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A Mini Review On L-asparaginase : Properties & Applications

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Abstract

L-asparaginase is a vital enzyme used in chemotherapeutic treatment against acute lymphoblastic (ALL) , Hodgkins lymphoma etc. The ability of L-asparaginase to convert essential amino acid L-asparagine (for tumor cells) into L-aspartic acid & ammonia , has made it an altogether a drug of choice for remmision of malignancies of lymphoid system.

The other major application of L-asparaginase is its prospective use in food industry as a mitigating agent. The addition of L-asparaginase before heating of starchy foods minimizes the risk of formation of acrylamide (a carcinogenic agent) & hence restricts its consequent intake. The acrylamide formed is converted into simple amino acids such as L-aspartic acid which is a necessary component in the foods. Apart from application in medicine & food industry , L-asparaginase has also been used efficiently as a biosensor. The distinction between normal cells & leukemic cells can be identified through L-asparaginase biosensors. Various organisms, including a few plants, animals, fungi, micro-organisms produce L-asparaginase. The physico-chemical properties, kinetic properties, optimum pH, temperature , molecular weights, differ from organism to organism. The present review is therefore, an attempt to highlight various sources of L-asparaginases, its applications, purification methods, optimizing conditions for production of L-asparaginases, kinetic properties & strategies employed for obtaining recombinant L-asparaginases.

Key words: L-asparginase, acute lymphoblastic leukemia (ALL), acrylamide, micro-organisms , properties , recombinant L-asparaginases .

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1. Introduction

L-asparaginase (L-asparaginase amidohydrolase ; EC 3.5.1.1)catalyzes the hydrolysis of L-asparagine to L- aspartic acid & ammonia.[1] Therefore, it has been widely used as an anti-cancerous agent to combat malignancies of the lymphoid system, Acute Lymphoblastic Leukmia(ALL), Hodgkins lymphoma & Melanosarcoma.[2] Other potential application of L-asparaginase lies in its use as a food-processing aid(mitigating agent) where, it reduces the formation of acrylamide. The distinction between normal cells & leukemic cells by using L-asparagine as a biosensor detected by colour visualization has also been its another vital application.[3]

L-asparaginase have been obtained from plants, animals, micro-organisms, fungi, yeast. Microbial L-asparaginases have been endowed with higher L-asparaginase activity, handling is easier, economical compared to other sources , with best therapeutic applications.[4] The microbial L-asparaginase (with higher therapeutic index) have been commercially marketed for medicinal use under the brand names as, Kidrolase, Elspar, Asparaginase & Medac.[5]

The current review summarizes, sources of L-asparaginase, optimizing conditions, purification methods & characteristics of purified L-asparaginases.

2. Historical Significance :

Lang S. (1904) was the first researcher to demonstrate the amido-hydrolytic activity of L-asparaginase.[6] This was later confirmed by Furth & Friedmann. (1910) [7] Clementi (1922) showed guinea pig blood serum contained L-asparaginase.[2,8]

Kidd J G. (1953) injected guinea pig serum invivo into mice & rat transplanted with lymphomas. In this experiment, Kidd J G.(1953) presented that the guinea pig serum consisted an active component necessary to display a selective necrosis of lymphoma cells. The inhibitory action towards lymphoma cells was because of enzyme L-asparaginase.[9] In 1964, researchers viz., Marshburn & Wriston brought a major development stating *Eschericia coli* L-asparaginase possesed anti-leukemic activity which inhibits tumiriogenic growth.[10]

3. Stucture of L-asparaginase :

Bacterial L-asparaginase (EC. 35.1.1) comprises of a tetrameric protein with identical subunits belonging to the family of amidohydrolase. Crystallographic X-ray analysis of this enzyme reveal molecular weight to be as 35.6 kDa.[11] The enzyme catalyzes the conversion of L-asparagine to L-aspartic acid & ammonia & to some extent to L-glutamine. There are two types of L-asparaginases viz.., Type I (cytoplamic) & Type II (periplasmic). Type II L-Asparaginases display higher affinity for substrate L-asparagine than Type I. Plant L-asparaginases (Type III) have also been identified ,but they confer lower specificity for substrate L-asparagine.[12]

4. Pharmacokinetics Of L-asparaginase :

The normal. healthy cells posses enzyme asparagine synthase (which the cancerous cells lack) responsible for L-asparagine synthesis . The healthy cells are not dependent upon non-essential amino-acid L-asparagine for growth & survival. This major source (of L-asparagine) is taken up

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by cancerous cells from healthy cells, which becomes a growth regulatory factor as they can enter into metastasis & proliferate. But, the activity of L-asparaginase converts L-asparagine to L-aspartic acid & ammonia making the tumorous cells deficient with this essential amino-acid thereby ultimately destroying them. [13, 14]

The commercially available enzyme drug therapy has exhibited certain untoward side-effects. Therefore, there is a need to obtain novel L-asparaginases from environment which can easily solve this problem.

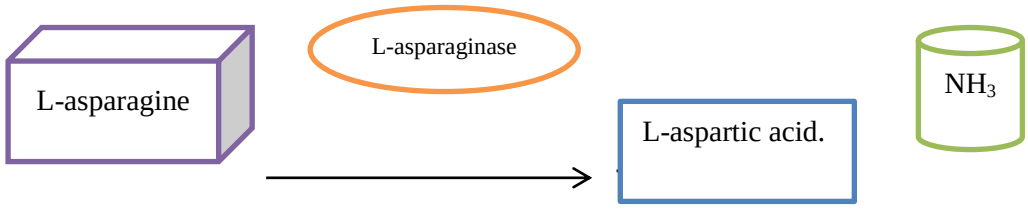

Figure 1. L-asparaginase amidohydrolytic activity.

