fermentation

MDPI

Article

Selection and Optimization of Medium Components for the Efficient Production of L-Asparaginase by *Leucosporidium scottii* L115—A Psychrotolerant Yeast

Ignacio S. Moguel [1], Celina K. Yamakawa [2], Larissa P. Brumano [1,3], Adalberto Pessoa, Jr. [1] and Solange I. Mussatto [2,*]

[1] Department of Biochemical-Pharmaceutical Technology, School of Pharmaceutical Sciences, University of São Paulo, Butantã, São Paulo 05508-000, Brazil
[2] Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads, Building 223, 2800 Kongens Lyngby, Denmark
[3] Center for Natural and Human Sciences, Federal University of ABC, Av. dos Estados, 5001, Bangú, Santo André 09210-580, Brazil
* Correspondence: smussatto@dtu.dk or solangemussatto@hotmail.com

Abstract: This study reports the production of L-asparaginase (ASNase), an enzyme mainly used for the treatment of acute lymphoblastic leukemia, by *Leucosporidium scottii* L115, a psychrotolerant yeast isolated from the Antarctic ecosystem. Focus was given to select the most appropriate medium components able to maximize the enzyme production by this yeast, as a first step for the development of a new process to produce ASNase. By combining knowledge in bioprocesses, statistical analysis and modeling, the medium composition that most favored enzyme production was established, which consisted of using a mixture of sucrose (28.34 g L^{-1}) and glycerol (15.61 g L^{-1}) as carbon sources, supplemented with proline (6.15 g L^{-1}) and the following salts (g L^{-1}): KCl, 0.52; $MgSO_4 \cdot 7H_2O$, 0.52; $CuNO_3 \cdot 3H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001; and $FeSO_4 \cdot 7H_2O$, 0.001. By using this medium, enzyme production of 2850 U L^{-1} (productivity of 23.75 U L^{-1} h^{-1}) was obtained, which represented a 28-fold increase in enzyme production per gram of cells (178 U $gdcw^{-1}$) when compared to the control (non-optimized medium), and a 50-fold increase when compared to a reference medium used for ASNase production.

Keywords: L-asparaginase; medium composition; optimization; *Leucosporidium scottii*; psychrotolerant

Citation: Moguel, I.S.; Yamakawa, C.K.; Brumano, L.P.; Pessoa, A., Jr.; Mussatto, S.I. Selection and Optimization of Medium Components for the Efficient Production of L-Asparaginase by *Leucosporidium scottii* L115—A Psychrotolerant Yeast. *Fermentation* **2022**, *8*, 398. https://doi.org/10.3390/fermentation8080398

Academic Editor: Donatella Cimini

Received: 16 July 2022
Accepted: 13 August 2022
Published: 17 August 2022

1. Introduction

Marine and psychrophilic yeasts have unique potential for the synthesis of new biomolecules, and their enzymes can offer biocatalysts with extraordinary properties [1–3]. Despite these attractive characteristics, strains from such environments have been little investigated so far. This is the case of *Leucosporidium scottii*, the psychrotolerant yeast used in the present study, which exists in cold environments around the world, including terrestrial and marine Antarctic ecosystems [4]. The investigation of *L. scottii* as an L-asparaginase (ASNase-EC 3.5.1.1) producer, as well as the selection of the most appropriate medium components able to maximize the production of this enzyme by the yeast, are interesting subjects of study since few yeast strains from marine and cold environments have been reported as good producers of ASNase [5]. These are the first steps for the development of a new process for the production of this enzyme.

ASNase is an enzyme mainly used as a biopharmaceutical for the treatment of acute lymphoblastic leukemia (ALL) [6,7] and in the food industry for acrylamide reduction in processed foods [8]. For the food industry, mainly the enzyme from filamentous fungi such as *Aspergillus* is used. Acrylamide is formed by the Maillard reaction, which is reduced with ASNase action, since L-asparagine is the main amino acid responsible for acrylamide

production in fried and baked foods when reducing sugars are condensed with a carbonyl source [9]. As a biopharmaceutical, ASNase acts in seric L-asparagine depletion, selectively inducing apoptosis in cancer cells. The importance of ASNase for the treatment of acute leukemia is recognized in the World Health Organization's List of Essential Medicines [10].

Currently, the production of the biopharmaceutical ASNase is carried out by bacteria, namely *Escherichia coli* and *Erwinia chrysanthemi* [11]. Four industrial ASNase expression systems are available: (1) the native ASNase produced from *Escherichia coli*; (2) recombinant ASNase produced in an *E. coli* expression system genetically altered with the DNA that encodes for *E. coli* ASNase (homologous expression)—this enzyme is commercialized in a PEGylated form (PEG-asparaginase); (3) native asparaginase derived from *E. chrysanthemi*; (4) the recombinant ASNase produced in a *Pseudomonas fluorescens* expression system genetically altered with the DNA that encodes for *Erwinia chrysanthemi* ASNase [12–16]. ASNase from *Erwinia* (ErA) and from *E. coli* (EcA II) have the same mechanism of action against tumor cells; however, their pharmacokinetics, affinity for the substrate (K_M) and immune system sensitization profile are different. Therefore, the change to ErA is an important option for patients that present an allergic response to treatment with *E. coli* ASNase (EcA II) (the first choice in the ALL treatment protocol) [17].

