Universidade de Aveiro Departamento de Química
2020

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Industrial e Ambiental, realizada sob a orientação científica do Doutora Ana Paula Mora Tavares, Investigadora Auxiliar do Departamento de Química, CICECO, da Universidade de Aveiro e coorientação da Doutora Ana Mafalda Rodrigues Almeida Rocha, Investigadora do Departamento de Química, CICECO, da Universidade de Aveiro.

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palavras-chave

Produção, otimização, biofármacos, L-Asparaginase, fermentação submersa, *Bacillus subtilis.*

resumo

Atualmente a enzima L-Asparaginase (L-ASNase) é utilizada nas indústrias alimentar e farmacêutica devido à sua capacidade de catalisar a reação de hidrólise do aminoácido L-Asparagina em amoníaco e ácido aspártico. Na indústria alimentar esta enzima é utilizada de forma a evitar a formação de compostos cancerígenos nos alimentos, como a acrilamida. Por outro lado, na indústria farmacêutica, a L-ASNase é usada no tratamento da leucemia linfoblástica aguda (LLA) pelo facto de impedir as células cancerígenas de crescerem, como resultado do decréscimo de L-Asparagina exógena. Como estas células (células cancerígenas) têm baixos níveis da enzima asparagina sintetase, encontram-se dependentes da absorção da L-Asparagina do meio fisiológico para sobreviver. Assim, a produção otimizada da L-ASNase, nomeadamente com alta pureza, é muito desejada, principalmente para aplicações médicas. Na produção desta enzima são utilizados diversos microrganismos, sendo os mais comuns as bactérias *Escherichia coli* e *Erwinia sp.* Contudo, associadas a estas bactérias está a produção de L-ASNase com baixos rendimentos e efeitos colaterais, o que leva ao aumento da procura de outras fontes de produção. Deste modo, a bactéria *Bacillus subtilis* surge como um microrganismo alternativo para a produção desta enzima terapêutica. Esta bactéria tem sido estudada por várias décadas, sendo o procariota mais compreendido em termos de biologia molecular e celular, desempenhando um papel importante como modelo de pesquisa de bactérias gram-positiva. Neste trabalho otimizaram-se vários parâmetros do processo fermentativo de produção da L-ASNase, tais como a concentração de indutor (0,5; 1; 2; 3 e 5% (v/v) de xilose), temperatura de fermentação após indução (25, 30, 35 e 40ºC) e o período de fermentação após indução (8, 12, 18, 24 e 36h). Nas concentrações ótimas de fermentação (3% de indutor, 30ºC durante 24h) obteve-se, após lise celular, uma L-ASNase com uma atividade enzimática de 0,756 U/mL, uma atividade específica de 0,107 U/mg e uma pureza de 21,97 %.

keywords

Production, Biopharmaceuticals, L-Asparaginase, Submerged fermentation, *Bacillus subtilis.*

abstract

Currently the enzyme L-Asparaginase (L-ASNase) is used in both food and pharmaceutical industries due to its ability to catalyze the hydrolysis reaction of the amino acid L-Asparagine in ammonia and aspartic acid. In the food industry, this enzyme is used in a way to prevent the formation of cancerous compounds in foods, such as acrylamide. In the pharmaceutical industry, L-ASNase is used in the treatment of acute lymphoblastic leukemia (ALL) once it prevents cancer cells from growing as a result of the decrease in exogenous L-Asparagine. As cancer cells have low levels of the enzyme asparagine synthase, they are dependent on the absorption of this amino acid from the physiological environment to survive. Thus, the optimized production of L-ASNase, particularly with high purity, is highly desired, particularly for medical applications. In the production of this enzyme, several microorganisms are used, such being the most commonly used *Escherichia coli* and *Erwinia sp*. However, associated with these bacteria are the L-ASNase production with low yields and side effects, which leads to increased demand for other sources of production. This way, *Bacillus subtilis* appears as an alternative microorganism for the production of this therapeutic enzyme. This organism has been studied for several decades, being the prokaryote most understood in terms of molecular and cellular biology, playing an important role as a model for gram-positive bacteria research. On this work, several factors of the fermentation process were optimized such as inductor's concentration (0.5, 1, 2, 3 and 5% (v/v) of xylose), temperature after induction (25, 30, 35 and 40ºC) and incubation times (8, 12, 18, 24 and 36h). In the optimum fermentation conditions (3% of xylose, 30ºC during 24h) it was obtained a L-ASNase, after cellular lysis, with an enzymatic activity of 0,756 U/mL, a specific activity of 0,107 U/mg and a purity of 21,97 %.

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List of abbreviations

ABS- aqueous biphasic system

ALL- lymphoblastic leukemia

AML- acute myeloblastic leukemia

C- cytosine

EcAI- *E. coli* cytosolic isoenzyme

EcAII- *E. coli* periplasmatic isoenzyme

G- guanine

GRAS- generally regarded as safe

HIC- hydrophobic interaction chromatography

IEX- ion exchange chromatography

ISE- ammonium selective electrode

Km- Michaelis-Menten constant

L-ASNase- L-Asparaginase

LLE- liquid-liquid extraction

Pl- isoeletric point

RDT- recombinant DNA technology

SEC- gel filtration chromatography

SEM- scanning electron microscope

SmF- submerged fermentation

SSF- solid state fermentation

TEM- transmission electron microscope

UV- ultraviolet

XPS- x-ray photoelectron spectroscope

XRD- x-ray powder diffraction

1.General Introduction

1.1. Scopes and objectives

In the past decades, technological progresses in bioprocess engineering have gained a high interest in the production of biopharmaceuticals due to their high sensitivity, specificity and lower risk and negative effects to the patient[1]. In fact, biopharmaceuticals are mostly therapeutic recombinant proteins obtained by biotechnological processes[2]. They are obtained from biological sources such as organs, tissues, microorganisms, animal fluids, or genetically modified cells and organisms[3,4]. Despite several different expression systems may be used including mammalian cell lines, insects and plants, new technological improvements are being made to increase microorganism production of biopharmaceuticals. This investment is justified by the well-characterized genomes, availability of different host strains and cost-effectiveness as compared to other expression systems[4,5]. Financial reports indicate that the global biopharmaceutical market size was valued at USD 369.63 billion in 2016 and is excepted to rise to USD 727.1 billion by 2025[6].

L-Asparaginase (L-Asparagine amidohydrolase, L-ASNase, (E.C. 3.5.1.1)) is the first therapeutic enzyme with antineoplastic properties that has been abundantly studied by researchers[7]. The discovery and development of the potential use 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[8]. 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, due to its anti-carcinogenic effect[9,10]. The anti-cancerous effect of this biopharmaceutical is related to the deficiency or lack of circulating L-Asparagine concentration in serum and cerebrospinal fluid[11]. Cancerous lymphoblasts are not able to synthesize this essential amino acid due to the deficiency in their L-Asparagine synthase, resulting on an exclusively dependence on the free amino acid in the blood by the lymphoblasts[12]. Intensive depletion of L-Asparagine by L-ASNase administered to the patient will lead to protein synthesis inhibition and ultimately death of lymphoblastic cells by apoptosis[13]. Therefore, there is a great demand for this protein, which usually is produced by *Escherichia coli* and *Erwinia sp*. However, the production of L-ASNase by these bacteria are associated with low yields and low immunogenicity, which leads to the search of a better-producing source[14].

Bacillus species, in particular *Bacillus subtilis*, have been intensively studied for several decades and as a consequence, is a well-characterized gram-positive bacterium[15]. Is also well known for its ability to differentiate into metabolically inactive spores that have a high resistance to environmental stresses and in addition to spores, populations of genetically identical *B. subtilis* comprise numerous distinct cell types[16]. Besides this, *B. subtilis* is one of the most understood prokaryotes in terms of molecular and cell biology, playing a major role as model for gram-positive research, and is widely used as industrial workhorse for the production of enzymes and metabolites with high value[15]. *B. subtilis* is predominantly used as a cell factory for the production of extracellular R-protein production, however, the accessibility to the well described genome sequence in addition to the extensive availability of biochemical and physiological data makes this bacteria a workhorse for both basic research and industrial scope[17,18]. Thus, some *Bacillus* species have been used for the production of L-ASNase, such as *B. licheniformis* isolated from the Red Sea, producing a glutaminase free L-ASNase[19]. Also, on the study by Jimat et al., *B. subtilis* was used for the production of L-ASNase, yet a low yield was obtained[20]. In this sense, this work aims the optimization of the production of L-ASNase using *B. subtilis* in order to obtain a higher yield of the process, an enzyme with greater biological activities and free of clinical toxicity.

1.2. L-Asparaginase (L-ASNase)

1.2.1. Types of L-ASNase, structure and biochemical properties

Several investigations have been carried out by many researchers to clarify the structure of L-ASNase in a molecular level. Commonly, this enzyme occurs as a tetramer, but monomeric, dimeric and hexameric forms can also be found when isolated from different sources[7]. L-ASNase produced by living organisms, microorganisms, plants and animals are classified based on their amino acid sequences, biochemical properties and structural and functional homology. Therefore, these enzymes are divided into three major groups: bacterial enzymes (type I and type II), plant-type enzymes (type III) and enzymes similar to *Rhizobium elti* L-ASNase[21]. Bacterial-type L-ASNase are structurally and evolutionarily distinct from the plant-type L-ASNase[22].

The bacterial-type L-ASNase is subdivided in type I and II based on their location in the cell and on the activity into L-Asparagine and L-Glutamine[23]. The type I L-ASNase is a cytosolic enzyme with relatively low affinity to L-Asparagine and high specific activity towards L-Glutamine. On the other hand, type II L-ASNase, which has been attracting much more attention, has a high affinity for L-Asparagine but a lower extent conversion of glutamine into glutamic acid[20,23]. Both types of L-ASNase can be produced from the same microorganism, such as *E. coli*, producing two isozymes of L-ASNase, however, only one possess anti-tumor activity[23]. This activity is related with the strains or culture conditions of microorganisms[25]. With the genome sequencing of *B. subtilis*, it became possible to establish that the ansZ gene encoded a L-ASNase which showed 59% identity to the L-ASNase I from *Erwinia chrysanthemi* and 53% identity to the L-ASNase II from *E. coli*[26]. Besides this, *B. subtilis* has another gene (ansA gene) that encodes type I L-ASNase[27].

The plant-type L-ASNase is characterized by hydrolyzing the side chain amide bond of L-Asparagine or its β-peptides. This type of enzyme belongs to the superfamily of N-terminal nucleophile hydrolases and are synthesized as inactive precursor molecules[28]. Also, in higher plants two forms of L-ASNase are labelled, the potassium-dependent and potassium-independent forms, which are immunologically distinct[22,29]. This last form has been more privileged for studies and researches due to its higher stability when compared to the potassium dependent form, even though the latter have displayed higher affinity for L-Asparagine[30]. Regardless of the distinct classification, the two groups of plant-type L-ASNase have significant levels of sequence similarity. However, there is no connection between them at sequence and mode of action level, and classic L-ASNase typified by the *E. coli* cytosolic (EcAI) and periplasmic (EcAII) isozymes[23,28].

The *rhizobial-type* L-ASNase includes enzymes displaying homologues sequences to the L-ASNase from *R. etli*, a symbiotic host of leguminous plants[21,31]. *R. etli* is a soil-living bacterium that uses as exclusive source of carbon and nitrogen the L-Asparagine through the action of L-Aspartase and L-ASNase. Regarding this bacteria, two L-ASNase activities were identified: L-ASNase I, characterized by its thermolability and constitutive activity, and L-ASNase II, thermostable induced by L-Asparagine and repressed by the carbon source[32]. Furthermore, *R. etli* L-ASNase II showed to be glutaminase-free, therefore having a potential application in chemotherapy[33].

Molecular structures of L-ASNase from *E. coli* and *Erwinia sp.* are deeply investigated and their structural information is easily available[34,35]. The molecular native L-ASNase type II isolated from *E. coli* possess a molecular weight of 138-141kDa (**Table 1**) and contains four identical subunits of 326 amino acids with one active center each, as shown in **Figure 1**[36]. The molecular weight of the *Erwinia*-derived L-ASNase is also 138kDa as described in **Table 1**[37]. In events of hypersensitivity reactions toward the native forms of L-ASNase, a PEG-modified L-ASNase from *E. coli* is often used[38]. For the preparation of this modified enzyme, units of monomethoxy PEG are attached to the *E. coli* derived enzyme by covalent bonds[37,39]. Therefore, due to the PEG weight (5000 Da) the molecular weight is only slightly higher than that of the native forms (**Table 1**)[40,41].

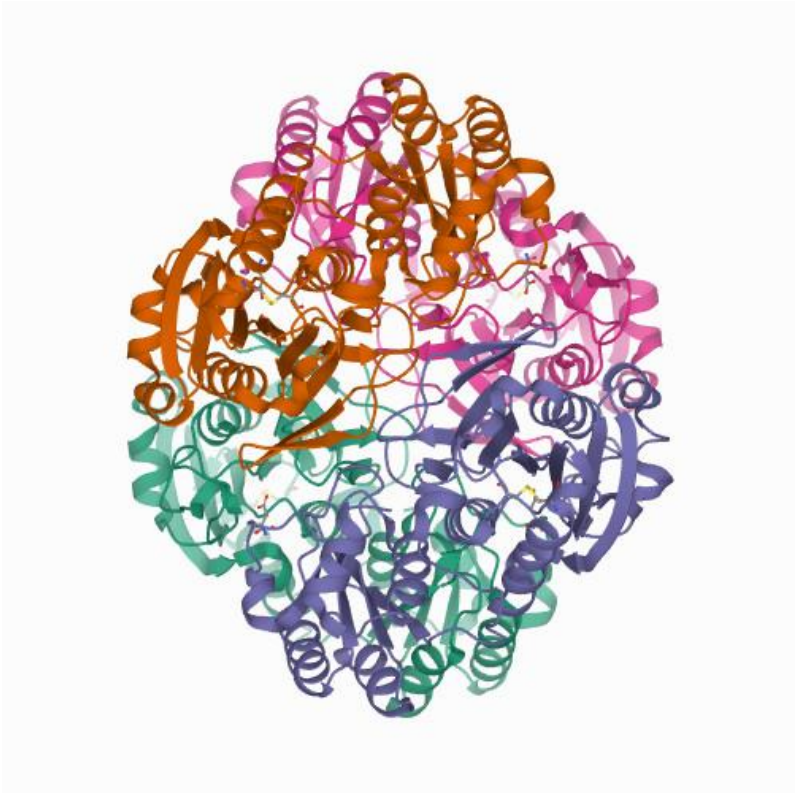

Figure 1. *E. coli* L-Asparaginase II homotetramer[42].

Almost every L-ASNase produced shows glutaminase activity, however, represents only 3-9 % of the L-ASNase activity, with exception of the one occurring in guinea pig serum[42]. Besides this, D-Asparagine and D-Glutamine are also metabolized in the same active center of the enzyme as the L-forms, and that both ester and amide bonds are hydrolyzed[43]. The considerable substrate specificity expresses itself a low Michaelis-Menten constant (K_m). For L-Asparagine the K_m is 6^{-15} µM, while the K_m for glutamine

is 100 times higher (**Table 1**)[37]. Even tough, a few minutes after the administration of the enzyme, an L-Asparagine depletion occurs, leading to an abundance of glutamine as substrate and consequently a depletion of serum from glutamine[42,44,45]. Besides this, the stability and low half-life of L-ASNase in the serum are of crucial concern in the pharmaceutical industry, thus, an enzyme with high stability and increased half-life can avoid the need for multiple dose administration which may lead to less chances of triggering hypersensitivity reactions[46]. Therefore, from the different L-Asparaginases listed on **Table 1**, the one from *E. coli* modified with PEG shows a higher half-life which will lead a shorter dose and reduced frequency of treatment to ensure adequate serum enzyme activity and complete serum L-asparagine depletion[47]. Besides this, the enzymes from *E. coli* and *Erwinia sp.* differ in their isoelectric point and show only medium immunological cross reaction[37].

Table 1. Properties of different L-Asparaginase preparations[40,48,49].