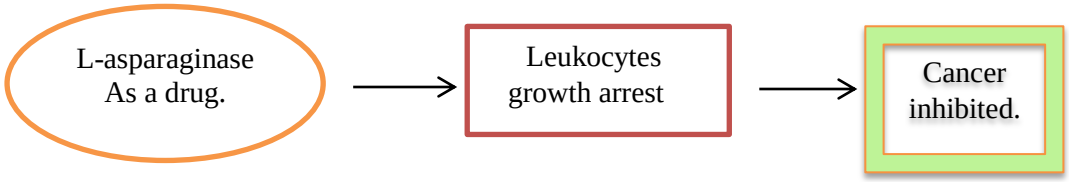

Figure 2. Anti-cancerous activity of L-asparaginase.

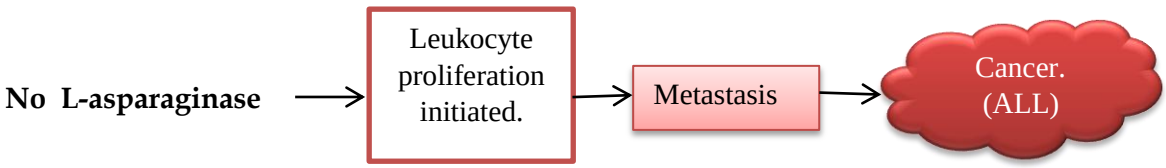

Figure 3. Effect of no L-asparaginase treatment

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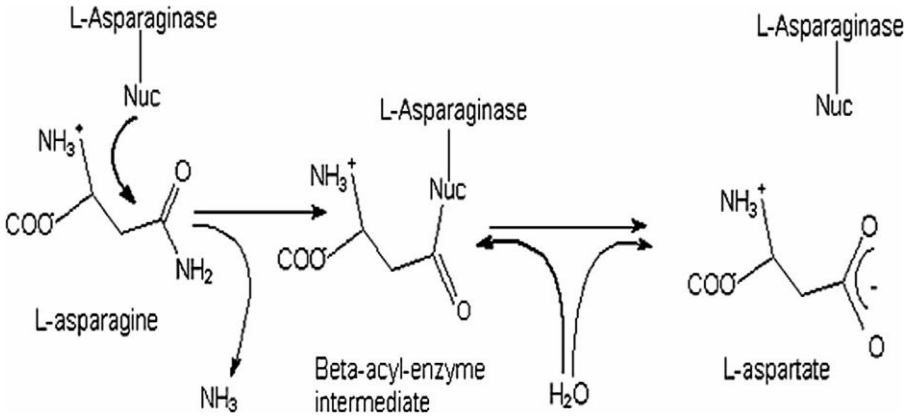

Figure 4. Diagramatic demonstration of pharmacokinetics of L-asparaginase [15]

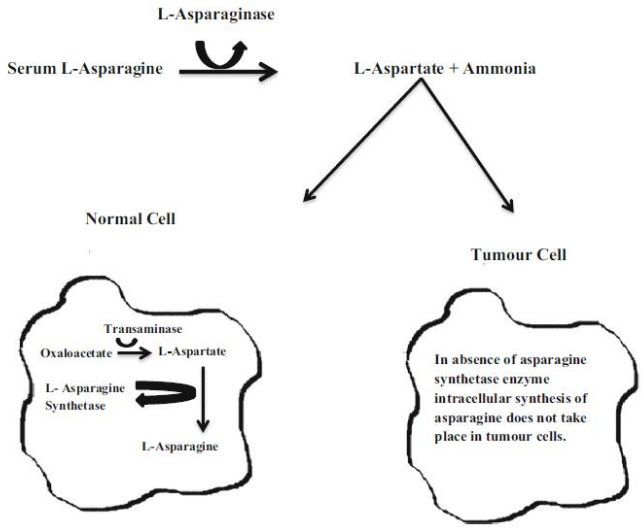

Figure 5. Mechanism of action in tumor & normal cells.[15]

5. Sources of L-

asparaginase :L-asparaginase is been produced by most micro-organisms, plants, fungi, yeast . The isolation of the enzyme from microbes has received significant importance, as it is eco-friendly & most affordable in synthesis by employing either solid-state or submerged fermentation.[16]

5.1 *Bacteria* :

Most microbial research has been focussed on *E.coli & Erwinia chrysanthemi* as commercially available L-asparginases (Elspar & Oncospar) have been isolated from these organisms.[17] Intracellular L-asparaginase from *Bacillus sp.* have shown industrial importance.[18] Santosh Kumar Jha *et al.*, (2014) , reported production of L-asparaginase from *Pseudomonas fluorescenes* through batch fermentation.[19]

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5.2 *Yeast* :

Candida sp. has shown best ability of L-asparaginase production through submerged fermentation.[20] Roon *et al.,*(1982) reported *Saccharomyces cerevisiae* to produce L-asparaginase.[21]

5.3 *Fungi* :

Aspergillus terrus MTCC 1782 through submerged fermentation produced L-asparaginase.[22] Radhika

Tippani et al.,(2012) reported *Fusarium* to be one of the industrially important fungus, which can produce L-asparaginase in higher amounts.[23]

5.4 *Actinomycetes* :

