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L-asparaginase – A promising biocatalyst for industrial and clinical applications

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ABSTRACT

L-asparaginase is a versatile enzyme with application in food and therapeutics and under constant quest for a reliable microbial source for commercial production. The review is an aid to comprehend the key milestones of L-asparaginase ever since its discovery, its potential sources, economical production strategies using cheaply available alternate substrates, its purification and downstream procedures reported so far, its characterization and the relevance of its application in food industry and therapeutics with an added clinical perspective.

1. Introduction

The serendipitous discovery of L-asparaginase was reported for the first time as the antitumor activity of the guinea pig serum against two strains of murine lymphoma and a strain of lymphosarcoma in rats (Kidd, 1953). However, it was later reported by Broome (1963) that the cytotoxicity of the guinea pig serum was due to presence of high concentrations of L-asparaginase in the sera of guinea pigs and other members of similar species. The principle involved in the cytotoxic activity of the enzyme was based on the fact that, leukemic cells cannot synthesize L-asparagine required for protein synthesis and must subsequently depend upon an external supply available in the plasma and tissues. The administration of the enzyme, L-asparaginase, quenches this free source of asparagine, starving and killing certain cancer cells. L-asparaginase was also used for treatment of solid tumors along with the cancers of the lymphatic system in children, and the efficacy of the treatment was reported by Tallal et al. (1970). The enzyme was reviewed as early as 1973 (McCredie et al., 1973), for its pharmacology, toxicity and the mechanism of resistance to L-asparaginase which involved immunological clearance. Ever since its discovery, L-asparaginase continues to be a constant quest for researchers who explored the microbial sources that utilized cheaper or easily available materials to produce the enzyme with relative ease in its purification. In the recent years it has been explored for stable and improved therapeutic efficacy as discussed in this review.

2. L-asparaginase- mechanism of action

The mechanism of hydrolysis process of L-asparagine by asparaginase occurs in two steps via an inter-mediate: beta-acyl-enzyme (Fig. 1). In the first step, the nucleophilic residue of the enzyme is activated by a strong base (NH_2) and hence attacks the amide carbon atom of L-asparagine, thus generating an intermediate product beta-acyl-enzyme. In the next step it acts on the ester carbon (R-C=O) made by a nucleophile activated by a water molecule, finally giving the product L-aspartic acid, with liberation of ammonia. Other hydrolysis reactions catalyzed by L-asparaginase are hydrolysis of L-glutamine and β-aspartyl peptide amide however the reaction yield is very low (Cachumba et al., 2016).

3. Sources of L-asparaginase

L-asparaginase has been reported to be produced by various plants, mammals and microbes. Table 1 enlists a large number of microbes producing L-asparaginase. (Peterson and Ciegler, 1969) screened over 123 bacterial species for L-asparaginase production and reported their enzyme activity. Among these bacteria, *Erwinia aroideae* NRRL B-138 provided the highest yields. Ever since, L-asparaginase was reported to be produced by many different bacteria including *Proteus vulgaris* (Tosa et al., 1971), *Pseudomonas aeruginosa* (El-Bessoumy et al., 2004), *E.coli* (Ghasemi et al., 2008), *Streptomyces gulbargensis* (Amena et al., 2010) etc.

Moreover, many bacterial enzymes of commercial importance are widely preferred and explored for the versatile properties of increased

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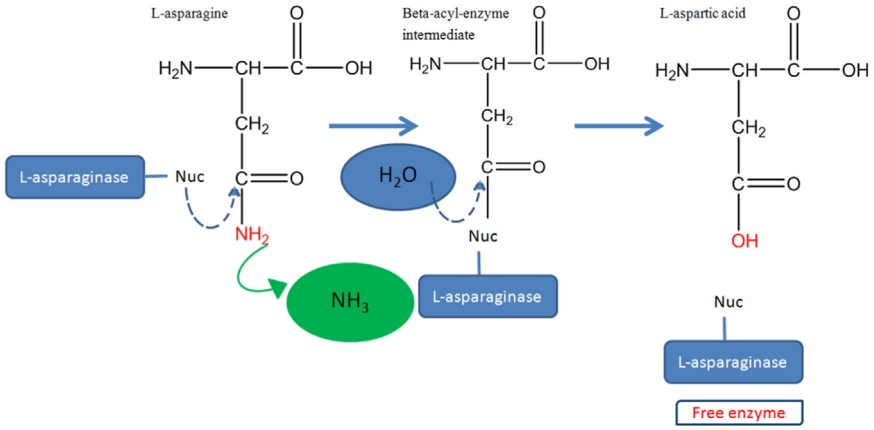

Fig. 1. Mechanism of action of L-asparaginase.

tolerance to temperature and pH attributed to the extreme habitats that these bacteria inhabit (Herbert, 1992). Many such habitats that are prospected for bacteria that produce L-asparaginase include, marine luminous bacteria (Ramaiah and Chandramohan, 1992) where the authors reported that though the production of the enzyme L-asparaginase is not uniform among the strain of the *Vibrio* species examined by them, and that none of the *Photobacterium phosphoreum* were reported to produce L-asparaginase before this report, and they also report that on a comparative basis the enzyme secreted by the luminous prokaryotes was higher than that reported for other bacterial species.

L–asparaginase producers from mangroves of Andaman Islands were reported by Shome and Shome in (2001), and among the isolates, 87.9% and 33.3% were capable of growing at pH 10 and 12 respectively and 6.6% were reported to be thermotolerant which makes them ideal candidates to be harnessed for industrial production of L-asparaginase which necessitates stability of the product under high pH and temperature production conditions (Shome and Shome, 2001). The reports of hyperproductive marine actinomycetes capable of greater L- asparaginase activity even while using a cheap low-cost substrate like soyabean meal is noteworthy in this context (Basha et al., 2009), Another type of extremophiles, the halophiles or the salt- loving organisms often inhabit hypersaline environments posess the capacity to balance the osmotic pressure of the harsh environment and hence they resist the denaturing effects of salts. *Bacillus* sp. BCCS 034 halophilic bacteria isolated from Maharloo Salt Lake displayed an attractive prospect to be harnessed for commercial production of L-asparaginase (Ebrahiminezhad et al., 2011).

Other than the clinical application, L-asparaginase being commercially important in food industry the need for thermostability of L-asparaginase used in high temperatures of food processing in order to prevent acrylamide formation is a requirement that cannot be under stated. The L-asparaginase produced by the *Bacillus subtilis* strain hswx88, isolated from Taptapani hotspring of Odisha, India, definitely is a potent candidate for such application (Pradhan et al., 2013) etc. Thus it can be prospected that geographical locations from which microbial asparaginase producers are isolated play an important role in the stability, withstanding of different conditions and properties of the L-asparaginases. Among the other habitats, marine microbes remains a very attractive prospect for exploring an unlimited potential for L-asparaginase producers (Izadpanah et al., 2018).