However, the enzyme produced by bacteria usually presents side effects such as immune inactivation and hypersensitivity [18]. Therefore, the search for new microorganisms able to produce ASNase with less adverse effects is of great interest, among which eukaryotic microorganisms have attracted attention due to their better compatibility with the human system [19,20]. Moreover, due to the wide use and important application of ASNase, especially in the health area, establishing a low-cost process for the production of this enzyme has also been the focus of research.

The composition of the culture medium is the most important parameter affecting the final cost of a bioprocess as it has a direct effect on microbial growth and metabolism, and on product formation. Carbon and nitrogen sources can dramatically affect the product formation, while changes in nutrients and their concentrations have different effects on the accumulation of metabolites, which are controlled by intracellular effectors. Nonetheless, each organism has different nutritional requirements for maximum production [21–24].

The type and concentration of carbon and nitrogen sources present in the culture medium play an important role in enzyme production by microorganisms. Carbon sources may cause catabolic repression, affecting, directly or through a regulatory cascade, the expression of genes subjected to the assimilation of different sugars. Although the pathways of sugar utilization follow the same theme in all yeasts, important biochemical and genetic variations exist among them [25]. On the other hand, the nitrogen source and concentration affect the regulation of enzymes responsible for the uptake, synthesis and interconversion of amino acids such as proteases, amidases and ureases [26]. Several genes in bacteria, yeast and filamentous fungi are regulated by this phenomenon, called nitrogen regulation or nitrogen catabolite repression. The objective of nitrogen regulation is to prevent or reduce the unnecessary divergence of the cells' synthetic capacity to the formation of enzymes needed for the utilization of nitrogen compounds that are non-preferred, when a preferred nitrogen source is available [27].

The screening of different sources and selection of the optimum concentration of medium components are therefore particularly important to maximize the production and activity of the target molecule, being different for each microbial strain. In addition, it has also an important impact on the economic feasibility of a production process. Some of the media commonly used at the laboratory scale are not cost-effective for application at a large scale, and the investigation of cheaper sources of carbon and nitrogen is important for industrial application.

Based on the above, the present study evaluated the production of ASNase by *Leucosporidium scottii* L115, a yeast strain recently isolated from the Antarctic ecosystem. Yeasts isolated from extreme environmental conditions have attracted attention for biotechnological applications since they have developed special mechanisms to survive under these

conditions, such as changes in enzyme characteristics that minimize the negative effects of low temperatures. As a consequence, their proteins are resistant to cold denaturation and their enzymes show higher activity [28,29]. In this context, the study of new enzymes produced by the psychrotolerant strain *L. scottii* is promising. This yeast is able to inhabit relatively extreme environmental conditions such as low temperatures, high salinity and high concentrations of aromatic compounds. In addition, this yeast has already been reported to have ability to metabolize alternative carbon sources such as xylose and glycerol, being able to produce lipases and coenzymes Q9 and Q10 [4], as well as to accumulate lipids [30]. Thus, *L. scottii* has great biotechnological potential.

Focus was given to selecting the most appropriate medium composition able to maximize the enzyme production by this yeast, as a first step for the development of a new process for the production of ASNase. In order to achieve this, an interdisciplinary approach was used, combining knowledge in bioprocesses with statistical analysis and modeling. Initially, screening of different components was carried out by using a Plackett–Burman design. This type of design is very useful for screening purposes because it makes it possible to study the main effects of different variables in a time-saving way, evaluating more than one variable in a single experiment. The orthogonality of the design implies that the effect of each variable is pure in nature and is not confused with interactions among variables [31,32]. Then, the optimum composition was established by mathematical modeling using a design of experiments with response surface methodology. At the end, the medium composition that most favored ASNase production by *L. scottii* was established and the results were compared with the current literature on the topic.

2. Materials and Methods

2.1. Microorganism, Inoculum Preparation and Fermentation Conditions

Leucosporidium scottii L115 was the yeast strain used in this study. This yeast was isolated from Antarctic marine sediments in the austral summer (2009 and 2010) and was provided by the Culture Collection of the Biochemical and Biochemistry Department of UNESP (Rio Claro, Brazil). Stock cultures of the yeast were maintained on potato dextrose broth (PDB, DifcoTM) with added glycerol (20% *v/v*), at −80 °C.

