VOLUME 10 NOMOR 2 DESEMBER 2023

ISSN 2548 – 611X

JURNAL
BIOTEKNOLOGI & BIOSAINS INDONESIA

Homepage Jurnal: http://ejournal.brin.go.id/JBBI/index

MICROBIAL L-ASPARAGINASES AND STRATEGIES TO IMPROVE THEM

L-Asparaginase dari Mikroorganisme dan Strategi untuk Mengembangkannya

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ABSTRACT

L-asparaginase is a type of hydrolase enzyme that has been used in anticancer treatment, mainly Acute Lymphoblastic Leukemia (ALL). L-asparaginase reduces the blood supply of L-asparagine needed by cancer cells to survive. The commercially approved L-asparaginase by the FDA originated from E. coli and E. chrysantemi. However, reports of immunogenic effects in more than 50% of cases due to the use of these enzymes have become the driving force for the need to explore other sources of L-asparaginase. In this review, various alternative sources of L-asparaginase other than these two microbes will be explained. Microbes from the group of Gram-positive bacteria, actinomycetes, and fungi produce L-asparaginase with a higher affinity for L-asparagine than L-glutamine. Protein engineering is an alternative strategy to produce L-asparaginase that is not recognized by antibodies to reduce the immune reaction. Besides, the fermentation process also needs to be considered to determine the appropriate substrate and bioprocess system to obtain the enzyme.

Keywords: *Anticancer, E. coli, E. chrysantemi, L-asparaginase, L-glutaminase*

ABSTRAK

L-asparaginase adalah enzim hidrolase yang digunakan sebagai agen terapeutik dalam pengobatan antikanker, khususnya Acute Lymphoblastic Leukemia (ALL). Pengobatan dengan L-asparaginase akan mengurangi pasokan L-asparagin dalam darah yang dibutuhkan oleh sel kanker, sheinbga secara selektif akan membunuh sel kanker. Kebutuhan akan L-asparaginase sangat tinggi, sedangkan sumber enzim yang telah disetujui oleh FDA berasal dari *E. coli* dan *E. chrysantemi*. Namun laporan adanya efek imunogenik hingga lebih dari 50% kasus karena efek raksi imun akibat penggunaan enzim tersebut menjadi pendorong dibutuhkannya eksplorasi sumber L-asparaginase yang lain. Dalam review ini akan dipaparkan berbagai sumber alternatif dalam memproduksi L-asparaginase selain kedua mikroba tersebut. Mikroba dari kelompok bakteri Gram-positif, aktinomisetes, dan fungi memiliki karakter L-asparaginase dengan afinitas terhadap L-asparagine yang lebih tinggi daripada L-glutamine. Rekayasa protein dari L-asparaginase juga menjadi satu alternatif untuk menghasilkan L-asparaginase yang tidak dikenali oleh antibodi sehingga akan menurunkan reaksi imun pada tubuh pasien. Selain itu, proses fermentasi juga peru diperhatikan untuk menentukan substrat, sistem bioproses, serta kondisi optimum yang sesuai.

Kata Kunci: Antikanker, *E. coli, E. chrysantemi,* L-asparaginase, L-glutaminase

***Received**: 10 November 2023* ***Accepted**: 21 December 2023* ***Published**: 29 December 2023*

INTRODUCTION

L-asparagine aminohydrolase known as L-asparaginase (E.C.3.5.1.1) is a hydrolysis enzyme that converts L-asparagine into aspartic acid and ammonia (Ameen et al. 2020). This enzyme is one of the most important anticancer drugs widely used since first found in the 1960s and commercially introduced in the 1970s in the treatment of Acute Lymphoblastic Leukemia (ALL), a type of cancer cell that attacks lymphoblast / lymphoid progenitor cells (Barros et al. 2021). This type of cancer is mainly found in children under 15 years and is the highest among children aged 2-3 years (Egler et al., 2016).

L-asparagine is crucial for the metabolic cycle and without this amino acid, the cells will undergo apoptosis (Barros et al. 2021). A normal cell can produce L-asparagine by converting L-aspartate into L-asparagine with the help of L-asparagine synthetase (Lomelino et al. 2017). While cancer cells are insufficient of L-asparagine synthetase, they rely on extracellular L-asparagine available in the plasma. As an anticancer agent, L-asparaginase hydrolyzes L-asparagine in the blood plasma, which malignant cells need to survive (Aisha et al., 2022; Schnuchel et al., 2023). Following deprivation of L-asparagine in the blood, cancer cells will lead to death (Fig. 1).

