REVIEW
OF
LITERATURE
2. REVIEW OF LITERATURE

2.1. L-ASPARAGINASE:

L-asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) has been the subject of more than 1000 papers in the last 10 years. Moreover, this enzyme has been the subject of a number of excellent reviews. What is unusual about L-asparaginase, and constitutes the feature responsible for considerable attention it has attracted, is its antineoplastic activity. L-asparaginase is a tetrameric protein (David, 2005) belonging to oncolytic enzymes (Verma et al., 2007). It catalyses the hydrolytic deamination of amino acid asparagine to yield aspartic acid and ammonium ion (Dominika and Jaskolski, 2001). Of all the chemotherapeutic agents that are active in treating human tumors, L-asparaginase is the only drug with a mechanism of action that is thought to depend on specific metabolic defect in the malignant cell (Hill, J. M et al., 1967). The basis of the antitumor activity of L-asparaginase is assumed to be a result of the depletion of L-asparagine from the circulation, thus depriving sensitive tumor cells of their sole source of amino acid asparagine (Oettgen, H. F et al., 1967). Most normal and resistant cells are thought to escape the cytotoxic effects of L-asparaginase because of their ability to synthesize L-asparagine (whitecar, J. P et al., 1970).
2.2. DISTRIBUTION (SOURCES OF L-ASPARAGINASE):

L-asparaginase is widely distributed, being found in animal, microbial and plant sources. Its presence in guinea pig serum was first reported by Clementi (1922), who also reported the absence of this enzyme from the sera of a number of other common animals. When interest in L-asparaginase increased as a result of its anti-lymphoma activity, the search of its distribution was broadened. Sera from three other genera of the superfamily Cavioidae, to which the guinea pig belongs, contain L-asparaginase (Old L. J. et al. 1963). The enzyme is also present in the liver and kidney of certain birds (Clementi, 1922) and also in rat (De Groot et al., 1960; McGee et al., 1971) and chicken (Ohnuma, T. et al., 1967). Among plants, L-asparaginase was reported in Testa of Immature Seeds of Pea, *Pisum sativum* L. (Wriston and Yellin, 1973 and Eliana P. Chagas and Ladaslav Sodek, 2007). Two isoforms of the enzyme have been isolated from the Bryophyte *Sphagnum fallax* (Heeshen et al., 1996). *Lupin araboreus*, plant parts such as leaves, flower buds, root tips and *Lupin angustiplus* which have the ability to produce L-asparaginase (Borek et al., 1999). L-asparaginase activity has also been reported in soil of roots of *Pinus pinaster* and *Pinus radiate* (Bell and Adams, 2004).

L-asparaginase is widely distributed in microbial sources Table-2.1, Table-2.2 and Table-2.3. Microbes can be easily cultured as well as extraction and purification of L-asparaginase from microbial sources is also convenient, facilitating for the large Industrial scale production.
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Jayaram <em>et al.</em>, (1968)</td>
</tr>
<tr>
<td><em>Erwinia aroideae</em></td>
<td>Peterson and Ciegler (1969)</td>
</tr>
<tr>
<td><em>Pseudomonas</em></td>
<td>Broome and Schenkein (1971)</td>
</tr>
<tr>
<td><em>Alcaligenes eutropus</em></td>
<td>Allison <em>et al.</em>, (1971)</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>Boyd and Philips (1971)</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>Tosa <em>et al.</em>, (1971)</td>
</tr>
<tr>
<td><em>Erwinia carotovora</em></td>
<td>Cammack <em>et al.</em>, (1972)</td>
</tr>
<tr>
<td><em>Vibrio succinogenes</em></td>
<td>Disteasio <em>et al.</em>, (1976)</td>
</tr>
<tr>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>Joner (1976)</td>
</tr>
<tr>
<td><em>Corynebacterium glutamicum</em></td>
<td>Mesas <em>et al.</em>, (1990)</td>
</tr>
<tr>
<td><em>Bacillus sp.</em></td>
<td>Mohapatra <em>et al.</em>, (1995)</td>
</tr>
<tr>
<td><em>Enterobacter cloacea</em></td>
<td>Nawaz <em>et al.</em>, (1998)</td>
</tr>
<tr>
<td><em>Enterobacteria aerogenes</em></td>
<td>Mukherjee <em>et al.</em>, (2000)</td>
</tr>
<tr>
<td><em>Thermus thermophiles</em></td>
<td>Pritsa <em>et al.</em>, (2001)</td>
</tr>
<tr>
<td><em>Erwinia sp</em></td>
<td>Borkataky and Bezarauah (2002)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Qin and Zhao (2003)</td>
</tr>
</tbody>
</table>
### TABLE-2.2: L-ASPARAGINASE DISTRIBUTION IN ACTINOMYCETES

<table>
<thead>
<tr>
<th>Actinomycetes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptomyces karnatakensis</em></td>
<td>Mostafa S. A. (1979 a)</td>
</tr>
<tr>
<td><em>Streptomyces venezuelae</em></td>
<td>Mostafa, (1979 b)</td>
</tr>
<tr>
<td><em>Streptomyces collinus</em></td>
<td>Mostafa and Salama, (1979)</td>
</tr>
<tr>
<td><em>Nocardia asteroids</em></td>
<td>Gunasekaran <em>et al.</em>, (1955)</td>
</tr>
<tr>
<td><em>Streptomyces griseus</em></td>
<td>De Jong P. J. (1972)</td>
</tr>
<tr>
<td><em>Streptomyces sp. PDK7</em></td>
<td>Dhevagi, P., Pooorani, E. (2006)</td>
</tr>
<tr>
<td><em>Streptomyces albidoflavus</em></td>
<td>Narayana K. J. P. <em>et al.</em>, (2007)</td>
</tr>
<tr>
<td><em>Streptomyces longsporusflavus</em></td>
<td>Narayana K. J. P. <em>et al.</em>, (2007)</td>
</tr>
<tr>
<td><em>Streptomyces gulbargensis</em></td>
<td>Amena S. <em>et al.</em>, (2010)</td>
</tr>
</tbody>
</table>
TABLE-2.3: L-ASPARAGINASE DISTRIBUTION IN FUNGI

<table>
<thead>
<tr>
<th>Fungi</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pichia polymorpha</em></td>
<td>Foda <em>et al.</em>, (1980)</td>
</tr>
<tr>
<td><em>Aspergillus nidulans</em></td>
<td>Drainas &amp; Drainas., (1985)</td>
</tr>
<tr>
<td><em>Candida guilliermondii</em></td>
<td>Stepanyan and Davtyan., (1988)</td>
</tr>
<tr>
<td><em>Cylidocapron obtusiporum</em></td>
<td>Raha <em>et al.</em>, (1990)</td>
</tr>
<tr>
<td><em>Candida utilis</em></td>
<td>Kil <em>et al.</em>, (1995)</td>
</tr>
<tr>
<td><em>Mucor sp.</em></td>
<td>Mohapatra <em>et al.</em>, (1997)</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Bon <em>et al.</em>, (1997)</td>
</tr>
<tr>
<td><em>Fusarium sp.</em></td>
<td>Nibha Gupta <em>et al.</em>, (2009)</td>
</tr>
<tr>
<td><em>Penicillium sp.</em></td>
<td>Nibha Gupta <em>et al.</em>, (2009)</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>Saranya S. <em>et al.</em>, (2012)</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>Radhika Tippani <em>et al.</em>, (2012)</td>
</tr>
</tbody>
</table>
### TABLE-2.4: L-ASPARAGINASE DISTRIBUTION IN PLANTS

<table>
<thead>
<tr>
<th>Plant</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sphagnum fallax</em></td>
<td>Heeshen <em>et al.</em>, (1996)</td>
</tr>
<tr>
<td><em>Lupin angustiplus</em></td>
<td>Borek <em>et al.</em>, (1999)</td>
</tr>
</tbody>
</table>
2.3. STRUCTURE OF L-ASPARAGINASE:

In 1976, Murthy and Knox performed small angle X-ray scattering studies on *E. coli* L-asparaginase solutions, which showed that the enzyme had a radius of gyration of 34.0±0.5 ÅNG at pH 7. The enzyme was found to be a tetramer with each monomer having a general shape of a prolate ellipsoid. In the same year, Homer and Allsopp discovered a new histidyl residue of L-asparaginase in *E. coli* that affected its properties. Amino acid sequence of tryptic peptides was determined by fragmentation with various enzymes and by Edman degradation (Miata *et al.*, 1979). Ionization/deionization of the functional groups of the active center of L-asparaginase was found to be responsible for enzyme activity (Frankenberger and Page, 1983). Danileichenko, Pavlii and Ruban (1989) studied the electron microscopic structure of L-asparaginase from *Erwinia carotovora* 268. The structure of *E. coli* L-asparaginase was studied by Swain *et al.*, (1993) and two domains were observed. Location of the active site was found to be between the N and C terminals. The tertiary structure and amino acid sequence of *Wollinella succinogenes* L-asparaginase has been compared with bacterial L-asparaginase by Lubkowski *et al.*, (1996) and a difference in structures was found and sub-specificity was affected due to differences between amino acid sequences. Kozak *et al.*, (2000) made crystallographic studies on a Y25 F mutant of periplasmic *E. coli* L-asparaginase and also studied its kinetic properties. The catalytic role of both Thr15 and Tyr 29 residues of L-asparaginase in bacteria has been confirmed by Aghaiypour, Wlodawer, and Lubkowski (2001). The crystal structure of Type-I L-asparaginase from *Pyrococcus harikoshii* has been described by Yao *et al.*, (2005) by diffraction. It has significant difference with the structure of Type-II L-asparaginase. Most catalytic residues are conserved except two
that recognize the amino group of substrate. Mezentsev et al., (2006) carried out a comparative molecular-graphic analysis of subunits interfaces and developed an experimental approach for an enzyme oligomerization study along with homology modelling of the spatial structure of L-asparaginase from *Erwinia carotovora*. In this study, L-asparaginase was immobilized on a CM5 chip surface of optical biosensor Biacore 3000 based on a surface Plasmon Resonance Technology.
Fig-2.1: Structure of L-asparaginase Purified from Bacteria (David S. Goodsell, 2005). The active sites grip asparagine (red) and use a well-placed threonine amino acid (green) to perform the cleavage reaction.
2.4. PROPERTIES OF L-ASPARAGINASE:

Several parameters such as Temperature, pH, Oxygen and several chelating agents such as EDTA play a key role for the maximum growth of the enzyme producing organism (Mesas et al., 1990). Some of the agents like 2-mercapto-ethanol, glutathione enhance the activity of enzyme (Raha et al., 1990). The physical and chemical properties of the enzyme may vary due to their different sources. Generally L-asparaginase from Guinea pig serum have pH 7.5-8.5 and molecular weight of 1,38,000 Da. The question of subunits has been examined in only few cases, but in every instance four subunits have been found. Differences do appear, however, with regard to the strength of the non-covalent bonding forces responsible for maintaining quaternary structure. L-asparaginase from Guinea pig serum is stable for at least six months at 20°C to repeated freezing and thawing, as well as heating to 55°C for 10 mins, which promotes surface denaturation (Mishra, 2006). The molecular weights of Guinea pig serum, Serratia and Erwinia L-asparaginases are fairly close, but several others are considerably different (e.g., Bacillus coagulans, Fusarium and yeast). The isoelectric points of L-asparaginases exhibit wide variation and depend upon different sources, however, ranging from a value of less than 4.5 for the Guinea pig serum L-asparaginase to 8.6 for the Alcaligenes eutrophus.

Effect of oxygen absorption rates on L-asparaginase-II production by E. coli cells was studied by Zubanovic and Johanides (1975) and it was shown that a definite O₂ absorption rate of 0.4 mm/l per min. resulted in good growth and high enzyme content. In Bacillus mesentericus 43A, the biosynthesis of L-asparaginase was inhibited by the addition of L-aspartic acid in the growing cultures (Eremenko et al., 1976). Comparative
Experimental evaluation of immune-depressive immuno-toxic effects of L-asparaginase from *E. coli* and from *Erwinia carotovora* showed that L-asparaginase from *E. coli* is more immune-depressive and immune-toxic than that from *Erwinia carotovora*. L-asparaginase of *Aspegillus nidulans* showed the clearest evidence of O$_2$ repression under N$_2$ metabolite derepressed conditions (Shaffer *et al.*, 1988). Four forms of L-asparaginase were isolated from *Tetrahymena pyriformis* (Tsirka and Kyriakidis, 1989) and regulation of enzyme activity was studied. Lipids activated the enzyme and phospholipase-C inactivated the enzyme. The pH optima of purified *Staphylococcal* L-asparaginase were found to be between 8.6 and 8.8 while the temperature optima were 30-32$^0$C. Two forms of L-asparaginase, L-asparaginase-I and L-asparaginase–II, were extracted and purified from *Thermus thermophiles* by Tsavdaridis, Triantafillou and Kyriakidis (1994). The two forms acted optimally at pH 8.6. An extracellular L-asparaginase from *Rhodosporidium toruloides* has been reported to be a homodimer having an optimum pH of 6.35 and an optimum temperature of 37$^0$C (Ramakrishnan and Joseph R, 1996).

