

Production, Purification and Applications of L-asparaginases

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ABSTRACT

L-Asparaginase is found in many living organisms including animals, plants and microorganisms. Owing to the difficult extraction procedures, microorganisms are preferred for its industrial scale production. Commonly L-asparaginase is present in three dimensional tetrameric structure but other forms also exist depending on its different sources. Its biochemical properties are also dependent on the organism from which it is obtained. L-asparaginase acts on amino acid L-asparagine in two main steps, first an intermediate is formed and then it is attacked by a water molecule and is hydrolyzed into ammonia and aspartic acid. Deficiency of L-asparagine causes inhibition of nucleic acid synthesis in malignant cells which results in cell death. L-Asparaginase can be produced by both solid state and submerged fermentation, but the SSF is more economical and eco-friendly. Enzyme purification is done either by protein precipitation, dialysis, and chromatography or liquid-liquid extraction procedures. L-Asparaginase is involved in treating acute lymphoblastic leukemia (ALL). It is also used essentially in food industry, biosensing and amino acid metabolism.

Keywords: ALL, SSF, SmF, Biosensors, Antineoplastic

I. INTRODUCTION

L-asparaginases (E.C. 3.5.1.1) are present in a variety of living organisms like plants, animals (mammals, birds), microorganisms (bacteria, fungi, algae, yeast, actinomycetes) and some rodents, but not in humans. It is an amido-hydrolase which hydrolyzes amide group of asparagine to ammonia and aspartate. In 1904, Lang first found this enzyme and in 1922 Clementi first observed it in the blood serum of guinea pigs (Clementi, 1922). The discovery of L-asparaginase as an antitumor began in 1953 by Kidd, when lymphomas of rat and mice were treated with serum of guinea pigs (Kidd, 1953). Later in 1961, Broome showed that guinea pigs serum actually contained L-asparaginase enzyme of comparative substrate specificity which caused deterioration of tumor cells (Broome, 1963). Normal body cells synthesize L-asparagines by asparagines synthetase, but the lymphoma cells need its huge amount from the external source for their quick malignant growth. L-asparaginase causes its starvation, blocks protein synthesis and inhibit DNA and RNA, hence; the death of cancerous cells results. At industrial level, L-asparaginase is being produced from microbial species both by submerged and solid state fermentation, and purified by crystallization,

dialysis, ion exchange and gel filtration methods. SSF is preferred over SmF due to its cost effectiveness and high yield for industrial scale enzyme production. Some of the commercially available asparaginase include Colapase, Crasnitin, Pasum, Elspar and Pegasparagasum. There are many challenges in the production of such enzyme which may have fewer side effects, because prokaryotic enzyme often shows hypersensitivity, anaphylaxis, hyperglycemia, hypo-albuminemia, coagulation abnormalities, azotemia and immune inactivation problems (Haskel *et al.*, 1969).

L-asparaginase plays a significant role in pharmaceuticals when used in combination chemotherapy drug for treating lymphoblastic leukemia, acute myelomonocytic leukemia, reticulosarcoma, malignant diseases, melanosarcoma and Hodgkin's lymphomas (Appel *et al.*, 2007). In food industry, it prevents acrylamide formation, a neurotoxin, during high temperature (at 120°C) processing of starch containing foods. It also plays an essential role in the biosynthesis of amino acids (e.g., Aspartic amino acids) such as lysine, threonine and methionine (Kumar *et al.*, 2013).

A. Bacterial and Fungal Sources

L-asparaginase is produced by Gram positive as well as Gram negative bacteria found both in terrestrial and marine environments, but Gram negative species has got more importance. There are its two types produced by Gram negative bacteria. Type I includes cytosolic enzymes are quantitatively expressed and show activity on L-asparagine and L-glutamine amino acids. Type II is specific for L-asparagine under anaerobic conditions (Sanches *et al.*, 2001). *E. coli*, *Erwinia chrysanthemi*, *E. carotovora* and many other bacterial species are in clinical use, over years, as antitumor agents in the treatment of ALL (Narayana *et al.*, 2008). Fungi are potential source of L-asparaginases along with bacteria. Fungi, being eukaryotic closely resemble humans, therefore; the chances of immunological reactions are lesser. The main reason of their importance is that they produce enzyme extracellularly, so it is easy to extract and purify it (Shrivastava *et al.*, 2012).

B. Actinomycetes, Yeast and Algal Sources

Actinomycetes are present throughout in nature, soil and water but those which are present in living organisms are known to be good for their enzymatic activity. They are also considered a better source as compared to fungi and bacteria. L-asparaginases are also produced from yeasts such as *Pichia* sp., *Saccharomyces* sp., *Aspergillus* sp., *Rhodotorula*., *Spobolomyces* etc., exhibiting antitumor activities (Nigarethinam *et al.*, 1995). *Chlamydomonas* species and a yellow green alga, *Vaucheria uncinata* are known to produce L-asparaginase under specific conditions of temperature, pH and other environmental factors (Usha *et al.*, 2011).

