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MINI-REVIEW

L-asparaginase production review: bioprocess design and biochemical characteristics

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Abstract

In the past decades, the production of biopharmaceuticals has gained high interest due to its great sensitivity, specificity, and lower risk of negative effects to patients. Biopharmaceuticals are mostly therapeutic recombinant proteins produced through biotechnological processes. In this context, L-asparaginase (L-asparagine amidohydrolase, L-ASNase (E.C. 3.5.1.1)) is a therapeutic enzyme that has been abundantly studied by researchers due to its antineoplastic properties. As a biopharmaceutical, L-ASNase has been used in the treatment of acute lymphoblastic leukemia (ALL), acute myeloblastic leukemia (AML), and other lymphoid malignancies, in combination with other drugs. Besides its application as a biopharmaceutical, this enzyme is widely used in food processing industries as an acrylamide mitigation agent and as a biosensor for the detection of L-asparagine in physiological fluids at nano-levels. The great demand for L-ASNase is supplied by recombinant enzymes from *Escherichia coli* and *Erwinia chrysanthemi*. However, production processes are associated to low yields and proteins associated to immunogenicity problems, which leads to the search for a better enzyme source. Considering the L-ASNase pharmacological and food importance, this review provides an overview of the current biotechnological developments in L-ASNase production and biochemical characterization aiming to improve the knowledge about its production.

Key points

- *Microbial enzyme applications as biopharmaceutical and in food industry*
- *Biosynthesis process: from the microorganism to bioreactor technology*
- *Enzyme activity and kinetic properties: crucial for the final application*

Keywords L-asparaginase · Biopharmaceutical · Food industry · Bioprocess

Introduction

Recent advances in enzyme technology empowered scientists to use, modify, and improve the efficiency of enzymes, leading to their maximum functionality (Muneer et al. 2020). L-asparaginase (L-asparagine amidohydrolase, L-ASNase) (EC 3.5.1.1) has been extensively used and studied because of its relevant potential as an oncological agent and as an acrylamide mitigation agent in the food industry, which is due to its ability to catalyze the hydrolysis of L-asparagine into L-aspartate and ammonia (Sharma et al. 2018; Chand et al. 2020). The discovery and development of potential uses of L-ASNase as an anti-cancer drug started in 1953, when Kidd first observed that lymphomas in rat and mice relapsed after treatment with guinea pig serum (Kidd 1953). Nowadays, L-ASNase is widely used in the treatment of acute lymphoblastic leukemia (ALL), acute myeloblastic leukemia (AML), and other lymphoid malignancies in combination with other drugs (Husain et al. 2016; Vala et al. 2018). However, formulation of this protein represents 40% of the total enzyme demands worldwide and one third of the global

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needs for anticancer agents, which is far more than for other therapeutic enzymes (Izadpanah et al. 2018). The biopharmaceutical world market, especially the healthcare market, is constantly growing. Therapeutical enzyme market was expected to reach $6.3 billion by 2021 as compared to $5.0 billion in 2016, corresponding to a 4.7% annual growth rate for the period (Chand et al. 2020).

L-ASNase is also widely used in food processing industries as an acrylamide mitigation agent since this compound is being classified as a probable carcinogen compound according to several agencies, namely the International Agency for Research on Cancer (Javier et al. 2016). Additionally, L-ASNase biosensor is a promising technology for the detection of L-asparagine in physiological fluids at nano-levels (Batool et al. 2016).

Several microorganisms and even a few plants and animals are endowed with L-ASNase producing ability. However, due to the complex process of extracting and purifying enzymes from plants and animals, the use of microorganisms is the most viable alternative (Moguel 2018). For instance, all L-ASNase drugs commercially authorized for clinical purposes are restricted to recombinant L-ASNase from *Escherichia coli* and *Erwinia chrysanthemi* (Muneer et al. 2020). Moreover, in order to have commercial and therapeutic value, L-ASNase must be stable over a wide range of pH and temperature and must have a low Michaelis-Menten constant (K_m) value (high substrate affinity in physiological conditions) and low collateral effects (Chand et al. 2020). Therefore, several investigations have been carried out in order to produce recombinant L-ASNase with improved characteristics and properties. Figure 1 presents an overview of L-ASNase production by recombinant microorganisms.

This review provides a deep overview of the literature regarding the microbial production of L-ASNase using different strategies, as well as its biochemical characterization.

Classification of L-asparaginase

Although L-ASNase can be produced by several sources, this enzyme is classified based on its amino acid sequence, biochemical properties, and structural and functional homology (Müller and Boos 1998). Currently, L-ASNases are divided into three major groups: (i) bacterial type L-ASNase (including the classification type I and type II), (ii) plant type L-ASNase (type III), and (iii) rhizobial type L-ASNase (Borek and Jaskólski 2001; Qeshmi et al. 2018). Bacterial type L-ASNases are structurally and evolutionarily distinct from the plant type (Michalska and Jaskolski 2006). The bacterial type L-ASNases are subdivided in types I and II based on their cellular localization and on the activity towards L-asparagine and L-glutamine (Izadpanah et al. 2018). L-ASNase type I is a cytosolic enzyme with relatively low affinity for L-asparagine and high specific activity towards L-glutamine. On the other hand, type II is a periplasmic or membrane associated L-ASNase with high affinity for L-asparagine and low activity towards L-glutamine, a combination highly attractive for oncologic application (Izadpanah et al. 2018). Both types of L-ASNase can be produced by the same microorganism; for example, *E. coli* is able to produce two isozymes of L-ASNase (Qeshmi et al. 2018). However, only the L-ASNase type II possesses anti-tumor activity. The characteristics of bacterial L-ASNase type I and type II are summarized in Fig. 2.

The structural feature differentiating type I and II isoenzymes is the active complex size. L-ASNase type I seems to form dimers (Yao et al. 2005; Yano et al. 2008), whereas the L-ASNase type II is widely reported as a tetramer. According to Aghaiypour et al. (2001) and Lubkowski et al. (2003) bacterial L-ASNases type II are active as homo-tetramers with 222 symmetry, each monomer consisting of about 330 amino acid residues forming 14 β-strands and eight α-helices, as shown in Fig. 3 for the three-dimensional structure of

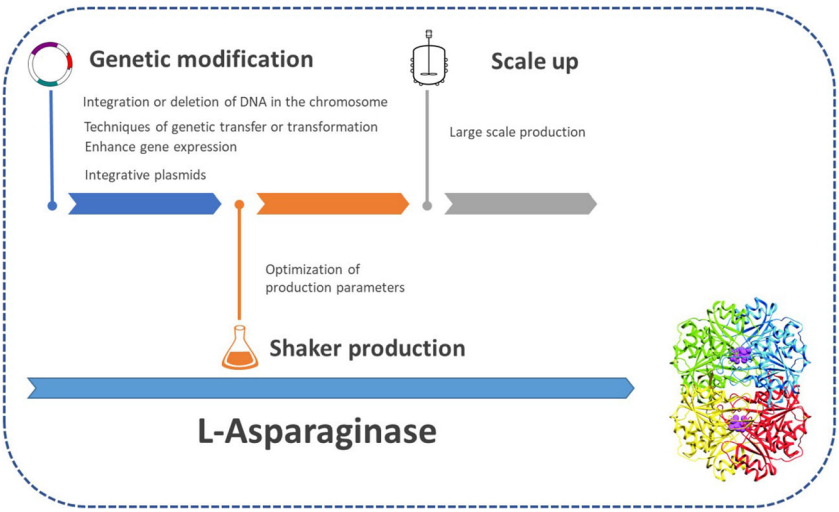

Fig. 1 Overview of L-asparaginase production by recombinant microorganisms

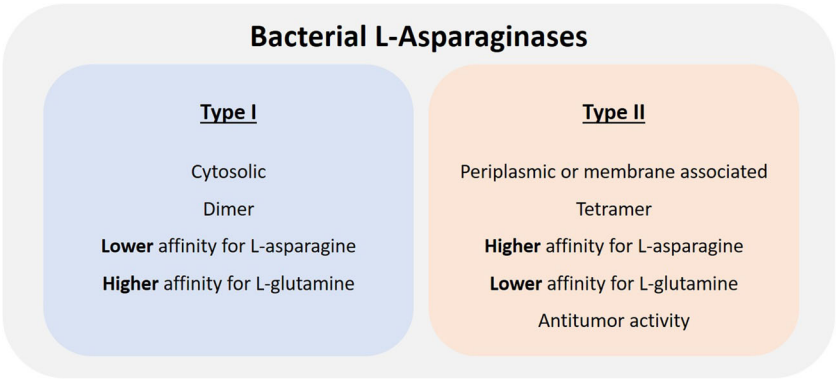

Fig. 2 Main characteristics of bacterial L-asparaginase type I and type II

E. chrysanthemi L-ASNase type II (Lubkowski et al. 2003). Studies on L-ASNase type-I structure are much scarcer in the literature than those for the type II.

