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MICROBIAL L-ASPARAGINASE: A REVIEW ON CURRENT SCENARIO AND FUTURE PROSPECTS

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ABSTRACT

L-Asparaginase (E.C. 3.5.1.1.), also known as L-asparagine amidohydrolase is the enzyme with anti-tumor activity and is well accepted as a chemotherapeutic agent against the acute lymphoblastic leukemia and lymphosarcoma. This article has briefly touched nearly all the industrial and clinical aspects of L-Asparaginase and provides the recent update of the topic. The article includes a brief introduction to the topics, mechanism of action, a little information about the structure, sources of enzyme, purification, optimum conditions for the enzyme production, recombinant strains for higher productivity and formulation of the enzyme.

INTRODUCTION: A remarkable achievement in the field of medicine was the development of the L-Asparaginase enzyme as an effective antitumor agent. It was demonstrated by the Mashburn *et al*., (1964) [1] that the L-Asparaginase produced by the bacteria *E. coli* possesses the same antitumor activity as recovered from the guinea pig serum.

L-asparagine is a nonessential amino acid, can be synthesized from central metabolic pathway intermediates within the cell by an enzyme called asparagine synthetase in humans and is not required from external source. L-asparagine is a major requirement by the cells for the production of protein.

Tumor cells, more specifically lymphatic tumor cells, requires huge amount of asparagine to keep up with their rapid malignant growth. Thus the asparagine from the diet as well as what can be made by themselves (which is limited) is utilized by them to satisfy their large asparagine demand. Therefore L-asparagine is an essential amino acid for the growth of tumor cells.

L-Asparaginase EC 3.5.1.1 is a tetramer protein that deaminates Asn and Gln. L-Asparaginase (ASNase) inhibits protein synthesis in T-cells by catalyzing the conversion of L-asparagine to L-aspartate and ammonia, and this catalytic reaction is essentially irreversible under physiological conditions.

The enzyme L-Asparaginase has the chemotherapeutic property against the tumor cells. It is an effective curable agent against the treatment of acute lymphoblastic leukemia and lymphosarcoma. The enzyme catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia.

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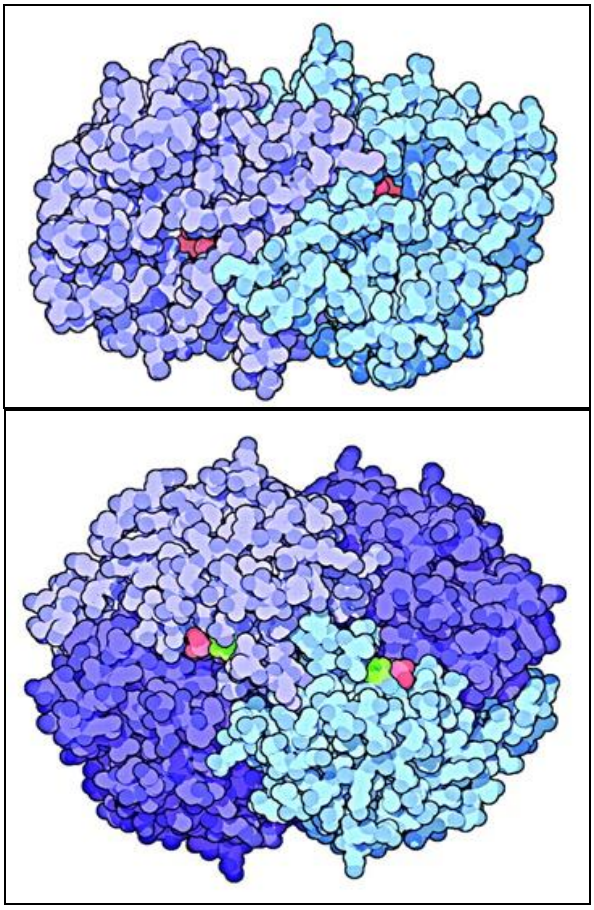
The principle behind the use of Asparaginase as an anti-tumor agent is that it takes advantage of the fact that all leukemic cells are unable to synthesize the non-essential amino acid asparagines their own, which is very essential for the growth of the tumor cells, whereas normal cells can synthesize their own asparagine; thus leukemic cells require high amount of asparagine. These leukemic cells depend on circulating asparagines for their ample nourishment and diet. Asparaginase, however, catalyzes the conversion of L-asparagine to aspartic acid and ammonia. This deprives the leukemic cell of circulating asparagine and prevents them from the rapid malignant growth (N. Verma e*t al.* 2007 2)

L-Asparaginase has its application in food industry also. It helps in reducing the content of acrylamide in baked food products by hydrolysing the L-asparagine (Mario Sanches *et al.* 2007 3).

The enzyme was isolated from the two bacterial sources: *Escherichia coli* and *Erwinia carotovora.* Bacterial source was helpful in producing high yield of the enzyme and a series of preclinical and clinical studies was able to be conducted. Today, L-Asparaginase is an essential drug that is in use for the treatment of acute lymphoblastic leukemia (ALL) in children all over the world. ELSPAR, ONCASPAR, KIDRPLASE, ERWINASE are the brand name of L-Asparaginase used as drug.