Streptomycetes sp. produced glutiminase free L-asparaginase which could serve as a potent enzyme drug.[24] Nocardia sp. produced L-asparaginase was reported by Gunasekaran.[25]

5.5 *Plant Sources* : Medicinal plant *Mentha spicata* was used,where bacteria *Staphylococcus capitus* was isolated that exhibited maximum L-asparaginase production.[26] *H. muticus* leaves showed higher L-asparaginase production with endophytic fungi *Aspergillus niger* .[27]

Sr, No	Organism	References
I	**Bacteria**	
1.	*Erwinia carotovora*	[28]
2.	*Paenibacillus barengoltzii*	[29]
3.	*Enterobacter aerogenes*	[30]
4.	*Corynebacterium glutamicum*	[31]
5.	*Bacillus licheniformis*	[32]
II.	**Yeast**	
1.	*Rhodosporidium toruloides*	[33]
III.	**Actinomycetes**	
1.	*Streptomyces albidoflavus*	[34]
IV.	**Fungii**	
1.	*Fusarium oxysporium*	[35]
2.	*Aspergillus tamari & Aspergillus terrus*	[36], [37]

Table 1: Other microbial sources of L-asparaginase.

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6. Production & optimization of L-asparaginase :

Intracellular production of L-asparaginase by using a novel strain , *Bacillus sp.PG02*. was reported by Qeshmi F I et al.(2015).[38] Shanthipriya et al.,(2015) investigated the 3 thermophilic caprophilous fungi where, *M. cinnamomea* produced maximum amount of both L-asparaginase & biomass.[39] Batch production & media optimization of anti-leukemic L-asparaginase was carried with *Pseudomonas fluorescens* by Taguchi DOE methodology . Here L-18 array was selected for media optimization. After

validation of result there was about 28.48% increase in production.[19]

Baskar G. et al., (2009) examined the effect of L-proline, L-glutamic acid & Sodium nitrate as nitrogen sources on production of extracellular L-asparaginase by *Aspergillus terrus* MTCC 1782 in shake culture fermentation through Latin Square Design. For this Data plot software,was used.[22] Deshpande N. et al.(2013), reported that *Streptomyces ginsengisoli* , by adopting one to one factor strategy & through submerged fermentation produce maximum L-asparaginase at pH 8 & temperature 30^0 c. Here, optimization of growth parameters showed significant increase over production of L-asparaginase from 2.52 to 3.23 µmol/l/min. was produced.[40]

A study carried out through submerged fermentation (by one to one factor strategy) revealed that glucose (210 IU/ml) and proline (404 IU/ml) were the best carbon and nitrogen sources for L-asparaginase production by *Fusarium sp.* [22] Pagalla U et al., (2013) used medicinal plant (*Mentha Spicata*) to produce L-asparaginase by endophytic bacteria. Media composition was optimized for the improvement of L-asparaginase activity by Response Surface Methodology.[26]

Ahmed H.M.El-Said et al.,(2016) tested leaves of *Datura innoxia* & *H muticus* medicinal plants for antibacterial & anticancerous activity. In this study, it was concluded that endophytic fungi serve as a reservoir of anticancerous & antibacterial compounds.[27]

A solid state fermentation strategy was employed for the production of L-asparaginase by *Pseudomonas stuzeri* PIMS6 using agro residues.[41] Sindhwad P & Desai K (2015) isolated a marine bacterium *Bacillus pumilis* that exhibited L-asparaginase production with 0.1% L-asparagine & 2% galactose. The purified L-asparaginase had molecular weight identical to the enzyme extracted from *E.coli*.[.42] Menegat et al.,(2016) reported a Gram negative bacterium *Zymomonas mobilis* that produced L-asparaginase (117.45 IU/L) using response surface methodology.[43] Saxena A. et al.,(2015) concluded that *Streptomyces phaechromogenes* by submerged fermentation produced highest glutiminase free extracellular L-asparaginase (19.2 IU/ml.).[44] Shukla D et al., reported , *Pseudomonas* proteolytic strain S13D produced maximum L-asparaginase (1.6 IU/ml).[45] Basker G. *et al* (2009) conjugated L-asparaginase with

nanobiocomposite of zinc oxide nanoparticle produced from *Aspergillus terrus*. This would serve as an alternative drug delivery system (compared to the routine intravenous injections) & would also reduce side-effects.[22]

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7. Purification of L-asparaginase :

Enzyme purification has always been a tidious task to achieve. But, it is the major requirement where, purified L-asparaginase(with no glutiminase activity) would yield an enzyme with attributes such as maximal anticancerous activity as well as excellent mitigation effect of starchy foods.[52] L-asparaginase was purified to about 98 folds by Juan M. et al., (1990) by applying methods of protamine sulphate precipitation, DEAE-Sephacel anion exchange chromatography, ammonium sulphate precipitation & Sephacryl S-200 gel filtration.[53]

Glutiminase free L-asparaginase was purified from *Pseudomonas otidis* by Husain I et al.,(2016), by employing ammonium sulphate precipitation, DEAE cellulose chromatography, Sephadex G chromatography. This resulted about 151.88 fold of purified enzyme with a yield of 38.90% & 107.84U/mg specific activity.[54] Narayana K J P (2008) reported multiple steps of purification viz., ammonium sulphate precipitation, Sephadex G-100 & CM-Sephadex C-50 gel filtration where recovery of enzyme from *Streptomyces albidoflavus* increased up to 106 folds with a total of 43% enzyme yield.[55]