Source	Molecular weight (kDa)	Isoeletric point (pl)	Km (μM) Asparagine	Km (mM) Glutamine	Half life
E. chrysanthemi	138	8.7	12	1.10	8-22 h
E. coli	141	5	10	6.25	8-30 h
PEG-E. coli	145	5	10	MD*	5-7 days
B. subtilis 168	40	MD*	5290	MD*	1 h

*MD – missing data

1.2.2. Sources of L-Asparaginase

Microorganisms capable of producing L-ASNase have been considered important, since this enzyme was discovered from *E. coli* and its antineoplastic activity demonstrated in guinea pig serum[37]. However, due to the complex process of extracting and purifying the enzyme from plants and animals, other sources such as microorganisms (**Tables 2**) are preferred. Microorgani´-sms are considered the best sources for L-ASNase because they can be easily grown and the processes of extraction and purification can be carried out on a large scale[7].

Table 2. Different sources of L-Asparaginase.

Source	Reference	Source	Reference
Bacteria		**Fungi**	
Bacillus licheniformis	[50]	*Aspergillus niger*	[51]
Bacillus sp.	[52]*-	*Aspergillus oryzae*	[53]
Erwinia carotovora	[54]	*Aspergillus terreus*	[55]
Erwinia chrysanthemi	[56]	*Cladosporium sp.*	[57]
Escherichia coli	[58]	*Fusarium sp.*	[59]
Pectobacterium carotovorum	[60]	*Penicillium sp.*	[61]
Pseudomonas aeruginosa	[62]	**Yeast**	
Pseudomonas fluorescens	[63]	*Candida utilis*	[64]
Serratia marcescens	[65]	*Pichia polymorpha*	[66]
Vibrio succinogenes	[67]	*Rhodotorula rubra*	[68]
Algae		*Rhodosporidium toruloides*	[69]
Chlamydomonas species	[70]	*Saccharomyces cerevisiae*	[71]
Actinomycetes		*Streptomyces gulbargensis*	[72]
Streptomyces ginsengisoli	[73]		

1.3. Applications of L-Asparaginase

1.3.1. Biopharmaceutical

L-ASNase is a biopharmaceutical that in combination with drugs, such as vincristine and a glucocorticoid[74], is used in the treatment of several types of blood cancer, specially ALL in children. Besides this, due to its antileukemic properties, this enzyme is a well-known chemotherapeutic agent used in the treatment of malignancies such as Hodgkin's disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma, reticulosarcoma and melanosarcoma[7].

The use of L-ASNase as a chemotherapeutic agent stands on its catalytic property of hydrolyzing the amino acid L-Asparagine[46]. Hence, following the administration of L-ASNase the non-essential amino acid is hydrolyzed into aspartic acid and ammonia (**Figure 2**). Regular (non-malignant) cells are able to synthesize L-Asparagine for their grown using transaminase enzyme that converts oxaloacetate into an intermediate aspartate, which afterwards transfers an amino group from glutamate to oxaloacetate originating α-ketoglutarate and aspartate[7,75]. Lastly, in healthy cells, asparagine synthetase uses aspartate to convert to L-Asparagine[7]. On the other hand, neoplastic cells are not able to synthesize L-Asparagine as a result of the absence of L-Asparaginase synthetase enzyme, resulting on a dependence on the exogenous supply of L-Asparagine for their existence and reproduction[76]. As a result, providing L-ASNase to malignant cells will result on a complete exclusion of circulating L-Asparagine, which leads to starvation of cancer cells and their dead (**Figure 2**). In fact, this L-Asparagine starvation leads to inhibition of the production of ribosomal precursor RNA and successively rRNA transcription, which limits peptide synthesis[46]. Depletion of this amino acid also causes the slowing of nucleotide biosynthesis and prolongation of the S-phase of the cell cycle[77]. The treatment with L-ASNase stops the cell cycle of the malignant cells at the G1 phase before DNA degradation. One of the by-products of L-ASNase activity is ammonium ions, capable of modifying the pH by diffusion into the cytosol leading to the activation of sign al transduction pathway associated with phosphorylation of substrates and apoptosis[46].

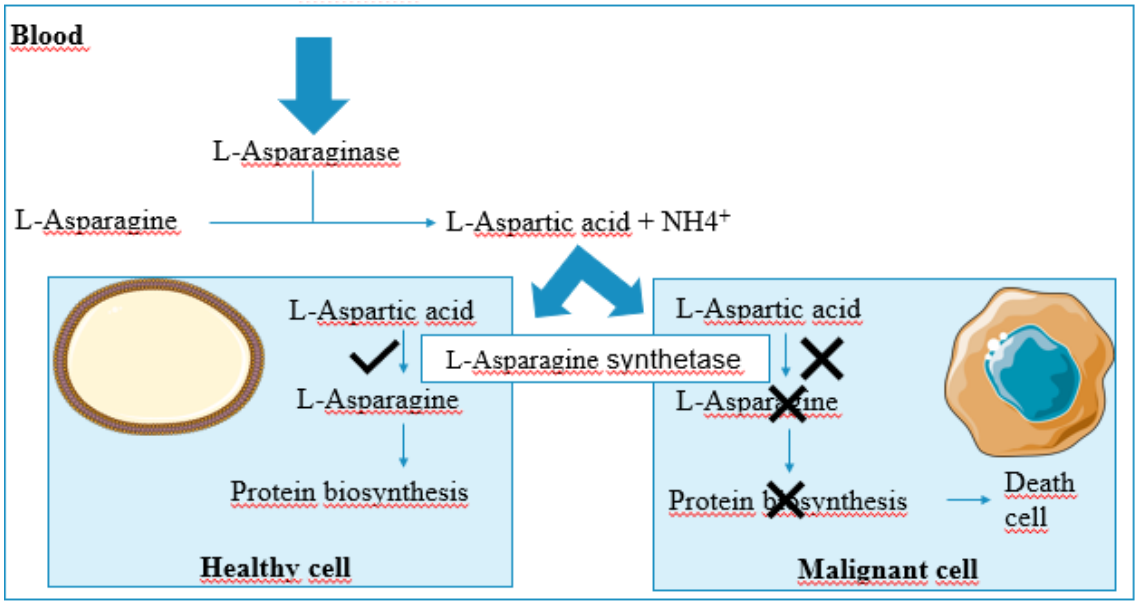

Figure 2. Schematic illustration of the enzymatic catalysis of L-Asparagine by the enzyme L-Asparaginase in healthy and malignant cells.

Despite the high therapeutic efficacy of L-ASNase, and although the L-ASNase type II from bacterial source has been used for more than 40 years, a clinically relevant toxicity has been detected[46]. This toxicity may occur in a sequence of doses, and in the form of hypersensitivity reactions and inactivation by antibodies production against the enzyme[46]. Hypersensitivity reactions to L-ASNase are categorized as either clinical or subclinical and are the most common reasons for the discontinuation of L-ASNase therapy[78]. Reports of clinical hypersensitivity describe on native *E. coli* a hypersensitivity up to 75% of patients with ALL, although rates normally range from 10-30 %. Treatments using PEG-L-ASNase appear to have less clinical hypersensitive reactions, with rates from 3-24%. In fact, these reactions are more common when patients have been previously exposed to native *E. coli* L-ASNase[78]. Rates of clinical hypersensitivity in patients receiving *Erwinia chrysanthemi* L-ASNase vary from 3-37 %[79]. Patients who exhibit a hypersensitivity reaction to an *E. coli* L-ASNase should cease their current therapy and be switched to *Erwinia sp.* L-ASNase, considered less toxic, but with a smaller half-life than *E. coli*[76]. This change, in a large number of patients with hypersensitivity, allows them to finish their prescribed treatment[78].

Hypersensitivity to L-ASNase can be expressed as an overt allergic reaction with symptoms such as anaphylaxis, pain, edema, urticaria, erythema, rash and pruritis[47]. Side effects like thrombosis, hepatic dysfunctions, acute pancreatitis, brain dysfunctional syndrome, coagulopathies and glycemia are a consequence of the glutaminase side activity presented by L-ASNase[80,81]. The structural difference between L-Asparagine and glutamine is only one methyl group, therefore L-ASNase have the dual substrate

specificity resulting in a drop of the concentrations of both amino acids in the body. Hence, the production of L-ASNase with a lower affinity by L-Glutamine is one of the major goals to achieve by researchers[82].

Some studies demonstrated that the incidence of hypersensitivity to L-ASNase is similar between age groups, although other investigations suggested that younger patients developed antibody and hypersensitivity reactions with less frequency than teenagers and adult patients[83,84]. Additionally, repeated administration may lead to reduction of enzyme activity and consequently of clinical efficacy, which is most likely due to immunological reactions[40].

1.3.2. Food industry

L-ASNase has aroused the attention of food processing industries as a promising acrylamide mitigating agent. Acrylamide (C_3H_5NO), also recognized as 2-propenamide, acrylic amide, ethylene carboxamide, propenamide, propanoic acid amide, monomer of acrylamide or acrylic acid amide, presents 71.08 g/mol, and is a neurotoxin categorized as a carcinogenic to the humans[7]. The formation of this organic compound has been quite studied in the past and it was demonstrated the occurrence of acrylamide in regular starch-based foods that were baked, roasted or fried[46]. Essentially, foods that are processed or cooked at high temperature like potato products, coffee, bakery products, roasted almonds, olives dry fruits are the prominent sources of acrylamide[46]. Researchers demonstrated that acrylamide is produced as a result of the Maillard reaction (**Figure 3**) from L-Asparagine and reducing sugars[85]. In fact, L-Asparagine and reducing sugars are used in a conjugation reaction resulting in the formation of N-glycosylasparagine. When treated at high temperature, a decarboxylated Schiff base is formed that may decompose directly to form acrylamide or hydrolyze to form 3-aminopropionamide, also an precursor of acrylamide (**Figure 3**)[85,86].

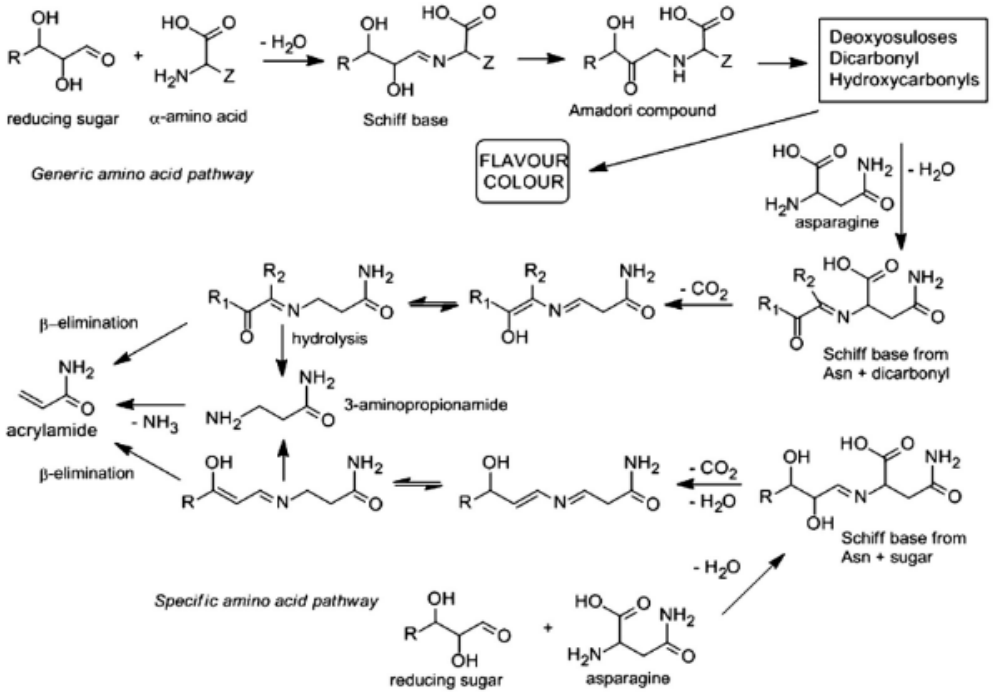

Figure 3. Mechanism of acrylamide formation in food processing[67].

Therefore, L-ASNase pre-treatment of food before heat treatment could be a suitable solution supported by several researchers for the reduction of free L-Asparagine and consequently the imminent risk of synthesis of acrylamide. Different tests were made, concluding that L-ASNase pre-treatment resulted on an acrylamide reduction of at least 90% in foods, such as crackers, french fries, potato, gingerbread, among others[46,87]. Regarding this, L-ASNase from *Aspergillus oryzae* and *A. niger* are often used in baking industries. These enzymes have an optimal temperature of 40-60ºC and a pH of 6.0-7.0 and since the baking temperatures go up to 120ºC, it is appropriate to have stable and active enzymes over a wide range of temperature and pH[88]. Although, complete removal of acrylamide is not possible due to other asparagine-independent formation[85]. Other studies allowed to know that L-ASNase pre-treatment does not only leave sensorial properties of the final food products unaffected, but also improves the flavor by increasing the percentage of glutamic acid on the food[46].

1.3.3. Biosensor

L-ASNase biosensors can be a promising technology for the detection of L-Asparagine in physiological fluids at levels as low as nano-levels, either in the treatment of leukemia or food industry[7]. Spectroscopy techniques such as X-ray Powder Diffraction (XRD), X-ray photoelectron spectroscopy (XPS), scanning electron microscope (SEM) and transmission electron microscopy (TEM) are currently used for L-Asparagine analysis[7,89]. Based on the deamination of L-Asparagine by L-ASNase and the formation of ammonia, an enzymatic method has been developed by Tagami and Mastsuda for the measurement of the enzyme's activity and L-Asparagine with an ammonia gas-sensing electrode[90]. Another study carried by Wang and Bachas, a thermostable recombinant L-ASNase from *Archaeoglobus fulgidus*, was expressed in *E. coli* as fusion protein[91]. Using an ammonium selective electrode (ISE) with the enzyme, it was possible to develop a biosensor for L-Asparagine[91]. Kim-Sun-Jin et al. have used garlic tissue electrode for the determination of L-Asparagine, where garlic tissue cells were responsible for conversion of the amino acid into ammonia using an ammonium gas electrode as detector[92]. Also, Verma et al. developed a whole-cell based fiber optic biosensor using a L-ASNase-producing coliform bacterial and phenol red indicator, which can monetarize L-Asparagine content in food samples[93].

In conclusion, several spectroscopy techniques are currently used for the analysis of L-Asparagine, however, their high cost and tedious procedures make them less favorable. Therefore, biosensor technology can become a reliable, cheap and user-friendly solution. The mechanism of action of the biosensor depends on L-ASNase activity, ammonium ions produced from the hydrolysis of L-Asparagine causing a change in pH and, thus, changes of color and absorption[7].

1.4. *Bacillus subtilis*

Bacillus subtilis has been studied over the last century and, as a result, is a well-characterized gram-positive bacterium with exceptional properties, such as the absence of an outer membrane along with and efficient sec-dependent secretion pathway[17]. This bacterium is broadly used as a cell factory for extracellular R-protein production due to its characteristics, like relatively high product yields (20-25 g/L), simplified downstream processes and lack of toxic byproducts. Moreover, a well-characterized genetic expression system and facility of manipulation are additional advantages of *B. subtilis*[18]. The rod-shaped endospore forming *B. subtilis* has been a model of the Firmicutes, a major phylum of bacteria. It became a reference for gram-positive microorganisms considering its amenability to genetic manipulation, like rapid grown, high natural competence for DNA uptake and stable integration of exogenous DNA into the chromosome. The availability of the well-annotated and compact genome sequence with an intermediate Guanine (G) + Cytosine (C) content as well as the wide physiological and biochemical data makes this bacterium a workhorse for research and industrial purposes[94].

In the past, *B. subtilis* was classified as a strict aerobe, however, following studies demonstrated that it can also grow anaerobically, using nitrate or nitrite as alternative terminal electron acceptor[95]. Microbiology textbooks commonly suggest as the main habitat of *B. subtilis* the soil, like another aerobic spore former. Still, recent research suggest that this could be a generalization since *Bacillus* endospore were found in the gut of various insects and animals. The presence of this endospores in gastrointestinal could be explained by the germination of the food and consequently proliferation in the medium[96]. Even though *B. subtilis* cannot be considered as a gut commensal, the gastrointestinal tract represents an important part of its life cycle. Besides this, *Bacillus* endospores can also be found in other locations including rocks, dust and aquatic environments.