Natural killer cells are found to be sensitive against various anti-tumor agents. Miki Ando *et al.* (2005 4) examined the effectiveness of various anti-tumor agents to natural killer (NK)-cell tumor cell lines and samples, which are generally resistant to chemotherapy, using flow cytometric terminal deoxynucleotidyl transferase mediated dUTP-biotin nickend labelling (TUNEL) assay. Two types of cells chosen by them, NK-YS and NK-92 were highly resistant to various anti-tumour agents, were sensitive to L-Asparaginase as the enzyme induced apoptosis in these two NK-cell lines. NK-cell leukemia/lymphoma and acute lymphoblastic leukemia (ALL) samples were selectively sensitive to L-Asparaginase and to doxorubicin (DXR) respectively, samples of chronic NK lymphocytosis, an NK-cell disorder with an indolent clinical course, were resistant to both drugs. They categorize the defined two NK-cell disorders and ALL according to the sensitivity to DXR and L-Asparaginase. Asparagine synthetase levels in the samples were checked by real-time quantitative polymerase chain reaction (RT-PCR) and immunostaining. They found a good correlation among asparagine synthetase expression, *in vitro* sensitivity and clinical response of nasal-type NK-cell lymphoma to L-Asparaginase. Although high level of expression of asparagine synthetase were found at both mRNA and protein levels in aggressive NK-cell leukemia, L-Asparaginase induced considerable apoptosis. Furthermore, samples of each disease entity occupied a distinct area in two-dimensional plotting with asparagine synthetase mRNA level (RQ-PCR) and *in vitro* L-Asparaginase sensitivity (TUNEL assay). It was confirmed rather specific anti-tumour activity of L-Asparaginase against NK-cell tumors *in vitro*.



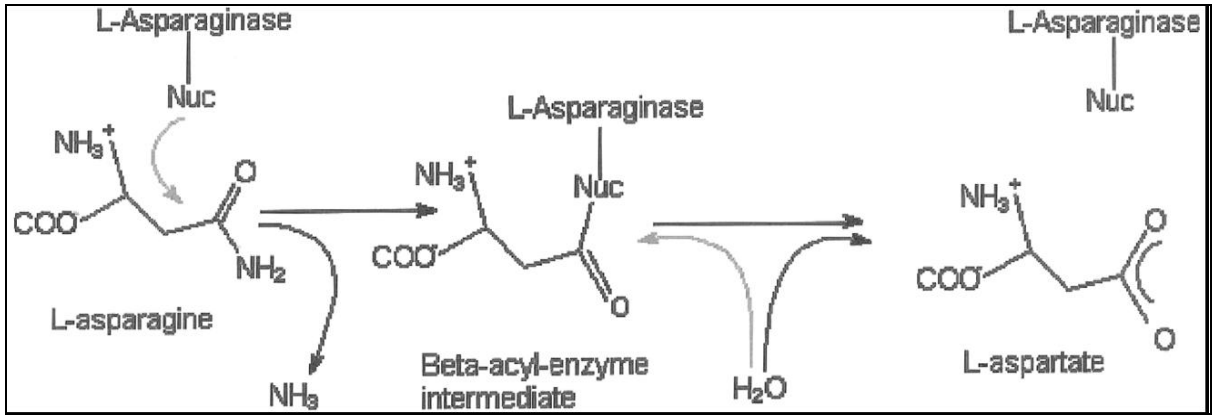
Asparagine synthetase (left) is a large enzyme composed of two identical subunits. The structure shown here is been referred to the enzyme from bacteria. It is responsible for the production of asparagine by combining an ammonia molecule directly to aspartate. In human the enzyme uses

glutamine to provide the amine instead of ammonia. L-Asparaginase (right) purified from bacterial cells is used for chemotherapy, composed of four identical subunits. The active sites grip asparagine (red) and use a well-placed threonine amino acid (green) to perform the cleavage reaction. The enzyme is also active with glutamine, cleaving its amino group off at a slower rate (David S. Goodsell 2005 5).

Comparison between the L-Asparaginase from *Erwinia carotovora* and *E. coli* has been reported by K. A. Cammack *et al.* (1972 6). According to them enzyme isolated from *Er. carotovora* is found to be more basic and the enzyme EC2 from *E. coli* contains neither tryptophan nor cystine, and disulphide bonds are therefore absent. The molecule is very stable in solution from pH 3.0 to about pH 12.0, and is somewhat more stable at alkaline pH than is the *E. coli* enzyme.

