L-asparaginase production review: bioprocess design and biochemical characteristics

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Abstract

In the past decades, production of biopharmaceuticals has gained high interest due to its high 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 empowers the scientists to use, modify and improve the efficiency of enzymes leading to a maximum functionality and high value are obtained (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 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, formulations of this protein represents 40% of the total enzyme demands worldwide and one-third of the global needs for anticancer agents, which is far more than other therapeutic enzyme (Izadpanah et al. 2018). The biopharmaceutical world market, especially the healthcare market, is constantly growing. Therapeutical enzymes 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).

Moreover, L-ASNase is 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 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, 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.

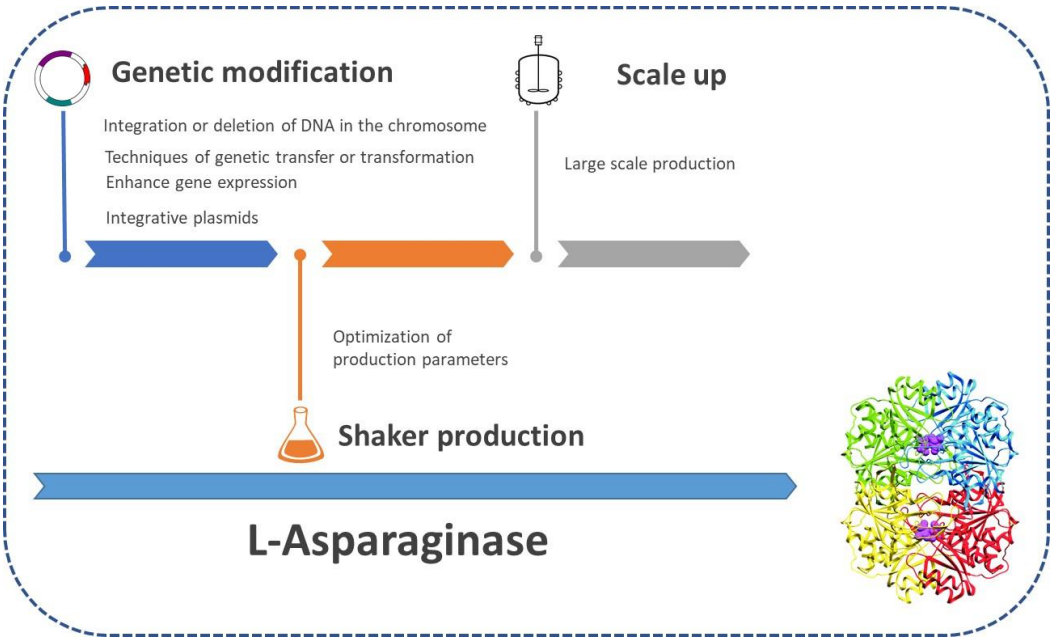

Figure 1. Overview of L-Asparaginase production by recombinant microorganisms.

Fig. 1

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-types L-ASNase (including the classification type I and type II), (ii) Plant-types L-ASNase (type III), and (iii) Rhizobial types L-ASNase (Borek and Jaskólski 2001; Qeshmi et al. 2018). Bacterial-types L-ASNases are structurally and evolutionarily distinct from the plant-type (Michalska and Jaskolski 2006). The bacterial-types L-ASNase are subdivided in type 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 the Figure 2.

