Chapter 2

Literature Review

2.1 L-asparagine amidohydrolase (EC 3.5.1.1)

L-asparagine amidohydrolase or L- asparaginase is an enzyme that catalyzes the hydrolytic reaction involving the breakdown of amino acid L-asparagine to L-aspartic acid and ammonia (Sharma *et al*., 2019). It is an indispensable enzyme that plays versatile roles ranging from the therapeutic drug to the food processing agent. L-asparaginase as a therapeutic finds usage for the treatment of acute lymphoblastic leukemia (ALL) and lymphomas, but the usage is accompanied by several immunological issues. The enzyme acts by depriving the tumor cells of asparagine resulting in inhibition of their growth and proliferation (Cecconello *et al.*, 2020). The enzyme also finds utilization in food industry applications for the mitigation of carcinogenic chemical acrylamide. The principal amino acid responsible for the generation of acrylamide is L-asparagine and is derived due to reactions involving the amino group of amino acid asparagine and the carbonyl group present in the reducing sugars during the course of baking and frying (Friedman, 2003). The reaction mechanism involves the hydrolysis of amino acid asparagine, thereby depleting the asparagine which acts as a precursor for the production of acrylamide (Hendriksen *et al.*, 2009).

The major producers of the enzyme used in clinical applications are bacterial sources (*Dickeya chrysanthemi* and *Escherichia coli*), while the fungal sources (*Aspergillus niger* and *Aspergillus oryzae*) are the workhorses for enzyme preparation in food applications (Radadiya *et al.*, 2020; Xu *et al.*, 2016). L-asparaginase is widely distributed among various micro-organisms (bacteria, fungi, yeasts, and actinomycetes), plants and animals. Among these different organisms, bacteria are the most preferred sources of the enzyme due to ease

of cultivation, higher growth rates, and achievement of higher productivity in short durations. L-asparaginase enzyme is produced by both the solid state fermentation (SSF) and submerged fermentation (SmF) strategies worldwide.

2.2 L-asparaginase reaction mechanism against cancer cells

The research on the antineoplastic activity of L-asparaginase began with studies on lymphoma-bearing mice. The mice when administered with guinea pig serum, showed complete regression of lymphomas. Serum from other animal species was devoid of anti-lymphoma activity (Kidd, 1953). Experiments performed by Broome proved that the substance accountable for the anti-lymphoma effects in guinea pig serum was the enzyme L-asparaginase. Furthermore, serum taken from newborn guinea pigs showed no asparaginase activity and failed to inhibit 6C3HED lymphoma cells (Broome, 1963). Mashburn and Wriston proved that L-asparaginase isolated from *Escherichia coli* shows a tumor inhibitory activity, while the L-asparaginase from *Bacillus coagulans* shows no tumor inhibitory effect (Mashburn and Wriston, 1964). Later, Campbell and his co-workers reported the presence of two L-asparaginases in *Escherichia coli* B, labeled as EC-1 and EC-2, and only EC-2 possess the anti-tumor effect (Campbell *et al.*, 1967). Later in 1968, other bacteria were examined as asparaginase producers and various species of *Erwinia*, particularly *Erwinia carotovora* was showing higher enzyme activity than *Escherichia coli* (Wade *et al.*, 1968). The first approval to treat ALL patients was given to the native preparation of L-asparaginase from *Escherichia coli* by FDA in 1978 but its use led to hypersensitive reactions in 10-30 % of patients.

L-asparaginase catalyzes the hydrolytic reaction involving the breakdown of the amino acid L-asparagine to L-aspartic acid and ammonia. Malignant lymphoblasts in comparison to normal cells have low levels/nearly complete absence of L-asparagine synthetase activity resulting in diminished endogenous production of L asparagine amino acid (Capizzi *et al.*,

1971; Haskell and Canellos, 1969). Thus, these leukemic lymphoblast cells become dependent on extracellular asparagine from blood serum for their viability and proliferation (Kiriyama *et al.*, 1989; Stams *et al.*, 2003) Administration of asparaginase hydrolyzes extracellular asparagine leading to asparagine starvation of leukemic lymphoblast cells. This asparagine depletion results in suppression of protein synthesis or the inhibition of mechanisms by which aspartate is accumulated (Broome, 1981).

Several experimental researches have been carried out to describe the biochemical reactions leading to apoptosis of tumor cells upon administration of L asparaginase. Story and others stated that the cells taken from murine and canine lymphoma when treated with L-asparaginase result in apoptosis characterized by changes in cellular morphology and internucleosomal cleavage of DNA. These characteristics are key features of cells undergoing apoptosis (Story *et al.*, 1993). Similarly, studies were performed on the treatment of murine leukemia cells with L- asparaginase. The various outcomes that were observed include DNA fragmentation, cell cycle arrest in G1 phase, eventually leading to apoptosis (Ueno *et al.*, 1997).

