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Review Article

L-Asparaginase from Microbes: a Comprehensive Review

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ABSTRACT
One of the prime candidates in the treatment of debilitating human cancers includes a family of enzymes referred to as L-asparaginases. The efficacious antitumor activity of these enzymes finds use in countering Acute Lymphoblastic Leukemia (ALL), a commonly diagnosed pediatric cancer. The enzyme's use is merited by the high remission rate and fairly rapid response with therapeutic index (1000). However, the downside to the use of this enzyme is the huge expenses involved in the treatment coupled with a high demand. Therefore, a great deal of interest has emerged in studying the possibilities of harnessing potential microorganisms that house this enzyme. Appropriate characterizations with low toxicity, less hypersensitivity without side effects are required for a large scale production. This review, hence, mainly focuses on the biochemical aspects of L-asparaginase production, aiming to comprehend the physiochemical characteristics, application and assay methods of L-asparaginase, enzyme properties and kinetics of recombinant enzyme production by fermentation. Processes central to these biochemical aspects, including Submerged Fermentation and Solid State Fermentation of L-asparaginase producing organisms and downstream processing of the enzyme are also discussed.
Keywords: L-asparaginase (L-asp); Submerged fermentation (SmF); Solid state fermentation (SSF); Antineoplastic agent; Acute Lymphoblastic Leukemia (ALL)

INTRODUCTION

Acute Lymphoblastic Leukemia (ALL) is the most common form of childhood cancer and it occurs about 6 in 10 cases among children [1,2]. Treatment of ALL is carried out by the enzyme L-asparaginase for remission induction. L-Asparaginase (EC 3.5.1.1) belongs to a group of homologous amidohydrolases family, which catalyses the hydrolysis of the aminoacid L-asparagine to L-aspartate and ammonia (Fig. 1). The history of the L-asparaginase enzyme dates back to 1922 when Clementi reported the presence of this enzyme in the blood of guinea pig serum [3]. Kidd, 1953 first reported that guinea pig serum inhibits the growth of lymphosarcoma in mice and rats, providing conclusive evidence of its antitumor activity [4]. However, the search for the constituents present in guinea pig serum that are responsible for the inhibition of tumors in mice and rats was studied by Broome (1963) and it was found that L-asparaginase is a substrate specific enzyme, responsible for the hydrolysis of L-asparagine required for tumor cells [5]. Later it was shown that the amino acid L-asparagine is necessary for the growth of Walker carcino sarcoma 256 i*n vitro* and it was also shown that cells of mouse leukemia had an absolute nutritional requirement of L-asparagine for growth *in vitro* [6, 7]. Thus, this enzyme plays a major role in treatment of Acute Lymphoblastic Leukemia (ALL) [8, 9]. Clinical trials carried out on ALL patients using L-asparaginase from guinea pig serum and from *Escherichia coli* showed positive results [10, 11]. The enzyme is widely distributed, being found in animal, plant and microbial sources. It was first reported from animal source such as guinea pig serum by Kidd (1953) and also found in tissues of several animals like liver of the rat, tissues of fish, pancreas, liver, brain, ovary, spleen, lung, testes and kidney of many of mammals and birds [12,13]. In plants it is found in barley rootlets [12, 14], *Pisum sativum* [15] etc. Microbial production of L-asparaginase from *E coli* was first reported by Mashburn and Wriston (1964) and compared with guinea pig serum enzyme [16]. It has further been reported by various authors from bacterial origin such as *Bacillus subtilis* [17]; *Corynebacterium glutamicum* [18]; *Erwinia chrysanthemi* [19, 20, 21]; *Escherichia coli* [22, 23, 24, 25]; *Rhodosporidium toruloides* [26]; *Serratia marcescens* [27]; *Thermus thermophilus* [28]; *Vibrio*

proteus [29]; *Zymomonas mobilis* [30]; and from fungal origin *Aspergillus terreus* [31, 32, 33]; *Aspergillus nidulans* [34]; *Candida utilis* [35]; and a few from protozoa, *Tetrahymena pyriformis* [36]. Though the enzyme is widely distributed, only some of these L-asparaginases possess anti-neoplastic activity and among the microbial sources, the most commercially notable ones are *Escherichia coli*, *Erwinia carotovora* and *Serratia marcescens* [37, 38]. Several authors have produced the enzyme in large quantities for the clinical purpose and it was found to be effective against several types of tumors [8, 12].

This review focuses on various conditions implemented for the production of L-asparaginase in sub-merged fermentation and solid state fermentation, applications and assay methods of L-asparaginase. Biochemical characteristics and purification aspects of the enzyme are dealt with briefly. The aim of the review is to give an overview on microbial production of L-asparaginase hitherto.

Biological role of L-asparagine in normal cells and tumor cells

L-Asparagine is a non-essential amino acid used by immature lymphocytes for their proliferation. It's biosynthetic pathway involves the conversion of oxaloacetate by transaminase enzyme to aspartate followed by transfer of amino group from glutamate to oxaloacetate producing α-ketoglutarate and aspartate. The enzyme, Human asparagine synthetase in healthy cell converts aspartate to asparagine by using ATP as energy source (Fig. 2). Tumor cells have an unusually high requirement for the amino acid asparagine and cannot synthesize sufficient endogenous L-asparagine due to very low levels of L-asparagine synthetase and therefore are dependent on serum levels of asparagine for their proliferation and survival [39, 40, 41, 42] or one more attributed reason is the inability of these cells to increase L-asparagine synthetase activity after L-asparaginase administration [43, 44, 45]. So they use both asparagine from the diet (blood serum) as well as what they make themselves (which is limited) to satisfy their large L-asparagine demand. Thus, administration of L-asparaginase deprives dependent tumor cells of their extracellular source of L-asparagine and lead to apoptosis [5, 46, 8, 47, 48]. However, healthy cells escape unaffected as they are capable of synthesizing asparagine de novo with the aid of the enzyme L-asparagine synthetase [49] (Fig.3).

MEDIA OPTIMIZATION FOR PRODUCTION OF L-ASPARAGINASE BY SmF

Many studies have been done to optimize culture conditions for L-asparaginase production both in batch and continuous fermentation. Production of this enzyme depends on various parameters like concentration of carbon and nitrogen sources, pH of culture medium, temperature, fermentation time and oxygen transfer rate. It has been observed that these parameters vary for different organisms [50]. L-asparaginase is mostly obtained by submerged fermentation (Table 1). In many countries, asparaginase either from *Escherichia coli* or *Erwinia carotovora* is used for the treatment of acute lymphoblastic leukemia [51]. Several research groups have studied L-asparaginase production and purification in attempt to minimize the impurities that produce allergenic reactions [52, 53-54].

2.1. Effect of nutrient medium

The nutritional requirements and culture conditions are critical for the biosynthesis of L-asparaginase and it varies from one microorganism to the other organism [55, 50-51]. Barnes et al. (1978) studied different culture conditions required for the growth as well as production of L-asparaginase enzyme. Studies conducted with medium consisting of brain heart infusion, nutrient broth, peptone and yeast extract produced maximum enzyme with good cell dry weight. Addition of L-glutamic acid, L-glutamine and monosodium glutamate did not enhance asparaginase activity. Further studies on minimal media like nutrient broth of 3.0% (w/v) and monosodium glutamate of 1.0% (w/v) has shown highest enzyme yield [55]. Heinemann and Howard (1969) carried medium optimization studies for growth with different nutrient sources like complete dehydrated media, protein hydrolysate, corn steep liquor and autolyzed yeast preparation (AYE). It was observed that maximum enzyme activity was achieved at 4 % (w/v) of AYE medium. The effect of carbon sources on enzyme production was studied and it was seen that when 0.4 % (w/v) of glucose, fructose, lactose, maltose and soluble starch were added to basal medium, the enzyme production was comparable to that in the basal medium. It is assumed that the depressive effect of carbohydrates