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Sr. No.	Organisms	Optimium parameters	L-asparaginase activity	References
1.	*Aspergillus Niger*	Bran of glycine Mix, pH 6.5, 37°C, moisture content 70%	17.52 U ml^{-1}	Abha 2006 [46]
2.	*Pichia pastoris*	BSM 2, for 2 L (flasks) : pH 5, 30°C	85.6 U ml^{-1}	Maria *et al.*, 2006 [47]
3.	*Vibrio sp.*	M9 medium , pH 7, 35 ° C.	28.7 U ml^{-1}	Saravanan *et al.*, 2014[48]
4.	*Cryptococus Nodaensis*	D-glucose: 3. Yeast extract: 0.5, pH 6, 28°C	2060 U	Sato *et al* ., 1999[49]
5.	*Bacillus pumilus*	Galactose:2.0, asparagine: 0.1, 30°C, 48 h, 100 rpm,	75.73 U ml^{-1}	Sindhwad and Desai, 2015 [50]
6.	*Bacillus cereus* MAB5	Soyabean meal 6.2 g l^{-1} , Wood chips 1.383 g l^{-1} L-asparagine 5.5 g l^{-1} & NaCl 4.535 g l^{-1}	51.54 U ml^{-1}	Thenmozhi *et al.*, 2011[50]
7.	*Actinobacterial sp.*	Asparagine, pH 8, 35 ° C	670.04 U mg^{-1}	Varma *et al.*, 2016 [51]
8.	*Serratia marcescens*	Sesame oil cake, pH 7.5, 37°C , 48 hr.	110.795 U ml^{-1}	Agarwal *et al.*, (2011) [31]

Table 2 : Optimizing conditions for L-asparaginase production.

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A study of effect of L-asparaginase isolated from local *Withania somnifera* plant over expression of CD8 & CD95 on surface of leukemia lymphocyte cells was carried out by Najwa Sh. et al.(2013) For this two purification steps, were undertaken with ion-exchange chromatography using DEAE-cellulose & further gel filtration chromatography using sephadex G-150.This raised the specific activity from 1.73 U/mg in crude extract to 2.29U/mg after ion-exchange while, 10.5U/mg after gel filtration. The purification fold here, increased from 1.32 after ion-exchange chromatography to about 6.06 times after gel filtration.[56]

Marine actinomycetes *Streptomyces sp.* isolated from Parangipettai & Cochin Coastal area produced L-asparaginase showing cytotoxic effect over JURKAT cells (Acute T-cell leukemia) & K562 cells (Chronic myelogenous leukemia).Purification of L-asparaginase comprised of steps such as, ammonium sulphate precipitation, dialysis, gel filtration using sephadex G 50 & G 200.

The yield observed was, 85-fold pure with 63.07IU/mg of L-asparaginase activity.[57]

7.1 Various properties of purified L-asparaginases.

Influence of temperature , pH , metal ions , chelators contribute to the overall properties of enzyme & its activity.Various characteristics , properties of enzymes, its environmental conditions help in understanding the catalyst behaviour of the enzyme , protein structure & optimum functions it can elucidate.[72]

7.1a *Influence of pH over activity of L-asparaginase & stability of L-asparaginase.*

Many researchers investigated the impact of pH on purified L-asparaginase activity, finding that enzyme activity increased when pH changed from alkaline to acidic (table 4). The pharmaceutical application of L-asparaginase necessitates optimum pH in the physiological range, but the food business seeks increased efficacy in the acidic pH range.

7.1b *Influence of temperature over L-asparaginase activity & its stability.*

The speed of catalysis and the stability of enzymes are both influenced by temperature.[58] Increase in temperature causes higher catalytic activity with higher rate of inactivation of enzyme.[59] As a result, it is required to maintain temperature changes in order to achieve higher enzyme activity and stability. Temperatures between 30 ^{O} C and 40 ^{O} C are ideal for elucidating L-asparaginase activity. (table 4). [5]

7.1c *Influence of metal ions & chelators*

To have a better knowledge of the mechanism of action of the enzyme, other impact or molecules that alter its activity can be examined. Metal ions, for example, are required for the maintenance of an enzyme's multimeric structure as well as the stabilization of reaction intermediates.[60]

L-asparaginase activity from *Cyclindrocarpon obtusisporum* MB-10 [60] & *Erwinia carotovora* [62] was increased by metal chelators like EDTA. As against to that *Pectobacterium caratovorum* MTCC-1428 showed no Impact over L-asparaginase activity with EDTA.[72] However, complete inhibition of activity was seen with L-

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asparaginase from *Erwinia*, suggesting it to be a metalloprotein.[63] The presence of sulfahydryl group have shown, inhibition Impact in most studies , like presence of Cd^{2+} , Zn^{2+}, as metal ion decreased L-asparaginase activity from *Cyclindrocarpon obtusisporum* MB-10. [61] The similar results were observed in case of *Erwinia carotovora* [62] & *Cladosporium sp.*[68]

Thiol group maintaining agents like 2-mercaptoethanol, glutathione, and dithiotritol were shown to stimulate L-asparaginase activity, whilst thiol group blocking drugs like iodoacetamide severely hampered it. This indicated the pivitol role of sulfahydryl groups to stabilize the confirmation of enzymes with maintaining catalytic activity. In a study by Warangkar & Khobragade (2010) the Km values were found to be decreased while Vmax values were elevated . This gave a proof of non-essential mode of activation , in presence of thiol groups (L-cysteine, reduced glutathione etc.) for L-asparaginase from *Erwinia carotovora*. [62]