A pH and dye-based rapid plate assay for screening L-Asparaginase producing micro-organisms was performed by R. Gulati *et al.* (1997 7). It takes 24 and 48 h to get the promising results for bacteria and fungi, respectively and the results obtained after assay corresponds to the quantity of product formation in culture broths. It is generally observed that L-Asparaginase production is accompanied by an increase in pH of the culture and that forms the basis of the assay by incorporating the pH indicator phenol red in medium containing asparagine (sole nitrogen source). Phenol red at acidic pH is yellow and at alkaline pH turns pink, thus a pink zone is formed around microbial colonies producing L-Asparaginase. Studies with different concentrations of the dye revealed that as the concentration of the dye increased, the clarity and visibility of the pink zone increased. Furthermore, the dye does not have any inhibitory effect on the growth of the test organism. A direct correlation existed between zone diameters and enzyme activities in culture broths.

Mechanism of Action: L-Asparaginase prepared by different sources has different half –lives. Different half-lives of the L-Asparaginase preparations lead to the different durations of the asparagine depletion.



SCHEMATIC ILLUSTRATION OF THE REACTION MECHANISM OF L-ASPARAGINASE. (HILL *ET AL*., 1967 8).

The action of L-Asparaginase on the leukemic cells makes them deprive of the asparagine by causing the hydrolysis of asparagine to asparatic acid and ammonia. This makes the serum free of asparagine. Normal cells possess the asparagine synthetase to fulfil the requirement of asparagines in their diet and asparagine is a non-essential amino acid for normal cells. The gene which encodes the asparagine synthetase is located on chromosome 7q21.3 (Andrulis I. L. *et al.* 1990 9) and is the only enzyme available for the asparagine synthesis. This enzyme leads to modification 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 (Kiriyama Y. *et al.* 1989 10). Protein and RNA synthesis is inhibited in the absence of asparagine (Goody H. E. and Ellem K. A. 1975 11) and as a consequence cell cycle arrest and apoptosis is induced in murine leukemia cell lines (Ueno T. *et al.* 1997 12). To achieve the complete asparagine depletion in the human circulation, the L-Asparaginase activity level in serum must be > 100 IU/L (Boos J. *et al.* 1554).

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 for asparagine synthesis. This offers the tumor cells a safe and confirm exit from the action of L-Asparaginase (Savitri *et al.* 2003 13.).

SOURCES OF ENZYME AND THEIR PHYSICOCHEMICAL PROPERTIES: Initially, L- Asparaginase enzyme could be isolated from the two bacterial sources: *Escherichia coli* and *Erwinia carotovora.* Bacterial source was helpful in producing high yield of the enzyme and a series of preclinical and clinical studies was able to be conducted. But today number of sources for the isolation of enzyme is present. They also include fungal sources of isolation, actinomycetes, plant sources and animal sources.

Some sources have been summarised under:

S. No.	Source	Temperature (°C)	pH	Reference
Bacterial sources				
1.	*Pseudomonas aeruginosa* 50071	37	9	El-Bessoumy A. A. *et al.* 2004 14
2.	*E. coli*	37	7-8	Howard Cedar and James H. Schwartz 1968 15
3.	*Pyrococcus furiosus*	Thermostable	9	Bansal S. *et al.* 2010 16
4.	*Bacillus sp.*	37	7	Vidhya Moorthy *et al.* 2010 17
5.	*Enterobacter aerogenes*	37	8	J. Mukherjee *et al.* 1999 18
6.	*Azotobacter vinelandii*	30-37	6.5-8.0	Gaffar and Shethna 1977 19
7.	*Pseudomonas stutzeri* MB-405	37	9.0	Manna S. *et al.* 1995 20
8.	*Thermus thermophilus*	77	9.2	Prista and Kyriakidis 2001 21
9.	*Cylindrocarpon obtusisporum* MB-10	37	7.4	Raha S. K. *et al.* 1990 22
10.	Mucor species associated with Marine Sponge *Spirastrella sp.*	50	4-8	Mohapatra B. R. *et al.* 1997 23
Fungal sources				
11.	*Aspergillus niger*	40	6.5	Abha Mishra 2006 24
12.	*Aspergillus*, *Penicillium*, and *Fusarium*	-	-	De-Angeli et al. 1970 25, Arima et al. 1972 26, Imada *et al.* 1973 27, Nakahama et al. 1973 28, Curran *et al*. 1985 29
13.	*Aspergillus terreus* MTCC 1782	-	-	Baskar and Renganathan 2009 30
Actinomycetes sources				
14.	*Streptomyces griseus* ATCC 10137	37	8.5	Peter J. Dejong 1972 31
15.	*Streptomyces tendae* TK-VL_333	30	7.0	Kavitha and Vijayalakshmi 2010 32
16.	Actinomycetes from estuarine fishes	37	7-8	Maloy Kumar Sahu *et al.* 2007 33
17.	Marine Actinomycetes	60-80	8-9	Dhevangi P. and Poorani E. 2006 34