may be a function of their ability to lower the pH value of the fermentation [38]. It was also shown that no yield was observed when 0.05 % (w/v) yeast extract medium was used. In case of 3 % (w/v) yeast extract, there was complete inhibition of the growth of cells and enzyme production [56]. But in case of *Klebsiella aerogenes* glucose does not inhibit the production of L-asparaginase [57]; the reasons could be the two distantly related bacteria exhibit comparable phenomena of carbon catabolic control or different molecular mechanisms which attribute this property. In case of *Bacillus* sp. with two different carbon sources viz., glucose and maltose used for enzyme activity glucose showed maximum while with maltose, low activity was observed [58]. Early studies reported that glucose inhibits the synthesis of L-asparaginase in *Escherichia coli*, *Erwinia carotovora, Erwinia aroideae and Serratia marscences* due to catabolic repression [23, 38, 59]. Further studies demonstrated that when 1% (w/v) glucose, 0.4 % (w/v) sucrose, D-mannitol, D-sorbitol and glycerol were added to the basal medium, asparaginase synthesis was inhibited, and the fermentation broth became acidic [38]. Similarly, other studies also provided conclusive evidence that glucose causes significant reduction in asparaginase activity when it was added to 3% nutrient broth with 1% (w/v) of monosodium glutamate [55]. Recent reports suggest that glucose at 0.1% (w/v) showed stimulatory effect as compared to glucose free medium, while glucose at 1% (w/v) had a complete inhibitory effect on enzyme activity [60]. In another study, Tryptone Glucose Yeast extract (TGY) medium was used for the enzyme production and it was observed that the growth of *Erwinia aroideae* has reached maximum of OD_{540} ~0.65 within 7 to 8 hr in TGY medium and the medium has shown optimum enzyme activity even at large scale production [59]. Similarly, yeast extract and lactose were also studied for enzyme production and it was observed that these nutrients not only affect growth, but also have a crucial role in enzyme activity. To obtain optimum yield, different concentrations of yeast extract medium were used and found that 1.5 % (w/v) yeast extract, 1.0 % (w/v) lactose has shown maximum enzyme production [56]. To study the effect of carbon and nitrogen on L-asparaginase production by *Enterobacter aerogenes*, different levels of carbon and nitrogen concentrations were tried to obtain maximum enzyme production. It has been found that sodium citrate of 1.0% (w/v) and diammonium hydrogen phosphate of 0.16% (w/v) showed the best L-asparaginase activity. But with sodium citrate as carbon source, it was observed that there was no intracellular asparaginase activity [61]. Dunlop and Roon (1975) studied the effect of carbon source by using 3 % (w/v) glucose and nitrogen free medium, the results of which indicated maximum activity in the absence of nitrogen source and in the presence of glucose as energy. But very low enzyme activity was reported when galactose was used as energy source [62]. Studies were performed with different growth media under anaerobic conditions with glucose of 1% (w/v) as an additional supplement. It was observed that maximum specific activity attained in the presence of Tryptone of 1% (w/v). Similarly, asparaginase II production was observed by supplementing asparagine as sole source of nitrogen under aerobic and anaerobic conditions and found that *E.coli* was capable of growing in both conditions [23]. Jones and Mortimer (1973) suggested from their studies the existence of a single intracellular yeast L-asparaginase synthesized constitutively and functionally affected by the products of it's activity [63]. Their studies on the genetic control of L-asparaginase synthesis showed a single structural gene called asp1 responsible for asparaginase synthesis. In case of *Bacillus* sp. isolated from intertidal marine alga, peptone was used sole source of nitrogen for the production of L-asparaginase and observed that maximum percentage of enzyme was synthesized at 0.1 % (w/v) peptone [64, 65]. Peter studied the effect of asparaginase in different media such as yeast malt glucose, without glucose and synthetic glucose-asparagine and it was observed that 4% peptone medium has shown good yield of L-asparaginase with *Actinomycetes*, *Streptomyces griseus* ATCC 10137 [66]. Maladkar *et al*. used different carbon and nitrogen sources for fermentative production. Different sources like lactose, monosodium glutamate, corn steep liquor, tryptone and yeast extract showed significant enhancement in the enzyme activity. Asparagine 0.2% (w/v) when used as substrate showed 6 times higher productivity indicating a distinct induction [67]. It has been reported that L-asparaginase of bacterial origin can lead to hypersensitivity in long term use as well as other side effects like allergic reactions and anaphylaxis [68]. The search of eukaryotic microorganisms led to the identification of L-asparaginase production with less side effects in filamentous fungi [31]. It also reported that eukaryotic microorganisms like yeast and fungi have

shown reasonable production of the L-asparaginase [69-70, 30, 32, 71-74]. It has been shown that L-asparaginase is nitrogen regulated and by altering different nitrogen sources, enzyme production could be enhanced [31]. First it was reported that growth of *Saccharomyces cerevisiae* is nitrogen regulated [75]. Cell growth and L-asparaginase production are usually stimulated by supplying complex forms of nitrogen such as peptone, yeast extract and casein hydrolysate [76]. It has been reported that yeast extract is essential for cell growth as well as L-asparaginase production from *Erwinia carotovora* [77, 67]. Studies were performed by varying different nitrogen sources for L-asparaginase production for two different fungal strains viz., *Aspergillus tamari* and *Aspergillus terreus* [31]. It is also reported that L-asparaginase from *Aspergillus oryzae* showed an optimum enzyme activity with L-asparagine and glucose as the sole source of nitrogen and carbon respectively. Similarly, bacterial strains also used the same medium for the enzyme production. The medium used for the fungal include L-asparagine of 1% (w/v) and glucose 0.2% (w/v). But in case of bacterial species the concentration of substrate L-asparagine was 0.5% (w/v) and same glucose concentration were used as that of fungal species [78]. In recombinant production of L-asparaginase several authors reported the use of peptone, yeast extract, beef extract and maltose as sole source of carbon and nitrogen for maximum enzyme activities [19, 79-80].

Effect of pH, temperature and agitation

L-asparaginase is commonly observed by semi-quantity plate assay, where pH plays a crucial role in determination of the enzyme [66]. For semi-quantification normally the medium is set in acidic or in neutral condition (around pH 5.5- 7.0). The pH indicator phenol red is incorporated in the medium containing asparagine as sole source of nitrogen. Phenol red, at acidic conditions is yellow in color but at alkaline condition it turns pink in color. Hence pink colonies are assayed for enzyme activity [78]. Temperature is one of the important process parameters for the enzyme production and it has been observed in several reports that optimum temperature ranges between 25 °C and 37 °C (Table 1). Similarly, aeration and agitation are also critical parameters for the maximum production of L-asparaginase. Geckil and Gencer, (2004) used *Vitreoscilla* hemoglobin (VHb) in *Enterobacter aerogenes* to evaluate the affect of such a highly efficient recombinant oxygen-uptake system on L-asparaginase production under different culture conditions. It was found that VHb expressing strain had lower L-Asparaginase activity than the wild type under different aeration conditions. The highest enzyme activity was observed in cultures under low aeration and low agitation. The enzyme activity decreased under both complete aerobic and anaerobic conditions with average pH 7.56±0.28. Therefore aeration and agitation were also found to be control factors for enzyme production [60]. The requirements for asparaginase production by submerged fermentation with *Serratia marcescens* ATCC 60 is limited aeration with a zero level of dissolved oxygen supply from 15 h to 32 h fermentation [38], the same also reported by other authors in *Erwinia aroideae* and *Citrobacter* sp. [56, 50]. From the study on affect of temperature (26 °C, 32 °C and 37 °C), it was found that an optimum temperature of 26 °C and pH of 5.0 would effect maximum enzyme production as well as peak cell population with autolyzed yeast extract medium [38]. It has been reported that the enzyme formed denovo when the pH is brought to 7.5 and consequently dissolved oxygen at zero level. So, asparaginase can be adjusted at any time simply by controlling the pH to 7.5 and dissolved oxygen to reach critical rate transfer [55]. Similar studies have shown optimum temperature of 28 °C for growth and 24 °C for enzyme production with activation energy of 8,500 cal $mole^{-1}$.

In continuous fermentation the enzyme concentration increased with decrease in dilution rate, the maximum enzyme yield achieved within 2 to 3 days. Temperature also showed effect on enzyme production and a 20% reduction in enzyme yield was reported due to shift in temperature [56]. Several studies were performed on enzyme production and obtained maximum enzyme yield at pH 8 and temperature 37 °C compared to that of marine *Vibrio* sp. [81]. Peter (1972) performed the experiment to find the effect of aeration on L-asparaginase production by volume to flask volume of 1:1.25 to 1:10 with 4% (w/v) of peptone. It was found that cells growing under conditions of greatest aeration had high specific L-asparaginase content. Consequently, studies were done with various pH ranges between pH 5.5 & 9.0 and found that the optimum pH was 8.5 for maximum enzyme activity [66]. In case of *Bacillus* sp, different pH optima were observed while single optimum in *Moraxella* sp. W2 (PH 5.6) and *Vibrio* sp. (pH 8.7) were reported [65]. Maya et al.