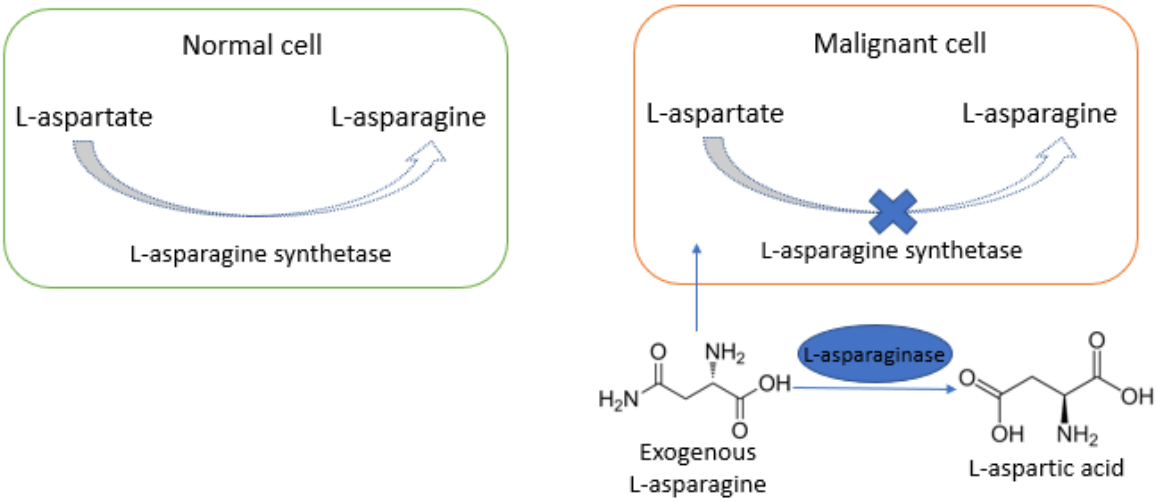

Figure 1. Healthy cells can produce their L-asparagine, while malignant cell needs exogenous L-asparagine to survive. L-asparagine hydrolysis by L-asparaginase will selectively attack cancer cells only.

The high need for L-asparaginase comprises 40% of the total world's enzyme demand, making this enzyme one of the three anticancer compounds widely used (Cachumba et al., 2016; Castro et al., 2021). Commercially available L-asparaginase and approved by the FDA obtained from Gram-negative bacteria *Escherichia coli* and *Erwinia chrysantemi* in various formulations. L-asparaginase from *E. coli* in its original form and PEG-modified are served under the names Elspar®, Kolase®, and Paronal®, while *E. chrysantemi* L-asparaginase named Erwinase® (Darnal et al., 2023; Effer et al., 2020; van den Berg, 2011).

Despite the significant use of L-asparaginase in patients, immunogenic effects from this treatment were reported in more than 50% of cases such as anaphylaxis, coagulation, renal impairment, thrombosis, glycemia, and pancreatitis (Mahajan et al. 2014; Doriya and Kumar 2016). The main causes of these cases were the use of Gram-negative bacteria as L-asparaginase producers and the presence of L-glutaminase activity in the enzyme. *E. coli* and *E. chrysantemi* along with other Gram-negative bacteria contain lipopolysaccharide (LPS) in the cell membrane that also known as bacterial endotoxin. The release of endotoxin is

constant from live bacteria and much higher at cell lysis(Sheehan et al. 2022). As cell lysis is crucial in enzyme purification and recovery, L-asparaginase preparation with free of any impurities and fewer adverse effects becomes more critical (El-Naggar et al. 2018). In addition, L-asparaginase possesses L-glutaminase activity that breaks down L-glutaminase alongside L-asparaginase. This led to the increase of ammonia concentration in blood as the result of amino acid hydrolysis (Patel et al. 2022; Sánchez-Moguel et al. 2023).