18.	Actinomycetes from Thai medicinal plant	37	7	Sutthinan Khamna *et al.* 2009 35
19.	*Streptomyces gulbargensis*	40	8.5	Amena S. *et al.* 2010 36
Plant sources				
20.	*Tamarindus indica*	37	8.5	Mozeena Bano and V.M. Sivaramakrishnan 1980 37
21.	*Capsicum annum* L.	37	8.5	Mozeena Bano and V.M. Sivaramakrishnan 1980 37
22.	*Withania somnifera* (Ashwagandha)	37	8.5	Oza V. P. *et al.* 2009 38
Yeast sources				
23.	*Candida utilis*	-	-	Kil *et al.* 1995 39
24.	*Pichia polymorpha*	-	-	Foda *et al.* 1980 40
25.	*Saccharomyces cerevisiae*	-	-	Bon *et al*. 1997 41

The L-Asparaginase II enzyme was isolated by Howard Cedar and James H. Schwartz from *Escherichia coli.* Tsuji was the first who identified and reported the deamidation of L-asparagine by *Escherichia coli* cells due to the presensce of L-Asparaginase II (Tsuji Y, 1957 42). L-asparagine amidohydrolase, EC 3.5.1 .1 was another enzyme, isolated from *E.coli*, reported later and possess different properties. Both the enzymes have different affinities for the asparagines (Campbell H. A. *et al.* 1967; Roberts J. *et al.* 1966; Stephenson M. and E. F. Gale 1937 43-45). The former has the higher affinity for asparagine which is normally found in periplasmic space (Cedar H. and J. H. Schwartz 1967 46).

Normally enzyme was produced under aerobic conditions but a significant greater amount of enzyme can be produced under anaerobic conditions by using media enriched with high concentration of various amino acids (Cedar H. and J. H. Schwartz 1968 15).

Some recent reports list *Tetrahymena pyriformis* (Triantafillou *et al.* 1988 47), *Corynebacterium glutamicum* (Mesas *et al.* 1990 48.), *Cylindrocarpon obtusisporum* (Raha *et al.* 1990 22), *Pseudomonas stutzeri* (Manna *et al.* 1995 20), and *Rhodosporodium toruloides* (Ramakrishnan and Joseph 1996 49) as sources.

It is considered that L-Asparaginase II form of the enzyme is responsible for the treatment of the cancer cells, which is found to be in active form in 5 strains among the different strains of coliform. Different growth conditions and media composition has been considered to isolate the enzyme in active form, so the advantages of enzyme can be possible. It may lead to high enzyme yield also. Optimum aerobic conditions have been proved useful for the higher yield, as the turbulent conditions has been shown to have higher growth of biomass with low yield of product. Purification of the enzyme in significant amount i.e. up to 40 folds can be achieved by ammonium sulfate and ethyl alcohol precipitation (M. H. Bilimoria 1969 50).

Hymavathi *et al.* optimized the conditions for the production of enzyme L-Asparaginase from the isolated strain of *Bacillus circulans* MTCC 8574 by solid state fermentation, using agricultural waste as the nutrient source. The conditions which affect the enzyme yield include incubation temperature, moisture content, inoculum level, glucose, and L-asparagine (Hymavathi M *et al.* 2009 51).

L-Asparaginase EC 3. 5. 1. 1 was purified to homogeneity from *Pseudomonas aeruginosa* 50071 cells that were grown on solid-state fermentation (Ashraf A. *et al.* 2003 52)

Fungal Sources: L-Asparaginase enzyme is also isolated from the various fungal sources else than the bacterial sources. Enzyme originated from the bacterial sources has been found to lead the allergic reactions and anaphylaxis (Reynolds & Taylor 1993 53). Enzyme isolated from fungal source does not have any allergic impacts.

T. Theantanal *et al.*(2007 54) went through more than 100 isolates of endophytic fungi and evaluate them for the quantitative and qualitative analysis of the production of Asparaginase. Very few of them, just about 25 of the isolates gave positive results by showing pink zones around the colonies on agar media plates. Enzyme isolated from *H. benghalensis* and *E. odoratum* were highly active, proved by spectrometric method.

Abha Mishra (2006 24) reported higher yield of the enzyme from a different isolate of *Aspergillus niger*, agrowaste from the leguminous crops as a source. She adopted the process of solid state fermentation (SSF). Bran of Glycine max as main source of nutrients give highest yield of enzyme, which was followed by the *Phaseolus mungo,* and *Cajanus cajan.*

Production of L-Asparaginase from Natural Substrates by Aspergillus terreus MTCC 1782 and the effects of Substrate, Supplementary Nitrogen Source and L-Asparagine had been studied by Baskar and Renganathan (2009 30). The maximum L-Asparaginase obtained from synthetic L-proline in addition with 1% sodium nitrate and 1% L-asparagine is 34.98 IU/ml.

Among the natural substrates studied such as groundnut oil cake powder, corn flour and cottonseed oil cake (mesh size of (80/120) were used as natural substrate as alternates to L-proline, groundnut oil cake is found to produce maximum L-Asparaginase.