2.1.1. Inoculum Preparation and Cultivation Conditions for the Plackett–Burman Design (PBD)

The inoculum was prepared by incubating the strain in 200 mL of PDB medium in a 500 mL baffled Erlenmeyer flask for 72 h (log growth phase), at 15 °C and 200 rpm. Thereafter, the cells were harvested by centrifugation (3400× *g*, 15 min, 5 °C), washed with sterile water and inoculated in the cultivation medium to obtain an initial concentration of 1% (*v/v*).

A total of 20 cultivation runs were performed at this step. The cultivation media were formulated according to the conditions and design presented in Tables 1 and 2. The concentration of each component was selected empirically based on a literature survey. All cultivation media were supplemented with the following salts (in g L^{-1}): KCl, 0.52; $MgSO_4 \cdot 7H_2O$, 0.52; $CuNO_3 \cdot 3H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001; and $FeSO_4 \cdot 7H_2O$, 0.001. pH was adjusted to 5.0. The added salts and their concentrations were based on salts' compositions from modified Czapek Dox medium.

Cultivations were carried out in 200 mL of medium (according to Tables 1 and 2) in a 500 mL baffled Erlenmeyer flask, at 15 °C and 200 rpm. During the cultivations, samples were taken at 24, 48, 72, 96 and 120 h. The samples were centrifuged at 3400× *g* for 15 min; the harvested cells were washed with distilled water and used for ASNase activity determination, while the supernatants were used for sugar analysis. The maximum yields of L-asparaginase and biomass (as dried cell weight, DCW), which were obtained at 120 h of cultivation, were used as response variables for the statistical analysis.

Table 1. Nitrogen, carbon and complex sources tested in the Plackett–Burman design and their levels.

Code	Variables	+1 (g L^{-1})	−1 (g L^{-1})
	Nutrients considered as nitrogen source		
A	Proline	5	0
B	Urea	5	0
C	L-Aspartate	5	0
D	Ammonium chloride	5	0
E	L-Arginine	5	0
F	L-Glutamine	5	0
G	L-Asparagine	5	0
H	L-Glutamate	5	0
	Nutrients considered as carbon source		
I	Maltose	10	0
J	Glycerol	10	0
K	Sucrose	10	0
L	Citric acid	10	0
	Complex sources		
M	Yeast extract	2.5	0
N	Soybean peptone	2.5	0
O	Corn steep solids	2.5	0
P	Potato dextrose broth	2.5	0
	Dummy variables *		
Q	D1	0	0
R	D2	0	0
S	D3	0	0
R	D2	0	0

* D1, D2 and D3 represent the dummy variables (variables without an assigned value) required to estimate the experimental error.

Table 2. Plackett–Burman design and responses of L-asparaginase (ASNase: U L^{-1} and U $gdcw^{-1}$) and biomass (as dried cell weight, DCW) production obtained for each experimental run.

Run	Variables *																			ASNase (U L^{-1})	DCW (g L^{-1})	ASNase (U $g_{dcw}{}^{-1}$)
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S			
1	-	+	-	-	-	+	+	-	+	+	+	-	+	+	-	-	+	+	-	166.6	5.96	28.0
2	+	-	+	-	-	-	+	+	+	+	-	-	-	+	+	-	-	+	+	141.6	6.18	22.9
3	-	+	-	-	-	-	-	+	+	+	+	+	+	-	+	+	-	-	+	206.0	7.04	29.3
4	+	-	+	-	+	+	-	+	-	+	+	-	+	+	-	+	-	-	-	181.8	10.36	17.5
5	+	+	-	+	-	+	+	+	-	-	+	+	-	+	+	-	-	-	-	212.0	4.23	50.1
6	+	+	+	-	+	-	+	-	+	-	+	-	-	-	+	+	+	-	-	152.7	5.97	25.6
7	+	+	+	+	-	+	-	-	+	+	-	+	-	-	-	+	-	+	-	161.9	3.64	44.5
8	-	+	+	-	+	+	+	+	-	+	-	+	-	-	-	-	+	-	+	73.1	1.29	56.7
9	-	-	+	+	+	-	+	+	+	-	+	+	+	-	-	-	-	+	-	119.5	5.37	22.3
10	+	-	-	+	+	-	-	-	+	+	+	+	-	+	-	-	+	-	+	296.8	11.33	26.2
11	+	+	-	+	+	-	-	+	-	+	-	-	+	-	+	-	+	+	-	117.8	3.24	36.4
12	-	+	+	+	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+	165.7	3.73	44.4
13	+	-	+	-	-	+	-	-	-	-	+	+	+	-	+	-	+	+	+	180.6	6.98	25.9
14	+	+	-	-	+	-	+	-	-	-	-	+	+	+	-	+	-	+	+	40.05	2.50	16.0
15	-	+	+	+	+	+	-	-	+	-	-	-	+	+	+	-	-	-	+	90.01	1.89	47.6
16	-	-	+	+	-	-	+	-	-	+	-	+	+	+	+	+	+	-	-	173.1	6.24	27.7
17	-	-	-	-	+	+	-	+	+	-	-	+	-	+	+	+	+	+	-	101.0	2.94	34.4
18	-	-	-	+	+	+	+	-	-	+	+	-	-	-	+	+	-	+	+	234.4	11.23	20.9
19	+	-	-	+	-	+	+	+	+	-	-	-	+	-	-	+	+	-	+	70.25	2.98	23.6
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	39.77	1.07	37.2