L-asparaginase inhibitory characteristic was first uncovered from guinea pig blood serum back in 1953 (Silva de Sousa et al., 2023). Now L-asparaginase is commonly found in various cells, including plants, animals, and yeasts. However, microorganisms still become preferable host cells to produce a wide variety of proteins as they are relatively easy to grow and obtain the product.

Over the years, various studies have been conducted to find L-asparaginase with a higher affinity for L-asparagine, higher availability, and fewer side effects on patients. Apart from *E. coli* and *E. chrysantemi,* various microorganisms potentially produce L-asparaginase with better characteristics. However, none of them have reached large-scale production. In this review, we summarize various sources of L-asparaginase from microbial producers. Several strategies like genetic and protein engineering of the target protein and optimization of the bioprocess system including medium composition are also significant in enhancing L-asparaginase yield.

Microbial Sources of L-Asparaginase

Microorganisms have become a favorable agent to produce proteins, enzymes, and other target compounds as they tend to be easier to grow and culture in large quantities. Besides, genetic manipulations can be done as several microorganisms are well identified. Microbial enzyme also tends to be more stable compared to plant and animal sources and have more diverse properties (Gopinath et al. 2013).

Two Gram-negative bacteria were considered safe and commercially approved by the FDA as L-asparaginase producers; *E. coli* and *E. chrysantemi.* These two microorganisms generated type I and type II L-asparaginase. Coded by *ansA,* type I L-asparaginase is a homodimeric enzyme that is produced in cell cytoplasmic and has a lower affinity to L-asparaginase. Meanwhile, type II L-asparaginase is a homotetrameric enzyme coded by *ansB*, a type of periplasmic protein, and has a higher affinity to L-asparaginase as a substrate (Micu et al., 2020; Sharafi et al., 2017). This led to a trivial challenge as periplasmic protein and cytoplasmic protein required different methods to be extracted so they do not contaminate each other. Hence, type II L-asparaginase is more frequently used as a therapeutic agent than type I L-asparaginase. In addition, *E. chrysantemi* L-asparaginase is known to be less toxic compared to *E. coli* L-asparaginase in terms of allergic reactions in patients (Qeshmi et al. 2018)

Aside from *E. coli* and *E. chrysantemi,* other microorganisms have been identified to produce L-asparaginase (Table 1). Some Gram-positive bacteria from the *Bacillus* group such as *B. licheniformis* and *B. velezensis* exhibited no or lower glutaminase activity. In addition, L-asparaginase produced by Gram-positive bacteria was dominated by type II extracellular L-asparaginase. In comparison, L-asparaginase-producing Gram-negative bacteria mainly retained the protein in the periplasmic area. While Gram-positive bacteria lack a periplasmic region, the protein will be secreted into the medium (Vimal and Kumar 2017). This gives the advantage of utilizing Gram-positive bacteria as an L-asparaginase producer to reduce economic costs in the industrial process.

Table 1. L-asparaginase sources from various microorganisms

Microorganisms	Enzyme Characteristics	References
E. coli MF-107	A 37 kDa L-asparaginase that is stable at a pH range of 7.5 to 8 at 35°C. Exhibit cytotoxic activity with IC_{50} toward MCF-7 at 5.7 IU/mL	(Shahnazari et al. 2022)