Mesas et al., (1990) , showed stable L-asparaginase from *Corynebacterium glutamicum* where ions & amino acids hardly showed any Impact over L-asparaginase activity. The presence of phenyl-methyl-sufonyl fluoride produced an inhibition impact over L-asparaginase activity from *Cladosporium sp.* [64]

Kumar & Manonmani (2013) , reported elevated L-asparaginase activity with N-bromosuccinimide as metal ion. This indicated, the modification produced in tryptophan residues. The active form of the enzyme is maintained by presence of serine, cysteine while existence of carboxylic residues & histidyl residues near active sites, would occur by chemically modifying L-asparaginase from *Thermus thermophiles* , was declared by Prista & Kyriakidis (2001). [65]

8. Applications of L-asparaginase :

8.1 Various Anti-cancer Drugs :

8.1a *Native E.coli Asparaginase* :

Type II *E.coli* asparaginases (periplasmic) effectively reduces the tumors than Type I asparaginase(cytosolic) was observed in mice transplanted with lymphoma cells.[76] Asparaginase formulation Medac^R has been used majorly (in children) for treatment purpose during chemotherapy.[77]

8.1b *Erwinia asparaginase* :

This has been used as an alternative to *E.coli* asparaginase as patients suffered intense hypersensitivity reactions during chemotherapy. Higher doses(>100U/L) of this asparaginase is been administered to ALL patients which had shown complete depletion of cancerous cells.[78]

8.1c *PEG-asparaginase* :

Covalent coupling of Asparaginase with Polyethylene-glycol(PEG) has reduced the side-effects, immunogenicity & antigenicity after administering this form of drug intramuscularly (2.500IU/m^2) for about 14 days to patients with ALL & Non-Hodgkins lymphoma. PEG-asparaginase remains stable in the plasma than *E.coli* or *Erwinia asparginases*.[70,77,78]

8.2 As a Food Processing Aid :

L-asparaginase pre-treatment alleviates acrylamide formation by converting it into simple L-aspartic acid during heating of starchy foods (Malliard reaction). Acrylamide a reactive molecule is formed

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Mini-Review
PP: 1-4
Peer Reviewed Journal

Sr. No.	Organisms	Purification steps	%yield / fold purification	References
1.	*Bacillus sp.*	Ammonium sulphate precipitation	100/1	[66]
2.	*Streptomyces Gulbargenesis*	Ammonium sulphate precipitation & Sephacryl S-200 gelfiltration	50.6/1.8 & 37.8/26.88	[67]
3.	*Cladosporium sp.*	DEAE cellulose chromatography & Methanol precipitation	190 folds	[68]
4.	*Bacillus licheniformis*	Acetone precipitation, DEAE cellulose chromatography & Gel filtration	100/1	[69]
5.	*Bacillus Megaterium* (H-1)	Ni-IDA (iminodiacetic acid) affinity chromatography	42.33/3.3	[70]
6.	*Yersinia pseudotuberculosis* (Q66CJ2)	DEAE-Toy pearl pooled peak	82%	[71]
7.	*Penicillium sp.*	Ammonium sulphate precipitation & Sephadex G-100 , 120 gelfiltration	36.2%	[35]
8.	*Streptomyces noursei* (MTCC 10469)	Ammonium sulphate precipitation & Sephacryl S-200 gelfiltration	100/1	[29]

Table 3 : Purification methods of microbial L-asparaginase.

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ISSN No: 2321-8681
Mini-Review
PP: 1-4
Peer Reviewed Journal

Table 4 : Characteristics of purified L-asparaginase.

Sr. No	Organisms	Mol. weight.	pH	Temp.	Influencing activators & enhancers.	Inhibitors	References
1.	*Bacillus sp.*	45	8.0	37 ^{O}C	$MgCl_2$	EDTA	[57]
2.	*Bacillus Megaterium* (H-1)	40	7.0	40 ^{O}C	-	-	[61]
3.	*Streptomyces gulbargensis*	-	9.0	40 ^{O} C	-	-	[67]
4.	*Cladosporium sp.*	121	6.3	30 ^{O}C	-	-	[68]
5.	*Yersinia pseudotuberculosis* (Q66CJ2)	36	6.0	60 ^{O}C	-	-	[71]
6.	*Pectobacterium carotovorm* (MTCC-1428)	144	8.0	40 ^{O}C	Fe^{3+} , Pb^{2+} , KI & detergents	Thiol blocking agents, SDS, Ca^{2+}	[72]
7.	*Thermococcus gammatolerans* (EJ3)	36.5	8.5	85 ^{O}C	-	-	[73]
8.	*Penicillium sp.*	66	7.0	37 ^{O}C	-	-	[35]
9.	*Aspergillus sp.*	56	6.0	47^{O} C	-	-	[29]
	Bacillus pumilis	71	7.0	45 ^{O} C	-	-	[42]
10.	*Pseudomonas Otidis*	34±1	7.5	40 ^{O}C	Na^{+} , K^{+} , L-cysteine	Inhibited by divalent cations & thiol group blocking reagents	[54]

Research & Reviews in Biotechnology & Biosciences
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ISSN No: 2321-8681
Mini-Review
PP: 1-4
Peer Reviewed Journal

Table 5 : Kinetic properties of purified L-asparaginase.

