L-Asparaginase a Biotherapeutic for Acute Lymphoblastic Leukemia – A Molecular Perspective

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Abstract

L-asparaginase (L-asparagine amino hydrolase) is an enzyme which was clinically proved as an antitumor agent to treat acute lymphoblastic leukemia. It catalyzes L-asparagine hydrolysis to L-aspartate and ammonia, and the depletion of asparagine causes cytotoxicity to leukemic cells. Microbial L-asparaginase (ASNase) production has attracted good attention regarding its cost effectiveness and eco-friendliness. The focus of this review is to provide a discussion regarding the microbial ASNase production, purification, its mechanism of action, sources, therapeutic side effects and focusing on the future prospects like protein engineering, recombinant microorganisms to develop a efficient therapeutics with significantly less side effects. This study is also focusing on the production of ASNases from new sources with improvement in the availability as a drug, and issues related to reducing the cost of the drug by improving the pharmacokinetics, pharmaco-dynamics and toxicological profiles in producing the ASNase enzyme.

Key words: Microbial L-asparaginase production, Biopharmaceutical drug, Acute Lymphoblastic leukemia.

Introduction

L-asparaginase (ASNase) is an enzymatic drug used in chemotherapy against diseases such as acute lymphoblastic leukemia (ALL), lymphosarcoma, Hodgkin's disease (1). Tumor cells, more specifically lymphatic tumor cells, requires high amount of asparagine to survive with their rapid malignant growth. This drug depletes L-asparagine (Asn) in to L-aspartate and ammonia in blood, blocking protein synthesis in T-cells and inhibiting DNA and RNA synthesis in cancer cells. As a result, cell functions are impaired resulting in apoptosis. However, normal cells are capable to synthesize their own Asn and are less affected by its depletion by treatment with ASNase. Nonetheless, when the drug was used for long-term treatment, it may cause hypersensitivity leading to allergic reactions such as respiratory disorders, skin rashes, low blood pressure, loss of consciousness (2). Various ASNase preparations from *Escherichia coli* [native and PEGylated form] or *Erwinia chrysanthemi* [native form] are available on the market (3). Moreover, researchers found that *Escherichia coli* yielded preparations that inhibited tumors, compared to the other bacterial ASNases are very less active or completely inactive state (4). Subsequently, the native *E. coli* ASNase was then used for developing a drug in the market.

Recently the interest in using ASNases to treat ALL in adults, specifically young adults has been increased (5). Among 4000 ALL cases diagnosed every year in the USA, approximately two-thirds are children and adolescents, making

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ALL the most common cancer among this age group (6). Around 80% of the children are reported to have a long-term improvement and an overall survival rate was 90%, where as in adults the figures are reduced to 38% and 50% respectively (7-8). In recent years, there has been reportedly good progress in leukemia treatment. ASNase is found widely among many different sources in nature, found not only in microorganisms, but also in plants and tissues of various animals like mammals, birds and fishes. However, microbes are known to be a better source compared to animals or plants, due to their ability to grow easily on simple and inexpensive substrates. Furthermore, they offer easy optimization of culture conditions for enzyme over production, easy genetic modification to increase the yield, commercially viable upstream and downstream techniques, good stability and consistency (9).

Many of the l-asparaginases are not suitable for therapeutic purposes, so many homologous ASNases have been selected are cloned and characterized to potentially reduce the side effects and less toxicity (10). Hence the ideal enzyme could persist for a longer time in the circulatory system with reduced antigenic properties. In order to meet these challenges, many trials have been made to solve the problem by attaching the ASNase enzyme with chemicals like polyethylene glycol (11-12), encapsulation to RBC's (13), deimmunization of T-cell epitope removal by neutral drift (14), trypsin resistance ASNase with increased stability was achieved by immobilization technique (15). When ASNase was used repeatedly due to its short half-life and instability leads to more serious side effects on the patients. However, due to chemical modification of the enzyme reduces the activity of the enzyme. So, to increase the stability of the enzyme thermo tolerant ASNase was cloned, purified from *E.coli* has been reported (16). Taking into account all this scenario, the main aim of this review is to provide a thorough discussion on microbial ASNase production. More particularly, it focuses on microbial related productions, recombinant microorganisms that are likely feasible for a better cost in the market.

Mechanism of Action

L-Asparaginase produced by different sources has different half –lives. Different half-lives of the ASNases preparations lead to the different durations of depletion of the asparagine. The action of L-Asparaginase on the leukemic cells makes them deprive of the asparagine by causing the hydrolysis of asparagine in to asparatic acid and ammonia (Fig.1).
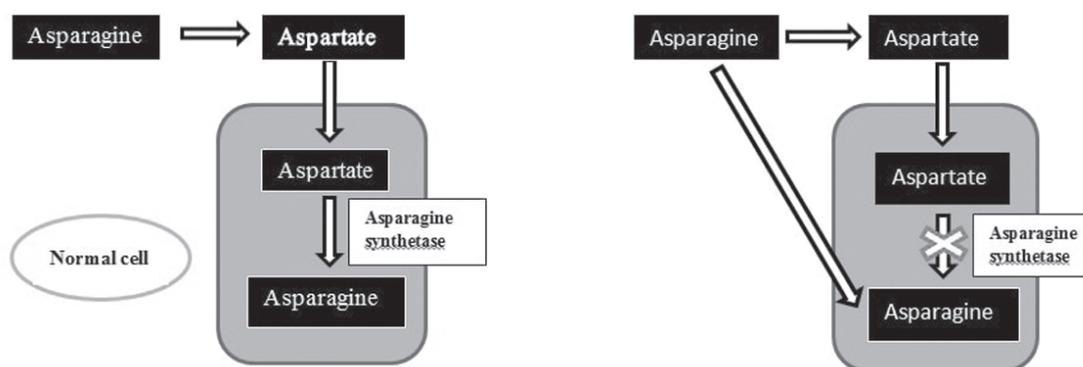