Microorganisms	Enzyme Characteristics	References
Pseudomonas sp. PCH199	A 37 kDa periplasmic L-asparaginase at pH 8.5 and temperature 60°C. Exhibit cytotoxic activity toward K562 cell line.	(Darnal et al. 2023)
Pseudomonas pseudoalcaligenes strain JHS-71	Produce up to 240 U/mL of L-asparaginase activity and increased in the presence of Co^{2+}	(Badoei-Dalfard 2016)
Bacillus sp.	5.9 times lower glutaminase activity compared to L-asparaginase activity and reached IC_{50} toward the MCF-7 cell line at 21µg/mL	(Roodposhti et al. 2023)
Streptomyces brollosae NEAE-115	Free glutaminase activity of L-asparaginase with a molecular weight of 67 kDa. Active in pH range from 4.5 to 10.5 with maximum activity at 37°C.	(El-Naggar et al. 2019)
Bacillus licheniformis	No glutaminase activity and endotoxin-free L-asparaginase approximately 37 kDa in molecular size. Stable at pH range 7.5 – 8.5, at 37°C	(Alrumman et al. 2019)
Bacillus velezensis	A 39.7 kDa of free glutaminase L-asparaginase with optimum activity at 37°C pH 7.5	(Mostafa et al. 2019)
Nocardiopsis alba NIOT-VKMA08	33 kDa L-asparaginase with no glutaminase activity. Optimum L-asparaginase activity (12.91 U/mL) recorded at 37°C pH 7 – 9	(Meena et al. 2015)
Bacillus australimaris NJB19	A 42.59 kDa of type II extracellular free glutaminase L-asparaginase	(Chakravarty et al. 2021)
Streptomyces ginsengisoli	An extracellular L-asparaginase with 3.23 µmol/mL/min of production rate optimum at pH 8 and temperature 30°C	(Deshpande et al. 2014)
Aspergillus flavus	An extracellular L-asparaginase with 112.67 U/100 mL of enzyme activity optimum at pH 6 and ammonium sulphate as nitrogen source	(Shuker 2019)
Leucosporidium muscorum CRM 1648	490.41 U/mL of L-asparaginase activity in 12-15°C and >25% of salinity	(Freire et al. 2021)
Talaromyces pinophilus	A heterodimer of 62.0 kDa and 39.0 kDa subunits with 120U/mL of L-asparaginase activity and 16% of glutaminase activity	(Krishnapura and Belur 2016; Krishnapura and Belur 2020)
Colletotrichum gloeosporioides	Tetramer L-asparaginase with 25 kDa of monomeric units. Pure L-asparaginase shows significant cytotoxic activity against Jurkat leukemic cells and H103 oral carcinoma cells.	(Yap et al. 2021)

Another Gram-positive bacteria that is widely known to produce secondary metabolites is actinomycetes, a type of filamentous Gram-positive bacteria. *Streptomyces* is the most abundant and widely known to produce several bioactive compounds. In this case, *Streptomyces is* also a dominant group known to generate L-asparaginase (Mangamuri et al. 2017; Selim et al. 2021). *S. brollosae* NEAE-15 yielded 87.81 U/mL of pure L-asparaginase at pH 37 °C with a higher cytotoxic effect towards Ehrlich ascites carcinoma cells (EAC) compared to commercial L-asparaginase (El-Naggar et al. 2018) *S. thermoluteus* especially subsp. *fuscus* NBRC 14270 and *S. griseus* were known to exhibit L-asparaginase and about 100-fold lower L-glutaminase than L-asparaginase activities. The low similarity in L-asparaginase sequence to both *E. coli* and *Erwinia* also indicates the distinct immunology effect (Hatanaka et al. 2011).

Fungi and yeast are eukaryotic cells that can express more complex proteins in terms of protein maturation and post-translational modifications (Garvey 2022). This capability of fungi and yeast cells makes it frequently used as a model to study mammalian cells. As an alternative for L-asparaginase producers, the less toxic and low immunogenic effect of the protein can be achieved using fungi or yeast (Freitas et al. 2021).

Not limited to the above, extremophile microorganisms have also been reported to produce L-asparaginase. *Pyrococcus abyssi,* a type of Archaea that can produce L-asparaginase in high temperatures up to 80°C (Nadeem et al. 2021). *Streptomyces fradiae* NEAE-82 produced L-asparaginase that reached optimum activity at pH 8.5 (El-Naggar et al. 2016). Other reports an extraordinary microorganism that can produce L-asparaginase, namely *Leucosporidium muscorum* CRM 1648, a type of yeast isolated from Antarctic marine sediment that can thrive on 25% of salinity exhibited 490.41 U/mL of L-asparaginase activity in 12-15°C (Freire et al. 2021). Although many different microbes are known to express L-asparaginase, up until now L-asparaginase production in the industry has only been limited to *E. coli* and *E. chrysantemi* platforms.

Recombinant L-Asparaginase

Microbial Host for Recombinant L-asparaginase

As previously described, the immunogenic effect on the treatment of L-asparaginase derived from bacterial endotoxin and L-glutaminase activity of the enzyme (El-Naggar et al. 2018). Several strategies including genetic and protein engineering approaches in L-asparaginase development have been investigated widely to offer less toxicity and increase the stability of the enzyme. This can be done by introducing a foreign gene encoding L-asparaginase into suitable microbial hosts (Table 2).