B. subtilis can be easily isolated from soil, using as energy source starch and high salt concentration. Preferably, the soil sample should be heated up to 100ºC for 30 min, allowing only endure spores to be cultured from the sample[18]. *B. subtilis* also presents a social behavior, meaning that the cells communicate with each other, forming multicellular structures in the form of swarming cells and biofilms. This bacterium can choose between three different genetic programs when in adverse conditions, such as,

nutrients or other resources become limited and cell density reaches a critical threshold. In order to its survival or adaptation, cells can either enter in stationary phase, characterized by the formation of singe motile cells; can become competent and incorporate DNA from the environment for acquisition of new genetical material; or can differentiate into enduring and metabolically inactive spores[16]. With the development of Genomics in the past decades, it was possible to determine the complete genome sequence of *B. subtilis* 168, a model for gram-positive microorganisms[97]. The 4,216 kb-genome of *B. subtilis* has an average G+C content of 43% and is made of 4,244 protein coding genes, 30 rRNA and 86 tRNA. Also, 3,662 regions transcriptionally active during mid-exponential growth were reported and a systemic inactivation of *B. subtilis* genes revealed that 271 genes were essential for growth[98].

1.4.1. Endospore formation

As well as other 200 species among 25 genera of aerobic bacteria, *B. subtilis* vegetative cells are able to produce ovoid-shape dorm[99]. Endospores display extraordinary resistance properties, such as being resistant to ultraviolet (UV) radiation, chemicals, extreme heat and other stresses, allow their survival in nutrient-free and harsh environments. This phenomenon called sporulation leads to a metabolically inactive and very resistant structure and the main stimulus for its activation is starvation[100]. In fact, sporulation (**Figure 4**) in these bacteria represent the best studied example of a prokaryotic differentiation process and has been widely studied in the past years[99]. Even though after an extended period of dormancy, spores have the ability to sense when environmental conditions become favourable for growth and convert to a regular vegetative cell cycle through germination (**Figure 4**)[101]. Their viability is unknown, however, some researchers reported the revival of spores from samples ranging in age from decades to several thousands of years[102].

Like in all other endospore forming organisms, sporulation in *B. subtilis* (**Figure 4**) follows a similar morphological sequence and spores have the same concentric architectural plan[103]. In fact, spores look quite different from growing cells and this morphological differentiation initiates with an asymmetric cell division near to one pole of the cell, resulting in the formation of a smaller cell and a larger cell, the forespore and mother cell, respectively[104]. A process denominated as “engulfment” is responsible for

the encasement of the smaller cell into the larger cell. After the complete asymmetric separation, the mother cell membranes move and will end up encircling the forespore. There are some similarities of this cell membranes moves with the ones occurring during phagocytosis in eukaryotic cells. However, this analogy may not be completely correct, since in sporulating cells there is a layer of peptidoglycan surrounding the forespore which separates the two compartments[104]. After the conclusion of engulfment, the forespore is a double membrane-bound cell inside the mother cell. This process is coordinated with the creation of two external protective structures, being one of them the cortex, composed by peptidoglycan and is assembled between the inner and outer forespore membranes[105]. The other one is the proteinaceous coat that comprises the outermost layer of the spore[101]. The coat is made of at least 70 single proteins that are produced by the mother cell and begin to localize to the spore surface during engulfment. Although most of these proteins are responsible for the formation of the coat structure, it is known that at least 20 of them possess an enzymatic function or present similarity to established enzymes. Therefore, these enzymes can take part in the assembly process (by posttranslational modifications), modulate germination or participate in spore protection. Besides this, the coat plays a role in the resistance of the spore to extreme physical stresses, such as high pressures, ultraviolet and gamma radiations, and chemical threats like lysozyme and oxidizing agents[106].

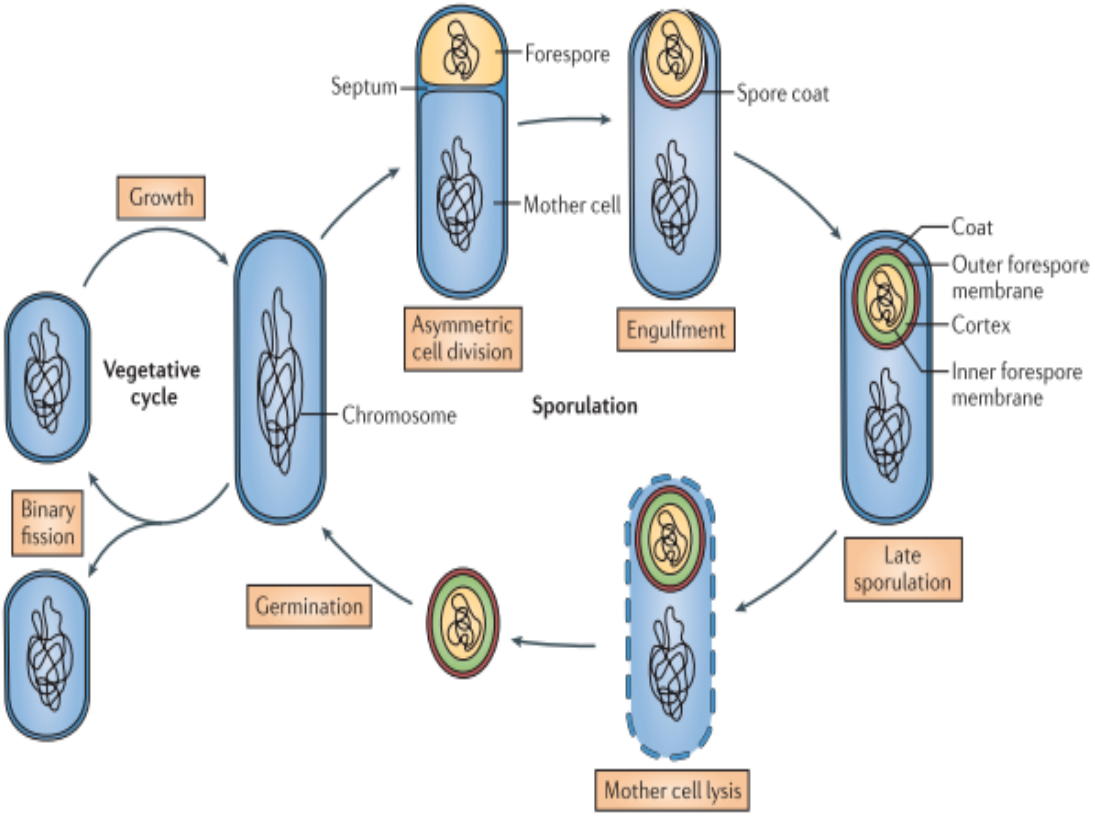

Figure 4. The sporulation and germination cycle in *B. subtilis*. After late sporulation, the mother cell lyses to release a mature spore into the environment. Spores are capable of quickly germinating and resuming vegetative growth in response to nutrients[91].

1.4.2. Genetic engineering

One of the major goals of genetic engineering in microorganisms involves the genetic optimization of biosynthetic pathways for the overproduction of commercially attractive metabolites through metabolic engineering. Strategies of metabolic engineering in *B. subtilis* consists on overcoming limitations of carbon flux in the metabolic routes, reducing maintenance metabolism and improving protein secretion and protein folding[107]. Rechanneling the carbon flux towards a metabolite of interest generally involves obstructing specific enzymatic reactions in other metabolic pathways[107]. However, carbon limitation in the metabolic pathway of interest is overcome by increasing gene expression. More precisely, or the transcriptional regulation can be reduced or either the amount or the stability of messenger RNAs available for ribosomal translation can be increased[108]. In the industrial production of metabolites, by high-cell density microbial processes, a low maintenance energy metabolism to reduce non-productive consumption of substrate is quite important. Once a specific biosynthetic pathway have been optimized for a certain metabolite, the reduction of the maintenance metabolism is a vital objective for improving the commercial process performance[109].

In *B. subtilis*, the export of proteins requires dedicated transport machineries constituted by channels and adenosine triphosphate-dependent or proton gradient-dependent proteins to drive active movements across the membrane. Exploring both engineering of protein translocation and folding would possible increase the capacity of this bacterium to secrete a novel class of pharmaceutically relevant proteins with high yields and activity[110].

For directed genetic modification in *B. subtilis* some tools and strategies should be adapted such as the integration or deletion of DNA in the chromosome and enhancement of gene expression. For the efficient gene insertion, deletion or modification, techniques of genetic transfer (bacteriophage-mediated transduction, protoplasts fusions) and transformation with various concentrations of naked or protoplast-protected DNA should be applied[111]. In order to enhance the gene expression, integrative plasmids can be used to add extra-copies of target genes into the bacterial chromosome. These recombinant plasmids can be integrated at different loci of the engineered recipient strain or in order to expand its number, the concentration of selective antibiotics should be increased[112,113]. Correct implementation of this strategy involves obtaining the optimal copy number and for large-scale fermentation, antibiotic selection

pressure must be maintained during the inoculum fermentation runs in order to stabilize the integrated plasmids.

1.4.3. Industrial biotechnology with *B. subtilis*

B. subtilis is non-pathogenic and has the GRAS (Generally Regarded As Safe) status, making this microorganism desirable for industrial purposes. Primarily, its short fermentation cycle times (high growth rates), relatively inexpensive and easy large-scale fermentation at high cell densities, allows the development of valuable industrial processes[94]. Secondly, the single membrane of the cells facilitates the direct transfer to the growth medium of proteins transported by secretion machineries, simplifying the downstream processes[94]. Moreover, proteins generated by *B. subtilis* are free of endotoxin lipopolysaccharide (LPS), a molecule present in the outer membrane of Gram-negative bacteria, that must be eradicated before using recombinant proteins for clinical purposes[94] .

More recently, the product portfolio of *B. subtilis* is enlarging from proteins to bio-products because of its distinct endogenous metabolism and development of new metabolic engineering technologies[108]. Some of this bio-products are riboflavins, N-acetylglucosamine, poly-ɣ-glucamic acid, hyaluronic acid, 2,3-butanediol and acetoin[108]. However, there are still some limitations with *B. subtilis* to produce heterologous proteins. The expression of cell wall-associated or secreted proteases causes significant degradation of secreted heterologous proteins[114]. Besides this, the bioinformatics of the bacteria need to be further developed since most of the available data was obtained from small-scale laboratory culture conditions, that are inaccurate to guide metabolic engineering for large-scale industrial applications. Nevertheless, compared to the well-studied *E. coli*, systems and synthetic biology technologies of *B. subtilis* are still behind. However, basic metabolic engineering efforts mostly focus on gene deletions and overexpression have been performed in order to improve the performances of the strains of the bacteria[108]. These static methods are unable to detect the pathway flux or the levels of pathway intermediates and adjust metabolism to accomplish maximum productivity[108,115]. Additionally, the production of cost-effective heterologous enzyme secretion to meet commercial production is still a challenge. In order to engineer *B. subtilis* into an highly efficient heterologous enzyme-secreting cell factory, several researches have been made to investigate the composition of protein secretion machinery,

the protein secretion pathways and tested various signal peptides to improve exportation of heterologous enzymes out of the cell[18,116]. However, the efficacy of the secretion pathway differs for different proteins and optimal signal peptide sequences cannot be generalized for all heterologous enzymes. Lastly, *B. subtilis* have some wild properties, such as production of a large amount of foam and high maintenance metabolism, which increases the conditions and difficulties in industrial operations[108].

Apart from this, industrial enzymes represent a major area of biotechnology, leading to the development of new products and improvements in the process and performance of several existing products. In fact, it is estimated that *B. subtilis* represent 50% of the total enzyme market[117]. Also, enzymes produced by these bacteria have several applications and can be classified as technical enzymes, used in textile, detergent and pulp and paper industries (50%), food (36%) and feed enzymes[118]. The main compounds produced from industrial fermentations of *B. subtilis* are summarized in **Table 3**.

Table 3. Main compounds produced by industrial fermentation of *B. subtilis*[117,118].

Products	Industrial Applications
α-Acetolactate decarboxylase	Beverage
α-amylase	Food, Paper, Starch, Textile, Brewing
β-Glucanase	Beverage
β-Glucosidase	Brewing
Cellulase	Detergents
Cyclodextrin glucanotranssferase	Food, Pharma, Cosmetics
Galactomannase	Feed, Beverage
Glutaminase	Food, Flavor
Lipases	Detergent
Neutral (metallo-) Protease	Detergent, Food
Alkaline (serine-) Protease	Detergent, Textile
Penicilin Acylase	Pharma
Pullulanase	Starch, Food, Beverage
Poly-gamma-glutamic acid	Food, Pharma, Cosmetics
Urease	Analysis, Beverage
Xylanases	Baking, Feed, Beverage, Brewing, Food
D-Ribose	Food, Feed, Cosmetics, Pharma
Poly-gamma-glutamic acid	Food, Feed, Pharma
Purine nucleosides	Food
Riboflavin	Food, Pharma
Streptavidin	Microarrays
Thaumatin	Food, Pharma
Surfactins	Pharma, Bioremediation

1.5. L-Asparaginase production and purification

1.5.1. Upstream process

Among the different species capable of producing L-ASNase, *E. coli* and *E. chrysanthemi* are the primary microbial agents used for the industrial-scale production of this enzyme. Fermentation is the principal technique to produce several enzymes, namely L-ASNase, since both bacteria and fungi are efficient microorganisms when fermented on appropriate (**Table 4**)[119]. L-ASNase production is performed by submerged fermentation (SmF), solid state fermentation (SSF) and recombinant DNA technology (RDT)[14]. Moreover, several cases demonstrated that enzymes produced in SmF have non-identical optimal temperature, pH stability and dissimilar kinetic parameters in comparison to the same enzyme when produced in SFF[120]. SmF is the main technique

usually employed for bacterial enzyme production and consequently, the most used to produce L-ASNase. This technique involves a process in which the microorganism grow up in a liquid broth medium which is enhanced with nutrients in order to have a better cultivation of microorganism, in other words, growing carefully the selected microorganisms in closed reactor containing the fermentation medium and a high concentration of oxygen[121]. Research evidences demonstrated that L-ASNase produced by SmF 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[119]. The production of L-ASNase from various microbial by SmF sources with optimized conditions are summarized in **Table 5**.

Table 4. Main bacteria employed in submerged fermentation for L-ASNase production. Adapted from Lopes et al[119].

Taxon	**Taxon**
Actinomycetes	*Pseudomonas spp.*
Bacillus spp.	*P. aeruginosa*
B.cereus	*P. aurantiaca*
B. licheniformis	*P. stutzeri*
B. subtillis	*Staphylococcus spp.*
Enterobacter spp.	*Streptomyces spp.*
E. aerogenes	*S. albidoflavus*
E. cloacae	*S. gulbargensis*
Erwinia spp.	*S. longsporusflavus*
E. aroideae	*S. phaeochromogenes*
E. carotovora	*S. plicatus*
E. chrysanthemi	*Thermus spp.*
Escherichia coli	*T. thermophilus*
H. pylori	*Vibrio spp.*
Nocardia spp.	*V. fisheri*
N. asteroides	*V. hawvevi*
Pectobacterium spp.	*V. proteus*
P. carotovorum	*Wolinella spp.*
Photobacterium spp.	*W. succinogenes*
P. leiognathi	*Yersinia spp.*
P. phoshoreum	*Y. pseudotuberculosis*
Z.mobilis	*Zymonas spp.*

Table 5. L-Asparaginase production by submerged fermentation at different operational conditions. Adapted from Vimal et al[14].