4. Screening of L-asparaginase

Screening for bacteria producing L-asparaginase has been prospected by many methods. The activity of L-asparaginase is accompanied by increase in pH due to liberation of ammonia. This property was used and explored in devising a rapid plate detection technique using a pH indicator incorporated medium containing L-asparagine as sole nitrogen source (Gulati et al., 1997). Phenol red indicator being yellow under acidic condition turns pink under alkaline condition due to increase in pH. Recently (Mahajan et al., 2013) reported an improved method for screening of the microorganisms producing extracellular L-asparaginase wherein bromothymol blue (BTB) is incorporated as pH indicator in L-asparagine-containing medium instead of phenol red. Furthermore, BTB gives a transient green colour at neutral pH, 7.0 and dark blue colour at higher pH 8.0–9.0, indicating the potency of the microorganism for L-asparaginase production.

The quantitative assay of L-asparaginase involves detection of liberated ammonia that is released during the reaction catalyzed by L-asparaginase on its natural substrate L-asparagine. Briefly, this nesslerization method involves, the enzyme source (crude supernatant if extracellular or cell lysate if intra cellular) is incubated with the substrate for 10 mins, the liberated ammonia is tested by addition of Nessler's reagent in the diluted enzyme substrate mixture. Formation of colouration due to presence of ammonia is used qualitatively and quantitatively to determine the enzyme activity (Mashburn and Wriston, 1964). One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of ammonia per minute at pH 8.6 and 37 °C. Other methods of detection include continuous assays of L-asparaginase by coupling it with the glutamic dehydrogenase reaction and by cationic glass electrode (Ferguson and Phillips, 1974). Table 2 enlists some of the significant activity of L-asparaginase from various organisms.

5. Carbon - nitrogen metabolism of L-asparaginase in microorganism

As previously reported, several microorganisms are presented as L-asparaginase producers; however, bacteria *E. coli* and *E. chrysanthemi* are the current main microbial agents for industrial-scale production in pharmaceutical area, while the L-asparaginase from the fungus *Aspergillus oryzae* is the most used in food industry. For production of L-asparaginase on large scale, many factors are considered optimize a process with higher yield and economic viability. The factors such as the type and concentration of carbon and nitrogen sources, pH, aeration, temperature, fermentation time, have great influence in the process. Different types of culture medium have been explored for L-asparaginase production. However, carbon source and nitrogen source are the more influencing components as the enzyme being an amino acid hydrolase is induced by the amino acid content in the production medium. For example, several studies have demonstrated that best inducers for higher yields of L-asparaginase are L-asparagine, L-glutamine and L-proline, and the most common carbon source is glucose, in addition to alter-native sources such as starch and maltose (Baskar and Renganathan, 2009; Tippani and Sivadevuni, 2012), and the importance of such medium components are reviewed by Cachumba et al.

Table 1
List of different microbial sources of L-asparaginase.

Gram positive bacteria	Gram negative bacteria	Actinomycete	Fungus	Algae
B. mesentericus (Tiul'panova et al., 1972)	*Acinetobacter calcoaceticus* (Joner et al., 1973)	*S. griseus* (DeJong, 1972)	*Fusarium roseum* (Nakahama et al., 1973)	*Chlamydomonas* sp. (Paul and Cooksey, 1979)
B. coagulans (Wriston and Yellin, 1973)	*Azotobacter agilis* (Wriston and Yellin, 1973)	*S. venezuelae* (Mostafa and Salama, 1979)	*Aspergillus nidulans* (Saxena and Sinha, 1981)	*Chlorella vulgarischlo* (Ebrahiminezhad et al., 2014)
Mycobacterium bovis (Wriston and Yellin, 1973)	*Citrobacter* sp. (Bascomb et al., 1975)	*S. karnatakensis* (Mostafa and Salama, 1979)	*Cylidrocapron obtusisporum* (Raha et al., 1990)	*Spirulina maxima* (Baky et al., 2016)
B. polymyxa (Nefelova et al., 1978)	*E. coli* (Netrval, 1977)	*S. collinus* (Mostafa and Salama, 1979)	*Candida utilis* (Kil et al., 1995)	*Synechococcus elongatus* (Kebeish et al., 2016)
Corynebacterium glutamicum (Mesas et al., 1990)	*E. cartovora* (Maladkar et al., 1993)	*S. plicatus* (Koshy et al., 1997)	*Mucor* sp. (Mohapatra et al., 1997)	
Bacillus sp. (Mohapatra et al., 1995)	*E. chrysanthemi* (Moola et al., 1994)	*S. aurantiacus* (Gupta et al., 2007)	*A. tamari* (Sarquis et al., 2004)	
B. subtilus (Fisher and Wray, 2002)	*E. aroideae* (Tiwari and Dua, 1996)	*Streptomyces albidoflavus* (Gupta et al., 2007)	*A. niger* (Mishra, 2006)	
B. circulans MTCC 8574 (Hymavathi et al., 2009)	*E. cloacae* (Nawaz et al., 1998)	*Actinomyces* sp. (Narayana et al., 2008)	*A. terreus* (Gurunathan and Sahadevan, 2009)	
Bacillus circulans (Hymavathi and Sathish, 2010)	*Enterobacter aerogenes* (Mukherjee et al., 2000)	*S. gulbargensis* (Amena et al., 2010)	*A. oryzae* (Hendriksen et al., 2009)	
B. licheniformis (Sudhir et al., 2016)	*Brevibacillus brevis* (Narta et al., 2011)	*S. tendae* (Kavitha and Vijayalakshmi, 2010)	*Alternaria* sp. (Nagarajan et al., 2014)	

(2016) (Fig. 2).

6. Fermentation media for L-asparaginase production by bacteria

With a profound knowledge of carbon and nitrogen source requirement for L-asparaginase production, it is an imperative need to devise a production medium that can have a balance of all the critical factors like nutrients, aeration, temperature and pH. Fermentation conditions vary from one organism to another for production of L-asparaginase, and it can be produced constitutively (Mesas et al., 1990) or after induction using suitable substrate or conditions. Fermentation kinetics for the production of L-asparaginase has been an essential part of study where the rate limiting factors and parameters are optimized to obtain a continuous production of the enzyme. Such study has been performed since past three decades for organism like *Erwinia* sp. (Deokar et al., 2010; Liu and Zajic, 1973)

The choice of method to be used for bacterial fermentation, by solid state (SSF) or by submerged (SmF) type, depends on the ability of the microorganism to utilize the substrate directly in solid state or whether the substrate may be pre-treated and provided to the microbes in easily accessible form respectively. However some marine actinomycetes have been reported for both SmF and SSF for production of L-asparaginase by Basha et al. (2009).