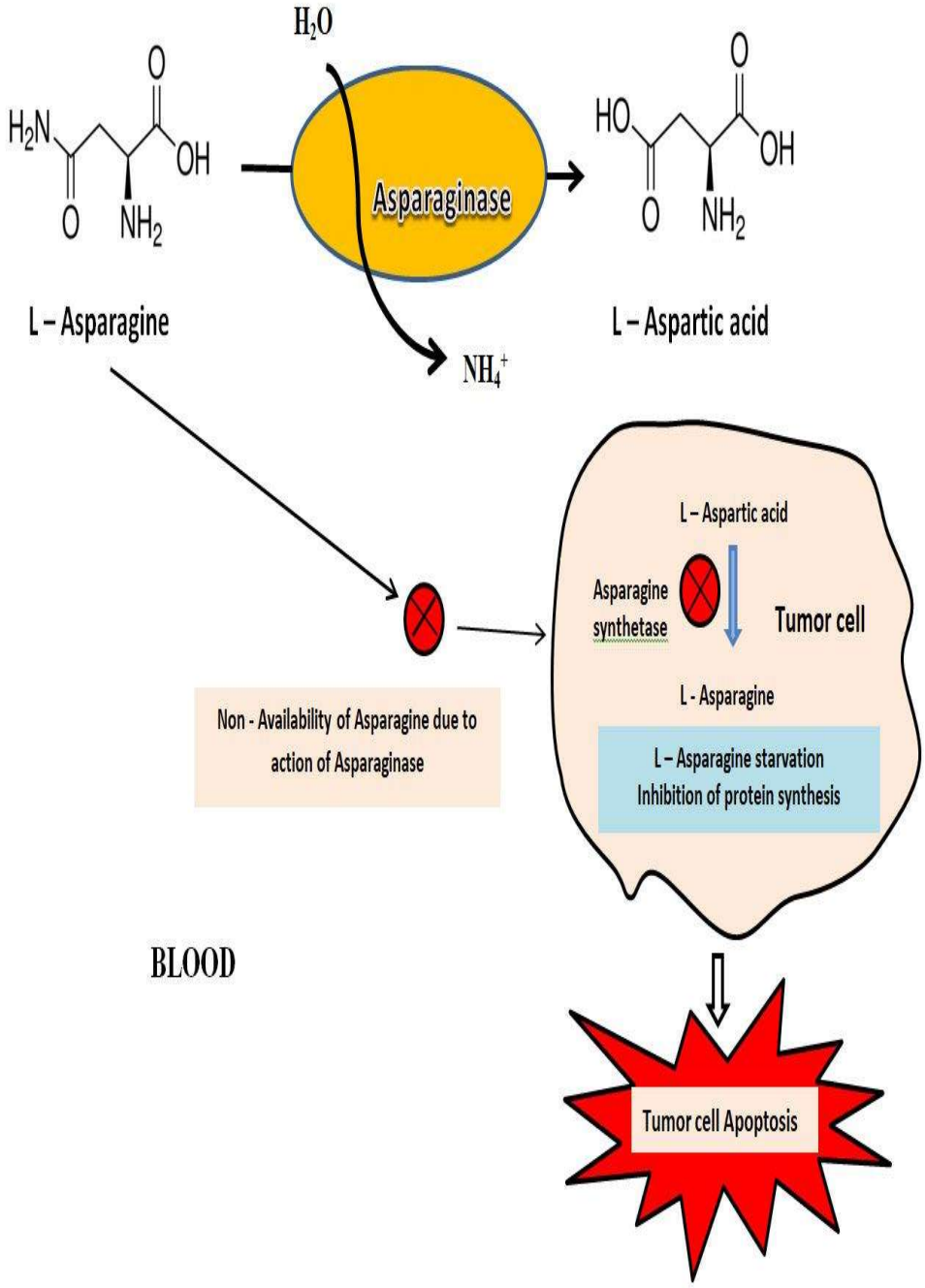

Figure 2.1: General reaction catalyzed and antineoplastic effect on tumor cells by L-asparaginase (Sharma *et al*., 2019).

2.3 L-asparaginase relevance in food industry

The formation of a suspected cancer-causing agent acrylamide in diverse types of heated foods particularly rich in carbohydrates has been confirmed by Swedish food regulatory authorities and research groups (Tareke *et al.*, 2002). Since then, the reduction of acrylamide from diverse foodstuffs has become a major aim for the food industry. Certain amino acids, particularly asparagine, when heated with a variety of reducing sugars, causes the Maillard reaction and the emergence of carcinogen acrylamide takes place (Stadler *et al.*, 2002; Knol *et al.*, 2010). No formation of acrylamide occurs when any of the carbohydrates is heated singly (Mottram *et al.*, 2002). Thus, the principal amino acid responsible for the generation of acrylamide is asparagine and is derived due to reactions involving the amino group of amino acid asparagine and the carbonyl group present in the reducing sugars during the course of baking and frying. (Friedman, 2003; Taeymans *et al.*, 2004; Yaylayan *et al.*, 2005).

Certain plant-based food products such as potatoes and cereals are rich in asparagine content and, therefore can easily correlate with the formation of acrylamide. The processed foods derived from these plant-based food products has been reported to have acrylamide content, including potato chips, french fries, crisp bread, tortilla chips and other baked and fried products (Baskar and Aiswarya, 2018; Maan *et al.*, 2022). The explanation of the reaction mechanism for the formation of acrylamide involves the formation of Schiff base between the α-amino group of asparagine and a carbonyl-containing compound. The Schiff base under the influence of heat, undergoes decarboxylation and elimination of either ammonia or a substituted imine to generate the undesirable carcinogen acrylamide (Zyzak *et al.*, 2003). The reaction mechanism of L- asparaginase involves the hydrolysis of amino acid asparagine, thereby depleting the asparagine, which acts as a precursor for the production of acrylamide (Hendriksen *et al.*, 2009).

Currently, there are two viable asparaginase products accessible for the mitigation of acrylamide in potato and cereal-based foodstuffs. These commercial asparaginases include the Acrylaway® from Novozymes A/S and PreventASe™ from DSM. Acrylaway® product is derived from the fungus *Aspergillus oryzae* and possesses a neutral profile with optimum pH of 7 and temperature of 37 ^{o}C (da Cunha *et al.*, 2019). PreventASe™, on the other hand, is derived from the *Aspergillus niger* strain and shows an acidic nature with pH and temperature optimum of 4-5 and 50^{o}C respectively (Andersen and Nielsen, 2009; Yim and Kim, 2019). Both the fungal strains, *Aspergillus niger* and *Aspergillus oryzae* possess a long history of safe usage and are considered as generally regarded as safe by JECFA (Khorshidian *et al.*, 2020). Over the last ten years, several studies reported on the reduction of acrylamide from various foodstuffs using the L- asparaginase enzyme treatment.

L-asparaginase enzyme from *Bacillus licheniformis* (RAM-8) was purified and reported a decrease in acrylamide levels by 80% in fried potatoes (Mahajan *et al.*, 2012). Kumar and others reported the use of L-asparaginase from *Cladosporium sp*. for the reduction of acrylamide during bread making process in the sweet bread. Both crust and crumb fractions were treated with varying concentrations (50-300 U) of the enzyme. 97% and 73% reductions in acrylamide formation were observed in crust and crumb regions of bread on the application of 300 U of asparaginase respectively. Hydroxymethylfurfural (HMF), a genotoxic compound and a common intermediate product of the Maillard reaction, was also estimated and found to decrease with L-asparaginase treatment (Kumar *et al.*, 2014).

Recombinant L-asparaginase was produced by cloning and expression of a gene encoding *Rhizomucor miehei* L-asparaginase in *Escherichia coli*. Upon application of 10 U per mg flour of the enzyme on baked products, the acrylamide levels reduced by 81.6% in biscuits and 94.2% in bread. Moreover, the enzyme showed very low glutaminase activity and was found to be optimally active at a pH 7 and temperature of 45 ^{o}C (Huang *et al.*, 2014). In

another recombinant study involving the cloning and expression of ansZ gene encoding asparaginase II from *Bacillus megaterium* H-1 in *Escherichia coli*, reduction of acrylamide formation by 92% was reported in fried potato strips (Zhang *et al.*, 2015).

The use of asparaginase offers the advantages as the enzyme is highly specific towards the substrate L- asparagine, thereby eliminating the major precursor responsible for acrylamide formation and does not affect the taste and physicochemical properties of the final product. As acrylamide is associated with lethal manifestations of neurotoxicity (causing peripheral neuropathy), reproductive toxicity and carcinogenicity, mitigation of acrylamide using L-asparaginase enzyme is a promising alternative approach in the food industry (Farahat *et al.*, 2020; Wang *et al.*, 2021).

2.4 Immunological reactions associated with L-asparaginase enzyme

Even though L- asparaginase usage as a therapeutic drug for the treatment of ALL has been long established, the currently used L-asparaginases suffer from various drawbacks. The current formulations of L-asparaginases induce multiple types of immunological reactions. These side reactions include hypersensitivity, allergenicity, coagulation disorders, hepatotoxicity, hyperglycemia and pancreatitis.

It has been reported in several studies that L-asparaginases with higher L-glutaminase co-activities cause more severe side effects. Many of the glutaminase-asparaginase (having equal glutaminase and asparaginase activities) have been explored in the past for the therapeutic usage, but none has been approved for therapeutic purposes as their usage led to severe side issues, including respiratory alkalosis, hepatotoxicity and depression of the central nervous system resulting in encephalopathies during the Phase I clinical trials.