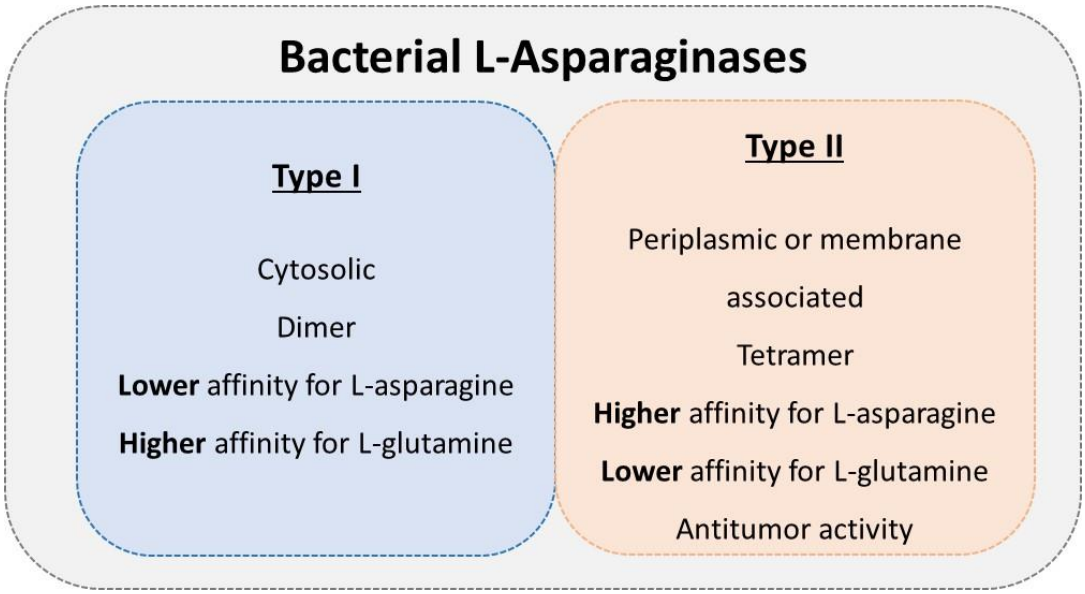

Figure 2. Mainly characteristics of bacterial L-Asparaginase type I and type II.

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 Figure 3 for the three-dimensional structure of *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 of type-II.

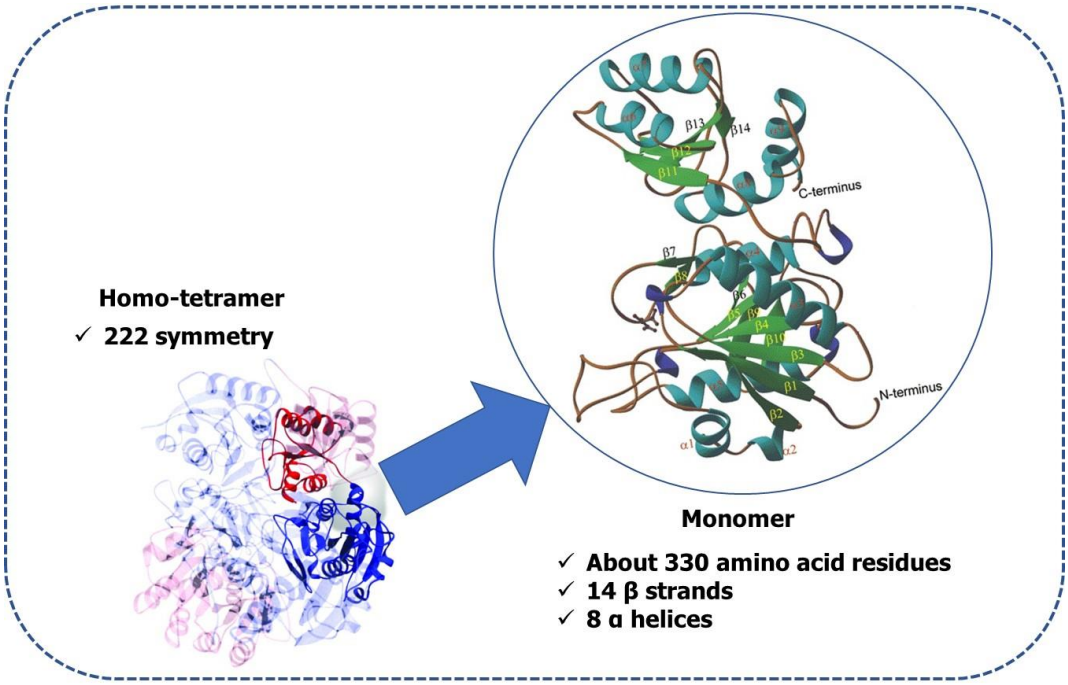

Figure 3. Three-dimensional structure of *Erwinia chrysanthemi* L-Asparaginase type II suggested Aghaiypour et al. (2001) and Lubkowski et al. (2003).

