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Optimization of L-Asparaginase Production from *Aspergillus caespitosus*: Solid-State and Submerged Fermentation Using Low-Cost Substrates and Partial Purification

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Abstract: This work aimed to optimize the production of L-asparaginase (L-ASNase) from *Aspergillus caespitosus* CCDCA 11593 using *Pereskia aculeata* (Ora-pro-nóbis) leaf fiber as a substrate for solid-state fermentation (SSF), along with powdered whey protein as a substrate in submerged fermentation (SmF) processes. A centered face design was applied to evaluate the effect of the different parameters. Additionally, L-ASNase was partially purified on an ion-exchange cryogel column. For SSF, the experimental condition, inoculum concentration 10^5 spores/mL, 120 h at 25 °C, 14% of substrate, and 1% of asparagine, corresponded to the highest enzymatic activity (2.75 U/mL) of L-ASNase. For SmF, the experimental condition of greater enzymatic activity (1.49 U/mL) was obtained in the medium containing 16% to 24% asparagine, 3.3% to 4.7% substrate, spore concentration of 7×10^6 to 10^7 spores/mL, temperature range of 29.8 to 34.8 °C, pH range of 5.7 to 6.3, and 87 to 105 h of fermentation. The L-ASNase obtained from SmF was subjected to adsorption tests, resulting in 4.4 U/mg of partially purified enzyme. This study suggested that whey protein and Ora-pro-nóbis leaf fiber could be a low-cost substrate for L-ASNase production. Additionally, using an ion-exchange cryogel column for enzyme purification holds promise for sustainable applications in the clinical and food industries.

Keywords: solid-state fermentation; submerged fermentation; L-ASNase; *Aspergillus caespitosus*; ora-pro-nobis; whey protein; ion-exchange purification



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1. Introduction

L-asparaginase (L-ASNase), also known as L-asparagine aminohydrolase, (EC 3.5.1.1) has become a prominent enzyme in the pharmaceutical and food industry for catalyzing the hydrolysis of the amino group on the side chain of the amino acid L-asparagine, in L-aspartic acid and ammonia [1,2]. In the food industry, this enzyme plays a role in the reduction of acrylamide in food, as it is one of the most efficient methods. In the pharmaceutical industry, it acts as a chemotherapeutic agent to treat a variety of diseases of the lymphatic system and leukemias, being used mainly in the treatment of acute lymphoblastic leukemia [3–5].

Microbial production of enzymes such as L-ASNase is primarily achieved using bacteria, yeasts, and filamentous fungi, wild or recombinant, through fermentation processes in liquid (submerged) or solid state. Among various microorganisms, filamentous fungi stand out as optimal agents for enzyme production. They are easily manipulable and have the capability to produce enzymes extracellularly. Alternatively, when needed, they can be subjected to straightforward and cost-effective unit operations for intracellular enzyme extraction [6]. In addition, several fungal species are Generally Recognized as Safe (GRAS). So far, the *Aspergillus caespitosus* species has been reported in the literature as a producer of enzymes such as amylase [7], alkaline phosphatase [8], laccase [9], invertase [10], and L-asparaginase, a crucial therapeutic enzyme that accounts for 40% of the global demand for enzymes [11] and was first described as a producer recently [12].

Despite these advancements, the production of L-ASNase remains challenging. Work carried out with fungi for the production of L-ASNase has led to better applications in the pharmaceutical and food industries, providing fewer side effects compared to synthetic enzymes and those produced by bacteria. However, there is still a need for further studies on the production of L-ASNase with high production yield and purity, while at the same time ensuring that the process is less expensive [13]. Moreover, a critical gap exists in utilizing alternative, low-cost substrates derived from agro-industrial by-products for enzyme production. These substrates are usually generated in agroindustry and often represent an environmental problem, in addition to the economic loss associated with the non-use of these by-products. The use and bioconversion of agricultural by-products contribute to improving the sustainability of the agro-industrial production chain [14].

According to this approach, the whey protein, a by-product of dairy products, has been used as a substrate in fermentation processes for the production of enzymes such as lactulose [15], β -galactosidase [16], protease [17], and L-ASNase [12]. Ora-pro-nobis (*Pereskia aculeata* Miller) is a non-conventional food plant (UFP) that has been extensively studied due to its high nutritional value, in addition to the presence of fiber-rich in cellulose, lignin, and ash, which can be extracted from the leaves and used as a substrate for the production of enzymes from microorganisms [12,18]. Notably, our research group has identified the feasibility of using these substrates for L-ASNase production by *Aspergillus caespitosus* CCDCA 11593. The enzyme produced was characterized by SDS-PAGE electrophoresis, presenting a dimeric structure, with average molecular masses of about 90 kDa and 45 kDa [12].

Given the urgent need for cost-effective and sustainable enzyme production methods, this work aimed to optimize the production of L-ASNase from *Aspergillus caespitosus* CCDCA 11593 using *Pereskia aculeata* (Ora-pro-nobis) leaf fiber (OPNF) as a substrate for solid-state fermentation (SSF), along with powdered whey protein as a substrate in submerged fermentation (SmF) processes. The optimization process focused on critical fermentation parameters, including inoculum concentration, substrate concentration, asparagine concentration, fermentation time, pH, and temperature. Furthermore, this study included partial purification of L-ASNase using an ion-exchange cryogel column, providing insights into both the production and downstream processing of this biotechnologically significant enzyme.

2. Materials and Methods

2.1. Microorganisms and Cultural Conditions

In the present work, the fungus *Aspergillus caespitosus* CCDCA 11593 was used, previously selected as a lineage that produces L-ASNase belonging to the Culture Collection of Microorganisms of the DCA/UFLA. The strain was grown in an inclined tube containing Potato Dextrose Agar (PDA) (Sigma–Aldrich, St. Louis, MO, USA) medium at 25 °C for

10 days. From the cultures, a spore suspension was obtained by adding 10 mL of sterile distilled water in test tubes. With the aid of a platinum loop, a scraping of the fungus growing on the surface of the medium was carried out. Subsequently, they were subjected to manual agitation. The number of spores per mL of the suspension was standardized using a Neubauer chamber, with the final concentration inoculated onto a 10 μ L plate, 10^5 spores/mL.