Sr.No.	Organisms	Vmax.	Km (mM)	References
1.	*Yersinia pseudotuberculosis*	-	0.017	[71]
2.	*Pectobacterium carotovorum* (MTCC 1428)	4.45 IU/µg	0.657	[72]
3.	*Thermococcus gammatolerans* (EJ3)	-	10	[73]
4.	*Mucor heimalis*	625 U/ml	4.3	[74]
5.	*Streptomyces fradiae*	51 IU/ml	10.07	[75]
6.	*Penicilium sp.*	-	4.00	[35]
7.	*Cladosporium sp.*	4.44 µmol/ml/min	0.100	[68]
8.	*Bacillus licheniformis*	1.03 IU	0.014	[69]
9.	*Bacillus megaterium* (H-1)	1.58 IU/ µg	0.8	[70]

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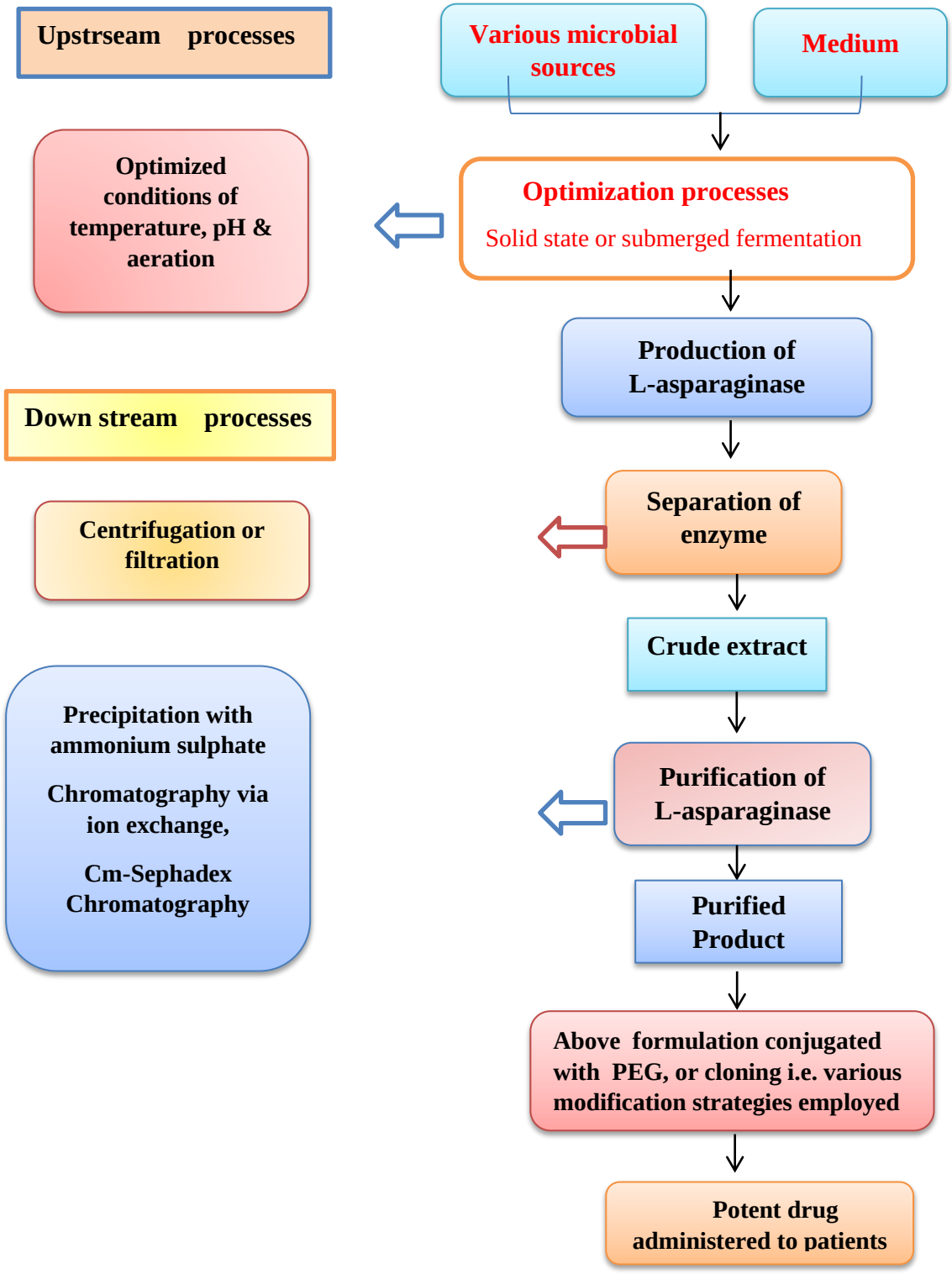

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Figure 6. L-asparaginase production