Microorganism	Medium	Rpm	Temp (º C)	pH	Incubation period (h)	Activity
Spirulina maxima	Modified Zarrouk medium	-	25	9.5	432	51.28 IU/L
Enterobacter aerogenes MTCC111	Trisodium citrate (0.75%)	-	33	6	40	18.35 IU/mL
Pectobacterium carotovorum MTCC 1428	Glucose (30 g/L) L-asparagine (30 g/L)	-	28	8.5	Fed-batch mode	38.8 U/mL
Penicillium digitatum	Czapek-Dox medium	-	37	-	96	363.80 IU/mL
Nocardia levis MK-VL 113	Asparagine-glycerol salts (ISP-5)	-	30	7	72	5.06 U/mg
Aspergillus terreus MTCC 1782	Wheat bran	-	30	-	72	110 U/gds
Aspergillus terreus MTCC 1782	Coconut oil cake	-	30	-	72	85 U/gds
Aspergillus terreus MTCC 1782	Czapek-Dox Medium + L-asparagine 1%, yeast extract 1%, peptone 0.6%, glucose 0.4%	160	35	6	72	24.10 IU/mL
Emericella nidulans	Czapel-Dox Medium	-	30	6	48	1.1 IU
Streptomyces Albidoflavus	Asparagine-maltose-yeast extract-salts broth	-	35	7.5	72	7.51 IU/mg
E. coli K-12	Lactose 10g/L; Tryptone 10g/L; yeast extract 5g/L; L-asparagine 2g/L; CaCl2 15g/L	200	37	6.5	-	3.82 IU/mL
Bacillus sp (DKMBT10)	KH2PO4 2.0, L-asparagine 6.0, MgSO4.7H20 1.0, CaCl2.2H20 1.0, glucose/maltose 3.0	200	37	7	24	0.1 U/mg
Pseudomonas fluorescens	Glucose, beef extract, L-asparagine, salt solution	-	37	8	48	168.4 IU/mL
Streptomyces sp (YA22)	Sucrose-potassium nitrate 1% asparagine	120	28	7	120	8.87 IU/mg

SmF is the most applied due to its well establishment, easy manipulation of medium components and high yields[121]. Besides this, the fact that no pre-treatment of substrate is required, facility on the manipulation of the medium parameters and easier purification of products makes SmF the major technique when compared with SSF[14,122]. Nevertheless, there are some shortcomings related with SmF that promote scientists to move toward SFF such as high energy demand, high risk of contamination, low yield, higher cost of production and vast amount of waste[14].

In the past years, SFF emerged as an alternative to SmF for the production of extracellular enzymes because it allows the direct use of crude fermented product as enzyme source and has the potential for the production of secondary metabolites[119]. However, when compared with SmF, only few reports are available on SFF for the L-ASNase production, being the most significant results found in the literature on SFF on **Table 6**.

Table 6. L-Asparaginase production by solid state fermentation at various operating conditions. Adapted from Vimal et al[14].

Micro-organism	Substrate	Moisture (%)	Temp (ºC)	pH	Incubation period (h)	Activity
Serratia marcescens (NCIM 2919)	Coconut oil cake	40	35	6	24	3.87 U/gds
Serratia marcescens (NCIM 2919)	Coconut oil cake	50	35.5	7.4	24	5.86 U/gds
Serratia marcescens (NCIM 2919)	Citrus limetta pulp	60	28	7.5	48	83.16 U/gds
Serratia marcescens SB08	Rice bran	50	30	7	36	79.84 U/gds
Pseudomonas aeruginosa 50071	Soya bean meal	50	37	7.4	96	1900 IU/mg
Aspergillus niger	Soya bean meal	70	30	6.5	96	40.9 U/gds
Aspergillus terreus MTCC 1782	Pomegranate	75	30		120	253 U/gds

Aspergillus terreus MTCC 1782	Wheat bran	75	30		72	110 U/gds
Aspergillus terreus MTCC 1782	Coconut oil cake	75	30		72	85 U/gds
Aspergillus terreus MTCC 1782	Bajra seed flour	70	30	8	96	273.3 U/gds
Aspergillus terreus MTCC 1782	Corn cob	70	30	8	96	55.66 U/gds
Aspergillus terreus	Carob pod	65	35	4.5	168	5.63 IU
Fusarium oxysporum	Wheat bran	60	30	7	120	8.14 IU
Aspergillus flavus	Orange peel	40	35	6	96	339.16 U/g
Aspergillus terreus MTCC 1782	Sesame oil cake(SOK)	40	30		96	68.49 U/gds
Aspergillus terreus MTCC 1782	Black gram husk (BH)	40	30		96	15.95 U/gds
Aspergillus terreus MTCC 1782	(SOC+BH) (7:3)	40	30		96	74.21 U/gds
Aspergillus terreus MTCC 1782	(SOC+BH) (7:3)	60	32	7	120	163.34 U/gds
Serratia marcescens - NCIM 2919	Sesame oil cake	68.64	37		96	110.80 U/gds

A comparison of SSF and SmF shows that SSF offers high yield and constitutes an eco-friendly process[121]. **Table 7** summarizes main advantages and limitation for both types of processes.

Table 7. Comparison of submerged and solid-state fermentation for enzyme production. Adapted from Doriya et al.[121].

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

RDT is another approach to produce L-ASNase with higher recovery. In a study conducted by El-Gendy et al., proplasts of two fungal isolates (*Trichoderma sp.* and *Cladosporium sp.*) were fused in order to get higher yields[123]. The recombinant strain developed has 2.58-fold more enzyme activity than original isolates[123]. In another study by Hegazy & Moharam, protoplast fusion technique was used with two strains (*B. subtilis* and *B. cereus*)[124]. The strains were maintained at 37ºC on lysogeny broth agar and supplemented when necessary with the antibiotic rifampcin 5 µg/mL. The protoplasts were induced by treating bacterial cells with 1 mg/mL lysozyme for 3 h in SMM buffer (0.5M sucrose, 0.02M malic acid and 0.02 M $MgCl_2$ pH 6.5) and consequently, for the protoplast fusion equal volumes from each parent's protoplast suspension was mixed with 40 % PEG 6000. The obtained results showed an recombinant strain with 2.5 times higher activity[124].

In order to obtain a higher yield, which subsequently leads to commercial success of L-ASNase, process parameters must be optimized. Since classical methods vary one factor at time, which leads to a long and expensive process, fast methods like strain improvement and a process for high-titter enzyme production should be used by researches with the objective achieving an higher production of L-ASNase[14]. For the economical production of the enzyme on industrial scale, cell immobilization can be applied since it has some advantages such as the efficiency to separate cell mass from the

bulk liquid to reuse, ease to operate in continuous operation for longer time and higher catalysis efficiency[125].

1.5.2. Downstream process

Since L-ASNase possess a high pharmaceutical value, the high purity is one of the major features that is desirable for its medical application. In general, L-ASNase with high purity leads to less toxic and allergic response in the patient[126].

Generally, the process of biomolecules purification consists in several individual steps that together accounts for 80 % of total production cost[14]. The major steps of purification include removal of insoluble material, concentration, fractionation and finally purification. This last step of purification consists in techniques such as filtration, centrifugation, salt precipitation followed by dialysis, liquid two-phase extraction and chromatographies (ion exchange, affinity, size exclusion, gel filtration)[127].

Separation by precipitation from an aqueous extract represents the most common method for recovering and purifying biomolecules. This technique leads to a temporary disruption of protein secondary and tertiary structures, causing its precipitation. Moreover, it is a technique easy to scale-up, with low costs and with the possibility to use several precipitants. Also, the precipitant agent can be recycled at the end of the process by distillation, evaporation or heat drying, decreasing the impact in the environment[119,128]. Several researchers have purified L-ASNase up to apparent homogeneity by ammonium sulphate precipitation (using a range of salts concentrations of 35 to 100%), obtaining different yields depending on the source of L-ASNase[129]. The study of Basha et al., used finely powdered ammonium sulfate and it was added to the crude enzyme extract[130]. The crude enzyme was brought to 45% saturation with ammonium sulphate at pH 8.4. The obtained results showed a specific activity of 536.6 IU/mg, 0.086 mg of proteins and 1.09 of enrichment purity-fold[130].

Besides precipitation, an attractive alternative for the extraction/purification of biomolecules is liquid-liquid extraction (LLE) by aqueous biphasic systems (ABS). LLE is known by the capacity of removing a solute from a liquid (or liquid mixture) phase when in contact with other immiscible or partially soluble liquid where the component (solute) is soluble. Its partition can be enhanced using simple tools like the addition of affinity ligands[119,131]. ABS can be formed when mixtures of water-soluble polymers are

mixed with another polymer or with certain inorganic salts above critical concentrations. In fact, the study of Magri *et al.* shows the potential of ABS for the purification of L-ASNase, achieving an purification level of 2.4 using ABS composed of PEG-2000 and potassium phosphate buffer[132]. Similarly, in the study of Santos et al. a purification factor of 173.8 was obtained ABS with an ammonium precipitation pre-purification factor[133].

Despite the new improvements in the development of low resolution separation methods, chromatography-based techniques remain the backbone of the biopharmaceutical industry[134]. These techniques are still widely used due to its scalability, robustness, selectivity, high clearance and easy validation compared to other purification processes. Amena et al., purified L-ASNase from *Streptomyces gulbargensis* using the different techniques previous described obtaining the results presented in **Table 8**[135]. Nevertheless, these techniques still allow to obtain the greater degree of purity which makes them essential for the biopharmaceuticals industry.

Table 8. Purification profile of L-ASNase from *S. gulvargensis*. Adapted from Amena et al.[135].

Step	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification fold	Yield (%)
Crude Extract	3200	128	25	0	100
Ammonium sulfate precipitation	1620	36	45	1.8	50.6
Sephacryl S-200 gel filtration	1210	1.8	672.2	26.9	37.8
CM Sephadex C-50 chromatography	1026.5	0.5	2053	82.1	32

2.Experimental Section

2.1. Materials

The recombinant bacteria used in this study was *Bacillus subtilis Δ6*, kindly provided by Professor Valéria de Carvalho Santos Ebinuma from the Department of Bioprocesses and Biotechnology, School of Pharmaceutical Sciences, São Paulo State University – UNESP Brazil. Briefly, the ansB gene from *Aliivibrio fischeri* (also called *Vibrio fischeri*) (NCBI: WP_011262105.1) was amplified using PCR of genomic DNA isolated from *A. fischeri* as a template. Specific primers were designed using Biobrick methodology. The PCR product was cloned in plasmid pBS0EXylRPxylA (V2) and used for thermal shock transformation in *B. subtilis Δ6*.

The remaining materials used in this study are listed in **Table 9**, along with the information of their degree of purity and its respective supplier.

Table 9. Materials used in this work with the respective degree of purity and supplier.

Reagent	Purity	Supplier
Agar Bios Special LL	-	Biolife
Bromophenol blue sodium salt	100 %	Merck
D (+)- Xylose	99 %	Merck
Erythromycin	-	
Glycerol	99,98 %	Fisher Chemical
L-Asparaginase lyophilized and purified from *E. coli* (P1321 -10000; 10000 IU)		Deltaclon S.L.
L-Asparagine	99 %	Acros Organics
Luria-Bertani Broth	-	Sigma
Nessler's Reagent	-	Sigma-Aldrich
Phosphate Buffered Saline (PBS)	-	Sigma
RunBlue 20x SDS run buffer TEO - Tricine -SDS	-	Expedeon
Sodium Phosphate dibasic heptahydrate	98 %	Sigma-Aldrich
Sodium phosphate monobasic	99 %	Panreac
Trichloroacetic acid	-	Prolabo
Tris(hydroxymethyl)aminomethane (TRIS)	PA	Pronalab

2.2. L-ASNase production

The enzyme production was carried out by submerged fermentation. Firstly, a pre-inoculum was manufactured by adding 5 mL of Luria-Bertani (LB) broth, 5 µL of Erythromycin (1 mg/mL) and 1 µL of the microorganism (*B. subtilis*) into a falcon tube. The mixture was incubated overnight at 37ºC at 250 rpm. Subsequently, 50 mL of LB broth, 50 µL of Erythromycin (1 mg/mL) and a pre-inoculum volume was added to an Erlenmeyer to start the inoculum. In order to know the total of volume of pre-inoculum (V_{Trans}) to add, the **Equation 1** should be considered:

$$OD_P * V_{Trans} = OD_I * V_T \quad (1)$$

where OD_P is the optical density (OD) of the pre-inoculum, V_{Trans} is the total volume of pre-inoculum to transfer to the Erlenmeyer (mL), OD_I is the optical density to start the inoculum (0.1) and V_T is the total volume of the inoculum (mL).

After, the inoculum is transferred for Erlenmeyers flasks of 250 mL and then incubated in an orbital shaker at 37ºC and 250 rpm. When the inoculum reaches an OD ranging between 0.7-10, an aqueous solution of xylose (50%) is added to the inoculum (final xylose concentration in the inoculum ranging between 0.5 and 5% (m/v)), according to the concentration studied), to induce the production of L-ASNase. The mixture was incubated in an orbital shaker during 8-36h at 25-40ºC (according to the operation conditions studied) and 210 rpm. All the materials and solutions used in this study were previously sterilized in autoclave (20 min at 121 ºC) and all the procedure was performed in a laminar flow chamber.

Since the production of L-ASNase by *Bacillus subtilis* used in the present work is intracellular, a cellular lysis was carried out after the fermentation process. The total fermentation volume was transferred to a falcon tube and centrifugated for 20 min at 5000 rpm. The supernatant was discarded, and the cells were resuspended in 5 mL of PBS. Following this, the solution was taken to Ultrasound and each sample was treated with 90 cycles of 5s of pulse and 10s of inactivity, using a Branson Digital Sonifier 250 & Sonifier Sound Enclosure. To conclude this process, the mixture was centrifuged during 10 min at 1789 g, and the supernatant containing L-ASNase was analyzed.

2.3. Quantification of cellular biomass

The cellular biomass quantification was carried out by weighing dry and wet weight of the cells. In first place, the Petri's dish with the cellulose acetate membrane was weight, followed by the vacuum filtration of 10 mL of the fermentation medium. Subsequently, the wet weight was registered, and the membranes were put in the oven at 80ºC. After 6 days, the dry weight was recorded.

For the calibration curve (*cf.* Supporting Information Figure S1), the same fermentation medium was diluted with different concentrations, followed by its OD measurement at 650 nm using a BioTeck Synergy HT microplate reader. The experiments were carried out in triplicate.

2.4. Optimization of L-Asparaginase activity quantification

The different steps to quantify the enzymatic activity were examined in order to accomplish the optimum procedure for this quantification. Regarding the enzyme reaction (catalysis of L-Asparagine by L-ASNase), different incubation periods (30, 90 and 165 min) and different volumes of L-Asparagine (50, 100 and 250 µL), were evaluated. Concerning the total volume of Nessler's reagent, several essays with different volumes (250, 500 and 1000 µL) were performed.

2.5. L-Asparaginase activity quantification

L-ASNase activity was measured using the colorimetric Nessler method according to the protocol described by Magri et al[136]. This method consists on the reaction of Nessler's reagent (dipotassium tetraiodomercurate (II)) with the ammonia released during the conversion of L-Asparagine into aspartic acid by L-ASNase, as shown in **Equation 2**. In this reaction, a yellow compound is formed providing a yellow color to the reaction mixture. The yellow color intensity of the solution is proportional to the ammonia released, allowing an indirect determination of the enzymatic activity. Briefly, this quantification is initiated by adding 0.5 mL of L-ASNase sample to a mixture containing 0.05 mL of 189 mM L-Asparagine solution and 0.5 mL of 50 mM of Tris(hydroxymethyl)aminomethane (TRIS)-HCl buffer (pH 8.6). The reaction mixture is incubated at 37ºC for 30 min and stopped by adding 0.25 mL of 1.5 M trichloroacetic acid (TCA). The total ammonia released in the enzymatic reaction was evaluated by adding 0.25 mL of Nessler's reagent to tubes containing 0.5 mL of the previous reaction mixture and 0.5 mL of distilled water. The absorbance was measured at 436 nm against the blank, which consisted of 0.25 mL of Nessler's reagent and 1 mL of distilled water, using a BioTeck Synergy HT microplate reader. In order to calculate the L-ASNase activity, a calibration curve was previously determined with ammonium sulfate (*cf*. Supporting Information Figure S2).

$$2\ K_2HgI_4 + 2NH_3 \longrightarrow NH_2Hg_2I_3 + NH_4I + 4\ KI \qquad (2)$$

The enzymatic activity (L-ASNase activity) was determined based on **Equation 3**. One unit of L-ASNase activity is defined as the amount of the enzyme liberating 1 μmol of NH_3 in 1 min at 37ºC.

$$\text{L-ASNase activity } \left(\frac{U}{mL}\right) = \frac{C_{[NH3]}\left(\frac{\mu mol}{mL}\right) * V_R\ (mL) * V_{Nessler}(mL)}{V_T(mL) * t_R(min) * V_E(mL)} \quad (3)$$

where $C_{[NH3]}$ is the ammonia concentration in solution (μmol/mL), V_R is the total volume of the mixture where the enzymatic reaction occurs (mL), $V_{Nessler}$ is the total volume of the mixture for quantification of ammonia with Nessler (mL), V_T is the volume of V_R transferred to the tube with Nessler and distilled water (mL), t_R is the Nessler's reaction time (min) and V_E is the total volume of L-ASNase sample (mL) introduced for the enzymatic reaction.