Clinical indications due to asparaginase hypersensensivity responses include broncospasm, itching and swelling of extremities, serum sickness, edema, local or generalized erythema, urticaria and rashes and anaphylactic reactions (rare) (Avramis *et al.*, 2006; Shrivastava *et al.*, 2016). Greater occurrence of major skin reactions appeared when the mode of administration is intramuscular (IM) as compared to intravenous (IV) route of administration of asparaginase (Nesbit *et al.*, 1979). The production of antibodies in response to asparaginase can cause rapid inactivation of the enzyme leading to suboptimal degradation of substrate asparagine. This phenomenon leads to reduced therapeutic potency due to higher plasma levels of asparagine and is known as silent inactivation or silent hypersensitivity (Panosyan *et al.*, 2004; Zalewska *et al.*, 2007).

L-asparaginase treatment can also lead to various coagulation disorders such as thrombosis, hypofibrinogenemia due to reduced synthesis of antithrombin III, fibrinogen and other clotting factors.(Alberts *et al.*, 1999; Beinart *et al.*, 2004).These changes in hemostatis were frequently perceived in patients with ALL accepting native *Escherichia coli* L-asparaginase alone or in combination with other drugs (Nowak-Göttl *et al.*, 2003).

Furthermore, asparaginase treatment can also cause organ toxicities, inflammation of the pancreas, glucosuria, liver dysfunction, cerebral impairment and ketoacidosis (Shrivastava *et al.*, 2016) Asparaginase-associated pancreatitis leads to vomiting, nausea, abdominal pain (Knoderer *et al.*, 2007) and enhanced serum levels of amylase and lipase acting as biomarkers for pancreatitis (Raja *et al.*, 2012).

2.5 Properties of a good L-asparaginase for diverse applications

The first crucial property is that the enzyme must show high binding affinity towards the substrate L-asparagine by depicting low Km. The low value of Km is a desirable feature as it is required for the efficient removal of L-asparagine amino acid during the L-asparaginase

drug treatment. The higher the enzyme affinity of the enzyme towards the substrate, the better the efficacy of the enzyme for diverse applications. Earlier studies conducted by different research groups on the L-asparaginases from different sources reported K_m values ranging from 0.074 mM to 3.5 mM (Yaacob *et al.*, 2014). Secondly, an L-asparaginase with a high level of accompanying L-glutaminase activity causes toxicity and immunological issues during drug treatment (Beckett and Gervais, 2019). So, a better L-asparaginase preparation must possess low/negligible L-glutaminase activity to prevent serious L-glutaminase-associated side-effects. Thirdly, a new L-asparaginase preparation must possess anti-cancer activity, as only a few L-asparaginases show anti-leukemic effects. The therapeutic efficacy of the L-asparaginase preparation must be checked against leukemia cells and other related types of cancer, as L-asparaginase is specifically used for the treatment of acute lymphoblastic leukemia (ALL).

2.6 Sources of L-asparaginase enzyme

L–asparaginase is widely distributed among various micro-organisms (bacteria, fungi, yeasts, actinomycetes), plants and animals. Among these different sources, bacteria are the most preferred sources of the enzyme due to ease of cultivation, higher growth rates, achievement of higher productivity in short durations and being amenable to genetic engineering techniques.

2.6.1 Bacterial sources of L-asparaginase

The production of L-asparaginase enzyme has been described in both gram-positive and gram-negative bacteria. The current industrial asparaginase preparations are derived from *Escherichia coli* and *Erwinia chrysanthemi*. Genus pseudomonads have also been explored and reported for the L- asparaginase by various research groups (Manna *et al.*, 1995; Husain *et al.*, 2016). The various sources that were explored previously include the *Escherichia coli*

(Mashburn and Wriston, 1964), *Enterobacter aerogenes* (Mukherjee *et al.*, 2000), *Erwinia aroideae* (Tiwari *et al.*, 1996), *Mycobacterium phlei* (Pastuzak and Szymona, 1975), *Serratia marcescens* (Boyd and Phillips, 1971), *Proteus vulgaris* (Tosa *et al.*, 1971). Table 2.1 depicts the recent studies performed by various researchers on diverse bacterial sources.

Table 2.1: Various bacterial sources of L-asparaginase enzyme.

Bacterial source	Product location	Reference
Pectobacterium carotovorum	Intracellular	Kumar *et al.*, 2011
Bacillus licheniformis	Extracellular	Mahajan *et al.*, 2014
Pseudomonas aeruginosa	Extracellular	Badoei-Dalfard, 2015
Enterobacter cloacae	Extracellular	Husain *et al.*, 2016
Streptomyces fradiae	Extracellular	El-Naggar *et al.*, 2016
Bacillus licheniformis	Extracellular	Alrumman *et al.*, 2019
Streptomyces brollosae	Extracellular	El-Naggar *et al.*, 2019
Streptomyces rochei	Extracellular	El-Naggar *et al.*, 2020
Bacillus australimaris	Extracellular	Chakravarty *et al.*, 2021

2.6.2 Fungal sources of L-asparaginase

Asparaginase from eukaryotic micro-organisms like fungi has also been reported by various research groups. Among fungi, the major genera investigated for L- asparaginase production are *Aspergillus, Mucor, Fusarium* and *Penicillium sp.* (Sarquis *et al.*, 2004; Mishra 2006; Huang *et al.*, 2014; Nakahama *et al.*, 1973, Shrivastava *et al.*, 2012). L-asparaginases from yeasts and algae have also been reported by research groups. *Saccharomyces cerevisiae* cells were grown on asparagine and a single, constitutively produced L-asparaginase was reported (Jones and Mortimer 1973). Several strains of the yeast *Saccharomyces cerevisiae* were found to possess both intracellular and extracellular forms of L-asparaginases. Asparaginase from *Aspergillus oryzae* and *Aspergillus niger* already finds practical applications in the food

industry for a decrease in levels of toxic carcinogen acrylamide. Fungal producers of L-asparaginases are listed in Table 2.2.

Table 2.2: Fungal producers of L-asparaginase enzyme.

Fungal source	Product location	Reference
Penicillium digitatum	Extracellular	Shrivastava *et al.*, 2012
Aspergillus oryzae	Extracellular	Dias and Sato, 2016
Aspergillus terreus	Extracellular	Costa-Silva *et al.*, 2019
Sarocladium strictum	Extracellular	Golbabaie *et al.*, 2020
Leucosporidium scottii	Intracellular	Moguel *et al.*, 2020
Ganoderma australe	Extracellular	Chakraborty and Shivakumar, 2021
Fusarium equiseti	Extracellular	El-gendy *et al.*, 2021

2.6.3 Other sources of L-asparaginase

Microalgal species were also examined for the production of L-asparaginase enzyme. *Chlamydomonas* L-asparaginase was purified and reported to possess minor anti-lymphoma activity (Paul, 1982). Also, some plant species were explored and reported for the production of L-asparaginase. L-asparaginase from *Capsicum annum L.* was purified but found to possess significant glutaminase and urease activities (Bano and Sivaramakrishnan, 1980). Similarly, another plant asparaginase from *Withania somnifera* was purified and found to possess a lower Km towards the substrate L-asparagine (Oza *et al.*, 2009).