Table 2. Recombinant microbial L-asparaginase

Sources of L-asparaginase	Modified Gene	Microbial Hosts	Enzyme characteristics	References
Pseudomonas sp. PCH199	Pg-asn II gene fused with Histidine tag and cloned into pET-47b(+) with *Sac*I and *Xho*I restriction sites	*E. coli* BL21 (DE3)	A 37.0 kDa of purified enzyme monomer with 75.8 U/mg of specific activity. Stable in the presence of metal ions (Cu^{2+}, Zn^{2+}, K^{+}, Co^{2+}, Ca^{2+}, and Na^{+}) and SDS.	(Kumar et al. 2022)
Aliivibrio fischeri	L-ASNase type II (*ansB* gene) cloned in pBS0E under PxylA promoter with *Eco*RI and *Pst*I restriction sites	*B. subtilis* KO7	A 37.5 kDA of enzyme subunit with affinity towards L-asparagine with optimum pH at 7.5 and 1.539 U/mL of enzymatic activity. Stable at 25°C and 37°C.	(Bento et al. 2022)
Anoxybacillus flavithermus	L-ASNase encoded gene from cloned into pET-22b(+) with *Nde*I and *Xho*I restriction sites	*E. coli* BL21 (DE3)-RIL	A 38.0 kDa of enzyme subunit with a specific activity of 165 U/mg. Wide thermostable enzyme between 30-90°C.	(Maqsood et al. 2020)
Bacillus cereus BDRD-ST26	L-ASNase gene fused with amyE signal peptide and cloned into pP43NMK plasmid under P43 promoter with *Kpn*I and *Pst*I restriction sites	*B. subtilis* WB600	An extracellular L-asparaginase with a monomeric molecular mass of 35.5 kDa. Yielded 374.9 U/mL of enzyme activity and 550.8 U/mg of specific activity towards L-asparagine.	(Feng et al. 2019)

Sources of L-asparaginase	Modified Gene	Microbial Hosts	Enzyme characteristics	References
Bacillus sonorensis	L-ASNase gene cloned into pET28a(+) with *Eco*RI and *Bam*HI restriction sites	*E. coli* BL21 (DE3) pLysS	A 36 kDa of cytosolic enzyme was obtained with optimum activity at 45°C, pH 7, and low glutaminase activity	(Aly et al. 2020)
Bacillus SL-1	ansA1 gene encoded L-asparaginase cloned into pET22b(+) with *Xba*I and *Sal*I restriction sites	*E. coli* Origami™ B	A 37.0 kDa of thermostable enzyme subunit and wide range of pH 4.5-10	(Safary et al. 2019)
Pyrococcus abyssi	L-ASNase I gene cloned into pET28a(+) with *Nco*I and *Bam*HI restriction sites	*E. coli* BL21 (DE3)	A 33 kDa of recombinant enzyme with optimum temperature at 80°C, pH 8 and exhibited 1175 U/mg of specific activity.	(Nadeem et al. 2021)
Penicillium sizovae	Synthetic partial L-ASNase gene cloned into pPICZα under P_{AOX1} promoter	*Komagataella phaffii* X-33 yeast strain Mut^+	A 37.0 kDa of enzyme subunit with 3.05 U/g cell of L-asparaginase activity, 5 times higher than the native enzyme.	(Freitas et al. 2021)
Aspergillus terreus	Reverse transcripted cDNA of L-ASNase cloned into pET-28a(+) with EcoRI and HindIII restriction enzymes	*E. coli* BL21 (DE3) PlysS	A 42.0 kDa of enzyme subunit with 42.46 U/mg of activity towards L-asparagine in soluble cytoplasmic fraction.	(Saeed et al. 2018)

E. coli still become the most favorable host for protein recombinant production as they are already well known, fast-growing, and relatively produce large quantities of product compared to plant and animal tissues (Rosano & Ceccarelli, 2014). L-asparaginase genes that have been cloned in recombinant *E. coli* might be derived from *Bacillus, Pseudomonas, Penicillin,* and *Aspergillus* (Feng et al. 2019; Aly et al. 2020; Freitas et al. 2021; Kumar et al. 2022). Commonly used recombinant protein production is using *E. coli* BL21 (DE3) as a microbial host and IPTG as an inducer under the lac operon system (Barros et al., 2021). However, IPTG is considered too expensive as an inducer for recombinant protein production on an industrial scale which covers 10% of the unit production cost (Ferreira et al. 2018)