Fig 1. Mechanism of action of ASNase on normal and tumor cell

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Normal cells contain the asparagine synthetase enzyme to fulfill the requirement of asparagines in their diet and asparagine is a non-essential amino acid for normal cells. This enzyme leads to the changes in the characteristics of asparatic acid by adding an amine group from the glutamine and hence leads to the production of asparagine. Asparagine is an essential amino acid for the tumor cells as they do not have the self-producing capability of asparagine due to the lack of asparagine synthetase (17). Protein and RNA synthesis is inhibited in the absence of asparagine (18) and as a consequence cell cycle arrest and apoptosis is induced in leukemia cell lines (19). To achieve the complete asparagine depletion in the human circulation, the L-Asparaginase activity level in serum must be > 100 IU/L (20). It has been reported that tumor cells can develop the potential to synthesize L-Asparagine intracellularly, which enables them to resist the action of the enzyme. The expression of asparagine synthetase is regulated by methylation of cytosine residues, which is responsible for the synthesis of asparagine synthesis. This offers the tumor cells a safe and confirms the exit from the action of L-Asparaginase (21).

Sources of L-asparaginase : Microorganisms have been proved to be very efficient and inexpensive sources of L-asparaginase. Large number of bacteria, fungi, yeast, actinomycetes and algae are reported as potential source of L-asparaginase (22).

Bacterial source : From *E.coli* L-asparginase II enzyme was isolated by Howard cedar and James H. Schwatrz (23). The deamidation of L-asparagine from *Escherichia coli*, due to the presence of L-asparaginase II was first reported and identified by Tsuji. Normally ASNase was produced under aerobic conditions but a significant higher yield of the enzyme was found under anaerobic conditions by using media enriched with high concentration of different amino acids .Optimum aerobic conditions have been proved useful for the greater yield of the enzyme compared to the turbulent conditions showed high amount of the biomass resulting in reduced yield of the enzyme. Ammonium sulfate precipitation and ethyl alcohol precipitation, purification techniques are proved to gain 40 folds amount of the enzyme.

Hymavathi et.al. (24) optimized the conditions for the production of ASNase from a strain *Bacillus circulans* MTCC 8574 by solid state fermentation, by using the agricultural waste as the suitable nutrient source. Incubation temperature, moisture content, glucose, L-asparagine, inoculum level are the conditions which affect the yield of the enzyme.

Production of recombinant *Erwinia caratovora* L-asparaginase II in *E.coli* cells by fed-batch cultures. Using the fed-batch technique with already determines exponential feeding rates; the bioreactor culture yielded 30.7 g of dry cell weight and 0.9 g of soluble rErAII protein per liter of culture broth (25).

Researchers produced a stable L-asparaginase enzyme which was tolerant at 45^0c. The cloning and expression of ASNase enzyme from thermo-tolerant strain *Escherichia coli* (KH027) was isolated from camel dung and could grow at 45^0c. Expression of recombinant asparaginase was performed by fusion of ASNase gene to pelB leader sequence and 6His residues at C-terminal under the inducible T7 promoter in DH5 cells. The protein that purified through nickel affinity chromatography showed optimum conditions at temperature of 43^0c and pH 6. Different other sources of bacteria which produce the enzyme are shown in (Table 1).

Yeast and Fungal sources : The production of Asparaginase from various strains of fungi has been reported using a different range of media. The production of ASNase by filamentous fungi such as *Aspergillus tamarii* and *Aspergillus terreus* has been reported with highest L-asparaginase production level in 2% proline medium from *A. terreus* (26). The production of asparaginases from fungi by various methods has been reported and L-asparaginase producing fungus, *Aspergillus terreus* was isolated and various parameters for

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Table 1. L-asparaginase production by various Bacterial speices.

S. No	Bacterial sp.	Substarte/Media	Operating conditions	Fermentation	Activity reported	Reference
1.	E.coli	Yeast extract – 4%, Peptone – 2%, L-Asparagine – 0.1%	37°C, 220 rpm, 12 h	SMF(submerged fermentation)	60.8 IU/mL	(42)
2.	Erwinia aroideae NRLL-B	Lactose – 1%, Yeast extract – 1.5%	24°C, pH – 7.5, 200 rpm, 12 h	SMF	4 IU/mL	(43)
3.	Pseudomonas aeroginosa 50071	Casein hydrolysate – 3.11%, Corn steep liquor –3.68%	37°C, pH – 7.9, inoculum – 1%, 4 days	SSF(solid state fermentation)	142.2 IU/mL	(44)
4.	Pseudomonas aeroginosa	Peptone 1%, yeast extract –0.5%, NaCl – 1%, glucose –0.1%	37°C, 200 rpm, 24 h	SMF	$210*10^3$ U/mg	(45)
5.	Recombinant E.coli BL 21	TB media with ampicillin100 µg/ml	37°C, 220 rpm, pH – 7.2, 24 h	SMF	22 IU/mL	(46)
6.	Zymomonas mobilis cp4	Molasses – 10%, Yeast extract – 0.2%	30°C, inoculum – 10%, 21 h	SMF	16.55 IU/mL	(47)
7.	Staphylococcu s sp	Ammonium chloride and glucose – 1:0.75	39°C, pH -7.5,100 rpm, 12 h, inoculum – 3%	SMF	55.6 IU/mL	(48)
8.	Pectobacteriu m carotovoram	Yeast extract – 2.08 %, Tryptone – 0.5 %, Monosodium glutamate – 9.89%, L-asparagine – 1%, Galactose – 0.9 %	30°C, pH – 6, inoculum – 5%, 120 rpm	SMF	3.25 IU/mL	(49)
9.	Bacillus circulans MTCC 8574	Red gram husk – 5 g, Glucose – 1.17 g, LA sparagine – 1.24%, Moisture – 99.5%	36.3°C, inoculum – 2.8 ml	SSF	2322 U/g	(50)
10.	Pectobacterim carotovoram	Glucose – 0.2%, L-Asparagine – 0.4% along with yeast extract and peptone	30°C, inoculum – 2%, rpm – 120 SMF (I/E)	SMF	14.56 IU/mL	(51)

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ASNase production through solid state fermentation were optimized (27). L-proline is the best nitrogen source for the production of ASNase from *A. terreus* for maximum asparaginase activity using latin square design (28). The *Penicillium* sp. from the soil producing ASNase with anti-oxidant properties has been found (29).