2.2. Solid-State Fermentation (SSF)

2.2.1. Ora-Pro-Nobis Fiber (OPNF) Used as a Substrate for SSF

The OPNF was obtained at the Laboratory of Refrigeration and Laboratory of Separation and Purification of Biomolecules of the Department of Food Sciences, at the Federal University of Lavras (UFLA). The fibers were obtained after extracting the mucilage present in ora-pro-nobis leaves [19]. Initially, the leaves were crushed and homogenized in water, in the proportion of 1 kg of raw material to 2.5 L of water, at a temperature of 100 °C for 10 min, using an industrial blender (Metvise LG10, Brusque, Santa Catarina, Brazil). Subsequently, the samples were placed in a beaker and remained under stirring in a thermostatic bath (Quimis, q-215-2) at 65 °C for 6 h. The resulting homogenate was manually filtered through organza fabric. The residual material retained on the organza filters, called ora-pro-nobis fiber (OPNF), was dried in a conventional oven at 60 °C for three days. Subsequently, the OPNF obtained was applied in solid fermentation tests.

2.2.2. Optimization of L-ASNase Enzyme Production by SSF

Optimized fermentations were carried out in 125 mL Erlenmeyer flasks, using a 1:1 (*m/v*) ratio of substrate and Khanna's salt solution. Khanna's saline solution was used as a wetting agent and has a composition of 0.2 g/10 mL NH_4NO_3 , 0.13 g/10 mL KH_2PO_4 , 0.0098 g/10 mL KCl, 0.00138 g/10 mL $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.0007 g/10 mL $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$, 0.00066 g/10 mL FeCl_3 , and 0.00062 g/10 mL CuSO_4 [20] (Sigma-Aldrich, St. Louis, MO, USA).

The factors and levels that were considered to affect the production of L-ASNase by SSF were chosen based on previous experiments of [12] and the results of [21]. To evaluate the effect of the temperature, substrate concentration (OPNF), asparagine concentration, fermentation time, and inoculum concentration in the production of L-ASNase by solid fermentation, a Centered Face Design (CFD) 25 with four replications and four central points was carried out to optimize extracellular L-ASNase production. This experimental design was chosen for its ability to evaluate quadratic effects and interactions among factors while minimizing the number of experiments required. The response variables were enzymatic activity (U/mL) and specific activity (U/mg), obtained through SSF. The values established for each parameter are shown in Table 1. Obtaining the crude enzymatic extract followed the methodology of [12,22]. The cell-free filtrates were subjected to extracellular enzymatic and total protein measurements.

Table 1. The values used in the Centered Face Design 25 were used to optimize the production of L-asparaginase in the solid-state fermentation.

Independent Variables	Code	Level		
		−1	0	+1
Inoculum concentration (spores/g)	x_1	10^5	10^6	10^7
Temperature (°C)	x_2	25	32.5	40
Time (h)	x_3	72	96	120

Table 1. *Cont.*

Independent Variables	Code	Level		
		−1	0	+1
OPNF concentration (% <i>m/v</i>)	x ₄	2	4	6
L-asparagine concentration (% <i>m/v</i>)	x ₅	1	2	3

x₁ = variable coded for inoculum concentration (spores/g), x₂ = variable coded for temperature (°C), x₃ = variable coded for time (h), x₄ = variable coded for OPNF concentration (% *m/v*), x₅ = variable coded for L-asparagine concentration (% *m/v*). The values of the real independent variables were used to optimize the production of L-asparaginase in the solid-state fermentation. All tests were performed in triplicate.

2.3. Submerged Fermentation (SmF)

2.3.1. Whey Protein

The whey protein used in submerged fermentation was donated by Porto Alegre Dairy Industry (Ponte Nova, Minas Gerais, Brazil). The physicochemical composition was provided by this establishment, with minimum of 65% of lactose, a minimum pH (sol. % a 20 °C) of 6, a maximum of 3.5% moisture, 11.0% of protein, 1.5% of fat, 9.0% of ash, 5.0% of chloride, 0.05 mg./Kg of arsenic, 0.05 mg/Kg of cadmium, 0.02 mg/Kg of lead, and 0.05 mg/Kg of mercury.

2.3.2. Optimization of L-ASNase Enzyme Production by SmF

The submerged fermentation optimization was carried out in 250 mL Erlenmeyer flasks containing 50 mL of modified Czapeck Dox medium, composed of 2 g of whey protein, 10 g of asparaginase, 1.52 g of KH₂PO₄, 0.52 g of MgSO₄, 0.52 g of KCl, 0.001 g of CuNO₃, 0.001 g of ZnSO₄, 0.001 g of FeSO₄ [23] (Sigma–Aldrich, St. Louis, MO, USA).

The factors that were considered to affect the production of L-ASNase by SmF were chosen based on the works of [12,24,25]. The parameters for optimization were temperature, substrate concentration, asparagine concentration, fermentation time, inoculum concentration, and pH. A complete Centered Face Design (CFD) 26 was performed, with four replications and 4 central points to optimize the production of L-ASNase, with the parameters presented in Table 2. Later, the crude enzymatic extract was obtained according to the methodology of [12]. Cell-free filtrates were subjected to enzymatic and total protein assays.

Table 2. Factors and levels of the Centered Face Design 26 + 4 central points were used to optimize the production of L-ASNase in the SmF.

Independent Variables	Code	Level		
		−1	0	+1
L-asparagine concentration (% <i>m/v</i>)	x ₁	10	20	30
Whey protein concentration (% <i>m/v</i>)	x ₂	2	4	6
Inoculum concentration (spores/mL)	x ₃	10 ⁵	10 ⁶	10 ⁷
Temperature (°C)	x ₄	25	32.5	40
pH	x ₅	5	6	7
Time (h)	x ₆	72	96	120

x₁ = variable coded for L-asparagine concentration (% *m/v*), x₂ = variable coded for whey protein concentration (% *m/v*), x₃ = variable coded for inoculum concentration (spores/g), x₄ = variable coded for temperature (°C), x₅ = variable coded for pH, x₆ = variable coded for time (h). Values of the real independent variables were used to optimize the production of L-asparaginase in the solid-state fermentation. All tests were performed in triplicate.

2.4. Determination of L-ASNase Activity

The enzymatic activity of L-ASNase was performed as described by [26], with modifications. The reaction mixture, consisting of 0.5 mL of L-asparagine (0.04 mol/L), 0.5 mL of Tris-HCl buffer pH 8.0 (50 mmol/L) (Himedia, Bengaluru, India), and 0.5 mL of extract

enzyme, was incubated at 37 °C for 30 min. The reaction was stopped by adding 0.5 mL of trichloroacetic acid (Himedia, Bengaluru, India) solution (1.5 mol/L). A 125 µL aliquot of the reaction mixture was diluted in 2 mL of distilled water and 125 µL of Nessler's reagent (Sigma–Aldrich, St. Louis, MO, USA). Absorbance was measured at 450 nm in a spectrophotometer. A standard curve with ammonium sulfate, using absorbance, was used to quantify released ammonia. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 µmol of ammonia per minute under standard assay conditions. The quantification of total proteins in the samples was carried out according to the [27], using Bovine Serum Albumin (Merck, Darmstadt, Germany) as the standard protein.