2.6. L-Asparaginase specific activity quantification

The total protein concentration was determined by measuring the absorbance at 280 nm of the solution containing L-ASNase. This method is based on the inherent absorbance of UV light by the aromatic amino acids tryptophan and tyrosine, as well as by cystine (disulfide-bonded cysteine residues) in proteins[137]. The measured absorbance of a protein sample solution is used to calculate the protein concentration by comparison with a calibration curve (*cf.* Supporting Information Figure S3) previously prepared from measurements with standard protein solutions, in this case bovine serum albumin (BSA). Therefore, with the total protein and the enzymatic activity previously obtained, it is possible to calculate the specific activity using the **Equation 4.**

$$L - ASNase\ Specific\ activity\ \left(\frac{U}{mg}\right) = \frac{L - ASNase\ enzymatic\ activity\ \left(\frac{U}{mL}\right)}{Total\ protein\ (mg)} \quad (4)$$

2.7. Determination of L-Asparaginase purity by Size-exclusion HPLC

To determine the L-ASNase purity in the extract size-exclusion high-performance liquid chromatography (SE-HPLC) was applied. A phosphate buffer solution containing NaCl was used as mobile phase and it was prepared using 47 mL of a Solution A (27.8 g of NaH_2PO_4), 203 mL of a Solution B (53.65 g $Na_2HPO_4 \cdot 7H_2O$) and 17.5 g of NaCl. Each sample with a total protein concentration of 3 mg/mL (previously determined UV-Vis spectroscopy) was diluted 1:9 (v/v) ratio in the phosphate buffer and then injected on a *Chromaster HPLC system* (*VWR Hitachi*). The SE-HPLC was performed on an analytical column *Shodex Protein KW-802.5* (8 mm x 300 mm). The mobile phase, a 50 mM phosphate buffer + NaCl 0.3 M, ran isocratically with a flow rate of 0.5 mL/min and the injection volume was 25 µL. The column oven and autosampler temperatures were kept at 40 ºC and at 10 ºC, respectively. The wavelength was set at 280 nm using a DAD detector. The obtained chromatograms were treated and analysed using the PeakFit version 4 software.

The L-ASNase purity was calculated based on **Equation 5.** The enzyme purity (% L-ASNase Purity) was determined by the ratio between the peak area of L-ASNase ($A_{L\text{-}Asparaginase}$) and the total area of all peaks of the chromatogram (A_{Total}), corresponding to other proteins produced during the fermentation process.

$$\%L - ASNase\ Purity = \frac{A_{L-Asparaginase}}{A_{Total}} \times 100 \quad (5)$$

2.8. Determination of fermentation protein extracts profile by SDS-PAGE

The proteins profile of the obtained extracts was determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The samples with a total protein concentration of 3 mg/mL (previously determined UV-Vis spectroscopy) were diluted at a 1:1 (v/v) ratio in a sample buffer composed by 2.5 mL of 0.5 M Tris-HCl pH 6.8, 4.0 mL of 10 % (m/v) SDS solution, 2.0 mg of bromophenol blue, 2.0 mL of glycerol and 310 mg of DTT. After this dilution, the samples were heated for 5 min at 95 ºC, to break up the quaternary structure and deconstruct part of the tertiary structure by reducing the disulfide bonds and denaturing the proteins. The diluted samples were loaded and run on a polyacrylamide gel (stacking: 4 % and resolving: 20 %). To stain the proteins the

gels were impregnated with BlueSafe and stirred in an orbital shaker at 50 rpm for 40 minutes at room temperature. GRS Protein Marker MultiColour (grisp Research Solutions) was used as molecular weight standards while lyophilized and purified L-ASNase from *E. coli* (P1321-10000; 10 000 IU) was used as a pure L-ASNase standard.

3.Results and discussion

3.1. Optimization of L-Asparaginase activity quantification

Among all the different reaction times, 30 min showed to be the optimal period time (**Figure 5**), revealing almost the double of the activity when comparing with the samples that reacted for 90 and 165 min. Concerning the L-Asparagine volume (L-Asparagine, 50 mM in phosphate buffer pH 8.6), the optimal volume was 0.05 mL, once higher volumes of the substrate will lead to the formation of a precipitate in the sample, when Nessler's reagent is added in more advanced phases of the quantification process. Finally, the optimal volume of Nessler's reagent was 0.25 mL since higher volumes will lead to a precipitation of the reagent (**Figure 6**).

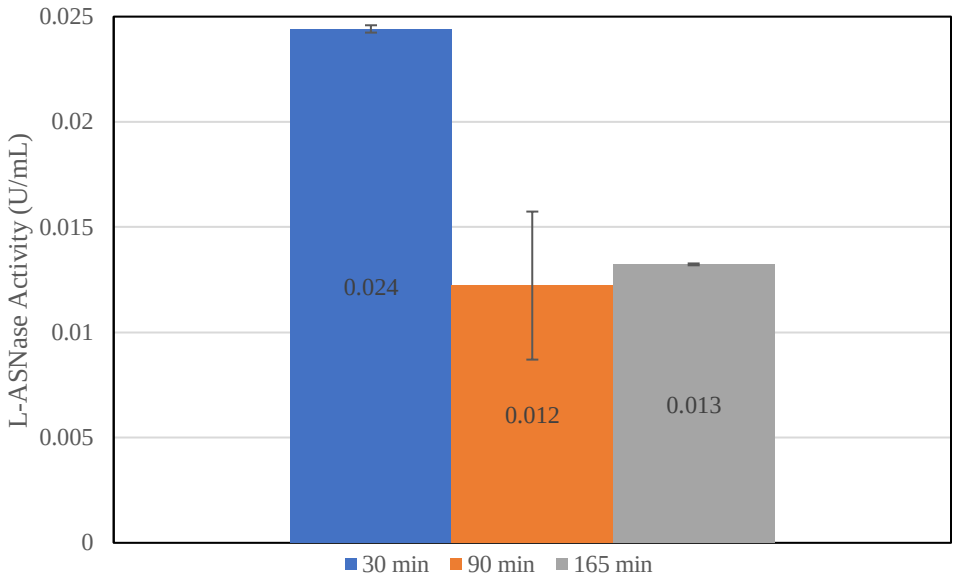

Figure 5. L-Asparaginase activity (L-ASNase activity (U/mL)) of fermentation extracts incubated with different periods of times using a total volume of L-Asparagine and Nessler's reagent of 0.05 and 0.25 mL, respectively.

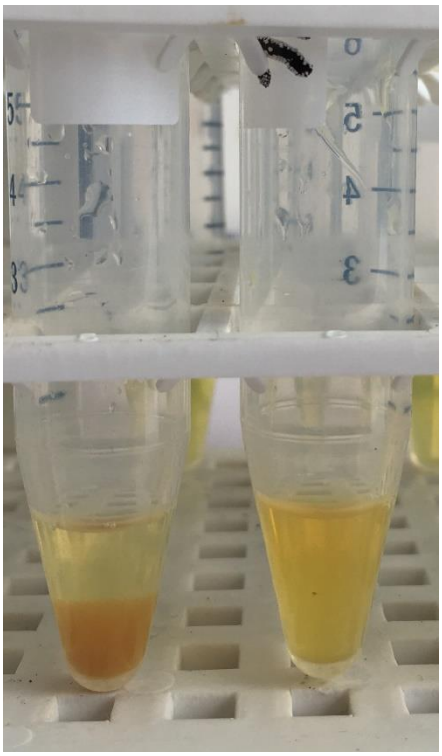

Figure 6. Macroscopic aspect of Nessler's reaction: Falcon tube on the left represents the samples treated with 0.5 and 1 mL of Nessler's reagent, which lead to its precipitation; falcon tube on the right represents the sample treated with 0.25 mL of Nessler's reagent, with no precipitated formed.

3.2. Optimization of L-Asparaginase production

The recombinant bacteria (*Bacillus subtilis*) used in this study was kindly provided by the Department of Bioprocesses and Biotechnology, School of Pharmaceutical Sciences, São Paulo State University – UNESP Brazil. Moreover, the primary fermentation conditions adopted were based on Professor Valéria Santos-Ebinuma's reports. Before the optimization of L-ASNase production, and with the purpose of future comparations, the pH value and optical density (OD) were monitored during the fermentation process applying the primary conditions (fermentation at 30ºC during 24h and using 0.5% (v/v) of xylose as the inductor for L-ASNase production).

3.2.1. Monitoring the pH in the fermentation process

pH monitoring was used to study the fluctuation of pH during the fermentation process and to understand if there are compounds being produced and exported to the extracellular medium influencing the pH. In **Figure 7** is displayed the graphic representation of the pH in function of time (h). This graphic representation allows us to conclude that the pH during the experiment slightly varied between 6.51 and 6.98, with the highest value of pH being 6.98, corresponding to the moment of the L-ASNase production using an aqueous solution of xylose. Moreover, the constant pH during the fermentation suggests the absence of compounds production to the extracellular medium that affect the pH medium. According to the literature, pH is a parameter which is optimized for the production of L-ASNase, varying from research to research in order to evaluate the final product[61,138–141]. Patro produced an L-ASNase from *Penicillium sp.* using a submerged fermentation, obtaining a maximum activity of 35.882 IU/mL at pH 7, which is quite similar to the pH of the fermentation process of this study[61]. Mihooliya et al. also produced a recombinant L-ASNase from *E. coli*, showing a maximum activity of 45.65 IU/mL at pH 7, which is in line with the earlier findings[140]. Contrarly, Kumar et al. reviewed the production and evaluation of L-ASNase obtained from *B. subtilis*, obtaining a maximum activity of the enzyme when the production medium was at pH 5[141].

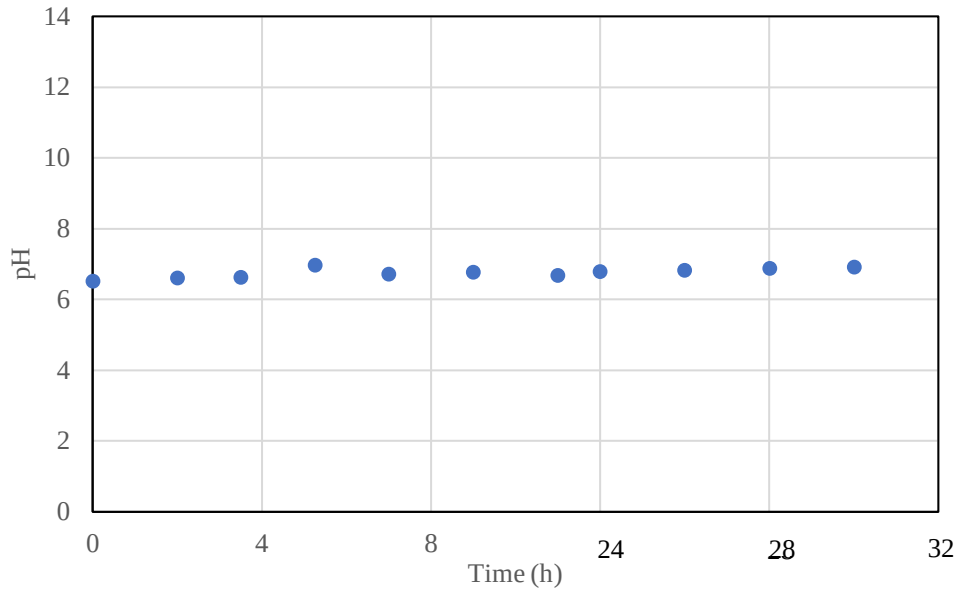

Figure 7. pH values of the fermentation process for the production of L-Asparaginase by *B. subtilis*.

3.2.2. Quantification of cell concentration

OD measurements were used to estimate the growth and metabolic activity of the bacterial cells kindly supplied by UNESP. The graphic representation of the OD in function of time (h) can be observed at supporting information (Figure S4). In order to quantify the cell concentration from the culture medium, the dry weight of the culture was calculated. The liquid culture was dried out and the amount of microbial mass was weighed on a scale, achieving a value of 0,583 g/L. Besides this, with the calibration curve made from the different dilutions of the fermentation medium it was possible to calculate the biomass concentration (g/L) along the fermentation process. From **Figure 8**, we can detect two different growing phases based on the biomass concentration during the experience. The first one until 10h of monitoring, classified as exponential phase in which there is a large increase in the biomass concentration, since the microorganism is perfectly adapted to the culture medium. After 10h of monitoring, a stabilization of the biomass is observed, this is, the microorganism consumed all the culture medium, reaching a stationary phase where there is no more increase of the biomass.

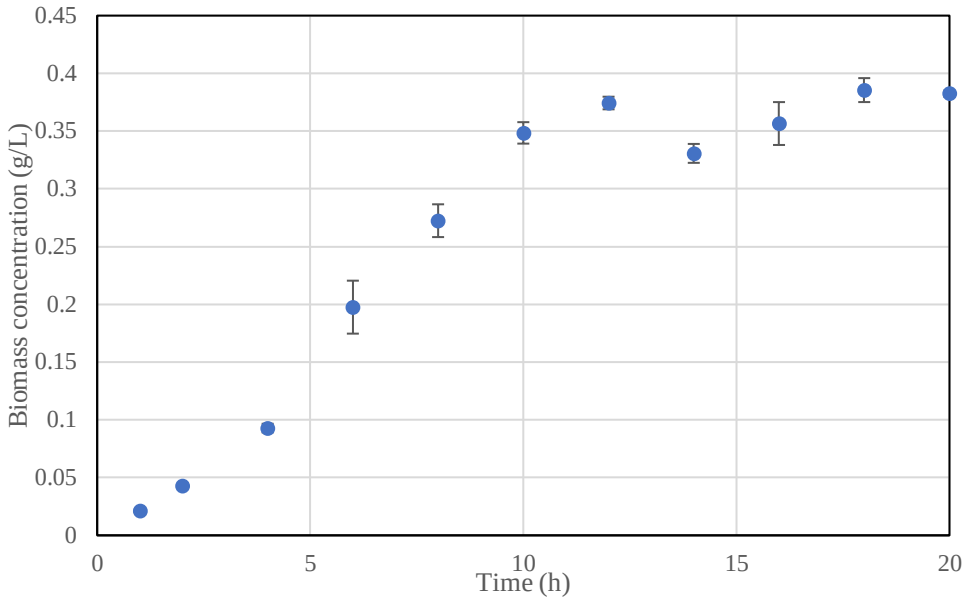

Figure 8. Biomass concentration during the fermentation process.

Besides this, a biomass growth curve was made which allowed to identify the exponential phase until 10h of monitoring, which goes in line **Figure 8.** Additionally, a stationary is observed after 10h of monitoring, where Ln values of biomass concentration remain almost constant due to the exhaustion of some compounds of the culture medium, such as carbon source, as shown in **Figure 9.** It was also possible to calculate the specific growth rate from **Figure 9** with a linear regression of the values corresponding to the exponencial phase (*cf.* Supporting Information Figure S5), obtaining a value of 0,3068 h^{-1}.

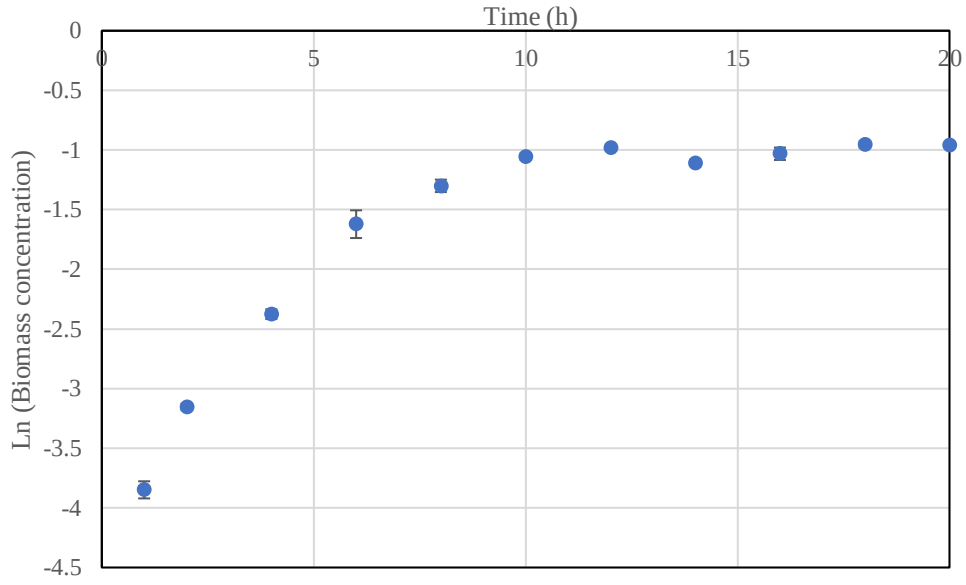

Figure 9. Ln values of biomass concentration during the fermentation process.