The maximum L-Asparaginase obtained for 2% groundnut oil cake, 1% sodium nitrate and 1.2% L-asparagine is 30.35 IU/mL and it is comparable with synthetic L-proline (34.98 IU/mL). The L-proline or natural substrates rich in nitrogen content along with sodium nitrate and L-asparagine enhances the L-Asparaginase production.

Actinomycetes as a Source: Sahu *et al.* extracted the L-Asparaginase from the actinomycete strain LA-29 source, isolated from the gut contents of the fish, Mugil cephalus of the Vellar estuary which was reported to have significant enzyme activity. The enzyme was purified 18-fold and from which 1.9% of protein was recovered and showed the activity of about 13.57 IU/mg of protein (Sahu *et al.* 2007 55).

Dhevagi and Poorani reported the isolation of marine actinomycetes from the Parangipettai and Cochin coastal areas of South India, having enough L-Asparaginase activity. The partially purified L-Asparaginase showed a specific activity of 64.07 IU/mg protein, 83-fold pure and yielded 2.18 per cent of protein. Molecular weight of enzyme was found to be 140 kDa (Dhevangi P. and Poorani E. 2006 34).

Gunasekaran *et al.* (1995 56) reported L-Asparaginase production by *Nocardia sp.* Production of intracellular and extracellular Asparaginase from *Streptomyces longsporusflavus* (F-15) has been described by Abdel-Fatah *et al.* (1998 57). *Streptomyces sp.* isolated from the gut of the fish *Therampon jarbua* and *Villorita cyprinoids* has L-Asparaginase activity (Dhevendaran and Anithakumari 58).

Plant Sources: L-Asparaginase as an anti-tumor agent can be isolated from a number of sources. To fulfil the demand of medicine industry search of new and novel sources is needed. Green chillies (*Capsicum annum* L.) and tamarind (*Tamarindus indica*) has been reported to have ample amount of L-Asparaginase. Sources were used to isolate the enzyme by Mozeena Bano and V.M. Sivaramakrishnan (1980 37). Enzyme isolated from the green chillies was purified up to 400-folds by different methods and it was observed that enzyme exist in two forms and only one of them showed the anti-tumor activity. Enzyme had a sharp pH of 8.5 and a temperature optimum of 37°C.

Seed of *Pisum sativum* was also used as a source of L-Asparaginase. It was shown by Lea and Miffin that its activity is dependent on the presence of K^+ ions. Activity was also observed by Na^+ and Rb^+ but it was not in ample amount. The concentration of K^+ at which activity was maximum was 20 millimolar. Presence of K^+ prevents the enzyme from denaturation on heating (Peter J. Lea and Benjamin J. Miflin 1980 59).

Different species of *Solancaceae* and *Fabaceae* were screened for the activity of L-Asparaginase. Out of 34 different species *Withania somnifera* was identified as L-Asparaginase producer. Purification of enzyme was followed by ammonium sulphate precipitation and as well as Sephadex-gel filtration.

Enzyme purified from the plant is obtained as homodimer with a molecular mass of 72+/- 0.5 kDa. Optimum pH and temperature for enzyme reported as 8.5 and 37°C, respectively (Oza V.P. *et al.* 2009 38).

Co-occurrence of both subtypes of L-Asparaginase has been seen in *Arabidopsis* by Bruneau *et al.* Their catalytic activity is dependent on K^+ dependent. It has also been reported that catalytic activity of L-Asparaginase is ten folds in recombinant At3g16150 in presence of K^+ and becomes 80 folds in presence of L-Asn (Bruneau L. *et al.* 2006 60).

Animal Sources: Guinea Pig serum has been accepted as an inhibitor of lymphoma cells. This was proved by Broome and M.B. in 1963. They culture the lymphoma 6C3HED cell line in eagle's medium. It showed the proliferation of few cells after a latent time of about two weeks and cells grew were not sensitive to Guinea Pig serum. But when, L-Asparagine was added to the media it provided a strong growth stimulus for cells freshly placed in culture, but the cells retained their original character of sensitivity to Guinea Pig serum even after prolonged periods of growth (Broome J. D. and M.B. 1963 61).

Activators and Inhibitors: It has been reported that there are some elements which affect the activity of the enzyme, some activates the enzyme and increase the activity up to many folds and some inhibit the activity. Various elements have been reported to this reference. Raha S. K. *et al.* (1990 22) isolated the enzyme from mesophilic fungus *Cylindrocarpon obtusisporum* MB-10, pI-5.5, optimum pH and temperature- 7.4 and 37°C. The enzyme was tetramer of four identical subunits, a conjugate protein wit 37.3% (w/w) carbohydrate. They reported metal ions, such as Zn2+, Fe2+, Cu2+, Hg2+ and Ni2+ potentially inhibited the enzyme activity, while metal chelators like EDTA, CN-, cysteine, etc., enhanced the activity indicating that the enzyme was not a metalloprotein.