The ASNase enzyme was purified to homogeneity from *Penicillium* sp. that was grown on submerged fermentation. This purified enzyme showed 13.97 IU/mg specific activity and 36.204% yield. The enzyme showed maximum activity at 7 pH and 37°C. This shows that the enzyme is independent on pH particularly from this organism (30).

Abha Mishra et.al., (31) was the one who reported higher yield of the enzyme from a different isolate of *Aspergillus niger*, agro waste from the leguminous crops as a source. She followed the process of solid state fermentation (SSF). Bran of Glycine max was used as a main source of nutrients gave highest yield of enzyme, which was further followed by the *Phaseolus mungo,* and *Cajanus cajan.*

Actinomycetes sources : The first L-asparaginase from actinomycetes was reported in *Nocardia* spp. (32). Production of intracellular and extracellular asparaginases from *Streptomyces* spp. was also studied (33, 34). Saleem et.al. (35) is the first to report on the production and partial purification of L-asparaginase from marine actinomycetes isolated via solid state fermentation (SSF). In the final purification step, the enzyme showed a specific activity of 662.61 IU/mg, which is approximately 2-fold purity. Optimum pH was found to be 7.5, which is close to blood pH, compared to L-asparaginases from other bacterial sources such as *Serratia marcescens*, *Mycobacterium* spp. and *Pseudomonas* spp. showed optimum pH in the range of 8.0 to 8.5. At 50 °C, the enzyme showed its optimum activity.

A potential extracellular ASNase was characterized from the *Streptomyces griseus* NIOT-VKMA29. Box-Behnken based optimization was used to determine the culture medium components to enhance the L-asparaginase production. In this report the authors have performed molecular characterization ad design of the ASNase gene. Further ASNase biosynthesis gene (*ans*A) from *Streptomyces griseus* NIOT-VKMA29 was heterologously expressed in *Escherichia coli* M15 and the enzyme production was increased threefold (123 IU mL"1) over the native strain (36). Few more actinomycetes sources with the enzyme activity are given in (Table 2).

Plant sources : A few variety of plant species are described with significant amount of asparaginase. Green chillies (*Capsicum annum* L.) and tamarind (*Tamarindus indica*) contain certain amount of ASNase and the enzyme was purified using ammonium sulphate precipitation, sephadex gel filtration and affinity chromatography (37). Enzyme that was isolated from the green chillies was purified up to 400-folds by various methods and it was observed that enzyme exist in two forms and only one of them showed the anti-tumor activity. Enzyme had a pH of 8.5 and a temperature optimum of 37°C. Gene encoding for ASNase enzyme was isolated from plant, *Lupinus angustifolius* (38). The low temperature inducible cDNA sequence that encodes ASNase was isolated from soybean leaves and cloned expressed in *E.coli* with almost has 3 times increased activity (39). *Withania somnifera* is the potential source of enzyme ASNase on the basis of high specificity of enzyme. The ASNase producing micro-organisms from *Ocimum sanctum L* were screened and characterized. ASNase from *Withania somnifera* was cloned and over expressed in *E. coli* with anti-cancer properties (40).

Algal sources : ASNase from a marine C*hlamydomonas* spp. has been purified in 1982. This L-asparaginase has shown limited antitumor activity in anti-lymphoma assay *in vivo.* Properties of this L-asparaginase varied with those of asparaginase from prokaryotic and eukaryotic microorganisms (41).

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Table 2. L-asparaginase production by various fungal speices.

S.No	Fungal sp	Substrate/ Media	Operating conditions	Fermen- tation	Activity reported	Reference
1.	Aspergillus niger	Bran of glycine max,Moisture- 70%	30°C, pH – 6.5, 96 h	SSF	40.9 U/g	(52)
2.	Aspergillus sp	L-Asparagine – 2%,Glucose – 1%, Ammonium sulphate – 1%	30°C, pH – 6.5, 160rpm, inoculum – 2%,48 h	SMF	19.5 IU/mL	(53)
3.	Aspergillus niger	Sesame cake	32°C, Aeration – 0.4vvm, Bed height 22cm	PBR-SSF	344.21 IU/g	(54)
4.	Aspergillus terreus	Carob pod with 65%moisture content,30 mm bed depth, particle size 2 mm	35°C, pH – 35°C, pH – 4.5, 72 h 4.5, 72 h	SSF	6.05 IU/g	(55)

Disadvantages of L-asparginase production from bacteria : Bacterial sources are better described for commercial production of L-asparaginase. Properties of ASNase vary from microorganism to microorganism and contrasted with those of prokaryotic and eukaryotic sources (56). ASNase from bacterial sources causes hypersensitivity in the long-term use, leading to allergic reactions and anaphylaxis. It has been observed that eukaryotic microorganisms like yeast and filamentous fungi genera have a potential for ASNase production with less adverse effects than prokaryotic microorganisms. The search for other ASNase sources, like eukaryotic microorganisms, can lead to an enzyme with less adverse effects (57).

