



Solid-State Fermentation vs Submerged Fermentation for the Production of L-Asparaginase

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Abstract

L-Asparaginase, an enzyme that catalyzes L-asparagine into aspartic acid and ammonia, has relevant applications in the pharmaceutical and food industry. So, this enzyme is used in the treatment of acute lymphoblastic leukemia, a malignant disorder in children. This enzyme is also able to reduce the amount of acrylamide found in carbohydrate-rich fried and baked foods which is carcinogenic to humans. The concentration of acrylamide in food can be reduced by deamination of asparagine using L-Asparaginase. L-Asparaginase is present in plants, animals, and microbes. Various microorganisms such as bacteria, yeast, and fungi are generally used for the production of L-Asparaginase as it is difficult to obtain the same from plants and animals. L-Asparaginase from bacteria

causes anaphylaxis and other abnormal sensitive reactions. To overcome this, eukaryotic organisms such as fungi can be used for the production of L-Asparaginase. L-Asparaginase can be produced either by solid-state fermentation (SSF) or by submerged fermentation (SmF). SSF is preferred over SmF as it is cost effective, eco-friendly and it delivers high yield of enzyme. SSF process utilizes agricultural and industrial wastes as solid substrate. The contamination level is substantially reduced in SSF through low moisture content. Current chapter will discuss in detail the chemistry and applications of L-Asparaginase enzyme and various methods available for the production of the enzyme, especially focusing on the advantages and limitations of SSF and SmF processes.



1. INTRODUCTION

L-Asparaginase is an enzyme found in a wide range of organisms including plants, microbes, animals, and in the serum of certain rodents but not in human beings. L-Asparaginase (E.C. 3.5.1.1) is an amidohydrolase, that catalyzes the hydrolysis of the amide group on the side chain of asparagine, an amino acid into aspartic acid and ammonia. This enzyme has relevant applications in the pharmaceutical and food industry. So, L-asparaginase has been reported to have tumor inhibitory properties and it is mainly used in the treatment of acute lymphoblastic leukemia (ALL). Normal cells can synthesize L-asparagine with the help of the enzyme asparagine synthetase, whereas certain sensitive malignant cells cannot synthesize it by itself and require an external source of L-asparagine for optimal growth. During the treatment of ALL with L-Asparaginase, all the circulating asparagine in the body of the patient get hydrolyzed to aspartic acid and ammonia preventing the absorption of asparagine by tumor cells and hence depriving the dependent tumor cells of their extracellular source of L-asparagine (Broome, 1963). L-Asparaginase is commonly used as a combination chemotherapy drug for the treatment of ALL in adults and children and non-Hodgkin's lymphoma in children. L-Asparaginase therapy may develop side effects such as anaphylaxis, coagulation abnormality, thrombosis, liver dysfunction, pancreatitis, hyperglycemia, and cerebral dysfunction. These side effects are developed either due to the production of anti-asparaginase antibody in the body or due to L-glutaminase activity of L-Asparaginase enzyme used (Haskell et al., 1969). This enzyme is also used in food industry to prevent the formation of acrylamide at high temperatures like frying or baking of food items containing starch.

L-Asparaginase is present in plants and mammals but in view of its difficult extraction, microorganisms are evaluated as potential sources of enzyme production (Arima, Sakamoto, Araki, & Tamura, 1972). L-Asparaginase production throughout the world is carried out either by submerged fermentation (SmF) or by solid-state fermentation (SSF). SSF is defined as the growth of microorganism on solid substrate which acts as an energy source in the absence of free flowing water (Moo-Young, Moreira, & Tengerdy, 1983). SSF is a substitute to SmF for the large-scale production of industrial enzymes. The solid substrates used in SSF are mainly agricultural or industrial wastes which are cheap and have resistance to contamination especially for the large-scale production of fungal enzymes. This chapter provides an overview of L-Asparaginase enzyme, its applications, production, and comparison of SSF over SmF for the enzyme production.

1.1 History of L-Asparaginase

In 1953, J.G. Kidd showed that transplanted lymphomas of mice and rat are repressed in vivo by repeated injections of guinea pig serum and found that some active constituent in serum is responsible for the selective necrosis of lymphoma cells (Kidd, 1953). Earlier in 1922, Clementi had observed that among mammals, guinea pig serum is unique and rich in L-Asparaginase (Clementi, 1922). Broome in 1961 went a step further and demonstrated that the enzyme L-Asparaginase present in guinea pig serum is responsible for inhibitory action on lymphoma 6C3HED cells (Broome, 1961). Furthermore, a major advancement resulted when Mashburn and Wriston in 1963 reported that asparaginase extracted from *Escherichia coli* could inhibit the growth of the tumor (Mashburn & Wriston, 1964).