It is also important to discuss the structure of L-ASNase at molecular levels. Commonly, the enzyme is found as a tetramer, but monomeric, dimeric, and hexameric forms have also been found for enzymes isolated from different sources (Batool et al. 2016). In fact, molecular structures of L-ASNases from *E. coli* and *Erwinia* sp. have been deeply investigated. The native L-ASNase type II isolated from *E. coli* has a molecular weight of 138–141 kDa and contains four identical subunits of 326 amino acids with one active center each (Kozak and Jurga 2002). The reported molecular weight of the *Erwinia*-derived L-ASNase is 138 kDa as described in Table 1 (Nguyen et al. 2016; Müller and Boos 1998). Information about the kinetic parameters of this L-ASNase formulation is also presented in Table 1. Different sources and post-translation modifications may strongly influence the molecular structure of the enzyme. For instance, Asselin et al. (1995) reported a PEG-modified L-ASNase from *E. coli* with increased half-lifetime (5–7 days) and molecular weight of 145 kDa.

The stability and half-lifetime of L-ASNase in the serum are of crucial concern for the pharmaceutical industry. An enzyme preparation with high stability and increased half-lifetime can avoid the need for multiple dose administration, which may lead to less chances of triggering hypersensitivity reactions (Krishnapura et al. 2016). Therefore, from the different L-ASNases analyzed for clinical trials, the ones from *E. coli* modified with PEG revealed a higher half-life when compared to non-modified *E. coli* L-ASNase, which ensure adequate serum enzyme activity and prevents complete L-ASNase serum depletion (Pieters et al. 2011; Asselin et al. 1995). An often used modification to prevent hypersensitivity

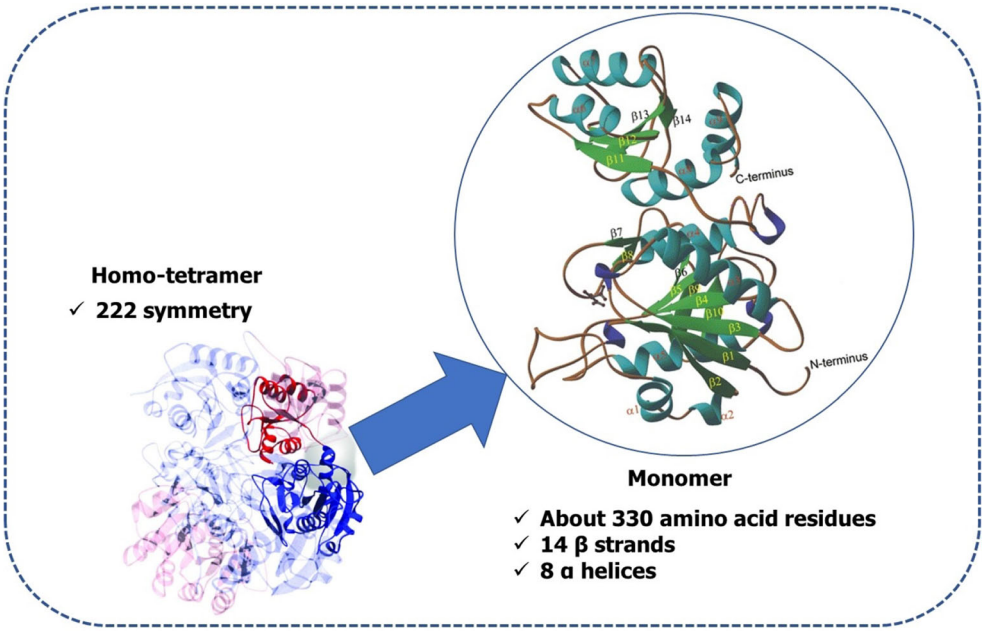

Fig. 3 Three-dimensional structure of Erwinia chrysanthemi L-asparaginase type II suggested by Aghaiypour et al. (2001) and Lubkowski et al. (2003)

Table 1 Properties of different L-asparaginase preparations

Source	Molecular weight (kDa)	Isoelectric point (pI)	K_m (μM) asparagine	K_m (mM) glutamine	Half-life time	Reference
E. chrysanthemi	138	8.7	12	1.10	8–22 h	Nguyen et al. (2016)
E. coli	141	5	10	6.25	8–30 h	Asselin et al. (1995)
PEG-E. coli	145	5	10	nd*	5–7 days	Asselin et al. (1995)
B. subtilis 168	40	nd*	5290	nd*	1 h	Feng et al. (2017)

**nd* not determined

reactions towards the native forms of L-ASNase is the PEG conjugation (Müller and Boos 1998; Pui et al. 2018). For preparation of the modified enzyme, units of monomethoxy PEG are attached to the derived enzyme (e.g., *E. coli*) by covalent bonds (Yoshimoto et al. 1986).

Sources of L-asparaginase

Production of L-asparaginase by wild-type species

As cited previously, L-ASNase is widely distributed in nature, being found in animals (fishes, mammals, and birds), in different tissues (such as liver, pancreas, brain, kidneys, and lungs), plants, and microorganisms, including bacteria, filamentous fungi, and yeast (Lopes et al. 2017; Brumano et al. 2019). However, as indicated by Savitri and Wamik, microorganisms are a better source of L-ASNase, considering their ability to grow easily on very simple and economical substrates (Savitri and Azmi 2003). Additionally, the biotechnological production process is usually easier to optimize and scale-up than other processes. Depending on the strain employed, it can be easily genetic modified in order to increase the yield, making the extraction and purification process economically feasible (Cachumba et al. 2016; Lopes et al. 2017).

Table 2 shows several microbial wild-type species able to produce L-ASNase and the corresponding enzyme characteristics. The best producers of L-ASNase belong to the Enterobacteriaceae family, followed by fungi species. The main bacteria producers of L-ASNase are *E. coli* and *E. chrysanthemi.* However, the main problem associated with L-ASNase produced by prokaryotic microorganism are hypersensitivity and immune inactivation (Javier et al. 2016). In this sense, different strategies with bacterial source have been studied and it will be further discussed in the "Production of recombinant L-asparaginase" section. Considering the production process, the L-ASNase produced by *E. coli* is intracellular, which inserts the unit operation of disruption cell in the downstream processes. Among the bacteria, wild types of *Bacillus* are also natural producers of L-ASNase, i.e., *Bacillus australimaris* NJB19 (MG734654) (Chakravarty et al. 2021), *Bacillus lichenformis* ((Mahajan et al. 2014), and *Bacillus* sp. (Singh and Srivastava 2012).

Other sources of L-ASNase are *Actinomycetes* strains, which are filamentous bacteria well known as a good source of antibiotics, with microorganisms such as *Streptomyces griseoluteus*, *Nocardia levis*, and *Streptomyces ginsengisoli* reported to be potential producers of L-ASNase (LopesOrabi et al. 2019; Qeshmi et al. 2018). The L-ASNase produced by actinomycetes is generally extracellular, which is an advantage for the production process. Saxena et al. (2015) studied 240 actinomycetes being 165 positives for L-ASNase activity. Among them, the strains *Streptomyces cyaneus* (SAP 1287, CFS 1560), *Streptomyces exfoliates* (CFS 1557), and *Streptomyces phaeochromogenes* (GS 1573) were L-glutaminase-free actinomycetes with a highlighted production of glutaminase-free L-ASNase by the last strain (Saxena et al. 2015). However, studies performed by Dhevagi and Poorani (2006) showed that L-ANSase from marine actinomycetes presented cytotoxic effect on acute T-cell leukemia and myelogenous leukemia, being this source of L-ASNase an alternative for the food industry.

Fungal L-ASNases are commonly produced extracellularly, simplifying the downstream purification process (Chand et al. 2020). *Fusarium*, *Aspergillus*, and *Penicillium* strains are the most common fungi genera reposted to produce L-ASNase (Orabi et al. 2019). L-ASNases from *Aspergillus oryzae* and *Aspergillus niger* are already commercially approved for use as processing agents in the food industry (Chand et al. 2020). The health sector requires a nobler source of L-ASNase with minimal or no cross-reactivity to minimize adverse reactions. Alike human cells and unlike bacterial cells, fungi cells can glycosylate proteins; therefore, enzymes isolated from fungi are expected to cause less immunogenicity (Chand et al. 2020). However, the fungal complex morphology can be critical for the feasibility of scaling up the process since fungal cultivation in bioreactor is sensible to several parameters, such as oxygen supply and transfer, inoculum size, pH, and stirring (De Oliveira et al. 2020).