(1992b) observed maximum activity at 37 °C in *Moraxella* sp. and in case of *Vibrio* sp. it was 60 °C. It has been reported that growth of *Streptomyces plicatus* was maximum at pH-8.0 but the enzyme activity was not highly enhanced; therefore it indicates that the growth is independent of the enzyme activity [82]. The influence of temperature on enzyme activity and growth of *Streptomyces plicatus* observed maximum at 29 °C which is almost equal to that of temperature of water column of the study area. In another report, using *Streptomyces* sp. from marine villorita cyprinoids showed optimum enzyme activity at pH-7.0 [83]. The maximum enzyme production from *Thermus thermophilus* HB8 has been reported at higher temperature of 70 °C with pH 7.0 [28]. In case of recombinant L-asparaginase production, the optimum pH and temperature are observed to be in between pH 6.0 & 7.0 and temperature 28 & 30 °C respectively [84,19,79]. In case of SSF, the authors maintained optimum pH and temperature at 7.4 and 37 °C respectively for the enzyme production [85-86]. Similarly, it has been reported from fungal species, for maximum enzyme activity, the parameters are set at pH 6.5 and temperature of 40 °C [87].

Activity by actinomycetes species

Major research has been focused on the production of L-Asparaginases from various microorganisms like bacteria, fungi and yeast [88]. The maximum yield of *Serratia marcescens* ATCC 60 showed optimum activity of 3.7 U ml^{-1} with productivity of 0.077 U ml^{-1} h^{-1} within 48 h in shake flask. It was also observed in scale up, which is carried out in 4L bottles and found highest enzyme activity of 4 U ml^{-1} with a productivity of 0.095 U ml^{-1} h^{-1} between 40-42 h. Further studies were performed in high volume of 60 L fermentor and found that the fermentor yielded 3.1 U ml^{-1} with productivity 0.088 U ml^{-1} h^{-1} in 35 h [38]. Similar conditions were studied using *Serratia marcescens* ATCC 60 and observed maximum specific activity of 0.7 U mg^{-1} of protein [89]. Peterson and Ciegler, (1969a) reported the production of L-asparaginase by submerged fermentation using *Erwinia aroideae* NRRL B-138. Maximum enzyme production was obtained with a yield of 1,250 IU g^{-1} and stable upto 24h with productivity of 0.218 U g^{-1} h^{-1}. After obtaining optimum yield in fed batch, further studies were performed on large scale production with similar fermentation conditions and found maximum enzyme yield of 960 IU g^{-1} with productivity of 0.056 U g^{-1} h^{-1} [77]. The purified enzyme tested from *Erwinia aroideae* NRRL B-138 has shown good response when compared with *E.coli* and *Serratia marcescens* [77]. Comparisons of batch and continuous processes were performed to obtain larger quantity of L-asparaginase from *Erwinia aroideae* NRRL B-138. The effect of growth rate kinetics and enzyme production were observed and found that 3%(w/v) yeast extract inhibited both cell growth as well as enzyme production. Kinetic data has shown the specific growth rate of $\mu \sim 0.526$ h^{-1} and saturation rate constant of $K_s \leq 1.18$ g l^{-1}. In batch and continuous fermentation, the maximum enzyme activity obtained was 4 U ml^{-1} with productivity of 0.25 088 U ml^{-1} h^{-1} and 3.7 U ml^{-1} respectively [56]. *Enterobacter aerogenes* was used to study the enzyme production and found a maximum enzyme activity of 0.60±0.02 U ml^{-1} with productivity of 0.021 U ml^{-1} h^{-1} at 37 °C within 24 h in shake flask. Further studies on scale up of the enzyme were performed in 2L batch fermentation and observed maximum enzyme activity of 1.2 U ml^{-1} with productivity of 0.2 U ml^{-1} h^{-1} at aeration 1.0 vvm with 700 rpm in 6 h [61]. But *Escherichia coli* A-1 has shown maximum enzyme production of 10.8 U ml^{-1} when compared with all bacteria reported above [55]. Gulati et al. (1997) used a semi-quantitative plate assay for screening of L-asparaginase producing microorganism and found that bacterial strain of *Bacillus licheniformis* has shown maximum activity of 0.14 U ml^{-1} under the optimal conditions at pH 6.2 and temperature 37 °C in 96 h [78]. An interesting observation and a potentially important one was that L-asparaginase produced from *Serratia marscences* is required considerably in lesser amounts of enzyme to induce complete regression of Gardener lymphosarcoma in mouse than compared to L-asparaginase from others sources [27]. The *Serratia marscences* enzyme was more resistant than the *Escherichia coli* and *Erwinia carotovora* enzymes to dissociation by sodium dodecyl sulphate, the reason could be due to the finding that the *Serratia* enzyme had a relatively high hydrophobicity [90]. In another report, bacterial strain *Pectobacterium carotovorum* MTCC 1428 was used for glutaminase-free L-asparaginase production and a maximum enzyme activity of 15.39 U ml^{-1} in shorter fermentation time of 12 h with volumetric productivity of 1.282 U ml^{-1} h^{-1} was found with optimized nutrient parameters [91].

Activity by actinomycetes species

Few reports are available on the actinomycetes species: *Streptomyces plicatus* strain has shown optimum amount of enzyme activity of 33.5 µg ammonia mol^{-1} h^{-1} at pH-7 and temperature of 29±2 °C, when the process was carried out in batch fermentation [82]. In another report with marine *actinomycetes*, maximum enzyme activity of 49.2 U ml^{-1} was found with soil isolate named S3 at fermentation conditions of pH-7.5 for 50 °C [92]. There are some reports on the production of L-asparaginase from marine sediments [93] and gut of estuarine fishes [94].

Activity by fungal species

In order to minimize the side effects shown by bacterial L-asparaginase such as hypersensitivity, the search of eukaryotic microorganism for L-asparaginase with less side effects was taken up in filamentous fungi [31]. It was reported that eukaryotic microorganisms like yeast and fungi have reasonable production of the enzyme [69-70, 30]. It has also been shown by various authors the production of L-asparaginase from the mitosporic fungal genera such as *Aspergillus*, *Penicillium* and *Fusarium* [32, 71-74]. Maximum enzyme activity of 0.058 U ml^{-1} was observed in *Aspergillus terreus* 10C217 with proline 2% (w/v) at an optimum pH 6.2 for 30 °C in 48 h [31]. Wherein, *Aspergillus tamari* maximum enzyme activity was found to be of 0.038 U ml^{-1} with same conditions as above. In their studies, it was found that production of L-asparaginase is nitrogen regulated and similar study was also reported using *Saccharomyces cerevisae* for the enzyme production [75]. Of 17 isolates of tulsi endophytes tested for the production of L-asparaginase by rapid plate assay technique [78], only three isolates showed positive for the production of L-asparaginase enzyme by forming a pink zone around the colony. The maximum zone was found in the isolate TRB4 (1.2 cm. dia) and the minimum zone was produced by the isolate TLB2 (0.8 cm dia). Also, determination of enzyme activity by the method of Imada et al. (1973) demonstrated the maximum activity of 0.45 U mg^{-1} ml^{-1} and the isolate TLB2 showed the least activity of 0.30 U mg^{-1} ml^{-1} [95]. Similarly, *Aspergillus oryzae* was used for L-asparaginase production and maximum enzyme activity of 0.14 U ml^{-1} was found within 96 h with semi-quantitative plate assay for detection of the enzyme [78]. In another report, *Streptomyces griseus* was used for the production of L-asparaginase and obtained maximum enzyme activity of 0.0117 U ml^{-1} with peptone as sole source of nitrogen [66]. Whereas, *Aspergillus terreus* showed maximum enzyme activity of 36.97 U ml^{-1} with productivity of 0.513 U ml^{-1} h^{-1} with ground nut oil cake, which could be used for SSF with optimized conditions [96].