Mode of optimization and production process : Researchers performed submerged fermentation for the production of the enzyme ASNase. They have taken the soil sample of *Bacillus* spp., culture conditions were optimized for producing the higher yield of the enzyme (58). The carbon sources like maltose and glucose were used for the production of the enzyme. The most adopted method of production of ASNase enzyme is from submerged

fermentation, which has been performed throughout the world. Some limitations of process were observed in later research and to overcome those disadvantages method of solid state fermentation has been adopted. It has several advantages compared to submerged fermentation like low capital cost, higher yield of the product, low energy consumption, usage of less water, simple fermentation media (59-60).

In SSF agricultural waste can be used as source of nutrients which is cost effective and environment friendly (61). SSF holds high potential for the production of secondary metabolites and has been increasingly applied in recent years (62). Abha et.al., reported higher yield of the enzyme from a different isolate of *Aspergillus niger*, agro waste from the leguminous crops as a source. An attempt was made to study the optimized production of L-Asparaginase by *Fusarium equiseti* using soya bean meal under solid state fermentation (SSF) by Hosamani et. al., (63). Solid state fermentation has emerged as a potential technology for the production of microbial products utilizing the cheaply available raw materials. Soya bean meal proved to be one of the best substrate

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for L-Asparaginase production. In the present study production of L-Asparaginase started at 24 hours and reached maximum at 48 hours and then decreased significantly with increase in the incubation time.

Optimization of production level of L-Asparaginase from *Erwinia carotovora* was done by Vaibhav et.al., (64). A Central composite Rotatable Design (CCRD) of Response Surface Methodology (RSM) was used to determine the combined effect of the three variables viz. Yeast Extract, Maltose and L-asparagine which were identified earlier using 'one-factor-at-a time' approach by them. The significant variable was Yeast Extract among three variables.

Extracellular L-asparaginase was produced using a fungi isolated from soil. The effect of various physical and chemical parameters was optimized for extracellular L-asparaginase production under submerged fermentation. The maximum L-asparaginase activity of 19.5 U/mL was obtained using MCD medium containing 2% (w/v) L-asparagine as substrate, 1% glucose as carbon source, 1% ammonium sulphate as an additional nitrogen source. The optimum process parameters for maximum L-asparaginase production were: Incubation time 96 h, incubation temperature at 30ºC, initial pH 6.5 and inoculum level 20% (v/v) with 48 h old inoculum (65). The filamentous fungi *Bipolaris* spp. isolated from brown rice in Thailand was identified to produce extracellular L-asparaginase. The maximum L-asparaginase activity of 6.3 U/mL was obtained using MCD media containing 1% L-asparagine and 0.4% glucose at 30ºC in 72 h of incubation. L-asparaginase from *Bipolaris* sp. was proved to be non-cytotoxic when tested against Vero cell lines and has potential application in food industry.

L-Asparaginase production by *Aspergillus* sp. under solid state fermentation was studied using different agro-industrial wastes such as rice bran, green gram bran, wheat rawa, wheat bran, bombay rawa, black gram bran, barley, saw dust, jowar flour, rice flour, castor oil cake, groundnut oil cake, coconut oil cake and sesame oil cake

as substrate. Of all the substrates studied that supported growth and enzyme formation by the fungi, groundnut oil cake showed the highest L-asparaginase production. The maximum production of L-asparaginase (60 U/gds) was achieved by using groundnut oil cake. The optimum process parameters for maximum L-asparaginase production were: Incubation period of 5 days, initial moisture content of solid substrate 90%, 1:10 (v/w) ratio of salt solution to weight of groundnut oil cake, inoculum level 20% (v/w), incubation temperature at 30°C and initial pH 6.5.

Enzyme in recombinant form : Due to very high cost of medicinal drug to be used against cancer and side effects of L-asparaginase isolated from *E. coli,* the enzyme is cloned in other vectors to improve the characteristics of the enzyme and reduce the side effects of the enzyme. As the enzyme is mainly used as anti-cancer drug, therefore, the recombinant techniques must be a part of the process adopted for the production of Asparaginase, which can help in reducing the allergic reaction produced by the enzyme and also the cost of the final product as well as the treatment. Considering the importance of implementation of recombinant techniques, Priscila Lamb Researchers have adopted a work plan:

a) Cloning of *E. Carotovora subsp. Atroseptica* L-Asparaginase II *era* gene,

b) Protein expression in *E. coli* cells,

c) Purification of the recombinant enzyme and

d) Measurement of Asparaginase activity and kinectic characterization of this enzyme

Another attempt for generating the recombinant form of the enzyme was made by the Harry et.al. They constructed the whole genomic library of *Erwinia chrysanthemi* in bacteriophage A1059 and purified, isolated anti-Asparaginase IgG were used to detect the recombinants expressing the enzyme. The gene was subcloned in pUC9 and sub-cloning was done to get the actual position of the gene. Recombinants were not observed to repress glycerol as their sole source rather they repress

glucose. Recombinants cells of *Erwinia carotovora* resulted in increased yield of the enzyme (66). To increase the production level of the enzyme, overproducing L-Asparaginase strains through protoplast fusion technique between two highly L-Asparaginase- producer local isolates, i.e., *Bacillus subtilis* and *B. cereus* was developed by Wafaa et.al., (67). *B. subtilis* was found to be sensitive to rifampcin (Rifs) and could utilize L-Asparagine as a sole source of nitrogen while *Bacillus cereus* was resistant to (Rifr), does not grow on minimal medium and cannot utilize L-asparagine.

