Aliivibrio fischeri L-Asparaginase production by engineered *Bacillus subtilis*: a potential new biopharmaceutical

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Abstract

L-Asparaginase (L-ASNase) is an enzyme applied in the treatment of lymphoid malignancies. However, an innovative L-ASNase with high yield and lower side effects than the commercially available preparations are still a market requirement. Here, a new-engineered *Bacillus subtilis* strain was evaluated for *Aliivibrio fischeri* L-ASNase II production, being the bioprocess development and the enzyme characterization studied. The pBS0E plasmid replicative in *Bacillus* sp and containing PxylA promoter inducible by xylose and its repressive molecule sequence (XylR) was used for the genetic modification. Initially, cultivations were carried out in orbital shaker, and then the process was scaled up to stirred tank bioreactor. After the bioprocess, the cells were recovered and submitted to ultrasound sonication for cells disruption and intracellular enzyme recovery. The enzymatic extract was characterized to assess its biochemical, kinetic and thermal properties using L-Asparagine and L-Glutamine as substrates. The results indicated the potential enzyme production in bioreactor achieving L-ASNase activity up to 1.539 U mL^{-1}. The enzymatic extract showed an optimum pH of 7.5, high L-Asparagine affinity (K_m = 1.2275 mmol L^{-1}) and low L-Glutaminase activity (0.568-0.738 U mL^{-1}). In addition, thermal inactivation was analyzed by two different Kinect models to elucidate inactivation mechanisms, low kinetic thermal inactivation constants for 25 ºC and 37 ºC (0.128 and 0.148 h^{-1}, respectively) indicate an elevated stability. The findings herein show that the produced recombinant L-ASNase has potential to be applied for pharmaceutical purposes.

Keywords: L-Asparaginase, *Bacillus subtilis*, Stirred tank bioreactor, Enzymatic thermal inactivation, Enzymatic Characterization

1. Introduction

L-Asparaginase (L-asparagine amidohydrolase, L-ASNase, EC 3.5.1.1) has been described as an enzyme with elevated industrial importance since it is widely used in the treatment of lymphoid malignancies as acute lymphoblastic leukemia (ALL) and similar diseases, and also for its application in the food industries as acrylamide mitigation agent. These high-value applications have made this enzyme to account for about 40% of the total enzyme demands worldwide, representing around 33% of all anticancer agents applied [1-3]. Potential applications are driven by the enzyme characteristics. Two types of bacterial L-ASNase are reported, type I is characterized by low affinity for L-Asparagine and high affinity for L-Glutamine, while type II is recognized by its antitumor activity due to high L-Asparagine affinity and low L-Glutamine affinity. For pharmaceutical purposes only type II shows anticancer activity, that for better performance must combine a high enzymatic activity at pH 7 (human physiological pH) and elevated stability (mostly at 25 ºC and 37 ºC) [4, 5].

As an oncological agent, this enzyme acts to decrease the concentration of L-asparagine in the blood plasma by catalyzing the hydrolysis of L-asparagine, preventing the proliferation of cancer cells that are unable to produce this amino acid due to silencing of the Asparagine Synthetase encoding gene[6-8]. Commercial L-ASNase used as biopharmaceutical are produced exclusively as recombinant enzymes from *Escherichia coli* and *Dickeya dadantii* (formerly named *Erwinia chrysanthemi*). Although those formulations are efficient as oncological agents, they have some limitations that prevent a broader use, such as hyper sensibility, side effects and low yields triggering high costs, which indicates the need for new enzyme sources and new anticancer drugs [9-11]. Some problems associated with the available treatments is the L-Glutamine hydrolysis that causes hepatotoxicity, coagulation dysfunction, immunosuppression and pancreatitis [12, 13]. These issues make the development of new L-ASNase source a key industrial requirement.

In this sense, several studies have been carried out to search for new wild microbial producers' and to create new recombinant L-ASNases with improved characteristics and properties, including reduced side effects, higher thermal and storage stabilities, easier purification and high production

yield. The advances in rDNA technology, computational biology, and biostatistics have highlighted recombinant protein production as the most promising alternative in the search for efficient enzymes [14, 15].

E. coli is extensively used as a bacterial workhorse for industrial purposes mostly due to its fast growth in a simple culture medium [16]. However, the use of other microbial hosts for recombinant protein production has received increasing attention. For instance, *Bacillus subtilis* is a Gram-positive bacterium that holds a Generally Recognized As Safe (GRAS) status and presents interesting attributes for an industrial workhorse, such as a stable expression system, clear inherited backgrounds, grows well on simple media and exhibits robustness in large-scale cultivation [17-20].

The need for novel L-ASNase sources has driven us to evaluate the production and characterization of the recombinant *Aliivibrio fischeri* L-ASNase produced by *B. subtilis*. In this work, we have evaluated the production in orbital shaker and the potential scaling up to bioreactor cultivations, also the enzyme's biochemical, kinetic and thermal characteristics were established, and a thermodynamic study was performed. Therefore, we present an engineered platform with potential to become a new industrial bioprocess to supply the biopharmaceutical market.