Its activity was also enhanced in the presence of reduced glutathione but not with dithiothreitol and 2-mercaptoethanol. From *Tetrahymena thermophila* two subtypes of L-Asparaginase have been isolated by Tsavdaridis I. K. *et al.* and they were purified to near homogeneity. Optimum pH of enzymes was reported as 8.6. Both forms cross reacted with antibodies raised against T. pyriformis L-Asparaginase and show isoelectric points 7.4 and 8.2. Among the metals tested, Ca2+ is the most effective in activating L-Asparaginase I or II activity (Tsavdaridis I. K. *et al.* 1994 62).

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Various other elements including Cu^{2+}, diphosphate, EDTA, I^-, $Li^{+,}$ Mg^{2+} has been reported to influence activity of L-Asparaginase, isolated from different sources, directly or indirectly.

Mode of Production and Optimization Process: L-Asparaginase production by submerged fermentation was performed by Vidhya Moorthy *et al.* (2010 17). They took a soil isolate belonging to the *Bacillus* species. The culture conditions were optimized for the higher yield of the enzyme. Two carbon sources, glucose and maltose, were used for the enzyme production. The former gave the better results. The enzyme was activated by $MgCl_2$ and inhibited by EDTA.

Nutritional requirement for the production of L-Asparaginase from *Fusarium* species was studied by Radhika Tippani and Girisham Sivadevuni (2012 63). They performed the production by submerged fermentation. The highest amount of enzyme production by *F. semitectum* (328 IU/ml), *F. moniliforme* (300 IU/ml) and *F. oxysporum* (210 IU/ml) was obtained with glucose as carbon source, with lactose presenting the second best carbon source for *F. semitectum* (218 IU/ml) and *F. oxysporum* (178 IU/ml) and mannose for *F. moniliforme* (213 IU/ml).

The most adopted method of production of L-Asparaginase enzyme is from submerged fermentation, which has been adopted throughout the world. Some limitations of process were observed later and to overcome those disadvantages method of solid state fermentation has been adopted. It has several advantages over submerged fermentation:

- Higher product yield
- Low capital cost
- Low energy input
- Simple fermentation media
- low water use and many more (Mukherjee *et al.* 1998; Mishra A. 64-65).

Moreover submerged fermentation leads to generation of effluents, which ultimately calls for treatment process (El-Bessoumy *et al.* 2004 14).

In SSF agricultural waste can be used as source of nutrients which is cost effective and environment friendly (Couto S. R. and Sanroman M. A. 2005 66).

SSF holds tremendous potential for the production of secondary metabolites and has been increasingly applied in recent years (Sangeetha *et al.,* 2004 67).

L – Asparaginase production by solid state fermentation (SSF) was studied by C. Venil & P. Lakshmanaperumalsamy (2009 68) from the source of *Serratia marcescens* SB08 and rice bran as substrate and broth was enriched with other nutrients.

Abha Mishra (2006 24) reported higher yield of the enzyme from a different isolate of *Aspergillus niger*, agrowaste from the leguminous crops as a source.

An Optimized Medium for Screening of L-Asparaginase II production by *Escherichia coli* was investigated by Ghasemi *et al* and also the results were revised by gene regulation. Samples collected by them were from different sources include samples from Khoshk river, some waste water samples and some clinical samples. A total of 130 isolates of *E. coli* were isolated (33 from waste water, 15 from the Khoshk River, 47 from urine samples and 35 from stool samples).

The concentration of media ingredients KH_2PO_4 (0.75 g), NaCl (0.5 g), L-asparagine (10 g), Maltose (1 g), agar (17 g) and phenol red (0.05 g) were found to be most promising among different sets of concentration. On optimized medium with above concentrations among the 130 isolates of *E. coli*, 35 isolates produced trace pink zone and 8 isolates produced (+) zone, while only 4 trace zones were produced on modified M9 medium. The *asnB* gene of *E. coli* is regulated by catabolite activator protein (CAP; also called cyclic AMP receptor protein or CRP). The low level of intracellular glucose results in a high level of cAMP, which means high cAMP-CAP complexes. cAMP-CAP binds to a specific site on the DNA that is located adjacent to the promoter for the genes regulated by CAP. Therefore, it was observed that glucose is an inhibitor for *asnB* gene. And the carbon source glucose was replaced by to disaccharide for reduction in the level of intracellular glucose and more *AnsB* production; maltose was the best (Y. Ghasemi *et al.* 2008 69).

An attempt was made to study to optimize the production of L-Asparaginase by *Fusarium equiseti* using soya bean meal under solid state fermentation (SSF) by Hosamani *et al.* 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 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.

L-Asparaginase production increased with moisture content and was observed to be maximized at 70% and the least activity was obtained at 100%. L-Asparaginase production increased readily with increase in the inoculum level and maximum enzyme activity was obtained at 20% (v/w) and least activity was obtained at 50% (v/w) inoculum level. Optimal glucose concentration was observed at 0.5% with maximum activity of 6.81 IU and the least activity of 4.25 IU for 1.25% sucrose. The L-Asparaginase production was studied supplementing the inorganic nitrogen forms such as ammonium sulphate, ammonium chloride, ammonium nitrate and organic nitrogen forms such as yeast extract, peptone and beef extract respectively.