Fig 3

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 formulations is also presented at **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.

Table 1. Properties of different L-Asparaginase preparations.

Source	Molecular weight (kDa)	Isoeletric point (pl)	K_m (μM) Asparagine	K_m (mM) Glutamine	Half lifetime	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	MD*	5290	nd	1 h	(Feng et al. 2017)

nd: not determined.

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 reactions toward 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 et al. 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 (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 section *Production of recombinant L-Asparaginase.* Considering the production process, the L-ASNase produced by *E. coli* is intracellular, which insert one unit operation in the downstream processes. Actinomycetes are filamentous bacteria well known as a good source of antibiotics and these microorganisms such as *Sreptomyces griseoluteus*, *Nocardia levis* and *Streptomyces ginsengisoli* were also reported to be potential producers of L-ASNase (Orabi 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 *S. cyaneus (*SAP 1287, CFS 1560), *S. exfoliates* (CFS 1557) and *S. 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 (Dhevagi and Poorani 2006) being this source of L-ASNase an alternative for the food industry.

Fungal L-ASNases are commonly produced extracellularly, easing 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 are 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 *Trichoderma* have been reported as L-ASNase producers (Chand et al. 2020; Kil et al., 1995; Foda et al., 1980). *S. 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.*, C*hlorella vulgaris*, *Spirulina maxima*, are considered as potential microalgal sources for novel enzymes production in several studies (Orabi et al. 2019; Ebrahiminezhad et al., 2014; Abd El-Baky and El-Baroty, 2016).

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

Microorganism	**Strain**	**Localization**	**K_m (μM)**	**k_{cat} (s^{-1})**	**V_{max}**	**Reference**
Bacteria	*Bacillus lichenformis*	Extracellular	14	2.68 x 10^3	4.03 IU	(Mahajan et al. 2014)
Bacteria	*Bacillus* sp.	Extracellular	nd	nd	nd	(Singh and Srivastava 2012)
Bacteria	*Serratia marcescens*	Intracellular	100	nd	nd	(Boyd and Phillips 1971)
Bacteria	*Vibrio succinogenes*	Intracellular	17	nd	nd	(Kafkewitz and Goodman 1974)
Bacteria	*Erwinia carotovora*	Intracellular	96	nd	1632.6 μmol mg^{-1} min^{-1}	(Warangkar and Khobragade 2010)
Algae	*Chlamydomonas species*	Intracellular	134	nd	nd	(Paul 1982)
Algae	*Spirulina maxima*	Intracellular	nd	nd	nd	(Abd El Baky and El Baroty 2016)
Actinomycete	*Streptomyces phaeochromogenes*	Extracellular	nd	nd	nd	(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 x 10^3	nd	nd	(Patro 2012)
Fungi	*Fusarium* sp.	Extracellular	443.98 x 10^3	nd	40 IU	(Asha and Pallavi 2012)
Yeast	*Candida utilis*	Extracellular	nd	nd	nd	(Kil et al. 1995)
Yeast	*Pichia polymorpha*	Extracellular	13.7 x 10^3	nd	nd	(Foda et al. 1980)
Yeast	*Saccharomyces cerevisiae*	Extracellular	nd	nd	nd	(Dunlop and Roon 1975)

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

Table 2

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 industry seeks for some characteristics, namely 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 disclosure 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 *B. 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, some work has been done

on alternative hosts such as *B. subtilis* (Feng et al. 2017; Sushma et al. 2017; Li et al. 2018) 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 cheaper. 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.

