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# Microbial L-Asparaginase and its future prospects

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## Abstract

L-asparaginase hydrolyses the free L-asparagine and depletes it in blood and selectively drives the leukemic cells to death as these cells cannot synthesize the amino acid on its own. It is used as a first line of therapy for the induction of remission in Acute Lymphoblastic Leukemia. Even though the enzyme selectively targets the cancerous cells its usage in the past is greatly affected due to two main reasons viz., immunogenic complications and glutaminase acitivity. Many prokaryotes and eukaryotes were found to produce the L-asparaginase with or without clinical application. Research oriented towards exploration of novel enzyme with clinical significance and without any immunogenic complication has to be accelerated. Challenges in the drug development to deliverable have to be confronted with available advances in the field of biotechnology. This review describes the key issues related to the use of L- asparaginase in therapy and discusses on the development of novel microbial enzyme by bioprocess technology.

**Key Words:** L- asparaginase,Acute Lymphoblastic Leukemia,immunogenic

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## INTRODUCTION

The compromised ability of leukemic cells to generate L-asparagine, a non-essential amino acid and subsequent dependency on extracellular L-asparagine paved way for use of L-asparaginase as anti-leukemic agent. L-asparaginase hydrolyses the free L-asparagine in blood, whereby depletion of this amino acid selectively drives the leukemic cells to death. The leukemic cells either have low expression levels of asparagine synthetase or low levels of its substrates, aspartic acid or glutamic acid.

Out of the different sources explored, L-asparaginase from *E.coli* gained momentum and after several studies and subsequent clinical trials, it was approved as drug for leukemia by FDA in 1978. Immunogenic complications were associated with the native enzyme and PEGylation (binding the enzyme covalently with Poly Ethylene Glycol) minimized the complications. Also, pegylated L-asparaginase (pegasparase) had extended half-life; thereby reducing the frequency of injections.

Many prokaryotes and eukaryotes were found to produce the L-asparaginase with or without clinical application. Research oriented towards exploration of novel enzyme with clinical significance and without any immunogenic complication has to be accelerated. Challenges in the drug development to deliverable have to be confronted with available advances in the field of biotechnology.

This review describes the key issues related to the use of L- asparaginase in therapy and discusses on the development of novel microbial enzyme by bioprocess technology.

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### Historical perspectives

The amido-hydrolytic activity of L-asparaginase was first observed by Lang S (1904) and further confirmed by Furth & Friedmann (1910) and Clementi (1922) . The potential of the enzyme in cancer was first explored by Kidd (1953), who observed the anti-lymphoma activity of the guinea pig serum . Later, Neuman and McCoy (1956) demonstrated the metabolic difference between normal and malignant cells *in vitro* in presence and absence of amino acid, asparagine . Taking clue from all these studies, Broome (1961) related the anti-lymphoma activity of guinea pig serum to the depletion of asparagine by the enzyme L-asparaginase .

Although the theory behind the use of the enzyme in malignancies was accepted, there were some difficulties in its clinical usage, as guinea pigs were the only source until then. It is only in 1964, Mashburn and Wriston explored an alternative source of the enzyme and L-asparaginase from *E.coli* came to prime focus . Two asparaginases were found to be produced of E. coli viz. EC-1(cytoplasmic) and EC-2 (periplasmic) and only EC-2 showed anti-lymphoma activity. *E.coli* L-asparaginase was produced in large quantities, which led to the initiation of preclinical and clinical studies of L-asparaginase. The clinical efficacy was confirmed by several clinical trials in patients and was finally approved as drug for leukemia by FDA in 1978.

In search of potential microbial sources for L-asparaginase, several studies were performed. Several prokaryotic and eukaryotic microorganisms were identified as potential sources. List is appended in Table 2-1. To date only two native (*E.coli* and *Erwinia caratovora*) and one pegylated *E.coli* L-asparaginase preparations are available for therapy. L-asparaginase from *Erwinia carotovora* is classified as orphan drug and is restricted for use in patients with allergic reaction towards *E.coli* L-asparaginase.