C. Biochemical Sources

L-asparaginase occurs in different types of animals, plants and also in microorganisms. Mostly L-asparaginases also have activity of glutaminases, only about 3-9% of its total activity. The metabolism of both D-glutamine and D-asparagine occurs at the same active site of enzyme by hydrolysis of ester and amide bonds (Nakamura *et al.*, 1971). The weight of L-asparaginase of *E.coli* is about 1,38,000-1,41,000 Da. The enzyme obtained from *Erwinia* has different iso-electric points and hence; exhibits average immunological activity. The Michaelis constant in Michaelis-Menten Kinetics of asparaginase is 100 times lower than glutamine.

However, high asparaginase concentration does not deplete glutamine's serum (Whelan and Wriston, 1979). The PEG modified enzyme is utilized in hypersensitivity reactions to natural form of asparaginase. As the PEG add up 5000 Da to the molecular weight so the molecular weight of natural enzyme is slightly lower than the modified. The activity of enzyme depends upon the biological sources and conditions of the reaction (Yap *et al.*, 1981).

V. PHARMACODYNAMICS

The asparagine is hydrolyzed and converted in ammonia and aspartate, serum asparagines are also exhausted with the administration of asparaginase. Malignant cells are incapable to supply their own asparaginase supply because of their lowered asparaginase synthase activity (Miller *et al.*, 1969). The deficiency of asparagine results in the disfunctioning of protein synthesis and also causes the delay in the inhibition of synthesis of nucleic acids. It results in the cell death. Asparaginase metabolizes the glutamine and also shows relative substrate specificity (Yoshimoto, 1986).

VI. PHARMACOKINETICS

L-asparaginase is intra-muscularly and intra-venously introduced in malignant treatment disease. According to the researches, the concentration time curve shows plasma half-lives of 4 to 15 h, which is against the 1st order pharmacokinetics. Researchers also propose that after the enzyme uptake, redistribution into plasma takes place. There is another plasma activity of asparagines that supports the 1st order pharmacokinetics (Ohnuma *et al.*, 1970). A scientist named Broome found that there is low activity of serum and spleen of mice with high hepatic activity followed by the treatment by L-asparaginase. It was suggested that the large amount of asparaginase is eliminated through the recirculatory system (Broome, 1968). A scientist named Hall concluded after observing plasma activity of the asparaginases (*E.coli* and *Erwinia* asparaginase) that elimination actually takes place by reticulo-endothelial system. Brueck *et al.* supported this hypothesis with his research on plasma and urine samples taken after administration of asparaginase. The plasma activities were half with intramuscular administration than that

with intravenous administration (Hall, 1970). It was reported that with dose of about 5000 U/kg per day there is low asparaginase activity in CSF. Investigations performed on asparaginase treatment in monkey and patient concluded that asparaginase activity of 2,100 U/L results in depletion of Cerebrospinal fluid and the serum (Riccardi *et al.*, 1981).

A. Intramuscular and Intravenous administration

Intravenous and intramuscular routes were compared to observe therapeutic efficiency and side effect. It was observed that there is no significant difference but some anaphylactic reactions were observed. They might have occurred because of some protein contamination. The severe reactions were considered to be the relative difference between two modes of administrations (Loos and Borsos, 1972).

B. Drug Interactions

The interaction between drugs and asparaginase depends upon the enzyme induced deficiencies. The administration of those substances that inhibit the synthesis of protein, anti-metabolites and the alkylating agents have shown the capacity to damage the toxicity of these agents (Capizzi, 1974). It was experimentally found that both time interval and sequence between doses of anti-metabolite, methotrexate and asparaginase are effective in therapeutic terms (Capizzi and Cheng, 1981). The optimal time interval between asparaginase and MTX is 9-10 days when asparaginase is given before the MTX (Capizzi and Castro, 1974). Some experiments on animals have shown that late administration of asparaginase is linked with the defensive effect of healthy cells in body. This effect can be compared with rescue function of decreasing tetrahydrofolic acid (Capizzi, 1975). Some animal base experiments have confirmed the direct relationship between MTX and asparaginase sensitivity.

VII. L-ASPARAGINASE PRODUCTION FROM MICROORGANISMS

Microorganisms can be easily modified for the production of desired substances. For the production of L-asparaginase, different organisms like yeast, actinomycetes and some marine organisms can be used. These sources are used to avoid immunological

reactions by bacterial L-asparaginase (Savitri *et al.*, 2003).