2.7 Microbial production of recombinant L-asparaginases from diverse sources

Genetic engineering techniques have also been utilized by certain researchers to express L-asparaginase genes from pathogenic organisms in safe microbial strains, to improve the productivities and to secrete the therapeutic L-asparaginase. The major basis for cloning and

expression of genes encoding L-asparaginase is to produce the therapeutic enzyme on a large scale. With the increasing knowledge of biosynthetic pathways and developments in molecular biology approaches, efficient producer strains can be synthesized having the capability to overproduce and secrete the desired therapeutic enzyme. Enhanced production of L-asparaginase enzyme has been described using the promoters of genes which are constitutively expressed or promoters of inducible genes in the enzyme producing microorganism. Some researchers have also reported overexpression of L-asparaginase utilizing heterologous host organisms. Most of the studies focussed on *Escherichia coli* as the suitable host strain and IPTG as an inducer for the recombinant expression of L-asparaginase. Another strategy employed for the asparaginase enzyme is the excretion of the enzyme out of the cells. This approach relieves the cells from metabolic stress which can cause inhibition of growth, and also confers easier downstream processing without cell disruption procedure. The asparaginase isozyme II from *E. coli* was secreted extra-cellularly by recombinant expression of enzyme using fusion tags comprising of pelB signal sequence and repeated aspartate residues (Kim *et al.*, 2015). They showed that the fusion of aspartate residues could be used for efficient secretion of asparaginase enzyme into a culture medium with a specific activity of 34.6 U/mg.

Majority of the genetic engineering studies focussed on the use of prokaryotic expression systems due to easiness of manipulation, ease of cultivation and less doubling time to achieve high yields of the asparaginase enzyme. Table 2.3 lists the detailed studies on the recombinant production of L-asparaginase enzyme from various microorganisms.

Table 2.3: Recombinant production of L-asparaginase enzyme from various micro-organisms.

Source	Host organism	Expression	Reference
Withania Somnifera L.	*E. coli* BL 21(DE3)	Inducible (IPTG)	Oza *et al.*, 2011
E. coli MTCC 739	*Escherichia coli* DE3	Inducible (IPTG)	Vidya *et al.*, 2011
Thermococcus kodakaraensis	*Escherichia coli*	Inducible (IPTG)	Chohan *et al.*, 2013
Rhizomucor Meihei	*E. coli* BL 21(DE3)	Inducible (IPTG)	Huang *et al.*, 2014
Nocardiopsis alba	*Escherichia coli*	Inducible (IPTG)	Meena *et al.*, 2014
Escherichia coli	*E. coli* BL 21(DE3)	Inducible (IPTG)	Ghoshoon *et al.*, 2015
Pseudomonas fluorescens	*E. coli* BL 21	Inducible (IPTG)	Kishore *et al.*, 2015
Bacillus subtilis 168	*Bacillus subtilis* WB 600	Constitutive	Feng *et al.*, 2016
Pseudomonas aeruginosa	*E. coli* BL 21(DE3)	Inducible (IPTG)	El-Sharkawy *et al.*, 2016
Lactobacillus reuteri	*E. coli* BL 21(DE3)	Inducible (IPTG)	Aishwarya *et al.*, 2017
Aspergillus terreus	*E. coli* BL 21(DE3)	Inducible (IPTG)	Saeed *et al.*, 2018
Lactobacillus casei	*E. coli* pET28a	Inducible (IPTG)	Aishwarya *et al.*, 2019
Pseudomonas resinovorans	*E. coli* BL 21(DE3)	Inducible (IPTG)	Mihooliya *et al.*, 2020
Anoxybacillus flavithermus	*E. coli* BL 21(DE3)	Inducible (IPTG)	Maqsood *et al.*, 2020

2.8 Submerged (SmF) and Solid state fermentation (SSF) processes

Submerged fermentation (SmF) for the production of L-asparaginase enzyme involves the growth of microbial cultures in liquid broths containing the desired production medium. The SmF is the most commonly utilized technique in which the whole process involves the production in closed vessels with careful monitoring of temperature, pH, and other necessary conditions required for optimal production of the enzyme (El-Naggar and El-Shweihy, 2020). These processes also have the advantages of easy scale-up operations and generally do not experience heat and mass transfer limitations. However, the submerged production processes also suffer from several drawbacks, including the lower concentration and higher dilution of the enzyme product, low yields, increased production costs, excessive generation of effluents, and disposal of wastewater in huge quantities (Mishra, 2006). Bacterial sources are mostly preferred under submerged conditions for the production of L-asparaginase enzyme. To reduce the production costs of L-asparaginase enzyme, various cost-effective nitrogen sources can be explored compared to costly yeast extract and peptones that are currently utilized for the submerged production of L-asparaginase.

SSF has emerged as a productive bioprocess method performed in lack or near absence of water content with the aim to provide an environment similar to natural conditions in which the desired microorganism exists. This is the major factor resulting in enhanced performance and increased product titres in SSF as compared to liquid fermentations (Pandey, 2003). However the substrate must supply adequate moisture essential to favor the growth and metabolic requirements of microorganisms. Other advantages associated with SSF include the higher concentration of end product, use of insoluble solid substrates, lower water and sterility requirements, lower catabolite repression, easier aeration, and higher stability of the product (Hölker *et al.*, 2004). Additionally several cases have been reported where the enzymes produced in SSF have a non-identical optimal temperature, pH stability and

dissimilar kinetic parameters in comparison to the same enzyme produced in submerged fermentation (Hölker *et al.*, 2005). Various physicochemical parameters must be regulated for the successful SSF fermentation of asparaginase enzyme. These include the selection of a suitable agro-industrial substrate as a source of nutrients, suitable microorganism for carrying out the process and optimization of various physicochemical and biochemical parameters. Table 2.4 list the SSF processes for asparaginase enzyme production using various fungal and bacterial sources.

Table 2.4: SSF processes for asparaginase enzyme production using various microbial sources.

Micro-organism	**Substrate**	**Enzyme Activity**	**Reference**
Pseudomonas aeruginosa	Soybean meal	-	El – Bessoumy *et al.*, 2004
Aspergillus niger	Bran of glycine max	40.9±3.35 U/gds	Mishra, 2006
Bacillus circulans	Red gram husk + L-asparagine	2322 U/gds	Hymavathi, *et al.*, 2006
Fusarium equiseti	Soybean meal	8.51 IU	Hosamani *et al.*, 2011
Cladosporium sp.	Wheat Bran	83.3 U	Kumar *et al.*, 2013
Serratia marcescens	Coconut oil cake	5.86 U/gds	Ghosh *et al.*, 2013
Fusarium culmorum	Soy bean meal	7.21 U/gds	Meghavarnam *et al.*, 2017

2.9 Cost-effective production of L-asparaginase enzyme

It has been reported in many studies that the production of L-asparaginase enzyme involves the utilization of glucose as a carbon source and yeast extract, peptone as nitrogen sources (Liu and Zajic, 1972; Verma *et al.*, 2007). The utilization of these sources makes the total production cost expensive in nature. So, there is certainly a need to lower down the expensive production costs of the L-asparaginase enzyme preparations. An excellent choice to lower the production costs is the exploration and utilization of cost-effective carbon and nitrogen sources for both the SmF and SSF production processes. For the submerged fermentation processes, low-cost soluble nitrogen sources need to be explored. The solid state fermentation is an attractive choice for the production of L-asparaginase enzyme using cheaper and nutrient-rich agro-industrial substrates in the near absence of water resulting in the formation of more concentrated products. Many previous studies on SSF have utilized different agro-industrial substrates in the form of soybean meal, wheat bran, coconut de-oiled cake, groundnut de-oiled cake and many other substrates for the production of L-asparaginase enzyme using fungal cultures (Meghavarnam and Jankiraman 2017; Kumar *et al.*, 2013; Ghosh *et al.*, 2013; Vala *et al.*, 2018; Mishra 2006).