Lymphatic tumor cells require huge amounts of L-asparagine to keep up with their rapid malignant growth and L-Asparaginase enzyme is focussed on the depletion of asparagine. Later several microbial species were studied and identified for the production of L-Asparaginase for clinical trials in ALL therapy. This mechanism was accepted as a promising approach for remission in leukemic patients (Hill et al., 1967).

1.2 Chemistry and Structural Aspects of L-Asparaginase

This enzyme can be generally classified into two groups, the bacterial-type and the plant-type L-Asparaginases, characterized by different structural and biochemical features. The bacterial-type enzymes are further grouped into type I and type II depending on their cellular localization and substrate specificity.

Type I includes cytosolic enzymes that exhibit low affinity for L-Asparaginase, whereas type II enzymes are localized in the periplasm and show considerably higher affinity for L-Asparaginase (Michalska & Jaskolski, 2006). These enzymes from various sources have been purified and its biochemical properties have been studied extensively over the last four decades. Type II asparaginase has a stable tetrameric structure composed of four identical subunits and each subunit contains 326 amino acid residues (Swain, Jaskolski, Housset, Rao, & Wlodawer, 1993). *E. coli* asparaginase has molecular weight of approximately 130 kDa and the affinity constants for L-asparagine and L-glutamine are 1.15×10^{-5} and 6.25×10^{-3} M, respectively. The isoelectric point of crystalline type varies from 4.8 to 5.6 (Ho, Frank, & Burck, 1969; Kumar & Sobha, 2012). Whereas the molecular weight of *Erwinia* L-Asparaginase is between 135 and 138 kDa and specific activity of the purified enzyme lies between 300 and 400 mol of the substrate per minute per milligram of protein. The isoelectric point ranges between pH 4.6 and 5.5 for *E. coli* enzyme, and is around 8.7 for the *Erwinia* enzyme (Warangkar & Khobragade, 2010).

1.3 L-Asparaginase Mechanism of Action

L-asparagine, a nonessential amino acid, is synthesized in human cells using L-asparagine synthetase. Tumor cells require huge amount of asparagine as an essential factor for their survival and growth. During the treatment of patients with ALL, L-Asparaginase that is injected (antineoplastic agent) into the body hydrolyzes L-asparagine into aspartic acid and ammonia leading to starvation of asparagine, resulting in the death of cancer cells (Broome, 1963). L-Asparaginase hydrolysis is known to proceed in two steps. In the first step a covalent intermediate, beta-acyl-enzyme intermediate is formed through nucleophilic attack by L-Asparaginase as shown in Fig. 1. In the

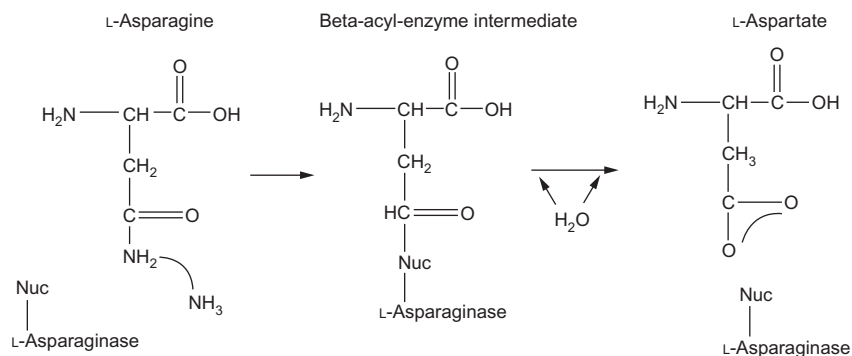


Fig. 1 Schematic illustration of hydrolysis of L-asparagine (Kumar & Sobha, 2012).

second step, a water molecule attacks the acyl-enzyme intermediate to produce L-aspartate and ammonia (Michalska & Jaskolski, 2006). Hydrolysis reaction is assayed by measuring the release of ammonia using Nessler's reagent or by measuring the release of L-aspartate. Catalytic mechanism of L-Asparaginase is similar to serine protease. The precise mechanism of L-Asparaginase action is still not clear because of its dual asparaginase and glutaminase activities (Gulati, Saxena, & Gupta, 1997; Wade, Robinson, & Phillips, 1971).