2.5. Partial Purification of L-ASNase Obtained by *SmF*

2.5.1. Batch Adsorption Tests

In order to determine the best pH condition and ionic strength of the saline solution in the medium for the adsorption of L-ASNase, batch adsorption tests were carried out using 15 mL Falcon tubes, to which 0.02 g of functionalized cryogel was added, along with 2-aminoethanesulfonic acid (Thermo Fisher Scientific, São Paulo, Brazil) and 3 mL of fermented medium solution in saline sodium phosphate solutions at different concentrations (0.025 mol/mL; 0.050 mol/mL and 0.100 mol/mL) and pH values (3.0 and 7.0), in a 1:1 (%v/v) ratio. The adsorption processes were carried out at 25 °C, under agitation at 50 rpm for 24 h. After adsorption, the tubes were centrifuged at $3248 \times g$ for 10 min and the supernatants were collected for quantification. All assays were performed in triplicate. The functionalization of the cryogel with 2-aminoethanesulfonic acid was performed via the glutaraldehyde method, according to the methodology by [28].

The adsorptive capacity (q , mg/g) of the adsorbent was determined according to Equation (1). The quantification of protein concentrations in the initial solution and the supernatants was measured using the Bradford method [27], with the reading of absorbance at 595 nm. An analytical curve using standard bovine serum albumin protein solution was constructed, with concentrations ranging from 0.10 mg/mL to 1.0 mg/mL.

$$q = ((c_0 - c) \times V) / M \quad (1)$$

where q is the number of proteins adsorbed on the functionalized cryogel (mg/g); c_0 and c are the initial and final concentrations of proteins in solution (mg/mL); M is the mass of dry cryogel (g), and V is the volume of fermented medium solution (mL).

A completely randomized design with three replications was carried out to evaluate the effect of the sodium phosphate solution concentration (0.025 mol/mL; 0.050 mol/mL and 0.100 mol/mL) and pH (3.0 and 7.0) on the adsorption of L-ASNase.

2.5.2. Partial Purification of L-ASNase

Partial L-ASNase purification assays were performed at room temperature using a constant flow rate of 1.0 mL/min. A UVis-920 Monitor (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with a UV detection filter at 280 nm and a compact peristaltic pump (Traceable® Products, Webster, TX, USA) were used. First, the 2-aminoethanesulfonic acid-functionalized cryogel column was equilibrated with 5 column volumes (VC) of sodium phosphate buffer (0.050 mol/L, pH 3.0). This method offers advantages over conventional purification techniques, such as faster processing times, lower backpressure, higher binding specificity for target proteins, smaller amount of energy, and costs lower than commercial chromatographic columns [29]. Subsequently, the fermented medium solution diluted in sodium phosphate (0.050 mol/L, pH 3.0) in a 1:1 proportion (%v/v) was pumped through the column until the rupture curve was formed. Then, the column was washed with 3 strokes of the equilibration buffer. Absorbance was monitored at 280 nm and recorded

using an Arduino Uno R3. Protein elution was performed in isocratic mode at a flow rate of 1 mL/min, pumping 5 VC of sodium phosphate aqueous solution (0.050 mol/L, pH 3.0) supplemented with sodium chloride (1.5 mol/L). Afterward, the spectrophotometer was read to evaluate the specific activity, enzymatic activity, and total protein. The enzymatic activity of the initial solution and the eluates were measured according to the previously described methodology.

2.6. Statistical Analyzes

The results obtained from the Centered Face Design for SSF and SmF were submitted to regression analysis at a significance level of 5%. All statistical analyzes were performed using the SAS[®] University Edition statistical package (SAS Institute Inc., Cary, NC, USA). The reliability of the mathematical model obtained was evaluated by checking the model's lack of fit ($p > 0.05$), the R^2 determination coefficient, and the significance level ($p < 0.05$) of the regression coefficients. Response surfaces were generated using SigmaPlot software version 12 (Systat Software, Inc., San Jose, CA, USA).

The results obtained from the L-ASNase enzyme batch adsorption assay were submitted to analysis of variance (ANOVA), and the means were compared using the Tukey test at 95% significance. The SAS University Edition statistical package (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses.

3. Results

3.1. Solid Fermentation

Optimization of the Parameters Used in the SSF Process for the Production of L-Asnase

A 25 CFD with four central points and four replications in each treatment was carried out in order to optimize the following parameters involved in the production of the enzyme L-ASNase through solid fermentation: inoculum concentration, L-asparagine concentration, the concentration of substrate, temperature, and fermentation time. The experimental conditions evaluated in this design are shown in Table S1.

The experimental results were submitted to ANOVA, and the significance ($p < 0.05$) of the parameters is shown in Table S2. The model obtained for the enzymatic activity of L-ASNase obtained by SSF is shown in Equation (2), which presents a coefficient of determination of 0.96 ($R^2 = 0.96$).

$$\begin{aligned} Y(\text{atv.vol.}) = & (-1638060869) - (0.00000036 \times x_1) - (0.282142220 \times x_2) \\ & + (0.12032465 \times x_3) + (2.61479370 \times x_4) \\ & + (0.0000000 \times x_1 \times x_1) + (0.00755849 \times x_2 \times x_2) \\ & - (0.10773418 \times x_4 \times x_4) - (0.00294059 \times x_2 \times x_3) \end{aligned} \quad (2)$$

where atv.vol. is the enzymatic activity (U/mL), x_1 is the inoculum concentration (spores/mL), x_2 is the temperature (°C), x_3 is the time (h), and x_4 is the OPNF concentration (% *m/v*).

The inoculum concentration, substrate concentration, fermentation time, and temperature significantly affected ($p < 0.05$) the production of the enzyme L-ASNase, while the concentration of L-asparagine did not exert any significant influence ($p > 0.05$) on the response variable. The contour curves (Figure 1) show the results of the enzymatic activity (U/mL), as a function of the independent variables studied.