2. Material and Methods

2.1 Material

The Thermosensitive Alkaline Phosphatase, the *Dpn*I, the T4 DNA Ligase and the restriction enzymes *Pst*I and *Eco*RI used in this work were obtained from Thermo Fisher Scientific™. The Polymerase Chain Reactions were performed by the high-fidelity TIME ECRA HIFI DNA Polymerase (ECRA Biotec™) using the thermocycler was the C1000 Touch™ Thermal Cycler (Bio-Rad). The PCRs and digestion reactions were purified by the GeneClean® turbo kit (MR BiomedicalsTM) and Wizard® SV Gel and PCR Clean-Up System (Promega). The plasmids were extracted by the PureYield™ Plasmid Miniprep System (Promega). The DNA was quantified in the microplate reader Tecan Infinite 200 Pro (Tecan™). In order to perform the Sanger sequencing, the samples were prepared using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied

Biosystems™, Thermo Fisher Scientific™) and the BigDye XTerminator™ Purification Kit (Applied Biosystems™, Thermo Fisher Scientific™). In addition, the in-silico analysis was performed in the softwares SerialCloner 2.6.1 and Benchling. L-Asparagine and L-Glutamine were acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Cloning and strain development

The L-ASNase type II gene *ansB* (NCBI GeneID: 3278692) was amplified by Polymerase Chain Reaction (PCR) from the *A. fischeri* genome using modifying oligonucleotides carrying the biobrick prefix (forward) and suffix (reverse). The Ribosome Binding Site R0 [21] was added to the forward primer (ansB_fw: CTGAGGAATTCAAAAAAAAATTCTAGAGAGCTGATTAACTAATAAGGAGGACAAACA TGAAAAAGAATGCCATTGC and ansB_rv: TTAGCTGCAGCGGCCGCTACTAGTACAACCTACATAAACATTTCATCCTTC). The resulting *ansB* amplicon was cloned into the replicative pBS0EXylRPxylA (BBa_ECE743) vector using the *Eco*RI and *Pst*I restriction sites [19]. That placed the *ansB* gene under control of the xylose induced PxylA promoter (BBa_K733002). *E. coli* TOP10 was used to propagate the constructed plasmid.

In order to perform the digestion of the plasmid and the *ansB* gene carrying the RBS by the restriction enzymes *Eco*RI and *Pst*I, the reactions were incubated at 37ºC for 1 h. In addition, the plasmid was dephosphorylated by a Thermosensitive Alkaline Phosphatase and the *Dpn*I was used to degrade the PCR template performed to amplify the *ansB* gene from the *A. fischeri* genome. The resulting plasmid and insert were ligated by the DNA Ligase and the ratio used in the reaction was one part of plasmid for five parts of insert. The reaction was incubated at 22ºC for 1 hour and the resulting plasmid was inserted into the *E. coli* TOP 10.

After sequence confirmation by Sanger sequencing, the plasmid pBS0EXylRPxylA-ansB was used to transform *B. subtilis* KO7 (BGSCID 1A1133), an extracellular protease-free strain. *E. coli* and *B. subtilis* were cultivated in Luria-Bertani (LB) (tryptone 10.0 g L^{-1}, yeast extract 5.0 g L^{-1},

sodium chloride 10.0 g L^{-1}, pH 7.0) medium supplemented with 100 µg/mL ampicillin and 1 µg/mL erythromycin, respectively. Cultivation was carried out at 37ºC and 220 rpm.

2.3 L-Asparaginase production

The L-ASNase production was evaluated in orbital shaker and in Stirred tank bioreactor. For both cases the inoculum was prepared by activation of a stock culture of the microorganism (20 v/v% in glycerol aqueous solution maintained at −80 °C) in 5 mL of LB medium supplemented with erythromycin 1 µg mL^{-1} in 10 mL test tubes. Cells were grown at 37 ºC, overnight (14-16 h) in an orbital shaker (INNOVA40, New Brunswick, USA). The production process started by transferring the inoculum culture of *B. subtillis* to 500 mL Erlenmeyer® type flasks containing 100 mL of the LB medium or to a Minifors II bioreactor (Infors, New Jersey/USA) with two Rushton impeller using a working volume of 4 L. The initial Optical density ($OD_{600\,nm}$) was adjusted to 0.1 Absorbance Units (A.U.), which corresponded to an inoculum volume of approximately 150 mL. The bioprocess was conducted at 30°C for 24 h. Orbital shaker cultivations were performed with 200 rpm while bioreactor cultivation conditions were 300 rpm, aeration rate of 1.5 L min^{-1} and the dissolved oxygen level was maintained at 30%. Aqueous xylose solution (50 wt.%) was used as inducing agent at 0.5 wt.% at different time points in the exponential growth phase. After cultivations, cells were harvested by centrifugation (12000 x G, 4 ºC, 10 min) and washed twice with saline-phosphate buffer pH 8.0 (PBS). Resulting pellets were stored at -20 ºC. The supernatants of fermented media were used to determine xylose consumption and, then, discarded. Recovered cells were submitted to ultrasound sonication (Eco-Sonics – Ultronic, SP, Brazil) with 45% power in 15 sets of 2 min of pulses and 1 min interval between them for cell disruption using 10 mL of PBS under ice bath. The enzymatic extract was recovered by centrifugation.

2.4 L-Asparaginase characterization

The enzymatic characterization was performed as a function of the optimum operating pH, using citrate-phosphate (pH 3.0-7.0) and TRIS-HCl (pH 7.5-9.5) buffers during the enzymatic hydrolysis reaction of L-ASNase at 37 ºC. Substrate affinity was evaluated considering different

concentrations of L-Asparagine and L-Glutamine solution in the enzymatic activity assays at 37 ºC for 10 min. The thermal stability characterization was performed by incubating the enzyme extracts (PBS buffer pH 8.0) under different temperatures in a thermostatic bath (model 521/2DE, New Ethics, SP, Brazil). Samples were withdrawn periodically for L-ASNase activity determination. The influence of temperatures of 25 (±1) ºC, 37 (±1) ºC, 45 (±1) ºC and 60 (±1) ºC was evaluated, with samples taken periodically to quantify the enzyme activity.