Generally L-asparaginase from Guinea pig serum have pH 7.8-8.5 and molecular mass of 1,38,000 Da. It is stable for at least 6 months at 20$^0$C to repeated freezing and thawing and to heating to 55$^0$C for 10 min, which promotes the surface denaturation (Mishra, 2006). L-asparaginase from *Bacillus sp.* exhibited maximal enzyme activity at pH 7 and temperature 37$^0$C (Vidhya Moorthy *et al.*, 2010). The enzyme was activated by MgCl$_2$ and inhibited by EDTA. L-asparaginase from *Aspergillus sp.* had optimal pH 7, optimal temperature 35$^0$C and molecular weight 94 KDa (Azad Chandrasekhar P., 2012).
**TABLE-2.5: PROPERTIES OF SOME MICROBIAL L-ASPARAGINASES**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>pH</th>
<th>Temp. (C°)</th>
<th>Km(M)</th>
<th>Specific activity (IU/mg)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. carotovora</em></td>
<td>8.0</td>
<td>50</td>
<td>1.8 x 10⁻³</td>
<td>167</td>
<td>Maladkar <em>et al</em>, 1993</td>
</tr>
<tr>
<td>Pseudomonas 7A</td>
<td>7.2</td>
<td>37</td>
<td>4.4 x 10⁻⁶</td>
<td>162</td>
<td>Roberts, 1976</td>
</tr>
<tr>
<td><em>C. glutamicum</em></td>
<td>7.0</td>
<td>40</td>
<td>2.5 x 10⁻³</td>
<td>2020</td>
<td>Mesas <em>et al</em>, 1990</td>
</tr>
<tr>
<td><em>V. succinogenes</em></td>
<td>8.5</td>
<td>37</td>
<td>1.7 x 10⁻³</td>
<td>5.6</td>
<td>Goodman, 1974</td>
</tr>
<tr>
<td><em>Bacillus sp.</em></td>
<td>8.0</td>
<td>37</td>
<td>2.4 x 10⁻⁴</td>
<td>–</td>
<td>Mohapatra <em>et al</em>, 1995</td>
</tr>
<tr>
<td><em>Aspergillus terreus</em></td>
<td>5 – 7</td>
<td>40 – 45</td>
<td>5.88 x 10⁻⁴</td>
<td>–</td>
<td>Ali <em>et al</em>, 1994</td>
</tr>
<tr>
<td><em>P. stutzeri</em></td>
<td>9</td>
<td>37</td>
<td>1.45 x 10⁻⁴</td>
<td>732</td>
<td>Manna <em>et al</em>, 1995</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em></td>
<td>9</td>
<td>37</td>
<td>0.147x10⁻³</td>
<td>1900</td>
<td>Ashraf. A, 2004</td>
</tr>
<tr>
<td>Actinomycetes sp.</td>
<td>8-9</td>
<td>60</td>
<td>_</td>
<td>64.07</td>
<td>P. Dhevagi <em>et al</em>, 2005</td>
</tr>
<tr>
<td><em>Bacillus sp.</em></td>
<td>7</td>
<td>37</td>
<td>_</td>
<td>1.12</td>
<td>Vidhya. M <em>et al</em>, 2010</td>
</tr>
<tr>
<td><em>Streptomyces sp.</em></td>
<td>7-8</td>
<td>50</td>
<td>_</td>
<td>662.6</td>
<td>Rekha <em>et al</em>, 2009</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>7</td>
<td>35</td>
<td>24 x 10⁻⁶</td>
<td>503.3</td>
<td>Chandrasekhar, 2012</td>
</tr>
</tbody>
</table>
TABLE-2.6: MOLECULAR WEIGHTS AND ISO-ELECTRIC POINTS OF L-ASPARAGINASES

<table>
<thead>
<tr>
<th>Source</th>
<th>MW(Da)</th>
<th>Isoelectric point</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>130,000</td>
<td>4.85</td>
<td>Cammack, K. A. <em>et al.</em>, 1972</td>
</tr>
<tr>
<td><em>Erwinia carotovora</em></td>
<td>135,000</td>
<td>7.6 – 8.9</td>
<td>Cammack, K. A. <em>et al.</em>, 1972</td>
</tr>
<tr>
<td>Guinea pig serum</td>
<td>138,000</td>
<td>3.6 – 4.5</td>
<td>Yellin, T. O. <em>et al.</em>, 1966</td>
</tr>
<tr>
<td><em>E. coli B</em></td>
<td>147,000</td>
<td>6.0</td>
<td>Whelan, H. A. <em>et al.</em>, 1969</td>
</tr>
<tr>
<td><em>Fusarium tricinctum</em></td>
<td>165,000</td>
<td>5.18</td>
<td>Scheetz, R. W. <em>et al.</em>, 1971</td>
</tr>
<tr>
<td><em>Alcaligenes eutropus</em></td>
<td>150,000</td>
<td>8.6</td>
<td>Allison, J. P. <em>et al.</em>, 1972</td>
</tr>
<tr>
<td><em>Acinetobacter sp.</em></td>
<td>130,000</td>
<td>8.43</td>
<td>Holcenberg, J. S. <em>et al.</em>, 1972</td>
</tr>
<tr>
<td>Yeast</td>
<td>800,000</td>
<td>_</td>
<td>Broome, J. D. <em>et al.</em>, 1965</td>
</tr>
<tr>
<td>Chicken liver</td>
<td>306,000</td>
<td>_</td>
<td>Ohnuma, T. <em>et al.</em>, 1967</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em></td>
<td>160,000</td>
<td>_</td>
<td>El-Bessoumy <em>et al.</em>, 2004</td>
</tr>
<tr>
<td><em>Streptomyces sp. PDK2</em></td>
<td>140,000</td>
<td>_</td>
<td>Dhevagi and Poorani, 2006</td>
</tr>
<tr>
<td><em>Bacillus sp.</em></td>
<td>45,000</td>
<td>_</td>
<td>VidhyaMoorthy <em>et al.</em>, 2010</td>
</tr>
<tr>
<td><em>Aspergillus sp.</em></td>
<td>94,000</td>
<td>_</td>
<td>Azad Chandrasekhar P., 2012</td>
</tr>
</tbody>
</table>
2.5. APPLICATIONS OF L-ASPARAGINASE:

The enzyme L-Asparaginase has the chemotherapeutic property against the tumor cells. It is an effective curable agent against the treatment of acute lymphoblastic leukemia and lymphosarcoma. The enzyme catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia. The principle behind the use of Asparaginase as an anti-tumor agent is that it takes advantage of the fact that all leukemic cells are unable to synthesize the non-essential amino acid asparagine on their own, which is very essential for the growth of the tumor cells, whereas normal cells can synthesize their own asparagine; thus leukemic cells require high amount of asparagine.

L-Asparaginase has a significant role in food industry. Acrylamide, reported as a significant toxic agent and cause neurotoxicity in humans, is present in ample amount in food items which are heat-derived and contain some reducing sugars. Formation of acrylamide is the result of Maillard reaction between the free amino acid asparagine and carbonyl group of reducing sugars like glucose. Maillard reaction is heat-induced reaction. Hence, food industry like baking food industry got an application of L-Asparaginase. The enzyme helps in hydrolysing the asparagine which significantly reduces the formation of acrylamide. The reported reduction in acrylamide content is about 90% (Mario Sanches et al., 2007). An example was conducted by Kukurova K et al., (2009) on dough resembling traditional Spanis rosquillas. They consider the different parameters which influence the reaction and formation of acrylamide and include temperature/time profile of frying process, moisture, sugars, amino acids, acrylamide, and some indicators of Maillard reaction. At different levels of asparagine they got a reduction of 96-97% in acrylamide content (Kukurova K et al., 2009).
2.6. ASSAY METHODS (L-ASPARAGINASE ASSAY):

Many attempts have been made by the researchers for the assay of L-asparaginase and monitoring its activity. Also, efficient methods for the assay of L-asparagine have been developed. Methods showing enzyme-substrate relation have been devised as well. In most of the assay methods, L-asparaginase activity was determined by Nesslerization method (Imada A. et al., 1973). An online gas analyser for automated enzymatic analysis with potentiometer ammonia detector has been described by Fraticelli and Meyerhoff (1983) where an ammonia electrode was incorporated in conjugation with pre-dialysis unit.

The direct measurement of L-asparagine in human plasma samples through the use of *E. coli* L-asparaginase in the soluble form has been a major clinical application of this system. A multi-analyte miniature conductance biosensor using enzymes such as urease and L-asparaginase and three-enzyme system consisting of urease, creatinase and creatininase for determining urea, L-asparagine and creatinine respectively, has been described by Cullen, Sethi and Lowe (1990). The device responded to changes in electrode double layer capacitance, as the ionic strength is increased by enzyme-catalysed generation of charged reaction products. An enzymatic method has been developed for the kinetic measurement for L-asparaginase activity and L-asparagine with an ammonia gas-sensing electrode. This method is based on deamination of L-asparagine by L-asparaginase resulting in the formation of ammonia (Tagami and Mastsuda, 1990). A specific quantitative colorimetric assay for L-asparagine by mixing it with dilute ethanol ninhydrin solution and noting its absorption (max.) at 340-350 nm has been reported by Sheng et al., (1993). Both L-asparaginase and asparagine synthetase activities can be
measured by this procedure, followed by HPLC amino-acid analysis. Determination of L-asparagine by garlic tissue electrode has been reported by Kim et al., (1995). Garlic tissue cells were employed for conversion of L-asparagine to ammonia. An ammonium gas electrode was used as an indicator. The combination of L-asparaginase in garlic tissue cells and gas electrode responds linearly to L-asparagine concentration. L-asparaginase from *Erwinia chrysanthemi* was assayed fluorometrically by incubating it with beta L-aspartic acid and measuring the release of 7-amino-4-methylcoumarin (Ylikangas and Mononen, 2000). Rapid analysis of L-asparaginase activity was made by this assay and it can be used for monitoring L-asparaginase activity in the serum of leukemic patients during L-asparaginase therapy. A thermostable recombinant asparaginase from *Archaeoglobus fulgidus* was cloned and expressed in *E. coli* as a fusion protein. It was later purified by an immobilized metal ion affinity chromatography method and its activity was determined by monitoring change in ammonia concentration in the solution. This enzyme was immobilized and used with an ammonium selective electrode to develop a biosensor for L-asparaginase (Wang and Bachas, 2002).

An automated kinetic enzymatic method for monitoring plasmatic L-asparaginase activity during therapy of acute lymphoblastic leukemia has been described by Orsonneau et al., (2004). The method is easy and requires less time to perform along with better specificity and precision than the Nessler’s method. Tsurusawa et al., (2004) demonstrated a highly sensitive enzyme coupling method to determine the minimum levels of L-aparaginase activity necessary for maintaining asparagine depletion under asparaginase treatment in acute lymphoblastic leukemia. It was shown that asparagine levels are strongly correlated with plasma L-asparaginase activity even at low enzyme activities (50 IU/ml).
Although various units have been used, the standard expression is in international units (IU). Assay method employed by Mashburn and Wriston (1963), where the rate of hydrolysis of asparagine is determined by measuring released ammonia. One unit releases one micromole of ammonia per minute at 37°C and pH 8.6 under the specified conditions. Thus one unit (IU) of L-asparaginase is defined as the amount of the enzyme that is capable of producing 1 µmole of ammonia per minute. L-Asparaginase activity can be measured by determining either ammonia or aspartic acid, or by following the disappearance of asparagine. In addition, an alternative assay method based on following the disappearance of 5-diazo-4-oxo-L-norvaline (DONV), an asparagine analogue has been devised.
2.7. CANCER THERAPY:

Cancer can be treated by surgery, chemotherapy, radiation therapy, immunotherapy, monoclonal antibody therapy or other methods. The choice of therapy depends upon the location and grade of the tumor and the stage of the disease, as well the general state of the patient (performance status). A number of experimental cancer treatments are also under development. Complete removal of the cancer without damage to the rest of the body is the goal of treatment.

Surgery:

In theory, non-hematological cancers can be cured if entirely removed by surgery, but this is not always possible. When the cancer has metastasized to other sites in the body prior to surgery, complete surgical excision is usually impossible. In the Halstedian model of cancer progression, tumors grow locally, and then spread to the lymph nodes, then to the rest of the body. This has given rise to the popularity of local-only treatments such as surgery for small cancers. Even small localized tumors are increasingly recognized as possessing metastatic potential.