Submerged fermentations suffer disadvantages of producing lesser yield while SSF are more cost effective. However, use of such cheaper waste nutrients in submerged fermentations can help enhance the cost effectiveness. An example of such a submerged fermentation study is where groundnut cake extract was optimized for production of L-asparaginase by *Streptomyces gulbargensis* (Amena et al., 2010). Solid state fermentation has been reported for organisms like *Serratia marcescens* (NCIM 2919) while using coconut oil cake (Ghosh et al., 2013) and *Bacillus subtillis* while using corn cob (Makky et al., 2014). Solid state fermentation (SSF) and submerged fermentation (SmF) are reported for different organisms by Batool et al. (2016).

Thus using different substrates for L-asparaginase production decides the type of fermentation conditions.

7. Waste utilization for cost effective production of L-asparaginase

In India, about 960 million tonnes of solid waste is being generated annually as by-products during industrial, agricultural and other processes. Of which, about 350 million tonnes are organic wastes from agricultural sources; 290 million tonnes are inorganic waste of industrial and mining sectors and nearly 4.5 million tonnes are hazardous in nature (Pappu et al., 2007). In 2017, Kumar et al., report an international seminar on 'Sustainable solid waste management for cities: opportunities in South Asian Association for Regional Cooperation (SAARC) countries' organized by the Council of Scientific and Industrial Research-National Environmental Engineering Research Institute and the Royal Society, where priority was to change from reliance on waste dumps that offer no environmental protection, to waste management.

Using cheaply available wastes as nutrient sources in the production medium for the bacteria can be another method of cost effective production of L-asparaginase. Different types of industrial and agro wastes can serve this purpose by being the nutritional substrate for bacterial fermentation as well as bringing about remediation of the waste thus solving the problems associated with its disposal. L-asparaginase activity is also reported in submerged rice soil amended with municipal solid waste compost and decomposed cow manure which suggests that wastes can indeed have a profound influence on L-asparaginase production by the microbes (Bhattacharyya et al., 2007). Thus microbes are also known to play a critical role in decomposition of organic matter and cycling of nutrients, and hence they can be tapped for commercial production of industrially important products by utilizing cheaper nutrients and a zero waste policy (Das et al., 2013).

Such an attempt for utilization of many agro wastes as alternate

Table 2
List of microorganisms producing L-asparaginase and their yield (Cachumba et al., 2016).

Name of organism	L-asparaginase activity	Reference
Pectobacterium carotovorum MTCC 1428	35.24 U mg^{-1}	(Kumar et al., 2011)
Bacillus licheniformis RAM-8	697.1 U mg^{-1}	(Mahajan et al., 2012)
Yersinia Pseudotuberculosis Q66CJ	62.7 U mg^{-1}	(Pokrovskaya et al., 2012)
Bacillus subtilis hswx88	23.8 U mL^{-1}	(Pradhan et al., 2013)
Bacillus aryabhattai ITBHU02	680.5 U mg^{-1}	(Singh et al., 2013)
Cladosporium sp.	83.3 U mg^{-1}	(Kumar et al., 2013)
Penicillium brevicompactum NRC 829	574.24 U mg^{-1}	(Thakur et al., 2013)
Photobacterium sp. J15	20 U mg^{-1}	(Yaacob et al., 2014)
Chlorella vulgaris	10 U/g	(Ebrahiminezhad et al., 2014)
Bacillus licheniformis MTCC 429	597.8 U mg^{-1}	(Sudhir et al., 2016)
Fusarium solani	121U/mL	(El-hadi et al., 2017)

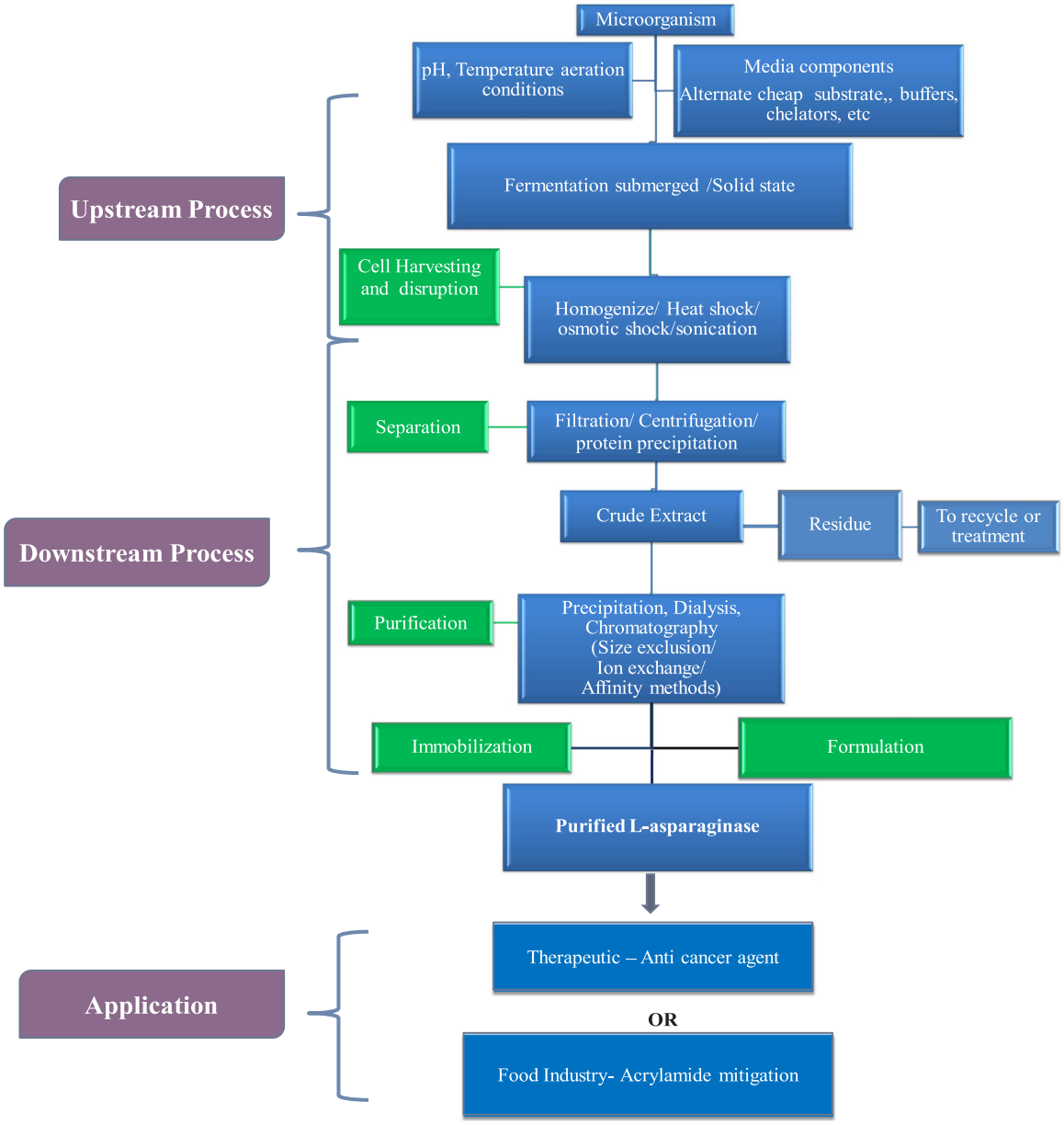

Fig. 2. Steps involved in L-asparaginase production.

substrates for the production of L-asparaginase like the corn cob have also resulted in increased L-asparaginase yield by the bacteria (Makky et al., 2014). Some such waste utilization strategies applied for L-asparaginase production are listed in Table 3. It is therefore a valuable prospect to utilize cheaply available wastes to produce an enzyme with such a commercial value while achieving bioremediation and having favourable economic concerns.