A. Bacterial L-asparaginase Fermentation

Different bacterial strains specially *Erwinia aroideae* and one *Hydrogenomonas eutropha* are very efficient in producing higher amount of L-asparaginase (Peterson and Ciegler, 1972). Bacterial L-asparaginase (strains of *E. chrysanthemi* and *E. coli*) are used for treating Acute Lymphoblastic Leukemia. Different bacterial L-asparaginases possess different affinity, stability and pH. *E. coli* and *E. chrysanthemi* are known as main microbial production source at industrial scale. Bacterial asparaginase differs in their pharmacokinetics, biochemical and serological nature (Kumar and Sobha, 2012). Different factors may affect its production rate i.e pH, concentration of C and N sources, temperature, aeration, time and microbial agent (Zia *et al.*, 2013). Among bacteria, the *Enterobacteriaceae* members are the best for asparaginase production. The activity of asparaginase is increased to 21% by adding 6% n-dodecane with 80 % increase in *E. coli* concentration via 12.7%. The asparaginase production is stimulated through adding Asn, yeast or tryptone and 10g/L lactose as carbon source for *Erwinia aroidae* batch cultivation (Alegre and Minim, 1993). The rod shape, gram negative bacteria *Enterobacter cloacae* can utilize Asn as C & N source and produce asparaginase intracellularly (Nawaz *et al.*, 1998). *Enterobacter aerogenes* is also known to produce asparaginase with different C sources like glycerol, lactose, glucose and mannitol (Geckil *et al.*, 2005). However, it has lower activity of about 0.60 U/mL. Recently, solid-state fermentation is good alternative of submerged fermentation.

VIII. L-ASPARAGINASE PRODUCTION BY FERMENTATION

L-asparaginase production involves different parameters i.e., temperature, potential of hydrogen, concentration of oxygen and carbon dioxide (Bascomb *et al.*, 1975). The commercial production includes the techniques of fermentation and strain selection. Production of L-asparaginase includes two techniques: Submerged fermentation (SmF) and Solid-state fermentation (SSF).

A. L-Asparaginase Production by Submerged Fermentation (SmF)

Submerged fermentation requires optimized liquid medium for the growth of microorganisms. This process includes a closed reactor containing oxygen concentration and a liquid broth medium. It uses existing microorganisms. Bacteria are used because high moisture content is required for their growth. Fungal sources include *Aspergillus niger*, *Aspergillus terreus*, *Fusarium* and *Penicillium* (Patro and Gupta, 2012). Synthesis of L-asparaginase includes different factors such as temperature, pH, composition of nutrients and the time of fermentation (Kumar and Sobha, 2012). Production of L-asparaginase by using fungal species exhibits the highest activity of about 58IU/ml. the fungal species includes *A. terreus*. The agricultural waste corn steep is used as a source for its production. *Saccharomyces cerevisiae* ASP3 gene was cloned in *Pichia Pastoris* and its production was about 800U/g per cell mass that reduces the immunological reactions (Ferrara *et al.*, 2006). Table1 shows the L-asparaginase production by submerged fermentation using different microorganisms.

TABLE I. L-ASPARAGINASE PRODUCTION BY SmF

Microorganisms	Medium	Factors	Production	Citation
<i>A.terreus</i>	Proline medium 2	Temp.30°C, pH. 6.2	58.8U/L	Serquis <i>et al.</i> , 2004
<i>T. thermophilus</i>	Tryptone, yeast extract, glucose	Temp.70°C, pH. 7.0	840IU/mg protein	Prits and kyria-Kidis, 2001
<i>Streptomyces griseus</i> NIOI-VKMA29	Asparagine dextrose salt Medium	Temp. 38°C pH. 8.0	56.78IU/mL	Meena <i>et al.</i> , 2015
<i>Streptomycesgriseus</i> ATCC10137	Peptone 4.0	pH. 8.5 batch	117IU/L	Dejong, 1972.
<i>E. aroideae</i>	Tryptone, glucose, yeast	Temp. 28°C pH. 7.0	1250IU/g dry weight of soil	Peterson and Ciegler, 1969

B. L-asparaginase Production By Solid-state Fermentation

Solid-state fermentation requires optimized solid substrate with minimal content of moisture for the growth of microorganisms. Solid substrate is used as a source. SSF is used in different processes like

bioremediation, bio detoxification, production of enzymes and secondary metabolites. It uses waste and residues as a cheap source for microorganisms (Watanabe *et al.*, 2002). L-asparaginase produced by actinomycetes shows high activity by this technique (Basha *et al.*, 2009). Substrate used in this technique includes wheat bran, sesame oil, soy bean, meal and sugar cane. *Aspergillus niger* use glycine as a solid substrate and produced high amount of L-asparaginase. Environmental and nutritional factors are very important in its production. *Cladosporium* species use wheat bran for the production of L-Asparaginase and involve different factors such as pH, Temperature, concentration of oxygen and moisture show enzyme activity of 3.74U. Production of L-asparaginase by using sesame oil includes temperature and aeration rate etc. and shows enzyme activity of 310U/g. L-Asparaginase was also produced by *Pseudomonas aeruginosa* and purification was done by ammonium sulphate fractionation (El Bessoumy *et al.*, 2004). Solid state fermentation has disadvantage because scaling of bioreactor is difficult due to the mass and heat transfer in the system. Table2 indicates the L-asparaginase production by solid state fermentation using microorganisms and their substrates.