Surgery is the primary treatment for brain tumors that can be removed without causing severe damage. Many benign (non-cancerous) tumors are treated only by surgery. Most malignant (cancerous) tumors, however, require treatment in addition to the surgery, such as radiation therapy and/or chemotherapy.
The goals of surgical treatment for brain tumors are multiple and may include one or more of the following:

- Confirm diagnosis by obtaining tissue that is examined under a microscope.
- Remove all or as much of the tumor as possible.
- Reduce symptoms and improve quality of life by relieving intracranial pressure caused by the cancer.
- Provide access for implantation of internal chemotherapy or radiation.

**Biopsy:** A biopsy is a surgical procedure to remove a small piece of tumor in order to confirm the diagnosis. The sample is examined under the microscope by a pathologist who determines the type of the tumor. A biopsy can be performed as part of the surgery to remove the tumor or as a separate procedure. There are two different approaches for performing a biopsy for brain tumors. An open biopsy involves exposing the tumor and then removing a small portion of it. A needle biopsy is performed by making an incision in the skin, drilling a small hole into the skull, inserting a narrow, hollow needle through the hole and into the tumor, and drawing up a small amount of tumor into the needle. A needle biopsy may also be conducted with the assistance of computers and scanning equipment, including MRI or CT, in order to increase the precision of the procedure. This procedure is called stereotactic needle biopsy and is useful for patients with deep or multiple tumors. Stereotactic techniques are discussed further in the Surgical Techniques section.
**Craniotomy:** The most commonly performed surgery for removal of a brain tumor is called a craniotomy. “Crani” means skull, “otomy” means cutting into. In preparation for a craniotomy, a portion of the scalp is usually shaved, and an incision is made through the skin. Using specialized equipment, a surgeon removes a piece of bone to expose the area of brain over the tumor. The dura-matter (the outermost layer of the brain tissue) is opened, the tumor is located and then removed (resected). After the tumor is removed, the bone is usually replaced and the scalp stitched shut. In a conventional craniotomy, surgeons guide themselves by what they see, their knowledge of anatomy, and their interpretation of the pre-operative scans. During stereotactic surgery, surgeons may rely on a computer to help direct the craniotomy.

When surgeons remove tumor tissue they try to leave a "margin" of healthy tissue to ensure all the cancer is removed. Sometimes this means the patient has to remain under general anaesthetic for another 30 minutes or so while tissue samples are sent for analysis to check if the margin is clear. Even then, it is still possible that some cancerous tissue remains, and the patient has to undergo further surgery to remove it. Now, a new technique based on an “intelligent knife,” called the “iKnife,” promises to remove the need for lab analysis and the accompanying delay, and it also helps avoid repeat surgeries. iKnife combines electrosurgery with new mass spectrometry techniques. The iKnife is a combination of an established technology called electro-surgery that was invented in the 1920s and a new technology that is still emerging, called rapid evaporative ionization mass spectrometry (REIMS). In electrosurgery, the surgeon’s knife delivers an electric current that heats the target tissue and cuts through it while
causing minimum loss of blood. The heat from the current vaporizes the tissue, which
gives off a smoke that is normally sucked away with an extractor. The mass spectrometer
technology behind REIMS almost instantly identifies the chemicals present in human
tissue by analyzing the smoke that is released during electrosurgery. The iKnife sniffs the
“smoke” created by the electrosurgical removal of cancerous tissue and tells the surgeon
almost immediately if the tissue it has come from is healthy or cancerous.

Colorectal cancer affects about 1,50,000 people every year, making it the third most
common cancer in both men and women. Colorectal cancer is most often treated with
surgery to remove the tumor, and possibly, chemotherapy and radiation. Different
surgical techniques to remove colorectal tumors:

Polypectomy is a procedure in which polyps-small growths on the inner lining of the
colon are removed during a colonoscopy, a procedure in which a special instrument (the
colonoscope) is inserted into the rectum to view the rectum and colon.

Local excision can be used to treat cancers in the rectum (the lowest portion of the
colon). The procedure involves removing the cancer and some tissue of the wall of the
rectum. It may be done through the anus (the opening of the rectum) or through a small
cut in the rectum. The procedure does not require major abdominal surgery.

Resection involves the removal of part, or all, of the colon along with the cancer and its
attaching tissues.
**Laparoscopic surgery:** The word “laparoscopy” means to look inside the abdominal cavity with a special camera or scope. To perform laparoscopy, between 3 and 6 small (5-10 mm) incisions are made in the abdomen. The laparoscope and special laparoscopic instruments are inserted through these small incisions. The surgeon is then guided by the laparoscope, which transmits a picture of the intestinal organs on a video monitor.

**Radiation therapy:**
Radiation therapy (also called radiotherapy, X-ray therapy or irradiation) is use of ionizing radiation to kill cancer cells and shrink tumors. Radiation therapy can be administered externally via external beam radiotherapy (EBRT) or internally via brachytherapy. The effects or radiation therapy are localized and confined to the region being treated. Radiation therapy injuries or destroys cells in the area being treated (the “target tissue”) by damaging their genetic material. Making it impossible for these cells to continue to grow and divide. Although radiation damages both cancer cells and normal cells, most normal cells can recover from the effects of radiation and function properly. The goal of radiation therapy is to damage as many cancer cells as possible, while limiting harm to nearby healthy tissue. Hence, it is given in many fractions, allowing healthy tissue to recover between fractions.

Radiation therapy kills cancer cells by damaging their DNA (the molecules inside cells that carry genetic information and pass it from one generation to the next Lawrence T.S et al, 2008). Radiation therapy can either damage DNA directly or create charged particles (free radicals) within the cells that can in turn damage the DNA. Cancer cells whose
DNA is damaged beyond repair stop dividing or die. When the damaged cells die, they are broken down and eliminated by the body’s natural processes. Radiation can come from a machine outside the body (external-beam radiation therapy) or from radioactive material placed in the body near cancer cells (internal radiation therapy, more commonly called-brachytherapy). Systemic radiation therapy uses a radioactive substance, given by mouth or into a vein that travels in the blood to tissues throughout the body.

The type of radiation therapy prescribed by a radiation oncologist depends on many factors, including:

- The type of cancer.
- The size of the cancer.
- The cancer’s location in the body.
- How close the cancer is to normal tissues that are sensitive to radiation.
- How far into the body the radiation needs to travel.
- The patient’s general health and medical history.
- Whether the patient will have other types of cancer treatment.
- Other factors, such as the patient’s age and other medical conditions.

Patients who receive most types of external-beam radiation therapy usually have to travel to the hospital or an outpatient facility up to 5 days a week for several weeks. One dose (a single fraction) of the total planned dose of radiation is given each day. Occasionally, two treatments a day are given.
Most types of external-beam radiation therapy are given in once-daily fractions. There are two main reasons for once-daily treatment:

- To minimize the damage to normal tissue.
- To increase the likelihood that cancer cells are exposed to radiation at the points in the cell cycle when they are most vulnerable to DNA damage (Connell PP, et al, 2009).
- In recent decades, doctors have tested whether other fractionation schedules are helpful, including.
  - Accelerated fractionation-treatment given in larger daily or weekly doses to reduce the number of weeks of treatment.
  - Hyperfractionation-smaller doses of radiation given more than once a day.
  - Hypofractionation-larger doses given once a day or less often to reduce the number of treatments.

Researchers hope that different types of treatment fractionation may either be more effective than traditional fractionation or be as effective but more convenient.

Radiation therapy may be used to treat almost every type of solid tumor, including cancers of the brain, breast, cervix, larynx, lung, pancreas, prostate, skin, stomach, uterus or soft tissue sarcomas. Radiation is also used to treat leukemia and lymphoma. Radiation dose to each site depends on a number of factors, including the radio sensitivity of each cancer type and whether there are tissues and organs nearby that may be damaged by radiation. Thus, as with every form of treatment, radiation therapy is not without its side
effects. Radiation therapy can cause both early (acute) and late (chronic) side effects. Acute side effects occur during treatment, and chronic side effects occur months or even years after treatment ends. The side effects that develop depend on the area of the body being treated, the dose given per day, the total dose given, the patient’s general medical condition, and other treatments given at the same time. Fatigue is a common side effect of radiation therapy regardless of which part of the body is treated. Nausea with or without vomiting is common when the abdomen is treated and occurs sometimes when the brain is treated. Medications are available to help prevent or treat nausea and vomiting during treatment.

Late side effects of radiation therapy may or may not occur. Depending on the area of the body treated, late side effects can include:

- Fibrosis (the replacement of normal tissue with scar tissue, leading to restricted movement of the affected area).
- Damage to the bowels, causing diarrhea and bleeding.
- Memory loss.
- Infertility (inability to have a child).
- Rarely, a second cancer caused by radiation exposure.

For example, girls treated with radiation to the chest for Hodgkin lymphoma have an increased risk of developing breast cancer later in life. In general, the lifetime risk of a second cancer is highest in people treated for cancer as children or adolescents.
Whether or not a patient experiences late side effects depends on other aspects of their cancer treatment in addition to radiation therapy, as well as their individual risk factors. Some chemotherapy drugs, genetic risk factors, and lifestyle factors (such as smoking) can also increase the risk of late side effects.

When suggesting radiation therapy as part of a patient’s cancer treatment, the radiation oncologist will carefully weigh the known risks of treatment against the potential benefits for each patient (including relief of symptoms, shrinking a tumour, or potential cure). The results of hundreds of clinical trials and doctors’ individual experiences help radiation oncologists decide which patients are likely to benefit from radiation therapy.

**Immuno-therapy:**

Cancer immunotherapy refers to a diverse set of therapeutic strategies designed to induce the patient’s own immune system to fight the tumor. By stimulating the body’s natural immune system, immunotherapy mobilizes the body’s own powerful anticancer mechanism to help achieve a durable response. Cancer immunotherapy activates T cells and B cells that target specific tumor antigens. The activated immune system is primed to recognize tumor antigens expressed by each patient's unique and frequently changing population of cancer cells. Some activated T cells kill tumor cells directly or indirectly. Some activated T cells activate B cells, which become antibody-producing plasma cells (Murphy K et al, 2008).

Cancer immunotherapy is designed to support the immune system’s ability to adapt its attack over time. Each patient's population of tumor cells mutates over time, which may result in resistance to traditional anticancer therapies. When a tumor cell is killed,
additional antigens are released, stimulating activation of new populations of T cells and B cells that recognize tumor antigens. This can result in an expanding cascade of immune cells able to recognize cancer cells bearing a variety of tumor antigens as the tumor mutates over time. Immunotherapy for cancer stimulates immunologic memory, which may lead to a prolonged antitumor response (Klebanoff CA et al, 2006).

Contemporary methods for generating an immune response against tumors include intravesical BCG immunotherapy for superficial bladder cancer, and use of interferons and other cytokines to induce an immune response in renal cell carcinoma and melanoma patients. Vaccines to generate specific immune responses are the subject of intensive research for a number of tumors, notably malignant melanoma and renal cell carcinoma. Sipuleucel-T is vaccine-like strategies in late clinical trials for prostate cancer in which dendritic cells from the patient are loaded with prostatic acid phosphatase peptides to induce a specific immune response against prostate-derived cells. Recent findings indicate that chemokines have therapeutic potential as adjuvants or treatments in antitumor immunotherapy (Bernhard Homey et al, 2002).

Allogeneic hematopoietic stem cell transplantation (“bone marrow transplantation” from a genetically non-identical donor) can be considered a form of immunotherapy; since the door’s immune cells will often attack the tumor in a phenomenon known as graft-versus-tumor effect. For this reason, allogeneic HSCT leads to a higher cure rate than autologous transplantation for several cancer types, although the side effects are also more severe.
Hormonal therapy:

The growth of some cancers can be inhibited by providing or blocking certain hormones. Common examples of hormone-sensitive tumors include certain types of breast and prostate cancers. Removing or blocking estrogen or testosterone is often an important additional treatment. In certain cancers, administration of hormone agonists, such as progestogens may be therapeutically beneficial. Several hormones like estrogens, progestins and adrenocorticosteroids are widely used in hormonal therapy of cancer.

Prostate cancer immunotherapy: Androgen Deprivation Therapy (ADT) is extensively used to treat prostate cancer. The goal is to reduce levels of male hormones, called androgens, in the body, or to prevent them from reaching prostate cancer cells. The main androgens are testosterone and dihydrotestosterone (DHT). Androgens, which are made mainly in the testicles, stimulate prostate cancer cells to grow. Lowering androgen levels or stopping them from getting into prostate cancer cells often makes prostate cancers shrink or grow more slowly for a time. However, hormone therapy alone does not cure prostate cancer.

Hormone therapy may be used:

- If you are not able to have surgery or radiation or can't be cured by these treatments because the cancer has already spread beyond the prostate gland
- If your cancer remains or comes back after treatment with surgery or radiation therapy
- Along with radiation therapy as initial treatment if you are at higher risk of the cancer coming back after treatment (based on a high Gleason score, high PSA level, and/or growth of the cancer outside the prostate)
- Before radiation to try to shrink the cancer to make treatment more effective

**Breast cancer immunotherapy:** There are certain hormones that can attach to breast cancer cells and affect their ability to multiply. The purpose of endocrine therapy, formerly called hormone therapy, is to add, block or remove hormones.