The thermal parameters were determined according to the enzyme deactivation models well described in the literature [22]: inactivation following consecutive reactions from an unique enzyme at zero time, eq. (2) and inactivation following elemental reactions from an unique enzyme at zero time, eq. (4);

$$E\ k_1 \rightarrow E^*\ k_2 \rightarrow D \qquad (1)$$

$$A = e^{-k_1 t} + \alpha \frac{k_1}{k_2 - k_1}(e^{-k_2 t} - e^{-k_1 t}) \qquad (2)$$

where A is the relative residual enzyme activity, α is the activity ratio between enzyme species, k_1 and k_2 are the kinetic thermal inactivation constants and t is the incubation time (h).

$$E\ k_1 \rightarrow E^* \qquad (3)$$

$$A = (1 - \alpha)e^{-kt} + \alpha \qquad (4)$$

The thermal parameters α and k_1 and k_2 of the model were estimated by a non-linear fitting using the GRG non-linear optimization method available in Microsoft Excel SOLVER tool, using as objective function the percentage absolute average deviations between the experimental and calculated activity

179 values. Biocatalyst half-life ($t_{1/2}$) was calculated from Eq. 2, using the estimated parameters (k and α)
180 and making A equal to 0.5.
181 All characterization experiments were performed in triplicate.

182 **2.5 Analytical methods**

183 *L-asparaginase activity*

184 L-ASNase activity was measured by the method described initially by Mashburn and Wriston
185 (1963) [23]. The assay is based on hydrolysis of L-asparagine by the enzyme preparation to release
186 ammonia. One unit of L-asparaginase activity is defined as the amount of enzyme required for the
187 release of 1 µmol of ammonia per min at 37 °C and pH 8.6 [24-26]. Enzymatic reactions were
188 performed by the addition of 0.5 mL of enzymatic extract obtained after cell disruption, 0.5 mL of
189 TRIS-HCl buffer pH 8.6 and 0.05 mL of L-asparagine solution 189 mmol L^{-1}. The reaction was
190 stopped after 10 min with addition of 0.25 mL of TCA 1.5 mol L^{-1}. After centrifugation, samples
191 were analyzed regarding NH_3 release using Nessler colorimetric quantification method.

192 L-asparaginase activity values were calculated according to equation 5.

193 $$L-ASNase\ activity\ (U\ mL^{-1}) = \frac{C_{[NH_4^+]} \times V_R \times V_{Nessler}}{V_T \times t_R \times V_E} \quad \text{Eq (5)}$$

194 Where: $C_{[NH_4^+]}$ = Ammonium concentration (µmol mL^{-1}), V_R = enzymatic reaction volume, $V_{Nessler}$
195 = sample volume for Nessler's ammonium quantification (mL), V_T = amount of V_R used for Nessler
196 quantification, t_R = enzymatic reaction time (min) and V_E = enzymatic extract volume used in the
197 reaction (mL).

198 Enzymatic activity colorimetric method was also confirmed by HPLC according to
199 methodology described by Magri et al. [24].

200 *Xylose concentration*

201 Culture supernatant aliquots were taken at the induction point and at the end of the cultivation.
202 Aliquots were then filtered i n a 0.45 µm filter (Millipore) and the xylose consumption was analyzed
203 by high performance liquid chromatography (HPLC-Shimadzu - LC 20AD) coupled with a RID
204 detector employing an AMINEX HPX-87H cation exchange column (300x 7.8 mm, 9 µm; BIO-

RAD©), at isocratic mode using as mobile phase 0.005 M H_2SO_4 for 13 min; flow rate of 0.6 mL min $^{-1}$ at 60°C.

Total protein concentration

Concentration of total protein in the extracts after cell disruption was quantified by the BCA protein assay test kit (Thermo-Scientific) by addition of 25 µL of diluted sample and 200 µL of BCA working reactant, the mixture was incubated at 37 ºC for 30 min in the absence of light and the absorbance was measured at wavelength of 562 nm. Quantification was calculated based in a standard calibration curve using bovine serum albumin.

Data analysis

All experiments were performed in triplicate and the data were statistically validated under 95% level of confidence according to Tukey test using the software OriginPro®2016 (OriginLab Corporation, MA-USA).

3. Results and discussion

3.1 *B. subtilis* as an expression platform for the *A. fischeri* L-Asparaginase

The L-ASNase II from *A. fischeri*, encoded by the *ansB* gene, shares 71% sequence identity with the *E. coli* L-ASNase and 52% with the *D. dadantii* L-ASNase. On the other hand, the *E. coli* and the *D. dadantii* L-ASNases share 49% amino acid sequence identity. The high identity score of the *A. fischeri* L-ASNase with the commercial enzymes indicates a potential candidate for a new efficient oncological drug while holding some sequence divergence that may elicit a different immune response. The divergence may also result in different enzyme characteristics such as stability. To determine the real potential of the *A. fischeri* L-ASNase, the *ansB* gene was overexpressed in *B. subtilis* KO7 driven by the xylose inducible promoter PxylA. Cell extracts from induced and non-induced cultures were analyzed through SDS-PAGE to identify the recombinant enzyme production. A visible protein band around 30-40 KDa indicates the successful production of the recombinant L-

ASNase (Figure 1). The *A. fischeri* L-ASNase has a molecular mass of 37.5 kDa calculated from the gene sequence. Therefore, the band found corresponds to the molecular weight of the L-ASNase monomeric form. L-ASNase are usually active as tetramers in native condition. The *E. coli* L-ASNase tetramer molecular weight is 141 KDa [27]. However, under denaturing conditions only the 30-40 KDa monomer band of the commercial *E. coli* L-ASNase appears (Figure 1). It is important to notice that the negative control did not show any significant band around 30-40 KDa confirming that the untransformed *B. subtilis* is unable to produce a detectable amount of L-ASNase.