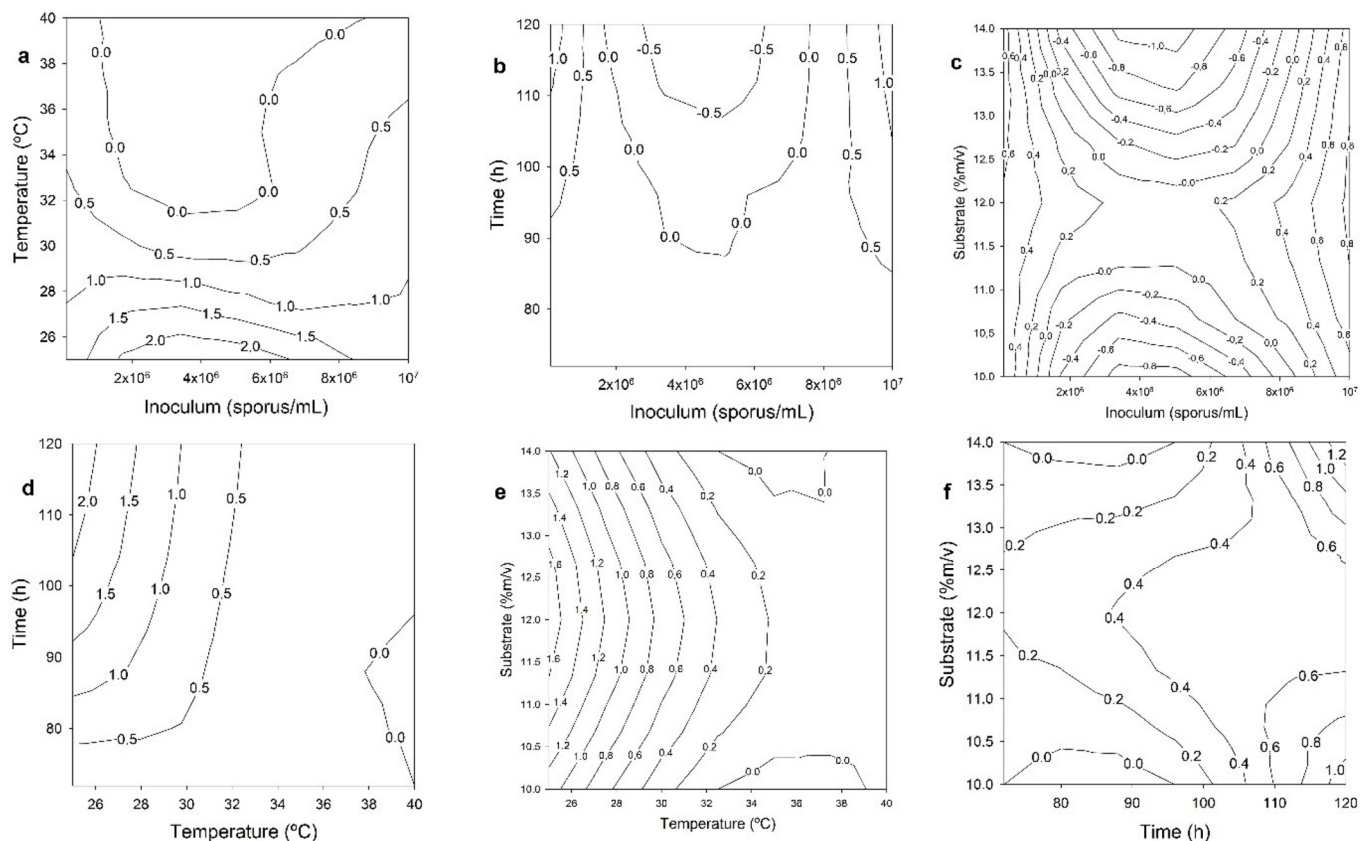


Figure 1. Experimental results of the enzymatic activity (U/mL) of L-ASNase as a function of significant independent variables ($p < 0.05$) for the production of L-ASNase by SSF. (a) Inoculum $\times$ temperature; (b) inoculum $\times$ time; (c) inoculum $\times$ substrate; (d) temperature $\times$ time; (e) temperature $\times$ substrate; (f) time $\times$ substrate. The results of the regression ANOVA using the full 25 factorial design with four replications and four central points are available in Table S2.

As can be seen in Figure 1a, the optimal temperature condition (at the interval tested in this study) for the production of L-ASNase by SSF using *A. caespitosus* CCDCA 11593 occurred when the inoculum concentration was between 2×10^6 to 6×10^6 spores/mL and the temperature was at 25 °C, producing 2.0 U/mL. However, when the temperature was above this value, the effect of this parameter was significantly reduced throughout the inoculum concentration range. In Figure 1a,d,e, the previous statement can be reinforced, considering that the optimal condition for enzyme production occurred when the temperature was at 25 °C, which was the lowest temperature tested in this study. However, to determine whether further increases in enzyme production could be achieved at temperatures below 25 °C, additional experiments would be necessary. It is important to note that such investigations would likely involve increased operational costs, as maintaining lower temperatures in industrial-scale processes requires greater energy expenditure and infrastructure adjustments.

Figure 1b,d,f, show the interactions between time $\times$ inoculum concentration, fermentation time $\times$ temperature, and substrate concentration $\times$ time, respectively. Note that the enzyme production was more efficient when the fermentation time was 120 h. However, further research would be needed to evaluate fermentation times longer than this limit to fully exploit the production potential. However, it is important to note that prolonged fermentation times can be economically unviable for industrial applications, due to the costs associated with increased energy consumption and process maintenance requirements. Figure 1c depicts the relationship between inoculum concentration and substrate concentration parameters affecting enzyme production. When the inoculum

concentration was in the range of values below 2×10^6 and above 8×10^6 spores/mL, the different substrate concentrations evaluated did not promote significant changes in the activity of the enzyme produced. According to the SSF mathematical model, only the temperature $\times$ time interaction was significant. Figure 1d illustrates the interaction between the two parameters, revealing that the optimal conditions (at the interval tested in this study) for L-ASNase production in SSF were achieved at a temperature of 25 °C and a fermentation time of 120 h, resulting in the production of 2.0 U/mL of the enzyme.

3.2. Submerged Fermentation

Optimization of the Parameters in the SmF for L-ASNase

The production of L-ASNase by the submerged fermentation process was evaluated using a complete factorial design of 26 with four replications and four central points in order to optimize the following parameters: L-asparagine concentration, substrate concentration, inoculum concentration, temperature, fermentation time, and pH. The enzymatic activity values obtained in this work are shown in Table S3.