Table 3

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 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 $\mu M\ min^{-1}$	(Jia et al. 2013)
L-ASNase	*Bacillus tequilensis*	*E. coli* BL21 (DE3)	pET28a+	IPTG-inducible	Intracellular	0.07	nd	7.79 $\mu M\ min^{-1}$	(Shakambari et al. 2018)
L-ASNase type II	*Cobetia amphilecti*	*E. coli* BL21 (DE3)	pQE80L	IPTG-inducible	Intracellular	2.05	nd	11641 $\mu M\ min^{-1}\ mg^{-1}$	(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; pET14b	Constitutive	Intracellular	0.029-0.080	440-798	nd	(Moola et al. 1994; 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; Nguyen et al. 2016)
L-ASNase type II	*Escherichia coli*	*Pichia pastoris* Super Man5 (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		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	162.2 µmol min^{-1} mg^{-1}	(Shi et al. 2017)
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 kodakarensis*	*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); 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 one µmol of ammonia per minute at specified conditions.

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 L-ASNase activity 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*, *B. megaterium* and *P. 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 Kumar et al. (2020), a pH value close to 8.0 is needed for a maximum activity for L-ASNase from *B. subtillis*. The enzyme produced by *Penicillum sp.* and *A. 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 *P. aeruginosa* PAO1 and *R. 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 ºC and 45 ºC (Chand et al. 2020). Nevertheless, the extreme thermophiles *T. kodaka* (TK1656) and *T. kodaka* (TK2246) produce L-ASNases with

optimal activity at 85 ºC (Chohan et al. 2020; Muneer et al. 2020). The study performed by Kumar et al. (2020), the authors concluded that the L-ASNase produced by *B. subtillis* shows an optimal activity at 37ºC. Additionally, authors showed that this enzyme is also active in a wide range of temperature from 30 ºC to 75 ºC; yet, at the maximum temperature will eventually lead to an unstable enzyme with no application (Kumar et al. 2020). Similarly, Patro and Gupta (2012) obtained a L-ASNase from *Penicillum sp* with optimal activity at 37 ºC. The authors determined the optimal temperature for the enzyme using a range of temperatures between 30 ºC and 50 ºC. The study performed by Borah *et al*. (2012), shows the production of L-ASNase from *E. coli* and the optimal enzyme activity was achieved at 55 ºC. As well, on this study the authors defined that the enzyme produced was able to tolerate high temperatures and hence can be considered an 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 application. L-ASNase immobilization and confinement techniques are interesting to maintain the enzyme biochemical properties.

Table 4

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. 2020)
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 aeruginosaPAO1	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)

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 to catalytic efficiency improvement and

consequently a higher yield for biotechnological purpose of the enzyme (Krishnapura et al. 2016). In fact, 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 *E. 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

The 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 hyper sensibility 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, a 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 *B. 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, e.g. 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 mechanisms systems and need to be deeply studied

before efficient and safe application. Kinect characterization has a key role in understanding enzymes 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 limitation for both fermentation types of processes.