2.10 Optimization approaches for enhancement of L-asparaginase production

2.10.1 Screening of process parameters using the Plackett Burman screening design

The first step involves screening the significant medium components or process parameters using the Plackett Burman (PB) screening design. Each factor is varied at 2 levels (high and low) in a PB design and is a widely utilized method with fewer experimental runs (Plackett and Burman, 1946). Only the specific process parameters that are having an influential effect on the production process are screened, while the rest of the parameters are eliminated. The output can be obtained in the form of a Pareto chart which is a diagrammatic approach to

visualize the results of Plackett Burman's screening design. It allows the identification of those factors that are majorly responsible for the design process.

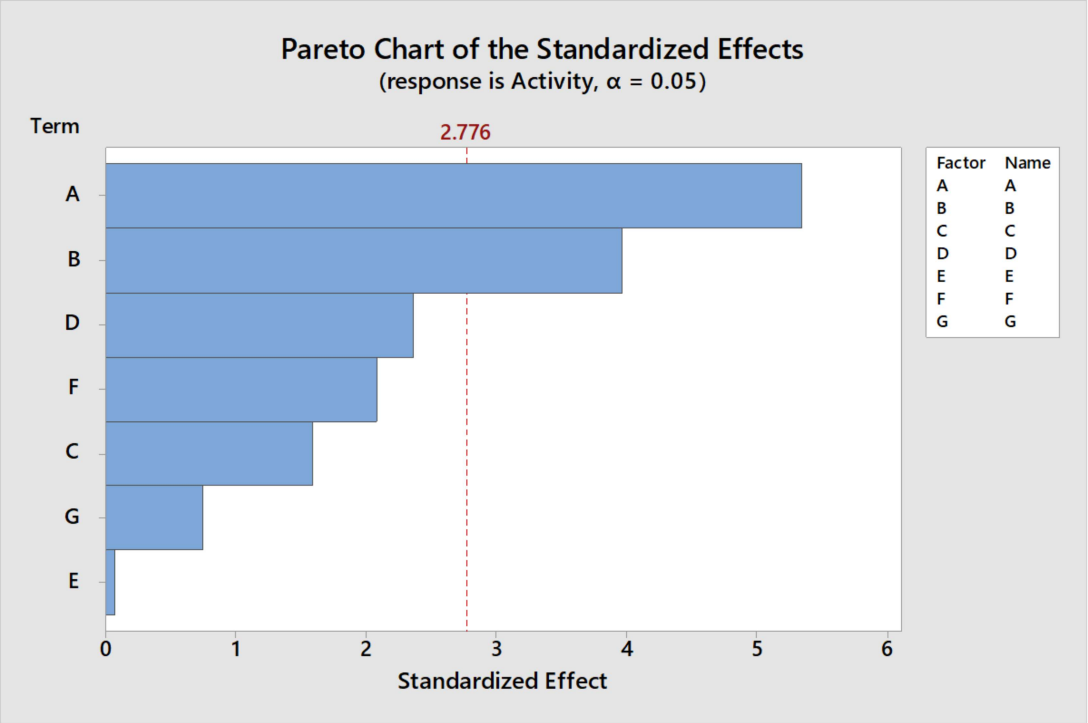

Figure 2.2: Pareto chart showing the significant and non-significant parameters obtained from the Plackett Burman screening design.

2.10.2 Optimization using Response surface methodology approach (RSM)

The response surface methodology is a statistical design of experiments approach that can be successfully utilized for finding out the optimum levels or the best conditions of the process variables under study. They are used to refine the levels of the important process parameters that are obtained after the initial screening design of experiments and are highly useful for experiments that involve curvature effects (Kalil *et al.*, 2000). There are two types of response surface experimental designs that are widely utilized for bioprocess optimization studies. A Central composite design (CCD) in which each process parameter is varied at five levels (-2, -1, 0, +1, +2) and a Box Behnken design in which each parameter was varied at

three levels (-1, 0, +1). Both these experimental designs generate second-order quadratic models and are used to predict the outcomes of the conditions at which no experimental runs are performed.

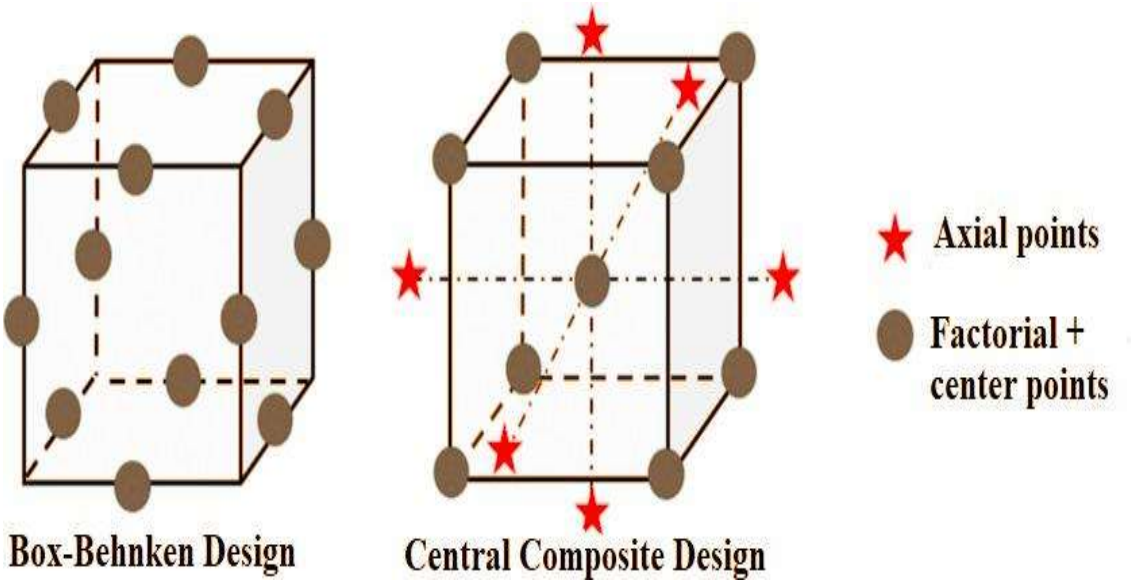

Figure 2.3: Schematic diagram of Response Surface Methodology (RSM) designs.

These models can be efficiently used to measure the variability (pure error) during the experiments. Moreover, these experimental designs have the ability to estimate the interaction effects among the various process parameters utilized during the optimization studies. A Box Behnken experimental design has fewer experimental runs than the central composite experimental design and is less expensive for the same number of process parameters. The results can be visualized in response surface plots or contour plots which are used to depict the overall range of each process parameter that is giving the desired response. Moreover the outcomes obtained during the experiments can be tested for their significance on the basis of their p-values and a p-value of < 0.05 is considered statistically significant. Thus, these major experimental designs can be efficiently used for optimization of bioprocesses with the aim to achieve higher yields and productivities and conducting fewer

experimental runs. These experimental designs can be implemented using the Minitab and Design-Expert statistical software packages.