Effect of metal ions and salt tolerance

L-asparaginase activity was varied in presence of enhancers or enzyme inhibitors. In order to study the synergistic effect on the enzyme production, different metal ions were studied and it was found that EDTA strongly inhibited the enzyme activity in some cases. Similar observations were made with $Fe^{2+}$and Ni^{2+} [97, 92], Cu^{2+} and Zn^{2+} [65]. In the salt tolerance, it was found that 2% (w/v) NaCl has relatively maximum enzyme production capability compared to other concentrations [64]. However, *E.coli* has shown good salt tolerance up to 5% (w/v) and this concentration did not have any effect on enzyme production with this strain [23]. It was shown that L-asparaginase was inhibited in presence of metal ions like Hg^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, and Zn^{2+}, while thiol-protecting reagents such as DTT, 2-ME, and glutathione acted as enhancers for the enzyme production [83, 98, 58, 92]. EDTA and some amino acids like L-cysteine and L-histidine have stimulatory effect on activity of L-asparaginase. Also, the activity was enhanced with some reducing agents like 2-mercaptoethanol (2-ME), DL-dithiothreitol (DTT), and GSH (reduced), and inhibited in the presence of thiol-group-blocking reagents such as p-chloromercuribenzoic acid (PCMB) and iodoacetamide [98]. All these facts indicated that L-asparaginase is not a metalloprotein. Hence, sulfhydryl group plays an important role in catalytic activity of L-asparaginase [43]. Furthermore, Gaffer and Shethna (1977) isolated L-asparaginase from various biological sources and compared their biochemical and biological activity against tumors. L-asparaginase from *Azotobacter vinelandii* had an optimum pH different from asparaginases of *Bacillus coagulans* [99], guinea pig serum [100], and *Serratia marcescens* [101], which have been reported to have broad pH optima. Sensitivity of *Azotobacter vinelandii* asparaginase to p-hydroxymercuribenzoate (pHMB), N-ethylmaleimide, iodoacetate, and heavy-metal ions (Hg^{2+}, Cu^{2+}, and Zn^{2+}) further confirmed the dependence of the activity of the enzyme upon sulfhydryl groups [43].

PRODUCTION OF L-ASPARAGINASE BY SSF

Few reports are available on solid state fermentation technique for the L-asparaginase production (Table 2). Ashraf et al. (2004) has reported the production of L-Asparaginase from *Pseudomonas aeruginosa* 50071 by solid-state fermentation using soya bean meal as substrate [85]. L-Asparaginase production was also reported from *Aspergillus niger* in solid state fermentation using agro wastes from three leguminous crops (bran of *Cajanus cajan, Phaseolus mungo* and *Glycine max*) [87]. In optimizing solid state fermentation for the production of L-asparaginase by *Pseudomonas aeruginosa* 50071, fifteen culture conditions were examined for their role in enzyme production and specific activity, using Plackett-Burman factorial design [86]. Factors such as pH, casein hydrolysate and corn steep liquor were the most significant factors improving enzyme production process. The optimal values of the 3 factors that bring about maximum L-asparaginase activity of 142.8 IU were identified as pH 7.9, casein hydrolysate 3.11% and corn steep liquor, 3.68% using Box-Behnken design. Ashraf et al. (2004) reported the L-asparaginase production by SSF using soya bean meal of particle size 0.4-0.8 cm as a substrate. The crude enzyme has shown maximum activity of 165.11 IU ml^{-1} under optimal conditions of 50 % (w/v) moisture content with pH 7.4 at 37 °C for 4 days. After purification steps the enzyme has shown maximum yield of 43 % with 106 fold times increase than the crude enzyme. It was observed that under optimum condition, the enzyme had maximum specific activity of 1900 IU mg^{-1} with *Pseudomonas aeruginosa* 50071 [85]. Abha, 2006 first reported on fungal strain *Aspergillus niger* using solid state fermentation technique for L-asparaginase production. By performing two-way ANOVA, the optimum process parameters were found to be pH 6.5 and temperature 30±2 °C. Initial moisture content of 70 % (w/v) with *Glycine max* bean size 1205-1405µ as a substrate for enzyme, the enzyme showed maximum titer value of 40.0±3.35 U g^{-1} dry substrate. The enzyme was purified with 80% ammonium sulfate then followed by DEAE cellulose method with a maximum enzyme yield of 92.04 % and 72.05 % respectively [87].

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF L-ASPARAGINASES

E.coli produces two distinct Asparaginases (I and II), designated as EC-1 and EC-2 [52, 102-103]. Asparaginase II differs from Asparaginase I by its broad pH activity profile and its higher substrate affinity. Asparaginase II (EC -2) has the anti-tumor properties. Its Michaelis Menten parameter K_m was estimated a 1.25 X 10^{-5} [104]. A low K_m value at physiological pH is an absolute requirement for anti-tumor activity since L-Asparagine concentration must be lowered to atleast 10^{-5} M in the medium to become rate-limiting in protein synthesis. Asparaginase I is usually eliminated during purification. Asparaginase II was produced optimally by bacteria grown between pH 7 and 8 at 37 °C. It was also shown that purified asparaginase II is effective inhibitor of cell free protein synthesis when compared to asparaginase I enzyme [23]. The enzyme is tetrameric with each subunit weighing 32-38 kDa: Molecular weight was estimated to be 141 kDa [24-25]. Its Isoelectric point is at pH 4.9 and it's extinction coefficient was estimated at 7.1 [104]. L-asparagine II (asn II) gene from *E. coli* W3110 was cloned into p^{GEX-2T} DNA vector under the tac promoter and was over expressed in *E. coli* BL21(DE3) and purified to homogeneity 238.4 fold by utilizing chromatography technique on DEAE-Sepharose fast flow, Glutathione S Sepharose 4B columns and thrombin. The molecular mass of the purified enzyme was found to be 40 kDa from SDS-PAGE [105]. The free recombinant enzyme from *E-coli* had an optimum pH at 7.5 while for the immobilized enzyme, it was shifted to 8.5. The optimum temperatures for free and immobilized enzymes were 37 °C and 50 °C respectively.

L-Asparaginase from *Cornyebacterium glutamicum* has been purified 98-fold by protamine sulphate precipitation, DEAE-Sephacel anion exchange, ammonium sulphate precipitation and Sephacryl S-200 gel filtration. Molecular mass as determined by SDS-PAGE was 80 kDa. The purified enzyme showed maximum activity at pH 7 and at temperature 40 ºC. A Lineweaver-Burk analysis showed a K_m value of 2.5 X 10^{-3} M [18]. Tosa et al. (1972) purified the enzyme from *Proteus vulgaris*. Sequentially the methods used were cell lysis by lysozyme and toluene, pH treatment, ammonium sulfate fractionation, Sephadex, G-100 gel filtration, DEAE-Sephadex chromatography and crystallization by the addition of ammonium sulfate. Its Michaelis Menten parameter K_m was estimated at 2.6 x 10^{-5} M. Optimum pH was between 7 and 8 and its isoelectric point was at pH