Table 3
L-asparaginase production utilizing waste materials.

Name of organism	Wastes used for production	Enzyme yield	References
Pseudomonas aeruginosa	Casein and corn steep liquor	142.8 IU	(Abdel-Fattah and Olama, 2002)
Aspergillus niger	Leguminous crops	0.9 ± 3.35U	(Mishra, 2006)
Bacillus cereus MAB5	Soyabean meal and wood chips	51.54 IU	(Thenmozhi et al., 2011)
Fusarium equiseti	Soybean meal	8.51 IU	(Hosamani and Kaliwal, 2011)
Bacillus subtilis	Ground nut cake	18.4 IU/mL	(Shukla and Mandal, 2012)
Cladosporium sp	Wheat bran, rice bran, and bagasse	3.74 IU	(Kumar et al., 2013)
Bacillus sp. KK2S4	Corn cob	0.261 ± 0.014 IU	(Makky et al., 2013)
Serratia marcescens (NCIM 2919)	Coconut oil cake	5.86 IU	(Ghosh et al., 2013)
Aspergillus wentii	Palm oil cake	89.954 IU	(Satya et al., 2014)
Pseudomonas plecoglossicida RS1	Industrial effluent	3.25 IU/mL	(Shakambari et al., 2015)
Bacillus tequilensis PV9W	Onion Garlic Peels	1.40 ± 0.04 IU/mL	(Shakambari et al., 2017)
Aspergillus niger LBA 02	Passion fruit peel flour	2380.11 U/gds)	(da Cunha et al., 2018)

8. Statistical optimization methods for media and parameters for L-asparaginase production

Conventional methods used to test individual fermentation parameters are laborious and time consuming and yet do not provide information on the interaction between factors for increase in overall productivity. The importance of statistical methods hence cannot be over ruled. Statistical operations help the assessment of each parameter as significant or insignificant in affecting the final yield and can be simply expressed in mathematical forms of equation. Statistical methods are rapid and reliable, can identify significant nutrients, help understand the interactions among the nutrients at various concentrations and also reduce the total number of experimental trials tremendously. One such earliest of the designs, the Plackett-Burman (PBD) helps in the identification of the most significant compounds and their concentrations from a large number of factors for process optimization (Plackett and Burman, 2018) .

The PB design allows evaluation of each variable at two levels namely, “high” (+1) and “low” (−1). ANOVA factors like the F and p values help decide the suitability of the model for the design. Graphical representation like the Paretos chart help understanding of the most significant factor in the descending order of importance. Plackett Burman factorial design was reported to optimize various media parameters and for multiple isolates thus becoming time saving and one such report was where pH, casein hydrolysate and corn steep liquor were the most significant factors improving enzyme production process on screening among 15 culture conditions for *Pseudomonas aeruginosa* to produce L-asparaginase (Abdel-Fattah and Olama, 2002). Venil et al. (2009) have reported optimization method that combined the Plackett-Burman design, a factorial design and the response surface method, which was used to optimize the medium for the production of L-asparaginase by *Serratia marcescens* SB08. Impact of carbon and nitrogen sources on the L-asparaginase production was reported using one such statistical tool called the Plackett and Burman (Hymavathi and Sathish, 2010).

Response Surface Methodology (RSM) involves use of different factors that are evaluated in $\alpha + 1$ and -1 level and the resulting output in terms of yield are analyzed statistically for their significance and the biological process can be given as a mathematical equation in a quadratic or a linear manner depending upon the interaction of the concerned factors. ANOVA further helps understanding of the significance of the model designed and the interacting factors in terms of the F and p values. As a step ahead in optimization, genetic algorithm coupled with RSM can be used. Optimization of culture conditions for L-asparaginase production by submerged fermentation of *Aspergillus terreus* MTCC 1782 was studied using a 3-level central composite design of RSM and artificial neural network linked genetic algorithm which was found to be more efficient than response surface methodology alone (Gurunathan and Sahadevan, 2012). The advantages of RSM over the conventional One factor at a time approach (OFAT) has been clearly demonstrated in a comparison study for L-asparaginase production by *Serratia marcescens*, using parameters coconut oil cake substrate amount, initial moisture content, pH and temperature, where RSM optimization approach enhances the enzyme production to 33% when compared to classical method (Ghosh et al., 2013). Central Composite rotatable design (CCRD) is more commonly reported as one of the steps in sequential optimization involving factors other than media constituents like optimum initial pH of the culture medium and inoculum concentration which show a remarkable increase in L-asparaginase yield (Dias and Sato, 2016). The most recent reports by da Cunha et al. (2018) also suggest that by process optimization an increase (57%) in L-asparaginase activity (3746.78 U/gds) was obtained using the optimized conditions of passion fruit peel flour with an initial moisture content and inoculum concentrations other factors, when compared with the initial values obtained in the “one factor-at-a-time” method.

Another design in response surface methodology, Box-Behnken designs is used usually when we have fewer design points than central composite designs and hence they are less expensive to run. Box-Behnken designs have 3 levels per factor, unlike the central composite designs which can have up to 5, as they do not include runs where all factors are at their extreme setting. Nevertheless the method has been reported for optimization of L-asparaginase production even recently for the factors of cultivation parameters such as pH, temperature and moisture content and yielded significant result (Doriya and Kumar, 2018)

Thus the statistical optimizations result in enhanced yield of production of L-asparaginase on commercial scale.

9. Purification and characterization of L-asparaginase

Purification of L-asparaginase to near homogeneity is reported from various bacterial sources and involves multiple steps depending on the intracellular or the extracellular nature of the enzyme. In either case, the proteins are further salted out by precipitation techniques like ammonium sulfate. The precipitated protein is then dialyzed and further subjected to column chromatography. The choice of the column chromatography varies with the source microorganism and the size of the L-asparaginase involved. Multiple steps of chromatography may be involved utilizing two or more types of column methods. Gel filtration chromatography based separation using materials like Sephadex G-100 gel is used where the enzyme is easily separated based only molecular size. The fractions thus obtained are analyzed for presence of the enzyme by assay protocols and then the fractions containing the enzyme may be pooled, concentrated and further applied further on to ion exchange chromatography matrix like CM-Sephadex C50 ion-exchange resin and eluted on a gradient of NaCl (0.1–0.5 M) concentration to achieve charge based separation. The methods of purification have thus been almost conventional for a long period of time (El-Bessoumy et al., 2004; Gaffar and Shethna, 1977). The general purification strategy can be as enlisted in Table 4.