2.10.3 Optimization using Artificial neural networks (ANN)

Recently, a novel methodology of machine learning using artificial neural networks (ANN) for non-linear multivariate modeling has been utilized to optimize process variables in several studies (Desai *et al.*, 2008; Haider *et al.*, 2008). The ANN architecture mimics the human brain learning process in which the information is passed through the network of interconnected neurons. The basic structure of ANN involves a collection of interconnected artificial neurons classified into three distinct categories: input layer neurons, output layer neurons, and hidden layer neurons.

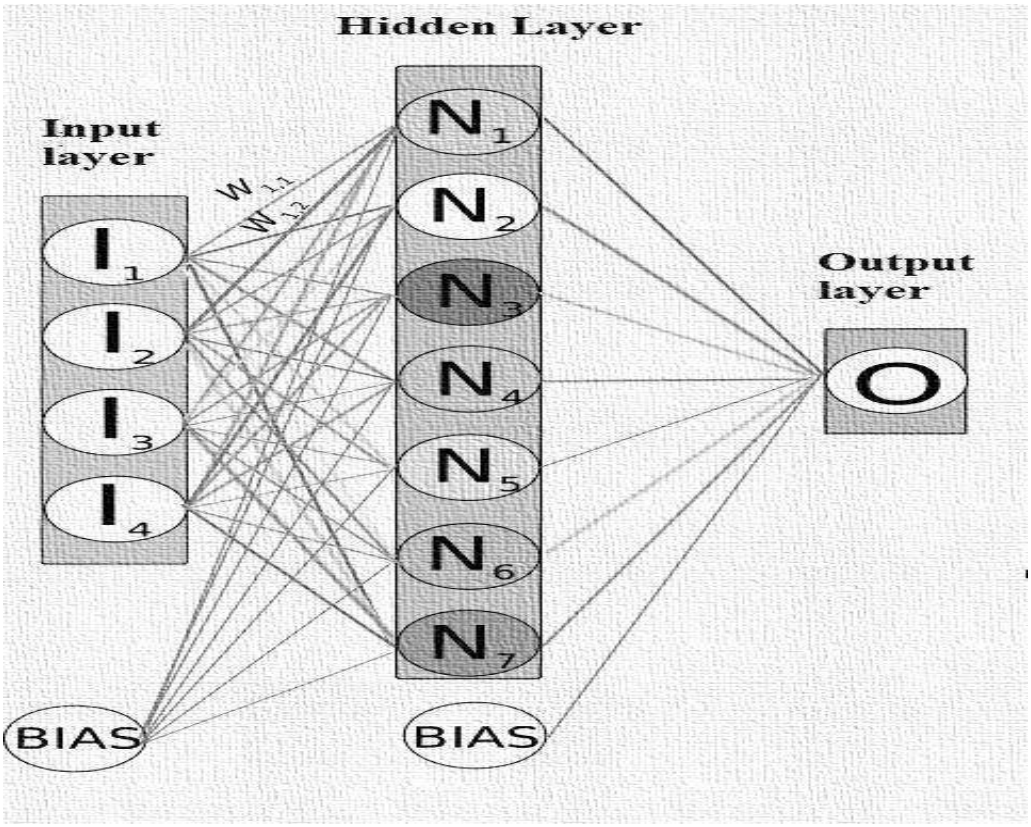

Figure 2.4: Schematic diagram of a neural network with four inputs and one output.

For fermentation optimization problems, feed forward neural networks are the most widely used as the transfer of information from input layer to output layer occurs only in a single direction. The first layer has the input variables or the various process parameters that need to be optimized. The output layer holds the things we want to predict using the neural network, like the predicted yield of L-asparaginase in our study. Thus, the input layer neurons present the input data, and the output layer neurons present the response to the input data.

The hidden layers are then used to connect the output and are optimized for the best fitting of the experimental data. Initially, the created neural network was trained by providing an experimental data set. The network then divided the data into three separate sets: training set, validation set and test set in the ratio of 70:15:15. The training data set represents the major portion of the input data and is used to make the neural network model learn the essential details from the input data. Then some data is utilized for validating the performance of the created model during the training and is known as the validation data. The test data set is mainly utilized for the testing of the ANN model after the training is completed. The training of the network is performed using a feed-forward neural network algorithm, and the results are obtained in the form of predicted values. During the training of created neural net, the training data set values are initialized with certain weights (w). These weights are then optimized during the training until the optimum weights are produced. These optimum weights are generated when the neural network is able to predict values close to the experimentally obtained values. After creating a successful neural network that predicts the output close to the experimental output values, an unknown data set values were then fed into the neural network to predict the response and the corresponding experimental runs were also performed on these unknown data set values. If the obtained experimental values were found to be close to the predicted values, then the accuracy and the effectiveness of the neural network are proved.

The ANN approach offers a significant edge over the previously used response surface methodology (RSM) models, as ANN can approximate all categorical types of non-linear functions. In contrast, RSM can only be used to solve quadratic functions (Santos *et al.*, 2017; Mukherjee *et al.*, 2019). Moreover, the ANN can efficiently learn from any data set with high accuracy. The output values generated using both the response surface methodology (RSM) and artificial neural network can be compared in terms of evaluation parameters such as root means squared error (RMSE), means squared error (MSE), and correlation coefficient.

2.11 Asparaginase Quantitative enzyme assays

2.11.1 Aspartyl β-hydroxamate (AHA) method

This method is based on the principle that L-asparaginase enzyme in the presence of hydroxylamine catalyzed the conversion of L-asparagine to Aspartyl β-hydroxamate (AHA). This AHA then reacts with the $FeCl_3$ to form a brown-coloured product, ferric-AHA that can be quantified spectrophotometrically at 500 nm wavelength. The standard curve is prepared by using multiple dilutions from a 5 mM stock solution of Aspartyl β-hydroxamate and the addition of appropriate amounts of $FeCl_3$/TCA/HCl solution, ranging from 0.01 to 3 µmol of ferric AHA mL^{-1} (Drainas *et al.*, 1977; Magri *et al.*, 2018). A unit of L-asparaginase activity (IU) corresponds to 1 µmol of AHA produced per minute at pH 8.6 and 37 °C.