Figure 1

Some reports have previously described the gene expression driven by the inducible promoter PxylA. For instance, Bhavsar et al. (2001) [28] studied the xylose-dependent expression system controlled by the PxylA in *B. subtilis* and compared it to an isopropyl-β-D-thiogalactoside (IPTG) dependent system using a thermostable β-galactosidase as a reporter. They found the induction with xylose resulted in an induction/repression ratio (279-fold) higher than the induction with IPTG and had lower expression levels without the inductor. These results show the expression system based on the XylR repressor and PxylA promoter is an interesting system for *B. subtilis* and can be a better option than the IPTG dependent systems. Moreover, IPTG is a consumable more expensive than xylose.

More recently, Larsen et al. (2018) [29] developed a system for protein expression and secretion by *B. subtilis* using the PxylA promoter. They confirmed the expression using the Green Fluorescent Protein (GFP) as reporter, then, to validate the protein secretion the subtilisin was produced. Therefore, it was proven that the proteins were produced and the subtilisin was secreted which confirms these system works and the inducible PxylA promoter is suitable for *B. subtilis*.

Considering the results herein, it was possible to confirm that L-ASNase II is being produced proving the PxylA expression system constructed is working as expected. This confirm the results from previous works, as mentioned before [28, 29] and shows that the XylR-PxylA dependent systems are a promising option for protein production by *B. subtilis*.

3.2 Heterologous production of *A. fischeri* L-asparaginase in orbital shaker and Stirred tank bioreactor

Genetically engineered *B. subtilis* growth and L-ASNase production were performed in orbital shaker and in Stirred tank bioreactor (STR) in order to evaluate the potential scale up of the bioprocess. The growth curves shown in Figure 2 indicate the cultivation in the bioreactor achieved higher cell density ($OD_{600\ nm}$ = 3.7 A.U.) than in an orbital shaker, which presented a slower growth reaching $OD_{600\ nm}$ = 2.7 A.U. in 24 h of bioprocess. These results indicate the potential of a high yield production at industrial scale of the engineered microorganism using bioreactors.

Figure 2

A previous report has pointed the middle of log phase (exponential phase) as the best time for induction of heterologous gene expression in *E. coli*, as most cells are alive and healthy at this stage [16]. Thus, it was evaluated the best induction point considering the middle of the exponential phase as the center point. The objective was to select the shortest time point for induction that causes the highest targeted protein production, maximizing the yield. First, the experiments were performed in orbital shaker through the analysis of L-ASNase production without induction and with induction at the beginning (4 h), in the middle (5 h) and at the end (6 h) of the exponential growth phase (indicated by the elevated linearity of these points corresponding to a linear fit R^2 = 0.9972). The experiments were carried out for 24 h. Table 1 shows the protein content, L-ASNase activity and L-Glutaminase activity in orbital shaker at different induction condition and in the bioreactor induced at OD_{600nm} = 0.7 A.U. According to Tukey test the cultivation without the inducing agent (xylose) addition has not promoted different L-ASNase production compared the negative control (*B. subtilis* strain with no genetic modification), presenting L-ASNase activities of 0.569 and 0.554 U mL^{-1}, respectively. This behavior indicates the promoter is successfully repressed in the absence of the inductor. Induction at the beginning and at the middle of the exponential growth phase (OD_{600nm} = 0.7 and 1.1 A.U.,

respectively) promoted the highest enzyme activities (1.418 and 1.427 U mL^{-1}, respectively) with no significant difference ($p<0.05$). The induction at the end of the exponential growth phase (OD_{600nm} = 1.5 A.U.) resulted in lower activity (1.185 U mL^{-1}), possibly due to the fact that cells were not in their maximum metabolic activity, or even due to the shorter production period.

Following, bioreactor cultivations were performed with xylose induction in the beginning of the exponential growth phase, OD_{600nm} = 0.7 A.U., which resulted in enzymatic extracts that presented L-ASNase activity of 1.539 ± 0.038 U mL^{-1}. This result shows that the scale-up process was performed satisfactorily and improved processual conditions can allow for high yield. It is quite common during this scale-up process to produce low amount of the target biomolecule, requiring a series of additional experiments to adjust the cultivation parameters. Specific activity achieved up to 0.336 U mg^{-1} protein.