TABLE II L-ASPARAGINASE PRODUCTION BY SSF

Microorganisms	Substrate	Factors	Enzyme activity	Citation
<i>P. aeruginosa</i> 50071	Soy bean meal	Temp.37°C, pH.7.4, 40% w/v	142.18IU	Abdel Fattah and Olama, 2002.
<i>A. Niger</i>	Glycine max	Temp.30°C pH. 6.5	40.9 ±3.35U/g of dry substrate	
<i>Cladosporium</i>	Wheat bran	Temp. 30°C pH. 5.8, 58%w/v	3.74U	Kumar <i>et al.</i> , 2013.
<i>A. Terreus</i> MTCC 1782	Oil cake	Temp. 30°C pH. 6.2	30.35IU/ml	Baskar and Ren-ganathan, 2009.
<i>F. equiseti</i>	Soy bean meal	Moisture content 70%	8.51IU	Hosamani and Kaliwal, 2011.

IX. PURIFICATION OF L-ASPARAGINASE

L-asparaginase purification includes chromatography, alkaline lysis, ion exchange etc. the purification was done through cation-exchange chromatography followed by affinity chromatography, finally through dialysis (Lee *et al.*, 1986). L-asparaginase free of glutaminase was purified by ammonium sulphate fractionation to

avoid the effects of glutaminase. The process of ammonium sulphate fractionation was followed by DEAE column chromatography. The value of V_{max} is 4.45U/ μ g and for K_m is 0.657mM (Kumar *et al.*, 2011). L-asparaginase normally shows activity at pH 7-9 but enzyme produced from *T. thermophilus* shows activity at 9.2.

X. IMMOBILIZATION OF L-ASPARAGINASE

Inhibitory effect of L-asparaginase involves two major factors, half-life of enzyme and its substrate affinity (Chang, 2013). Polyethylene glycol and L-asparaginase conjugate does not bind to the active site but asparaginase attaches there to avoid allergic reactions. As a result, production of antibodies against asparaginase reduces and half-life of enzyme increases (Zhang *et al.*, 2004).

A. Downstream Processing

Downstream processes contribute to about 50-80% of the total yield of proteins. This can be improved by combination with other techniques of purification (Dutra-molino *et al.*, 2014).

B. Protein Precipitation

Precipitation is one of the oldest methods to purify the molecules. It causes disruption of the protein structure and forms precipitates. This process has advantages of low cost and simple equipment. Precipitant can also be recycled. Asparaginase from different sources required precipitation. The process start with addition of $(NH_4)_2SO_4$ to the enzyme extract. 35-100% of the salt concentration was added to the solution. Precipitation increased by about 26.9 fold with centrifugation. This process was followed by dialysis (Amena *et al.*, 2010). Ethanol can also be used as a precipitation agent.

C. Liquid-liquid Extraction

This process was achieved by aqueous two-phase systems (ATPS). It is defined as the removal of solute from the liquid phase during contact with the mixture of liquid. Affinity ligands are used for partition. ATPS is used for Asparaginase purification (Qin and Zhao, 2003). Cells are treated with 9.4%w/v K_2HPO_4 and 15% triton

X-100. Cell disruption is combined with homogenization for purification of this enzyme. This process increases the yield 52-73% and the enzyme activity by 78.60-94.80U/mg (Jian-Hang *et al.*, 2007).

D. Chromatography

High level of purification is achieved by this technique. About 68.9 fold asparaginase, from *Bacillus* sp., is purified by ammonium sulphate fractionation and column chromatography (Singh *et al.*, 2013). Asparaginase by *Cladosporium* was purified by cellulose ion exchange and enzyme activity was 83.3U/mg.