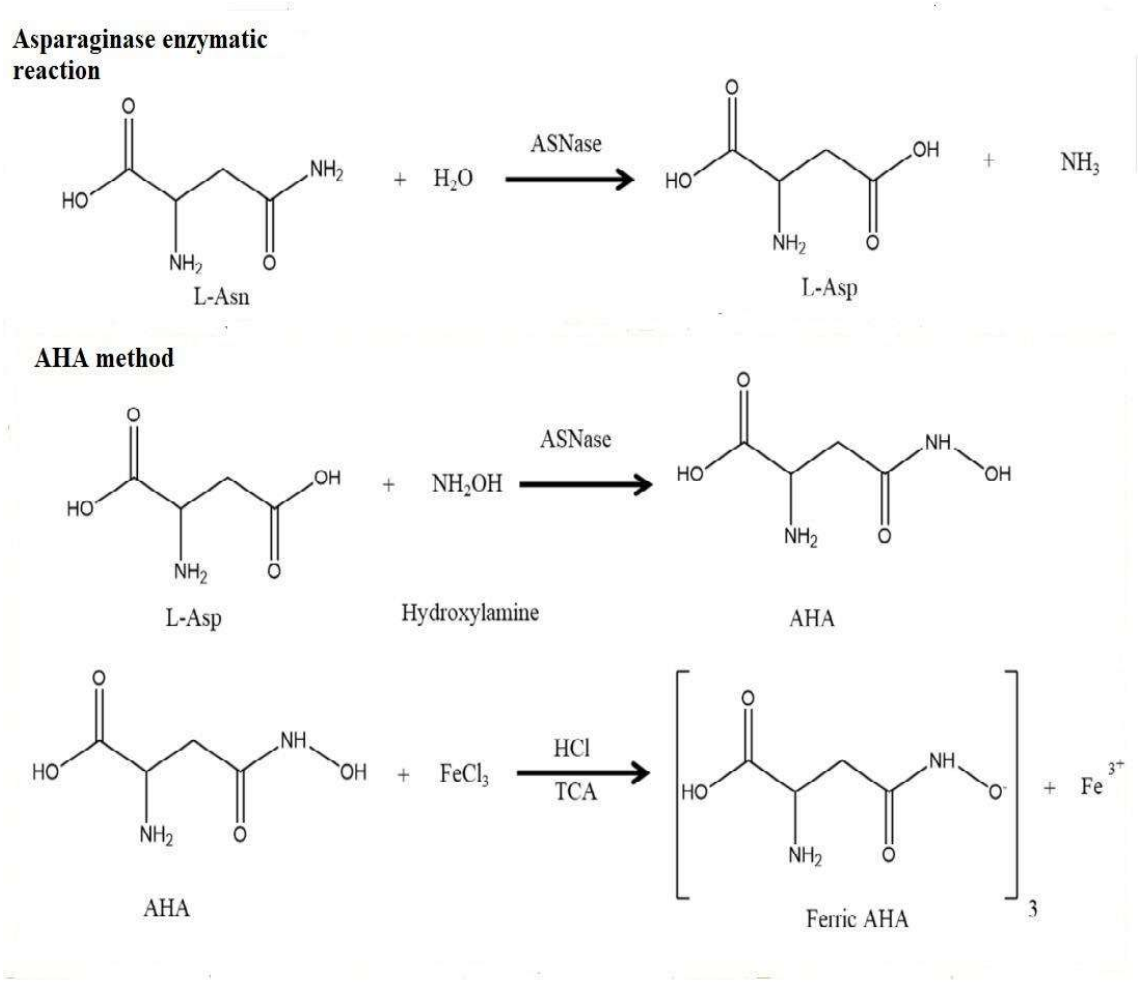

Figure 2.5: Aspartyl β-hydroxamate (AHA) method for quantitative enzyme estimation (Magri *et al*., 2018).

2.11.2 Nesslerization method

The nesslerization method is based on the detection of ammonia released by the catalytic reaction of L-asparaginase on the L-asparagine substrate. The quantification was based on the method in which the ammonia released was quantified spectrophotometrically at 436 nm wavelength (Shifrin *et al.*, 1974). The calibration curve was prepared through the reaction of the Nessler reagent with multiple dilutions of ammonium sulfate $(NH_4)_2SO_4$ stock solution. A

unit of L-asparaginase activity (IU) corresponds to 1 µmol of NH_4^+ produced per minute at pH 8.6 and 37°C.

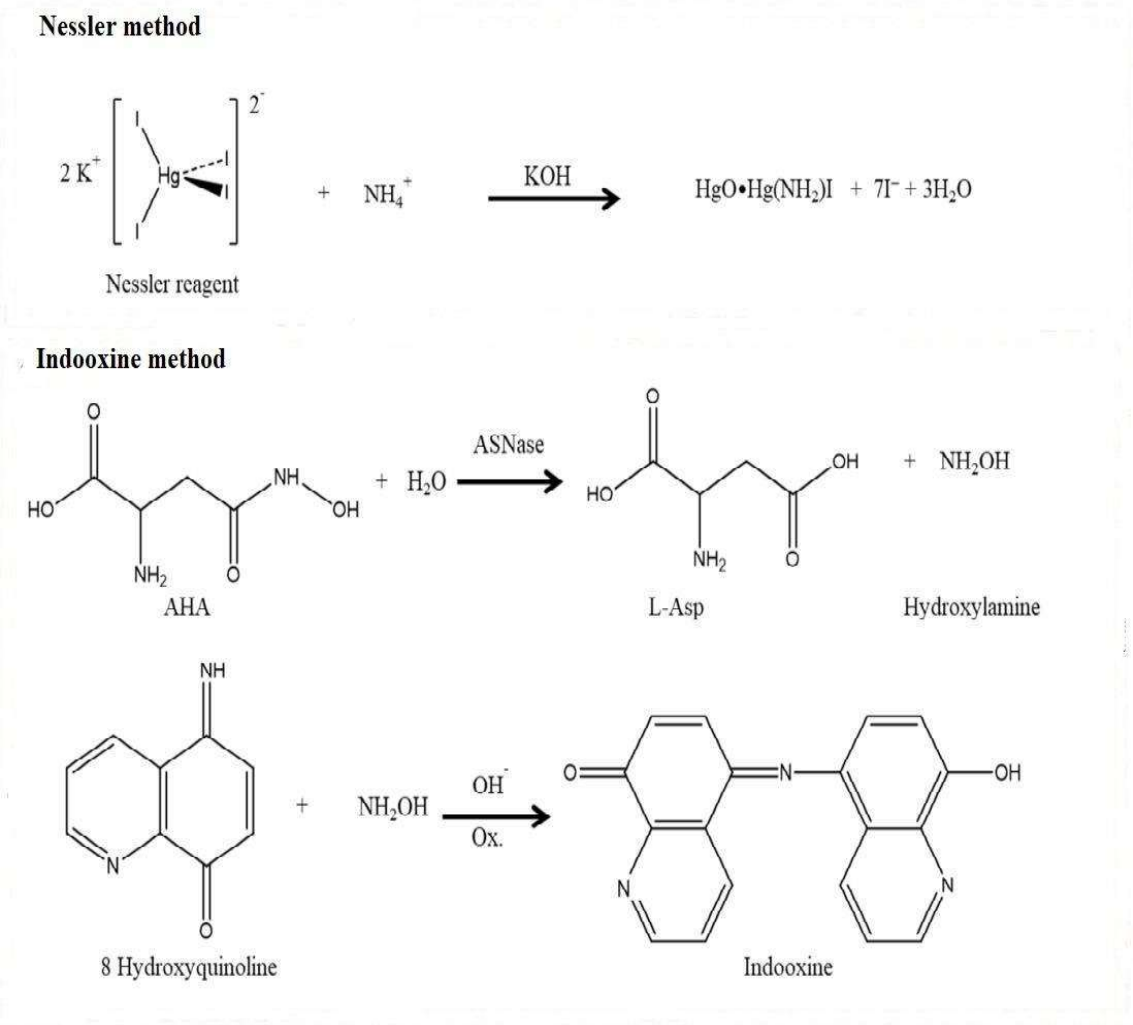

Figure 2.6: Nesslerization and indooxine method for quantitative enzyme estimation (Magri *et al*., 2018).

2.11.3 Indooxine method

The quantification using the Indooxine method relies on the utilization of aspartyl β-hydroxamate (AHA) as substrate (Lanvers *et al.*, 2002). The reaction mixture containing

AHA, Tris buffer, bovine serum albumin (BSA) and L-asparaginase enzyme is allowed to incubate for 10 minutes. After stopping the reaction using TCA solution, 8-hydroxyquinoline solution is added, and the absorbance of the solution containing the indooxine produced is measured using a plate reader at 710 nm. The calibration curve was constructed using green indooxine stock solution at 710 nm. A unit of L-asparaginase activity (IU) corresponds to 1 µmol of indooxine produced per minute at pH 7.3 and 37°C.