1.4 L-Asparaginase as a Therapeutic Agent and Its Toxic Effects

Current practice of L-Asparaginase chemotherapy is to inject the enzyme intravenously or intramuscularly to deplete L-Asparagine concentration in plasma, thus selectively distressing the malignant cells. Because of this mechanism L-Asparaginase is used in the treatment of a variety of lymphoproliferative disorders and lymphomas, particularly pediatric ALL (Piatkowska-Jakubas et al., 2008). Clinically, three asparaginase formulations are available, two from bacterial sources *Escherichia coli* (*E. coli* asparaginase) and *Erwinia chrysanthemi* (*Erwinia* asparaginase) and one is pegylated form of *E. coli* asparaginase (PEG-asparaginase). *E. coli* and *Erwinia* asparaginases have identical mechanisms of action but their kinetic properties are different, and patients sensitive to one drug have often shown tolerance to the other. *Eriwinia* asparaginase is recommended to patients having high allergic reactions (Muller & Boos, 1998). Available commercial forms of L-Asparaginase are listed in Table 1.

Table 1 Commercial Forms of L-Asparaginase

Source	Marketed By	Trade Name
<i>E. coli</i> asparaginase	EUSA Pharma, Oxford, UK	Kidrolase
	Ovation Pharmaceuticals, Deerfield, Illinois	Elspar
	Bayer AG, Leverkusen, Germany	Crasnitin
	Sanofi-Aventis, Paris, France	Leunase
	Kyowa Hakko, Tokyo, Japan	Asparaginase Medac
PEG-asparaginase	Sigma-Tau Pharmaceuticals, Inc. Gaithersburg, Maryland	Oncaspar
<i>Eriwinia</i> asparaginase	EUSA Pharma, UK	Erwinase

Unusual response and toxicity of L-Asparaginase has been a challenge to researchers who have done extensive studies over the past 40 years on clinical trials of oncolytic enzyme L-Asparaginase. L-Asparaginase influence toxicity mainly due to inhibition of protein synthesis or nucleotide synthesis that results in abnormal sensitive reactions. Depletion of L-asparagine results in increased levels of aspartic acid and glutamic acid causing central nervous system dysfunction and liver dysfunctions manifested by increased amounts of ammonia. Major side effects attributed to the administration of *E. coli* and *E. chrysanthemi* asparaginases are anaphylaxis, hyperglycemia, hypolipoproteinemia, hypoalbuminemia, coagulation abnormalities, azotemia, hepatotoxicity, and pancreatitis (Haskell et al., 1969).

Asparaginase-associated pancreatitis occurs rarely as side effect among pediatric patients. A patient with extranodal natural killer/T-cell lymphoma (ENKTL), nasal type, and the chronic hepatitis B virus (HBV) infection that received L-Asparaginase as part of a chemotherapy treatment, acute pancreatitis occurred and the patient failed to resist 72 h after completion of chemotherapy. Though L-Asparaginase has been demonstrated to be an effective drug for leukemic patients, it induces pancreatitis independently (Wu, Qu, Tan, Zhang, & Hu, 2014). Overall survival rate depends on the age, stage of disease, and performance status of the enzyme (Yong et al., 2009).

1.5 L-Asparaginase as Food Processing Agent

Acrylamide present in fried or baked starchy food products have attained worldwide concern as it is categorized as “probable human carcinogen” by International Agency for Research on Cancer. Formation of acrylamide in food is mainly the result of heat induced Maillard reaction (or non-enzymatic browning reaction) between the amino acid group of asparagine and the carbonyl group of reducing sugar which provides desirable flavor to the food. The increases in temperature and pH induce the formation of acrylamide (Friedman, 2003).

L-Asparaginase mitigates acrylamide formation in starchy food by selectively hydrolyzing asparagine to aspartic acid and ammonia without affecting other amino acids there by retaining food quality. Application of L-Asparaginase enzyme (2 U/g) successfully reduced acrylamide content by 90% in potato products which has high asparagine content (Ciesarová, Kiss, & Boegl, 2006).

Recombinant *Aspergillus oryzae* asparaginase was tested on different food products such as semisweet biscuits, ginger biscuits, crisp bread, French fries, and sliced potato chips, and was found that acrylamide content was reduced by 34–92% (Hendriksen, Kornbrust, Ostergaard, & Stringer, 2009).