Table 1

In fact, *B. subtilis* cultivations in bioreactors have the potential for higher production yields once important parameters as dissolved oxygen can be easily controlled. Feng et al. (2017) [30] have increased the cell concentration of *B. subtilis* in 3-fold in the scaling up from flasks to a 3 L bioreactor using a fed-batch strategy. Regarding the enzymatic activity obtained, Einsfeldt et al. (2016) [31] have achieved similar results in the heterologous production of *Zymomonas mobilis* L-ASNase in *E. coli* in bioreactor, achieving activity values up to 3.6 U mL^{-1}. Hegazy et al. (2020) [32] have indicated higher activity values from 9.0 to 22.0 U mL^{-1} when expressing a *B. subitlis* L-ASNase in *E. coli*. A wide range of L-ASNase activity can be found in the literature since the quantification method and units are not standardized, also, several purification levels are reported. It is also worth to highlight those other parameters besides enzymatic activity are of highly important for comparison and evaluation, as substrate affinity and enzymatic stability.

To ensure low toxicity as a biopharmaceutical, the enzyme must present high specificity to L-asparagine and low or no activity on L-glutamine. In all enzyme extracts tested, the L-Glutaminase activity was 2 to 2.5-fold lower compared to the L-ASNase. In fact, the L-Glutaminase activity seems

307 to maintain the same basal level observed for the non-induced cells, which is most possibly displayed
308 by a native L-Glutaminase from the host cell, as we a crude cell extract was use as enzyme source.
309 Therefore, it is likely that the *A. fischeri* L-ASNase has no or very low activity on L-Glutamine. A
310 slightly higher value was only observed in the bioreactor samples, probably due to the higher cell
311 density achieved. These results indicate the analyzed enzymatic extracts presented characteristics
312 worth to be explored as a biopharmaceutical process with successful expression of *A. fischeri* L-
313 ASNase type II (corroborated with the low L-Glutaminase activities). No significant xylose
314 consumption was observed in any experiment (data not reported). Moreover, the presence of L-
315 ASNase can be noticed in the electrophoresis gel (Figure 1) in both cases (orbital shaker and
316 bioreactor production) by a band in the region between 30 and 35 kDa.

317 Recent studies have indicated *B. subtilis* as a model host for recombinant protein expression
318 for many reasons (as already mentioned), e.g., easy and cheap cultivation media and methods, well-
319 known genetic background, fast growing and the GRAS status [33, 34]. For industrial purposes it is
320 important the use of a robust microorganism as workhorse for the development of a feasible and
321 efficient bioprocess. The engineered strain developed in this study demonstrated the potential of the
322 *A. fischeri* L-ASNase II production by *B. subtilis* in orbital shaker and in bioreactor, achieving similar
323 results of enzyme activities for both cases at the same induction time (1.418 and 1.539 U mL^{-1},
324 respectively). These results indicate the potential of scaling up the process without production losses,
325 and even able to achieve higher yields. In fact, scaling up to bioreactor assays usually shows increase
326 in the enzyme production. Kumar et al. (2017) [35] have reported a significant increase from 0.76 U
327 mL^{-1} to 18 U mL^{-1} in L-ASNase production by *Pectobacterium carotovorum* MTCC 1428 when
328 moving from shaken flasks to bioreactor. Mihooliya et al. (2020) [36] also reported elevated L-
329 ASNase production in bioreactors, the authors have achieved L-ASNase activity up to 38.88 U mL^{-1}
330 by *Pseudomonas resinovorans* IGS-131 enzyme expressed in *E. coli*. Some tools and techniques may
331 be applied in order to increase productivity in bioreactor cultivations. The fed-batch strategy is one

main alternative reported as an efficient strategy to increase production in *B. subtilis* cultivation in bioreactors [37].

3.3 L-Asparaginase characterization

3.3.1 Biochemical and Kinetics characteristics

The characterization of bioproducts is essential to evaluate their potential applications and industrial production feasibility. The biochemical and kinetic characteristics are key parameters to prove the potential application of an enzyme as biopharmaceutical. L-ASNase activities over a wide range of pH can be seen in Figure 3. It is possible to conclude that the enzymatic extract was active in the pH range from 3.0 to 9.5, presenting best performance between pH 7.0-8.0 and optimum pH at 7.5.

The optimum pH 7.5 for L-ASNase activity and good activity range from pH 7.0 to 8.0 is in accordance with those found in the literature. Alrumman et al. (2019) [38] have also reported a maximum activity of *B. licheniformis* L-ASNase at pH 7.5, while El-Fakharany et al. (2020) [39] studied a newly isolated *B. halotolerans* and observed a maximum enzyme activity at pH 8.2.

Figure 3

Since a low L-Glutaminase activity is desired for L-ASNase preparations to be used as biopharmaceutical, the study of substrate affinity is important to evaluate the enzyme application. Figure 4 shows the behavior of the enzymatic reaction rate over increasing concentrations of both substrates (L-Asparagine and L-Glutamine). It is possible to observe that in both cases the Michaelis-Menten kinetic could be applied with significant fits (R^2 = 0.98075 and R^2 = 0.99267, for L-Asparagine and L-Glutamine, respectively).

Figure 4

Michaelis-Menten constants (K_m) revealed the enzymatic extract exhibited a significant higher affinity for L-Asparagine (K_m = 1.2275 mmol L^{-1}) than for L-Glutamine (K_m = 24.0330 mmol L^{-1}), indicating once again the successfully expression of L-ASNase type II and the potential to be applied

as a biopharmaceutical for anticancer treatment. For instance, commercial preparations of L-ASNase have been reported to present higher L-Glutamine affinity (K_m = 3.0 mmol L^{-1} for *E. coli* native and PEGylated L-ASNase), indicating the potential of the herein produced enzyme cause less side effects in the leukemia treatment [40]. The produced L-ASNase also showed catalytic efficiency up to k_{cat}/K_m = 0.302.