2.12 Purification procedures for L-asparaginase enzyme

The L-asparaginase enzyme is widely used for diverse applications as therapeutics and well as a food processing agent. Therefore, high purity of the enzyme is required for both applications. The purification of the L-asparaginase irrespective of the microbial source or the type of fermentation involved requires a combination of techniques in order to achieve the desired purification. Initially, precipitation techniques including both salt and solvent precipitation have been reported by several researchers. The precipitated enzyme is further subjected to diverse chromatography techniques performed in tandem for the complete purification to homogeneity. However, the increase in the number of purification techniques will lead to an additional and increased overall cost of the final L-asparaginase enzyme preparations. Moreover, the more the number of steps, the less will be the final enzyme yield or recovery obtained due to the enzyme losses at each purification process step. High purity of the enzyme is also required to assess the biochemical, kinetic properties of the enzyme and to determine the structural properties. Also, as the L-asparaginase enzyme has anti-cancer applications, appropriate and accurate dose requirements in terms of enzyme activity are an absolute requirement. Both the higher dose as well as the suboptimal dose will lead to inappropriate treatment in both in-vitro and in-vivo conditions.

Table 2.5: Purification procedures reported for the L-asparaginases from different sources.

S.No.	Microbial source	Purification steps	Yield (%)	Purification fold	Reference
1.	*Streptomyces albidoflavus*	$(NH_4)_2SO_4$ ppt.	72.4	4.7	Narayana *et al.*, 2008
		Sephadex G-100	43.5	23	
		CM- Sephadex C-50	40	99.3	
2.	*Streptomyces tendae*	$(NH_4)_2SO_4$ ppt.	-	-	Kavitha *et al.*, 2010
		Sephadex G-100	30.5	17.23	
		CM- Sephadex C-50	25.7	82.2	
3.	*Pectobacterium carotovorum*	$(NH_4)_2SO_4$ ppt.	70.15	6.86	Kumar *et al.*, 2011
		DEAE- cellulose	48.38	44.63	
		Sephadex G-100	42.05	72.12	
4.	*Penicillium digitatum*	$(NH_4)_2SO_4$ ppt.	43.32	0.78	Shrivastava *et al.*, 2012
		G-100 column	4.35	60.94	
5.	*Bacillus licheniformis*	Ultrafiltration	94.81	1.13	Mahajan *et al.*, 2014
		Acetone ppt.	82.06	1.85	
		DEAE- cellulose	49.28	18.09	
		Sephadex G-150	32.95	30.17	
6.	*Pseudomonas aeruginosa*	$(NH_4)_2SO_4$ ppt.	-	-	Badoei-Dalfard, 2015
		Q - Sepharose	-	-	
		Sephadex G-100	46	-	
7.	*Enterobacter cloacae*	$(NH_4)_2SO_4$ ppt.	68	6.19	Husain *et al.*, 2016
		DEAE- cellulose	47.42	57.67	
		Sephadex G-100	33	119.39	
8.	*Pseudomonas otidis*	$(NH_4)_2SO_4$ ppt.	66.96	6.15	Husain *et al.*, 2016
		DEAE- cellulose	53.56	75.78	
		Sephadex G-100	38.90	151.88	

S.No.	Microbial source	Purification steps	Yield (%)	Purification fold	Reference
9.	*Bacillus tequilensis*	$(NH_4)_2SO_4$ ppt.	22.67	3.52	Shakambari *et al.*, 2016
		Sephacryl S-200	19.5	13.79	
10.	*Aspergillus oryzae*	$(NH_4)_2SO_4$ ppt.	39	6.6	Dias *et al.*, 2016
		Q Sepharose	12	9.3	
		SP Sepharose	7	11.8	
		CM- Sepharose	6	28.6	
11.	*Bacillus megaterium*	$(NH_4)_2SO_4$ ppt.	60.14	4.21	Roy *et al.*, 2018
		DEAE-Sephacel	36.15	8.72	
		P-100 gel filtration column	29.05	31.52	
12.	*Bacillus velezensis*	$(NH_4)_2SO_4$ ppt.	83.53	1.29	Mostafa *et al.*, 2019
		Sephadex G-100	36.41	4.98	
13.	*Bacillus altitudinis*	Ultrafiltration	22.67	1.92	Prakash *et al.*, 2020
		Sephadex G-200	19.5	14.76	
14.	*Fusarium equiseti*	$(NH_4)_2SO_4$ ppt.	75	1.47	El-gendy *et al.*, 2021
		Q - Sepharose	63	2.07	
		Sephacryl 200	48	2.67	
15.	*Penicillium crustosum*	$(NH_4)_2SO_4$ ppt.	92.5	1.20	Khalil *et al.*, 2021
		DEAE- sephadex	70.13	4.07	
		Sephadex G-100	36.3	6.47	

It can be inferred from all the studies that the L-asparaginase enzyme was partially purified using the ammonium sulphate precipitation at 60-90 % saturation. After ammonium sulfate precipitation, most of the studies have utilized anion exchange chromatography for further purification of the enzyme.

2.13 L-asparaginase role in the virulence and survival of pathogenic microorganisms

Several recent studies have reported that certain pathogenic microorganisms specifically utilize the L-asparaginase enzyme to cause virulence and pathogenicity. These pathogenic microorganisms include *Mycobacterium tuberculosis* and *Salmonella typhimurium*. Recent studies have shown that the mycobacterial genome employs a single asparaginase that is crucial for survival in the multiple stress environments (Cole *et al.*, 1998). *Mycobacterium tuberculosis* hydrolyzes L-asparagine to L-aspartate using the enzyme L-asparaginase to assimilate nitrogen that can be further utilized to fulfill downstream metabolic requirements. Moreover, the hydrolysis results in release of ammonia that helps the microorganism cope with the acid-stress environment inside the mycobacterial phagosome. Thus L-asparaginase acts as a virulence factor and a crucial component in preventing acidification of phagosome, thereby enhancing the pathogenicity and the survival of the intracellular *Mycobacterium tuberculosis* pathogen inside the macrophages.

Similarly, it has been reported in a study that the pathogenic microorganism *Salmonella typhimurium* specifically utilizes the L-asparaginase-II enzyme to inhibit the T-cell responses of the host organism (Kullas *et al.*, 2012). The production of L-asparaginase enzyme by the pathogen is extremely crucial both for survival and for causing inhibition of T-cells. The specific removal of the L-asparagine by the enzyme L-asparaginase II of *Salmonella typhimurium* leads to the downregulation of TCR-β of the host cells.

The identification of critical role of L-asparaginase of these microorganisms has elucidated the reaction mechanism by which these pathogenic microorganisms can evade the immune cell responses and can establish the pathogenicity in the mammalian host. Also, with the emergence of multiple drug-resistant strains of these microorganisms, the enzyme L-asparaginase is a promising novel therapeutic drug target that can be targeted for selective

inhibition of many pathogenic organisms. The identification of the L-asparaginase enzyme as a drug target can also lead to the growth of novel therapeutics targeting the specific L-asparaginases from these pathogenic microorganisms.

Thus, it can be interpreted that L-asparaginase can act as a drug in some cases or as a drug target in others.