Several yeast genera (Table 2), including *Saccharomyces*, *Candida*, *Pichia*, *Rhodotorula*, *Rhodosporidium*, and

Table 2 Microbial wild-type species producer of L-asparaginase and corresponding properties

Microorganism	Strain	Localization	K_m (μM)	k_{cat} (s^{-1})	V_{max}	Reference
Bacteria	*Bacillus australimaris*	Extracellular	nd*	nd*	nd*	Chakravarty et al. (2021)
Bacteria	*Bacillus licheniformis*	Extracellular	14	2.68×10^3	4.03 IU**	Mahajan et al. (2014)
Bacteria	*Bacillus* sp.	Extracellular	nd*	nd*	nd*	Singh and Srivastava (2012)
Bacteria	*Erwinia carotovora*	Intracellular	96	nd*	1632.6 μM mg^{-1} min^{-1}	Warangkar and Khobragade (2010)
Algae	*Spirulina maxima*	Intracellular	nd*	nd*	nd*	Abd El Baky and El Baroty (2016)
Actinomycetes	*Streptomyces phaeochromogenes*	Extracellular	nd*	nd*	Nnd*	Saxena et al. (2015)
Fungi	*Aspergillus niger*	Extracellular	16	nd*	66.66 μM mL^{-1} min^{-1}	Luhana et al. (2013)
Fungi	*Aspergillus terreus*	Extracellular	nd*	nd*	nd*	Farag et al. (2015)
Fungi	*Cladosporium* sp.	Extracellular	100	nd*	4.00 μM mg^{-1} min^{-1}	Mohan Kumar and Manonmani (2013)
Fungi	*Penicillium* sp.	Extracellular	4.0×10^3	nd*	nd*	Patro and Gupta (2012)
Fungi	*Fusarium* sp.	Extracellular	443.98×10^3	nd*	40 IU**	Asha and Pallavi (2012)
Yeast	*Candida utilis*	Extracellular	nd*	nd*	nd*	Kil et al. (1995)

*nd not determined. **International unit (IU) of asparaginase activity is defined as the amount of enzyme required to release 1 μmol of ammonia per minute at specified conditions

Trichoderma, have been reported as L-ASNase producers (Chand et al. 2020; Kil et al. 1995). *Saccharomyces cerevisiae* strains were found to produce both the intracellular and extracellular forms of L-ASNase, whereas production of the extracellular form seems to be triggered under nitrogen starvation (Sharma et al. 2018).

An alternative source of L-ASNase is blue-green microalgae, an attractive option due to its no seasonal variation, low cost of medium formulation, and easy cultivation and harvesting characteristics (Orabi et al. 2019). *Chlamydomonas* sp., *Chlorella vulgaris*, and *Spirulina maxima* are considered as potential microalgal sources for novel enzyme production in several studies (Orabi et al. 2019; Ebrahiminezhad et al. 2014; Abd El Baky and El Baroty 2016).

As demonstrated, several microorganisms presenting particular characteristics can act as potential producers of L-ASNase. However, before designing and scaling up the bioprocess, pharmaceutical and food industries seek for high productivity, easily handling and scaling up, highly stable enzymes (temperature, pH, storage), high enzymatic activity, low toxicity, easy product purification, and low production costs (Chand et al. 2020; Brumano et al. 2019). Therefore, in-depth studies are essential to disclose the best sources of the enzyme for industrial applications.

Production of recombinant L-asparaginase

Recently, many efforts to produce recombinant L-ASNase from different sources have been made, as depicted in Table 3. Each system presents its own characteristics regarding production capacity, cost, safety, complexity, and processing impact (Santos et al. 2018). It is important to highlight that in the last years there was significant progress in synthetic biology through the development of molecular tools and methods for engineering biological systems, which facilitate the construction of efficient chassis for industrial relevant bioprocesses, including the production of L-ASNase (Corrêa et al. 2020). To exemplify, Corrêa et al. (2020) presented the engineering of tunable and modular devices for autonomous control of gene expression in *Bacillus subtilis* that requires no inducer and no human supervision. The device developed can be applied for heterologous protein production (Corrêa et al. 2020).

The preferred host for overproduction of recombinant L-ASNase is *E. coli*, and the pET system with isopropyl β-D-thiogalactoside (IPTG) induction is the most used gene expression system (as summarized in Table 3). However, additional work has been done on alternative hosts such as *B. subtilis* (Feng et al. 2017; Sushma et al. 2017; Li et al. 2018; Niu et al. 2021) and *Pichia pastoris* (Sajitha et al. 2015; Rodrigues et al. 2019; Lima et al. 2020). Unlike *E. coli*, these hosts hold the GRAS (Generally Regarded as

Safe) status, and can be engineered to secrete the enzyme to the medium, which may turn the downstream process easier and of lower cost. From the gene sequence it is possible to establish that the *ansZ* gene from *B. subtilis* encodes a L-ASNase with 59% identity to the L-ASNase type I from *E. chrysanthemi* and 53% identity to the L-ASNase type II from *E. coli* (Fisher and Wray 2002). Moreover, *B. subtilis* has another gene (*ansA*) that encodes a L-ASNase type I (Yano et al. 2008). Feng et al. (2017) were able to successfully overproduce and secrete a recombinant L-ASNase type II in *B. subtilis*, reaching 2.5 g L^{-1} of enzyme in a 3-L bioreactor through a fed-batch strategy. More than protein secretion, *P. pastoris* is able to add post-translation modifications to the overproduced enzyme. Lima et al. (2020) used *P. pastoris* to engineer a L-ASNase with a human-like glycosylation pattern, which lowered the immunogenicity of the protein tested in vitro compared to the non-glycosylated.

Biochemical characterization of L-asparaginase

Effect of pH and temperature in L-asparaginase activity

In order to guarantee the best possible performance of an enzyme, biochemical characterization regarding temperature and pH are essential parameters to define its application (Krishnapura et al. 2016). Different studies have been performed in order to evaluate the effect of pH on activity of L-ASNase produced by different microorganisms (Table 4). In general, the L-ASNase maximum activity ranges from acidic to alkaline pH values (Chand et al. 2020). The pH affects not only the enzyme structure but also its affinity for the substrate. For therapeutic use, optimal pH for the L-ASNase must lie in the physiological range, while for the food industry, the L-ASNase must keep enough activity even at acidic pH (Krishnapura et al. 2016). L-ASNase produced by bacteria such as *E. coli*, *Bacillus megaterium*, and *Pseudomonas fluorescens* presents optimum activity at pH of 6.0, 7.0, and 7.5, respectively (Borah et al. 2012; Zhang et al. 2015; Sindhu and Manonmani 2018b). According to Jeyaraj et al. (2020), a pH value close to 8.0 is needed for a maximum activity for L-ASNase from *B. subtillis* (Jeyaraj et al. 2020). The enzyme produced by *Penicillum* sp. and *Anoxybacillus flavithermus*, a fungus and a bacterium, respectively, both demonstrate an optimal activity at pH of 7.0 (Chand et al. 2020; Maqsood et al. 2020). On the other hand, the Gram-negative bacteria *Pseudomonas aeruginosa* PAO1 and *Rhizobium etli* produce enzymes with maximum activity in acidic and alkaline conditions, 5.5 and 9.0, respectively (Angélica et al. 2012; Dutta et al. 2015).

Temperature also affects the pace of catalysis and stability of an enzyme (Daniel et al. 2010). Temperature tolerance and stability of L-ASNases differs from species to species (Table 4); however, the enzymes often have optimal activity in a temperature range between 25 °C and 45 °C (Chand et al. 2020). Nevertheless, the extreme thermophiles *Thermococcus kodaka* (TK1656) and *T. kodaka* (TK2246) produce L-ASNases with optimal activity at 85 °C (Chohan et al. 2020; Muneer et al. 2020). In the study performed by Kumar et al. (2017), the authors concluded that the L-ASNase produced by *B. subtillis* shows an optimal activity at 37°C. Additionally, authors showed that this enzyme is also active in a wide range of temperature from 30 °C to 75 °C; yet, at the maximum temperature will eventually lead to an unstable enzyme with no application (Kumar et al. 2017). Similarly, Patro and Gupta (2012) obtained a L-ASNase from *Penicillum* sp. with optimal activity at 37 °C. The authors determined the optimal temperature for the enzyme using a range of temperatures between 30 °C and 50 °C. The study performed by Borah et al. (2012) shows the production of L-ASNase from *E. coli,* whereas the optimal enzyme activity was achieved at 55 °C. As well in this study, the authors defined that the enzyme produced was able to tolerate high temperatures and hence can be considered a thermostable enzyme (Borah et al. 2012).