2. L-ASPARAGINASE PRODUCTION FROM MICROBES

Microorganisms are considered as effective sources for the production of therapeutic enzymes since they are easy to manipulate. Broad range of microorganisms such as filamentous fungi, yeast, actinomycetes, and marine organisms has been isolated and screened for the production of L-Asparaginase to provide an alternative solution to immunological reactions caused by bacterial L-Asparaginase (Savitri, Asthana, & Azmi, 2003).

2.1 Bacterial L-Asparaginase

L-Asparaginase production from several bacterial sources is extensively studied as they are easy to manipulate. Numerous bacterial sp. were screened for the production of L-Asparaginase by Peterson and Ciegler and showed that four strains of *Erwinia aroideae* and one *Hydrogenomonas eutropha* showed higher L-Asparaginase activity (Peterson & Ciegler, 1969a, 1972). Bacterial L-Asparaginases are mainly used for the remission induction therapy of ALL. *E. coli* and *E. chrysanthemi* are clinically used for the treatment of ALL. L-Asparaginase derived from various bacteria sources differ in pH, stability, and affinity (Imada, Igarasi, Nakahama, & Isono, 1973) and are briefly summarized in Table 2. Hence approaches are made to find microbial sources that can reduce the allergic reactions and toxic effects of bacterial L-Asparaginase.

2.2 Fungal L-Asparaginase

In spite of numerous investigations on bacterial L-Asparaginase, treatment with this enzyme sometimes results in hypersensitive reactions such as anaphylactic shock. L-Asparaginase from isolated filamentous fungi, *Aspergillus terreus* showed a greater carcinostatic effect on static tumor (De-Angeli et al., 1970). Similar effect was observed with purified L-Asparaginase from deuteromycetes *Fusarium tricinctum* that also reduced lymphosarcoma in mice (Scheetz et al., 1971). Imada et al. observed that amidase activity is present in fungal strains, *Penicillium claviforme* and *P. expansum* (Imada et al., 1973).

Table 2 Various Microbial Sources of L-Asparaginase
References

Bacteria	
<i>E. coli</i>	Mashburn and Wriston (1964)
<i>Erwinia aroideae</i>	Peterson and Ciegler (1969b)
<i>Serratia marcescens</i>	Heinemann and Howard (1969)
<i>Erwinia carotovora</i>	Lee, Ross, Gustafson, Wroble, and Muschik (1986)
<i>Enterobacter aerogenes</i>	Mukherjee, Joeris, Riechel, and Scheper (1999)
<i>Enterobacter aerogenes</i>	Mukherjee, Majumdar, and Scheper (2000)
<i>Thermus thermophilus</i>	Pritsa and Kyriakidis (2001)
<i>Pseudomonas aeruginosa</i> 50071	Abdel-fattah and Olama (2002)
<i>Erwinia carotovora</i>	Kamble, Rao, Borkar, Khobragade, and Dawane (2006)
<i>Bacillus circulans</i> MTCC 8574	Hymavathi, Sathish, Rao, and Prakasham (2009)
<i>Serratia marcescens</i> SB08	Venil and Lakshmanaperumalsamy (2009)
<i>Bacillus</i> sp.	Moorthy, Ramalingam, Sumantha, and Shankaranaya (2010)
<i>Pectobacterium carotovorum</i> MTCC 1428	Kumar, Venkata Dasu, and Pakshirajan (2011)
<i>Serratia marcescens</i>	Ghosh, Murthy, Govindasamy, and Chandrasekaran (2013)
<i>Bacillus licheniformis</i>	Mahajan et al. (2014)
Fungi	
<i>Aspergillus terreus</i>	De-Angeli et al. (1970)
<i>Fusarium tricinctum</i>	Scheetz, Whelan, and Wriston (1971)
<i>Aspergillus niger</i>	Mishra (2006)
<i>Aspergillus terreus</i> MTCC 1782	Baskar and Renganathan (2009)
<i>Fusarium equiseti</i>	Hosamani and Kaliwal (2011)
<i>Penicillium</i> sp.	Patro and Gupta (2012)
<i>Penicillium digitatum</i>	Shrivastava, Khan, Shrivastav, Jain, and Singhal (2012)
<i>Cladosporium</i> sp.	Mohan Kumar and Manonmani (2013)

Table 2 Various Microbial Sources of L-Asparaginase—cont'd
References

Actinomycetes	
<i>Streptomyces albidoflavus</i>	Narayana, Kumar, and Vijayalakshmi (2008)
Marine actinomycetes	Basha, Rekha, Komala, and Ruby (2009)
<i>Streptomyces noursei</i> MTCC 10469	Dharmaraj (2011)
<i>Streptomyces</i> ABR2	Sudhir, Dave, Trivedi, and Subramanian (2012)