0.5% ammonium sulphate and 0.5% yeast extract gave the optimum activity. Hence in the present study ammonium sulphate and yeast extract can be used as best nitrogen source (Hosamani and Kaliwal 2011 70).

Optimization of production level of L-Asparaginase from *Erwinia carotovora* was done by Vaibhav D. Deokar *et al.* 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.

Using Response Surface Methodology (RSM) it was found that a medium containing 11.33 g/L of Maltose, 17.44 g/L of Yeast Extract, 1.97g/L of L-asparagine was optimum for production of intracellular L-Asparaginase .The optimized medium was predicted to give 107.46 IU of L- Asparaginase activity. The applied methodology was validated by using optimized medium and L-Asparaginase activity of 108.06 IU was obtained with 1.32 fold increase in L-Asparaginase activity (Vaibhav D. Deokar *et al.* 2010 71).

M. Sunitha *et al.* used the Plackett-Burman design to screen the media components influencing the production level of enzyme. They used the *Bacillus cereus* MNTG-7 as the source. A total of 67 nutrients comprising of fifteen each of carbon, nitrogen, mineral/salt and eleven each of inorganic nitrogen sources and amino acid sources were screened for the production of L-Asparaginase by submerged fermentation.

This design involves screening of up to 'n-1' variables in just 'n' number of experiments. Regression coefficients and t-values were calculated by subjecting the experimental data to statistical analysis. Among the 67 nutrients based on their performance in terms of product yield and cost, tapioca starch, L-Asparagine, ammonium oxalate, gelatin and $CaCO_3$ were identified as most effective by them (M. Sunitha *et al.* 2010 72).

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 use 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 Wink et al. 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

PCR technique was adopted by the scientists to amplify the *E. carotovora* Asparaginase gene (*ErA*) and it was cloned into a pCR-Blunt vector and subcloned to pET-30a(+), an expression vector. The best conditions optimized for the best expression were at 30°C after 6 hours, incubated with IPTG 1mM induction. SDS-PAGE technique was used to confirm the results.

For purification purpose cation exchange chromatography and size exclusion columns were used (Priscila Lamb Wink *et al.* 2008 73).

Another attempt for generating the recombinant form of the enzyme was made by the Harry J. Gilbert 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 (Harry J. Gilbert *et al.* 1986 74). To increase the production level of L-Asparaginase enzyme, as it is well accepted as the anti-tumor agent, a technique by constructing recombinant

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 K. Hegazy and Maysa E. Moharam. *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 2.5fold more L-Asparaginase (Wafaa K. Hegazy and Maysa E. Moharam 2010 75).

Different patterns of expressions of recombinant L-Asparaginase in different *E. coli* hosts were studied by Gustavo Roth and his colleagues. *E. coli* was chosen as it is the most well studied microorganism. Mutant strains 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. 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. For 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 polyacrylamide gels (SDS-PAGE). All *E. coli* strains tested showed the superexpression 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 (Gustavo Roth *et al.* 2010 76). To discover the physiology of the L-Asparaginase enzyme in recombinants, W.R. Barnes *et al.* mate the Escherichia coli 4318 (thi leu Las- Hfr) and E. coli A-1 (Met- Las' F-) which resulted in the formation of prototrophic recombinants having L-Asparaginase activities at three distinct levels. They described the physiology of L-Asparaginase synthesis in these recombinants.

One class of recombinants produced significantly more L-Asparaginase than E. coli A-1. Oxygen was the factor which has the negative effect on the L-Asparaginase production in the medium and was transiently repressed by the presence of glucose in the same manner as that observed in the parental strains. L-Asparaginase activity was increased by the addition of oxalacetate as well as other members of the tricarboxylic acid cycle (W.R. Barnes *et al.* 1978 77).

Purification of Enzyme: Juan M. Mesas *et al.* discovered L-Asparaginase in a significant active form when lysine is over produced in cultures of *Corynebucterium 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 (Juan M. Mesas *et al* 1990 78). The enzyme, sometimes, produced intracellularly. To purify the intacellular product Horst Bireau perform bead milling followed by the adsorption of captured intracellular products and enzyme by fluidised bed adsorption (Horst Bierau 2000 79). Sonication method was also approached by Supriya D. Saptarshi and S.S. Lele for the purification purpose of L-Asparaginase enzyme from the *E. carotovora* species. Outcome of this method is again significant in figures. Yield of the enzyme was further optimized by a classical factor at a time of approach followed by evolutionary optimization (EVOP) technique.

The recovery of enzyme increased from 25.615 to 781 IU/ml in EVOP optimized sonication protocol (Supriya D. Saptarshi and S. S. Lele 2010 80). A simple sonication was implemented to purify the enzyme from crude cells of *Enterobacter aerogenes.* The enzyme was separated by gel filtration with sephcryi S-100, followed by ultra filtration (J. Mukherjee *et al* 1999 18).