One way to preserve and/or improve the enzyme characteristics including L-ASNase activity and stability is to confine or to immobilize the enzyme in nanomaterials. The process can enhance thermal, pH, storage, and operational stabilities, and can even improve the pharmacological properties, as high enzyme selectivity. This modification process may also prevent enzyme deactivation (Nunes et al. 2020). Cristovão et al. (2020) studied the application of multi-walled carbon nanotubes (MWCNTs) as support for ASNase immobilization by adsorption method. According to the results, MWCNTs are efficient supports for ASNase immobilization, with no chemical modification or covalent binding required, opening up the possibility for ASNase–MWCNT bioconjugates in several applications. L-ASNase immobilization and confinement techniques are interesting to maintain the enzyme biochemical properties.

Influence of effector molecules on L-asparaginase activity

Metal ions are essential for the structural regulation of a protein as they act as electron donors or acceptors (Buchholz et al. 2012). In some cases, the presence of a metal ion is mandatory for the preservation of the multimeric structure of the enzyme and also to stabilize the reaction intermediates (Krishnapura et al. 2016). For a better understanding of the mechanism of enzyme action it is important that the influence of various effectors that activate or inhibit (or in any other way affect) the protein is well described and studied. These data may lead

Table 3 Recombinant L-asparaginase production and corresponding properties

Identification	Gene source	Host	Plasmid	Gene expression	Localization	K_m (mM)	k_{cat} (s^{-1})	V_{max}	Reference
L-ASNase type I	*Acinetobacter soli*	*E. coli* BL21 (DE3)	pET30a	IPTG-inducible	Intracellular	3.22	nd*	1.55 IU μg^{-1}**	Jiao et al. (2020)
L-ASNase	*Anoxybacillus flavithermus*	*E. coli* BL21-CodonPlus (DE3)	pET-22b (+)	IPTG-inducible	Intracellular	nd*	nd*	nd*	Maqsood et al. (2020)
L-ASNase	*Aspergillus terreus*	*E. coli* BL21 (DE3)	pET-28a (+)	IPTG-inducible	Intracellular	nd*	nd*	nd*	Saeed et al. (2018a)
L-ASNase type I	*Bacillus licheniformis Z-1*	*Bacillus subtilis RIK 1285*	ET-30a	Constitutive	Intracellular + extracellular	nd*	nd*	nd*	Niu et al. (2021)
L-ASNase type II	*Bacillus* sp. SL-1	*E. coli* BL21 (DE3)	pET22b+	IPTG-inducible	Intracellular	0.0103	23.96	nd*	Safary et al. (2019)
L-ASNase type II	*Bacillus subtilis*	*B. subtilis* WB600	pMA0911	Constitutive	Extracellular	5.3	54.4	nd*	Feng et al. (2017)
L-ASNase type II	*Bacillus subtilis*	*B. subtilis* WB800N	pHT43	IPTG-inducible	Intracellular	nd*	nd*	nd*	Sushma et al. (2017)
L-ASNase II	*Bacillus subtilis*	*B. subtilis* 168	pMA5	Constitutive	Extracellular	0.43	nd*	77.51 μM min^{-1}	Jia et al. (2013)
L-ASNase	*Bacillus tequilensis*	*E. coli* BL21 (DE3)	pET28a+	IPTG-inducible	Intracellular	0.07	nd*	7.79 μM min^{-1}	Shakambari et al. (2018)
L-ASNase type II	*Cobetia amphilecti*	*E. coli* BL21 (DE3)	pQE80L	IPTG-inducible	Intracellular	2.05	nd*	11641 μM min^{-1} mg^{-1}	Farahat et al. (2020)
L-ASNase	*Erwinia carotovora*	*E. coli* BL21 (DE3)	pET28a and pET22b	IPTG-inducible	Intracellular	nd*	nd*	nd*	Faret et al. (2019)
L-ASNase type II	*Erwinia carotovora*	*E. coli* BL21 (DE3)	pET22b	IPTG-inducible	Intracellular	nd*	nd*	nd*	Goswami et al. (2019)
L-ASNase type II	*Erwinia chrysanthemi*	*E. coli BL21*	pJ401, pMTL22p, and pET14b	Constitutive	Intracellular	0.029–0.080	440–798	nd*	Moola et al. (1994) and Gervais and Foote (2014)
L-ASNase type II	*Escherichia coli*	*E. coli*	pMTL22p	Constitutive	Intracellular	0.015–0.050	100	nd*	Moola et al. (1994), Derst et al. (2000), and Nguyen et al. (2016)
L-ASNase type II	*Escherichia coli*	*Pichia pastoris* SuperMan5 (his-)	pJAG-s1	Methanol-inducible	Extracellular (wt), periplasm (mut)	nd*	nd*	nd*	Lima et al. (2020)
L-ASNase II	*Lactobacillus casei*	*E. coli* BL21 (DE3)	pET28a (+)	IPTG-inducible	Intracellular	0.01235	nd*	1576 μM min^{-1}	Aishwarya et al. (2019)
L-ASNase	*Lactobacillus reuteri*	*E. coli* BL21 (DE3)	pET28a (+)	IPTG-inducible	Intracellular	0.3332	nd*	14060 μM min^{-1}	Susan Aishwarya et al. (2017)
L-ASNase	*Nocardiopsis alba*	*E. coli* M15	pQE30	IPTG-inducible	Intracellular	nd*	nd*	nd*	Meena et al. (2016)
L-ASNase	*Paenibacillus barengoltzii*	*E. coli* BL21 (DE3)	pET-28a (+)	IPTG-inducible	Intracellular	3.6	nd*		Shi et al. (2017)

Table 3 (continued)

Identification	Gene source	Host	Plasmid	Gene expression	Localization	K_m (mM)	k_{cat} (s^{-1})	V_{max}	Reference
								162.2 μmol min^{-1} mg^{-1}	
L-ASNase type I	*Pseudomonas aeruginosa*	*E. coli* BL21 (DE3)	pET28a (+)	IPTG-inducible	Intracellular	nd*	nd*	nd*	Saeed et al. (2018b)
L-ASNase	*Pseudomonas fluorescens*	*E. coli* BL21 (DE3)	pET-32a	IPTG-inducible	Intracellular	nd*	nd*	nd*	Sindhu and Manonmani (2018a)
L-ASNase	*Pseudomonas resinovorans*	*E. coli* rosetta DE3	pET-28a (+)	IPTG-inducible	Intracellular	nd*	nd*	nd*	Mihooliya et al. (2020)
L-ASNase	*Pyrococcus furiosus*	*E. coli* BL21 (DE3) pLysS	pET26b (+)	IPTG-inducible	Intracellular	1.623	nd*	105 μmol min^{-1} mg^{-1}	Saeed et al. (2020)
L-ASNase	*Pyrococcus yayanosii*	*B. subtilis* 168	pMA5	Constitutive	Intracellular + extracellular	6.5	nd*	2929 μM min^{-1}	Li et al. (2018)
L-ASNase type II	*Saccharomyces cerevisiae*	*E. coli* BL21 (DE3)	pET28a (+)	IPTG-inducible	Intracellular	nd*	nd*	nd*	Lopes et al. (2019)
L-ASNase	*Saccharomyces cerevisiae*	*E. coli* BL21 (DE3)	pET15b	IPTG-inducible	Intracellular	nd*	nd*	nd*	Santos et al. (2017)
L-ASNase type II	*Saccharomyces cerevisiae*	*Pichia pastoris*	pPIC9K	Methanol-inducible	Periplasmic	nd*	nd*	nd*	Rodrigues et al. (2019)
L-ASNase type II	*Saccharomyces cerevisiae*	*E. coli* BL21 (DE3)	pET15b	IPTG-inducible	Intracellular	0.075	217	0.042 μmol min^{-1}	Costa et al. (2016)
L-ASNase	*Streptomyces griseus*	*E. coli* M15 pREP4	pQE30	IPTG-inducible	Intracellular	nd*	nd*	nd*	Meena et al. (2015)
L-ASNase type II	*Thermococcus kodakaraensis*	*E. coli* strain BL21-CodonPlus (DE3)	pET-21a	IPTG-inducible	Intracellular	3.1	nd*	833 μmol min^{-1} mg^{-1}	Chohan et al. (2020)
L-ASNase type II	*Vibrio cholerae*	*E. coli* BL21 (DE3)	pMCSG7	IPTG-inducible	Intracellular	1.1	4424	1006 μM min^{-1}	Radha et al. (2018)
L-ASNase type II	*Zymomonas mobilis*	*E. coli* BL21 (DE3)	pET26a and pET28b	IPTG-inducible	Extracellular (pET26b) and intracellular (pET28a)	nd*	nd*	nd*	Einsfeldt et al. (2016)