Bacillus subtilis is an alternative microbial host for recombinant protein production as they are considered generally recognized as safe (GRAS) and have stable expression systems (Su et al. 2020). As part of Gram-positive bacteria, *B. subtilis* does not have lipopolysaccharide (LPS) which is considered an endotoxin in its cell wall and trigger an immune response in human (Wang and Quinn 2010). In addition, they can secrete recombinant protein into the media and eliminate the need to break the cells. Xylose can be used as an inducer to produce recombinant protein in *B. subtilis* under the PxylA promoter system and gives a higher expression level compared to the IPTG induction system (Bento et al. 2022).

Yeast is a eukaryotic cell that is feasible to use as a protein expression host. However, the utilization of yeast as an expression host is not as common as *E. coli*. As eukaryotic cells, yeast can undergo protein folding and post-translational modifications and thus become standard tools in protein engineering (Karbalaei et al. 2020). *Saccharomyces cerevisiae* and *Pichia pastoris* previously known as *Komagataella phaffii* are the two most used yeast in recombinant protein production. As a methylotrophic yeast, *P. pastoris* can consume sugar and methanol as sources of carbon and energy. Under the AOX promoter,

they will produce the recombinant protein if glycerol is depleted and methanol is added (Freitas et al., 2022).

Erwinase, a type of PEGylated formulation of L-asparaginase that has been authorized by the FDA was made to prevent the recognition by the immune system. However, antibodies against PEG will be generated simultaneously (Kolate et al. 2014). *Pichia pastoris* became the alternative to produce glycosylated L-asparaginase, a more organic molecule than synthetic PEG, and produced active monomer and tetrameric glycosylated L-asparaginase (Effer et al. 2020)

Although other microbial hosts were available as a host of choice for recombinant L-asparaginase production, *E. coli* remains the most used host. However, this does not eliminate the occurrence of bacterial endotoxin produced by *E. coli* and other challenges like inclusion bodies formation due to protein overexpression, intracellular or periplasmic protein localization, and purification of the target protein (Rosano and Ceccarelli 2014; Upadhyay et al. 2014).

Genetic and Protein Engineering of L-asparaginase

All bacterial L-asparaginase including type I and type II consists of a tetrameric structure (a double dimer) whose size varies in the range of 25 kDa to 40 kDa for each monomer (Schalk et al. 2014; Aly et al. 2020; Yap et al. 2023) They form tight structure from each monomer with more extensive interface within the dimer and each dimer established two active regions known as active-site flexible loop (ASFL). The catalytic motives of L-asparaginase are highly conserved and consist of Tyr25, Thr89, Asp90, Lys162, and the primary nucleophile Thr12 (Lubkowski and Wlodawer 2021; Maggi et al. 2021).

Improving protein or enzyme characteristics can be done by mutagenesis, whether random or site-directed mutagenesis. Site-directed mutagenesis simplifies the work to create specific mutants with the desired characteristics and is commonly done by Polymerase Chain Reaction (PCR) with designated primers. While the catalytic residues should not be changed to maintain the activity, other non-conserved residues that are not related to the center of catalytic activity and bind more to Gln than Asn was subjected to mutagenesis. Using *E. coli* L-asparaginase type II as a model, the V27F mutant type successfully yielded in higher affinity towards Asn (Ardalan et al. 2021). Another report generated a quintuple mutant strain of *B. licheniformis* named mutant IL-RAC with mutations as follows: E102I, V104L, A180R, T223A, and T325C. This type of mutant exhibited 841.62 IU/mg of L-asparaginase activity, or about 4.24-fold higher than the wild type (Zhou et al., 2022).

Another strategy to decrease the hypersensitivity effect of L-asparaginase treatment is using protein re-surfacing. This method allows to substitution of the surface residues on the target proteins and avoids pre-existing Anti-Drug Antibodies (ADAs). Still, allowing the protein to maintain the enzymatic activity is an obligation, thus the mutations only target amino acids that have high solvent accessible area and do not directly interact in the catalytic center and high (Chapman & McNaughton, 2016). Using L-asparaginase *E. coli* as a model organism, 26-58 site mutations reduce 50% of ADAs binding relative to the wildtype L-asparaginase (Bootwala et al. 2022).