Table 4
Purification and characterization of L-asparaginase from different microorganisms.

Name of organisms	Purification step/method	Yield (%)	Purification fold	Optimum pH	Optimum temp (°C)	Mol Wt kDa	References
Azotobacter vinelandii	Protamine sulfate	90	–	8.6	48	84 kDa	(Gaffar and Shethna, 1977)
	A. sulfate	63	1.6				
	Sephadex G150	21	4.5				
	DEAE	5	12.5				
Thermus aquaticus strain T351	DEAE-Sepharose CL-6B column	86	18	9.5	–	80 ± 2 kDa	(Curran et al., 1985)
	QAE-Sephadex A 50	83	29				
	Hydroxylapatite	38	75				
	Sephadex G-150	21	225				
Corynebacterium glutamicum	Protamine sulfate	105	1.2	7.0	40	80 ± 1 kDa	(Mesas et al., 1990)
	DEAE Sephacel	35	6.6				
	A. sulfate	16	16.3				
	Sephacryl S 200	12.5	98				
Pseudomonas aeruginosa 50071	A. sulfate	85	5.2	9.0	37	160 kDa	(El-Bessoumy et al., 2004)
	Sephadex G100	60.8	27.7				
	CM Sephadex C50	43	106				
Erwinia carotovora	A. sulfate	85	6	8.6	35	33.5 kDa	(Kamble et al., 2006)
	DEAE	76	88				
Marine actinomycetes PDK2	A. sulfate	65.83	1.09	8.0	60	140 kDa	(Dhevagi and Poorani, 2006)
	Sephadex G50	8.61	33.68				
	Sephadex G200	2.18	82.98				
Bacillus sp.	Ammonium sulfate	96.2	10.9	7.0	37	45 kDa	(Moorthy et al., 2010)
	IEC- DEAE	43.1	11.2				
Streptomyces gulbargensis	A. sulfate	50.6	1.8	9.0	40	85 kDa	(Amena et al., 2010)
	Sephacryl S 200	37.8	26.88				
	CM Sephadex C50	32	82.12				

Many novel methods have been explored to purify L-asparaginase. One such method being, *in situ* extraction of intracellular L-asparaginase utilizing a thermo-separating aqueous two-phase system that produced greater yield and higher specific activity when compared with the conventional aqueous two-phase extraction process, (Zhu et al., 2007). Further, Zhu et al. (2007) reported enhanced recovery of the enzyme on combined high-pressure homogenization with aqueous two-phase extraction for the purification of intracellular L-asparaginase I. (Ferrara et al., 2010) reported the extraction of periplasmic L-asparaginase from recombinant *Pichia pastoris* containing the *Saccharomyces cerevisiae* ASP3 gene. They obtained high extraction yields and enzyme recovery using freeze–thaw cycles, ethanol treatment and alkaline extraction in the presence and absence of cysteine.

Similar protein purification strategies are devised to obtain purified L-asparaginase which is further confirmed by electrophoretic techniques. The homogeneity of the purified protein is confirmed by single band of protein in native PAGE followed by the subunit size obtained on SDS PAGE. The sizes thus inferred give an idea of the character of the enzyme either as a homo- or a hetro-mer.

10. Molecular mass of purified L-asparaginase

The L-asparaginase sizes vary from every organism to the other in terms of genus and species. In case of the Gram negative bacteria *Serratia* sp. exists as pentamer or hexamer with an average molecular weight of 170–180 kDa as reported by (Stern et al., 1976). Whereas the L-asparaginase was found to be a homotetramer with identical subunits (38,000 Da) in *E. coli* (Muller and Boos, 1998). The molecular size and subunits of L-asparaginase in *Pseudomonas* vary from a single subunit of 34–33 kDa under non-denaturing and denaturing conditions respectively. L-asparaginase also exists as a monomer with a size of 160 kDa in *Pseudomonas* as reported by (El-Bessoumy et al., 2004). Thus, L-asparaginase shows a wide structural variation in the subunits of the above mentioned bacteria. Table .4 enlists the purification steps and the properties of the purified enzyme.

11. Molecular genetics of L-asparaginase and cloning and over-expression of L-asparaginase

L-asparaginase is regulated by different molecular elements in different organisms. *Escherichia coli* produce two L-asparaginases with remarkably different properties, where L-asparaginase I, has a low affinity for L-asparagine, it is cytoplasmic, and is produced constitutively while, the L-asparaginase II is a high-affinity enzyme and is secreted into the periplasm, and its expression is positively regulated by differeent inducers namely, the cyclic AMP receptor protein and anerobiosis (the Fumarate and Nitrate Reductase or the FNR protein) (Jennings and Beacham, 1990). The sequences of both these L-asparaginase coding genes are quite dissimilar. Molecular understanding of L-asparaginase genetics have revealed that *ans*A encodes L-asparaginase I, while gene *ans*B encoding L-asparaginase II. Since asparaginase II has high affinity towards L-asparagine, it is of great interest in chemotherapy.

Attempts at cloning *ans*B and over expression of L-asparaginase have been successfully made and resulted in production of a functional enzyme (Vidya and Vasudevan, 2011). Cloning of the gene Tk1656 coding the thermophilic L-asparaginase of *Thermococcus kodakarensis* KOD1 was performed in *Escherichia coli* BLR (DE3). The recombinant protein was successfully over-expressed, purified and characterized (Hong et al., 2014).

In *Bacillus* sp. the L-asparaginase is regulated by two differentially controlled genes. Studies with *Bacillus subtilis* revealed, expression of the two set of genes encoding L-asparaginase is controlled by independent regulatory factors, where, the *ans*Z gene was shown to encode a functional L-asparaginase whose expression is activated during nitrogen-limited growth by the TnrA transcription factor. TnrA regulates *ans*Z expression by binding to a DNA site that lies upstream of the *ans*Z promoter.

However, the expression of the *ans*A gene, which encodes another L-asparaginase, was induced by asparagine. The *ans*A gene is located in an operon along with *ans*B, which encodes L-aspartase and the

Table 5
Recombinant DNA technology in cloning of L-asparaginase genes from microbial origin.