Figure 4

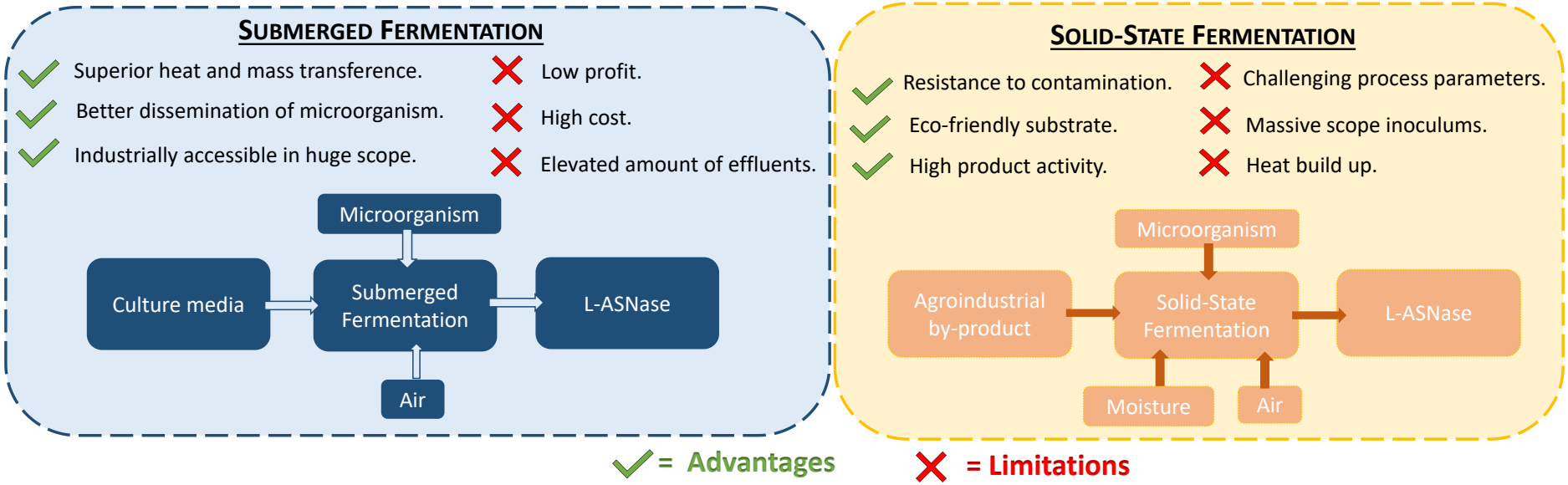

Figure 4. Comparison between submerged and solid-state fermentations for the production of L-ASNase. Adapted from Doriya et al. 2016.

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 reactor 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 flasks 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, microelements 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 amounts 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 and 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, 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 a bottleneck to be explored. Production of L-ASNase from various microbial sources by SmF and the respective optimized conditions reported in the literature are summarized in **Table 5**.

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

Microorganism	Culture medium type	Temperature (ºC)	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)
Enterobacter aerogenes MTCC11	Trisodium citrate (75%), Ammonium chloride (15%)	33	6	40	-	18.35 IU mL^{-1}	(Erva et al. 2017)
*Pectobacterium carotovorum MTCC 1428**	Glucose (30 g/L) L-Asparagine (30 g/L)	30	8.5	Feb-batch mode	-	38.8 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)
Azotobacter vinelandii	Sucrose	30	7.4	-	-	4 IU mg^{-1}	(Gaffar and Shethna 1977)
Emericella nidulans	Czapek–Dox Medium	30	6	48	-	1.1 IU	(Jayaramu et al. 2010)
E. coli	-	37	7.2	24	220	67 IU mg^{-1}	(Khushoo et al. 2004)
E. coli BL21*	LB medium, Glucose 1%, kanamycin 50 µg/mL	37	7	-	200-800	3.6 IU mL^{-1}	(Einsfeldt et al. 2016)
E. coli K-12	Lactose; 10 g/L, Tryptone; 10 g/L, yeast extract; 5 g/L,	37	6.5	-	200	3.82 IU mL^{-1}	(Vimal and Kumar 2017)

	L-Asparagine; 2 g/L and $CaCl_2$; 15 g/L						
Erwinia aroideae	Tryptone, glucose, yeast extract broth	28	7	-	-	-	(Peterson and Ciegler 1969)
Erwinia chrysanthemi 3937	Tryptone, glucose, yeast extract broth	28	7	-	-	7.7 IU mg^{-1}	(Kotzia and Labrou 2007)
Erwinia carotova	LB 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)
Acinetobacter glutaminasificans (ATCC 27197)	L-Glutamic acid	-	-	-	-	150 IU mg^{-1}	(Steckel et al. 1983)
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)
Corynebacterium glutamicum	Tryptone soya broth	30	7.3	24	-	20.6 IU mg^{-1}	(Mesas et al. 1990)

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)
Serratia marcescens	4% (w/v) autolyzed yeast media	26	5	40	-	0.7 IU mg^{-1}	(Boyd and Phillips 1971)
Serratia marcescens	Glycerol, peptone, yeast extract	37	6.8-7.0	24	-	2.4 IU mL^{-1}	(Sukumaran et al. 1979)
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)
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	LB broth	37	-	-	150-180	8.7 IU mg^{-1}	(Pokrovskaya et al. 2012)