Key issues and approaches related to the therapeutic use of L-asparaginase

**Immunogenic complications**

Immunogenicity and L-asparaginase are inseparable. This is attributed to its complex structure (tetramer with 4 identical sub units) and molecular weight (130-140 kDa). L-asparaginase from *E.coli* is a homotetramer of 142 kDa with 326 amino acid residues per monomer with four identical subunits A, B, C and D. Among the subunits, interaction between A & B and C & D are most extensive forming two intimate pairs of subunits. Thus, the tetramer is a dimer of intimate dimers. Even though each dimer has two active sites, only the tetramer has been reported to show activity.

Immunogenicity results in generation of antibodies against L-asparaginase and subsequent high rate of enzyme inactivation. Immunogenicity may be with significant clinical reactions including systemic anaphylaxis or without any clinical signs of hypersensitivity often termed as 'silent inactivation' .

Many studies have clearly established the relationship between hypersensitivity, decrease in enzyme activity and anti-asparaginase antibodies . High levels of IgG and/or IgM antibodies were found in patients with hypersensitivity reactions. But apparently, IgE was found to have very less role in hypersensitivity. With increased number of doses there was increase in probability for hypersensitivity reactions. Moreover immunogenic complications are observed more likely in adults when compared to infants and younger patients .

Other than immunogenic complications, the enzyme was found to possess immunosuppressive potential, since it slightly suppresses normal bone marrow functions. Higher incidences of infections were observed in patients under therapy with L-asparaginase .

With administration of steroid prior to the treatment, the clinical allergic reactions have been taken care to some extent. Also, since L- asparaginase from different sources have limited cross-reactivity to each other, continuation of therapy in case of immunological reaction becomes easier by replacing the enzyme from different source.

Chemical modification by PEGylation has greatly reduced the immunogenic complications and also increased the half-life, thus reducing the frequency of administration . But, L-asparaginase when coupled with dextran was found to be less effective in reduction of immunogenicity . Use of poly –DL-alanyl peptides to block immunogenic epitopes or conjugation of the enzyme to human serum albumin were found to be effective, but have to be confirmed with in vivo studies . Other approaches like acylation, entrapment in red blood cells and elimination of immune-dominant epitopes by site directed mutagenesis were studied .

**Asparaginase resistance**

Apart from inactivation by antibodies, resistance to the drug is mainly attributed to the de-repression of Asparagine synthetase gene . Many studies have observed the increase in expression of asparagine synthetase gene in patients with clinical resistance to drug . *In vitro* resistance to the drug was found in cells from patients with relapsed ALL . Few studies also relate the observed *in vitro* resistance towards L-asparaginase to monotherapy with the drug .

Formation of anti-asparaginase antibodies and early clearance of L-asparaginase have also been related to the resistance observed. Moreover, the inactivation of caspase-3 or PARP [Poly (ADP-ribose) polymerase] was also found to be responsible for resistance to L-asparaginase . Very recently, lysosomal cysteine proteases were found to degrade L-sparaginase and this leading to treatment failure. However, considerable data are needed to understand the mechanism of L-asparaginase resistance.

**Leukaemogenicity**

In combination chemotherapy, L-asparaginase was found to potentiate the leukemogenicity of epipodophyllotoxins . This is mainly attributed to the inhibition of albumin synthesis by the enzyme, which leads to increase in unbound fraction of epipodophyllotoxins and its metabolites. In addition, the enzyme depletes the enzymes and proteins of DNA repair and apoptosis, whereby potentiates the leukemogenecity of epipodophyllotoxins, thus exacerbate the risk of leukemia.

This problem can be overcome by avoiding the concomitant use of such combinations in therapy.

**Glutaminase activity**

Substrate specificity and affinity of the enzyme are considered to be the most important factors responsible for the antitumor property of an L-asparaginase. The *E.coli* L-asparaginase is associated with significant glutaminase activity. This glutaminase activity has both advantageous as well as deleterious effect.

The use of inhibitors of glutamine or asparagine biosynthesis in combination with L-asparaginase considerably increases its therapeutic efficiency. This is mainly because asparagine synthesis occurs by trans-amidation of aspartic acid, where glutamine serves as amino group donor. Theoretically, glutaminase activity enhances asparagine depletion, and hence it is advantageous for L-asparaginase to have added glutaminase activity.