Bioprocess and Fermentation Strategies

Obtaining high biomass and protein yield through fermentation can be achieved through fermentation by optimizing the bioprocess system and nutrients (Ekpenyong et al. 2021). Producing L-asparaginase through fermentation can be achieved by submerged or solid-state fermentation and can be chosen depending on the microorganisms and desired target metabolites (Fig. 2). But in general, solid-state fermentation is a relatively new method to reduce the cost of production (Sankar et al. 2023). Choosing a suitable carbon or nitrogen source as substrate in fermentation media is required to enhance the synthesis of target metabolites (Table 3).

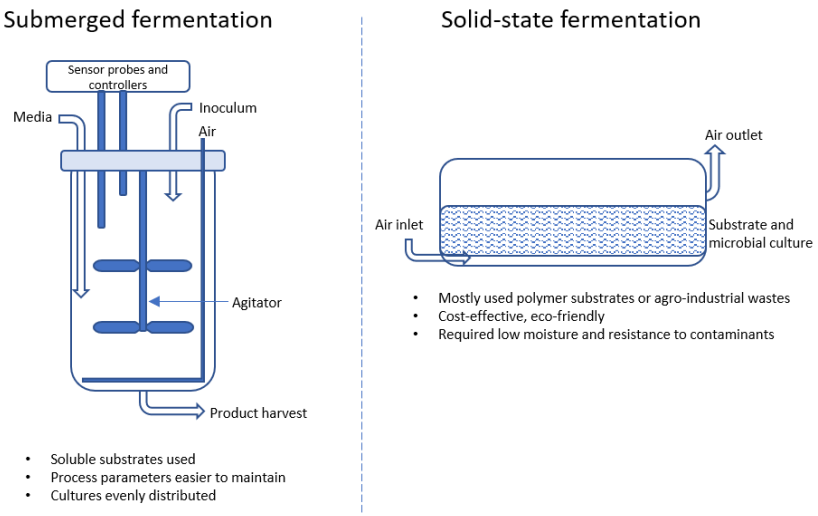

Figure 2. Differences in submerged and solid-state fermentation strategies

Table 3. Fermentation strategies of microbial L-asparaginase

Microorganisms	Fermentation strategies	Enhancement in Enzyme Characteristics	References
Myroides gitamensis BSH-3	Solid-state fermentation using wheat bran and nitrogen extract as a substrate for 47 hours	295.6 IU of L-asparaginase activity (increased in 3.4 folds)	(Talluri et al. 2019)
Acinetobacter baumannii ZAS1	Submerged fermentation with L-asparagine, peptone, glucose, and Na_2HPO_4 influenced L-asparagine production	45.59 U/mL of L-asparagine activity detected after optimization (2.41 folds higher)	(Abhini et al. 2022)
Aspergillus caespitosus CCDCA 11593	Solid state fermentation using *Pereskia aculeata* residue as fiber substrate and saline solution as moisturizing agent. Submerged fermentation using glucose as a substrate.	0.0245 U/mL of L-asparaginase activity 0.0960 U/mL of L-asparaginase activity	(Fernandes et al. 2021)
Aspergillus candidus UCCM 00117	Submerged fermentation in media containing 49.55 g/L molasses, 64.98% corn steep liquor, 44.23 g/L asparagine, 1.73 g/L potassium, 0.055 g/L manganese and 0.043 g/L chromium (III) ions	5216.95 U of yield L-asparaginase and exhibited 15-fold more cytotoxicity toward myeloid leukemia cell line	(Ekpenyong et al. 2021)
Fusarium oxysporum F-S3	Solid-state fermentation using irradiated pomegranate peel and other agro-based materials	Increased in L-asparaginase activity for 1.74 folds (280.4 U/gds) after irradiation-treated substrate	(Abdel-Hamid et al. 2022)
Penicillium sp. LAMAI505	Multiple reactors system with immobilized cells to maintain solid state fermentation in inert solid support	Resulted in 29.45 U/mg of specific activity	(Vieira et al. 2020)