Treating the cells with 1 mg/ml lysozyme for three hours in SMM buffer caused the protoplast fusion. Protoplast regeneration was successfully obtained on sodium-succinate medium where protoplast regeneration rates were 39.8 and 25.6% for *Bacillus subtilis* and *B. cereus*, respectively. Protoplast fusion was performed between the two parental protoplasts in the presence of 40% PEG 6000. Among forty five fusants, 18 showed significant higher L-Asparaginase activity, they produced approximately 2.5 fold more L-Asparaginase.

L-Asparaginase production by *Aspergillus* sp. under solid state fermentation was studied using different agro-industrial wastes such as rice bran, green gram bran, wheat rawa, wheat bran, bombay rawa, black gram bran, barley, saw dust, jowar flour, rice flour, castor oil cake, groundnut oil cake, coconut oil cake and sesame oil cake as substrate. Of all the substrates studied that supported growth and enzyme formation by the fungi, groundnut oil cake showed the highest L-asparaginase production. The maximum production of L-asparaginase (60 U/gds) was achieved by using groundnut oil cake. The optimum process parameters for maximum L-asparaginase production were: Incubation period of 5 days, initial moisture content of solid substrate 90%, 1:10 (v/w) ratio of salt solution to weight of groundnut oil cake, inoculum level 20% (v/w), incubation temperature at 30°C and initial pH 6.5 (68).

Different patterns of expressions of recombinant L-Asparaginase in different *E. coli* hosts were studied by Gustavo et.al. Mutant strains of *E. coli* were constructed by genetic manipulations with the help of recombinant tools. After amplification of the L-Asparaginase gene from *Erwinia carotovora*, the gene was cloned into the expression vector pET30a (+) and used to transform different *E. coli* strain by electroporation method. A control was kept to verify the results. The *E. coli* strains used were: BL21 (DE3) NH, BL21 (DE3) Star, C41 (DE3), C43 (DE3), Rosetta (DE3), and BL21 (DE3). The cultures were maintained on TB medium at 37°C. As a control, the *E. coli* strain was transformed with the plasmid lacking the L-Asparaginase gene. For expression analysis, 1 mL of the cultures were submitted to electrophoresis on SDS-PAGE. All *E. coli* strains tested showed the higher expression of the L-Asparaginase at 37°C. At the moment, the cultures are being tested at 30°C in order to observe if the temperature will influence in the expression of the protein. The results were promising and can be used for the scale up process to increase the production level of the enzyme (69).

Purification of Enzyme : Juan et.al., (70) discovered L-Asparaginase in a significant active form when lysine is over produced in cultures of *Corynebacterium glutamicum*. Purification measures adopted by them include:

- Protamine sulphate precipitation,
- DEAE-Sephacel anion exchange,
- Ammonium sulphate precipitation and
- Sephacryl S-200 gel filtration

98-fold purification was adopted by these methods and the purified enzyme was eluted from gel, when subjected to PAGE, in an active form. The enzyme, sometimes, produced intracellularly.

Purification of L-Asparaginase from *Acinetobacter calcoaceticus* was done by precipitation with streptomycin, chromatography on DEAE-cellulose and CM-cellulose, gel filtration on agarose and chromatography on phosphocellulose. The enzyme catalyzed the

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deamination of L-glutamine to about the same extent as L-asparagine and showed a weak tumor inhibitory power (71).

Bacterial L-asparaginases catalyze the conversion of L-asparagine to L-aspartate and ammonia. Kotzia et.al., (72) reported the cloning and expression of L-asparaginase from *Erwinia chrysanthemi* 3937 (ErLASNase) in *Escherichia coli* BL21(DE3) and purification of the enzyme was done by a single-step procedure involving cation exchange chromatography on an S-Sepharose FF column which showed comparatively high activity.

L-Asparaginase (Isozyme II) from *E. coli* was cloned and expressed extracellularly. The resulting recombinant protein was purified by a single step using Ni-NTA affinity chromatography which gave an overall yield of 95 mg/L of purified protein, with a recovery of 86%. This is approximately 8-fold higher to the previously reported data in literature (73). Gladilina et.al., (74) reported the cloning and expression of recombinant protein from *Helicobacter pylori* J99 in *E. coli* (BL21) which was first purified up to 1.8 fold, then sonicated for the preparation of cell free extract and the enzyme was purified from the soluble fraction of cell free extract by chromatography on SP-Sepharose which gave more than 60% yield.

Statistically based experimental design was done to assess the physical process parameter was applied to maximize the production of glutaminase-free L-asparaginase from *Pectobacterium carotovorum* MTCC 1428 which after purification via a three step process enhanced the production and productivity of L-asparaginase by 26.39% (specific activity) and 10.19%, respectively (75).

Purification of the L-asparaginase enzyme to homogeneity from *Pseudomonas aeruginosa* 50071 cells that were grown on solid-state fermentation was done by applying different purification steps including ammonium sulfate fractionation followed by separation on Sephadex G-100 gel filtration and CM-Sephadex C50crude culture filtrate. The enzyme was purified 106-fold

and showed a final specific activity of 1900 IU/mg with a 43% yield by Khushoo et.al., (74).

The recombinant L-asparaginase enzyme of *Pyrococcus koshii* was expressed in *E.coli* (BL21) and purified by anion exchange chromatography and gel filtration followed by hydrophobic interaction chromatography and ultrafiltration. L-asparaginase of *Streptomyces tendae* isolated from laterite soil samples of Guntur region and the crude enzyme was purified to homogeneity by ammonium sulfate precipitation, Sephadex G-100 and CM-Sephadex G-50 gel filtration (77).

Immobilization of the enzyme : Some of the major limitations in the use of the enzyme are its severe immunological reactions and a very short serum half-life. Modifications like formulation and immobilization of the enzyme onto a suitable matrix can greatly reduce the immunogenicity of the enzyme; increase its half-life and its therapeutic potential.