3.2.3. Effect of inductor's concentration in L-ASNase production

The fermentation process can be affected by several parameters, namely temperature, incubation time, agitation speed, pH, percentage of moisture, culture medium, type of inductor and its concentration. In order to optimize the L-ASNase production, the effect of inductor's concentration was firstly evaluated, using different concentrations of xylose ranging between 0.5-5% (v/v) and maintaining constant the remaining operational parameters, namely temperature, incubation time, agitation speed and culture medium. The L-ASNase production was monitored through the determination of the enzymatic activity (U/mL), L-ASNase specific activity (U/mg) and L-ASNase purity (%) (SE-HPLC analysis) in the different cell extracts. Based on **Figure 10**, it is possible to conclude that the induction of L-ASNase production with 3% of xylose is the optimum concentration to be used, leading to a higher L-ASNase activity and L-ASNase specific activity. In fact, as we increased the concentration of xylose, the enzymatic activity grows until a maximum of 0.756 U/mL for 3% of xylose, followed by a decrease to 0.415 U/mL corresponding to the concentration of 5% of inductor. Moreover, 1% and 2% of inductor's concentration revealed a quite similar enzymatic activity, while for the lowest concentration of inductor, a value of 0.269 U/mL of enzymatic activity was obtained. Moorthy et al., produced L-ASNase from *B. subtilis* DKMBT 10, and reported a maximum activity of 0.26 U/mL when glucose was the carbon source[142]. Comparing this work with the present results, it is possible to conclude that a higher enzymatic activity was obtained with *B. subtilis Δ6* genetically modified with the ansB gene from *A. fischeri*. This divergency on the enzymatic activity could be due to the different strain of *B. subtilis* used or due to the different volumes used for the quantification using Nessler's reagent. In another study, Makky et al., using the KK2S4 strain reported a maximum L-ASNase activity of 0.460 U/mL applying sodium nitrate as a inductor[143]. On a similar study by Jia et al., where *B. subtilis* was transformed using a gene that encodes for L-ASNase (ansZ), a value of 9.98 U/mL of enzymatic activity was obtained for the recombinant enzyme, which is significantly higher than the one from the gene's origin and from the present work [144]. Feng et al. on an analogous work, reported a L-ASNase from *B. subtilis* WB600 with genes from *B. subtilis* 168 with an enhanced enzymatic activity of 407.60 U/mL[145]. This superior activity compared with the one achieved on this work may be due to the combined strategy adopted, where plasmids expressing L-ASNase with different signal peptides were used[145]. Besides this, from **Figure 10** it is

possible to conclude that the maximum specific activity obtained was 0.011 U/mg for the samples treated with 3% of xylose, indicating the presence of a low content of other proteins, this is, the sample is hardly pure. Moorthy et al., reported a L-ASNase from the crude extract with a specific activity of 0.1 U/mg, however, after the purification steps, which included precipitation with ammonium sulphate and dialysis and ion exchange chromatography, obtained an enzyme with a specific activity of 1.120 U/mg[142]. Studies by Makky et al., revealed a L-ASNase with a maximum specific activity of 0.504 U/mg and 0.157 U/mg when treated at pH 5 and using lactose as a carbon source, respectively[143].

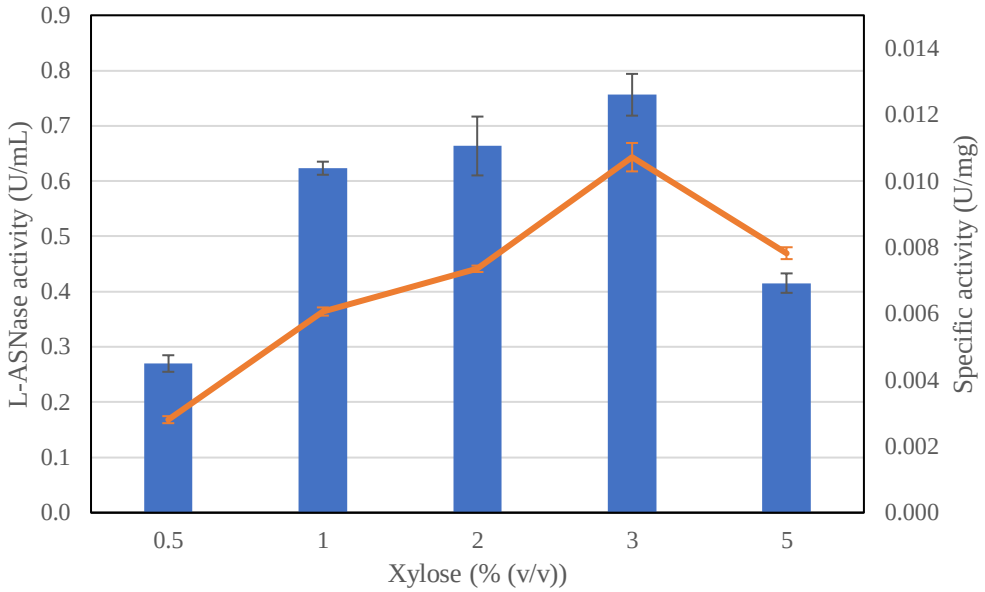

Figure 10. Effect of inductor's concentration (xylose) in the L-Asparaginase production at 30ºC during 24h: L-ASNase activity (U/mL) and L-ASNase specific activity (U/mg) represented by blue bars and orange line, respectively.

After quantification of enzyme activity and specific activity, the L-ASNase purity in the extracts from fermentations with different inductor's concentration, was determined by SE-HPLC analysis (**Figure 11** and **Table 10**). Besides this, the protein profile of the extracts was investigated by SDS-PAGE to observe the proteins profile and compare with the SE-HPLC chromatograms (**Figure 12**). From the chromatograms below (**Figure 11**), it is possible to observe two peaks between 10 and 15 min in all samples, except in L-ASNase standard produced by *E. coli*, which correspond to other proteins produced during the fermentation. Besides this, around the 16th min we can observe the peak of the commercial L-ASNase, thus, it is possible to conclude that the samples treated with 1% and 2% of xylose reveal a higher peak when compared with others, which may indicate an higher production of L-ASNase. However, associated with this higher peak at 16th min in these samples, there is also higher peaks at different times, which affects L-ASNase

purity. The samples treated with 0.5%, 3% and 5% of xylose reveal a 2-3 minutes deviation for the L-ASNase peak when compared to the commercial L-ASNase. Additionally, these last two samples, as well as the sample treated with 2% of xylose, have smaller peaks at other times, which may indicate that they could have a higher purity.

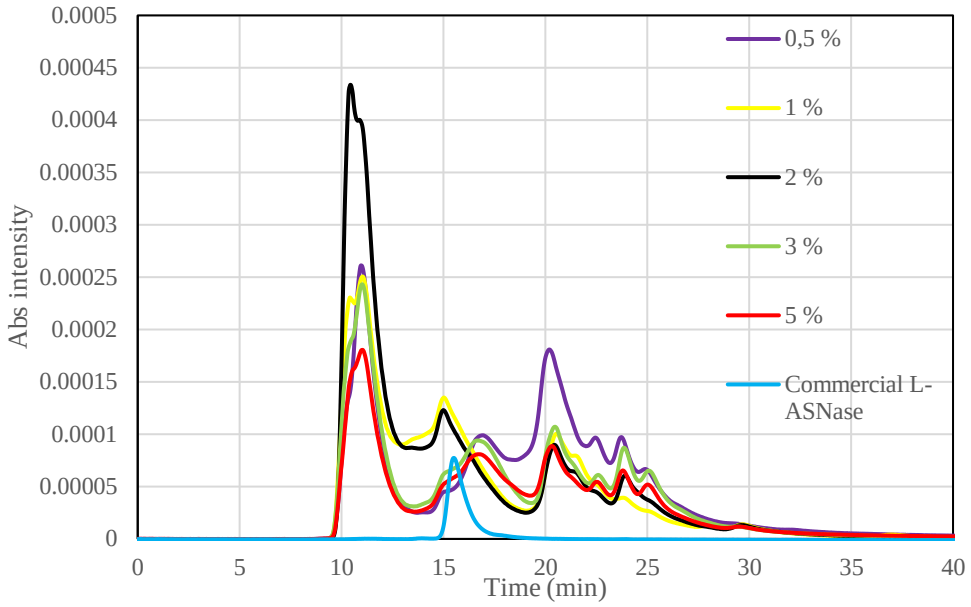

Figure 11. SE-HPLC chromatograms of extracts obtained after the fermentation process with different inductor's concentration and cellular lysis.

From **Table 10**, it is possible to conclude about the L-ASNase purity depending on the inductor's concentration used. In fact, with a concentration of 5% (v/v) of xylose, a higher L-ASNase purity is achieved, followed by the fermentations where a 1; 3; 2; 0,5 % (v/v) of xylose was applied. These results are not in agreement with the values of L-ASNase activity and specific activity from **Figure 10**, apart from the fermentation where a 0,5 % (v/v) of xylose was used (lower purity may correspond to a lower quantity of L-ASNase and therefore a lower specific activity), and we can conclude that probably some of the L-ASNase produced doesn't have any catalytic active. However, the higher purity obtained by the extract with a concentration of 5% (v/v) could be explained by the inferior production of proteins, which can be observed in **Figure 11**.

Table 10. Effect of inductor's concentration (xylose) in the purity of L-Asparaginase.

%Xylose	Purity (%)
0,5%	15.42
1%	23.97
2%	18.99
3%	21.97
5%	28.41

SDS-PAGE gel and the protein profile of the fermentation extracts with different concentrations of xylose were investigated and are depicted in **Figure 12**. The results demonstrated the presence of L-ASNase (a band between 45 and 37 kDa) as well as the presence of other proteins, which corroborate the previous results from the chromatograms of **Figure 11** and the purity from the **Table 10**. The commercial enzyme from *E. coli* (lane 2) showed a single band indicating a pure standard as expected. Usually, L-ASNase is known as a tetramer protein molecule, this is, having four protein subunits[36]. However, as reducing conditions were applied to perform the SDS-PAGE, only a single band corresponding to L-ASNase is noticed. Moreover, in every extract (lane 2 to lane 7) we can observe three bands in SDS-PAGE at approximately 37 kDa (L-ASNase), 60 kDa and 180 kDa. The presence of these three bands are in accordance with **Figure 11**, where 3 main peaks are in every chromatogram.

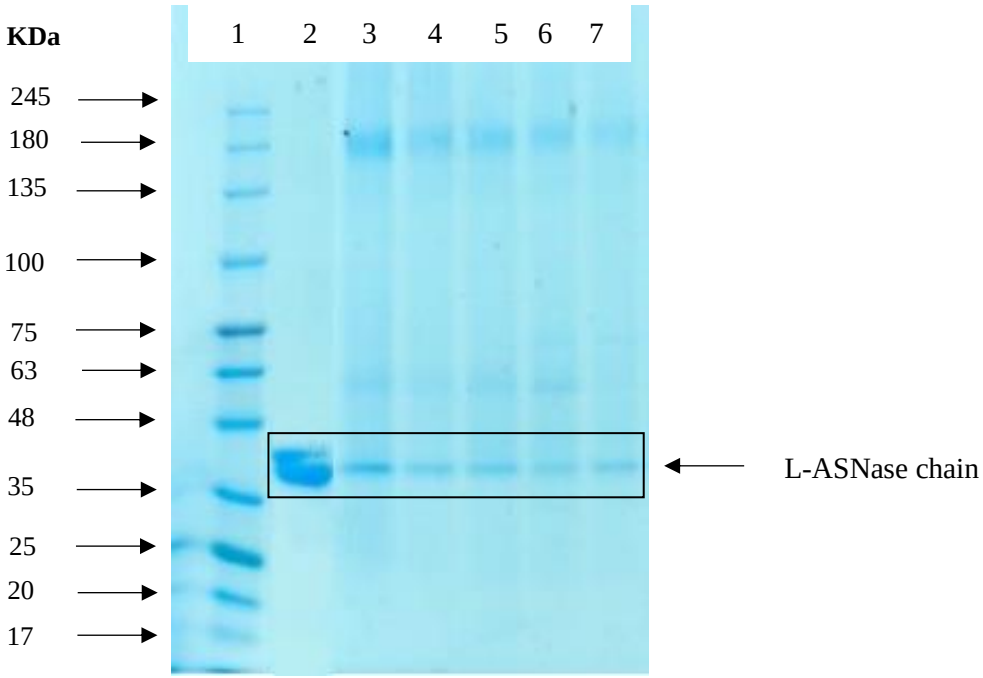

Figure 12. SDS-PAGE loaded with 1.5 µg of protein stained with Blue Safe.Lane 1: Protein marker; Lane 2: Commercial L-ASNase from *E. coli*; Lane 3: L-ASNase fermentation extract induced with 0,5% (v/v) of xylose; Lane 4: L-ASNase fermentation extract induced with 1% (v/v) of xylose; Lane 5: L-ASNase fermentation extract induced with 2% (v/v) of xylose; Lane 6: L-ASNase fermentation extract induced with 3% (v/v) of xylose; Lane 7: L-ASNase fermentation extract induced with 5% (v/v) of xylose.

3.2.4. Effect of temperature in L-ASNase production

Based on the previous results, where the concentration of inducer was tested, the best values for both enzyme and specific activity were obtained with 3% (v/v) of xylose. Beyond the effect of xylose concentration in the L-ASNase production, the temperature of fermentation after the induction step was also studied to optimize the production of L-ASNase. In this way, fermentation temperatures after induction between 25 and 40ºC were evaluated. In these assays, a 3% (v/v) of xylose to induce the L-ASNase production was applied according to the explanation given above. The L-ASNase activity (U/mL) and specific activity (U/mg) depending on the fermentation temperature are displayed on **Figure 13**. From the different temperatures investigated the fermentation at 30ºC revealed to be the optimal temperature after the induction for the L-ASNase production, reporting the highest enzymatic activity of 0,756 U/mL. Besides this, a slight difference of the L-ASNase activity between the samples treated at 30ºC and 35ºC is observed. Still, when applying a temperature of 40ºC leads to a loss of the enzymatic activity of more than 50%. The optimal fermentation temperature obtained in this work is in line with the earlier findings by Kumar et al., that reported a L-ASNase production with the same optimal temperature using *B. subtilis*[141]. However, studies by Chityala et al., demonstrated an ideal temperature of 37ºC, achieving an L-ASNase with a maximum enzymatic activity of 55 U/mL[146]. Similarly, Shukla and Mandal, revealed an optimum production of L-ASNase with an activity of 18.4 U/mL after 48h of incubation at 37ºC, like Hussein et al., with an optimal temperature for L-ASNase production by *B. subtilis* mutant B1U1 at 37ºC, reporting an enzyme with an specific activity of 6 U/mg[138,147]. Jia et al., also demonstrated an optimal temperature for cultivation of 37ºC with a specific activity of 23.85 U/mg, using *B. subtilis*[144]. On the other hand, Erva et al., optimized the production of L-ASNase from *B. subtilis* VUVD001, achieving a 2.88 U/mL with an ideal temperature of 49.9 ºC[148]. Regarding the L-ASNase specific activity, higher values are attained in the fermentation occurred at 40ºC (0,013 U/mg), and similar values are achieved for the fermentations at 25 and 30ºC (0.010 U/mg and 0,011 U/mg). Although the L-ASNase activity is lower at 40ºC, the absence of other proteins leads to an increase in the L-ASNase specific activity.