Microbial sources of the foreign gene	GenBank accession no. of the foreign gene	GenBank accession no. of the enzyme	Host cells	Plasmid for recombinant	Reference
E. chrysanthemi 3937	AY560098.1	AAS67028.1	*E. coli* BL21(DE3) pLysS	pCR®T7/CT- TOPO®	(Kotzia and Labrou, 2007)
H. pylori CCUG 17874	AIHX01000025.1	EIE30409.1	*E. coli* BL21(DE3)	pET101	(Cappelletti et al., 2008)
S. thermoluteus subsp. *fuscus* NBRC 14270	AB469678.1	BAJ25701.1	*S. lividans* 1326	pTONA5a	(Hatanaka et al., 2011)
Escherichia sp. NII	KF059845.1	AGO43921.1	*E. coli* BL21(DE3)	pET-20b(+)	(Vidya and Pandey, 2012)
Y. pseudotuberculosis Q66CJ2	BX936398.1	CAH20651.1	*E. coli* BL21(DE3)	pBAD24	(Pokrovskaya et al., 2012)
T. kodakaraensis KOD1	NC_006624.1	YP_184069.1	*E. coli* BL21 Codon Plus(DE3) RIL	pET-21a(+)	(Chohan and Rashid, 2013)
B. subtilis B11-06	KF444946.1	AGT62618.1	*B. subtilis* 168	pMA5	(Jia et al., 2013)
R. miehe	KF290772.	AHF50151.	*E. coli* BL21(DE3	pET-28a(+	(Huang et al., 2014)
T. gammatolerans EJ3	NC_012804.1	YP_002959808.1	*E. coli* BL21(DE3)	pET-22b(+)	(Zuo et al., 2014)
V. cholerae	clone (ID: VcCD00584780)	–	*E. coli BL21 (DE3)*	pMCSG7	(Radha et al., 2018)

expression of the *ans*AB operon is repressed by AnsR, and the activity of AnsR has been hypothesized to be regulated by either asparagine or aspartate (Fisher and Wray, 2002). The cloning, of the gene encoding L-asparaginase (*ans*Z) from a non-pathogenic strain of *Bacillus subtilis* B11-06 and its overexprssion and purification of the thermostable protein was successfully demonstrated (Jia et al., 2013). The list of organisms whose genes have been cloned for L-asparaginase over expression have been listed in Table 5.

Thus recombinant DNA technology has been applied successfully to yield many fold increased L-asparaginase production and to possess improved properties of activity and stability (Zuo et al., 2015).

12. Effects of pH, temperature and effectors on L-asparaginase activity and L asparaginase kinetics studies

L-asparaginases from different organisms differ in their biochemical properties. the general optimum temperature for L-asparaginase activity is between 30 and 40 °C; however, several *Yersinia* and *Streptomyces* species have higher optimum temperatures (40–60 °C), while the hyperthermophilic L-asparaginases from archaea *Thermococcus kodakarensis* KOD1 and *P. furiosus* have optimum temperatures at 85 °C and 80 °C, respectively. L-asparaginase is generally active across a wide pH range, with optimum activity in the range of 6.0–9.5; however, most of them are active at alkaline pH. *Streptomyces gulbargensis* L-asparaginase was stable at alkaline pH, while it retained 55% activity at 80 °C for 1 h (Amena et al., 2010).

L-asparaginase purified from *Bacillus licheniformis* was found to be active over the range of pH 6.0–10.0 and temperature of 40 °C (Mahajan et al., 2014). L-asparaginase produced by *Streptobacillus sp*. KK2S4. showed the optimum activity of purified enzyme was recorded at pH 8.5 and 35 °C (Makky et al., 2014). L-asparaginase from *Pseudomonas otitidis* showed optimum activity of asparaginase was achieved at 40 ºC and pH 7.5, which is close to the internal environment of the human body (Husain et al., 2016).

On the other hand, different ions have different influences on the activity of L-asparaginase and the knowledge of such effectors help in the understanding of the active site of the enzyme, that can further lead to studies in engineering its substrate specificity or activity. Warangkar and Khobragade (2010) reported the enhancing effect of EDTA on L-asparaginase activity which was interpreted that the enzyme was not a metalloprotein. This report was also supported by the reports of Jia et al. (2013), Kumar et al. (2011), Singh et al. (2013).

Similarly, inhibition of enzyme activity in presence of Hg^{2+}, Cd^{2+} and Zn^{2+}, increased activation with reducing agents like 2-mercaptoethanol (2-ME), dithiothreitol (DTT), and reduced glutathione (GSH) and inhibition in the presence of thiol group blocking reagents, namely, PCMB (*p*-chloro mercury benzoate) were indicative of role of sulfhydryl groups of the enzyme for productive catalysis. Kumar et al. (2011) documented complete inhibition of L-asparaginase activity from *P. carotovorum* MTCC 1428 in the presence of di-valent cations Cu^{2+}, Cd^{2+} and Hg^{2+}.(Jia et al., 2013) reported Fe^{3+} strongly inhibited *Bacillus subtilis* B11-06 L-asparaginase activity.

Study of kinetics of L-asparaginase with respect to its substrate L-asparagine provides an insight into the affinity for the substrate. The V_{max} and K_m value are indication of the reaction velocity and the substrate affinity in low concentrations. K_m values of asparaginases from different sources vary widely. The kinetic parameters K_m are calculated by non-linear regression analysis of experimental steady-state data. Knowledge of the enzyme affinity for L-asparagine can address the potential of the enzyme to interact with its substrate at the least concentration available as in the human sera. Thus the enzyme with lowest K_m is the most sought for real time therapeutic application. The K_m value thus serves as a denomination of comparison of the enzyme from various source for its substrate affinity. Table 6 enlists some of the organisms producing L-asparaginase and its kinetic parameters.

Table 6
Kinetic parameters of L-asparaginase for its natural substrate L-asparagine.

Name of organism	Km	V max	Reference
Pseudomonas aeruginosa 50071	0.147 mM	35.7 IU	(El-Bessoumy et al., 2004)
Pectobacterium carotovorum MTCC 1428	0.657 mM	4.45 U/μg	(Kumar et al., 2011)
Thermococcus kodakaraensis	5.5 mM	3300 μmol/min/mg	(Chohan and Rashid, 2013)
Bacillus aryabhattai ITBHU02	0.257 mM	1.537 U/ μg	(Singh et al., 2013)
Bacillus licheniformis	1.46×10^{-5} M	4.03 IU	(Mahajan et al., 2014)
Pseudomonas plecoglossicida RS1	2.25 mM	8.9 IU/mL/min	(Shakambari et al., 2015)
Bacillus tequilensis PV9W	0.045 mM	7.46 μmol/mL/min	(Shakambari et al., 2016)
Streptomyces fradiae NEAE-82	0.01007 M	95.08 U/mL/min	(El-Naggar et al., 2016)

13. Applications of L-asparaginase in food industry

Acrylamide is classified by the IARC as "probably carcinogenic to humans" and is formed as a result of the Maillard reaction between asparagine and carbonyl compounds, such as reducing sugars in products such as the fried potato products and other food commodities, including cereals products, coffee, chocolate and potato snacks (Vinci et al., 2011).