XI. APPLICATION OF L-ASPARAGINASE

Pharmaceutical Industry; Antineoplastic Action

L-asparagine hydrolyzes into ammonia and aspartate was observed in bovine's tissues. Later on L-asparaginase hydrolase was also confirmed and detected in horse and pig organs (Furth and Friedmann, 1910). Further L-asparaginase detection was carried out in guinea pig serum (Clementi, 1922). The antitumor activity of the enzyme was identified later on. L-asparaginase of *E. coli* had inhibitory capacity on tumors in rats (Mashburn and Wriston, 1964). L-asparaginase is also a chief chemotherapeutic substance for treating lympho-proliferative and lymphoma diseases. Figure 4, shows processing its processing in normal and cancerous cells.s

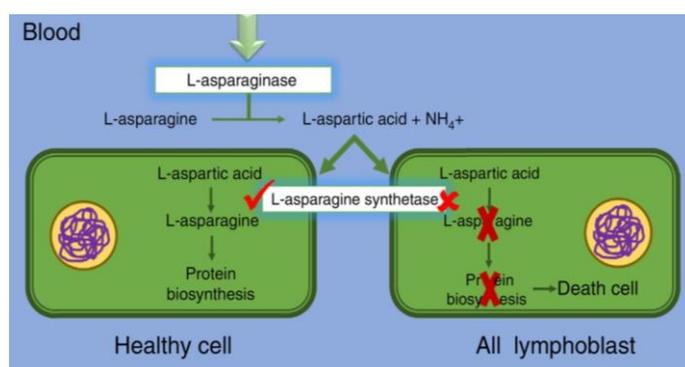


Figure 4: Antineoplastic action in blood

A. Anticancer Drugs

Use of L-Asparaginase as anticancer drugs is one of its most widely used applications. It is used in combination with vincristine and glucocorticoid to treat ALL. It is a

very important antitumor drug. It is used widely in treatment of malignancies such as Hodgkin's diseases, ALL (Schrappe *et al.*, 2000), Myelomonocytic leukemia, Melanosarcomas, Reticulosarcomas, Myelocyticleukemia, and Lymphosarcomas (Kidd, 1953; Broome, 1968). L-Asparagine is involved in protein synthesis and tumor cell growth. L-asparaginase has capacity to change L-Asparagine to aspartic acid. Due to the existence of L-Asparaginase, malignant cells become deficient in important growth factors, the depletion of asparagine, die off the cells. Although L-Asparaginase is present in many living organisms like animals, microorganisms and plants but asparaginase used in treatment of ALL, is taken only from *E. coli* species. Asparaginase is used for curing acute leukemias and non-Hodgkin lymphomas in 226 children aged between 6.1 and 14.0 years who suffered from stage III and IV lymphoblastic non-Hodgkin lymphoma. An analysis was carried out on the relationship between outcome and a variety of factors involving gender, disease stage, degree of WBC rise, Platelet count, CNS involvement, bone marrow involvement, primary tumor site, degree of LDH increase and before treatment allocation. On the basis of origination from the lymphatic cell system various analysis on patients with acute myelogenous leukaemia were reported who experienced protocol treatment. Another treatment was given to adult patients with myelomonocytic leukaemia. When they were treated with asparaginase monotherapy, some patients get a complete decrease, some achieved good partial reduction and duration reduced to only 1–3 months. L-asparaginase also performs an essential role in the biosynthesis of aspartate amino acids family. Amino acids that are synthesized by it are lysine, threonine, methionine. Krebs's cycle is used in its synthesis, but on the other hand it can also be produced by the activity of L-asparaginase enzyme (Sinha *et al.*, 2013).

XII. L-asparaginase Role In Biosensor

In order to analyze the levels of asparagine in leukemia or food industry, L-Asparaginase is used for expansion of a biosensor. Although there are various techniques for analysis of L-asparagine such as XPS, SEM, TEM and XRD, but use of biosensor is most effective one. Biosensor technology has many advantages over spectroscopy techniques, as it is more reliable and cheap while the spectroscopy techniques are high cost and less

favorable. Mode of action of biosensor is based on the activity of asparaginase. Hydrolysis of asparagines results into the production of ammonium ions and thus causes change in potential of hydrogen. This results into change in color (Kumar *et al.*, 2013).

XIII. L-asparaginase Role In Food Industry; Acryl amide Formation

It is also used in food industry. Acrylamide is neurotoxin (Gokmen and Plazoglu, 2008), known by various other names like 2-propenamide, ethylene carboxamid and propenamide. Its molar mass is 71.08 g/mol (Friedman, 2003). It is a colorless odorless, crystalline solid and carcinogenic to human. It is used as a food processing agent. The development in food industry have shown to acrylamide is synthesized by Millard reaction (Tareke *et al.*, 2000). L-asparaginase has the ability to convert L-asparagine to L-aspartate. It is commonly used to decrease the number of precursors for Millard reaction. Some fungal asparaginases utilised in food industries now a day's involve L-asparaginase by different sources such as *Aspergillus niger* and *Aspergillus oryzae* (Morales *et al.*, 2008). Acrylamide is produced in fried and baked foods when reducing sugars are condensed with a carbonyl source. L-asparagine is the main amino-acid that is involved in this phenomenon. Acrylamide is mainly obtained by heat induced reactions. Reaction is carried out between the α -Amino group and Carbonyl groups of reducing sugars (Zyzak *et al.*, 2003). The amide chain present in the acrylamide structure is provided by L-asparagine. Reduction and removal of L-asparagine is an important approach for decreasing acrylamide amount in foods (Pedreschi *et al.*, 2008). Several different ways are involved in reduction of L-asparagine, selection of vegetal species with lower level of L-asparagine, deletion of enzymes involved in biosynthesis of L-asparagine by suppression of specific genes, acid hydrolysis of L-asparaginases and acetylation process of L-asparagine to form N-acetyl-L-asparagine, preventing creation of acrylamide (Friedman, 2003).