But, the main consequence of glutaminase activity is decreased synthesis of protein, which has deleterious effects. Protein synthesis was found to be reduced when the glutamine is reduced to a critical level.

The levels of several important proteins including albumin, insulin, fibrinogen, protein-C, protein-S, serum thyroxine binding globulin, serum sex hormone binding globulin, proteins necessary for cholesterol transport, plasminogen and antithrombin-III were found to be reduced with L-asparaginase therapy . The consequences are ketonic hyperglycemia, hypocholesterolemia glycosuria, thrombosis and prolonged bleeding time. Moreover, immuno suppressor property observed with therapy is due to the presence of L-glutaminase activity .

Most of these abnormalities are because of decreased protein synthesis, due to L-glutamine depletion, brought about by L-glutaminase activity of L-asparaginase. Therefore, an L-asparaginase preparation which lacks L-glutaminase activity would be significant in cancer chemotherapy.

**Half-life**

In patients with ALL, 200-1000 IU/kg/day of L-asparaginase is administered. Due to lower plasma half-life, frequent injections are needed and such higher doses may lead to toxicity.

To increase the clinical effectiveness and to reduce the frequent injections, L-asparaginase with a prolonged plasma half-life is preferred . The half-life of L-asparaginase is greatly affected by specific and non-specific antibodies .

Immobilization of the enzyme in polymers such as polyethylene glycol has not only prolonged the plasma half-life, but also has reduced the immunogenicity and thus has greater therapeutic efficiency than the native enzyme . Similar effects were observed with encapsulation into the erythrocytes or entrapping into liposomes .

Alternatively, L-asparaginase from newer sources like fungi with improved plasma half-life and safety could result in a better therapeutic enzyme.

**Development of L-asparaginase using fungi as alternative source**

**Search for an ideal candidate**

An ideal candidate of L-asparaginase should have improved stability and lesser side effect. The ideal candidate should have high substrate affinity, low $K_m$ value, sufficient half-life, lower immunogenicity and high stability.

The occurrence of extracellular L-asparaginases in fungi led to in-depth investigations on the distribution of this enzyme amongst various genera of fungi. The extracellular enzymes are advantageous as they could be purified more easily than intracellular ones. L-asparaginase from fungi has gained importance because of this fact. Several yeasts including *Saccharomyces, Hansenula, Cryptococcus, Candida, Sporobolomyces, Rhodotorula* and *Pichia* were found to produce extracellular L-asparaginase . Asparaginase from several mold including *Fusarium, Penicillium*, and *Aspergillus* were reported. All these fungal asparaginases exhibited anti-lymphoma activity and also the enzyme was not found to act on L-glutamine .

Earlier studies were only restricted to screening of known microorganism for their ability to produce L-asparaginase. A breakthrough in search of newer isolates producing L-asparaginase was established by Gulati *et al*, by developing novel rapid plate assay for screening microorganism producing L-asparaginase .

**Optimization and production**

The production of enzyme can be carried out by submerged (SmF) and solid state (SSF) fermentation (detailed list is appended in Table-1). Although SSF is cost effective, which uses agricultural wastes as source of energy, the uncertainty of nutrient content makes it less suitable. On contrast, in SmF, the manipulation of medium components leading to high and quality yield of the product is possible. .

In bioprocess development, optimization of the process parameters to achieve higher yield of the product is of interest. A small improvement as a result of process optimization may lead to commercial success in industry. Traditional one-factor-at-a time approach is tedious, time consuming and expensive. Moreover, the effects of interaction among the factors may be masked which might lead to misinterpretation of results. In contrast, statistical methodologies are economical, fast and the interactions among the factors are well assessed.

Factorial designs allow the screening and selection of key factors from a large number of process variables. Among factorial designs, Plackett-Burman Design (PBD) is useful in preliminary studies and helps in selection of variables that can be fixed or eliminated for further optimization processes. But, the interaction of the selected factors is not assessed by this model. Hence, in order to assess the interaction and to further precise the role of selected factors, Response Surface Methodology (RSM) can be used.