5.08.The enzyme is inactivated by heat, organic solvents, and chymotrypsin treatments. The presence of L-asparagine or its analogs protects the enzyme from the inactivation caused by these treatments [106]. Purified enzyme from mesophilic fungus *Cylindrocarpon obtusisporum* showed about 65 fold with an overall yield of 11%. It is constituted of four identical subunits and had an apparent molecular weight of 216 kDa. The pI of the enzyme was at pH 5.5. The pH and temperature optima for the enzyme activity were 7.4 and 37 ºC, respectively. The K_m of the L-asparaginase was found to be 1 x 10^{-3} M. Metal ions, such as Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+} and Ni^{2+} potentially inhibited the enzyme activity, while metal chelators like EDTA, CN-, and cysteine enhanced the activity [97]. Rozalska (1989) purified 400-fold L-asparaginase from *Staphylococcus* with 40% recovery. The procedure involves ammonium sulphate precipitation and a column chromatography on Sephadex G-200 gel filtration. The enzyme had isoelectric point at pH 4.4 with an approximate molecular weight of 125 kDa which was estimated by Sephadex G-200 gel filtration. The enzyme is composed of non identical subunits. The polyacrylamide-SDS gel electrophoresis indicated two non –identical subunits with molecular weights 18 and 22 kDa [107]. A homogeneous preparation of L-asparaginase was obtained from *Vibrio succinogenes*, an anaerobic bacterium from the bovine rumen by ammonium sulfate fractionation followed by chromatography on columns of hydroxylapatite, CM-Sephadex, and DEAE Sephadex. The yield was about 40-45 %. The enzyme has a molecular weight of 146 kDa and a subunit molecular weight of approximately 37 kDa. Lineweaver-Burk analysis showed a K_m value of 4.78 x 10^{-5} M. The isoelectric point of the L-asparaginase is 8.74 and the pH optimum was 7.3 [108]. L-asparaginase from the Deuteromycete *Fusarium tricinctum* has been purified to apparent homogeneity by ammonium sulfate fractionation followed by chromatography on DEAE cellulose Ampholine and the final step was preparatory disc electrophoresis. The molecular weight of the enzyme, as determined by sucrose density gradient ultracentrifugation was between 161 and 170 kDa. A K_m value of 5.2 x 10^{-5} M was obtained by use of the GLDH coupled-assay system. The pH optimum was between 7.5 and 8.7.The enzyme was stable to freezing but was inactivated completely and irreversibly upon exposure to 8 M urea [109] (Table.3).

Clinically used L-asparaginase is available in three forms: two are native forms purified from bacterial sources and one is the modified form of the native one (Table 4). The native enzyme from *E.coli* is marketed commercially as Elspar by Merck and Co and that from *Erwinia carotovora* is available as Erwinia L-asparaginase from Ogden Bioservices Pharmaceutical Repository in the United States. The *Erwinia* product is commercially available as Erwinase in Canada and Europe marketed by Porton. In Europe, two different preparations of *E.coli* L-asparaginase (L-asparaginase Medac and Crasnitin) are available. A fourth new recombinant *E-coli* asparaginase is engineered to have an amino acid sequence identical to that of Asparaginase Medac. Efficacy and toxicity profile of this recombinant drug are comparable to those of the other *E-coli* asparaginases and is currently under clinical evaluation [110]. A new approach to maintain enzyme activity and reduce formation of antiasparaginase antibodies is the encapsulation of the enzyme into homologous red blood cells [111]. Also, a Pegylated form of recombinant *Erwinia* asparaginase is under pre-clinical study [112]. The parenteral administration of asparaginase results in rapid and complete deamination of the amino acid asparagine, especially in the plasma and, in part, the cerebrospinal fluid (CSF). Results of the studies carried out by Dana-Faber Cancer Institute (DFCI) ALL consortium and Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP) suggest that high-dose native *E-coli* asparaginase (25,000 IU-m^2 weekly for weeks) significantly improve complete continuous remission in pediatric patients with T-cell ALL and lymphoblastic lymphoma compared with patients treated with a lower dose asparaginase regimen. A study carried out by Rizzari et al. (2001) revealed a significantly finding that no significant difference in disease-free survival could be observed between patients who received standard treatment (10,000 IU-m^2 asparaginase for 4 doses during reinduction) and those who received high-dose treatment (25,000 IU-m^2 asparaginase weekly for 20 weeks during reinduction and early continuation) [113].

L-ASPARAGINASE AND ITS DEVELOPMENT AS A CHEMOTHERAPEUTIC AGENT

L-asparaginase is used as a chemotherapeutic drug in the treatment of a variety of lymphoproliferative disorders and lymphomas, more particularly acute lymphoblastic leukemia

(ALL) where it continues to be of benefit when used in sequential chemotherapy [114-115]. Most of the drug applications include use in cancers [54] like pancreatic carcinoma [116], Bovine lymphomosarcoma [117], diagnosis of acute pancreatitis [118] and childhood ALL [119]. It is also used in variety of lymphoproliferative disorders and lymphomas [120]. Bacterial L-asparaginase is widely used in pediatric Acute Lymphoblastic Leukemia (ALL) [121]. It also used in treating meningeal leukemia successfully [122]. L-asparaginase, first observed in the guinea pig serum by Clementi, (1922) was identified to be a cytotoxicant and growth inhibitor [3, 123, 4, 6-7, 5]. The therapeutic potential of this enzyme as an anti-lymphoma agent was established by Broome, 1961 [124]. Out of the two isoforms of L-asparaginase that were partially purified from guinea pig serum by Yellin and Wriston (1966), one exhibited anti-lymphoma activity [100]. Interestingly, L-asparaginase of bacterial origin was also demonstrated to have tumoricidal activity [16, 102]. Since then several studies have been attempted for large-scale production of enzyme for pre-clinical and clinical studies [52, 125]. The efficacy of L-asparaginase in the treatment of human leukemic subjects was first successfully demonstrated by Oettgen et al. (1967) and for more than three decades from then [126], L-asparaginase is employed as a drug in combination chemotherapy for pediatric ALL as well as in multiagent regimens for adult ALL [127-129]. Although the drug proved to be effective chemotherapeutic agent without cross-reactivity, yet it's use is limited due to the manifestation of clinical hypersensitivity in about 80% of patients when administered in the native form. For this reason, attempts are being made to develop alternative forms of the enzyme without the loss of its tumoricidal activity [130-131].

The application of the drug as a chemotherapeutic agent is mainly based on its catalytic property of hydrolyzing L-asparagine to L-aspartic acid and ammonia [5, 132-133]. Tumor cells have an unusually high requirement for the amino acid asparagine but have very low levels of L-asparagine synthetase and therefore are dependent on serum levels of asparagine for their proliferation and survival [39-42]. The enzyme L-asparaginase causes depletion of serum asparagine resulting in the death of tumour cells owing to nutritional deprivation. However, healthy cells escape unaffected as they are capable of synthesizing asparagine *de novo* with the aid of the enzyme L-asparagine synthetase [49]. The enzyme is usually considered to be cell cycle phase nonspecific and also reported to arrest the cell cycle in G1 phase in the murine and human T- lymphoblastoid cell lines [134-135, 118] and lead to apoptosis. Although the general therapeutic benefit of L-asparaginase is attributed to its ability to cause nutritional deprivation, yet some studies emphasize the requirement of a functional p53 protein for causing apoptosis. Therapy for the ALL with the native L-asparaginase is limited because of its immunogenicity in approximately 25% of the patients [136] and its very short half-life. The shortened plasma half life of the enzyme appears to be more important especially since the maximum initiation of catalytic activity by immune serum has been found to be 45-53% [139-141]. Several attempts have been made to improve the half-life of the enzyme and reduce its immunogenicity by chemical modification. PEGylation of the enzyme has so far been found to be the most successful method of chemical modification as it reduced the immunogenicity without compromising the anti-tumor activity [142-143, 42]. In pre-clinical studies, L-asparaginase was found to be effective against more than 50 murine tumors including rat and canine lymphosarcoma, rat fibrosarcoma, walker carcinosarcoma and Jensen's sarcoma [133, 132]. In humans, only one tumor viz., childhood and adult ALL was found to respond consistently. Inconsistent response to the drug was shown by acute myeloblastic leukemia (AML), Non-Hodgkin's lymphoma (NHL) and chronic leukemias. Thus, tumors of lymphoid origin were found to be more susceptible to the drug than the solid tumors. Pegaspargase can be used in the reinduction and maintenance therapy of patients with ALL and it has been reported that polyethylene glycol asparaginase decreases the toxicity and can be administered for a longer time [144-146]. This form of L-asparaginase is very effective as it needs only to be administered once every 2 weeks unlike the native form which has to be administered every 2-5 days. Another limitation of L-asparaginase is that it has a distinct toxicity profile, ranging from acute hypersensitivity (immunological sensitization) and hyperglycemia to hepatocellular dysfunction and pancreatitis (inhibition of protein synthesis) [147]. All commercially available asparaginase preparations are reported to have similarity in the frequency of toxicity except in the case of pegaspargase where suppression in allergic reaction is observed. L-asparaginase therapy is also

reported to be associated with imbalances in the formation of clotting factors and neurotoxicity. The enzyme work proceeded in several laboratories and clinics leading to clinical trials [148-149].