With breast cancer, the female hormones estrogen and progesterone can promote the growth of some breast cancer cells. Therefore in some patients, endocrine therapy is given to block the body’s naturally occurring estrogen to slow or stop the cancer’s growth.

There are two types of hormone therapy for breast cancer.

- Drugs that inhibit estrogen and progesterone from promoting breast cancer cell growth.
- Drugs or surgery to turn off the production of hormones from the ovaries.

Hormone therapy for cancer treatment, endocrine therapy, stops hormones from getting to breast cancer cells. Hormone therapy for post-menopausal women without cancer-called "hormone replacement therapy" -adds more hormones to the body to counter the effects of menopause.
As part of the diagnosis process, tests are done to determine if the breast cancer cells have estrogen or progesterone receptors. If so, that means that growth of the cancer can be stimulated with these hormones. If a cancer is found to have these receptors, endocrine therapy is recommended as part of the treatment plan.

There are several common hormone therapy drugs used for breast cancer:

- Tamoxifen
- Fareston
- Arimidex
- Aromasin
- Femara
- Zoladex/Lupron

**Angiogenesis Inhibitors:**

Angiogenesis inhibitors prevent the extensive growth of blood vessels (angiogenesis) that tumors require to survive. Some, such as bevacizumab, have been approved and are in clinical use. One of the main problems with anti-angiogenesis drugs is that many factors stimulate blood vessel growth, in normal cells and cancer. Anti-angiogenesis drugs only target one actor, so the other factors continue to stimulate blood vessel growth. Other problems include route of administration, maintenance of stability and activity and targeting at the tumor vasculature.
For tumors to develop a neovascular blood supply, tumor and host cells must secrete pro-
angiogenic factors that offset the activities of inhibitory angiogenic factors. In addition,
the newly derived tumor endothelium must respond to survive in a relatively caustic
microenvironment. Thus, endothelial-cell survival factors are essential in the
maintenance of this neovasculature. Because redundant factors and pathways regulate
angiogenesis, inhibition of any single pathway is unlikely to lead to prolonged response
in most patients with solid malignancies. Since anti-angiogenic therapy is unlikely to
induce tumor regression, the criteria for efficacy must be evaluated by means other than
the standard criteria used to evaluate cytotoxic chemotherapy regimens. Understanding
the basic principles that drive tumor angiogenesis will lead to the development of
therapies that will likely prolong survival without the toxicity associated with standard
chemotherapy (Ellis LM et al, 2005).

In oncological practice, angiogenesis inhibition, mainly through the blockade of the
VEGF family and its receptors, has been robustly demonstrated to produce clinical
benefits and, in specific disease subsets such as colorectal cancer, to extend the overall
survival of treated patients. VEGF is a multifunctional growth factor that mediates its
functions through cognate receptors on endothelial cells and it has been discovered for its
capability to induce macromolecule hyper-permeability in veins and venules. Several
approaches have been taken to target angiogenesis in cancer: drugs that target one or
more soluble ligands of the VEGF family, drugs that selectively inhibit one or more
receptors of the VEGF receptor family, and drugs that inhibit VEGF receptor(s) among
other, non VEGF-related targets. At present, two compounds have shown significant
clinical activity, bevacizumab, Avastin® and aflibercept, Zaltrap®, and only one of these (bevacizumab) has so far been registered for use in clinical practice. The molecular pathways that characterize angiogenesis, focusing on VEGF family, the current applications (particularly in colorectal cancer) and limitations of its blockade in oncology, and the hypothetical future perspectives of anti-angiogenic therapy was overviewed by Bagnasco L et al, 2012.

**Combination Chemotherapy:**

Multiple drug therapy in the case of neoplastic diseases has been proved to be superior to that of single drug (Henderson and Samaha, 1969). Researchers have achieved an experimental breakthrough in the simultaneous use of two or more agents for treating cancer, known as “combination therapy”. If it is shown to be safe and effective in humans, the therapy outlined by the new study is promising general strategies for treating many cancers that do not respond to current therapies. The treatment of acute myelogenous leukemia is more difficult and less successful than the treatment of acute lymphoblastic leukemia. Complete remissions may be obtained relatively easily in almost patients with acute myelogenous leukemia. Therefore, combination chemotherapy has proved more efficient than single drug treatment (Henderson, 1969).

Gee *et al.*, (1969), using a combination of cytosine arabinoside and thioguanine in 38 adult patients with acute myelogenous leukaemia, achieved a complete remission in 15 and good partial remission I further four. Some researchers reported similar results with a combination of cyclophosphamide, vincristine, cytosine arabinoside and prednisone
(COAP), 53% patients achieving complete remission. Among the 227 evaluable cases the highest complete and partial remission rates were obtained with cytosine arabinoside in combination with either Thioguanine or dannorubicin. The remission rates, including partial remissions in both series, where about 50%, but the complete remission rates were somewhat lower.

L-asparaginase (L-asparagine amidohydrolases, E.C.3.5.1.1) is an effective agent for treatment of acute lymphocytic leukemia but the enzyme has little or no therapeutic affective against any other neoplasm in man (Clarkson, et al., 1970).

Roberts et al., (1970), reported that asparaginase-resistant Ehrlich carcinomas regressed completely when tumor bearing mice were injected with purified bacterial glutamiase or glutaminase-aspararginase preparations.

Resistance to cancer chemotherapy is a common phenomenon especially in metastatic breast cancer (MBC), a setting in which patients typically have had exposure to multiple lines of prior therapy. The subsequent development of drug resistance can result in rapid disease progression during or shortly after completion of treatment. Moreover, cross-class multidrug resistance limits patient treatment choices, particularly in a setting where treatments options are few. One attempt to minimize the impact of drug resistance has been the concurrent use of two or more chemotherapy agents with unrelated mechanisms of action and differing modes of drug resistance, with the intent of blocking the development of multiple intracellular escape pathways essential for tumor survival.
Within the past decade, an array of mechanistically diverse agents has augmented the list of combination regimens that may be both synergistic and efficacious in pretreated MBC. The mechanisms of resistance to common chemotherapy agents and to consider current combination treatment options for heavily pretreated and/or drug-resistant patients with MBC were overviewed (Denise A. Yardley, 2013).

Combination of antiangiogenesis with chemotherapy for more effective cancer treatment was reviewed by Jie Ma and David J. Waxman (2008). Antiangiogenic drugs such as the anti-vascular endothelial growth factor antibody bevacizumab can induce a functional normalization of the tumor vasculature that is transient and can potentiate the activity of co-administered chemo-radiotherapies. However, chronic angiogenesis inhibition typically reduces tumor uptake of co-administered chemotherapeutics, indicating a need to explore new approaches, including intermittent treatment schedules and provascular strategies to increase chemotherapeutic drug exposure. In cases where antiangiogenesis induced tumor cell starvation augments the intrinsic cytotoxic effects of a conventional chemotherapeutic drug, combination therapy may increase antitumor activity despite a decrease in cytotoxic drug exposure. As new angiogenesis inhibitors enter the clinic, reliable surrogate markers are needed to monitor the progress of antiangiogenic therapies and to identify responsive patients. New targets for antiangiogenesis continue to be discovered, increasing the opportunities to interdict tumor angiogenesis and circumvent resistance mechanisms that may emerge with chronic use of these drugs.
Combination drug regimens with newer cytotoxic and biologic therapies are an effective strategy in fighting tumor growth and/or progression. These combinations can facilitate the attack on multiple intercellular processes, which may result in more efficient tumor responses. These strategies may also delay or circumvent mechanisms of drug resistance by interfering with cell survival or tumor growth pathways and the cross-talk established between them. Unfortunately, mechanisms of drug resistance to both cytotoxic and biologic therapies may ultimately limit the therapeutic efficacy of any anticancer drug, especially in heavily pretreated patients who have already exhausted many of their options. Although it is hoped that tumor gene expression profiles can help us select appropriate patients for specific treatments, development of drug resistance to chemotherapy or biologic therapy remains a major limitation. To this end, numerous ongoing and future trials are investigating novel effective combinations consisting not only of chemotherapy but also of biologic agents designed to target identified cell-signaling pathways that are active in settings of disease progression. Identifying additional biomarkers and potential drug targets may lead to the development of novel biologic/chemotherapy combinations that will ultimately extend the utility of these combinations. These advances in therapy will continue to help us overcome tumor resistance and disease progression in any given patient with breast cancer.
2.8. DISCOVERY OF ANTI-TUMOR PROPERTIES OF L-ASPARAGINASE:

The discovery of L-asparaginase has a useful place in the treatment of some forms of malignant disease in man was prompted by Kidd (1953). However, Clementi as early as in 1922 showed that guinea pig serum alone, among many mammalian sera, is rich in L-asparaginase. The action of guinea pig serum in bringing about \textit{in vivo} regression of three transplanted mouse and rat lymphomas was first described by Kidd (1953). Broome (1961) noticed similar inhibitory effects in tissue culture experiments and further proved that the active principle in Kidd’s observation was the enzyme L-asparaginase. Mashburn and Wriston (1964) demonstrated that the same enzyme from \textit{E. coli} can exert inhibitory effect on the growth of mouse leukemia. This discovery led to large scale production of the enzyme from microbial sources and also to the study of its experimental and clinical effects on different types of tumors.
2.9. PRODUCTION OF L-ASPARAGINASE FROM VARIOUS SOURCES:

L-asparaginase can be produced by Solid State Fermentation (SSF) as well as Submerged Fermentation (SmF). L-asparaginase is produced throughout the world by submerged fermentation (SmF). The submerged fermentation technique is dominated by industrial enzymologists because it is well adopted to fermentation of aerobic bacteria which are used to produce different types of enzymes like amylase, protease and other bacterial enzymes. SmF utilizes free flowing liquid substrates, such as molasses and broths. Additional advantage of SmF technique is that purification of products is easier. SmF is primarily used in the extraction of secondary metabolites that need to be used in liquid form.

There have been many reports about the production of L-asparaginase under different conditions by different micro-organisms. Several parameters especially physical and chemical parameters for L-asparaginase production vary with the species of microbial source (Barnes et al., 1977). Other constituents such as media composition can affect the growth as well as production of V. succinogenes was studied (Albanase and Kafkewitz, 1978). L-asparaginase from Pseudomonas ovalis was reported by Badr and Foda (1976). L-asparaginase from Serratia marcescens (Stern, Phillips and Gottleib, 1976) has also been reported. The synthesis of L-asparaginase in E. coli –W and E. coli K-12 was almost completely suppressed if glucose was added at a concentration of 0.5% to the growth medium. This was because glucose caused catabolic repression and catabolic inhibition of the components involved in lactate transport (Garaev and Golub, 1977) and lactate stimulated L-asparaginase synthesis.
Organic acids and amino acids such as L-leucine and L-methionine were found to enhance production of L-asparaginase in *E. coli* (Netrval *et al.*, 1977). *S. cerevisiae* synthesises two forms of L-asparaginase, L-asparaginase-I and L-asparaginase-II. The two enzymes are genetically and biochemically distinct. The interactions between both were studied by Jones (1977). L-asparaginase-I is constitutive and L-asparaginase-II is secreted in response to Nitrogen starvation (Dunlop *et al.*, 1978). High L-asparaginase activity has been observed in bacterial cultures growing in ample nitrogen (Paul and Cooksey, 1981). In *Lupin araboreus*, plant parts such as leaves, root-tips, flower buds and developing seeds have been found to be sources of L-asparaginase (Chang and Farnden, 1981).

Use of keiselguhr composite and CM sepharose for large scale production of L-asparaginase from *Erwinia chrysanthemi* has been described by Goward *et al.*, (1989). Cell growth and enzyme formation were studied in batch fermentation for the purpose of obtaining therapeutic L-asparaginase from *Erwinia aroidae* by Minim and Alegre (1992). The optimum lactose concentration was 10g/l and the addition of asparagine into the medium enhanced L-asparaginase production. Yeast extract was important for the cell mass formation and L-asparaginase synthesis, but in high concentration L-asparaginase production was inhibited. Strains of luminous bacteria (43 strains) belonging to four species (*Vibrio hawveyi, V. fisheri, Photobacterium leiognathi and P. phosphoreum*) isolated from different marine samples were examined for L-asparaginase production. Amounts of enzyme secreted by luminous prokaryotes were found to be higher than those reported from other bacterial species (Ramaiah and Chandramohan, 1992).
Regulation mechanism of production of Staphylococcal L-asparaginase showed that carbon sources such as sucrose, maltose, galactose, lactose, mannitol and mannose inhibited, while exogenous c-AMP in presence of carbon sources stimulated L-asparaginase enzyme production (Rozalska and Mickucki, 1992). Cheese whey supplementation with tryptone (0.3%) and asparagine (0.5%) was used (Minim, 1992). Fermenter operator conditions had a significant effect over *Erwinia arodiae* NRR LB-138 L-asparaginase production. *Nocardia asiroides*, an aerobic actinomycete, was grown in three different media, namely sabouraud dextrose broth (SD), tryptic soy broth and synthetic medium, as a shaker culture at 37°C for six days. The SD broth yielded maximum growth and maximum L-asparaginase production (Gunasekaran *et al.*, 1995). A pH and dye based fast procedure for screening L-asparaginase producing microorganisms has been reported by Gulati, Saxena and Gupta (1997). The compound n-dodecane at 6% increased cell concentration by 12.7% and production of L-asparaginase by 21% to give 60.8 IU/ml in the fermentation medium of E. coli (Wei and Liu, 1998).