Since L-asparaginase is also used in food processing, a demand for L-asparaginase is expected in coming years. Hence development of an economically viable fermentation process either using SmF or SSF is highly significant.

**Purification of L-asparaginase**

Cost effective downstream process would considerably reduce the final cost of any product, thus leads to commercial success. The more the number of sequential operation in achieving the desired purity of a product, the more will be its cost. Additionally, with each additional process step, there is a reduction in overall yield of the product. This is mainly because of the inherent handling losses of product and/or product activity. Moreover, purification of an extracellular product is comparatively easier and cheap than intracellular one. Thus, extracellular L-asparaginase produced from fungi definitely gains momentum in all these aspects, and thus is a potential candidate for further exploration.

**Table :1 Microbial sources of L-asparaginase and its properties**

| Organism | Production conditions | | | | Purification | | | Properties | | | Ref |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Method | Substrate/ medium | Temp (°C) | pH | Steps | Yield | Purification fold | Optimum pH | Optimum temp (°C) | Mol. wt SDS-PAGE | |
| *Aspergillus nigerAK-10* | SSF | Soyabean meal | - | - | - | - | - | 8.6 | 37 | 91.4 kDa | [71] |
| *Bacillus sp.* | SmF | Glucose | 37 | - | Ammonium sulfate | 96.2 | 10.9 | 7.0 | 37 | 45 kDa | [72] |
| | | | | | IEC DEAE | 43.1 | 11.2 | | | | |
| *Marine actinomycetes S3* | SSF/SmF | Soybean meal/Tryptone glucose yeast extract broth | 37 | 7 | Ammonium sulfate | 75* | 1.09 | 7.5 | 50 | - | [73] |
| | | | | | Sephadex G 100 | 69.97* | 2 | | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Erwinia carotovora* | SmF | Nutrient broth | 25 | 6.9 | Ammonium sulfate | 85 | 6 | 8.6 | 35 | 33.5 kDa | [74] |
| | | | | | DEAE | 76 | 88 | | | | |
| *Marine actinomycetes PDK2* | - | - | - | - | Ammonium sulfate | 65.83 | 1.09 | 8.0 | 60 | 140 kDa | [75] |
| | | | | | Sephadex G50 | 8.61 | 33.68 | | | | |
| | | | | | Sephadex G200 | 2.18 | 82.98 | | | | |
| *Pseudomonas aeruginosa 50071* | SSF | Soyabean meal | 37 | 7.4 | Ammonium sulfate | 85 | 5.2 | 9.0 | 37 | 160kDa | [76] |
| | | | | | Sephadex G100 | 60.8 | 27.7 | | | | |
| | | | | | CM Sephadex C50 | 43 | 106 | | | | |
| *Corynebacterium glutamacium* | SmF | Tryptone soya broth | 30 | 7.3 | Protamine sulfate | 105 | 1.2 | 7.0 | 40 | 80 ±1 kDa | [77] |
| | | | | | DEAE Sephacel | 35 | 6.6 | | | | |
| | | | | | Ammoniumn sulphate | 16 | 16.3 | | | | |
| | | | | | Sephacryl S 200 | 12.5 | 98 | | | | |
| *Azotobacter vinelandii* | SmF | Sucrose | 30 | 7.4 | Protamine sulfate | 90 | - | 8.6 | 48 | 84KDa | [78] |
| | | | | | Ammonium sulfate | 63 | 1.6 | | | | |
| | | | | | Sephadex G150 | 21 | 4.5 | | | | |
| | | | | | DEAE-Cellulose | 5 | 12.5 | | | | |
| | | | | | Affinity chromatography | 2.8 | 18.5 | | | | |
| *Vibrio succinogenes* | - | - | - | - | - | - | - | 7.3 | 37 | 146kDa | [79] |
| *Streptomyces gulbargensis* | SmF | Groundnut cake extract, 0.5% maltose | 40 | 8.5 | Ammonium sulphate | 50.6 | 1.8 | 9.0 | 40 | 85 KDa | [80] |