The immobilization of *E.coli* L-asparaginase into a hydrogel matrix made of poly (ethylene glycol) PEG and BSA showed a 200 fold increase in its Km value and a wider pH range for the optimal activity of the enzyme with 90% of activity at physiological pH of 7.3 as compared to 43% activity for the native form. Also the half-life of the immobilized enzyme enhanced to 50 days with 90% activity at 37 °C as compared with the half-life of 2 days for the native enzyme. In an another experiment, they coupled *E.coli* asparaginase in a biocompatible hydrogel made of rat serum albumin and PEG and assessed for its effectiveness to deplete the serum L-asparagine *in vivo*. It was found that 85-90% of serum asparagines got depleted in 2 days with 5 units/rat and also 80% activity of the enzyme was still retained even after 10 days (78). Vina et.al., (79) and other reported the immobilization of L-asparaginase from *Erwinia carotovora* on a biologically active fructose polymer levan of different molecular mass (75 and 2000 kDa) obtained from *Zymomonas mobilis*. They employed the method of periodate oxidation of the polysaccharide followed by reductive alkylation which retained.

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Immunological side effects and enzyme instability : ASNase administration can promote a number of harmful side effects including immunological responses, ranging from allergic reactions to fatal anaphylactic shock, pancreatitis, coagulation, hyperglycemia, protein synthesis inhibition and hepatotoxicity (80). As far as the immunological side effects are present, the decay of ASNase antitumoral activity is directly associated with the production of L-asparginase antibodies by the patient, which leads to the drug clearance from the bloodstream and reduces the treatment efficacy. In this respect, early studies have shown high circulating levels of ASNase by ELISA with low enzyme activity, which was initially attributed to L-asparginase denaturation (81). However, more recent studies suggest that ASNase clearance may be a result of protease cleavage (82). Immunogenic effects and Protein stability are apparently closely related. The proteolytic cleavage of ASNase may be responsible for additional epitopes exposure, which is involved in the patients' immune response . Hypersensitivity occurs more frequently when the treatment is interrupted, with children presenting less hypersensitivity and antibody production when compared to adolescents and adults (83-84).

Formulation and Modification of the Enzyme : L-Asparaginase has certain side effects and very less half life. Its dosage activates the immune response in the body. It is the need of time to reduce the immunogenicity of the drug with some modification in the structure of the drug. Various formulations and modifications has been tried to improve the influence of the drug. The enzyme from the *E. coli* was modified by Kodera et.al., (85). They manipulated the enzyme by coupling it with two types of comb shaped copolymer of polyethylene glycol derivative and maleic anhydride with multivalent reaction sites. This coupling improves the half life of the modified drug and stabilizes it. The serum retained in the body for a longer period.

Another experiment was performed by Yoshihiro et.al. They modified the enzyme from *E. coli* A-1-3 with activated polyethylene glycols with molecular weights of 750, 1900 and 5000. The modification of enzyme did not show any significant results and the retained enzymic activity was just 7% mPEG2 (2,4 bis(Omethoxypolyethyleneglycol)- 6-chloro-S-triazine) is also used to modify the enzyme by Zhang *et al.* (86), it is performed in the presence of L-asparagine and the molar ratio maintained was mPEG2/-NH2 was 10. The modified enzyme retained 33% of initial enzymatic activity with complete abolishment of immunogenicity and *in vitro* half-life get increased from 4.6 h to 33 h has been obtained (86).

Cross linkage was the technique adopted by Handschumacher et.al., to modify the enzyme to reduce its side effects. They used the dimethyl suberimidate to cross link the tetrameric form of enzyme from *E. coli*. The cross linkage cause the reduction in the activity of enzyme and only 17% of the total activity was retained after modification. Approximately 60% of the enzyme is converted to dimers and higher oligomers (87).

The modified L-Asparaginase from *E. coli* retained the activity of 8% after the modification with monomethoxypolyethylene glycol, reported by Kamisaki et.al., (88). Cyanuric acid chloride was used as a coupler in the reaction. The modified enzyme did not react with the anti- L-Asparaginase antibody in precipitin reaction. It has the same Km value for L-Asparagine and the same optimal pH as the native enzyme. The immunogenicity of the modified enzyme was substantially reduced because mouse antiserum to it showed no significant increase in hemagglutinin titer of L-Asparaginase-coated sheep red blood cells (88).

Fermentation kinetics of L-asparaginase production : Although the production of bacterial ASNases from wide range of bacterial sources has been studied extensively by various researchers during the last few decades, the kinetics of production of this enzyme has not been explored in detail. Liu et al in reported the kinetics of ASNase production by *E. aroideae* and Arrivukkarasan et.al., reported the kinetics of L-

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asparaginase production by *P. carotovorum*. The fermentation kinetics and continuous production of ASNase was first studied in the bacterial source *E. aroideae*. Cell growth and ASNase production were investigated in both batch, fed-batch and continuous fermentation using yeast extract as a growth limiting substrate. The relationship between specific growth rate and substrate concentration was found to fit the Monod model. The optimum temperature for enzyme production was 24°C, though cell growth was higher at 28°C. The enzyme yield reached its maximum of 4 IU/mL during the negative acceleration growth phase which occurs just prior to stationary growth. Compared to batch fermentation, the continuous fermentation process gave a lower ASNase yield. The optimum temperature for L-asparaginase production in batch process was 24°C, which was the same as in continuous process. Increasing the temperature from 24°C to 28°C resulted in a 20% loss of L-asparaginase yield .