High affinity for the substrate L-Asparagine is necessary for therapeutic L-ASNase in order to ensure sufficient reduction of the endogenous L-ASNase at safe doses [4, 5]. Although the commercial preparations of L-ASNase presents K_m values close to 0.012 mmol L^{-1} [40], several studies showed the potential of enzymes with lower L-Asparagine affinity. Li et al. (2018) [41] have characterized a recombinant *Pyrococcus yayanosii* L-ASNase expressed in *B. subtilis* and obtained a similar Michalis-Menten constant (K_m = 6.5 mmol L^{-1}). Likewise, Feng et al. (2017) [30] reported a K_m = 5.3 mmol L^{-1} for an extracellular recombinant *B. subtillis* L-ASNase. Kante et al. (2019) [42] have also reported interesting results of recombinant human asparaginase expressed in *E. coli* presenting elevated substrate affinity (K_m = 2.25 mmol L^{-1}). Since L-Glutaminase activity has been related with possible adverse effects, including a contribution to increase the cytotoxicity of the drug on leukemic cells [43], L-ASNase with low L-Glutamine affinity have been widely reported as potential alternatives for commercial L-ASNase. Nguyen et al. (2018) [43] have studied different strains of *E. coli* and *D. dadantii* and indicated similar results for the two lowest L-Glutamine affinity analyzed strains (K_m = 15.80 and 47.46 mmol L^{-1}). However, the L-ASNase affinity observed by the authors for these strains were higher than the obtained in this present study (K_m = 0.095-0.1853 mmol L^{-1} and 1.22754 mmol L^{-1}, respectively).

3.3.2 Thermal stability and deactivation characteristics

The thermal parameters were calculated according to two deactivation models, well described in the literature [22]. The two kinetic models were based on the enzyme inactivation following consecutive (model Eq. 1) and elemental (model Eq. 3) reactions from a unique enzyme [22]. The thermal kinetic parameters and the enzyme half-life are presented in Table 2. Considering the thermal

inactivation parameters, it is clear that the model from Eq.1 does not proper describe the inactivation of L-ASNase since k_2 values are zero or approximately zero. These results indicate that thermal inactivation of L-ASNase does not follow consecutive reactions, *ie*, no disruption of possible enzyme aggregates occurs since the initial phase in the inactivation process can be related to the disaggregation step [22]. On the other hand, the model from Eq. 2 used to fit the experimental data using Eq. 4 and represented in Figure 5, and the respective calculated parameters, presented in Table 1, shows that the kinetic constant of enzyme deactivation (k_1) increases with the increase ine temperature. At low temperatures of 25 and 37ºC, k_1 take similar values (0.128 and 0.148 h^{-1}, respectively), while, as the temperature increases, k_1 values becomes dependent on the temperature, taking increasing as the temperature increases (0.701 and 1.187 h^{-1}, for the temperatures of 45 and 60 ºC, respectively). This result indicates that the enzyme thermal stability decreases with the temperature increase and that it becomes more pronounced for temperatures above 45ºC. Additionally, as expected, it was found that the $t_{1/2}$ decreases with temperature, from 13h for 25ºC to 0.7h to 60ºC confirming the lower stability of L-ASNase at high temperatures.

The biopharmaceutical stability at room (25ºC) and at the application temperature (37ºC) are also key characteristics to establish the potential of new drugs. The produced L-ASNase extract has shown a typical increase in the deactivation parameter (k_1) value when the enzyme was exposed to higher temperatures. The analyses denote similar k_1 values for temperatures ranging from25 to 37ºC which indicate good stability for biopharmaceutical applications from the enzymatic extract.

Figure 5

Table 2

4. Conclusions

L-ASNase from *A. fischeri* was successful cloned and produced by engineered *B. subtilis.* Moreover, the main properties of the recombinant enzyme have been determined. The results showed elevated L-ASNase activity produced on cultivation in orbital shaker and stirred tank bioreactor

(1.427 - 1.539 U mL^{-1}), indicating the potential of yield improvement under bioreactor cultivation. The characterization of the enzymatic extract has demonstrated the enzyme potential application as biopharmaceutical in leukemia treatment, since the recombinant enzyme preparation (crude extract) showed low glutaminase activity (0.568-0.738 U mL^{-1}), indicating the produced enzyme is L-ASNase type II. Enzymatic extract also showed good stability at 25 and 37 ºC (half-life times = 15.21 and 6.52 h, respectively) and elevated affinity for L-Asparagine (K_m = 1.2275 mmol L^{-1}). In this way, through production and characterization studies, a new recombinant L-ASNase bioprocess is presented, with potential application in the pharmaceutical industry.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Tables

Table 1. L-Asparaginase and L-Glutaminase production by recombinant *B. subtillis* KO7 in Orbital shaker at different induction times and in Stirred Tank Bioreactor

	Total protein content (mg L^{-1})	**L-Asparaginase activity (U mL^{-1})**	**L-Glutaminase activity (U mL^{-1})**	**L-Asparaginase specific activity (U/mg protein)**
Orbital Shaker				
Induction time				
No induction	3948.18 ± 102.02^{a}	0.569 ± 0.026^{d}	0.583 ± 0.055^{h}	0.144
OD = 0.7	4221.33 ± 139.90^{b}	1.418 ± 0.103^{e}	0.591 ± 0.082^{h}	0.336
OD = 1.1	4291.49 ± 125.28^{b}	1.427 ± 0.131^{e}	0.568 ± 0.070^{h}	0.332
OD = 1.5	4168.04 ± 144.05^{b}	1.185 ± 0.097^{f}	0.602 ± 0.078^{h}	0.284
Bioreactor	4634.55 ± 163.89^{c}	1.539 ± 0.038^{g}	0.738 ± 0.099^{h}	0.332
Control*	4248.18 ± 201.51^{b}	0.554 ± 0.042^{d}	0.678 ± 0.064^{h}	0.130

*Induction = induction in the OD_{600nm} = 0.7 A.U.; **Control = *B. subtilis* prior the genetic modification. [a, b, c, d, e, f, g, h] Same letters indicate results with no statistical significative difference according to Tukey test (95% confidence level).