| | | | | | Sephacryl S 200 | 37.8 | 26.88 | | | | |
| | | | | | CM Sephadex C50 | 32 | 82.12 | | | | |
| *Pseudomonas stutzeri MB-405* | - | - | - | - | - | - | - | 9.0 | 37 | 34kDa | [81] |
| *Fusarium equiseti* | SSF | Soyabean meal | 45 | 7 | - | - | - | - | - | - | [82] |
| *Flammulina velutipes* | SmF | Minimal Medium | | | CTAB | - | - | 7.0 | 40 | 13 kDa, 85 kDa[@] | [83] |
| | | | | | Superose 6 | - | - | | | | |
| *Streptomyces sp. TA22* | SmF | Sucrose | 28 | 7 | Ammonium sulfate | - | - | 7.0 | - | - | [84] |
| *Staphylococcus s. 6A* | SmF | Glucose | 39 | 7.5 | - | - | - | - | - | - | [85] |
| *Thermus aquaticus strain T351* | - | - | - | - | DEAE-Sepharose CL-6B column. | 86 | 18 | 9.5 | - | 80±2 kDa | [86] |
| | | | | | QAE-Sephadex A 50 | 83 | 29 | | | | |
| | | | | | Hydroxylapatite. | 38 | 75 | | | | |
| | | | | | Sephadex G-150 | 21 | 225 | | | | |
| *Erwinia chrysanthemi 3937 (Erl-* L-asparaginase*) in Escherichia coli BL21(DE3)pLysS* | SmF | - | - | | S-Sepharose FF column | 69.8 | 15.4 | - | - | 37.2 kDa | [87] |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Vibrio succinogenes* | SmF | Sodium fumarate, Cysteine | 37 | 7.3-7.4 | - | - | - | - | - | - | - | [88] |
| *Escherichia coli* | SmF | Terrfic broth | 37 | 7.2 | Ni–NTA affinity chromatography | - | 3.3 | - | - | 37 kDa | | [89] |
| *Mycobacterium tuberculosis (tiasparagine amidohydrolases)* | SmF | Youman's Medium | - | - | Ammonium sulphate | - | 7 | - | - | - | | [90] |
| *Acinetobacter glutaminasificans (ATCC 27197)* | SmF | L-glutamic acid | - | - | - | - | - | - | - | - | | [91] |
| *Staphylococcus aureus strain NCTC413* | SmF | cas Amino acids, | - | - | - | - | - | - | - | - | | [92] |
| *Fusarium tricincfum* | SmF | Nutrient broth with asparagine | - | - | Ammonium sulphate | 67 | 16 | 8 | - | 161-170 kDa# | | [62] |
| | | | | | DEAE-cellulose | 35 | 33 | | | | | |
| | | | | | Ampholine | 22 | 110 | | | | | |
| | | | | | Prep-disc electrophoresis | 15 | 200 | | | | | |
| *Acinetobacter calcoaceticus* | SmF | Xylose and L-asparagine | - | - | Precipitation with streptomycin | - | - | 7-9 | - | 130 kDa# | | [93] |
| | | | | | DEAE-cellulose column | - | - | | | | | |
| | | | | | CM-cellulose chromatography | 58 | 4 | | | | | |
| | | | | | Agarose filtration | 35 | 14 | | | | | |
| | | | | | Phospho-cellulose | 11.6 | 140 | | | | | |
| *Acinetobacter calcoaceticus* | SmF | Xylose and L-asparagine | - | - | Streptomycin precipitation | | | 8.4-8.8 | 20-30 | 25 kDa, 105 kDa@ | | [94] |
| | | | | | DEAE-cellulose, | 50 | 3 | | | | | |
| | | | | | Sephadex G-2oo | 31 | 6 | | | | | |
| | | | | | Affinity chromatography Sepharose 6B | 15 | 80 | | | | | |
| *Serratia marcescens* | - | Trypticase soy broth | - | - | Ammonium sulpahte | 80 | 4.09* | 8.5 | - | 37 kDa, 147 kDa# | | [95] |
| | | | | | P-150 Bio-Gel filtration | 47 | 95.15* | | | | | |
| | | | | | Isoelectric focusing | 50 | 153.03* | | | | | |
| | | | | | Prep-disc electrophoresis | 27 | 684* | | | | | |
| *Serratia marcescens* | - | Trypticase soy broth | 37 | - | DEAE-cellulose, | 28.85# | 33.5 | - | - | - | | [96] |
| | | | | | BioGel P-300, | 13.45# | 130 | | | | | |
| | | | | | Hydroxylapatite | 9.5# | 240 | | | | | |