The enzyme from various sources has been purified to crystallized form by various researchers. Bacterial L-Asparaginase has anti-neoplastic property and as well some side effects are there. L-Asparaginase from *Erwinia carotovora* has been reported to show low glutaminase activity. Recombinant *Er. carotovora* l--Asparaginase was crystallized in the presence of l-glutamate by the hanging-drop vapour-diffusion method using 10 mg ml^{-1} purified enzyme, 16–18%(*w*/*v*) PEG 3350 and 0.2 *M* NaF. X-ray diffraction data were collected to 2.6 Å at 293 K using an in-house rotating-anode generator. The crystals belong to the monoclinic $P2_1$ space group, with unit-cell parameters *a* = 78.0, *b* = 112.3, *c* = 78.7 Å, β = 101.9° and a homotetramer in the crystallographic asymmetric unit (Linnea E. K. Wikman *et al.* 2005 81).

The analysis of a monoclinic crystal modification of L-Asparaginase from *Escherichia coli* was done by Otto Epp *et al.* They reported the fact that pseudo-symmetries occurring in the crystal structure provide evidence that the molecules supports the chemical and physico-chemical evidence for a tetrameric subunit structure lie on positions with approximate point symmetry 222 (Otto Epp *et al.* 1971 82).

An exracellular expression of recombinant therapeutic enzyme L-Asparaginase II from *Escherichia coli* was obtained by Amardeep Khushoo *et al*. by fusing the different gene coding sequences. Again scientist induced the cells with 0.1 mM IPTG during the exponential phase. 4-fold higher activity was observed with this technique in TB media as compared to LB media. The recombinant form of extracellular enzyme was purified in 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% (Amardeep Khushoo *et al* 2004 83).

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

L-Asparaginase has a significant role in food industry. Acrylamide, reported as a significant toxic agent and cause neurotoxicity in humans, is present in ample amount in food items which are heat-derived and contain some reducing sugars. Formation of acrylamide is the result of Maillard reaction between the free amino acid asparagine and carbonyl group of reducing sugars like glucose. Maillard reaction is heat-induced reaction. Hence, food industry like baking food industry got an application of L-Asparaginase. The enzyme helps in hydrolysing the asparagine which significantly reduces the formation of acrylamide. The reported reduction in acrylamide content is about 90% (Mario Sanches *et al.* 2007 3).

An example was conducted by Kukurova K *et al.* (2009) on dough resembling traditional Spanis rosquillas. They consider the different parameters which influence the reaction and formation of acrylamide and include temperature/time profile of frying process, moisture, sugars, amino acids, acrylamide, and some indicators of Maillard reaction. At different levels of asparagine they got a reduction of 96-97% in acrylamide content (Kukurova K *et al.* 2009 84).

Formulation and Modification: 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 Yoh Kodera *et al.*

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 (Yoh Kodera *et al.* 1994 85).

Another experiment was performed by Yoshihiro Ashihara *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% (Yoshihiro Ashihara *et al.* 1978). mPEG2 (2,4-bis(O-methoxypolyethyleneglycol)-6-chloro-S-triazine) is also used to modify the enzyme by Zhang J. F. *et al.* it is performed in the presence of L-asparagine and the molar ratio maintained was mPEG2/-NH_2 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 (Zhang J. F. *et al.* 2004 86).

Cross linkage was the technique adopted by Handschumacher and Gaumond to modify the enzyme to reduce its side effects. They use the dimethylsuberimidate 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 (R. E. Handschumacher and C. Gaumond 1972 87).

The modified L-Asparaginase from *E. coli* retained the activity of 8% after the modification with monomethoxypolyethylene glycol, reported by Y. Kamisaki *et al.* 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 (Y Kamisaki *et al.* 1981 88).

CONCLUSION: The discovery of the fact that L-Asparaginase is responsible for the action of the guinea-pig serum against the acute lymphoblastic leukemia has set a milestone in the field of medicine. After this discovery detailed information about the enzyme has been dug out. It has been proved that L-Asparaginase from *Escherichia coli* and *Erwinia carotovora* has anti-neoplastic activity against cancer and is being used as anti-cancer drug. But, it is observed that the action of enzyme is coupled with some side effects. Moreover, the yield of enzyme was not enough to fulfil the demand of the drug. Solid state fermentation is being adopted all over the world for the production of the enzyme as it has many advantages over submerged fermentation. So, it created the need to discover new sources and techniques to enhance the yield and decrease the side effects of the enzyme. Enzyme isolated from different sources has different optimized conditions for production and activity. The structure of enzyme predicted from *E. coli* has four identical units. Recombinant work and formulation of enzyme is also in progress, yet there is still a long way to go. Moreover, L-Asparaginase has spread its arms in food industry also, as an ingredient which reduces the toxicity of baked food by significantly reducing the amount of acrylamide in food items. It is a need of the time, medicine and food industry to dig a lot more to satisfy the thirst of the drug.

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