XIV. CONCLUSION

L-Asparaginase is an industrially important enzyme, produced by animals, plants, bacteria, fungi, algae and actinomycetes. It has a three dimensional tetrameric

structure consisting of four monomers of different molecular weight. In tumorous cells it is produced by asparagine synthetase. It acts by hydrolyzing the amino acid L-asparagine to aspartic acid and ammonia. Mostly bacterial species are used for its extraction due to the easy extraction procedures. It can be produced by fermentation, submerged or solid state, and purified by cation exchange chromatography, filtration, precipitation and dialysis. It is used to treat leukemia and other malignant diseases. In food industry, it prevents acrylamide formation and carries out amino acid metabolism.

XV. REFERENCES

- [1]. Abdel-Fattah, Y. R. and Z. Olama. 2002. L-asparaginase production by *Pseudomonas aeruginosa* in solid state culture. *Biochem.*, 38:115-122.
- [2]. Alegre, R. M., L.A. Minim. 1993. Cheese whey utilization for L-asparaginase production from *Erwinia aroideae* NRRL B-138 in pilot plant. *Arq. Biol. Tecnol.*, (36): 525-34.
- [3]. Appel, I. M., C. Bakvis, R. Stigter and R. Pieters. 2007. Influence of two different regimens of concomitant treatment with Asparaginase and dexamethasone on haemostasis in childhood acute lymphoblastic leukaemia. *Leukaemia*, 21(11): 2377-2380.
- [4]. Amena, S., N. Vishalakshi and M. Prabhakar. 2010. Production, purification, characterization of L-asparaginase from *Streptomyces*. *Braz. J. microbial.*, 41:173-78.
- [5]. Basscomb, S., G.T.Banks, A. Fleming, K. A. Bettelheim and T. A. Connors. 1975. The properties and large scale production of L-asparaginase from *Citrobacter*. *J. gen. microbial.*, 91(1): 1-16.
- [6]. Baskar, G. and S. Renganathan. 2009. Production of L-asparaginase from natural substrates by *Aspergillus terreus* MTCC1782. *Intern. J. Chem. Eng.*, 7(1): 1-16.
- [7]. Broome, J. D. 1968. Studies on the mechanism of tumour inhibition by L-asparaginase. *J. Exp. Med.*, (127):1055-72.
- [8]. Basha, N., R. Rekha and S. Ruby. 2009. Production of L-Asparaginase from actinomycetes by Solid-state and submerged fermentation: purification and characteristics. *J. pharma. Res.*, 353-360.
- [9]. Broome, J. D. 1961. Evidence that the L-asparaginase activity of guinea pig serum is responsible for its anti-lymphoma effects. *Nature*, 191(4793):1114-1115.
- [10]. Capizzi, R. L. and Y. C. Cheng. 1981. Therapy of neoplasia with asparaginase. In: Holcenberg J, Roberts J, (Ed.), *Enzymes as Drugs*. New York: Wiley, pp. 1-24.
- [11]. Capizzi, R. L. 1975. Schedule-dependent synergism and antagonism between methotrexate and L-asparaginase. *Biochem. Pharmacol.* 23(2):151-61.
- [12]. Capizzi, R. L., O. Castro, and G. Aspnes. 1974. Treatment of acute lymphocytic leukaemia with intermittent high dose methotrexate and Asparaginase. *Proc. Am. Assoc. Cancer. Res. ASCO.*, (15):182.
- [13]. Chang, T. M. 2013. Biomedical applications of immobilized enzymes and proteins. *Nut. Res.*, 105-114.
- [14]. Dejong, P. J. 1972. L-asparaginase production by *Streptomyces griseus*. *App. Micbiol.*, 23(6): 1163-64.
- [15]. Dutra-Molino, J. V., V. Araujo and L. C. Novaes. 2014. Biomolecules extracted by ATPS. *Rev. Max. Ing.*, 39:359-77.
- [16]. El-Bessoumy, A. Sarhan and J. Marisour. 2004. Production, isolation and purification of L-asparaginase from *Pseudomonas aeruginosa* 50071 using solid state fermentation. *J. Biochem.Mol. Bio.*, 37(4): 387-393.
- [17]. Ferrara, M. A., N. Severino, J. Mansure and A. G. Siani. 2006. Asparaginase production by recombinant *Pichia pastoris* strain harbouring *Saccharomyces cerevisiae* ASP3 gene. *Enz. Microbial technol.*, 39(7): 1457-63.
- [18]. Furth, O. and M. Friedman. 1910. Ueber die Verbreitung asparaginspaltender Organismen. *J. Biochem.*, 26:425-44.
- [19]. Friedman, M. 2003. Chemistry, biochemistry and safety of acrylamide. *J. Food Chem.*, 51(16):4504-4526.
- [20]. Geckil, H., B. Ates, and S. Gencer. 2005. Membrane permeabilization of Gram-negative bacteria with potassium phosphate/hexane aqueous phase system for the release of L-