However, Milliard reactions do impart many favourable changes in the food industry apart from the acrylamide formation and because of which its formation cannot be completely eradicated. Application of the L-asparaginase however, provides a possible alternative method for acrylamide mitigation that should have a very limited effect on the general formation of Maillard products. L-asparaginase from various sources have been evaluated for acrylamide mitigation. L-asparaginase from *Aspergillus oryzae* was tested in a range of food products, including dough-based applications (semisweet biscuits, ginger biscuits, crisp bread) where 34–92% reduction of acrylamide content was seen, in French fries about 60–85%, and sliced potato chips up to 60% reduction was documented (Hendriksen et al., 2009). Mahajan et al. (2012) demonstrated acrylamide degradation by L-asparaginase from *Bacillus licheniformis* that inhibited poly-acrylamide formation in 10% acrylamide solution and reduced acrylamide formation in fried potatoes by 80%. Further, recombinant thermophilic L-asparaginase from *Thermococcus kodakarensis* KOD1 showed reduction in acrylamide contents in baked dough were reduced to sixty percent after treatment with recombinant TkAsn as compared to the untreated control (Hong et al., 2014). The impact of acrylamide on neurological potencies and the mitigating effect of L-asparaginase was demonstrated efficiently using *C.elegans* as animal model by studying effect of acrylamide exposure on chemotactic ability of the nematodes. This study demonstrated the applicability of the L-asparaginase and the necessity of the acrylamide mitigation (Shakambari et al., 2017). Thus, L-asparaginase finds an application in food industry for preventing accumulation of acrylamide (Fig. 3).

14. Application of L-asparaginase as cancer therapy regimen

Anti lymphoma effects of L-asparaginase has been reported since 1968 (Broome, 1968). Cancer cells, mainly of the lymphoid origin, require high amount of asparagine for fast and malignant growth. In this way, cancer cells are in need of the amino acid from diet. However, leukemic lymphoblasts and some others tumor cells are auxotrophs for L-asparagine and have low level of L-asparagine synthetase required for L-asparagine synthesis. Thus, these malignant cells are dependent for exogenous supply of L-asparagine from blood serum for their proliferation and survival (Fig. 4). L-asparaginase hydrolyzes asparagine from blood serum, leading the tumor cells to death by lacking of an essential factor for protein synthetases (p53-dependent apoptosis). However, healthy cells are not affected, because they are able to produce asparagine using L-asparagine synthetase present in enough quantities.

Extensive clinical trials for treatment of many types of malignancies including those for haematopoietic diseases, acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphomas was tried where L-asparaginase was one of the combinations in chemotherapy. Treatment of ALL in pediatric patients showed a high survival rate (70%) along with very low reports of hypersensitivity reactions and immunological interferences. (Muller and Boos, 1998)

The sensitivity of the cancer cells to L-asparaginase treatment has been attributed to more than the auxotrophy for L-asparagine synthetase. The major limitation of L-asparaginase treatment in ALL is the rapid development of clinical resistance, either due to rapid clearance of L-asparaginase by immunological mechanism, or active synthesis of L-asparagine leading to enough supply of the amino acid necessary for the cell survival. In human leukemic cells, the resistance is at least in part related to the asparagine synthetase activity, where there is a seven fold increase in asparagine synthetase activity where asparaginase was given as treatment (McCredie et al., 1973).

The recent decade has lead to the deeper insights into the molecular understanding of the reason for the sensitivity of the ALL to L-asparaginase treatment. The t(12;21) translocation resulting in TEL/AML1 gene fusion is present in approximately 25% of patients with precursor B-lineage pediatric acute lymphoblastic leukemia (ALL), which may cause sensitivity to L-asparaginase alone, at doses that secure complete asparagine depletion (Woerden et al., 2000).

Thus L-asparaginase is currently available for treatment in three forms namely, native L-asparaginase from *Escherichia coli* (*E. coli* asparaginase), a PEGylated (PEG: polyethylene glycol) form of L-asparaginase (PEG-asparaginase), and L-asparaginase from *Erwinia chrysanthemi* (*Erwinia* asparaginase). *E. coli* asparaginase or PEG-asparaginase is used as a first-line treatment of childhood ALL in current treatment protocols and *Erwinia* asparaginase has been adopted in European and US protocols for second- or third-line treatments (Zuo et al., 2015). Till date, L-asparaginase continues to be prospected in treatment of many malignancies owing to the mechanism of depletion of L-asparagine pool in the cells leading to cell death (Shrivastava et al., 2016).

Recent research on mechanism of action of L-asparaginase by asparagine depletion revealed that a novel low L-glutaminase variant which was developed on the backbone of the FDA-approved L-asparaginase from *Erwinia chrysanthemi* was highly efficacious against both T and B cell ALL, while displaying reduced acute toxicity features. These results pave the way for the development of a new generation of L-asparaginases without L-glutaminase activity for safer treatment of human ALL (Nguyen et al., 2018).

15. Immobilization and formulation of L-asparaginase for increased efficacy

Immobilization of L-asparaginase is done with a perspective of increasing its half-life, lower immunological reactions and efficiency. The use of site directed mutagenesis technique followed by covalent coupling of methoxypoly (ethylene glycol) succinate N-hydroxysuccinimide ester to L-asparaginase from *E. carotovora* showed improved resistance to trypsin and higher thermostability.

Kotzia and Labrou immobilized the recombinant L-asparaginase

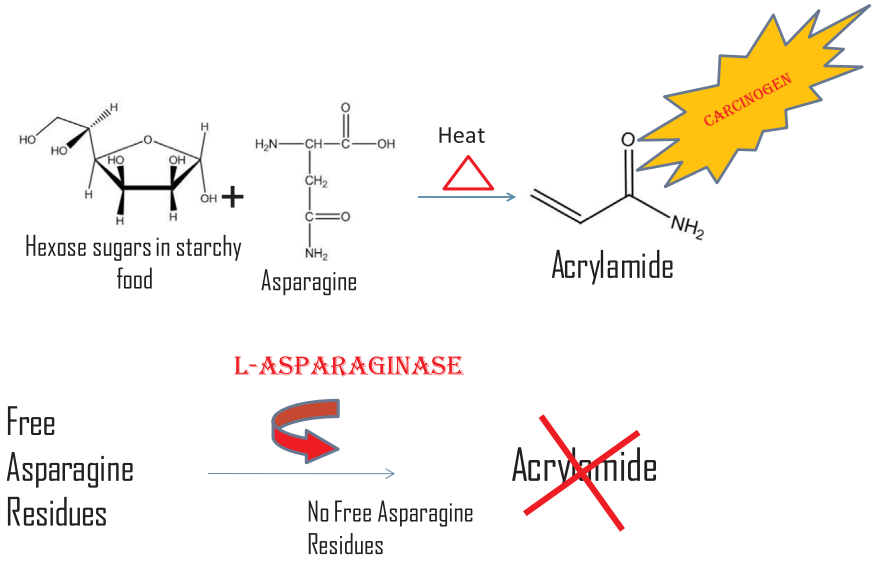

Fig. 3. Application of L-asparaginase in food industry for mitigation of acrylamide, which is formed spontaneously during baking and exposures to high temperature.

from *E. chrysanthemi* 3937 on epoxy activated Sepharose CL-6B, which exhibited high stability at 4 °C (Kotzia and Labrou, 2011). Zhang et al. (2008), devised a method of preparing silk fibroin nanoparticles and immobilized L-asparaginase by glutaraldehyde cross-linking. Further, Zhang et al. (2011) reported another novel and method to process fine crystalline silk fibroin nanoparticle harbouring L-asparaginase bio conjugates in the presence of organic solvents. Whereas the metal oxide nanoparticles suffer with the disadvantages of acute toxicity at times, the L-asparaginase fatty acid bio-conjugates showed applicability for intravenous delivery of L-asparaginase, it was evaluated and its kinetics were found to be better than native L-asparaginase. Moreover, this modified form of enzyme has improved biological half-life, negated the acute toxicity and preserved the antitumor activity when even tested *in vivo* with respect to the native l-ASNase (Ashrafi et al., 2013). Table 7 enlists methods for immobilization of L- asparaginase.