555 **Table 2.** Thermal stability parameters of *A. fischeri* L-ASNase produced by cultivation of engineered *B.*
556 *subtilis.*

	Model 1			Model 2		
Temperature	α	k_1 (h^{-1})	k_2 (h^{-1})	α	k_1 (h^{-1})	**Half-life time (h)**
25 ºC	4.566	0.202	0.016	0.382	0.128	13.0
37 ºC	2.276	0.187	0.043	0.093	0.148	5.0
40 ºC	439.8	0.262	0.001	0.088	0.262	3.0
45 ºC	1521	0.727	0.000	0.138	0.701	1.2
60 ºC	16.49	23.37	0.047	0.137	1.187	0.7

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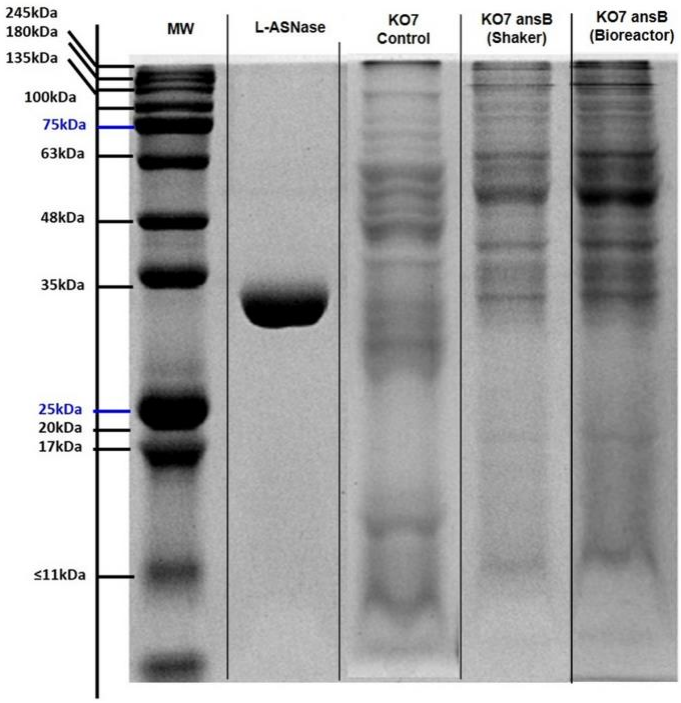
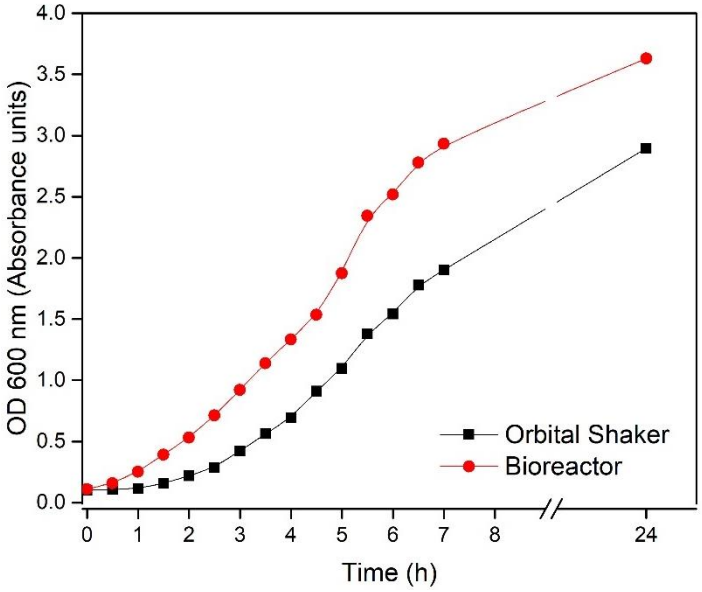


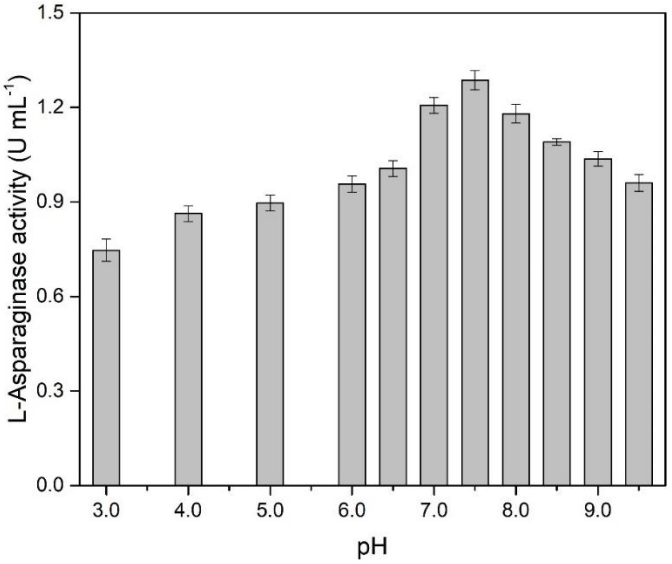
Fig. 1 Electrophoresis gel (SDS-PAGE) of commercial L-ASNase, engineered *B. subtilis* and non-engineered *B. subtilis*



566

567 Figure 2- Growth curves of engineered *B. subtilis* KO7 *ansB* cultivated in orbital shaker and stirred
568 tank bioreactor at 30 ºC for 24 h.