- asparaginase: an enzyme used in cancer therapy. *Process Biochem.* (40): 573–9.
- [21]. Hall, J. G. 1970. The partitioning of L-asparaginase between blood and lymph. *Recent Results. Cancer. Res.*, (33): 75–80.
- [22]. Jian-Hang, Z., Y. Xi-luan and C. Hong-Jun. 2007. In-situ extraction of intracellular asparaginase by the two-phase system. *J. Chromatogr.*, 1147:127–34.
- [23]. Kumar, D. S. and K. Sobha. 2012. L-asparaginase from microbes: A comprehensive review. *Adv. Biores.*, 3(4): 137-157.
- [24]. Kumar, M. and H. K. Manonmani. 2013. Purification, characteristics and kinetic parameters of L-asparaginase from *cladosporium* sp. *J. micro. Biotechnol.*, 29(4): 577-587.
- [25]. Kidd, J. G. 1953. Regression of translated lymphomas induced in vivo by means of normal guinea pig serum I. course of transplanted cancers of various kinds in mice and rats, given guinea pig serum, horse serum or rabbit serum. *J. Exp. Med.*, 98(6):565-582.
- [26]. Kumar, D.S. and K. Sobha. 2012. L-asparaginase from microbes: A comprehensive review. *Adv. Biores.*, 3(4):137-157.
- [27]. Kumar, M.N.S., C.A. Shimray, D. Indrani and H.K. Manonmani. 2013. Reduction of acrylamide formation in sweet bread with L-Asparaginase treatment. *Food Bioprocess Technol.*, 7(3):741-743.
- [28]. Lubkowski, J., M. Dauter, K. Aghaiypour, A. Wlodawer and Z. Dauter. 2002. Atomic resolution structure of *Erwinia chrysanthemi* L-Asparaginase. *Acta Crystallographic D.*, 59: 84-92.
- [29]. Lee, S., J. T. Ross, M. H. Wroble and G. M. Muschik. 1986. Large scale production of L-asparaginase from *Eriwiniacarotovra*. *App. Biochem. Biotechnol.* 12: 229-247.
- [30]. Loos, M., and T. Borsos. 1972. Inactivation of complement by L-asparaginase not correlated with enzyme content. *Nat. New. Biol.*, 237(71): 55–7.
- [31]. Michalska, K. and M. Jaskolski. 2006. Structural aspects of L-asparaginase, their friends and relations. *Acta Biochem. Pol.*, 53:627–40.
- [32]. Mashburn, L. and J. Wriston. 1964. Tumour inhibitory effect of L-asparaginase from *Escherichia coli*. *J. Biochem. Biophysiol.* 105:450-452.
- [33]. Nakamura, N., Y. Morikawa, M. Tanaka. 1971. L-asparaginase from *E. coli*. Part II: substrate specificity studies. *Agric. Biol. Chem.*, 35(5): 743–51.
- [34]. Nawaz, M. S., D. Zhang, and A. A. Khan. 1998. Isolation and characterization of *Enterobacter cloacae* capable of metabolizing asparagine. *Appl. Microbial. Biotechnol.* (50): 568–72.
- [35]. Narayana, K.J., K.G. Kumar and M. Vijayalakshami. 2008. L-Asparaginase production by *Streptomyces albidoflavus*. *Indian J. microbial.* 48(3):331–336.
- [36]. Nagarethinam, S., A. Nagappa, N. Udupa and V. Rao. 1995. Microbial L- Asparaginase and its future prospects. *Asian J. Med. Res.*, 1:159-168.
- [37]. Ohnuma, T., F. Rosner, and R. N. Levy. 1970. Treatment of adult leukaemia with L-asparaginase (NSC 109229). *Cancer. Chemother. Rep.*, (55): 269–75.
- [38]. Patro, K. R. and N. Gupta. 2012. Extraction, purification and characterization of L-asparaginase from *Penicillium* sp. by submerged fermentation. *Intern. J. biotechnol. Molbiol. Res.*, 3(3): 30-34.
- [39]. Peterson, R. E. and A. Ciegler. 1972. Factors influencing L-asparaginase production by *Erwinia aroideae*. *App. Microbiol.*, 23(3): 671-673.
- [40]. Pritsa, A. A. and D. A. Kyriakidis. 2001. L-asparaginase of *Thermus thermophilus*: purification, properties and identification of amino acids for its catalytic activity. *Mol. Cell. Biochem.* 216:93-101.
- [41]. Pedreschi, F., S. Mariotti, K. Granby and J. Risum. 2008. Acrylamide reduction in potato chips by using commercial asparaginase in combination with conventional blanching. *J. Food Sci. Technol.*, 44(6):1473–1476.
- [42]. Qin, M. and F. Zhao. 2003. L-asparaginase release from *E. coli* with aqueous two-phase system. *Appl. Biochem. Biotechnol.* 110:11-22.
- [43]. Riccardi, R., J. S. Holcenberg, D. L. Glaubinger. 1981. L-asparaginase pharmacokinetics and asparagine levels in cerebrospinal fluid of rhesus monkeys and humans. *Cancer. Res.*, (41): 4554–8.
- [44]. Sanches, M., K. Krauchenco and I. Polikarpov. 2001. Structure substrate complexation and