The probability values obtained using ANOVA, presented in Table S4, indicate that the variables inoculum concentration, substrate concentration, fermentation time, pH, asparagine concentration, and temperature significantly influenced the production of the enzyme L-ASNase ($p < 0.05$). The calculation of the regression analysis presented the coefficient of determination value of $R^2 = 0.64$. The mathematical model obtained by regression analysis (Equation (3)) describes the relationship between the independent variables (parameters $x_1, x_2, x_3, x_4, x_5, x_6$) and the dependent variable (enzymatic activity), considering the statistically significant terms ($p < 0.05$).

$$\begin{aligned} Y(\text{atv.vol.}) = & (-2.041002563) - (0.030530055 \times x_1) \\ & + (0.0218688758 \times x_2) + (0.0000000034 \times x_3) \\ & + (0.083746041 \times x_4) + (0.355620550 \times x_5) \\ & - (0.007607069 \times x_6) - (0.000000002 \times x_3 \times x_1) \\ & - (0.000000002 \times x_3 \times x_4) + (0.000000012 \times x_3 \times x_5) \\ & + (0.000000006 \times x_3 \times x_2) - (0.006119979 \times x_4 \times x_2) \\ & + (0.000334705 \times x_6 \times x_1) - (0.010805979 \times x_4 \times x_5) \end{aligned} \quad (3)$$

where atv.vol. is the enzymatic activity (U/mL), x_1 is the asparagine concentration (% m/v), x_2 is the whey powder concentration (% m/v), x_3 is the inoculum concentration (spores/mL), x_4 is the temperature (°C), x_5 is the pH, and x_6 is the fermentation time (h). Contour plots provide a better representation of ideal conditions for L-ASNase enzyme production. Figure 2 presents the interactions between the significant independent variables, representing the enzymatic activities (U/mL).

The interactions between the independent parameters by the enzymatic activity obtained by the SmF process using *A. caespitosus* CCDCA 11593, displayed in Figures 1a and 2b–f, revealed that the optimal inoculum concentration (at the interval tested in this study) for enzyme production was 7×10^6 to 10^7 spores/mL. Figure 1a demonstrates that when the temperature increased to 29.8 to 34.8 °C, a temperature interval considered optimal, among those tested in this study, and the inoculum concentration was above 7×10^6 spores/mL, the production condition was more significant, with an enzymatic activity of 0.5 U/mL. However, when the temperature remained lower (between 25 and 26 °C) enzyme production decreased (Figure 2g–j). When the substrate concentration remained lower (between 3.3 and 4.7% w/v), and there was an increase in the inoculum concentration, enzyme production was higher (Figure 2b), reaching 0.5 U/mL. This result demonstrates that the amount of substrate and its composition were sufficient for the conversion of substrate into metabolites to occur, even with

high amounts of inoculum, thus demonstrating that the substrate is a promising candidate for the production of L-ASNase from *A. caespitosus*.

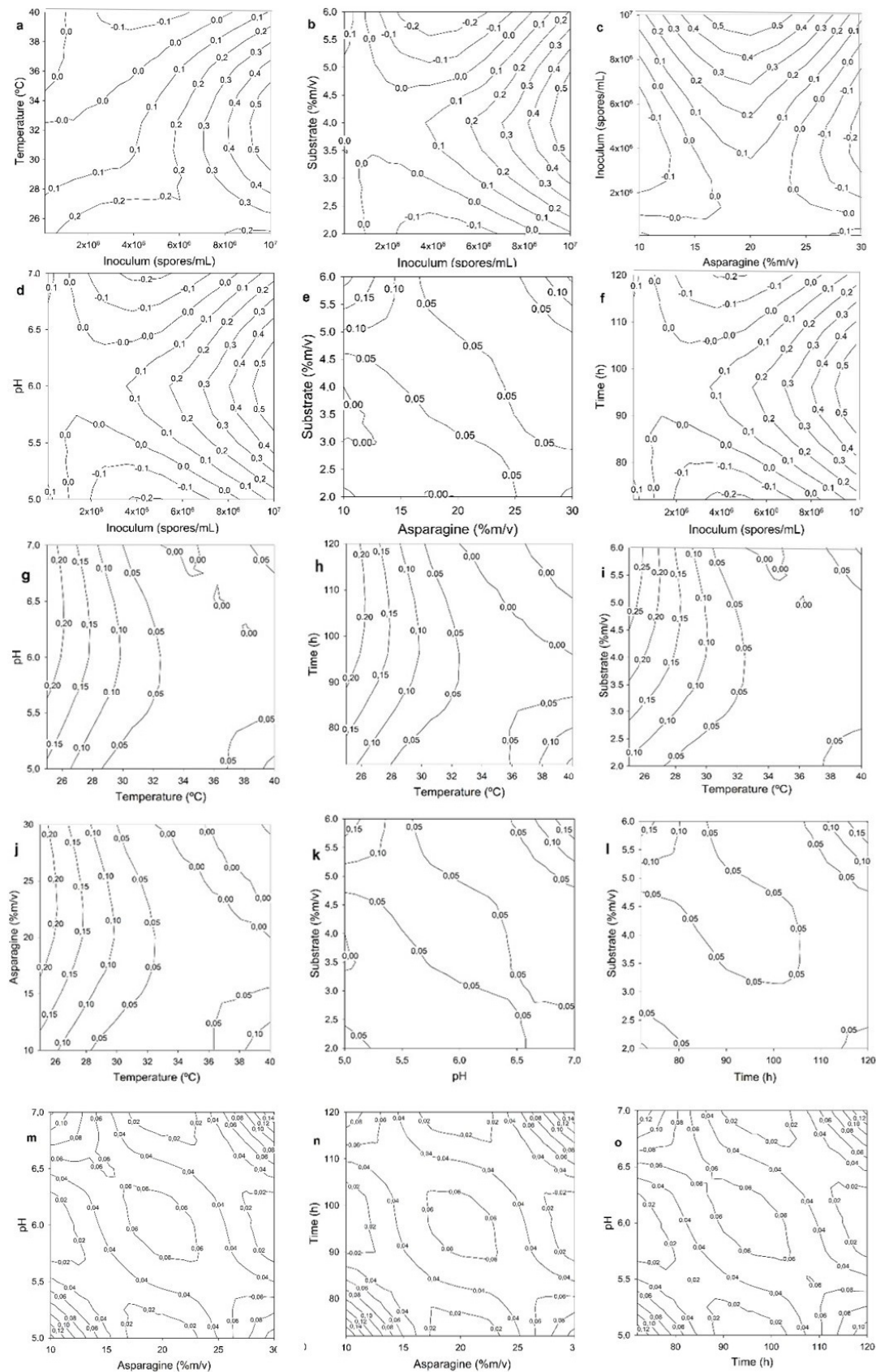


Figure 2. Enzymatic activity (U/mL) generated for the interactions of the significant parameters for the production of L-ASNase by SmF. (a) Inoculum $\times$ temperature; (b) inoculum $\times$ substrate;

(c) asparagine $\times$ inoculum; (d) inoculum $\times$ pH; (e) asparagine $\times$ substrate; (f) inoculum $\times$ time; (g) temperature $\times$ pH; (h) temperature $\times$ time; (i) temperature $\times$ substrate; (j) temperature $\times$ asparagine; (k) pH $\times$ substrate; (l) time $\times$ substrate; (m) asparagine $\times$ pH; (n) asparagine $\times$ time; (o) time $\times$ pH. The results of the regression ANOVA using the full 26 factorial design with four replications and four central points are available in Table S4.