An intracellularly expressed L-asparaginase was detected from *Enterobacter cloacae* by Nawaz, Zhang and Cerniglia (1998). These gram negative, rod-shaped bacteria utilized L-asparagine as the sole source of carbon and nitrogen. High L-asparaginase activity was found in cells cultured on L-fructose, D-galactose, sucrose or maltose, and in cells cultured on L-asparagine as the sole nitrogen source. The optimum pH and optimum temperature of L-asparaginase were found to be 8.5 and 37-42°C, respectively. Production of L-asparaginase from a new *Erwinia* *sp.* has been reported by Borkotaky and Bezbaruah (2002). The optimum pH was 9.2 and Km for L-asparaginase was 2.8 mM. It was thermo-stable and followed linear kinetics, even at 77°C. The enzyme at
native state state is a hexamer and does not hydrolyse L-glutamine. Optimization of solid-state fermentation for the production of L-asparaginase by *Pseudomonas aeruginosa* 50071 has been reported by Abdel, Yasser and Olama (2002). A pH of 7.9, casein hydrolysate (3.11%) corn-steep liquor (3.68%) were the most significant factors improving the enzyme production process. Release of L-asparaginase from *E. coli* cells with aqueous two-phase micellar systems (Qin *et al.*, 2003) by using K$_2$HPO$_4$ and Triton X-100 has also been reported.

That L-asparaginase production by filamentous fungi is under nitrogen regulation was reported by Sarquis *et al.*, (2004). *Aspergillus tamari* and *Aspergillus terreus* were cultivated in a medium containing different nitrogen sources. *Aspergillus tamari* showed the highest L-asparaginase production in 2% proline medium, while the lowest L-asparaginase production levels in both the fungi were found in the presence of glutamine and urea as the nitrogen sources. L-asparaginase production in *Erwinia aerogenes* expressing *Vitreoscilla* haemoglobin for efficient oxygen has been described by Geckil and Gencer (2004). *Vitreoscilla* haemoglobin in *Erwinia aerogenes* and *Pseudomonas aeruginosa* respond differently to catabolic and oxygen repression for L-asparaginase production (Geckil, Gencer and Uckun, 2004). Also, recovery of L-asparaginase from *P. aeruginosa* and *E. aerogenes* by membrane permeabilization by 50 mM potassium phosphate with 1% hexane has been carried out (Geckil *et al.*, 2005). To understand how different carbon sources and *Vitreoscilla* haemoglobin (VHb) affect the production of this enzyme in *P. aeruginosa* and its VHb-expressing recombinant strain (PaJC), both strains were grown in various carbon sources such as glucose, lactose, mannito and glycerol (Geckil *et al.*, 2006). Compared to no carbohydrate-supplemented
medium, glucose caused a slight repression of L-asparaginase in the host strain, while it stimulated it in the PaJC strain.

L-asparaginase is produced by submerged fermentation (SmF). This methodology has some limitations like low net yield and cost intensive. Moreover submerged fermentation leads to generation of effluents, which ultimately calls for treatment process (El-Bessoumy et al., 2004). Another alternative solution to SmF is solid state fermentation (SSF) which is offering a wide range of advantages compared to SmF (Lonsane et al., 1985). SSF methodology is a very effective technique, as the yield of the product is many times higher when compared to that in SmF. SSF also offers many other advantages including resistance to contamination, ease of product extraction, simpler methods for treating the fermented residue, low capital investment, low energy requirement and less water output, better product recovery and reported to be most appropriate process for developing countries (Carrizales and Jaffe, 1986). In SSF agricultural waste can be used as source of nutrients which is cost effective and environment friendly (Couto S. R. and Sanroman M. A.2005). SSF holds tremendous potential for the production of secondary metabolites and has been increasingly applied in recent years (Sangeetha et al., 2004).

*Staphylococci* had the maximum yield during the stationary phase of growth on a batch culture where Carbon and Nitrogen sources can be supplied as Casein hydrolysate and yeast extract (Mikucki et al., 1997). Maximum yield of L-asparaginase was found when cultures were aerated during the accelerated log phase or exponential phase of growth.
and further incubated in the stationary phase. Repression by L-asparagine and L-aspartic acid was absent but glucose inhibited the enzyme formation (Savitri et al., 2003).

Various types of media can be used as N and C source especially synthetic media with maximum production than normal media by Streptomyces (Mostafa and Salma, 1979). For the production of maximum yield of L-asparaginase, starch (1.0%) as Carbon and asparagine (0.8%) as Nitrogen source was optimum for enzyme production at pH 8.5. Incubation was done at 28-30°C for six days. S. cerevisiae has ability to produce L-asparaginase under Nitrogen starvation condition (Bon et al., 1997). L-asparaginase produced by cultivating the cells of Condida utilis medium containing glucose, yeast nitrogen base and peptone at 30°C. After 18 hours, cells were collected by centrifugation and L-asparaginase activity was measured (Kil et al., 1995).

Prakasham et al., (2006) evaluated the fermentation process-parameter interactions for the production of L-asparaginase by isolated Staphylococcus sp. -6A to optimize nutritional (Carbon and Nitrogen sources), physiological (incubation temperature, medium pH, aeration and agitation) and microbial (inoculum level) fermentation factors. They reported that incubation temperature, inoculum level and medium pH, among all fermentation factors, were the major influential parameters at the individual level, and contributed to more than 60% of total L-asparaginase production. The production of high levels of L-asparaginase from a new isolate of Aspergillus niger in solid state fermentation (SSF), using agro-wastes from three leguminous crops (bran of Cajans cajan, Phaseolus mungo and Glycine max), has been reported by Mishra (2006).
Production of extracellular L-asparaginase from marine actinomycetes by both solid state fermentation (SSF) and submerged fermentation (SmF) was reported by N Saleem Basha et al, 2009. Production of L-asparaginase was carried out in three different media, namely, solid-state media, Tryptone Glucose Yeast Extract (TGY) broth and Tryptone Fructose Yeast Extract (TFY) broth. It was reported that among the three media solid-state media gave the highest enzyme activity. Production of extracellular L-asparaginase from Bacillus sp. was reported by Vidhya Moorthy et al, 2010. The enzyme production was carried by using submerged fermentation. Two different carbon sources, glucose and maltose were used for the enzyme production and glucose was found to be better source. Withania somnifera L. has been traditionally used as a sedative and hypnotic. The study was carried out for the purification, characterization, and in vitro cytotoxicity of L-asparaginase from W. somnifera L. (Oza V. P. et al, 2010). L-Asparaginase was purified from the fruits of W. somnifera L. up to 95% through chromatography. The purified L-asparaginase was characterized by size exclusion chromatography, polyacrylamide gel electrophoresis (PAGE), and 2D PAGE. The antitumor and growth inhibition effect of the L-asparaginase was assessed using [3-(4,5-dimethyl-thiazol-2yl)-2, 5-diphenyl-tetrazolium bromide] (MTT) colorimetric dye reduction method. The purified enzyme is a homodimer, with a molecular mass of 72 +/-0.5 kDa, and the pI value of the enzyme was around 5.1. This is the first report of the plant containing L-asparaginase with antitumor activity. Data obtained from the MTT assay showed a LD (50) value of 1.45 +/-0.05 IU/ml. W. somnifera L. proved to be an effective and a novel source of L-asparaginase. Further more, it shows a lot of similarity with bacterial L-asparaginases EC-2.
L-asparaginase was isolated from *Aspergillus* species and grown by using liquid-state fermentation technology by Azad Chandrasekhar P, 2012. Potato Dextrose Agar (PDA) medium was used for the growth of microbial colonies and Modified Czapek Dox medium was used for screening of L-asparaginase producing micro-organisms. The optimum pH and temperature for L-asparaginase were found to be 7 and 35 respectively.
2.10. PURIFICATION AND CHARACTERIZATION OF L-ASPARAGINASE:

L-asparaginase from *Mycobacterium phlei* was purified by fractionation with ammonium sulphate, absorption of contaminating proteins on calcium phosphate gel and chromatography on Sephadex G-150 and DEAE cellulose. The apparent Km for L-asparaginase was 0.7 mM and the energy of activation was 9800 cal/mol (Pastuszak and Szymona, 1976). Most of the microbial L-asparaginase is intracellular in nature except few, which are secreted outside the cells (Kil *et al*, 1995; Mohapatra *et al*, 1995).

KSCN, NaClO₄ and Triton X-100 have been used for the solubilisation of enzyme purified from *Tetrahymena pyriformis* (Triantafillou *et al*., 1988). Maladkar *et al* (1993) extracted an intracellular L-asparaginase from the cells of *Erwinia carotovora* and purified about 30-fold to apparent homogeneity employing polyacrylamide gel electrophoresis. The methods used in the sequence were DEAE cellulose chromatography, sephadex G-200 gel filtration, hydroxylapatite ion-exchange and affinity chromatography on sepharose CL-6B. Extraction of L-asparaginase (yield 60%) was done by treating enzyme with acetone. This acetone treated powder of cells was extracted in potassium phosphate buffer (pH 8.0) containing NaCl for obtaining cell free preparation. Crude extracts of *Thermoactinomyces vulgaris* 13 MES (Mostafa and Ali, 1983) were prepared after filtration of cultures and grinding the cells with sand, alumina or glass beads, by rapid freezing and thawing, by rapid mixing and also by exposure to ultrasonic waves. Tiwari and Dua (1996) purified L-asparaginase from *Erwinia aroideae* NRRL B-138 to apparent homogeneity by ammonium sulphate precipitation, chromatography on sulphopropyl-sephadex C-50 and G-200 with 22% recovery and
567-fold purification. L-asparaginase from *Pseudomonas stutzeri*, after initial ammonium sulphate fractionation, was purified by consecutive column chromatography on sephadex G-200, calcium hydroxyapatite and DEAE sephadex A-50 (Manna *et al*, 1995). Lee *et al* (1985) purified L-asparaginase form crude acetone treated extracts of *Erwinia carotovora* by CM-sepharose fast flow column chromatography with a pre-column of CDR (Cell Debris Remover) modified cellulose. An affinity column of L-asparagine coupled epoxy-activated sepharose CL-4B further purified the partially purified enzyme. In a patent report (Goward, 1994), a detailed process of *E. chrysanthemi* L-asparaginase (59%) purification comprises alkaline extraction of enzymes from the cells, acid precipitation followed by Sepharose fast flow chromatography. *Corynebacterium glutamicum* L-asparaginase was purified 98-fold by protamine sulphate precipitation, DEAE -sephacel anion exchange, ammonium sulphate precipitation and sephacryl S-200 gel filtration. Partially purified preparations kept their activity when frozen at -80°C but lost their 50% activity at 4°C (Mesas *et al*, 1990).

The intracellular L-asparaginase of *Cylindrocarpon obtusisporum* MB-10 was purified 65-fold from cell free extract with an overall yield of 11.4% by acetone fractionation, ammonium sulphate precipitation and two chromatographic runs on DEAE Sephadex A-40 columns (Raha *et al*, 1990). Guy *et al* (1984) reported a method for separation of stereo specific D-asparaginase from a mixture of D-asparaginase and L-asparaginase. Joner (1976) purified L-asparaginase from *Acinetobacter calcoaceticus* using various techniques, which include precipitation with streptomycin, chromatography on DEAE-cellulose, gel filtration on agarose and chromatography on phosphocellulose.
Asparagine catabolism in Bryophytes has been studied and purification sand characterization of two forms of L-asparaginase, L-asparaginase-I and L-asparaginase-II, obtained from *Sphagnum fallax* was carried out by anion-exchange chromatography by Heeschen *et al*., (1996). They observed that the pH optimum of the enzyme was 8.2 and its molecular weight was 126,000 Da. It had characteristics that were intermediate between those from higher plants and those from microorganisms. L-asparaginase from *Thermus thermophilus* had a dual L-asparaginase as well as kinase activity. It was purified and its apparent molecular weight by SDS-PAGE was found to be 33 kDa by Prista and Kyriakiodis (2001). Purification of the L-asparaginase from *Pseudomonas aeruginosa* by Sephadex G-100 gel filtration and SDS-PAGE analysis of the protein was performed by El-Bessoumy, Sarhan and Mansour (2004) and its molecular weight was determined to be 160 kDa.