TOXICITY OF THE DRUG L-ASPARAGINASE

In general, hypersensitivity reactions due to antiasparaginase antibody production were found in up to 60 % of patients, particularly with native *E-coli* asparaginase as compared to the Pegylated enzyme [150-151]. It has been observed that the route of administration determines the clinical symptoms with a greater incidence of major skin reactions observed with intra muscular (IM) administration compared with intravenous (IV) administration. Clinical hypersensitivity is reported to occur almost exclusively in post induction regimens (intensification, reinduction) when asparaginase has not been given for weeks or months. The various possible explanations for the rarity of allergic reactions during remission induction include delayed complement activation and antibody production, suppression due to intensive corticosteroid treatment, frequent dosing causing a desensitizing effect etc. There is a divided opinion on the incidence of hypersensitivity between age groups; some authors reporting that they are similar between age groups [152-153] while others suggest that infants and younger patients develop antibody and hypersensitivity reactions less frequently than teenagers and adult patients [154].

Studies reporting that antibodies, instead of leading to clinical hypersensitivity, might cause rapid inactivation of the asparaginase resulting in sub-optimal asparagine depletion, often referred to as "silent hypersensitivity" or "silent inactivation" have also been cited and is estimated to occur approximately in 30 % of the patients [155]. Generally, patients exhibiting clinical allergy symptoms to one formulation of asparaginase are switched to another product to ensure that they receive the most efficacious treatment regimen possible. However, the switch may not be optimal because antibodies against *E-coli* asparaginase can cross-react with PEG-asparaginase [156-157]. Also pegasparaginase may induce silent inactivation with antibodies resulting in a fast decline in asparaginase activity. Therefore, switching from pegasparaginase to *E-coli* asparaginase after an allergic reaction is not a viable treatment option [158-159]. Pancreatitis, abnormalities of hemostasis, hyperglycemia, abnormalities of lipid metabolism etc. are other asparaginase-related toxicities that are more common in adolescents and adults than in younger children [153]. Liver toxicity with elevated liver enzymes or increased bilirubin is a frequent clinical problem in adult patients [160]. It has been recommended that measuring triglycerides (TG) before and during asparaginase treatment could prevent further increase of TG and decrease the risk of potential complication [161].

ASSAYS FOR DETERMINATION OF L-ASPARAGINASE ACTIVITY

There are different methods for estimating the activities of L-asparaginase quantitatively and qualitatively are presented in Table 5, appropriate references could be referred to.

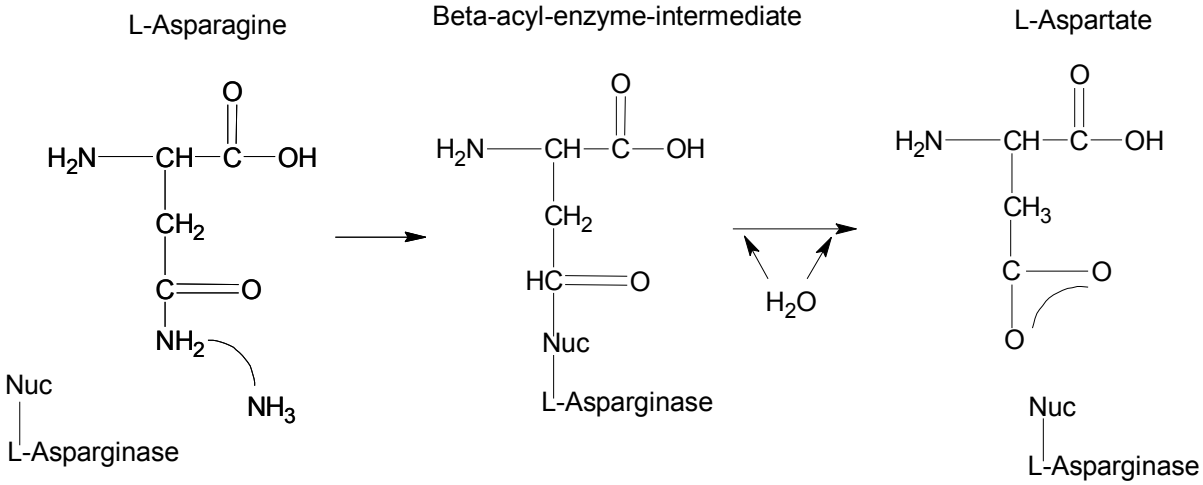

Fig.1. Degradation of asparagine by L-asparaginase enzyme. Nuc indicates nucleophilic change.

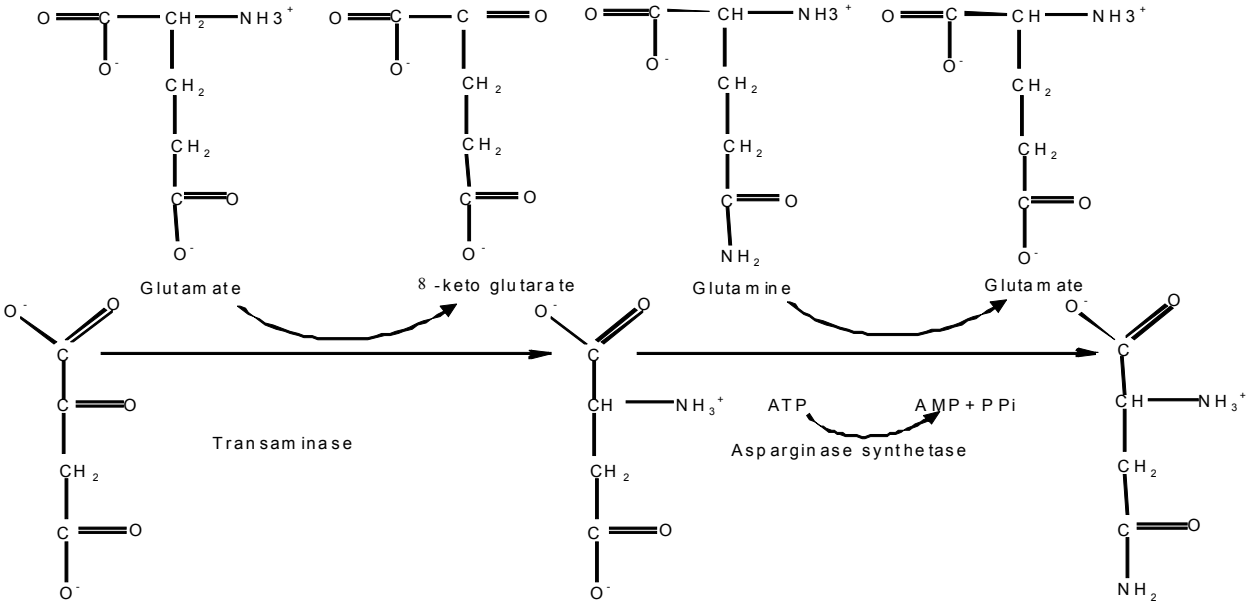

Fig.2. Biosynthesis of L-asparagine

Table 1 : Summary of fermentation conditions for production of L-asparaginase by Sub-merged fermentation (SmF)