Kinetics of L-asparaginase production by *P. carotovorum* MTCC 1428 in submerged fermentation (SMF) was studied using yeast extract–tryptone–galactose media, keeping constant fermentation conditions at temperature 30°C, initial pH 7.0 and agitation speed of 120 rpm. The production medium was inoculated with 5% v/v of seed culture in its mid-exponential phase at 24 h. The maximum production of periplasmic and extracellular ASNase was 3.25 and 0.83 U/mL, respectively, at 30 h of fermentation. Unstructured kinetic models such as logistic model for cell growth, incorporated Luedeking–Piret model for L-asparaginase production and logistic incorporated modified Luedeking–Piret model for substrate utilization kinetics were satisfactorily described the fermentation profile of *P. carotovorum*. Kinetics of L-asparaginase production by *P. carotovorum* in submerged fermentation (SMF) was studied using YET (yeast extract–tryptone) media, keeping constant fermentation conditions at temperature 30°C, initial pH 7.0 and agitation speed of 120 rpm. The maximum intracellular and extracellular L-asparaginase activity of 2.28 U/mL and 0.58 U/mL, respectively, were obtained at the late logarithmic phase. The unstructured models predicted the cell growth and product formation profile accurately with high coefficient of determination. No report has been found on kinetics of fungal ASNase production. The information on kinetics of ASNase production was unexplored over a long period of time in spite of its increased commercial need as high value therapeutic protein. The cost of this therapeutic enzyme is high due to the lack of efficient production of this enzyme at large scale. Hence it is important to focus on the kinetic modeling of ASNase production with less adverse effect by fungal sources in submerged fermentation to develop economically viable and efficient bioprocess.

Statistical optimization of L-asparginase production : Screening and evaluation of environmental and nutritional requirements of microorganism is an important step for bioprocess development. Optimization studies involving one factor at a time approach is tedious and tends to overlook the effect of interacting factors but may lead to misinterpretations of the results. In contrast statistically designed experiments tackle the problem effectively, which involves the specific pattern of experiments, which minimizes the error in determining the effect of parameters, and the results are acquired in an economic manner (89). The statistical design of experiments is an efficient procedure for designing experiments so that the data acquired can be analyzed to yield valid and informative conclusions. The planning of experiments begins with determining the objectives and choosing the process variables for the study.

An experimental design is the complete layout of a detailed experimental plan in process of doing the experiment. A screening experiment is performed in order to determine the experimental variables that have significant influence on the response variables. Plackett–Burman (PB) design is an effective screening design when main parameters are to be considered. This is a very economical design with the run number being a multiple of four and

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comprises of two-level screening designs. PB experimental design does not consider the interaction among the variables on response variable and it is based on the first-order model. This design is very useful in finding the importance of the factors affecting the product yield in bioprocesses (90).

Response surface methodology (RSM) is a statistical technique based on the fundamental principles of statistics, randomization, replication and duplication, which simplifies the optimization process by studying the mutual interactions among the variables over a range of values in a statistically valid manner. It is an efficient statistical technique for optimization of multiple variables in order to predict the best performance condition with a minimum number of experiments. These designs are used to find improved or optimal process settings, troubleshoot process problems and weak points and make a product or process more robust against external and non-controllable influence. Central Composite Design (CCD) is one of the RSM usually utilized to obtain data that fits a full second-order polynomial model. Variables are coded as ±1 for factorial points, 0 for the center points and ± 2 for axial points. The effect of process variables on response is fit into the second-order polynomial model and it is solved for optimum level of process variables to give maximum response. Statistically based experimental design was applied for optimization of a solid-state fermentation for the production of L-asparaginase by *Pseudomonas aeruginosa* 50071.

PB factorial experimental design was used for evaluation of various culture conditions for their significance on L-asparaginase production. Casein hydrolysate, corn steep liquor and pH were identified as the most significant factors for improving L-asparaginase production process. Box–Behnken design, a type of RSM was used to find the optimum value significant factors for maximum L-asparaginase activity. The maximum L-asparaginase activity of 142.8 IU was obtained at the predicted optimum conditions of pH 7.9, casein hydrolysate 3.11%, and corn steep liquor 3.68%. L-Asparaginase production by

Zymomonas mobilis by sugarcane molasses fermentation was optimized using factorial experimental design. A model obtained by the response surface methodology was good fit of the experimental data. Maximal enzyme activity of 16.55 IU/L was predicted under the optimum conditions of molasses 100.0 g/L of total reducing sugars, yeast extract 2.0 g/L and fermentation time 21 h. The effect of fermentation process parameters for the production of L-asparaginase by isolated *Staphylococcus* spp. was evaluated using Taguchi methodology, a type of fractional factorial experimental design. The carbon source, nitrogen source, incubation temperature, medium pH, aeration, agitation and inoculum level were evaluated and it was reported that incubation temperature, inoculum level and medium pH were the major influential parameters at their individual level, and contributed to more than 60% of total L-asparaginase production .

The interaction effect of incubation temperature, moisture content, inoculum level, glucose and L-asparagine on L-asparaginase production by *Bacillus circulans* MTCC 8574 under solid state fermentation was optimized using fractional factorial central composite design. L-asparagine and incubation temperature were found to have significant linear and quadratic effect on L-asparaginase production. L-Asparaginase production was improved from 780 to 2,322 U/gds. The effect of various carbon and nitrogen sources on production of L-asparaginase by isolated *B. circulans* MTCC 8574 under submerged fermentation was studied using PB experimental design. Ammonium chloride and glucose were found to be the most significant carbon and nitrogen source respectively for L-asparaginase production. Statistically planned experimental designs were applied to maximize the production of glutaminase free L-asparaginase from *P. carotovorum* MTCC 1428 under submerged fermentation. The fermentation media components such as glucose, L-asparagine, KH_2PO_4 and $MgSO_4.7H_2O$ were identified to have significant influence on the production of L-asparaginase using the PB experimental design. The optimum

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levels of glucose, L-asparagine, KH_2PO_4 and $MgSO_4.7H2O$ were found to be 2.07, 5.20, 1.77 and 0.37 g/L, respectively, using the central composite experimental design. The maximum specific activity of L-asparaginase in the optimized medium was 27.88 U/mg of protein, resulting in an overall 8.3-fold increase in production. Effect of various medium components on the production of L-asparaginase under submerged fermentation by *P. carotovorum* was studied for optimal nutrient requirements. Maximum intracellular and extracellular L-asparaginase activity was obtained in the medium containing tryptone, yeast extract, monosodium glutamate, K_2HPO_4 and L-asparagine. These medium components were optimized by central composite experimental design.