However, when the substrate concentration was above 5.4% m/v , production dropped by at least half (Figure 2e,i,k,l). This result corroborates that the use of powdered whey substrate is promising, since a low amount of it is enough to produce the enzyme. Regardless of the asparagine concentration, when the concentration in the medium remained intermediate (between 16 and 24% w/v), enzyme production was more relevant, totaling 0.5 U/mL of the enzyme (Figure 2c). When it was higher (between 28 and 30% m/v), enzyme production decreased considerably, with an enzymatic activity of 0.14% m/v (Figure 2m,n). Regarding pH (Figure 2d), the optimal condition (in the range tested in this study) for production occurred when the pH value was between 5.7 and 6.3. However, when the pH was below or above these values, production decreased significantly (Figure 2k,m,o). Figure 2o shows that when the pH was kept low, at 5.2, and above 6, with a fermentation time below 75 h and above 118 h, the lowest enzyme activity was observed, producing 0.12 U/mL.

Finally, it can be seen that fermentation time plays an essential role in the production of L-ASNase. The ideal fermentation time range, in the interval evaluated, is between 87 and 105 h. During this period, enzyme activity reached its peak, with values close to 0.5 U/mL (Figure 2n,o). On the other hand, shorter fermentation times, below 75 h or higher than 118 h, resulted in substantially lower yields.

Among the results, those presented in Figure 2a–d and f corroborate the results of the SmF mathematical model. In these, optimal enzymatic activities are observed (in the interval tested in this study), with production of 0.5 U/mL. Values above 7×10^6 spores/mL, between 3.3 and 4.7% m/v of substrate, 16 to 24% m/v of asparagine, a pH range of 5.7 to 6.3, and 87 to 105 h of fermentation proved to be significant compared to the other evaluated values.

3.3. Purification of the L-ASNase Enzyme

3.3.1. Adsorption Tests

The average results for the adsorptive capacity ($\text{mg}_{\text{protein}}/\text{g}_{\text{cryogel}}$) of the cryogel functionalized with 2-aminoethanesulfonic acid under the different analyzed conditions are presented in Table 3. The results indicate that treatments 4, 5, and 6 exhibited the highest adsorptive capacities, without statistical differences between them. Among these, treatment 5 (a sodium phosphate concentration of 0.050 mg/mL and a pH of 3.0) was selected for the batch adsorption process of the L-ASNase enzyme. This choice was guided by its slightly higher adsorptive capacity (26.62 mg protein/g cryogel) and its optimal balance between the buffer concentration and pH.

Interestingly, the selected conditions of 0.050 mg/mL sodium phosphate and pH 3.0 deviate from those reported in other studies, where higher pH values and buffer concentrations are commonly employed. This highlights the importance of tailoring the adsorption conditions to the specific enzyme and cryogel system to maximize efficiency.

Table 3. Average adsorptive capacities of cryogel functionalized with 2-aminoethanesulfonic acid.

Treatments	Sodium Phosphate Concentration (mg/mL)	pH	q (Mgprotein/Gcryogel)
1	0.025	7.0	21.91 ± 2.38 b
2	0.050	7.0	23.83 ± 0.42 ab
3	0.100	7.0	14.65 ± 1.59 c
4	0.025	3.0	24.38 ± 1.19 ab
5	0.050	3.0	26.62 ± 0.51 a
6	0.100	3.0	26.12 ± 0.51 a

The average values followed by the same letter for each type of salt indicate that there is no statistical difference between them using Tukey's test at the 5% probability level.

3.3.2. Purification Test

The elution test was carried out from the crude extract obtained by optimizing the submerged fermentation, using whey powder as a substrate. This assay was performed using an ion-exchange cryogel column for purification, and the results obtained from the crude extract and the eluate are shown in Table 4.

Table 4. Purification of L-ASNase using an ion-exchange column with cryogel at room temperature.

Samples	Total Protein (mg/mL)	Specific Activity (U/mg)	Enzymatic Activity (U/mL)
Initial solution	0.088	2.78	0.22
Eluted	0.008	4.40	0.77

The initial solution refers to the substance added to the ion-exchange column, while eluted pertains to what has been extracted or come out of the column during the process.

The specific activity of the enzyme increased from 2.78 U/mg in the initial solution to 4.40 U/mg in the eluted fraction, indicating a significant improvement in enzyme purity. This represents a 1.58-fold purification of L-ASNase.

Despite the reduction in total protein concentration (from 0.088 mg/mL to 0.008 mg/mL), the enzymatic activity increased from 0.22 U/mL to 0.77 U/mL in the eluate. This demonstrates that the ion-exchange cryogel column effectively concentrated the active enzyme while removing impurities. The reduction in total protein content highlights the column's capacity to selectively retain the enzyme while removing non-specific proteins.

4. Discussion

The optimization of the parameters influencing the production of L-asparaginase (L-ASNase) through solid-state fermentation (SSF) using *Aspergillus caespitosus* CCDCA 11593 revealed critical insights into the process dynamics. The values chosen for the experiments were based on previous studies carried out by our research group using the same fungus, *Aspergillus caespitosus*, in particular, the work of [12], who evaluated the best conditions for *A. caespitosus* in the production of L-asparaginase. The results show that glucose as a carbon source, an initial pH of 6.0, and a culture duration of 96 h, produced the highest production of L-asparaginase, with an activity of 0.0960 U-mL⁻¹.

The central composite design (25 CFD) demonstrated a robust experimental framework, with the model showing a high coefficient of determination ($R^2 = 0.96$), indicating that the independent variables explain 96% of the variability in enzymatic activity. The ANOVA results highlighted that the inoculum concentration, substrate concentration, fermentation time, and temperature had a significant effect ($p < 0.05$) on L-ASNase production. In contrast, the concentration of L-asparagine did not significantly influence the enzymatic activity. This indicates that the metabolic pathway for L-ASNase synthesis may not be directly dependent on the presence of its substrate, L-asparagine, under the conditions

evaluated, confirming that filamentous fungi normally require a repression of nitrogen in relation to the carbon source for the production of secondary metabolites [30].