*: Bioreactor experiments

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 ºC and fermentation times extending 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). Using *Serratia marcescens* and glycerol, peptone and yeast extract as medium, Sukumaran et al. (1979) recovered an enzyme with 2.4 IU mg^{-1} activity, optimal temperature of 37 ºC, optimal pH ranging from 6.8 to 7.0, and fermentation time of 24h (Sukumaran et al. 1979). Boyd and Phillips (1971) recovered an activity of 0.7 IU mg^{-1} at 26 ºC, pH of 5.0 and fermentation time of 40 h when studying the same enzyme from the same source (Boyd and Philips 1971). 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 33ºC (Erva et al. 2017). Reports from Gaffar and Shethna (1977) revealed a L-ASNase with a specific activity of 4 IU mg^{-1} when applying sucrose as carbon source at 30 ºC and pH of 7.4 (Gaffar and Shethna 1977). 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 ºC 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 stablished 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 at **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 makes the procedure not only less cost effective but also it 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 an high water activity, such as bacteria (Babu and Satyanarayana 1996; Nadu 2012). Additionality, 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 making concentrating unit operations unnecessary (Holker

and Lenz 2005). However, when compared with SmF, only few reports are available on SFF (**Table 6**) for the L-ASNase production.

Table 6

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

Microorganism	Culture medium	Moisture (%)*	Temperature (ºC)	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 2011a)
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. 2011)
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.

Venil and Lakshmanaperumalsamy (2009) produced an L-ASNase with an activity of 79.84 IU g^{-1} using a modified strain of *S. mercescens* grown in rice bran for 36 h with 50% moisture, at 30 ºC 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 ºC to 32 ºC, 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 30ºC 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 ºC 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 ºC and pH of 6.0 to reach an enzyme activity of 339.16 IU g^{-1} (Rani et al. 2011).

Operational 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). Mesas et al. (1990) used *Corynebacterium glutamicum* to produce an L-ASNase with an activity of 20.6 IU mg^{-1}, value that did not change significantly with the time of cultivation suggesting that this enzyme might be constitutive in this strain (Mesas et al. 1990).

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 ºC to 37 ºC. 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 *B. licheniformis* presented high enzyme yield (7.78 IU mL^{-1}) at an optimum temperature of 37 ºC, while increasing the temperature to 47 ºC results on a reduction in the enzyme activity up 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 ºC, while when incubated with a medium made up of coconut oil cake and sesame oil cake revealed an optimal temperature of 35 ºC and 37 ºC, respectively, indicating once again the complexity of stablishing an optimized industrial bioprocess (Ghosh et al. 2013). Nevertheless, there are organisms like *Streptomyces gulbargensis* and *Fusarium equiseti* with higher optimal temperatures (40 ºC and 45 ºC, 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 et al. (2010) and Narta el 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 et al. 2010, Narta el 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 the bacteria *Vibrio succinogenes*, Kafkewitz and Goodman (1974) revealed an optimal pH between 7.0 and 7.2 for L-ASNase production using succinate as medium, while Krautheim et al. (1982) demonstrated that using sodium fumarate and cysteine as medium the optimal pH is 7.2-7.3.

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 stablished 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), and Crasnitin® (Bayer AG, Germany) (Shrivastava et al., 2016; Pieters et al., 2010).

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 present 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 the 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. The enzyme is naturally produced by a high number of microorganisms; nevertheless, only a few provide enough enzyme with the desired and improved biochemical properties that makes 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 the 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, thermal and storage stability, characteristics that diverge from the optimal for stablishing 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 to 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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Author's contributions

DC, MRA, MGF, APMT and VCSE: designed and lead the writing process, DC, ASM GBP, HBSB, DBP, MRA and VCSE jointly performed the literature search and wrote the manuscript. All authors read and approved the manuscript.

Compliance with Ethical Standards

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