Maximum intracellular and extracellular L-asparaginase activity of 2.28 U/mL and 0.58 U/mL were obtained respectively in optimized media. Yeast extract, galactose, monosodium glutamate, $MgSO_4.7H_2O$ and $CaCl_2.2H_2O$ were identified to be the most significant components on production of L-asparaginase by *P. carotovorum* MTCC 1428 under submerged fermentation. The significant components were further optimized using CCD. Maximum periplasmic and extracellular L-asparaginase product yields of 3.25 U/mL and 0.83 U/mL, respectively, were obtained using the optimized medium components of yeast extract 20.8 g/L, galactose 9.16 g/L, monosodium glutamate 9.89 g/L, $MgSO_4.7H_2O$ 0.304 g/L and $CaCl_2.2H_2O$ 0.042 g/L (91). Although there are many reports found on statistical optimization of bacterial L-asparaginase production, there is no report has been found on statistical optimization of fungal L-asparaginase production. Hence it is important to focus on statistical optimization of media components and process conditions for the production of fungal L-asparaginase to develop cost effective and efficient bioprocess.

Applications of L-Asparaginase

The enzyme ASNase has the chemotherapeutic property against the tumor cells. It is an effective therapeutic enzyme against the treatment of cancers like acute lymphoblastic leukemia. This enzyme helps in catalyzing the hydrolysis of L-asparagine into L-aspartic acid and ammonia. The principle behind the use of ASNase as an anti-tumor agent is that it takes the fact that all leukemic cells cannot synthesize the non-essential amino acid ASN on their own, which is very essential for the growth of the tumor cells, whereas the normal cells can synthesize their own asparagine; thus tumor cells require high amount of asparagine.

L-Asparaginase has a significant role also in food industry. L-Asparaginase from fungal sources is used as food processing aid in food and allied industries to reduce the formation of acrylamide. JECFA (Joint Expert Committee on Food Additives) has recommended the use of L-asparaginase to reduce acrylamide formation for its carcinogenicity and neurotoxicity during processing of high-starch food products (JECFA 2001). Acrylamide is formed as a reaction product between asparagine and reducing sugars contained in starchy food products such as potato chips, frech fries, gingerbreads, roasted coffee and wheat dough based products such as biscuits and crisp breads when heated above 120°C during baking or frying. The heat induced reaction between a reducing sugar and asparagine, which is one of the reaction pathways of the Maillard reaction, forms acrylamide. The Maillard reaction is the process that gives the brown colour and tasty flavour of baked, fried and toasted foods. Incubation of unbaked or un-fried starchy foods with L-asparaginase solution at 37ºC reduces acrylamide level in fried foods up to 90% by converting asparagine into aspartic acid and ammonia, without altering the appearance, taste and quality of the final product (92).

In biosensors: L-asparaginase is applicable in asparagine levels sensing in mammalian and hybridoma cells or the food industry . L-asparaginase used as antioxidant: capable to reduce free oxygen radicals.

Conclusion

The discovery of the fact that ASNase is responsible for the action of anti-tumor activity

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against the acute lymphoblastic leukemia has set a good mark in the field of medicine. After this discovery clear information about the enzyme and its mode of action has been dug out. It has been already proved that L-Asparaginase from *E.coli* and *Erwinia carotovora* has anti-neoplastic activity against leukemia and is being used as anti-cancer drugs. But, thorough research it is observed that the action of enzyme is leading to some side effects. Moreover, the yield of enzyme from the present discovered sources was not sufficient to fulfill the demand of the drug. Solid state fermentation has more advantages compared to submerged fermentation to it is adopted at larger scale all over the world. So, there was a need to discover new sources and production techniques to enhance the yield and reduce the side effects of the enzyme. Enzyme isolated from various sources has different optimized conditions for production and activity. The structure of enzyme that was predicted from *E. coli* has four identical units. Recombinant and formulation of enzyme is already in progress, yet there is still a long way to go to increase the yield of the enzyme. Moreover, L-Asparaginase has also have its applications in food industry, as an essential ingredient in reducing the toxicity of baked food by minimizing the amount of acrylamide in food items. So, there is a need to fulfill the thirst of the enzyme because there is a lot of demand for the enzyme in medicine and food industry.

Rational protein engineering based on protein structure is another upcoming strategy to produce ASNases with improved pharmacodynamics, pharmacokinetics and toxicological profiles. Further, approaches involving site directed mutagenesis of residues in the enzymes active site were able to produce recombinant enzyme with good ASNase activity, and negligible GLNase activity. Additional procedures involving the introduction of structural disulfides and cutting of proteases cleavage sites may allow the production of more robust enzymes. There is little information on *Saccharomyces cerevisiae* ASNase and, giving the easy possibility of cultivation and possibility of genetic manipulation

of this yeast, they believe that such an enzyme is possible to be better investigated as an alternative to the existing bacterial ASNases. In particular, special attention has to be paid to its better structural and kinetic characterization as well as to the rational engineering of the yeast enzymes by means of site-directed and random mutations. Another interesting technological approach that may contribute to improve the yield of ASNase production by recombinant microorganisms is the metabolic flux analysis (MFA), it is a powerful tool to postulate the metabolic state constrained by exchange of nutrient fluxes between cells and environment.

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