Later purified extracellular L-Asparaginase from *A. terreus* was conjugated with polyethylene glycol and evaluated for its antiproliferation activity. It was observed that L-Asparaginase from *A. terreus* showed anti proliferative effects on two leukemic cell lines, with a molecular weight similar to that of *E. coli* asparaginase with no glutaminase activity (Loureiro, Borges, Andrade, Tone, & Said, 2012). To reduce the toxic effects associated with bacterial L-Asparaginase, fungi is the best choice. Being eukaryotic and evolutionarily closer to human it can minimizes the chances of immunological reactions (Shrivastava et al., 2012). L-Asparaginase from fungal sources is summarized in Table 2.

2.3 Actinomycetes L-Asparaginase

Recently, the microorganisms focus has been shifted to marine microbes for the production of bioactive compounds. Marine biosphere is a potential source of actinomycetes from which various antibiotics and bioactive compounds can be derived. L-Asparaginase from marine actinomycetes showed cytotoxic effects on acute T-cell leukemia and myelogenous leukemia (Dhevagi & Poorani, 2006). Dharmaraj (2011) reported the production of L-Asparaginase from marine actinomycetes, and the purified enzyme showed a final specific activity of 78.88 IU/mg at pH 8. L-Asparaginase production from numerous actinomycetes such as *Streptomyces* ABR2 and *Streptomyces albidoflavus* have been explored and the sources are summarized in Table 2 (Narayana et al., 2008; Sudhir et al., 2012).



3. PRODUCTION OF L-ASPARAGINASE

Large-scale production of the enzyme involves control of typical parameters like pH, temperature, dissolved oxygen concentration, and

CO₂ evolution (Bascomb et al., 1975). For the commercial production of L-Asparaginase selection of strain and type of fermentation technique are crucial.

3.1 Production of L-Asparaginase by SmF

SmF is a process in which the growth of microorganisms takes place in liquid broth medium which is optimized with required nutrients to have a better cultivation of microorganisms. This involves growing carefully the selected microorganisms in closed reactor containing the fermentation medium and a high concentration of oxygen. SmF has well-established equipment that makes use of the existing microorganisms. Bacteria are commonly used as a source in this process as it requires high moisture content. Production of L-Asparaginase from various microbial sources using SmF is listed in Table 3. Fungal species such as *Aspergillus niger*, *A. terreus*, *A. tamarii*, *Fusarium*, and *Penicillium* are reported for L-Asparaginase production by SmF (Patro & Gupta, 2012), and studies on optimization of culture media and environment conditions in both batch and continuous SmF have been reported. Synthesis of L-Asparaginase hinge on type of the organism and various parameters such as pH of medium, temperature, nutrient composition, inoculation concentration, dissolved oxygen concentration, and fermentation time (Kumar & Sobha, 2012).

Production and purification of L-Asparaginase from *E. aroideae* NRRL B-138 by SmF-yielded enzyme with an activity of 275 IU/mL (Peterson & Ciegler, 1969b). Presence of L-Asparaginase in marine bacteria was screened and found positive (Mohapatra, Sani, & Banerjee, 1995). Production of L-Asparaginase was investigated using fungi *A. tamarii* and *A. terreus* and found that *A. terreus* showed the highest L-Asparaginase activity of 58 U/L when cultured in a 2% proline medium (Sarquis et al., 2004). L-Asparaginase production was studied using agricultural waste corn steep liquor as economical source (Zia, Bashir, Ahmed, & Iftikhar, 2013). To reduce immunological reactions from Bacterial L-Asparaginase *Saccharomyces cerevisiae* ASP3 gene was cloned and expressed in *Pichia pastoris*, and further SmF production of recombinant strain resulted in enzyme yield of 800 U/g per dry cell mass (Ferrara et al., 2006). SmF is extensively used for large-scale production of L-Asparaginase. A comparison of SSF and SmF shows that SSF provides high yield and constitutes an eco-friendly process that avoids environmental pollution. Main advantages and limitations for both types of processes are summarized in Table 4.

