Krejci et al., (2005) reported that up regulation of asparagine synthetase does not avert cell-cycle arrest induced by L-asparaginase in leukemic cells. The 45 genes that are differentially expressed in ALL and exhibiting cross-resistance to prednisolene, vincristine, asparaginase and duanorubicity have been identified (Lugthart et al., 2005). Targeting particular genes involved in response to amino acid starvation in ALL cells may provide a novel way to overcome L-asparaginase resistance (Fine et al., 2005). Epigenic changes (changes involved in the expression of genes) in the repression and induction of asparagine synthesis in human leukemic cell lines have been studied may be targeted for studying L-asparaginase resistance (Ding and Broome, 2005). Fine et al., (2005) also described that L-asparaginase resistance can be studied by targeting epigenetic changes. Li et al. (2006) have reported that the down regulation of asparagine synthetase expression can increase the sensitivity of cells resistant to L-asparaginase.
1.7 L-asparaginase – A Subject of considerable Medical Interest and Persistent Research

L-asparaginase has been a major research subject for many researchers worldwide. Its chemotherapeutic potential in treating acute lymphoblastic leukemia and lymphosarcoma has been one of the most eminent discoveries of modern times. Its therapeutic potential is now well established, as it has remarkably induced remission in most of the patients suffering with ALL. A comparative examination of preparations of *E. coli* L-asparaginase produced in the USSR, Germany and Japan was made by Kondrat’Eva (1984) and it revealed that the clinical characteristics of the preparation made in the USSR and the preparation made in Germany (crasnitin) were identical. The antileukemic action of the preparation made in the USSR was superior to the preparation (leumase) made in Japan. L-asparaginase made in USSR and Germany was recommended for clinical use.

In India, the team of Neelam Verma, Biosensor Technology Lab, Dept. of Biotechnology, Punjabi University, Patiala (2007), is the first to be engaged in the use of L-asparaginase for the development of a novel diagnostic biosensor for the detection of levels of asparagines in leukemia cells.

Thus, L-asparaginase and the research being carried out on it may only be the tip of the iceberg. Although, much has been unraveled, it appears that there is still a long way to go in exploring this amazing enzyme.

1.8 Modeling as a tool for protein structure prediction

Protein structure prediction is the prediction of the three-dimensional structure of a protein from its amino acid sequence — that is, the prediction of its secondary, tertiary, and quaternary structure from its primary structure. Structure prediction is fundamentally different from the inverse problem of protein design. Protein structure
prediction is one of the most important goals pursued by bioinformatics and theoretical chemistry; it is highly important in medicine, in drug designing and in biotechnology for the design of novel enzymes.

Homology modeling is based on the reasonable assumption that two homologous proteins will share very similar structures. Because a protein's fold is more evolutionarily conserved than its amino acid sequence, a target sequence can be modeled with reasonable accuracy on a very distantly related template, provided that the relationship between target and template can be discerned through sequence alignment. It has been suggested that the primary bottleneck in comparative modeling arises from difficulties in alignment rather than from errors in structure prediction given a known-good alignment (Zhang and Skolnick 2005). Unsurprisingly, homology modeling is most accurate when the target and template have similar sequences.

Macromolecular docking is the computational modeling of the quaternary structure of complexes formed by two or more interacting biological macromolecules. Protein–protein complexes are the most commonly attempted targets of such modeling, followed by protein–nucleic acid complexes.

The ultimate goal of docking is the prediction of the three dimensional structure of the macromolecular complex of interest as it would occur in a living organism. Docking itself only produces plausible candidate structures. These candidates must be ranked using methods such as scoring functions to identify structures that are most likely to occur in nature.

The term "docking" originated in the late 1970s, with a more restricted meaning; then, "docking" meant refining a model of a complex structure by optimizing the separation between the interactors but keeping their relative orientations fixed. Later, the relative orientation of the interacting partners in the modeling was allowed to vary, but the
internal geometry of each of the partners was held fixed. This type of modeling is sometimes referred to as "rigid docking". With further increases in computational power, it became possible to model changes in internal geometry of the interacting partners that may occur when a complex is formed. This type of modelling is referred to as "flexible docking".