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|---|---|---|---|---|---|---|---|---|---|---|---|
| *Bacillus coagulans* | - | - | - | - | DEAE-cellulose chromatography at pH 8.6 (11.8), | 75.5 | 11.8 | 8.8-9.7 | 55 | 84 kDa@ | [97] |
| | | | | | DEAE-cellulose chromatography at pH 6.6 (1.2), | 55.2 | 1.2 | | | | |
| | | | | | Sephadex G-150 (1.2), | 68.5 | 1.8 | | | | |
| | | | | | Hydroxyapatite-cellulose, pH 6.6 | 47.7 | 13 | | | | |
| | | | | | Overall | 13.6 | 332 | | | | |
| *Pseudomonas sp Strain GG13* | - | Monosodium Glutamate and Glucose | - | - | butanol, ammonium sulfate, and zone electrophoresis | - | - | - | - | 25 kDa | [98] |
| *Bacillus brevis* | SmF | Fructose, Liquid paraffin | 30 | 7 | - | - | - | - | - | - | [99] |
| *Erwinia carotovora (cloned in E.coli)* | - | Yeast extract, Soya bean meal, Glucose, Wood chips, Aspargine, Nacl | - | - | cation-exchange chromatography and af?nity chromatography | - | - | - | - | - | [100] |
| *Pectobacterium carotovorum MTCC 1428* | | Glucose and L-asparagine | 30 | 6.5 | Ammonium Sulphate, | 70.15 | 6.86 | 8.5 | 40 | 36.5 kDa, 146 kDa@ | [101] |
| | | | | | DEAE cellulose | 48.38 | 44.63 | | | | |
| | | | | | Sephadex G-100 | 42.05 | 72.12 | | | | |
| *Pseudomonas aeruginosa* | SmF | Corn steep liquor, Asparagine, Tryptone and Casein | 37 | 8 | - | - | - | | - | - | [102] |
| *Escherichia coli ATCC 11303* | SmF | Lactose, tryptone, yeast extract and L-asparagin | 37 | 7.2 | - | - | - | - | - | - | [103] |
| *Helicobacter pylori CCUG 17874.* | - | - | - | - | Superdex 200 10/300 GL column | - | - | - | - | 37 kDa, 140 kDa@ | [104] |
| *Pseudomonas PO7111 (cloned in E coli)* | SmF | Glucose, peptone and beef extract | - | - | - | | - | - | - | - | [105] |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Aspergillus niger* | SmF | Bran of *Glycine max* | 35 | 6.5 | Ammonium sulfate | 92.05 | 1.14 | 6.5 | 40 | | [106] |
| | | | | | DEAE Cellulose | 72.04 | 1.44 | | | | |
| *Aspaergillus nidulans* | - | - | - | - | - | - | - | - | - | - | [107] |
| *Serratia marcescens* | SmF | Glycerol peptone teast extract medium | 37 | 6.8-7.0 | - | - | - | - | - | | [108] |

*- calculated, @-Native PAGE, #- Gel filtration, mol.wt- Molecular weight, Temp- temperature

## CONCLUSION

L-asparaginase has applications in medical and non-medical areas. The immunogenic complications, resistance, leukemogenicity are limiting the application of the enzyme. The demand for novel L-asparaginase is necessity due to such drawbacks. Fungi as an alternative source of L-asparaginase need to be explored.

A systematic and more in-depth investigation on screening, optimization of production parameters and purification of the enzyme from alternative sources is need of the day. Such exploration, when supported by adequate preclinical and clinical studies would definitely result in a safe and efficient L-asparaginase preparation.

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