- reaction mechanism of bacterial Asparaginase. *Curr. Chem. Bio.*, 1:175-186.
- [45]. Savitri, S., N. Asthana, & W. Azmi. 2003. Microbial L-asparaginase: A potent antitumor enzyme. *Ind. J. Biotech.*, 2(2): 184–194.
- [46]. Serquis, M., E. M. Oliveira and G. Costa. 2004. Production of L-asparaginase by filamentous fungi. 99(5): 489-92.
- [47]. Singh, Y., R. K. Gundampati and M. V. Jagannadham. 2013. Extracellular L-Asparaginase from a protease-deficient *Bacillus*: purification, characterization and evaluation of activity in-vitro. *Appl. Biochem. Biotechnol.* 171: 1759-74.
- [48]. Shrivastava, A., A.A. Khan, M. Khurshid, M.A. Kalam, S.K. Jain and P.K. Sanghal. 2016. Recent developments in L-Asparaginase discovery and its potential as an anticancer agent. *Crit. Rev. Onco.Hematol.*,100:1-10.
- [49]. Schrappe, M., A. Reiter, and W. Ludwig. 2000. Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90. *J. Blood.*, 95(11):3310–3322.
- [50]. Tareke, E., P. Rydberg, P. Karlsson and S. Eriksson. 2000. Törnqvist M. Acrylamide: a cooking carcinogen. *J. Chem. Toxicol.*, 13(6):517–522.
- [51]. Usha, R., K.K. Mala, C.K. Venil and M. Palaniswamy. 2011. Screening of actinomycetes from mangrove ecosystem for L-asparaginase activity and optimization by response surface methodology. *Polish J. microbial.* 60(3):213-221.
- [52]. Van den Berg, H. 2011. Asparaginase revisited. *Leuk. Lymphoma*, 52(2):157-167.
- [53]. Whelan, H., and H. Winston. 1979. Purification and properties of asparaginase from *E. coli*. *B. Biochem.*, (8): 2386–93.
- [54]. Watanabe, I., N. Miyata and T. Namakura. 2002. Biotechnological advantages of laboratory scale solid-state fermentation with fungi. *J. Biochem. Eng.*, 93(1): 92-95.
- [55]. Yap, B. S., K. B. McCredie, M. J. Keating. 1981. asparaginase and methotrexate combination chemotherapy in relapsed acute lymphoblastic leukaemia in adults. *Cancer. Treat. Rep.*, 65(1): 83–7.
- [56]. Yoshimoto, T., H. Nishimura, and Y. Saito. 1986. Characterization of polyethylene glycol-modified L-asparaginase from *E. coli* and its application to therapy in leukaemia. *Jap. J. Cancer. Res.*, (77): 1264–70.
- [57]. Zhang, Y., M. L. Tao, W. Shen and Y. Ding. 2004. Immobilization of L-Asparaginase on protein and its characters. *Biomaterials*, 25(17): 3751-3759.
- [58]. Zia, M. A., R. Bashir, and I. Ahmed. 2013. Production of L-asparaginase from *Aspergillus niger* using agro wastes by-products in submerged fermentation process. *J. Teknol.*, (62): 47–51.
- [59]. Zyzak, D., R. Sanders and M. Stojanovic. 2003. Acrylamide formation mechanism in heated foods. *J. Agri. Food Chem.*, 51(16):4782–4787.