The interaction between temperature and fermentation time was particularly significant, as shown in contour curves (Figure 1). The optimal temperature (at the interval tested in this study) of 25 °C and a fermentation time of 120 h yielded the maximum enzymatic activity (2.0 U/mL). However, increasing the temperature beyond 25 °C significantly reduced enzyme production, underscoring the thermosensitivity of the fungal system. Similarly, an inoculum concentration between 2×10^6 and 6×10^6 spores/mL was optimal for enzyme production, with no significant improvements observed outside this range. On the other hand, higher temperatures (35 °C), fermentation time (144 h), and yield of L-ASNase (12.57 U/mL) were shown in the optimization work of [31] using *Aspergillus* sp. in solid fermentation, with agro-industry substrates such as cottonseed (2/3), wheat bran (1/6), and red grass husk (1/6).

In the context of this study, the term “optimal conditions” refers specifically to the parameters tested within the experimental range established. While the results indicate that these conditions yielded the highest observed enzymatic activity, it is important to note that the investigation did not extend beyond the tested boundaries. Therefore, the possibility of further enhancements in enzyme production under conditions outside this range cannot be excluded. Future studies are encouraged to explore broader ranges of temperature, cultivation time, and other factors to fully uncover the potential for maximizing enzyme activity, while prioritizing a comprehensive economic evaluation and optimizing the utilization of by-products to enhance the scalability and industrial viability of enzyme production [32].

The optimization of L-asparaginase production through SSF was also studied by [33], who evaluated the production of L-ASNase using *Aspergillus terreus* CCT7693 by the SSF process under conditions of 30 °C for 96 h. The procedure involved the utilization of sugarcane bagasse as a support material. Various sources of carbon and nitrogen were used in the medium to produce the enzyme of interest. Statistical analysis indicated that using 0.54% starch, 0% maltose, 0.44% asparagine, and 1.14% glutamine in the medium resulted in 120.72 U/L of enzyme produced, demonstrating that the independent variables used in the statistical analysis were efficient for the production of the enzyme of interest. The same study showed higher production compared to our current work with *A. caespitosus*. Despite both studies employing the same fungus genus, lignocellulosic substrate, and SSF process, the discrepancy in results may stem from differences in carbon and nitrogen source supplementation introduced in the fermentation medium for *A. terreus*.

The optimization of L-ASNase production by SmF using *A. caespitosus* CCDCA 11593 provided valuable insights into the influence of six key parameters: L-asparagine concentration, substrate concentration, inoculum concentration, temperature, fermentation time, and pH. A factorial design (2^6) revealed the statistical significance of all six variables ($p < 0.05$), with a regression model yielding a coefficient of determination ($R^2 = 0.64$). Although the model's predictability is moderate, it identifies critical trends and optimal conditions for enzyme production.

The experimental results revealed that the highest enzymatic activity (0.5 U/mL) was achieved with an inoculum concentration between 7×10^6 and 10^7 spores/mL, a temperature of 29.8 to 34.8 °C, and a substrate concentration of 3.3 to 4.7% (w/v). These findings suggest that SmF conditions favor a relatively high fungal biomass and moderate nutrient availability for efficient enzyme production. From these results, it can be concluded that the production of L-ASNase through *A. caespitosus* by the SmF process, using whey powder as a substrate, can be a commercially advantageous option. This is due to the decrease in production cost and the low amount of asparagine required in the medium.

Another important factor reported in the literature is that the deprivation of asparagine in the fermented medium for the production of L-ASNase is beneficial because it causes ammonium depression and, therefore, an increase in enzymatic activity [30].

According to [34], pH is an important parameter for the cultivation of microorganisms and the production of metabolites because it can influence nutrient availability, which is crucial for synthesis, since a pH below the optimum values can decrease the activity of the enzyme. This is due to the dissociation of some groups of ionizable enzymes, leading to its denaturation.

The ideal fermentation time range, in the interval evaluated, is between 87 and 105 h. Shorter fermentation times, below 75 h, resulted in substantially lower yields. This can be attributed to the initial growth phase of the fungus, in which the production of secondary metabolites, such as L-ASNase, is not yet optimized. Similarly, longer times, higher than 118 h, led to a significant reduction in enzyme activity, possibly due to nutrient depletion in the medium, the accumulation of toxic metabolites, or changes in the pH value of the medium, which negatively impact the fungus' metabolism. The optimal incubation period for fungal strains, including those of the *Aspergillus* genus, could differ significantly based on factors such as their inherent growth rates, nutrient availability, strain-specific preferences for substrates, and other critical environmental conditions [35]. For *Aspergillus* species, studies have consistently indicated that the optimal production of L-asparaginase typically occurs within 4–5 days of incubation. However, some research has identified an extended incubation period of 8 days as ideal, under specific conditions, for maximizing L-ASNase production [36].

The works found in the literature present production conditions close to those presented in this present work. However, the enzymatic activities obtained in the literature were higher than those presented here. Ref. [37] used the fungus *Aspergillus oryzae* LBA 01 and the Czapeck Dox medium modified from SmF to produce the same enzyme, and the optimal production occurred under conditions of pH 6.5, 30 °C for 5 days, obtaining 20 U/mL of the enzyme. The production conditions presented were very close to those obtained in the present work. However, the enzymatic activity obtained was higher, which can be attributed to several factors, including differences in the fungal strain, as *Aspergillus oryzae* is known for its high enzyme production potential compared to *Aspergillus caespitosus*. In addition, the alternative substrates used in our study, such as ora-pro-nobis leaf fiber and whey protein, although sustainable and economical, may lack certain nutrients present in the chemically defined medium used by [37]. Furthermore, variations in fermentation conditions, including aeration, agitation, and extraction methods, along with potential differences in enzyme assay protocols, could also contribute to the disparity observed. These factors highlight the trade-off between using sustainable substrates and achieving optimal enzyme activity, suggesting that further optimization of fermentation and substrate composition is required to improve yields.

The work carried out by [38] obtained 43.29 U/mL of L-ASNase, produced by the fungus *Aspergillus terreus* in the modified Czapeck Dox medium for the mBSP process, and the best condition was 35 °C and a pH of 6.3. The species belong to the same genus; however, the optimal temperature results of *A. terreus* in the present work differed. The pH values presented by both works were similar. However, this present work, which used whey powder as a support substrate for production, presented a less satisfactory production.