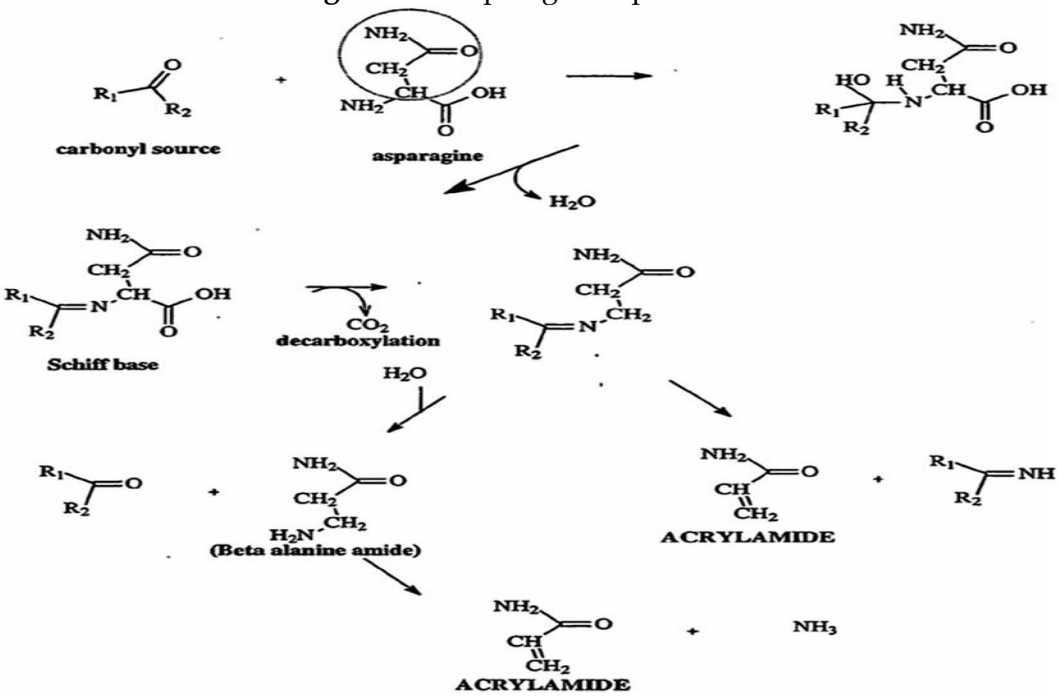

Figure 7. Acrylamide formation [99]

by various cascade reactions between carbohydrates and free asparagine at higher temperatures. Free asparagine is responsible for acrylamide formation . But, L-asparaginase pre-treatment causes 88% reduction of free asparagine thereby eliminating 99% of acrylamide from the final potato product.[93] . The international agency for research on cancer (IARC) has claimed acrylamide to be carcinogenic.[94] 92% reduction in acrylamide formation in final products of French fries, ginger biscuits & semisweet biscuits.[97] Vass *et al.,* (2004) reported 70% of acrylamide reduction after

L-asparaginase biosensors have been widely used in the medicine & in the food industry. These biosensors measure the L-asparagine levels in foods & in leukemic patients.[100]

Acrylamide intake can lead to ovarian , endometrial & breast cancer was reported recently.[95]

Pedreschi *et al.,*(2008). carried out a detailed study of optimum temperature (60 ^{0}c) as well as pH(7.0) required for acrylamide reduction(67%) in French fries.[96] Kumar & Manonmani (2013) revealed 96% acrylamide reduction.[68] Hendriksen *et al.,* (2009) showed addition of L-asparaginase during preparation of dough & cracker products.[98]

8.3 As a Biosensor :

Kumar *et al.,* (2013) reported 10^{-1} to 10^{-10} M of L-asparagine levels could be detected in normal & leukemic patients blood samples. Detection of asparagine , aspartate, glutamine, glutamate are accurate

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& consistent with biosensors compared with the regular Nesselerization assays. The mechanism of action of biosensors comprises of detection of L-asparaginase activity, where, hydrolysis of L-asparagine liberates ammonium ions , causing a change in pH . thereby changing the colour & absorption spectra. Also, plant based biosensors are observed to be more efficient & reliable , over microbial biosensors. [101]
Verma *et al.,* (2007a) developed a biosensor which could measure 10^{-9} M of L-asparagine levels in serum samples. This biosensor could detect leukemic samples by colour visualization. The biosensor consisted of a phenol red indicator & L-asparaginase co-immobilized together over beads of silicone gel & calcium alginate.[102]

9. Resistance to L–asparaginase :

Tumor cells become resistant to L-asparaginase by synthesizing intracellular L-asparagine on their own without seeking from normal cells. This basically can take place, by increasing the degree of cytosine residue methylation in DNA , that is responsible for the expression of asparagine syntetase . This enzyme therefore, increases & regulates the L-asparagine synthesis level in tumorigenic cells.[89] The increased level of specific antibodies developed to counteract with accelerated rate of cleaved L-asparaginase , has also been a potential reason for L-aspraginase resistance.[90] Some reporters have claimed that the drugs efficacy remains unimpaired despite of patients immunization.[91] It is also observed that L-asparaginase sensitive cells often produce cytokines controlling & regulating the expansion of resistant cells. The sensitive cells when get killed by virtue of L-asparaginase action , the resistant cells bypass from the regulatory control & eventually proliferate to other tissues.[92]

10. Disadvantages of currently used L-asparaginase in cancer treatment.

Although many microorganisms generate L-asparaginase, pure enzymes derived from *Erwinia sp.* and *E. coli* have been utilised in the treatment of ALL and other cancers. [103] Complications associated with the use of L-asparaginase derived from *Erwinia chrysanthemi* and *E.coli* majorly include allergic responses, [104] anaphylactic shock, [105] and drug resistance , [106] all of which result in decreased success of therapy of L-asparaginase. [107] About 30% of people experience side Impacts as a result of the medication. The negative consequences of hypersensitivity in patients can be reduced when pharmacologically different L-asparaginases are substituted in the first-line therapy. [108]

Research & Reviews in Biotechnology & Biosciences
Website: www.biotechjournal.in
Volume: 8, Issue: 2, Year: 2021
DOI: https://doi.org/10.5281/zenodo.5775421