Table 3 Summary of Fermentation Conditions and Microbial Cultures for Production of L-Asparaginase Using SmF

Fermentation Medium (w/v)	Microorganism	Fermentation Parameters	L-Asparaginase Yield	Specific Activity	References
Autolyzed yeast extract	<i>Serratia marcescens</i> ATCC 60	Temperature 26°C pH 5.0	3.7 IU/mL		Heinemann and Howard (1969)
Tryptone 0.05; Glucose 0.1; Yeast 0.05	<i>Erwinia aroideae</i> NRRL B-138	Temperature 28°C, pH 7.0	1250 IU/g dry weight of cells		Peterson and Ciegler (1969b)
Peptone 4.0	<i>Streptomyces griseus</i> ATCC 10137	pH 8.5; batch	117 IU/L		DeJong (1972)
Tryptone 0.5; Yeast extract 0.3; NaCl 0.2; glucose 0.1; FeCl ₃ 2 µM; 1 mM MgCl ₂ ; 0.2 mM CaCl ₂	<i>Thermus thermophilus</i>	Temperature 70°C, pH 7.0	–	840 IU/mg protein	Pritsa and Kyriakidis (2001)
Proline medium 2	<i>Aspergillus terreus</i>	Temperature 30°C, pH 6.2	58.8 U/L		Sarquis, Oliveira, Santos, and Da Costa (2004)
Asparagine dextrose salt medium	<i>Streptomyces griseus</i> NIOT-VKMA29	Temperature 35°C, pH 8.0	56.78 IU/mL		Meena et al. (2015)

Table 4 Comparison of SSF and SmF for Enzyme Production

Advantages		Limitations	
Submerged Fermentation	Solid-State Fermentation	Submerged Fermentation	Solid-State Fermentation
Better heat and mass transfer can be achieved	Low water requirement, resistance to contamination	Complex in operation, low yield	Heat build up
Better diffusion of microorganism	Substrate are agricultural wastes	High energy consumption and cost intensive	Large-scale inoculums and difficult to control process parameters
Commercially available in large scale	High yield and product activity	High release of effluents	Difficulties in scale-up

3.2 Production of L-Asparaginase by SSF

L-Asparaginase is usually produced through SmF technique. But this method has several disadvantages such as low product yield, high cost, and the generation of large amounts of waste water effluent. SSF is extensively used in various processes such as bioremediation, biodetoxification of various hazardous compounds, production of various therapeutic enzymes and secondary metabolites, and as an effective alternative to SmF. SSF is defined as the process in which the growth of microorganism takes place on a solid substrate in the absence or presence of little water or with minimal moisture content. In this technique, solid substrate is considered as the only source for the microorganisms. SSF uses agricultural wastes and industrial residues that are cheap and readily available material as source of growth for microorganism for the production of low volume–high-cost products (Watanabe et al., 2002). L-Asparaginase was produced from marine actinomycetes by SSF and SmF and a comparison showed that L-Asparaginase with higher activity was isolated from SSF process (Basha et al., 2009). Substrates such as wheat bran, coconut oil cake, black sesame oil cake, soy bean meal, and sugar cane bagasse are commonly used for the SSF production of the enzyme as shown in Table 5. Mishra (2006) reported maximum production of L-Asparaginase from *A. niger* using *Glycine max* as substrate followed by that of *Phaseolus mungo* and *Cajanus cajan*. Many statistical methods have been reported for the optimization of the extraction process of L-Asparaginase from microbes. Estimation of nutritional and environmental factors is essential for the production of enzyme. Statistically designed and planned experiments screen and evaluate the response of different parameters which

Table 5 Summary of Microbial Cultures and Fermentation Conditions for Production of L-Asparaginase Using SSF