**nd* not determined. ***International unit (IU) of asparaginase activity is defined as the amount of enzyme required to release 1 μmol of ammonia per minute at specified conditions

Table 4 Optimum pH and temperature of microbial L-asparaginase

Microorganism	pH	Temperature (°C)	Reference
Acinetobacter baumannii	7.2	37.0	Muslim (2014)
Anoxybacillus flavithermus	7.0	60.0	Maqsood et al. (2020)
Aspergillus niger	6.5	40.0	Sharma et al. (2018)
Aspergillus oryzae (CCT 3940)	7.0–8.0	40.0	Dias et al. (2016)
Bacillus subtillis	8	37.0	Kumar et al. (2017)
Bacillus licheniformis	6.0–10.0	40.0	Mahajan et al. (2014)
Bacillus megaterium	7.0	40.0	Zhang et al. (2015)
Bacillus firmus (AVP 18)	9.0	37.0	Rudrapati and Audipudi (2015)
Cobetia amphilecti AMI6	7.0	60.0	Farahat et al. (2020)
Cornyebacterium glutamicum	7.0	40.0	Kumar and Sobha (2012)
Escherichia coli	6.0	55.0	Borah et al. (2012)
Penicillum sp.	7.0	37.0	Chand et al. (2020)
Pseudomonas aeruginosa PAO1	5.5	50.0	Dutta et al. (2015)
Pseudomonas fluorescens	7.5	37.0	Sindhu and Manonmani (2018b)
Stenotrophomonas maltophilia	6.9	38.1	Abdelrazek et al. (2020)
Streptomyces rochei	6.7	37.0	El-Naggar and El-Shweihy (2020)
Rhizobium etli	9.0	37.0	Angélica et al. (2012)
Thermococcus kodaka (TK1656)	9.5	85.0	Muneer et al. (2020)
Thermococcus kodaka (TK2246)	7.0	85.0	Chohan et al. (2020)
Vibrio cholerae	7.1	38.5	Radha and Gummadi (2020)

to catalytic efficiency improvement and consequently a higher yield for the biotechnological purpose of the enzyme (Krishnapura et al. 2016).

The same metal chelator can have different influence on L-ASNase isolated from different sources. Ethylenediamine tetra acetic acid (EDTA) enhances the activity of the enzyme from *Erwinia carotovora* but has no effect on the L-ASNase from *Cladosporium* sp. (Krishnapura et al. 2016). Divalent ions, such as Ca^{2+}, Co^{2+},Cu^{2+} ,Mn^{2+}, Hg^{2+}, Mg^{2+}, Fe^{2+}, Sn^{2+}, Pb^{2+}, and Ba^{2+}, were proved to have an inhibitory effect on L-ASNase from *Bacillus aryabhattai* ITHBHU02, while Na^{+} and K^{+} enhance the enzymatic activity (Singh et al. 2013). For L-ASNase from *Thermococcus gammatolerans* EJ3, Mg^{2+} acts as an activator, while Zn^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, and Ba^{2+} are considered inhibitors of the enzyme (Zuo et al. 2014).

Kinetic properties of L-asparaginase

Efforts to produce recombinant L-ASNases and the search for new different wild sources are mostly directed towards developing alternatives for treating ALL patients that develop hypersensibility reactions to the available commercial L-ASNase. The underlining idea is that enzymes from different sources provide different protein sequences that may present different immunogenicity profiles. However, there are other requirements for a new enzyme to become an efficient new oncogenic biopharmaceutical, such as the kinetic parameters. Because L-asparagine is present at ~50 μM in the human blood, therapeutic L-ASNase must have a substrate affinity in the lower micromolar range (Ollenschläger et al. 1988; Nguyen et al. 2016). Low Michaelis-Menten constant (K_m) associated with high turnover number (K_{cat}) ensure that the therapeutic L-ASNase will sufficiently reduce the endogenous L-asparagine at safe doses (Beckett and Gervais 2019). Apart from this, kinetic parameters are crucial for the efficient use of enzymes in different industrial processes (Choi et al. 2017). Most of the mesophilic L-ASNase reported to date have low K_m values while the thermophilic ones show relatively high K_m (Hong et al. 2014). The values of kinetic parameters for L-ASNases obtained from wild type and recombinant microorganisms are listed in Table 2 and Table 3, respectively.

In that regard, few promising sources of recombinant enzymes were recently characterized: L-ASNase type II from *Lactobacillus casei*—K_m 12.3 μM (Aishwarya et al. 2019), L-ASNase A1 from *Bacillus* sp. SL-1—K_m 10.3 μM (Safary et al. 2019), AnsA from *Bacillus tequilensis*—K_m 70 μM (Shakambari et al. 2018), and ScASNase1 from *S. cerevisiae*—K_m 75 μM (Costa et al. 2016). These K_m values closely match the *E. coli* (K_m 10 μM) and the *E. chrysanthemi* (K_m 12 μM) enzymes affinity for asparagine, as shown in Table 1, that present some L-ASNase characteristics of typical commercial sources (Krishnapura et al. 2016; Nguyen et al. 2016; Gervais and Foote 2014; Moola et al.

1994). Expressive high values of K_{cat} were achieved by the recombinant L-ASNase from *Vibrio cholerae*, *S. cerevisiae*, and *E. chrysanthemi* (values ranging from 217 to 4424 s^{-1}). For comparison, commercial L-asparaginase Erwinase® and Elspar® present K_{cat} around 286.2 s^{-1} and 126.5 s^{-1}, respectively (Krishnapura et al. 2016).

Regarding wild-type microorganism, several authors have reported L-ASNases with elevated substrate affinity; for example, Warangkar and Khobragade (2010) produced an efficient enzyme from *Erwinia carotovora* presenting K_m value of 0.096 mM. Elevated substrate affinity was also obtained by Mahajan et al. (2014) when studying the enzyme produced by *Bacillus licheniformis*, presenting a K_m = 0.014 mM (Mahajan et al. 2014). For instance, Asha and Pallavi (2012) reported an enzyme from *Fusarium* sp. presenting V_{max} = 40 IU and K_m = 443.98 mM and indicated its potential in cancer therapy since the enzyme did not elicit any immunostimulatory response in human lymphocytes in vitro, unlike most of the reported prokaryotic asparaginases (Asha and Pallavi 2012). Enzymes generally present complex action mechanism systems and need to be deeply studied before efficient and safe application. Kinetic characterization has a key role in understanding enzyme activity and in designing the most efficient application routes. Additionally, as shown in Tables 2 and 3, the values of K_m and K_{cat} are intrinsically related to the enzyme source and represent important comparison parameters in order to evaluate the potential application of the protein.

Bioprocess for L-asparaginase production

The effective application of a bioprocess for the production of the target enzyme requires a meticulously selection of the microorganism as the basis of the process, as it affects directly the characteristics of the final product (Brumano et al. 2019). Among the different species capable of producing L-ASNase and as previously mentioned, *E. coli* is the main microbial host used for the industrial-scale production of recombinant L-ASNase. However, other species have been studied and are promising candidates. L-ASNase production can be performed by submerged fermentation (SmF) and solid state fermentation (SSF) (Lopes et al. 2017). Figure 4 summarizes the main advantages and limitations for both fermentation process types.

SmF is the main type of fermentation employed for bacterial enzyme production and, consequently, the most used to produce L-ASNase. In fact, SmF is well established and the manipulation of medium components is comparatively easier, leading to high production yields (Vimal and Kumar 2017). Moreover, no requirement for pre-treatment of substrate, easiness of manipulation of the reaction parameters and easy purification of products strongly contribute for the widely application of this type of fermentation. This type of fermentation allows the microorganism to grow in closed reactors containing a liquid broth medium. High concentration of dissolved oxygen is usually required (Doriya et al. 2016).

As for other biomolecules, the process to obtain L-ASNase is considerably influenced by several factors, such as type and concentration of carbon and nitrogen sources, pH, temperature, fermentation time, aeration, and mainly the microbial agent (Lopes et al. 2017). The productivity of microbial metabolites is related to the process variables such as type and concentrations of nutrients, and operation conditions (Marques et al. 2014). Submerged fermentations can be performed in laboratory scale (shaken flasks culture and bioreactor up to 10 L) and industrial scale (bioreactor larger than 10 L). The shaken flask experiments are important to study the performance of microorganisms with minimal costs and material; therefore, it is extensively used to optimize some conditions for the biotechnological process, such as carbon and nitrogen source and concentration, microelement presence, among others. However, the production in shaker incubator presents several limitations such as limited oxygen transfer, and inability to control pH and dissolved oxygen tension. Moreover, for industrial application, high amount of product is necessary and the production in bioreactor can improve the process reducing the product final cost (Gamboa-Suasnavart et al. 2013).