Microorganism	Nutrition requirements % (w/v)	Fermentation conditions	Activity (U ml^{-1})	Volumetric Productivity (U ml^{-1} h^{-1})	References
Erwinia aroideae NRRL B-138	Tryptone (0.05); Glucose (0.1); Yeast (0.05)	pH 7.0; 28 °C ; 200 rpm; 8 h; (2.8L); fernbatch flasks pH 7.0; 28 °C ; 300 rpm; 24 h; aeration 0.5 (vvm); (20L) Semi-pilot	1250[a] 960[a]	156.25[b] 40 [b]	[77]
Bacillus sp.	Peptone (0.1)	pH 8.0; 37 °C ; 200 rpm; rotary shaker	RA	-	[64]
Serratia marcescens ATCC 60	AYE (4)	pH 5.0; 26 °C; 250 rpm; 48 h; (0.5L); EF pH 8.5; 40 to 42 h; (4L); P pH 8.4; 35 h; F	3.7 4 3.1	0.077 0.095 0.088	[38]
Erwinia aroideae NRRL B-138	Lactose (1.0); Yeast extract (1.5); K_2HPO_4 (0.1)	pH 7.5; 24 °C; 550 rpm; 16 h; aeration 1.0 (vvm); antifoam 0.02 (% v/v); B pH 7.5; 24 °C; D=0.05 h^{-1} ; activation energy- 8,500 cal $mole^{-1}$	4 3.7	0.25 -	[56]
Enterobacter aerogenes NCIM 2340	Sodium citrate (1); Diammonium hydrogen phosphate (0.16)	pH 7.0; 37 °C; 24 h; (0.25L); B Aeration 1.0 (vvm); 700 rpm; 6 h; (2L); B	0.60±0.02 1.2	0.021 0.2	[61]
Erwinia Chrysanthemi 3937	Peptone (1.0); Yeast extract (0.5); NaCl (1.0)	pH 5.5-7.0; 24 °C; B	2.25	-	[19]
Streptomyces plicatus	NaCl (0.5)	pH 7.0; 29±2 °C; B	-	33.5[d]	[82]
Streptomyces griseus ATCC 10137	Peptone (4.0)	pH 8.5; B	0.01	-	[66]
Thermus thermophilus HB8	Tryptone (0.5); Glucose (0.1); Yeast extract (0.3)	pH 9.2; 70 °C	494[e]	-	[28]
Pectobacterium carotovorum MTCC 1428	Glucose (0.207); L-asparagine (0.52)	pH 7.0; 30 °C; 180 rpm; 12 h; EF Uncontrolled pH; 30 °C; 200 rpm; aeration 1.5 (vvm); 12 h; (4 L) fermenter	14.71 15.39	1.225 1.282	[91]

Bacillus sp.DKMBT10	L-Asparagine (0.6); glucose/maltose (0.3)	pH 7; 37 °C; 200 rpm; 24 h	1.12[c]	-	[58]
Pichia pastoris	BSM2	pH 5.0; 30 °C; aeration 5 (l min^{-1}); (2L); antifoam (polypropylene oxide); B	85.6	1.083	[79]
Aspergillus terreus IOC 217	Proline (2.0)	pH 6.2; 30 °C; 160 rpm; 48 h; B	0.058	0.0012	[31]
Aspergillus oryzae	L-asparagine (0.5)	pH 6.2; 37 °C; 250 rpm; 96 h; B	0.14	0.0014	[78]
Aspergillus terreus MTCC 1782	L-Asparagine (1.0); Corn flour (1.5); Glucose (0.2)	pH 6.2; 30 °C; 160 rpm; 72 h.	33.25	0.462	[163]
Aspergillus terreus MTCC 1782	L-asparagine (1.0); Yeast extract (1.0); Peptone (0.6); Glucose (0.4)	pH 6.0; inoculum size (1.5); 30 °C; 160 rpm; 72 h.	24.10	0.334	[164]
Aspergillus terreus MTCC 1782	Ground nut oil cake Flour (3.99); Sodium nitrate (1.04); L-asparagine (1.84); Sucrose (0.64)	pH 6.0; 35 °C; 160 rpm; 72h; spore count of $2 \times 10^7 \sim 10^8$ ml^{-1}	36.97	0.513	[96]

EF, Elerenmeyer flask; F, Fermentor; P, Bottle; B, Batch; a, U g^{-1}; b, U g^{-1} h^{-1}; c, Specific activity; d, µg ammonia ml^{-1} h^{-1}; E, International units (IU); RA, Relative activity; AYE, Autolyzed Yeast Extract; BSM2, [117]

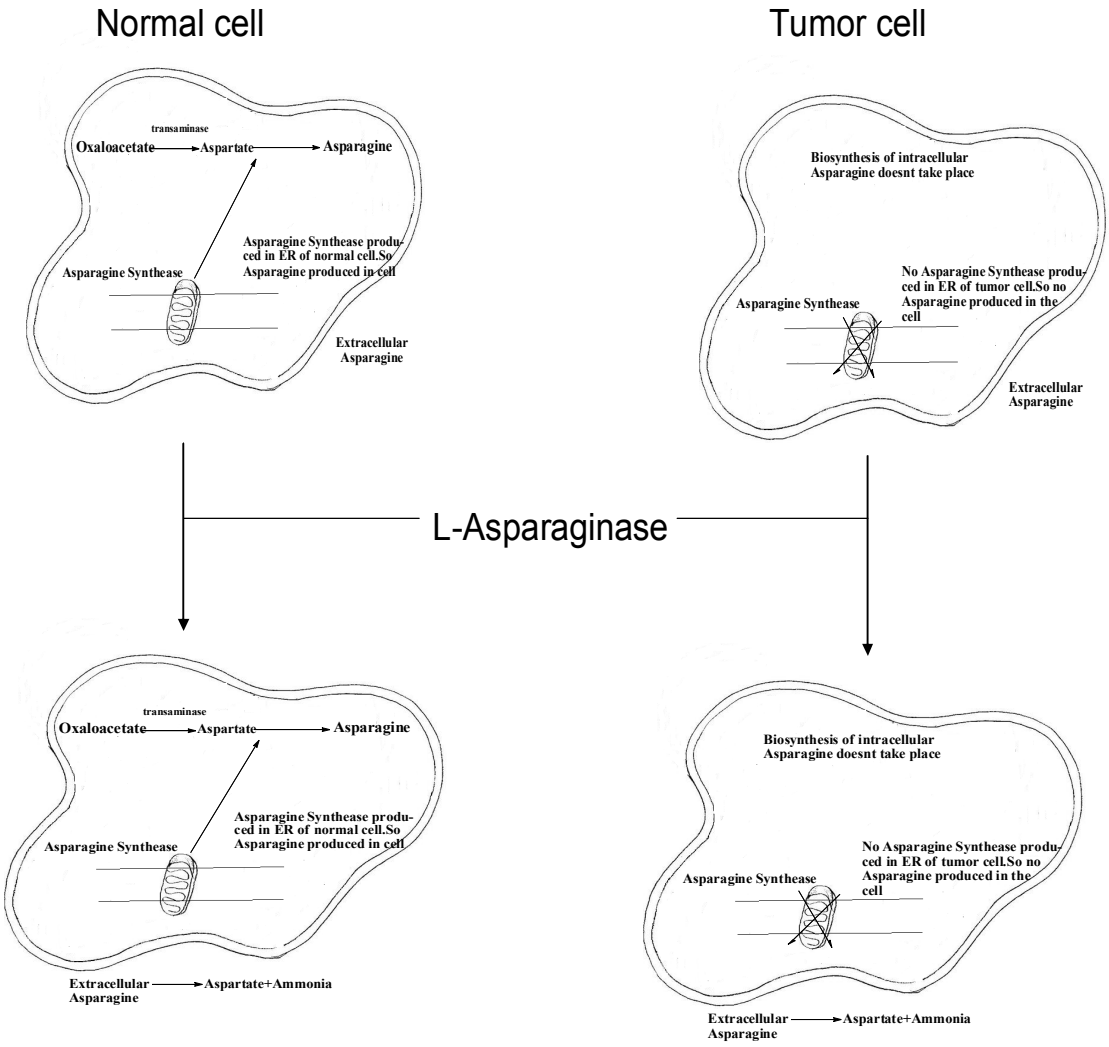

Fig.3. Role of L-asparagine in normal cells and tumor cells

Table 2: Summary of fermentation conditions for production of L-asparaginase by Solid state fermentation (SSF)