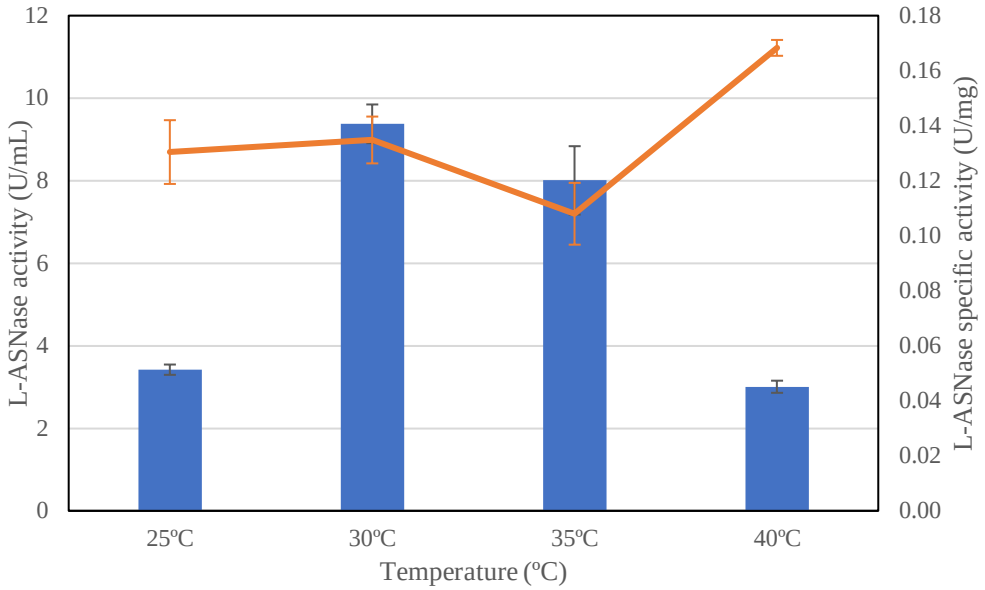

Figure 13. Effect of fermentation temperatures after induction in the L-Asparaginase production using 3% (v/v) of xylose during 24h: enzymatic activity (U/mL) and specific activity (U/mg) of L-Asparaginase are represented by blue bars and orange line, respectively.

The different fermentation extracts were also analyzed by SE-HPLC was used in order to determine the L-ASNase purity (**Figure 14 and Table 11**). From **Figure 14**, we can conclude that the fermentation at 35ºC after induction discloses the highest peak around the 16th min, this is, the time that matches with the commercial L-ASNase peak, which may indicate a higher production of L-ASNase when compared with other extracts. Nevertheless, the sample treated at 30ºC reveals a peak, slightly delayed when compared with the previous sample, which correspond also to L-ASNase, and a high production of L-ASNase. Still, this last sample has smaller peaks along the chromatogram, which suggests greater purity. In fact, from **Table 11**, it is possible to conclude that the fermentation at 30ºC lead to a high L-ASNase purity (21.97%). Regarding the extracts from fermentations at 25 and 40ºC, both samples present smalls peaks at different times, including the peak corresponding to L-ASNase, indicating that these temperatures are not the ideal for the production of L-ASNase by *B. subtilis*, and lower L-ASNase purity values were attained (13.96 and 15.33%). These results are in agreement with the data present in **Figure 13**, with fermentations at 30 and 40ºC leading to a high and low L-ASNase activity, respectively. These results support the report from Kumar et al., where a L-ASNase was produced from the same microorganism, with the maximum activity being achieved at an optimal temperature of 30ºC[141].

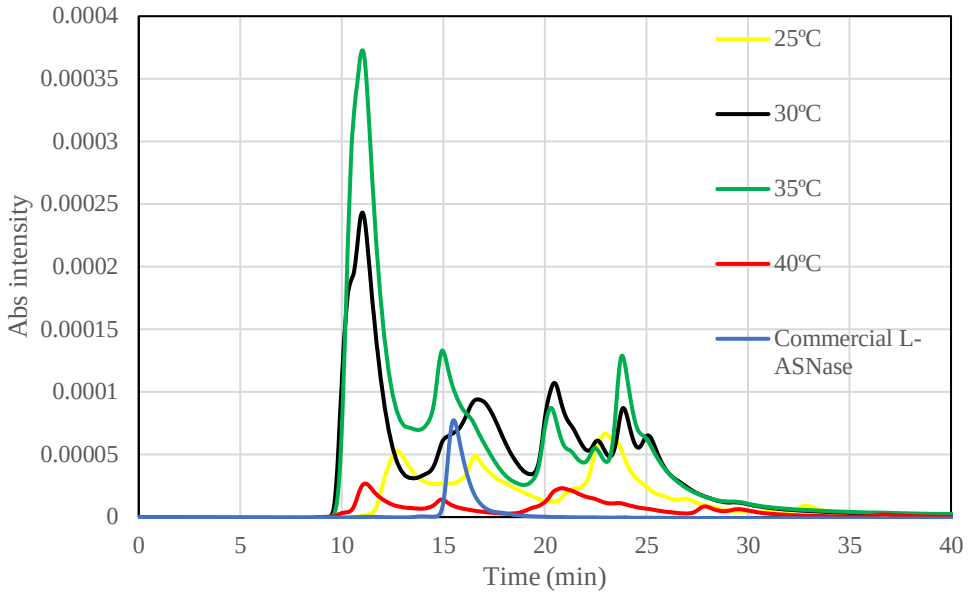

Figure 14. SE-HPLC chromatograms of extracts obtained after the fermentation process at different temperatures and cellular lysis.

Table 11. Effect of fermentation temperature after induction in the L-Asparaginase production using 3% (v/v) of xylose during 24h.

Temperature (ºC)	Purity (%)
25	13.96
30	21.97
35	16.55
40	15.33

The protein profile of L-ASNase extracts is depicted in the SDS-PAGE gel shown in **Figure 15**. The results proved the presence of L-ASNase as well as other proteins in most of the extracts, validating the results from **Figure 14** and the purity values from the **Table 11**. From **Figure 15**, it is possible to observe the presence of a very clear band around 37 kDa on the extract treated at 25ºC (lane 3), corresponding to L-ASNase. This clearness of the band could be explained by the low concentration of the sample. Besides this, comparing to the samples treated at 30ºC and 35ºC, lane 4 and 5, respectively, it is possible to notice more intense bands on the extract treated at higher temperature (35ºC), which corroborates the data from **Figure 14**, since this last sample has higher peaks at different times and consequently more production of proteins and less purity. Regarding the sample treated at 40ºC, we can perceive the more intense bands between 63 kDa and 75 kDa and the absence of the band corresponding to L-ASNase at 37 kDa. This intensity

of bands goes in line with the chromatogram from **Figure 14** once the higher peaks correspond to other proteins instead of L-ASNase.

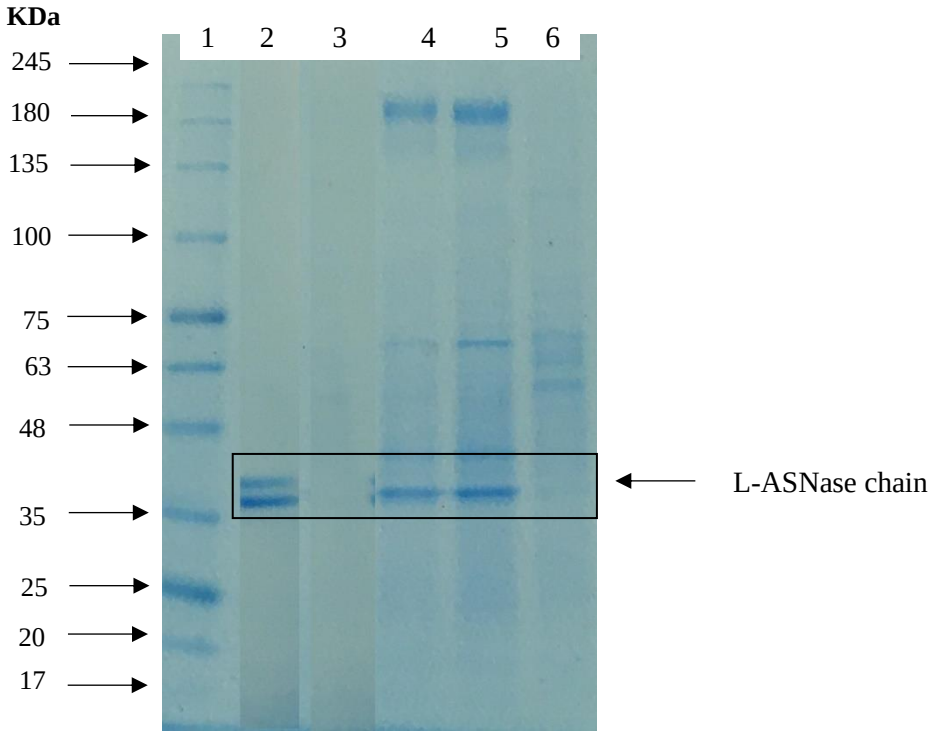

Figure 15. SDS-PAGE loaded with 1,5 µg of protein stained with Blue Safe.Lane 1: Protein marker; Lane 2: Commercial L-ASNase from *E. coli*; Lane 3: L-ASNase fermentation extract treated at 25ºC after induction; Lane 4:L-ASNase fermentation extract treated at 30ºC after induction; Lane 5:L-ASNase fermentation extract treated at 35ºC after induction; Lane 6: L-ASNase fermentation extract treated at 40ºC.

3.2.5. Effect of incubation time in L-ASNase production

Taking in account the results from the previous section, we can conclude that the best results for enzymatic activity and purity were achieved at a temperature of 30ºC after induction. Apart from the effect of inductor's concentration and temperature in the L-ASNase production, the incubation time of fermentation was another factor that was studied to optimize the production of L-ASNase. Therefore, incubation periods between 8 and 36h were tested. In these trials, a 3% of xylose to induce the L-ASNase production and a temperature of 30ºC after the step of induction were applied, since the previous data revealed this concentration and temperature as optimum. The L-ASNase activity (U/mL) and specific activity (U/mg) of L-ASNase depending on the incubation periods are exhibited on **Figure 16**. Among the different incubation periods investigated, the fermentation that lasted 24h revealed to be the optimal period for the L-ASNase production, reporting the highest enzymatic activity of 0,756 U/mL. Aside from this, a slight difference of the L-ASNase activity between the samples with an incubation time of 24h and 36h is detected. However, when the fermentation process took 8, 12 or 18h, the enzymatic activity dropped more than 50%. In fact, the sample with the shortest incubation time registered the smallest enzymatic activity. Between these three last extracts, the one which the fermentation process took 18h reported a maximum activity of 0,326 U/mL. The optimal incubation time in this work is in line with the earlier findings by Hussein et al., that reported an optimal period of fermentation of 24h, achieving an L-ASNase with a specific activity of 6 U/mg from a *B. subtilis* mutant isolate[147]. This high value of specific activity compared with the one obtained on this work may be due to the lack of other proteins in solution once this enzyme was produced extracellularly. Similarly, Moorthy et al., demonstrate that using glucose as carbon source in a 24h fermentation process, a L-ASNase from *B. subtilis* DKMBT10 was produced with an activity of 0.26 U/mL[142]. This value of activity compared with the one obtained on this work for the same incubation period, suggests that a higher yield was achieved on the present work. On the other hand, Li et al. described the production of a L-ASNase from *B. subtilis* with an enzymatic activity of 5321 U/mL, after 42h of fermentation[149]. Shukla and Mandal also reported the L-ASNase production from *B. subtilis* with a maximum activity of 18.4 U/mL after 48h of incubation[138]. This higher value compared with the one

obtained on this work may be explained by the different type of fermentation used on the work from the previous authors[138].

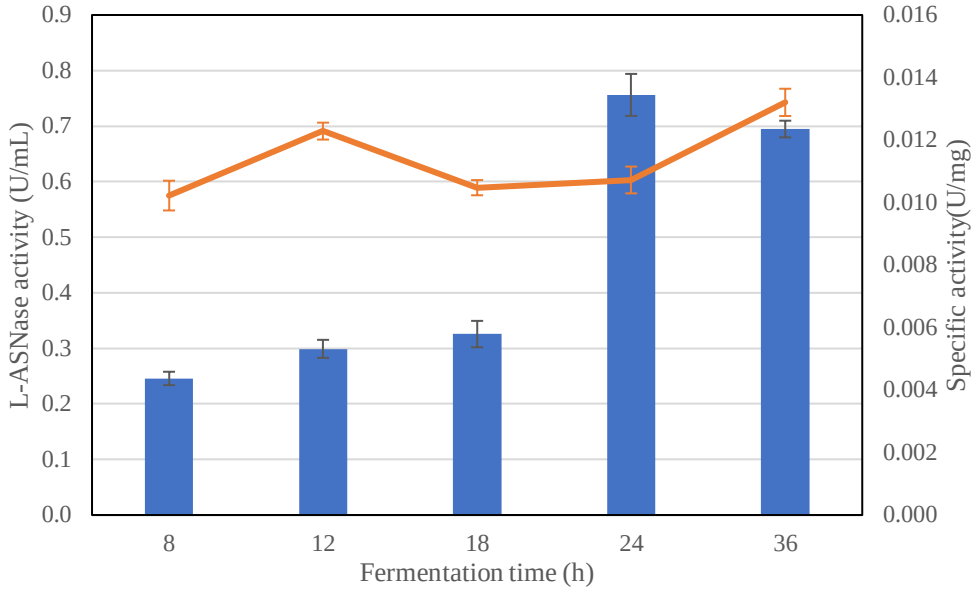

Figure 16. Effect of fermentation time in the L-Asparaginase production using 3% (v/v) of xylose with an incubation temperature of 30ºC: enzymatic activity (U/mL) and specific activity (U/mg) of L-Asparaginase are represented by blue bars and orange line, respectively.

Towards the calculation of L-ASNase purity, all fermentation extracts were analysed by SE-HPLC (**Figure 17 and Table 12**). **Figure 17** showed that the extract with the fermentation period of 36h has the highest peak around the time corresponding to L-ASNase peak and consequently the higher value of L-ASNase purity (**Table 12**). This result corroborates the data of **Figure 16**, as it is the sample with the highest specific activity (0.133 U/mg). Regarding the extract from the fermentation during 24 h, it is possible to conclude that it has the second highest peak around the 16th min (peak corresponding to L-ASNase), as well as other peaks along the chromatogram, which makes it less pure (21.97%) when comparing with the previous one. The remaining fermentation extracts with fermentation periods of 8, 12 and 18h reveal a small production of L-ASNase, as we can see from the small peaks on **Figure 17**, which goes in line with the low enzymatic activity from **Figure 16**. Moreover, the fermentation during 18h, has higher peaks corresponding to other proteins when comparing with the peak around the 16th min, explaining the low L-ASNase specific activity in the comparison with other extracts from **Figure 16**.

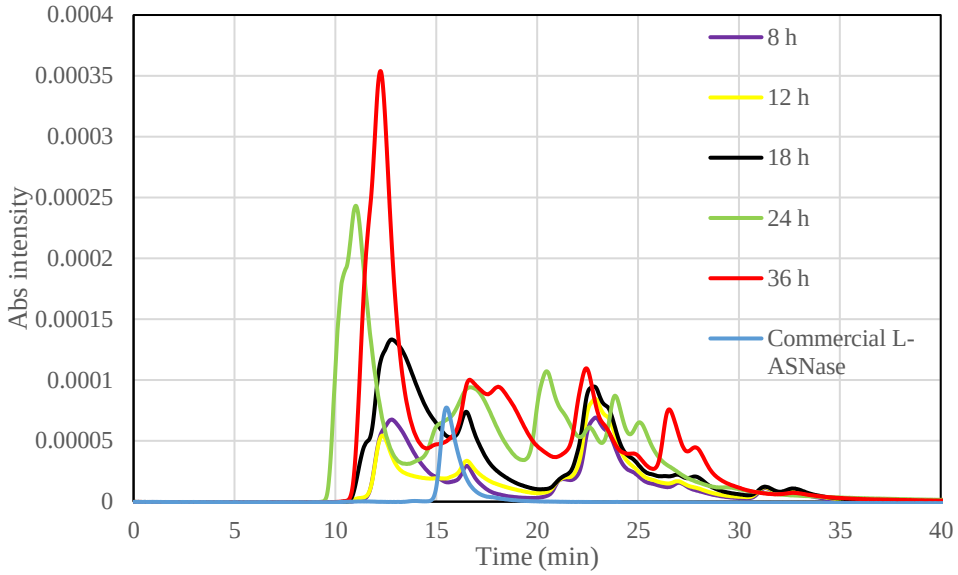

Figure 17. SE-HPLC chromatogram of extracts obtained after the fermentation with different incubation periods and cellular lysis.