Submerged fermentation is relatively a more established method to produce biomolecules and medium modification is easier (Vimal and Kumar 2017). For fermentation using wild-type strains of microorganisms, L-asparagine is needed as an inducer to produce the enzyme. Other factors such as carbon, nitrogen, and ion source or trace elements are also required to enhance the production of the enzyme (Rizzari et al. 2000). *Acinetobacter baumanii* ZAS1 needs L-asparagine, peptone, glucose, and Na_2HPO_4 in L-asparagine production (Abhini et al. 2022). *Streptomyces koyangensis* SK4, a type of actinomycetes isolated from Arctic sediment also produces this enzyme. Maximum activity of 136 IU/ml was achieved in asparagine broth media at 20°C and pH 7.5, with concentration of L-asparagine at 7.5 gr/L (Saleena et al. 2023).While fungi like *Aspergillus candidus* UCCM 00117 reportedly exhibit 5216.95 U of L-asparaginase in submerged fermentation in media containing molasses, corn steep liquor, asparagine, potassium, manganese and chromium (III) ions (Ekpenyong et al., 2021). Another report showed that Modified Czapek's Dox Broth containing dextrose, ammonium sulfate and wheat bran in concentrations of 3.0 gr/L, 20 gr/L, and 2.5 gr/L respectively is optimum to produce higher activity of L-asparaginase from *Fusarium* sp*.* LCJ273 (Jenila and Gnanadoss 2018).

To facilitate easier purification and reduce economic costs relative to submerged fermentation, solid-state fermentation was carried out with various substrates. Fungus is the most suitable organism for solid-state fermentation as it can penetrate the substrate with minimal water, thus increasing the enzyme titer (Doriya and Kumar 2018). Agricultural residues can also be used as substrate for large-scale fermentation. *Fusarium oxysporum* F-S3 yielded 280.4 U/gds in solid-state fermentation using irradiated pomegranate peel (Abdel-Hamid et al. 2022). *Aspergillus caespitosus* CCDCA 11593 produced L-asparaginase with *Pereskia aculeata* residue as the fiber substrate (Fernandes et al. 2021)

The microbial cultivation system is a laborious task, in which product accumulation in the system can feedback inhibit the cell metabolism. A long-term bioprocess system can be achieved using cell immobilization. Similar to enzyme immobilization, this allows the cells to be used several times and reduces the accumulation of the product (Lu et al. 2020). Recombinant *E. coli* that have been immobilized in an alginate matrix released L- L-asparaginase with lower initial cell density and more stable plasmid (Ghoshoon et al. 2022). After 3 days, 315.8 U of enzyme activity was achieved, which was 2 times higher than the free cells *Penicillium* sp. LAMAI 505 immobilized in polyurethane foam in four reactor systems. Product withdrawal and nutrient cycle were done for 48 hours, resulting in 29.45 U/mg of L-asparaginase-specific activity (Vieira et al. 2020).

Further Development of L-Asparaginase

L-asparaginase has been introduced since the 1960s to treat ALL patients in pediatric treatment protocol (Egler et al. 2016). To date, only two sources of L-asparaginase have been approved by FDA, namely *E. coli* and *E. chrysantemi.* However, both L-asparaginase and their derivatized form were reported to show immunogenic effects in humans.

Various cells were known to produce L-asparaginase, but none of them were subjected to large-scale production. From this review, we conclude that finding alternative sources to produce L-asparaginase that have a lower immunogenic effect in humans is not impossible. The first key point is to find L-asparaginase enzymes that have lower to no affinity towards L-glutamine as substrate, which can be achieved by selecting microbial sources like *Bacillus* and *Streptomyces* (El-Naggar et al. 2018; Alrumman et al. 2019). To further reduce the chance of immunogenic effects, fungi or yeast can be used as they are eukaryotic organisms and considered evolutionarily closer to humans (Doriya and Kumar 2016).

Two of the above challenges can also be solved by recombinant L-asparaginase. Mutations of the surface area of the protein are needed to avoid antibody recognition and to enhance the affinity of the enzyme towards L-asparaginase. Producing recombinant L-asparaginase coupled with selecting appropriate fermentation systems like cell immobilization to reduce cost production is

a necessary approach to obtain a new L-asparaginase source with enhanced characteristics.

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