Microorganism	Substrates	Fermentation conditions	Activity (U ml^{-1})	Volumetric Productivity (U ml^{-1} h^{-1})	References
Pseudomonas aeruginosa 50071	Soya bean meal (10g); size (0.4-0.8 cm)	pH 7.4; 37 °C; 96 h; moisture content 50 % (w/v); SC	165.11	1.719	[85]
Pseudomonas aeruginosa 50071	Soya bean meal (5g); Inducers- casein hydrolysate (3.11) % (w/v); corn steep liquor (3.68) % (w/v)	pH 7.4; 37 °C; 96 h; moisture content 40 % (w/v); SC	1.428	0.0148	[86]
Aspergillus niger	Bran of glycine max	pH 6.5; 40 °C; 96 h; moisture content 70 % (w/v); SC	17.52±1.43	0.183	[87]
Serratia marcescens	Sesame oil cake	pH 7.0 - 7.5; 37 °C; 48 h; moisture content 68.64 % (w/v); SC	110.795[a]	2.308[b]	[165]
Serratia marcescens SB08	Rice bran (10g); L-asparagine (0.01) %; yeast extract (0.5) %	pH 7.0; moisture content 40 % (w/v); particle size (0.5 mm); 30 °C; 36 h	79.84 [a]	2.217 [b]	[166]
*Marine Actinomycetes**	Soya bean meal (5g);	pH 7.0; 37 °C; 96 h;	49.2	-	[92]
Bacillus circulans MTCC 8574	Red gram husk; Bengal gram husk; Coconut; Ground nut cake; L-asparagine (0.5) %	pH 7.0; 37 °C; 24 h; fractional factorial central composite design (FFCCD); SC	2322[a]	-	[167]
Fusarium equiseti	Soya bean; di potassium hydrogen phosphate (0.5) %; Manganese (0.01) %	pH 7.0; 37 °C; 48 h; SC	6.85[e]	0.142[b]	[168]
Fusarium equiseti	Glucose (0.5 %); Ammonium sulphate (0.5 %); Yeast extract (0.5%)	pH 7.0; 37 °C; 48 h; moisture content 70% (v/w); particle size (3 mm); inoculum volume (20%); SC	8.51[e]	0.177 [b]	[169]

*, Production L-asparaginase performed both in SSF and SmF; SC, Static conditions; a, U g^{-1}; b, U g^{-1} h^{-1}; e, International units (IU)

Table 3: Biochemical properties of some microbial L-Asparaginases

Microbial Source	pH optima	Temperature Optima (°C)	K_m (M)	pI	Specific Activity (µmol/min/mg)	Molecular Weight (kDa)	References
Pseudomonas aeruginosa	9	37	0.147 x 10^{-3}	-		160	[85]
Pseudomonas stutzeri	9	37	1.45 x 10^{-4}	6.38	732.3	34	[170]
Pseudomonas fluorescens	8.0-9.0	-	4.1 x 10^{-4}	4.5	-	70	[171]
Azotobacter vinelandii	8.6	48	1.1 x 10^{-4}	-	2.47	84	[43]
E.coli	7-8	37	1.25 x 10^{-5}	4.9	-	141	[104, 23-25]
Serratia marcescens	6.8	-	1.0 x 10^{-4}	5.2	255	171-180	[89, 27]
Tetrahymena pyriformis	8.6	-	2.2 x 10^{-3}	6.8	-	230	[36]
Erwinia aroideae	7.5	-	3 x 10^{-3}	6.8	256	155	[77, 172]
Erwinia carotovora	8	50	1.8 x 10^{-5}	-	-	125-145	[67, 173]
Saccharomyces cerevisiae	8.5	-	7.4 x 10^{-3}	-	-	400	[174]
Asparaginase I	6.8	-	3.5 x 10^{-4}	-	-	800	
AsparaginaseII							
Corynebacterium glutamicum	7	40	2.5 x 10^{-3}	-	-	80	[18]
Cylindrocarpon obtusisporum	7.4	37	1 x 10^{-3}	5.5	-	216	[97]
Mycobacteriun phlei	8.8-9.2	-	0.7 x 10^{-3}	-	-	126	[107]
Bacillus coagulans	8.5-9.5	55	4.7 x 10^{-3}	-	10.9	85	[99]
Fusarium tricinctum	7.5-8.7	-	5.2 x 10^{-4}	5.18	-	161-170	[109]
Vibrio succinogenes	7.3	-	4.78 x 10^{-5}	8.74	202	146	[108]
Proteus Vulgaris	7-8	57	2.6 x 10^{-5}	5.08	300	-	[106]

Table 4: Available clinical forms of L-asparaginase

Source	Marketed by	Commercial name	Country
E-coli asparaginase	EUSA Pharama	Kidrolase	UK
	Ovation pharmaceuticals Deerfield, Illinois	Elspar	US
	Bayer AG, Leverkusen		
	Sanofi-Aventis Paris	Crasnitin	Germany
	Kyowa Hakka, Tokyo	Leunase	France
		Asparaginase Medac	Japan
PEGylated form of Native *E.coli* Asparaginase	Sigma-Tau pharmaceuticals Inc., Gaithersburg	Oncaspar	MD
Erwinia Asparaginase	EUSA pharma, Oxford	Erwinase	UK

Note: Some of the preparations are not available in all countries.

Table 5: Assays for determination of L-asparaginase activity

S. No	Assay	Substrate used	Product analyzed	Principle	Remarks	References
1	Spectrophotometry (Colorimetry)	L-Asparagine	Ammonia Released	The Optical density at 500nm after the colored reaction is measured and compared to a standard curve prepared from solutions of ammonium sulphate as nitrogen source	Method involves the use of highly toxic reagents. Simple and quantitative but low activities of L-asparaginase enzyme cannot be measured	[77]
		L-Asparagine	Ammonia Released	Ammonia produced is degraded by glutamate dehydrogenase with concomitant oxidation of beta-NADH; disappearance of beta-NADH is then monitored spectrophotometrically at 340 nm	Continuous (and rather simple) detection of asparaginase activity	[175]
		L-Asparagine	Aspartate	The aspartate concentration measured spectrophotometricallly through coupling with L-glutamic oxaloacetic transaminase and L-malic dehydrogenase	Linear results for ammonia concentrations between 0 and 200 μM.	[176]
2	Fluorometry	L-aspartic acid Beta- (7-amido-4-methylcoumarin)	7-amino-4-methylcoumarin	The excitation and emission wavelengths of the released 7-amino-4-methylcoumarin are measured fluorometrically at 37°C using a fluorometer	Rapid assay but expensive substrate and equipment can be used monitoring L-asparaginase activity in patients during L-asparaginase therapy.	[177]

3	Titrimetry (Direct Nesslerization)	Asparagine	Ammonia Released	The ammonia released is quantitatively measured by titrations.	Good reproducibility is achieved but the method requires meticulous care and disparate results are obtained from different labs analyzing the same samples	[178]
4	Kinetic enzymatic method (gas sensing electrode)	Asparagine	Ammonia Released	The ammonia released is measured using an ammonia gas-sensing electrode	Expensive but very accurate and instantaneous quantification of ammonia	[179]
5	Paper electrophoresis	[14C]L-asparagine	[14C]L-aspartic acid	L-Aspartate was well separated from L-asparagine by electrophoresis. the section of the strip bearing aspartate was cut out and counted in a scintillation counter	Good sensitivity but Time Consuming	[180-181]
6	Chromatography	[14C]L-asparagine	[14C]L-aspartic acid	The conversion of [14C]L-asparagine to [14C]L-aspartic acid is followed by rapid chromatography on ion exchange paper	Detection of as small as a few pmoles of [14C] L-aspartic Acid. Therefore very less costly substrate required. But the method is Time consuming and not continuous	[23]
7	Conductimetry	asparagine or aspartate hydroxamate	ammonia and/or aspartate	The increase of conductivity due to the production of ammonia and/or aspartate in the reaction mixture containing cell-free extract and asparagine or aspartate hydroxamate	Measurement is simple because conductivity is linear with time and enzyme concentration and it follows Michaelis kinetics	[182]

CONCLUSIONS

Optimal formulation and dosage of asparaginase in the treatment of ALL is still to be understood thoroughly. Critical minimum value of serum asparaginase for effective control of malignancy has to be established. Literature suggests that a serum level of asparaginase greater than 100 IU l^{-1} corresponds to depletion of asparaginase below the level of quantification and is therefore considered the target trough asparaginase level [162]. However, some evidence points out that trough asparaginase level of below 50 IU l^{-1} can also result in serum and CSF asparagine depletion. These revelations prompt a thorough investigation to ensure administration of right doses of the drug and thereby minimize the side effects [113]. Also route of administration (IM or IV) is to be evaluated further for better outcomes of the treatment. Patients should be screened for silent hypersensitivity and if necessary should be switched over to second-line and third–line therapy. For this, effective monitoring of asparaginase levels should be adopted. At present, only 3 main preparation of asparaginase are used in treatment protocols (*E-coli* asparaginase, its PEGylated form and *Erwinia* asparaginase) although a lot of studies on different other sources of asparaginase have yielded encouraging outcomes. Further studies and regulatory approvals will enable the introduction of new asparaginase drugs with potential benefits to patients. Measurement of Triglycerides during the course of asparaginase therapy is strongly recommended to avoid complications.

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