L-asparaginase from marine actinomycetes, *Streptomyces sp.* PDK2 was partially purified using Ammonium Sulphate precipitation, Dialysis and Gel Filtration on Sephadex G-50 and Sephadex G-200 (Dhevagi P and Poorani E., 2006). The purified L-asparaginase was characterized by SDS-PAGE and its molecular weight was found to be 140 kDa. Purified L-asparaginase from *Streptomyces gulbargensis* (Amena *et al*., 2010) and from *Streptomyces albidoaviris* (Narayana *et al*., 2007) exhibited a molecular weight of 85 kDa and 112 kDa respectively. L-asparaginase from marine *Actinomycetes sp.* designated as S3, S4 and S6 was purified and characterized by Ammonium Sulphate Precipitation, Dialysis, Gel Filtration on Sephadex G-100 and SDS-PAGE (Saleem Basha *et al*., 2009). The optimum pH was found 7.5 and optimum temperature as 50°C. The *Km*
and $V_{\text{max}}$ determined by Lineweaver-Burk plot were approximately 24 µM and 51 IU/ml respectively. Furthermore, it was found that the enzyme was activated by Mg$^{2+}$ but inhibited by Cu$^{2+}$, Zn$^{2+}$ and EDTA. L-asparaginase from *Bacillus sp* was partially purified by Ammonium Sulphate Precipitation, Dialysis and Ion-exchange chromatography using DEAE column (Vidy Moorthy *et al.*, 2010). The purified enzyme was characterized by SDS-PAGE and its molecular weight was determined approximately 45 kDa. L-asparaginase from *Aspergillus sp*. under submerged fermentation was purified using various steps including Ammonium sulphate precipitation, dialysis and column chromatography (Azad Chandrasekhar P, 2012). The purified enzyme was characterized by SDS-PAGE and its molecular weight was determined to be above 94 kDa.

The production of L-asparaginase enzyme was carried out by using L-asparagine as a substrate with the help of *E. coli*, isolated from sewage (Debajit Borah *et al.*, 2012). The enzyme was purified by salt precipitation, dialysis and ion exchange chromatography. The enzyme activity was found to be 0.039 U/ml/min and the molecular weight was determined as 153 kDa with the help of SDS-PAGE. The Optimum temperature and pH was recorded as 55°C and 6 respectively. The study has shown the capability of the enzyme to withstand high temperature and hence can be considered thermo-stable enzyme.

The expression, purification, and characterization of L-asparaginase (Ans-A) from *Rhizobium etli* was reported (Moreno-Enriquez A *et al.*, 2012). The enzyme was purified
to homogeneity in a single-step procedure involving affinity chromatography, and the kinetic parameters K(m), V(max), and k(cat) for L-asparagine were determined. The enzymatic activity in the presence of a number of substrates and metal ions was investigated. The molecular mass of the enzyme was 47 kDa by SDS-PAGE. The enzyme showed a maximal activity at 50 degrees C, but the optimal temperature of activity was 37 degrees C. It also showed maximal and optimal activities at pH 9.0. The values of K(m), V(max), k(cat), and k(cat)/K(m) were $8.9 +/-.0967 \times 10^{-3}$ M, $128 +/-.2.8$ U/mg protein, $106 +/-.2$ s$^{-1}$, and $1.2 +/-.0.105 \times 10^4$ M$^{-1}$s$^{-1}$, respectively. The L-asparaginase activity was reduced in the presence of Mn$^{2+}$, Zn$^{2+}$, Ca$^{2+}$, and Mg$^{2+}$ metal ions for about 52% to 31%. In addition, it was found that NH$_4^+$, L-Asp, D-Asn, and beta-aspartyl-hydroxamate in the reaction buffer reduced the activity of the enzyme, whereas L-Gln did not modify its enzymatic activity. This is the first report on the expression and characterization of the L-asparaginase (AnsA) from *R. etli*. Phylogenetic analysis of asparaginases reveals an increasing group of known sequences of the Rhizobial type asparaginase-II family.
2.11. ACTIVATORS AND INHIBITORS OF L-ASPARAGINASE:

It has been reported that there are some elements which affect the activity of the enzyme, some activates the enzyme and increase the activity up to many folds and some inhibit the activity. Various elements have been reported to this reference. Raha S. K. et al., (1990) isolated the enzyme from mesophilic fungus *Clyndrocarpon obtusisporum* MB-10, pI-5.5, optimum pH and temperature - 7.4 and 37°C. The enzyme was tetramer of four identical subunits, a conjugate protein wit 37.3% (w/w) carbohydrate. They reported metal ions, such as Zn$^{2+}$, Fe$^{2+}$, Cu$^{2+}$, Hg$^{2+}$ and Ni$^{2+}$ potentially inhibited the enzyme activity, while metal chelators like EDTA, CN-, cysteine, etc., enhanced the activity indicating that the enzyme was not a metalloprotein.

Its activity was also enhanced in the presence of reduced glutathione but not with dithiothreitol and 2-mercaptoethanol. From *Tetrahymena thermophila* two sub types of L-Asparaginase have been isolated by Tsavdaridis I. K. et al., and they were purified to near homogeneity. Optimum pH of enzymes was reported as 8.6. Both forms cross reacted with antibodies raised against T. pyriformis L-Asparaginase and show isoelectric points 7.4 and 8.2. Among the metals tested, Ca$^{2+}$ is the most effective in activating L-Asparaginase I or II activity (Tsavdaridis I. K. et al., 1994). Co-occurrence of both subtypes of L Asparaginase has been seen in Arabidopsis by Bruneau *et al*., The catalytic activity of L-Asparaginase subtypesis found to be dependent on K$^+$. It has also been reported that catalytic activity of L-Asparaginase is ten folds in recombinant At3g16150 in presence of K$^+$ and becomes 80 folds in presence of L-Asn (Bruneau L. *et al*., 2006). Various other elements including Cu$^{2+}$, diphosphate, EDTA, I$, Li^+$ and Mg$^{2+}$ have been
reported to influence activity of L-Asparaginase, isolated from different sources, directly or indirectly.

The enzyme activity was determined in the presence of different thiol compounds (Suchita C et al., 2010). A lower concentration of GSH (reduced glutathione) and NAC (N-acetyl cysteine) in the medium has exhibited stimulatory effects on L-asparaginase catalytic activity but inhibited by inhibited by p-chloromercurybenzoate (PCMB) and iodoacetamide (IA).
2.12. MECHANISM OF ANTI-NEOPLASTIC ACTION OF L-ASPARAGINASE:

L-asparaginase converts L-asparagine to L-aspartic acid (Fig-2.2). As several types of tumour cells require L-asparagine as an essential amino acid for protein synthesis, they are deprived of an essential growth factor in presence of L-asparaginase and they fail to survive. Effective depletion of L-asparagine results in cytotoxicity for leukemic cells. Asselin et al., (1989) have quantified killing of acute lymphoblastic leukemic cells both in vitro and in vivo in patients with acute lymphoblastic leukemia undergoing treatment with L-asparaginase as a single agent. Besides these specific effects, L-asparaginase can exert immuno-suppressive effects in general. Chakrabarty and Friedman (1970), using an animal model, demonstrated the depression of both humoral and cellular immune reactions.

In humans, acute lymphoblastic leukemia cell lines are markedly inhibited by Asparaginases, the effect being 10-fold higher for Erwinia carotovora L-asparaginase (Han and Ohnuma, 1972). Cell cycle arrest in G1 phases, which results in apoptosis of leukemia cells, is induced by L-asparaginase (Ueno et al., 1997). E. coli L-asparaginase has been found to phosphorylate endogenous polypeptides in immune cells. Products of L-asparaginase especially NH₄⁺ ions diffuse into the cytosol and modify the pH, which activates the signal transduction pathways associated with phosphorylation of substrates (Mercado and Arenas, 1999). Kelo et al., (2002) have reported L-asparaginase action on polypeptides and their effect on metabolism in human body. While these in vitro results indicate significant differences among preparations in vivo, the immuno-suppressive effects in animals are comparable.
Fig-2.2: Schematic illustration of the mechanism of action of L-asparaginase (Narta et al., 2007)
2.13. TREATMENT WITH L-ASPARAGINASE:

Current treatment protocols of acute lymphoblastic leukemia and lymphosarcoma do not employ L-asparaginase as a single agent. In fact, it is always a part of multiple agent regiments and thus combined with drugs having definitive immune-suppressive effects. Enhanced polymerization rate of fibrin monomers or fibrin clottability by L-asparaginase treatment has been reported by Strapinni 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). There has been a modification of anti-leukemic treatment by means of injecting doses of ECA bound to patient’s own isolated immune cells. L-asparaginase in combination with methotrexate has shown synergistic anti-leukemic activity in a schedule dependent fashion (Aguayo et al., 1999).

In humans, acute lymphoblastic leukemic cell lines have been markedly inhibited by asparaginases. Cell cycle arrest in G1 phases, which results in apoptosis of leukemic cells, is induced by L-asparaginase (Ueno et al., 1997). *E. coli* L-asparaginase has been found to phosphorylate endogenous polypeptides in immune cells. Products of L-asparaginase specially NH$_4^+$ ions diffuse into the cytosol and modify the pH, which activates signal transduction pathways associated with phosphorylation of substrates (Mercado and Arenas, 1999). Kelo et al., (2002) have reported L-asparaginase action on peptides and their effect on metabolism in human body. L-asparaginase in lidocaine decreases the pain intensity of an intramuscular injection in children without changes in bio availability and absorptive rates of the enzyme. L-asparaginase has been found to be effective in nasal type leukemia treatment as well (Young et al., 2003). Hyankuna et al., (2004) have reported successful asparaginase treatment followed bone marrow transplantation in
leukemia patients. Deamination of glutamine may enhance the anti-leukemic effect of L-asparaginase. Asparagine levels have been found to be strongly correlated with plasma L-asparaginase activity, even at low enzyme activities of < 50 IU/l (Tsurusawa et al., 2004). Asparagine levels have an inverse relation with L-asparaginase activity and chemotherapy. L-asparaginase results in decreased asparagine, glutamine and five other amino acid levels in pediatric patients with acute lymphoblastic leukemia. Correlation between the presence of anti-asparaginase antibodies and L-asparaginase activity has been observed by Zalewska, Beata and Bodalski (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).

The view that selectivity to treatment with L-asparaginase is due to lower asparagine synthetase expression has been supported by studies carried out by Leslie et al., (2006), where they reported that asparagine synthetase mRNA levels were higher in acute myeloid leukemia (AML) than acute lymphoblastic leukemia (ALL) blasts in both children and adults, with intermediate levels in normal peripheral blood mononuclear cells (NPBMC). The effect of two different L-asparaginase preparations, native E. coli L-asparaginase (Crasnitin; Bayer AG, Leverkusen, Germany; n=10) and L-asparaginase derived from Erwinia chrysanthemi (Erwinase; Porton Products, London, UK; n=10), on the changes in parameters concerning hypercoagulability was evaluated in ALL patients by Appel et al., (2006). A significant decrease in [alpha] 2-antiplasmin and plasminogen levels was measured in E. coli L-asparaginase but not in Erwinase-treated patients. Results of the Dana-Farber Cancer Institute ALL Consortium Protocol 95-01 for children
with acute lymphoblastic leukemia have shown that *Erwinia* L-asparaginase is less toxic than *E. coli* L-asparaginase. Steiner *et al.*, (2006) have 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.

GCN2 protein kinase is required to activate amino acid deprivation responses in mice treated with L-asparaginase (Piyawan Bunpo *et al.*, 2009) L-asparaginase reduces protein synthesis by increasing phosphorylation of eukaryotic initiation factor 2 (eIF2). Phosphorylation of eIF2 requires eIF2 kinase GCN2 (general control nonderepressible 2) and PERK (protein kinase R-like endoplasmic reticulum kinase).