ISSN No: 2321-8681
Mini-Review
PP: 1-4
Peer Reviewed Journal

Sr. No	Modification strategies.	References
1.	The immobilization of polyaniline nanofibers was done with L-asparaginase resulting into enhanced activity as well as stability	[79]
2.	Dextran was coupled with L-asparaginase. The effect observed was, increase in catalytic activity , high retension & enhanced half-life with bioavalaibility	[80]
3.	A study was carried out where, Levan (a glycoprotein extracted from Zymomonas mobilis) was coupled with L-asparaginase (from Erwinia carotovora). This resulted into better kinetic properties & higher enzyme stability	[81]
4.	Fusion of *E. coli* gene with single chain antibody obtained from a protective (non-inhibitory) Monoclonal antibody(MABs) exhibited resistance to trypsin digestion & proteolysis.	[82]
5.	Site - directed mutagenesis reduced the immunogenicity caused after treatment with L-asparaginase from *Erwinia chrysanthemi* . The critical proline residues residing within epitopes (immunologically active) were changed with threonine placed at the C – terminus of the enzyme.	[83]
6.	Site - directed mutagenesis was carried out where proline was introduced in place of Asp 178 residue at hydrogen bonded turn of L-asparaginase. This generated a thermostable mutant of *E.coli* L-asparaginase. The thermostability of enzyme was therefore elevated.	[84]
7.	Cladosporium sp. L-asparaginase was chemically modified using bovine serum albumin & ovalbumin respectively. This increased the activity as well as stability of L-asparaginase	[85]
8.	Glutaraldehyde was used to covalently immobilize L-asparaginase over surface of microparticles (particularly at polar side groups) of sericin. This raised the substrate affinity (about 8 times) of the immobolized enzyme as compared to the native L-asparaginase	[86]
9.	Succinimidyl-succinate(a derivative of polyethylene glycol) was conjugated with L-asparaginase. This completely eliminated denaturation of enzyme when exposed to organic solvents or sonication.	[87]
10.	RBC's have also been used as a biocompatible & biodegradable tool. The administration of L-asparaginase bound with RBC's, have caused higher enzyme efficiencies with reduction in major side–effects.	[88]

Table 6 : Various modification strategies for enhancement of anticancerous activity of L-asparaginase.

Research & Reviews in Biotechnology & Biosciences **ISSN No: 2321-8681**
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Volume: 8, Issue: 2, Year: 2021 **PP: 1-4**
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Therefore, mostly use of asparaginase by *Erwinia* [109] is used instead of asparaginase from *E.coli* [104] to suppress hypersensitivity in patients. Still, side-Impacts are observed after administration of both the drugs suggest an alternative to these L-asparaginase preparations. Mostly the major problem encountered against PEG-asparaginase, *Erwinia* & *E.coli* are cross-reactivity between antibodies . [110] Duval et al., (2002) discovered percentages of hypersensitivity, liver disease, frequency of pancreatitis, and serious complications from both L-asparaginase derived from *E. coli* & *E. chrysanthemi*.[111] PEG-conjugation have shown many advantages , but drug toxicity & its silent inactivation are major drawbacks .[112] The development of anti-PEG asparaginase antibodies also have rendered treatment inImpactive by rapid clearance of the enzyme .[113] PEG- L-asparaginase have caused toxicity when administered by a multiagent regimen & ill effects have found to be similar with that of native L-asparaginases.[114] This lead Holle (1997) [115] to come to a conclusion , that PEG stands inactive as a regular substitute, since many side-effects have complicated the therapeutic treatment. [116]

11. Use & need of novel L-asparaginase during cancer therapy :

Limitations of *E.coli* , *Erwinia* & PEG-asparaginases have raised the demands of newer L-asparaginases possessing variable antigenic properties to be used for remission of cancer cells (induction therapy). When induction and reinduction treatments are carried out using the identical L-asparaginase preparation (prednisone, vincristine, L-asparaginase, and anthracycline), the rate of immunological responses sharply increases . [117] This means , that the L-asparaginases other than currently available, would be more useful in therapeutic treatment. Also, significant properties such as stability & low half-life of L-asparaginase are critically concerning issues considered by pharmaceutical industry . [103]

The high stability & half-life prevents the necessity of multiple & continuous dose administration thereby reducing the hypersensitivity reactions .[5]

Factors such as general clinical condition & immunological response of patients contribute to the overall outcome of cancer treatment with L-asparaginase .[118] Also, structural uniqueness & their activities confer anticancerous properties with immunogenicity of L-asparaginase.

12. Future :

Research for obtaining physiologically, pharmacokinetically active L-asparaginases have been in operation since several years. By employing modifications at genetic level or at structure level , the possibility of obtaining more unique and novel enzymes enhances. This would induce the enzymatic activity thereby increasing the utility as well as applications at a wider scale. But , there have always been certain drawbacks to the processes being employed which revert back the expected outcome consequently lowering the product yield. The shorter half-life , untoward side-effects , after administering the drug constitute major issues to be resolved. The mitigation strategy that diminishes acrylamide from

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Mini-Review
PP: 1-4
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food would demand for diversified L-asparaginase to minimize its risk of intake.

There is a need of advance research in obtaining a serologically different enzyme that would provide more efficacy and higher enzymatic activity to the existing enzyme drugs. Modifications through site directed mutagenesis , cloning , recombinant techniques are required to be employed which would improve the stability against proteolysis with reduction in immunogenicity.

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14. Conflicts of Interest :

The authors declare that there is no conflict of interest.

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