Data presented by [39], for the production of L-ASNase by SmF using the modified Czapeck Dox medium, indicated the best enzymatic production under the conditions of pH 6 and a temperature of 27 °C, managing to produce 32.54 U/mL of the enzyme from *Aspergillus* sp. These results shown above are closer to the conditions obtained in this present work. The same genus of fungus and humidifying medium were used, and the

temperatures and pH values were similar. However, the production of the enzyme obtained in our work was smaller, due to the alternative substrate used.

In contrast, the work of [36] identified the ideal growth conditions for *Aspergillus tubingensis*, which included a pH of 6, a temperature of 30 °C, glucose as the carbon source, ammonium sulfate as the nitrogen source, rice husks as a natural L-asparagine source, and a significantly longer incubation time of 8 days. Under these optimized conditions, *A. tubingensis* demonstrated a high capacity for L-asparaginase production. These variations highlight the influence of fungal species, fermentation type, and substrate composition on enzyme production efficiency.

This study underscores the potential of SmF for L-ASNase production using *A. caespitosus* and whey powder as a substrate. The optimal conditions at the interval tested in this study—moderate substrate and L-asparagine concentrations, high inoculum, and temperatures near 30 °C—promoted effective enzyme synthesis. Importantly, whey powder demonstrated promise as an economical substrate, requiring low concentrations for optimal results. These findings provide a foundation for scaling up SmF processes while minimizing resource inputs and costs.

The adsorptive capacity of the cryogel functionalized with 2-aminoethanesulfonic acid was evaluated under various conditions, as shown in Table 3. The selection of treatment 5 demonstrates an effective balance between buffer concentration and pH, leading to the optimized adsorption of L-ASNase. This approach can serve as a benchmark for future studies aiming to purify this enzyme, as it combines high adsorptive efficiency with relatively low buffer requirements. The work carried out by [40] used the conditions of 0.05 M Tris-HCl buffer at a pH of 8.5 to purify the enzyme L-ASNase obtained by the process of submerged fermentation by *Penicillium* sp. The study conducted by [41] purified the L-ASNase enzyme obtained by the fungus *Trichoderma viride* by SmF, under the conditions of 0.05 M phosphate buffer with a pH of 7.0.

The use of an ion-exchange cryogel column at room temperature is a promising approach for the purification of L-ASNase from crude extracts. This method combines simplicity with efficiency, making it a valuable tool for scaling up enzyme production for industrial applications. Among the eluates, the highest enzymatic activity obtained after the adsorption assay of the enzyme produced from the fungus *A. caespitosus* was 4.4 U/mg. The work carried out by [42] obtained the enzyme from the fungus *Aspergillus flavus*, produced through SmF. The ion-exchange purification process was also used, and 12.69 U/mg of the L-ASNase enzyme was obtained. Therefore, the purification obtained a better result than that of this present work. However, ref. [43] purified the L-ASNase enzyme by the ion-exchange process produced by *Fusarium solani* using the solid fermentation process and obtained 4.314 U/mg, a value close to that obtained in this present work. The purification process by ion exchange takes place due to the difference in electrostatic charge between the biomolecule of interest (L-ASNase) and a charged group, which was added to give support in the chromatographic column. In this case, it was 2-aminoethanesulfonic acid. The interaction between both is dependent on properties such as pH, temperature, type of buffer, and the ionic strength of the medium. The cryogel column was initially immobilized by 2-aminoethanesulfonic acid, also known as taurine. This compound has the function of binding the biomolecule of interest to the column for enzyme adsorption.

Therefore, the possibility of using cryogel for the purification of biomolecules is an alternative that presents a very good cost benefit ratio. The ion-exchange cryogel method is particularly advantageous for industrial scale-up due to its high porosity, selectivity, and ability to function effectively under various conditions, such as varying pH and ionic strengths. This flexibility results from the immobilization of 2-aminoethanesulfonic acid (taurine), which binds to the biomolecule of interest through electrostatic interactions,

allowing for efficient enzyme capture. Cryogels, as highlighted by [44,45], have demonstrated superior performance in the purification of biomolecules, offering high purity, yield, and binding capacity compared to traditional columns such as Sepharose. Their high porosity and structural robustness make cryogel a promising and scalable alternative for protein purification in integrated processes, supporting their use as a cost-effective solution for industrial L-ASNase production applications.

5. Conclusions

This work highlights the cost-effectiveness and sustainability of producing the L-ASNase enzyme using low-cost alternative substrates, ora-pro-nóbis leaf fiber, and whey protein. These substrates not only provide a favorable composition for the growth of microorganisms, but are also renewable, cheap, and easily accessible, making the enzyme production process economically viable.

In the solid fermentation process, parameter optimization revealed that inoculum, substrate concentration, fermentation time, and temperature significantly affect enzyme production, while the L-asparagine concentration had no notable effects. The optimal conditions (at the interval tested in this study) were an inoculum concentration of 2×10^6 to 6×10^6 spores/mL and a temperature of 25 °C. For the submerged fermentation process, various parameters were found to significantly influence L-ASNase production. The optimal conditions for enzyme production were an inoculum concentration of 7×10^6 to 10^7 spores/mL, a substrate concentration of 3.3% to 4.7% (*w/v*), an asparagine concentration of 16% to 24% (*w/v*), a pH of 5.7 to 6.3, and a fermentation time of 87 to 105 h.

The purification of L-ASNase using an ion-exchange cryogel column proved to be a viable and economical method for the separation of biomolecules, further increasing the economic viability of this process.

To the best of our knowledge, this is the first study to focus on optimizing the production and purification of L-ASNase from *Aspergillus caespitosus*. Although our findings provide a promising basis for sustainable applications in the clinical and food industries, more research is needed to explore the industrial scalability of these methods. Future work could focus on large-scale validation, assessing long-term stability and exploring potential modifications to improve the enzyme's efficiency.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/applmicrobiol5010019/s1>, Table S1. Extracellular L-ASNase activity for each experiment in the 25 full factorial designs, produced by *Aspergillus caespitosus* in solid-state fermentation, Table S2. ANOVA results of the regression for the production of L-ASNase (volumetric activity U/mL), with the parameters that exerted influence using the complete factorial design of 25, with four replications and four central points, Table S3. Extracellular L-ASNase activity for each experiment in the full factorial design 26 produced by *Aspergillus caespitosus* in SmF. Table S4. ANOVA result of the regression for the production of L-ASNase (volumetric activity), with the parameters that exerted influence using the complete factorial design of 26, with four replications and four central points.

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