In bioreactor, the operation mode can generate high productivities. It can be carried out as batch (all nutrients required for the culture are added at the beginning of the cultivation, whereas the product, by-products, and non-consumed components are removed at the end of each batch), fed-batch (some nutrients are provided during the process until a limitation of volume, and the product is removed at the end of each batch), and continuous fermentation (nutrients are added continuously, and product is removed at the same speed of the feeding flow, with the volume inside the bioreactor remaining constant) (Torres et al. 2016). Currently, there are several reports exploring the production of L-ASNase in shaken flasks and a few in bioreactor. However, with the market need for this enzyme, further studies in bioreactor are mandatory. To exemplify how the production step is important, de Oliveira et al. (2019) studied the production of natural colorants with antimicrobial properties, obtaining a 30-fold increase varying only the culture media in shaken flask experiments after 168 h of bioprocess. Later on, the same authors working with bioreactor stirred tank under batch cultivation reduced the time of bioprocess for 120 h, achieving similar amount of colorants (De Oliveira et al. 2020). Regarding L-ASNase production, Kumar et al. (2011a, b) with *Pectobacterium carotovorum* MTCC 1428 produced an enzyme with 17.81 IU·mL^{-1} of activity in shake flask level. Kumar et al. (2011a, b) working with the same microorganism but in batch and fed-batch mode feeding L-asparagine and/or glucose, produced 18 and 38.8

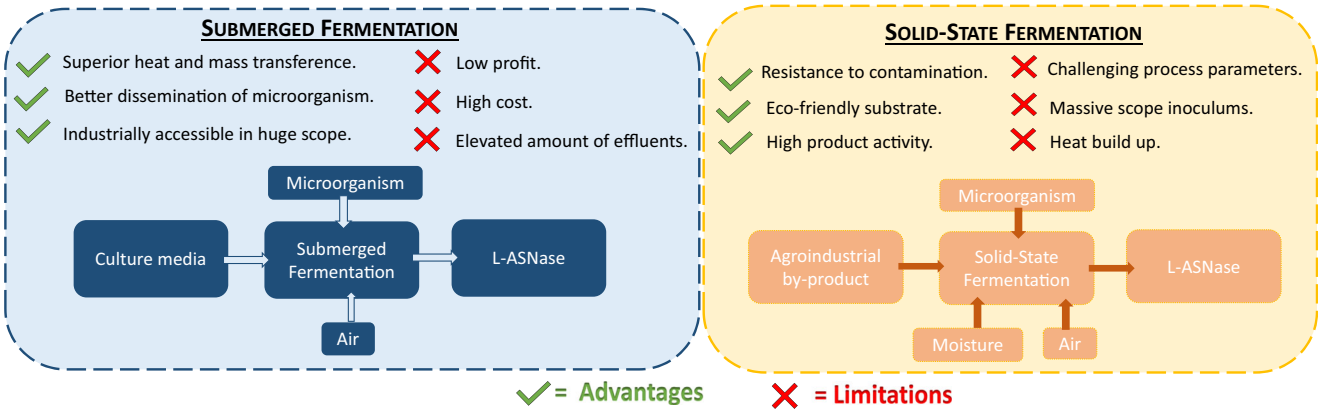

Fig. 4 Comparison between submerged and solid-state fermentations for the production of L-asparaginase. Adapted from Doriya et al. (2016)

IU·mL^{-1}, respectively, demonstrating the importance of studies in bioreactor.

Production of L-ASNase from various microbial sources by SmF and the respective optimized conditions reported in the literature are summarized in Table 5.

The most frequently reported culture media for L-ASNase production by SmF are Luria-Bertani (LB) medium, tryptone glucose yeast extract broth, and modified Czapek-Dox medium with optimal pH ranging from 6.2 to 7.5, temperature from 28 to 37 °C, and fermentation times ranging from 24 to 168 h depending upon the type of the employed microorganism (Dharmaraj 2011; Usha et al. 2011; Gurunathan and Renganathan 2012; Einsfeldt et al. 2016; Vimal and Kumar 2017; El-Naggar et al. 2018). Using LB broth as medium, a L-ASNase with an activity of 8.7 IU mg^{-1} and 23.85 IU mg^{-1} was obtained from *Yersinia pseudotuberculosis YpA* and *B. subtilis* hswx88, respectively (Pokrovskaya et al. 2012; Jia et al. 2013).

According to Singh et al. (2013), the production of L-ASNase from *B. aryabhattai ITBHU02,* using M9 medium and L-asparagine as nitrogen source, reached an enzymatic activity of 9.88 IU mg^{-1} with optimal temperature and pH of 40 °C and 8.5, respectively (Singh et al. 2013). The M9 medium and L-asparagine as nitrogen source was also used by Chakravarty et al. (2021) in the study of L-ASNase production by *B. australimaris*. The authors performed the experiments in incubator shaker and using Box-Behnken design achieved an enzyme production of 37.93 IU mL^{-1} at the following conditions: 48 h of incubation time, 35°C, 1.25% (*w*/*v*) of inoculum, and 2.5% (*w*/*v*) of L-asparagine (Chakravarty et al. 2021). The authors also identified the L-ASNase gene and cloned it in *E. coli* using pET30b vector and demonstrated that the L-ASNase produced was type II (Chakravarty et al. 2021).

Erva et al. (2017) produced L-ASNase from *Enterobacter aerogenes MTCC111* with an activity of 18.35 IU mL^{-1} applying trisodium citrate (0.75% (*m*/*v*)) and ammonium chloride (0.15% (*m*/*v*)) for 40 h at 33°C (Erva et al. 2017). Using *Emericella nidulans* to produce L-ASNase, Jayaramu et al. (2010) obtained a protein with an activity of 1.1 IU mL^{-1} with fermentation period of 48 h, at 30 °C, and pH of 6.0 (Jayaramu et al. 2010). These results indicate that each potential producing strain requires its own specific conditions, and there are no established fixed parameters for Smf. Thus, specific optimization studies need to be performed after the microorganism selection.

As aforementioned, L-ASNase production by SmF from recombinant microbial strains, such as *E. coli*, has been employed aiming to meet the current market demand. However, as depicted in Table 5, there are several studies performed in SmF with other potential microorganisms that can result in high yields of L-ASNase and these enzymes can be applied in the food industry.

Considering SFF, it emerged as an alternative to SmF for the production of extracellular enzymes as it allows the direct use of crude fermented product as enzyme source and has the potential for the production of secondary metabolites (Lopes et al. 2017). Generally, this process uses cheap agriculture waste such as rice bran, wheat bran, sesame oil cake, corn cob, soybean meal, gram husk, coconut oil cake, groundnut cake, and tea waste (Vimal and Kumar 2017). The use of agricultural wastes not only makes the procedure less cost effective but also reduces the environmental pollution (Vimal and Kumar 2017). In this fermentation process, substrates are used slowly and steadily by the microorganism. That means the same substrate can be used for long fermentation periods (Nadu 2012). In fact, SSF is more relevant for fermentation processes involving fungi and microorganisms that require less moisture content. It does not suit fermentation processes involving organisms that require a high water activity, such as bacteria (Babu and Satyanarayana 1996; Nadu 2012). Additionally, this process offers benefits such as low energy and equipment requirement, cheaper growth substrates, and the downstream processes can be easier since the fermentation process can provide more concentrated solutions, turning unnecessary the use of concentrating unit operations (Holker and Lenz 2005). However, when compared

Table 5 L-asparaginase production by submerged culture at various operating conditions