16. Pharmaceutical and clinical perspective of current L-asparaginase therapy

Biobetter or biosuperior is a generic term, to refer to value-added biologics, poses an advantage of an established mechanism of action, safety, and efficacy profile of a known biologic. Such a bio better approved for L-asparaginase is the Oncaspar approved by FDA, for treatment of acute leukemia patients hypersensitive to asparaginase. This Oncaspar is an *E. coli*-derived L-asparaginase covalently conjugated to monomethoxy polyethylene glycol (mPEG) to reduce dosing frequency to once every 2 weeks and also reduce the incidence of hypersensitivity reactions in patients (Singh, 2017).

Pegcristantaspase, a recombinant PEGylated asparaginase from *Erwinia* with improved pharmacokinetics, was developed for patients with hypersensitivity to pegaspargase, However, when pediatric patients with ALL or lymphoblastic lymphoma and hypersensitivity to pegaspargase, received intravenous pegcrisantaspase, on Children's Oncology Group trial AALL1421 (Jazz 13-011), results showed pre-existing immunogenicity against the PEG moiety of pegaspargase that led to hypersensitivity to pegcrisantaspase manifested and rapid clearance of the serum asparaginase activity. This poses the question, if PEGylation may indeed be an effective strategy to optimize *Erwinia* asparaginase administration (Rau et al., 2018).

Recent reviews on this subject to tackle the problems associated with hypersensitivity suggest, native *E. coli* asparaginase be used for patients without anti-asparaginase antibodies and pegcrisantaspase for

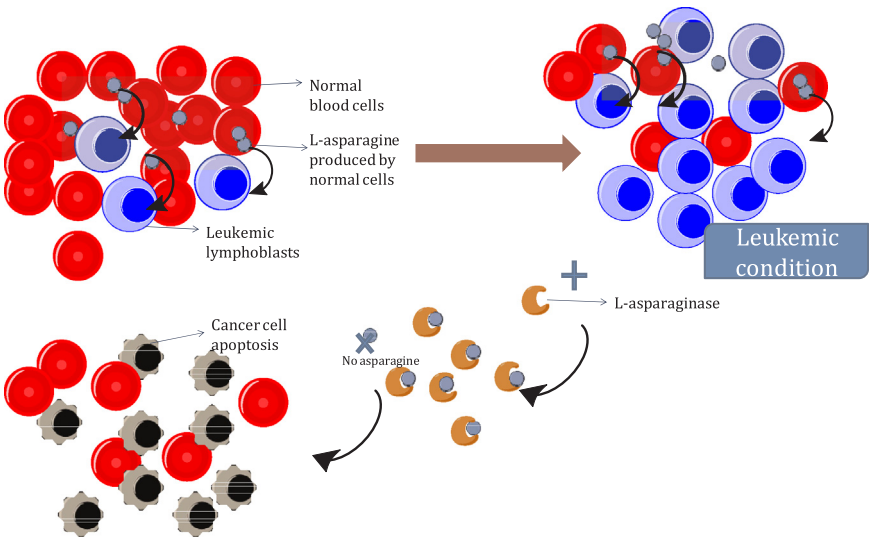

Fig. 4. L-asparaginase mode of cytotoxicity in cancer cells.

Table 7
Immobilization of microbial L-asparaginase Source: (Zuo et al., 2015).

Microbial source of L-asparaginase	Preparation method	Reference
E. carotovora 1526	Covalent coupling of methoxypoly (ethylene glycol) succinate *N*-hydroxysuccinimide ester	(Kotzia et al., 2007)
E. chrysanthemi 3937	Cross-linked epoxy-activated Sepharose CL-6B	(Kotzia and Labrou, 2007)
E. coli	Glutaraldehyde cross-linking silk fibroin nanoparticles	(Zhang et al., 2008)
B. circulans	Immobilized by polyaniline nanofiber	(Ghosh et al., 2012)
Cladosporium sp.	Bovine serum albumin, ovalbumin by crosslinking using glutaraldehyde, *N*-bromosuccinimide, and mono-methoxy olyethylene glycol	(Mohan Kumar et al., 2014)

those without antibodies to PEG, however, the use of additional immunosuppressive drugs preceeding the administration of pegaspargase, was warranted to reduce the frequency of hypersensitivity reactions or decrease silent inactivation of pegaspargase (Pui et al., 2018).

As a substantiation for the suggestion, Combined Modality Treatment with “Dexamethasone, Methotrexate, Ifosfamide, L-Asparaginase, and Etoposide ” chemotherapy followed by radiotherapy for early stage natural killer/T cell lymphoma with local tumor invasiveness was evidenced as a tolerable protocol with efficacy without mortality (Gupta et al., 2018).

On a similar note, a novel induction therapy named Tandem HiCHOP-LA which involved administration of cyclophosphamide intravenously on days 2 and 23; daunorubicin intravenously on days 3, 4, 24, and 25; vincristine intravenously on days 1, 8, 15, and 22; L-asparaginase intravenously or intramuscularly on days 8, 10, 12, 15, 17, 19, 22, 24, and 26; prednisolone orally on days 7–28; and methotrexate, cytarabine, and prednisolone intrathecally on days 1 and 21 to adolescents and young adults with Philadelphia-negative ALL patients and it was to be followed by Cord Blood Transplantation (CBT). The study reported all the patients achieved complete remission and proceeded to cord blood transplantation without cases of observed mortality or recurrence (Kojima et al., 2018).

Conclusively it can be perceived that L-asparaginase is an effective candidate in a treatment regimen for malignancies of the lymphatic system. This review thus contends that the microbiological, molecular biological and the clinical studies made so far leave a huge quest for researchers to explore to unearth a potential L-asparaginase source of organism, cheaper and cost effective production strategy by waste utilization, statistical optimization and a biocompatible preparation for sustained release and increased stability of the enzyme. These strategies cumulatively make L-asparaginase a cost-effective, reliable and a proficient candidate for the pharmaceutical application.

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Declarations of interest

None.

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