Table 12. Effect of fermentation time after induction in the L-Asparaginase production using 3% of xylose at 30ºC.

Fermentation time (h)	Purity (%)
8	8.35
12	3.03
18	12.3
24	22.0
36	28.6

According to the SDS-PAGE depicted in **Figure 18**, we can notice the presence of contaminant proteins besides the L-ASNase, especially in the samples with higher enzymatic activity, this is, the ones with incubation periods of 24 and 36h. In fact, the default of intense bands on lane 3, 4 and 5 suggests the use of a minor concentration as well as the limited production of proteins, which corroborates with the small peaks of the extracts with fermentation times of 8, 12 and 18h from **Figure 17**. Nonetheless, it is possible to see a more intense band in lane 4 (fermentation during 18h) around 37 kDa (L-ASNase band), when comparing with extracts with shorter fermentation times, which could be explained by the higher enzymatic and peaks, from **Figure 16** and **Figure 17**, respectively. Besides this, the presence of bands around 180 kDa and 65 kDA, reveal the presence of other proteins, which goes in line with the peaks obtained on the chromatogram from **Figure 17**. Also, the more intense bands around 48 kDA on lane 7, comparing with lane 6, verify the higher peaks along the chromatogram from above (**Figure 17**).

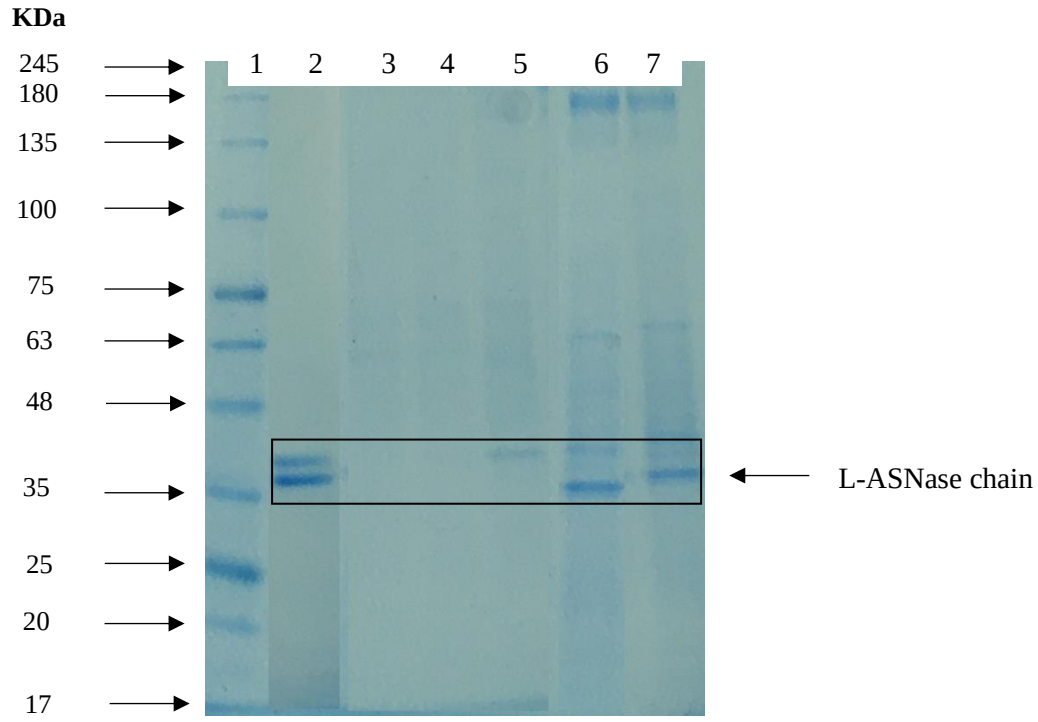

Figure 18. SDS-PAGE loaded with 1,5 ug of protein stained with Blue Safe. Lane 1: Protein marker; Lane 2: Commercial L-ASNase from *E. coli*; Lane 3: L-ASNase fermentation extract with an incubation period of 8h; Lane 4: L-ASNase fermentation extract with an incubation period of 12h; Lane 5: L-ASNase fermentation extract with an incubation period of 18h; Lane 6: L-ASNase fermentation extract with an incubation period of 24h; Lane 7: L-ASNase fermentation extract with an incubation period of 36h.

Based on the results obtained on this section, we can conclude that an incubation period of less than 24h is not ideal for the production of L-ASNase. Regarding the samples with an incubation period of 24 and 36h, both showed to be promising for the production of L-ASNase, achieving values of purity above 20 %. Furthermore, besides the higher enzymatic activity obtained with the extract with a fermentation time of 24h, the sample treated for 36h revealed a higher specific activity, which goes in line with its higher purity.

4. Final remarks and future work

Currently, L-ASNase is widely used in the treatment of different types of lymphoid malignancies due to its anti-cancerous effect, which leads to a great requirement of this protein. In fact, the microorganisms associated with the production of this enzyme are *E. coli* and *Erwinia sp.*, which are also linked to low yields and low immunogenicity, making of big interest the search for a new and better producing source.

In conclusion, the present study confirms that the genetically modified *B. subtilis* used shows the capacity to produce L-ASNase. Since the main aim of this work was the optimization of the production of this enzyme by the *B. subtilis*, several factors were optimized such as inductor's concentration, temperature after induction and incubation times, which allowed to obtain an enzyme with higher enzymatic activity in comparison with several current reports. In fact, with all these parameters optimized, this is, an induction with 3 % (v/v) of xylose at 30ºC for 24h, it was possible to produce a L-ASNase with an enzymatic and specific activity of 0,756 U/mL and 0.010 U/mg, respectively, and a purity of 21.97 %. Still, when comparing the specific activity obtained on this work with the ones reported on the literature, it was concluded that the samples have a low degree of purity. Therefore, the study of other fermentation's parameters such as agitation speed, culture medium, type of inductor and pH should be consider for future works with the purpose of obtaining a higher yield on the process as well as a L-ASNase with higher enzymatic activity. Besides this, in order to increase the specific activity, purification steps should be taken in account such as extraction and precipitation, solubilization, ultracentrifugation or chromatographic methods.

5.References

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6. Supporting information

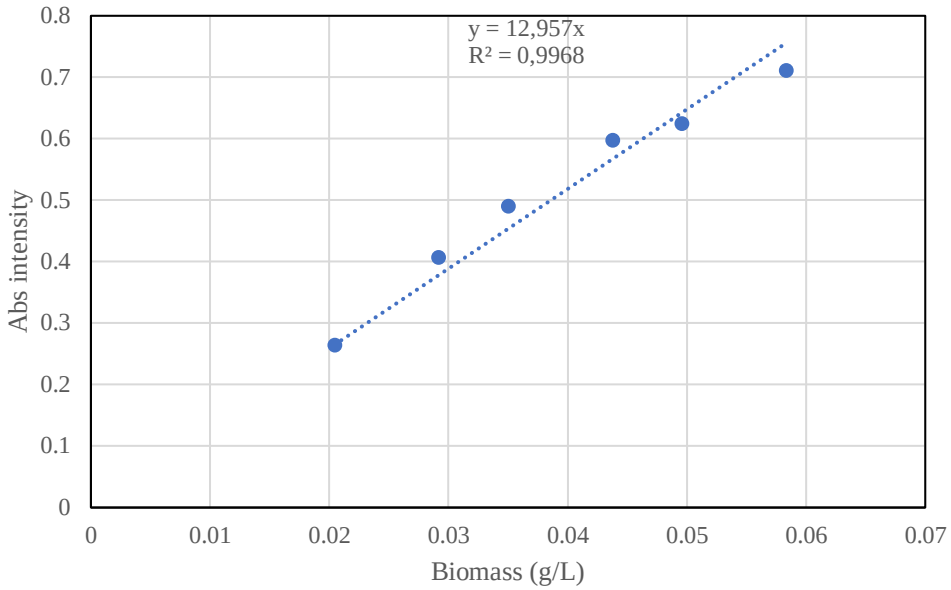

Figure S1. Calibration curve for the quantification of biomass concentration at 650 nm.

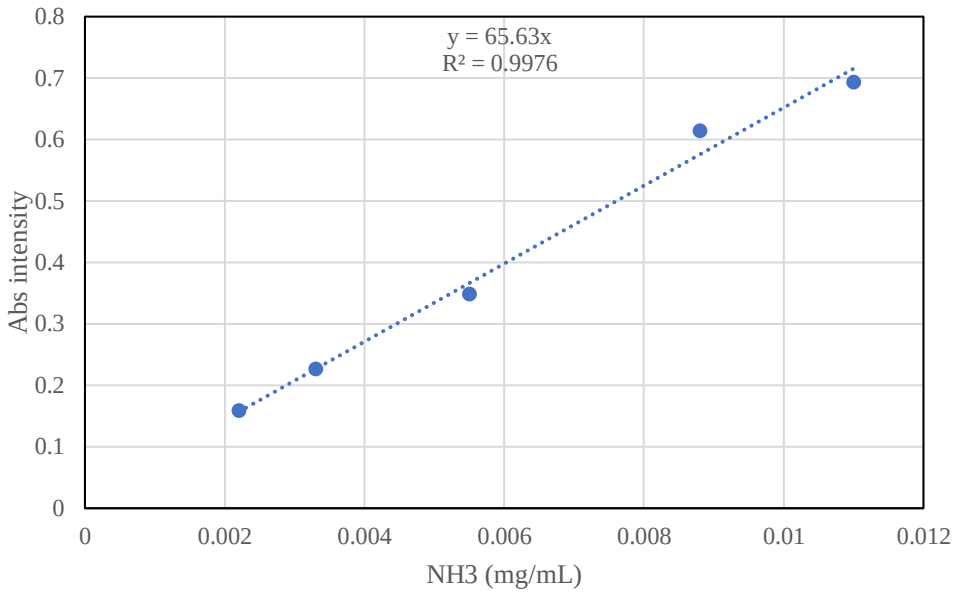

Figure S2. NH3 calibration curve for the quantification of L-ASNase enzymatic activity at 436 nm.

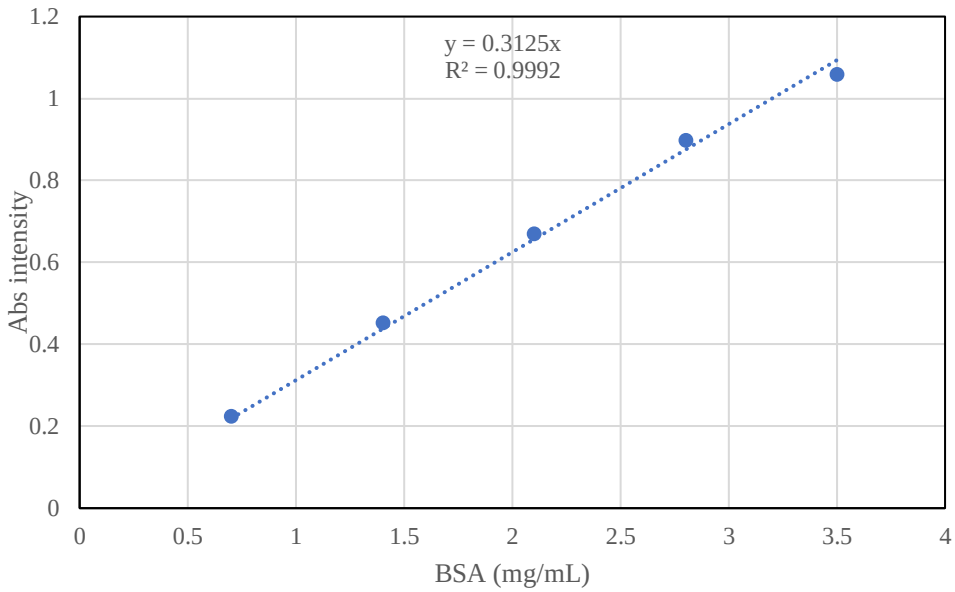

Figure S3. BSA calibration curve for the quantification of L-ASNase specific activity at 280 nm.

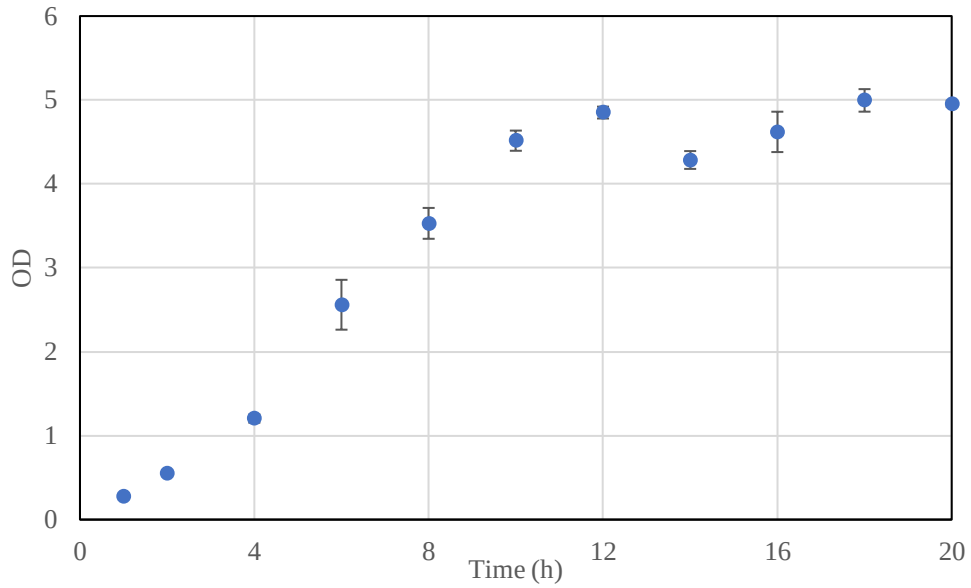

Figure S4. Monitoring optical density (OD) during 20h of fermentation for the production of L-Asparaginase by *B. subtilis*.

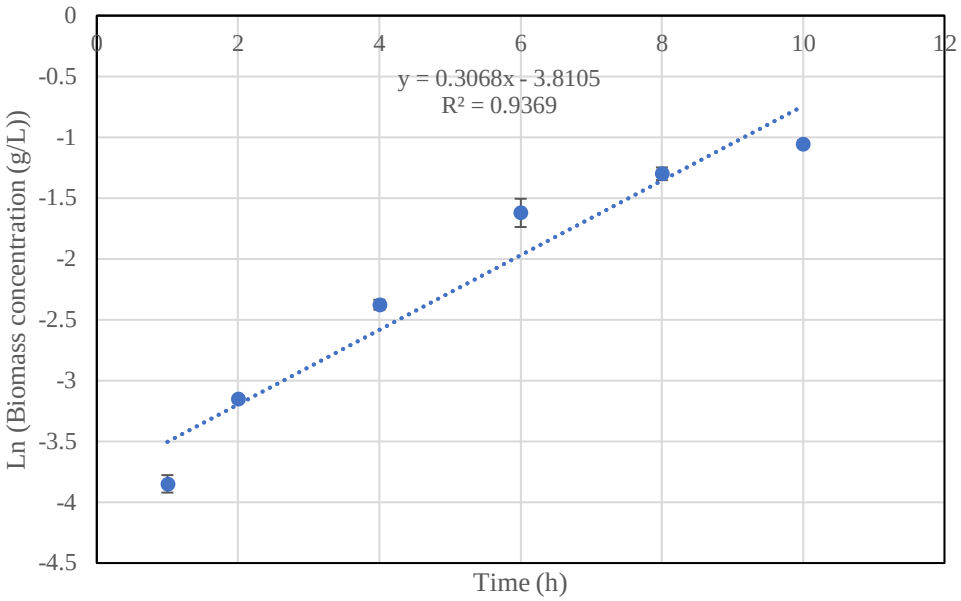

Figure S5. Linear regression of the Ln (biomass concentration (g/L)) and time (h) values corresponding to the exponential growth phase of *B. subtilis*.