Microorganism	Culture medium type	Temperature (°C)	Initial pH	Cultivation period (h)	Stirring (rpm)	L-ASNase activity	Reference
*Spirulina maxima**	Modified Zarrouk medium	25	9.5	432	-	51.28 IU L^{-1}	Abd El Baky and El Baroty (2016)
Pectobacterium carotovorum MTCC 1428	Glucose 2.076 (g/L), L-asparagine 5.202 (g/L), $Na_2HPO_4.2H_2O$ 6.0 (g/L), KH_2PO_4 1.772 (g/L), NaCl 0.5 (g/L), $MgSO_4.7H_2O$ 0.373 (g/L), $CaCl_2.2H_2O$ 0.015 (g/L), yeast extract 1.0 (g/L), and peptone 1.0 (g/L)	30	7.0	30	-	38.8 IU L^{-1}	Kumar et al. (2017)
Enterobacter aerogenes MTCC11	Trisodium citrate (75%), ammonium chloride (15%)	33	6	40	-	18.35 IU mL^{-1}	Erva et al. (2017)
Bacillus australimaris NJB19	M9 medium**, 2.5% (*w/v*) L-asparagine	33.5	6.77	48	-	37.93 IU mL^{-1}	Chakravarty et al. (2021)
Bacillus subtilis WB800N	Luria-Bertani medium	37	8.5	-	-	105 IU mL^{-1}	Chityala et al. (2015)
Nocardia levis MK-VL 113	Asparagine-glycerol salts (ISP-5) broth	30	7	72	-	5.06 ± .002 IU mg^{-1}	Kavitha and Vijayalakshmi (2012)
Aspergillus terreus MTCC 1782	Czapek–Dox medium + L-asparagine 1%, yeast extract 1%, peptone 6%, glucose 4%	35	6	72	160	24.10 IU mL^{-1}	Gurunathan and Renganathan (2012)
Emericella nidulans	Czapek–Dox medium	30	6	48	-	1.1 IU	Jayaramu et al. (2010)
Escherichia coli	-	37	7.2	24	220	67 IU mg^{-1}	Khushoo et al. (2004)
Escherichia coli BL21*	Luria-Bertani medium, glucose 1%, kanamycin 50 μg/mL	37	7	-	200–800	3.6 IU mL^{-1}	Einsfeldt et al. (2016)
Escherichia coli K-12	Lactose 10 g/L, tryptone 10 g/L, yeast extract 5 g/L, L-asparagine 2 g/L, and $CaCl_2$ 15 g/L	37	6.5	-	200	3.82 IU mL^{-1}	Vimal and Kumar et al. (2017)
Erwinia chrysanthemi 3937	Tryptone, glucose, yeast extract broth	28	7	-	-	7.7 IU mg^{-1}	Kotzia and Labrou (2007)
Erwinia carotova	Luria-Bertani medium	37	-	-	-	0.72 IU mg^{-1}	Kotzia and Labrou (2005)
Bacillus sp. (DKMBT10)	KH_2PO_4 2.0, L-asparagine 6.0, MgSO4.$7H_2O$ 1.0, $CaCl_2.2H_2O$ 1.0, and glucose/maltose 3.0	37	7	24	200	1 IU mg^{-1}	Moorthy, V; Ramalingam, A: Sumantha 2010
Pseudomonas fluorescens	Glucose, beef extract, L-asparagine, salt solution	37	8	48	-	168.4 IU mL^{-1}	Prema et al. (2013)
Bacillus aryabhattai ITBHU02	M9 medium**, L-asparagine	40	8.5	-	200	9.88 IU mg^{-1}	Singh et al. (2013)
Bacillus brevis	Fructose, liquid paraffin	30	7	-	-	2.036 IU mg^{-1}	Narta et al. (2011)
Bacillus licheniformis	Asparagine broth medium	37	6.5	72	250	7.78 IU mg^{-1}	Alrumman et al. (2019)
Bacillus subtilis hswx88	LB medium	37	7	-	160	23.85 IU mg^{-1}	Jia et al. (2013)
Marine actinomycetes S3	TGY extract broth	28	7	24	200	384.6 IU mg^{-1}	Saleem Basha et al. (2009)
Penicillium brevicompactum NRC 829	Czapek–Dox medium	-	-	-	-	574.24 IU mg^{-1}	Elshafei et al. (2014)
Pseudomonas stutzeri MB-405	L-asparagine and Na-succinate	30	7	20	160	1.1 IU mg^{-1}	Saleem Basha et al. (2009)
Streptomyces brollosae NEAE-115	Asparagine dextrose, starch	37	7	168	150	9.79 IU mg^{-1}	El-Naggar et al. (2018)
Streptomyces noursei MTCC 10469	Tryptone, glucose, yeast extract broth	28	7	24	200	0.803 IU mg^{-1}	Dharmaraj (2011)

Table 5 (continued)

Microorganism	Culture medium type	Temperature (°C)	Initial pH	Cultivation period (h)	Stirring (rpm)	L-ASNase activity	Reference
Streptomyces parvalus KUA 106	Tryptone, glucose, yeast extract broth	28	7	24	-	146 IU mL^{-1}	Usha et al. (2011)
Vibrio succinogenes	Sodium fumarate, cysteine	37	7.3–7.4	-	-	-	Krautheim et al. (1982)
Vibrio succinogene	Succinate	37	7.0–7.2	18–22	-	8.4 IU mg^{-1}	Kafkewitz and Goodman (1974)
Yersinia pseudotuberculosis YpA	Luria-Bertani broth	37	-	-	150–180	8.7 IU mg^{-1}	Pokrovskaya et al. (2012)

*Bioreactor experiments

**M9 medium: 0.6% $Na_2HPO_4.2H_2O$ (*w/v*), 0.3% KH_2PO_4 (*w/v*), 0.05% NaCl (*w/v*), 2.5% L-asparagine/L-glutamine (*w/v*), 1 M $MgSO_4.7H_2O$, 0.1 M $CaCl_2.2H_2O$, and 20% (*w/v*) glucose. International unit (IU) of asparaginase activity is defined as the amount of enzyme required to release 1 μmol of ammonia per minute at specified conditions

with SmF, only few reports are available on SFF (Table 6) for the L-ASNase production.

Venil and Lakshmanaperumalsamy (2009) produced an L-ASNase with an activity of 79.84 IU g^{-1} using a modified strain of *Serratia marcescens* grown in rice bran for 36 h with 50% moisture, at 30 °C, and pH of 7.0 (Venil and Lakshmanaperumalsamy 2009). Suresh and Raju (2012) optimized the production of L-ASNase by SSF from *Aspergillus terreus* MTCC 1782 using different culture media, such as sesame oil cake (SOC), black gram husk (BH), and a mix of both, with temperature and moisture ranging from 30 °C to 32 °C, and 40% to 60%, respectively. The fermentation period ranged between 96 h and 120h. The optimal culture medium ended up being the mixture of SOC and BH (7:3), reaching an enzymatic activity of 163.34 IU g^{-1}, while using just black gram husk resulted in an enzyme with 15.95 IU g^{-1} of activity (Suresh and Raju 2012). Reports from Mishra (2006) revealed an L-ASNase with an activity of 40.9 U g^{-1} using bran of *Glycine max* with a 70% moisture for 96 h at 30°C and pH of 6.5 (Mishra 2006). On a similar study, El-Bessoumy et al. (2004) used soy bean meal as a culture medium with a 50% moisture during 96 h, at 37 °C, and pH of 7.4 to produce an L-ASNase with an activity of 1900 IU mg^{-1} from *Pseudomonas aeruginosa* 50071 (El-Bessoumy et al. 2004). Using a genetically modified fungi (*Aspergillus flavus* KUFS20), Rani et al. (2011) applied orange peel with a moisture of 40% during 96 h, at 35 °C, and pH of 6.0 to reach an enzyme activity of 339.16 IU g^{-1} (Rani et al. 2011).

Operating conditions influencing L-asparaginase production

The optimum production period for L-ASNase from microbial sources varies from 24 to 72 h, depending on the microorganism employed. The lowest optimum cultivation time for production of this enzyme was reported in *Staphylococcus aureus* strain NCTC413 corresponding to a total of 10 h (Chand et al. 2020). On the other hand, among the analyzed studies, the highest optimum period for L-ASNase production bioprocess was registered for *Spirulina maxima* with 432 h (18 days), which allowed to obtain an activity of 51.28 IU L^{-1} (Abd El Baky and El Baroty 2016). Among the reported actinomycetes, the maximum enzyme production of 8.79 U mg^{-1} was obtained after a culture period of 144 h for *Streptomyces brollosae* NEAE-115 using dextrose starch as production medium (El-Naggar et al. 2018).