Substrate	Microorganism	Fermentation Parameters	Enzyme Activity	Specific Activity	References
Soy bean meal	<i>Pseudomonas aeruginosa</i> 50071	Temperature 37°C; pH 7.4; moisture content 40% (w/v)	142.18 IU		Abdel-fattah and Olama (2002)
Soy bean meal	<i>Pseudomonas aeruginosa</i> 50071	Temperature 37°C; pH 7.4; moisture content 50% (w/v)		1900 IU/mg	El-Bessoumy, Sarhan, and Mansour (2004)
Glycine max	<i>Aspergillus niger</i>	Temperature 30 ± 2°C; pH 6.5; moisture content 70% (w/v)	40.9 ± 3.35 U/g of dry substrate		Mishra (2006)
Red gram husk Bengal gram husk Ground nut cake Coconut oil cake	<i>B. circulans</i> (MTCC 8574)	Temperature 30°C; pH 7.0; moisture content 40% (w/v)	780 U/g of dry substrate 600 U/g of dry substrate 360 U/g of dry substrate 380 U/g of dry substrate		Hymavathi et al. (2009)
Groundnut oil cake	<i>Aspergillus terreus</i> MTCC 1782	Temperature 30°C; pH 6.2	30.35 IU/mL		Baskar and Renganathan (2009)
Rice bran with nutrients	<i>Serratia marcescens</i> SB08	Temperature 30°C; pH 7.0; moisture content 50% (w/v)	79.84 U/g		Venil and Lakshmanaperumalsamy (2009)
Soya bean meal	<i>Fusarium equiseti</i>	Initial moisture content 70%	8.51 IU		Hosamani and Kaliwal (2011)
Wheat bran	<i>Cladosporium</i> sp.	Moisture content of 58%; pH 5.8; temperature 30°C	3.74 U		Mohan Kumar, Ramasamy, and Manonmani (2013)
Coconut oil cake	<i>Serratia marcescens</i>	Initial moisture content of substrate 50%; temperature 35.5°C; pH 7.4	5.86 U/g of dry substrate		Ghosh et al. (2013)

enhances the enzyme production. L-Asparaginase production on wheat bran using *Cladosporium* sp. was studied with five experimental design variables like pH, temperature, moisture content, inoculum concentration, and time using response surface methodology giving an enzyme yield of 3.74 U (Mohan Kumar & Manonmani, 2013). L-Asparaginase production on sesame oil cake using *A. niger* C4 in a SSF column bioreactor was optimized for three variables (aeration rate, bed thickness, and temperature) which resulted in maximum L-Asparaginase yield of 310 U/gds (Uppuluri, Dasari, Sajja, Jacob, & Sri Rami Reddy, 2013). El-Bessoumy et al. produced this enzyme from *Pseudomonas aeruginosa* 50071 using SSF and purified enzyme 106 fold using ammonium sulfate fractionation followed by Sephadex gel filtration (El-Bessoumy et al., 2004).

Continuous efforts toward improving design and modeling characteristics led to the development of various bioreactors such as tray, packed bed, rotating drum, and fluidized bed. The limited use of SSF is due to its disadvantage when considering large-scale extraction and purification of the enzyme because the scaling up of SSF bioreactor is difficult due to heat and mass transfer problem in heterogeneous system.

3.3 Purification of L-Asparaginase

L-Asparaginase purification involves alkaline lysis, ammonium sulfate fractionation, ion-exchange, gel filtration, dialysis, and crystallization. *Erwinia* L-Asparaginase purification was achieved by cation-exchange chromatography which was further purified by affinity chromatography followed by dialysis. Several substantial purification was attained by cation-exchange chromatography as bacterial enzymes are acidic (Lee et al., 1986). To alleviate the side effects caused by glutaminase, L-Asparaginase free of glutaminase was purified through ammonium sulfate precipitation followed by DEAE cellulose column chromatography and Sephadex G-100 chromatography. Kinetic parameters, K_m and V_{max} of purified L-Asparaginase from *P. carotovorum* MTCC 1428, were 0.657 mM and 4.45 U/ μ g, respectively (Kumar et al., 2011). Usually, microbial L-Asparaginase shows activity at pH 7–9 but enzyme derived from *T. thermophilus* shows optimal activity at pH 9.2 and a K_m value of 2.8 mM (Pritsa & Kyriakidis, 2001). A lot of work has been done on the production and purification of L-Asparaginase from bacteria although the yield of bacterial enzyme is low. Cultivation method and main properties of the purified enzyme from various sources are summarized in Table 6. Fungal strain *Cladosporium* sp. cultivated on

Table 6 Cultivation Method, Purification Procedure and Properties of L-Asparaginase from Different Sources