569



570

571 Figure 3 – Characterization of *A. fischeri* L-ASNase produced by cultivation of engineered *B. subtilis*
572 in relation to optimal pH. These experiments were carried out at 37°C. The error bars represent the
573 standard deviation of triplicates.

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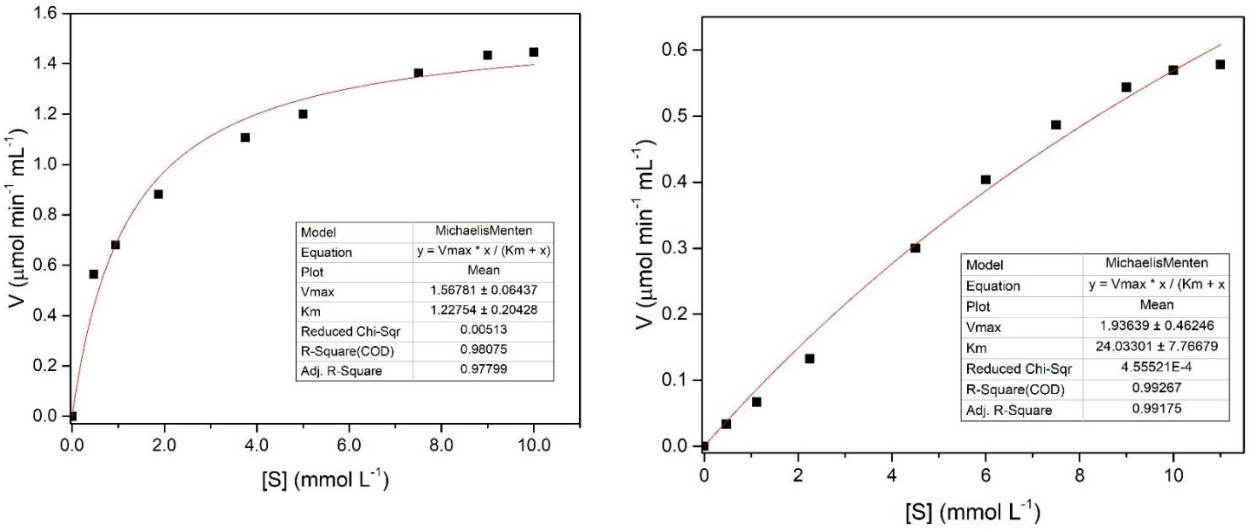

575 Figure 4 – L-Asparagine (a) and L-Glutamine (b) affinities considering the Michaelis-Menten fit for

576 the *A. fischeri* L-ASNase produced by cultivation of engineered *B. subtilis.*

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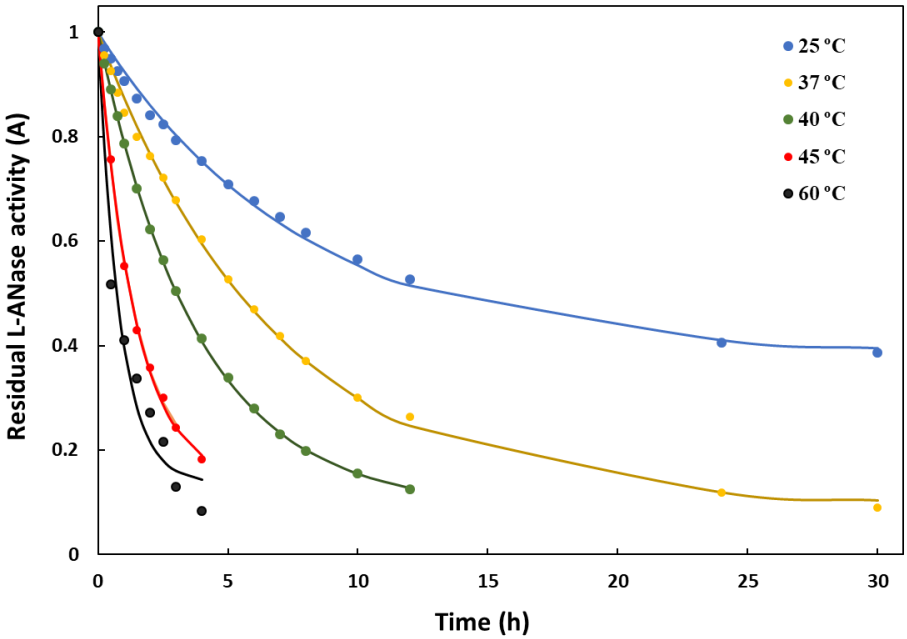

578

579 Figure 5 - Thermal stability of *A. fischeri* L-ASNase produced by cultivation of engineered *B. subtilis*
580 at temperatures from 25 to 60 ºC. Experimental data are shown as points while lines are the fitting
581 from Eq. (2).

582