The dose-and time-dependent antitumor and cytotoxic effects of L-asparaginases from *Erwinia carotovora* (ECAR LANS) and *Escherichia coli* (MEDAC) have been investigated using human leukemic cells and human and animal solid tumor cells (O. Yu. Abakumova, *et al.*, 2012). These included human T-cell acute lymphoblastic leukemia cell lines (Jurkat, Jurkat/A4, Molt-4), human chronic myeloid leukemia K562 cells, human promyelocytic leukemia HL-60, and also human solid tumor cells (prostate carcinoma LnCap, breast adenocarcinoma MCF7, ovarian adenocarcinoma SCOV-3 and carcinoma CaOV, hepatocarcinoma Hep G2, fibrosarcoma HT-1080) and animal solid tumor cells (rat Gasser’s ganglion neurinoma cells GGNC-1, mouse glioblastoma EPNT-5). It was investigated that sensitivity of tumor cells (seeded at different density) to L-asparaginases, as well the effect of L-asparaginases on cell growth rate, protein and
DNA synthesis in the presence of various cytostatics. Cell cycle analysis by flow cytofluorimetry and detection of apoptotic cells before and after treatment with L-asparaginases indicate that ECAR LANS L-asparaginase suppressed growth of all tested solid tumor cells. Evaluation of leukemic cell number after treatment with L-asparaginases for 24 hr, 48 hr and 72 hr demonstrated that asparagine deficiency did not kill cells but stopped normal cell division. The cytofluorometric study of solid and leukemic cells revealed that except HL-60 cells the treatment with L-asparaginase for 72 hr did not change cell cycle phase distribution and did not increase the number of apoptotic cells. Combined treatment of cells using a combination of L-asparaginase and doxorubicin significantly increased the number of apoptotic cells up to 60% (MCF-7 cells), 40% (Jurkat cells) and even 99% (HL-60). High levels of DNA and protein synthesis rates in asparaginase-treated tumor cells suggest lack of massive entry of tumor cells to apoptosis. This conclusion is based on the fact of sensitivity of multi-resistant Jurkat/A4 cells to L-asparaginases (it is nearly impossible to induce apoptosis in these cells). Since ECAR LANS did not influence growth of normal human fibroblasts it appears that the enzyme cytotoxicity is associated only with asparagine deficiency.

Yu. M. et al., 2012 reported that L-asparaginase inhibits invasive and angiogenic activity and thus induces autophagy in ovarian cancer. Their work identified L-asparaginase, as a putative therapeutic target for ovarian cancer. They suggested that L-asparaginase, a dysregulator of glycosylation, would interrupt the local microenvironment, affecting the ovarian cancer cell-endothelial cell interaction and thus angiogenesis without cytotoxic effects.
2.14. SIDE EFFECTS OF L-ASPARAGINASE THERAPY:

Besides minor side effects such as an allergic reaction and vomiting, L-asparaginase therapy of acute lymphoblastic leukemia has some serious side effects. Onset of venous thrombosis in children undergoing histopathologic disease due to L-asparaginase 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 enterocolitis (NE) has been observed as an unusual complication of neutropenia, associated with leukemia and lymphoma (Radulovic et al., 2004). Ikarashi et al., (2004) reported tubular and glomerular dysfunction due to ALL chemotherapy. Myocardial ischemia has been reported in a patient with acute lymphoblastic leukemia (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 patients has been attributed to L-asparaginase therapy (Imamura et al., 2005). Acute hepatic dysfunction (Aoki, Bowlus and Rossaro, 2005) and immune deficiency in children with ALL have been other major side effects of L-asparaginase therapy.

L-asparaginase therapy can be associated with some side effects including hyperglycemia, hyperlipidemia, hypofibrinogenemia, coagulation abnormalities, hypoalbuminemia, allergic reactions and thromboembolic events (Abdullah Bani-Hashem et al., 2009).
Treatment with L-asparaginase is often times accompanied by secondary complications including hepatic dysfunction, neurologic seizures, pancreatitis and immune-suppression. These side effects are largely due to inherent glutaminase activity of the enzyme, causing depletion of the conditionally essential amino acid Glutamine (Bunpo P. et al., 2009). The glutaminase activity of the L-asparaginase can be eliminated by modification of the enzyme.

The possible side effects of L-asparaginase in the treatment of acute lymphoblastic treatment were investigated and the correlation of these side effects was explored at different therapeutic stages by means of retrospective analysis, so as to reduce the incidence of side effects and improve the safety of chemotherapy and long term survival of patients.

An allergic response to L-asparaginase is not unusual because L-asparaginase is derived from *Escherichia coli* and is often recognized as a foreign protein. The hypersensitivity induced by L-Asp is of the immediate type in most cases. It was reported on a 5-year-old girl who was hospitalized for precursor T-cell lymphoblastic leukemia (Hidehiko Narazaki et al., 2012). She was treated according to a Tokyo Children’s Cancer Study Group protocol (TCCSG ALL L09-1603 HEXBFM). During the intensification phase, blisters with erythema developed on the arm proximal to the catheter insertion site owing to a delayed-type hypersensitivity reaction caused by intravenous L-Asp administration. She was treated with additional methylprednisolone, tapered dexamethasone, and an antihistamine for the allergic reaction. No asparaginases other than *E. coli* L-Asp have been approved for use in Japan. Other asparaginases, such as polyethylene glycol L-Asp
and *Erwinia* L-Asp should be quickly approved for use as alternative chemotherapy reagents in Japan.

Hypersensitivity reactions to asparaginase preparations are not rare and can be serious. The risk of a severe allergic reaction changes depending on prior exposures and concomitant medications (corticosteroids). In induction regimens that use high-dose steroids, allergic reactions to asparaginase are infrequent. In 1500 adults treated on the Medical Research Council UKALL XII/Eastern Cooperative Oncology Group E2993, a 90% induction success was reported for a well-tolerated induction regimen that employed high-dose prednisone and multiple injections of L-asparaginase. There was no mention of allergic reactions to asparaginase. Similarly, multiple authors have noted no severe allergic reactions to PEG-asparaginase in adults during asparaginase therapy (Rijneveld AW *et al.*, 2011). It seems likely that with repeated exposures and while patients are not receiving corticosteroids, allergic reactions become more prominent and probably occur in approximately 5%–10% of patients. An allergic reaction rate of 10% or greater is more in keeping with the larger pediatric experience with asparaginase. A purported advantage of PEG-asparaginase is that it is less immunogenic than L-asparaginase. Still, severe allergic reactions clearly occur in patients who get repeated doses of PEG-asparaginase.

Patients who have allergic reactions to L-asparaginase or PEG-asparaginase may be successfully treated with *Erwinia* asparaginase. In the main, antibodies to PEG-asparaginase and L-asparaginase do not cross-react with *Erwinia*. Allergic reactions to *Erwinia* can occur in up to one third of children (Vrooman LM *et al.*, 2010). Much less is known about the incidence of severe allergic reactions in adults treated with *Erwinia* L-asparaginase after prior severe allergic reactions to the other asparaginases.
2.15. RESISTANCE TO L-ASPARAGINASE:

A cell line resistant to L-asparaginase expressed high levels of asparagine synthetase activities reported by Andrulis, Argonza and Cairney (1990). This was due to increased expression but without amplification of the genes encoding asparagine synthetase. Tumour cells can develop the potential to synthesize L-asparagine intracellularly, which makes the cells resistant to the action of L-asparaginase (Broome, 1963). The degree of methylation of cytosine residues in DNA regulates the expression of L-asparagine synthetase, the enzyme responsible for L-asparagine synthesis. Holleman et al., (2004) reported that drug resistance in acute lymphoblastic leukemia (ALL) is associated with impaired ability of cells to induce apoptosis. Also, PARP and Procasptase-2 is related to drug resistance in childhood ALL. 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 anthracyclines, vincristine and L-asparaginase. An in vitro chemo-sensitivity assay is a good indicator of cellular response to chemotherapy. In rat serum cells and in ARJ cells, L-asparaginase treatment depletes cellular asparagine; also, cellular glutamine levels have been severely found to be 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. GS may thus form a target for the suppression of 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. 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). Epigenetic changes (changes involved in the expression of genes) in the repression and induction of asparagine synthesis in human leukemic cell lines have been studied and may be targeted for studying L-asparaginase resistance. Fine et al., (2005) also described that L-asparaginase resistance can be studied by targeting epigenetic changes. Researchers have reported that the down regulation of asparagine synthetase expression can increase the sensitivity of cells resistant to L-asparaginase.

Obesity impairs efficacy of L-asparaginase in leukemia treatment as reported by Michael Kaufman, May 20, 2013. Findings suggest that obesity impairs the ability of L-asparaginase to kill leukemic cells reported online in the journal cancer Cancer Research by investigators from the Saban Research Institute, Children’s Hospital Los Angeles (April, 2013). In their study, Ehsanipour EA and colleagues (2013) investigated the adipocytes which produce significant amounts of Glutamine counteract the effects of L-asparaginase. Their findings suggest that adipocytes work in conjugation with other cells of the leukemia microenvironment to protect leukemia cells from L-asparaginase treatment.

L-asparaginase strongly affects the bioenergetics pathways of leukemic cells (Ivana Hermanova et al., 2012). The data shows that L-asparaginase inhibits mTORC1 inhibitor. It decreased c-myc-regulated glycolysis and increased oxidative phosphorylation. These
metabolic changes are mainly caused by the effort of the cells to mobilize another mitochondrial pathway as Krebs cycle and β-oxidation, with the aim to supply depleted amino acids. We conclude from these data that resistance of the cells is caused by better biochemical adaptability to the nutrient deprived environment.
2.16. MODIFICATION OF NATIVE L-ASPARAGINASE:

The native enzyme has very less half-life value and high immunogenicity, which has coerced the researchers to modify the enzyme, resulting in its increased half-life, reduced immunogenicity and better storage stability. Attempts were made for the chemical modification of the enzyme by Bluma, Zina and Zhagat (1975). They modified the enzyme with N-bromosuccinimide, which inhibited activity. Covalent attachment to the fibres of a hollow fibre hemodialyzer resulted in ready access of the substrate to the enzyme (Jackson et al., 1979). Reductive alkylation had no effect on thermal stability and pI of *E. coli* L-asparaginase. However optimum pH and catalytic activity were altered (Shprunka et al., 1980). Immobilization of the enzyme by polyacrylamide increased its stability to denaturation and proteolysis (Galaev, Chuplygina Klement’Eva, 1981). Covalent attachment of poly-D-alanine peptides to lysyl residues on the surface of *Erwinia carotovora* L-asparaginase decreased the immunogenicity of the enzyme (Uren, Hargis and Beradsley, 1982). This led to the increase in the efficiency of the action of the enzyme and acted as adjuvants for the production of antibodies. However, a decrease in the activity of the enzyme was reported when modification of the tyrosyl residues and carboxyl groups was carried out as reported by Qian et al., (1984). Immobilization on polyglucin led to greater thermal stability and storage stability as well as greater antigenic affinity. The therapeutic potential of L-asparaginase was greatly improved by complexing with dextran sulphate. The enzyme had good substrate specificity, increase thermal stability, better storage stability and was resistant to proteolysis (Karsakevich et al., 1986).
Nerkar and Gangadharan (1989) coupled *Erwinia* L-asparaginase with human serum albumin. The complex was enzymatically active but had a 10-fold lower Km value. Acylation has also been applied as a method L-asparaginase modification. Immunological and pharmacokinetic data regarding this procedure is not yet available.

Immobilization of the enzyme on soluble LM cellulose by using azide binding was carried out by Karsakevich *et al.*, (1987). Electrophoretic, enzymatic and immunologic properties of the CM cellulose asparaginase depend on the amount of the enzyme in the polymer conjugate of the enzyme. Yoshimoto *et al.*, (1987) chemically modified the enzyme by coupling a magnetic modifier to amino groups of L-asparaginase and the anti-leukemic activity of the of enzyme was further improved by immobilizing the enzyme on water-soluble vinylpyrrolidone and acrolein copolymer and the enzymatic activity depended on the amount of the acrolein radicals in the copolymer. The anti-leukemic activity of L-asparaginase was studied when it was covalently bound with water-soluble CM-cellulose (Karsakevich *et al.*, 1988) and the activity depended on the amount of polymer bound to the enzyme. Modification of L-asparaginase by acetic anhydride, dextran and monomethoxy PEG has been reported by Cao *et al.*, 1990. Chemical modification of the enzyme by acylation increased the catalytic activity of the enzyme (Martins *et al.*, 1990). There had been a noticeable increase in the half-life of the enzyme when it was chemically modified by N, O-carboxy methyl chitosan in the presence of L-aspartic acid (Qian *et al.*, 1996). Holle (1977) reported that PEG modified L-asparaginase was extremely efficient, so much so that when given intracellularly or intravenously it proved to be a good alternative to native L-asparaginase in patients
allergic to acute lymphoblastic leukemia treatment. PEG and Dextran, being macromolecular modifiers, are better than the small molecular modifier acetic anhydride. Fernandes and Gregoriadis (1997) reported that chemical modification by polysialic acid improved therapeutic use by enhancing potentially beneficial anti-tumor activities of L-asparaginase. The modified enzyme had improved functionality. The biologically active fructose polymer levan, from \textit{Z. mobilis}, was covalently coupled to L-asparaginase from \textit{Erwinia carotovora} and the Km of the native enzyme and the pH-optima range widened, thermo-stability increased but the electro-phoretic mobility was reduced (Vina, Karsakevich and Bekers, 2001).