Source	Cultivation Method	Purification Method	Properties of Purified L-Asparaginase	References
<i>Pseudomonas aeruginosa</i> 50071	Solid-state fermentation	Ammonium sulfate fractionation Sephadex G-100 gel filtration CM-Sephadex C50 ion-exchange chromatography	pH 9; Temperature 37°C; Specific activity 1900 IU/mg; Mr 160 kDa; K _m 0.147 mM; V _{max} 35.7 IU	El-Bessoumy et al. (2004)
<i>Erwinia carotovora</i>	Submerged fermentation	Ammonium sulfate precipitation DEAE-cellulose anion-exchange chromatography	pH 8.6; Temperature 35°C; Specific activity 3.1 U/mg	Kamble et al. (2006)
<i>Bacillus</i> sp.	Submerged fermentation	Ammonium sulfate precipitation: DEAE column	pH 7; Temperature 37°C; Mr 45 kDa	Moorthy et al. (2010)
<i>Enterobacter aerogenes</i>	Submerged fermentation	Gel filtration ultrafiltration	pH 8; Specific activity 55 IU/mg K _m 1.25 mmol/L	Mukherjee et al. (1999)
<i>Thermus thermophilus</i>	Submerged fermentation	Ammonium sulfate precipitation DEAE-52 column Phenyl Sepharose column, Cibacron Blue Sepharose column, Reactive Red Agarose column, Hydroxyl apatite column Heparin Sepharose column	pH 9.2; K _m 2.8 mM; Mr 200 kDa	Pritsa and Kyriakidis (2001)

Continued

Table 6 Cultivation Method, Purification Procedure and Properties of L-Asparaginase from Different Sources—cont'd

Source	Cultivation Method	Purification Method	Properties of Purified L-Asparaginase	References
<i>Streptomyces gulbargensis</i>	Submerged fermentation	Ammonium sulfate precipitation CM-Sephadex chromatography	pH 8.5; Temperature 40°C; Mr 85 kDa; Specific activity 2053 IU/mg	Amena, Vishalakshi, Prabhakar, Dayanand, and Lingappa (2010)
<i>Penicillium</i> sp.	Submerged fermentation	Ammonium sulfate precipitation: Sephadex G-100-120 gel filtration: ion-exchange chromatography	K_m 4×10^{-3} M	Patro and Gupta (2012)
<i>Penicillium digitatum</i>	Submerged fermentation	Ammonium sulfate precipitation Gel filtration chromatography	K_m 1×10^{-5} M	Shrivastava et al. (2012)
<i>Cladosporium</i> sp.	Solid-state fermentation	Methanol precipitation Ion-exchange chromatography, Size exclusion chromatography	V_{max} 4.44 μ mol/ mL/min; K_m 0.1 M	Mohan Kumar and Manonmani (2013)

wheat bran by SSF was purified with V_{max} of 4.44 μ mol/mL/min and K_m of 0.1 M indicating that the purified enzyme has greater affinity toward L-Asparagine ([Mohan Kumar et al., 2013](#)).

3.4 Immobilization of L-Asparaginase

Substrate affinity of the L-Asparaginase and the half-life of the enzyme are the two major factors that influence the tumor inhibitory effectiveness of L-Asparaginase ([Chang, 2013](#)). In order to limit the allergic reactions caused by L-Asparaginase, polyethylene glycol is conjugated to this enzyme at a site other than active site thereby allowing access of asparagine to the active site of the enzyme. This reduces the possibility of developing antibodies against

asparaginase and increases half-life of the enzyme. PEG L-Asparaginase administered at less frequent intervals and clinical trials of this enzyme have achieved remission with ALL (Keating, Holmes, Lerner, & Ho, 1993). To increase the half-life of the enzyme and to reduce the side effects caused by L-Asparaginase, it is often chemically modified by covalent conjugation to macromolecules or physically embedded with several biocompatible polymers to produce immobilized enzyme. L-Asparaginase covalently conjugated on the microparticles of sericin protein showed eight times lower K_m value than that of native L-Asparaginase and this bioconjugation also enhanced the enzyme stability (Zhang et al., 2004). Several attempts are made to covalently immobilize L-Asparaginase on to activated supports such as agarose-glutaraldehyde, dextran, and monomethoxypoly ethylene glycol to increase the half-life of the native enzyme (Balcão, Mateo, Fernández-Lafuente, Xavier Malcata, & Guisán, 2001; Baskar & Renganathan, 2009; Kamisaki et al., 1982).



4. CONCLUSION

In conclusion, L-Asparaginase from various microbial sources exhibits properties that acts as an antineoplastic agent and reduces acrylamide levels in food. Even though bacterial L-Asparaginase is clinically applied for treatment of ALL, it induces adverse reactions. For food and pharmaceutical industry applications, further studies to extract L-Asparaginase from novel sources such as eukaryotic organisms and marine sources should be explored in order to get an extracted enzyme with higher specificity towards L-asparagine and prolonged half-life. Media components and other process variables can be optimized by statistical design for improving the yield of L-Asparaginase. Further studies on L-Asparaginase production using SSF of agroindustrial waste and controlling the factors during synthesis should improve the yield of the enzyme.

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