Besides the fermentation period, one of the most essential parameters in bioprocessing is the temperature. Optimum temperatures reported for L-ASNase production by most microorganisms ranged from 25 °C to 37 °C. In fact, cultivation temperature has a direct effect on the development of microorganisms and, consequently, affects the enzyme production and its activity (Ghosh et al. 2013). L-ASNase produced from

Table 6 L-asparaginase production by solid-state culture at various operating condition

Microorganism	Culture medium	Moisture (%)*	Temperature (°C)	pH	Cultivation period (h)	L-ASNase activity	Reference
Aspergillus niger	Soya bean meal	70	30	6.5	96	40.9 IU g^{-1}	Mishra (2006)
Aspergillus flavus (KUFS20)	Orange peel	40	35	6	96	339.16 IU g^{-1}	Rani et al. (2011)
Aspergillus terreus MTCC 1782	Sesame oil cake (SOC)	40	30	-	96	68.49 IU g^{-1}	Suresh and Raju (2012)
Aspergillus terreus MTCC 178	Black gram husk (BH)	40	30	-	96	15.95 IU g^{-1}	Suresh and Raju (2012)
Aspergillus terreus MTCC 1782	(SOC+BH) (7:3)	40	30	-	96	74.21 IU g^{-1}	Suresh and Raju (2012)
Aspergillus terreus MTCC 1782	(SOC+BH) (7:3)	60	32	7	120	163.34 IU g^{-1}	Suresh and Raju (2012)
Cladosporium sp.	Wheat bran	-	30	-	120	0.096 IU g^{-1}	Mohan Kumar and Manonmani (2013)
Fusarium solani AUMC 8615	Maltose	60	30	8	-	438.4 IU mL^{-1}	Isaac and Abu-Tahon (2016)
Fusarium equiseti	Soybean meal, glucose, yeast extract	-	45	7	48	3.26 IU mL^{-1}	Hosamani and Kaliwal (2011)
Pseudomonas aeruginosa 50071	Soya bean meal	50	37	7.4	96	1900 IU mg^{-1}	El-Bessoumy et al. (2004)
Serratia marcescens (NCIM 2919)	Sesame oil cake	68.64	37	-	96	110.80 IU g^{-1}	Ghosh et al. (2013)
Serratia marcescens (NCIM 2919)	Coconut oil cake	40	35	6	24	3.87 IU g^{-1}	Ghosh et al. (2013)
Serratia marcescens (NCIM 2919)	Citrus limetta pulp	60	28	7.5	48	83.16 IU g^{-1}	Kumar et al. (2011a, b)
Serratia marcescens SB08	Rice bran	50	30	7	36	79.84 IU g^{-1}	Venil and Lakshmanaperumalsamy (2009)

*Ratio of the water mass in the sample to the mass of solids in the sample. International unit (IU) of asparaginase activity is defined as the amount of enzyme required to release 1 μmol of ammonia per minute at specified conditions

B. licheniformis presented high enzyme yield (7.78 IU mL^{-1}) at an optimum temperature of 37 °C, while increasing the temperature to 47 °C results on a reduction in the enzyme activity down to 32.19% (Alrumman et al. 2019). Aside from this, the same microorganisms may have distinct optimum temperatures when supplied with different culture medium. Ghosh et al. (2013) demonstrated that *Serratia marcescens* NCIM 2919 incubated with *Citrus limetta* pulp showed an optimal temperature of 28 °C, while when incubated with a medium made up of coconut oil cake and sesame oil cake revealed an optimal temperature of 35 °C and 37 °C, respectively, indicating once again the complexity of establishing an optimized industrial bioprocess (Ghosh et al. 2013). Nevertheless, there are organisms like *Streptomyces gulbargensis* and *Fusarium equiseti* with higher optimal temperatures (40 °C and 45 °C, respectively), which when applied to other enzymes results in lower production or lower activity (Amena et al. 2010; Hosamani and Kaliwal 2011). These microorganisms (*S. gulbargensis* and *F. equiseti*) can be considered as sources to be explored further for production of heat-resistant L-ASNase for food processing.

The pH of the culture also affects the bioprocessing of the enzyme alongside the transport of several components across the cell membrane (Chand et al. 2020). The regulation of pH is indispensable while using carbon sources like glucose, fructose, or mannitol once it decreases the pH of the medium. This drop occurs as a result of acid production in the fermentation process which leads to inhibition of L-ASNase production (Alrumman et al. 2019). Several studies report that the optimum pH to produce L-ASNase from bacterial sources is usually close to 7.

According to Moorthy and Sumantha (2010) and Narta et al. (2011), both *Bacillus* sp. and *Bacillus brevis* produced L-ASNases with maximal enzyme activity at pH 7.0, revealing a specific activity of 1 IU mg^{-1} and 2.036 IU mg^{-1}, respectively (Moorthy and Sumantha 2010; Narta et al. 2011). Prema et al. (2013) demonstrated that *Pseudomonas fluorescens* produced L-ASNase with an activity of 168.4 IU mL^{-1} at an optimal pH of 8 (Prema et al. 2013). Regarding to *Vibrio* species, there are some reports from 70s and 80s such as the bacteria *Vibrio succinogenes* studied by Kafkewitz and Goodman (1974) which revealed an optimal pH between 7.0 and 7.2 for L-ASNase production using succinate as medium, and Krautheim et al. (1982) which demonstrated that using sodium fumarate and cysteine as medium the optimal pH is 7.2–7.3. Recently, L-ASNase from *Vibrio* species has been overexpressed recombinantly in *Escherichia coli* (Radha et al. 2018; Radha and Gummadi 2020).

Overall, new species or recombinant microorganism that overexpress L-ASNase with improved characteristics such as glutaminase free, stability, and promote fewer collateral effects are key factors to enlarge the application of L-ASNase.

Industrial developments to produce L-asparaginase from microbial source

Despite the growing demand for L-ASNase worldwide in the last decades, only a few commercial products have been widely spread throughout the market and are well established in major industries and biotechnology companies.

Considering therapeutical applications, some of the most successful formulations for use against ALL are the native *E. coli* L-ASNase Elspar® (Merck & Co., Inc., USA), its pegylated form Oncaspar® (Sigma-Tau Pharmaceuticals, USA), and the *E. chrysanthemi* L-ASNase Erwinaze® (Jazz Pharmaceuticals, USA). These L-ASNase formulations have been already produced in several countries under different brand names, such as Medac® (Kyowa Hakko, Japan), Crasnitin® (Bayer AG, Germany) (Shrivastava et al. 2016; Pieters et al. 2011), Aginasa (Medac, GmbH), and Leuginase ((Beijing SL Pharmaceutical, China) (Araujo et al. 2021). Both L-ASNase preparations share the same therapeutic mechanism but differ considerably in their pharmacokinetics and immunological properties (Araujo et al. 2021).

Due to the increasing health concerning by authorities and industrialized food consumers, food industries have suffered pressure to offer healthier products. In that context, alternatives for prevention of acrylamide formation begin to emerge in the market. PreventASe™ from DSM (the Netherlands) is produced by *Aspergillus niger* and present indications to reduce up to 90% in acrylamide levels. The commercial product was released in the market in 2007 and presents an acidic profile (optimum pH 4.0–5.0, temperature 50°C). Other often applied commercial L-ASNase is the branded Acrylaway® from Novozymes A/S (Denmark), obtained from *Aspergillus oryzae,* that presents neutral biochemical characteristics (optimum pH 7.0, temperature 37°C) (Xu et al. 2016).

Pedreschi et al. (2008) pioneered in publishing results using a commercial L-ASNase for food process purposes (Acrylaway®). The authors established that the optimum temperature and pH for this enzyme are 60°C and 7.0, respectively. In these conditions, it was reached a 67% reduction in acrylamide in tested French fries. The authors highlighted the importance of blanching and temperature control in the food treatment (Pedreschi et al. 2008). Hendriksen et al. (2009) reported the use of Acrylaway® and its effectiveness in a wide range of foods, e.g., gingerbread, crispbread, semi-sweet biscuits, french fries, and crisps (Hendriksen et al. 2009). New similar products had been launched since then, such as Acrylaway® HighT in 2013 (Novozymes A/S, Denmark), an enzyme specifically designed for higher temperature processing, expanding its applicability (Xu et al. 2016).

Conclusion and future perspectives

L-ASNase catalyzes the hydrolysis of L-asparagine which is of high importance in healthcare and food industries. This enzyme is naturally produced by a high number of microorganisms; nevertheless, only a few provide enough enzymes with the desired and improved biochemical properties that make them commercially and economically viable. Microbial L-ASNases are different from each other in terms of biochemical parameters, such as optimal pH and temperature, molecular weight, kinetic properties, and stability. Currently, *E. coli* and *E. chrysanthemi* genes are the main sources of commercial L-ASNase. However, both enzymes face the challenge of resilient immunogenicity and clinical resistance, which consequently affect its application. Thus, L-ASNases from new sources stand out as promising alternatives. However, their use faces several obstacles, such as enzyme activity, kinetic parameters, and thermal and storage stability, characteristics that diverge from the optimal for establishing applicable and feasible bioprocesses. Still, sources such as *A. flavus*, marine *Actinomycetes*, *P. aeruginosa*, and recombinant *Bacillus* show promising characteristics for industrial production. Moreover, studies in bioreactor are still required aiming to improve L-ASNase yields from potential novel sources. Following the wide application and importance of L-ASNase preparations, further studies should be conducted in order to reduce production costs, adverse reactions, and clinical side effects.

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Declarations

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