Intravenous infusion in mice resulted in an effective elimination of L-asparagine for a period of two weeks (Kravtzoff \textit{et al.}, 1990). However, the immunogenicity of the enzyme seems to be only slightly diminished. The doses of 150-200 IU per kg body weight results in effective elimination of L-asparaginase for 50 days.

L-asparaginase entrapped in liposomes can be expected to display reduced immunogenicity and toxicity. Encapsulation of L-asparaginase in small liposomes with a median diam of 158-158 nm resulted in marked prolongation of \textit{in vivo} half-life in animals, avoidance of induction of L-asparaginase antibodies and enhanced antitumor activity. Encapsulation of L-asparaginase into PHB nanocapsules and study of adverse effects and anaphylaxis in mice was performed by Baran, Ozer and Hazirki (2002). Soares \textit{et al.}, (2002) have reported that modification with PEG changed physio-chemical and biological properties of the enzyme resulting in less immunogenicity and a longer
half time of plasmatic life. The stability of enzyme improved as well. In polynanospheres, biological activity of L-asparaginase improved but the release profile was altered significantly (Wolf et al., 2003) by co-encapsulation of stabilizers. Improved biological activity and improved stability was also observed. Zhang, Shi and Wei (2004) studied immobilization of L-asparaginase on silk particles (Sericin) from Bombyx mori silkworm, resulting in covalent binding of the enzyme. It was also reported that modification by PEG resulted in increased half-life and abolished immunogenicity. Also, the modified enzyme had more activity than native L-asparaginase. Bio-conjugation of silk fibroin significantly helps to reduce the immunogenicity and antigenicity of the enzyme. Zhang et al., (2004) also reported the covalent attachment of the silk sericin peptides to L-asparaginase (ASNase) producing silk sericin-L-asparaginase (SS-ASNase) bioconjugates that are active, stable, have a lower immune response, and have extended half-lives in vitro in human serum.

The use of L-asparaginase has been limited by a high rate of hypersensitivity reactions and development of neutralizing anti-asparaginase antibodies, and by the need of frequent administration. To overcome these limitations modified versions of L-asparaginase (such as asparaginase from other sources, pegylated formulations, and asparaginase loaded into erythrocytes) have been recently proposed (Thomas X et al., 2010).

Asparaginases are a cornerstone of treatment protocols for acute lymphoblastic leukemia (ALL) and are used for remission induction and intensification treatment in all pediatric regimens and in the majority of adult treatment protocols. Extensive clinical data have
shown that intensive asparaginase treatment improves clinical outcomes in childhood ALL (Rob Pieters MD et al, 2011). Three asparaginase preparations are available: the native asparaginase derived from *Escherichia coli* (*E. coli* asparaginase), a pegylated form of this enzyme (PEG-asparaginase), and a product isolated from *Erwinia chrysanthemi*, i.e, *Erwinia* asparaginase. Clinical hypersensitivity reactions and silent inactivation due to antibodies against *E. coli* asparaginase, lead to inactivation of *E. coli* asparaginase in up to 60% of cases. Current treatment protocols include *E. coli* asparaginase or PEG-asparaginase for first-line treatment of ALL. Typically, patients exhibiting sensitivity to one formulation of asparaginase are switched to another to ensure they receive the most efficacious treatment regimen possible. Erwinia asparaginase is used as a second-or third-line treatment in European and US protocols. Despite the universal inclusion of asparaginase in such treatment protocols, debate on the optimal formulation and dosage of these agents continues.
2.17. RECOMBINANT L-ASPARAGINASE:

Recombinant L-asparaginase has been produced by the use of molecular cloning and genetic engineering techniques. *E. coli* mutants resistant to substrate of L-asparaginase were studied by Spring *et al.*, (1986). It was found that the genes encoding L-asparaginase-I and L-asparaginase-II were not sequence related. Cloning of *E. coli* gene ANS-Bencoding L-asparaginase-II, using strategy based on PCR, and sequencing the gene was discussed by Bonthron (1990). The amino acid sequence differed from 11 positions from the data previously derived by direct amino acid sequencing. In plants, mainly *Lupin arborens*, isolation and characterization of cDNA clone encoding L-asparaginase from the developing seeds were reported by Lough *et al.*, (1992).

Expression of L-asparaginase-II encoded by ANS-B in *Salmonella enterica* was found to be positively regulated by a cAMP receptor protein (cRP) and anaerobiosis (Jennings and Beecham, 1993). The trypsin sensitive L-asparaginase can be made trypsin resistant by genetically fusing its gene with that of a single chain antibody derived from preselected antibody capable of providing protection against trypsin (Newsted *et al.*, 1995). The formation of the fusion protein L-asparaginase ScFv expressed in *E. coli* in the form of inclusion bodies was reported by Guo *et al.*, (2000). The fusion protein conferred steric hindrance, blocked cleavage sites and changed the electrostatic potential surface of the enzyme. Wang *et al.*, (2001) discussed the cloning and expression of L-asparaginase in *E. coli*. Cloning was done as a DNA fragment generated by PCR. The recombinant plasmid PASN, containing ASN gene and expression vector PBV 220, was transferred in *E. coli* host strains. Greater enzyme activity was observed in recombinant enzymes.
The L-asparaginase gene of the *E. coli* alpha-acetyl acetate decarboxylate gene (ALDC) of *B. bravis* were amplified by PCR and cloned into new vectors transformed into *S. cerevisia*. Most of the enzyme activities were secreted into the medium and the new vectors had excellent segregation stability (Zhao *et al.*, 2002). Abundance of asparagine synthetase (AS) mRNA was measured by RQ-PCR as reported by Irino *et al.*, (2004). The AS mRNA level paralleled the AS enzyme activity and the AS protein level. Cellular levels of AS synchronized with cellular resistance to L-asparaginase in cell lines. Recombinant L-asparaginase from *Erwinia carotovora*, and purified by Krasotkina *et al.*, (2004). The kinetic properties showed that recombinant L-asparaginase combined the main advantages of *Erwinia chrysanthemi* and *E. coli* L-asparaginase-II. Recombinant L-asparaginase was developed by cloning L-asparaginase from *Erwinia carotovora* NCYC 1526 (Er A) and expressing in *E. coli*. The enzyme was purified by anion-exchange chromatography and affinity chromatography on immobilized asparagine (Kotzia and Labrou, 2005). The Km and Vmax of the enzyme were also estimated. Its activation energies were found to be dependent on the substrate.

Recombinant human AS, that is C-terminally tagged has been prepared in a *Baculovirus* based expression system. The recombinant enzyme has high catalytic activity and offers a major possibility in identifying and characterizing inhibitors that may be used to treat asparagine resistant cells (Ciustea *et al*, 2005). Expression of recombinant L-asparaginase fused to pub leader sequence under the inducible T7 lac promoter in BLR (DE) host cells resulted in optimum extracellular production in shake flasks. The enzyme had 80% activity of the native (Khushoo, Pal and Mukherjee, 2005). Mapping B-cell epitopes and
ascertaining which of them are clinically relevant for triggering immune reactions, due to L-asparaginase-II of *E. coli* treatment of acute lymphoblastic leukemia, has been reported by Werner, Rohm and Muller (2005).

L-asparaginase from *Erwinia chrysanthemi* 3937 (Erl-ASNase) has been expressed in *E. coli* B L21 (DE3) pLysS (Kotzia and Labrou, 2006). The enzymatic and structural properties of the recombinant enzyme were investigated and the kinetic parameters [K(m), K(cat)] for a number of substrates were determined. The enzyme was later immobilized on epoxy-activated Sepharose CL-6B. The immobilized enzyme retained most of its activity (60%) and showed high stability at 4°C. Recently, studies have been carried out by Tang et al., (2006) for the development of a novel growth hormone releasing hormone (hGHRH) analog by 127 amino acid residues of the C-terminus from L-asparaginase, which act as a fusion partner gene for recombination with the Asp-Pro-hGHRH (1-44) gene synthesized by PCR method to form one kind of fusion protein with the unique acid labile linker Asp-Pro. The peptide so formed has been found to have good function-selectivity and species-specificity.

L-asparaginase production was improved by constructing recombinant over producing L-asparaginase strains through protoplast fusion technique between two highly L-asparaginase-producer local isolates, i. e., *Bacillus subtilis* and *B. cereus* (Wafaa K et al., 2010). The results obtained confirmed that protoplast fusion technique is an important tool in strain improvement. It was used to combine genes from different organisms for creating strains with desired properties. It also concluded that although the two parents
are the same, their fusants showed different levels of stability and expression of L-asparaginase which reflect the overall genetic background in these fusants. Depending on fusants genetic background, different environmental effects were identified to influence L-asparaginase activity.
2.18. SHORTAGE OF THE DRUG (L-ASPARAGINASE):

According to Silvestrini Anderson (December, 2013), president of Brazilian Society of Clinical Oncology, cure rates of cancer may fall if there is a shortage of L-asparaginase. According to Silvestrini, every year about 3000 people, both children and adults using this drug to cure different types of cancers. He adds that there is concern of shortage of this drug used in the treatment of cancer and believes that lack of interest to manufacture this drug may be why because it does not give the returns expected by the laboratories. In 2012, the U. S. president, Barak Obama, Congress was to report cases of shortage of this drug and it was reported that there is a substantial shortage of L-asparaginase drugs in some cases.

Lundbeck, a Danish international Pharmaceutical company discontinued the sale of Elspar in December 2012. Elspar is an asparagine specific enzyme, used as a chemotherapeutic in patients with acute lymphoblastic leukemia. The company stated that the decision to discontinue the sale of Elspar was a business decision. Recordati (an international pharmaceutical group) acquired several products including Elspar from Lundbeck in late 2012. However, the company made a business decision in early 2013 to discontinue Elspar and not to pursue its further manufacturing.

There should be an agreement between the countries regarding the manufacturing of this drug and should be produced for entire world, otherwise the cancer cure rates will fall if this drug is discontinued.
L-asparaginase has been a major research subject for many researchers world-wide. 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 acute lymphoblastic leukemia. A comparative examination of preparations of \textit{E. coli} L-asparaginase produced in USSR, Germany and Japan was made by Kondrat Eva (1984) and it revealed that the clinical characteristics of the preparation made in USSR and the preparation made in Germany (crasnitin) were identical. The antileukemic action of the preparation made in USSR was superior to the preparation (leumase) made in Japan. L-asparaginase made in the USSR and Germany was recommended for clinical use.

Extensive studies have been on the therapy by eliminating L-asparaginase, which is non-essential for normal cells from blood. This therapy is expected to cause no damage to normal cells. It is demonstrated that a variety of L-asparaginases are useful for the therapy of certain leukemias and solid tumors including acute lymphoblastic leukemia (Masao, 1986). This therapy got attention as a specific and favourable therapeutic idea, which represents inhibition of growth of leukemic cells without damaging normal cells. If means provided for avoiding the immune-reaction caused by the foreign protein, it is expected that the therapy will recover great hope. As a solution, a scheme has been proposed, in which blood is temporarily drawn out of the body, contacted with
immobilized L-asparaginase to decompose the asparagine dissolved in blood and then returned to the body (Maladkar et al., 1993). The whole potential of L-asparaginase has probably not been fully elucidated due to its treatment-limiting immunogenicity. Two major ways might be able to reduce this problem. One is to reduce the drug immunogenicity by chemical modification of relevant epitopes. The other would be to activate immune mechanisms involved in immune tolerance. If the details for such an approach will work out in the future, that will lead to an even more effective L-asparaginase.

A novel asparaginase, GLLAP localized to rat brain astrocytes and involved in astroglial production of the neuro-active amino acid aspartate, has been identified and characterized by Dieterich et al., (2003).

An innovative way of eliminating cancer-causing acrylamide from bread has been developed by researchers of the Dutch chemical firm (DSM), using gene technology to degrade L-asparagine, the free amino acid that is precursor to acrylamide, prior to baking. This involved cloning a strain of asparagine busting L-asparaginase from food-grade Aspergillus niger. The resulted strain was fermented and purified before being added to the dough. This resulted in considerable decrease in acrylamide levels (Dunn, 2004). A study by Taeymans et al., (2005) has shown the application of L-asparaginase in the food industry, where it is being used to determine acrylamide presence and acrylamide formation in food products.
A chimeric enzyme, \textit{AnsB-TTP-CETPC}, comprising asparaginase, tetanus toxin helper T-cell epitope and human CETP B-cell epitope, has been expressed as a soluble protein in \textit{E. coli} (Gaofu \textit{et al.}, 2006). The purified chimeric enzyme exhibited approximate 83\% activity of the native asparaginase. High yields of anti-CETP antibodies were induced in mice after immunization with three doses of the chimeric enzyme. The chimeric enzyme may have future use as vaccine against atherosclerosis.

In India, the team of Neelam Verma is the first to be engaged in the use of L-asparaginase for the development of novel diagnostic biosensor for the detection of levels of asparagine in leukemic cells.

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