* Variables coded as described in Table 1. (+): higher level of the variable; (−): lower level of the variable.

2.1.2. Inoculum Preparation and Cultivation Conditions for the Central Composite Design (CCD)

To reactivate the cells, a pre-inoculum was prepared by incubating the strain in 200 mL of PDB medium in a 500 mL baffled Erlenmeyer flask for 72 h, at 15 °C and 200 rpm. To adapt the cells to the minimal medium (composed according to the PBD results), the cells were transferred to 200 mL of a new medium containing (g L^{-1}) proline, 5; sucrose, 10; and glycerol, 10, in a 500 mL baffled Erlenmeyer flask. Then, the cells were added to this medium in a concentration of 5 g L^{-1} and the flask was kept at 15 °C and 200 rpm, for 72 h.

Thereafter, the cells were harvested and inoculated in the cultivation medium to obtain an initial concentration of 1% (*v/v*).

A total of 17 cultivation runs were performed at this step. The cultivation media were formulated according to a 2^3 CCD. All cultivation media were supplemented with the following salts (in g L^{-1}): KCl, 0.52; $MgSO_4 \cdot 7H_2O$, 0.52; $CuNO_3 \cdot 3H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001; and $FeSO_4 \cdot 7H_2O$, 0.001. pH was adjusted to 5.0. The cultivations were performed in 200 mL of medium (according to the 2^3 CCD) in a 500 mL baffled Erlenmeyer flask, at 15 °C and 200 rpm. During the cultivations, samples were taken at 24, 48, 72, 96 and 120 h. The samples were centrifuged at 3400× *g* for 15 min; the harvested cells were washed with distilled water and used for ASNase activity determination, while the supernatants were used for sugar analysis. The maximum yields of L-asparaginase and biomass (as dried cell weight, DCW), which were obtained at 120 h of cultivation, were used as response variables for the statistical analysis.

2.2. Statistical Analysis and Modeling

2.2.1. Plackett–Burman Design (PBD)

The effect of 16 different medium components on ASNase production by *L. scottii* was initially evaluated through a PBD, which resulted in 20 independent experimental runs. The variables tested (medium components) and their levels (−1 and +1), or concentrations, are presented in Table 1. Three unassigned variables (D1, D2 and D3), commonly referred to as dummy variables, were also included in the design in order to estimate the experimental error [33].

The effect of each variable on the enzyme and biomass production was evaluated using Equation (1), where X_{i+} is the yield of ASNase and biomass from the experiments when the variables were tested at high levels (+1); X_{i-} is the yield of ASNase and biomass when the variables were tested at low levels (−1); and N is the number of experiments.

$$Effect_{(x_i)} = \frac{\sum X_{i+} - \sum X_{i-}}{N} \quad (1)$$

The significance of the variables was determined by Student's *t*-test and *p*-values. Variables significant at a 90% confidence level ($p < 0.1$) were considered to have a significant impact on ASNase production.

2.2.2. Central Composite Design (CCD)

The variables with the highest influence on ASNase production, identified in the previous PBD, were studied in more detail through a 2^3 CCD, with the aim of selecting their concentrations able to maximize the enzyme production. A set of 17 experiments was performed at this time, where three variables were tested at five different levels ($-\alpha$, −1, 0, 1, α; with $\alpha = 1.6817$). The experimental error was estimated by performing three replicates at the central point (0, 0, 0–runs 15–17).

The response variations as a function of the studied variables were described as a quadratic model, as in Equation (2), where Y is the predicted response; β_0 the intercept; β_i the coefficients for linear effects; β_{ii} the coefficients for quadratic effects; β_{ij} the cross-product coefficient for the effects; X the independent variables; and ε the experimental error.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \varepsilon \quad (2)$$

To validate the composition of the medium, an additional experiment was performed in triplicate under the optimum selected conditions, using 200 mL of optimized medium in a 500 mL baffled Erlenmeyer flask, at 15 °C and 200 rpm, for 168 h.

Statistica version 10.0 (Statsoft, Inc., Tulsa, OK, USA) was the software used for statistical analysis of the data, response surface plots and modeling.

2.3. Analyses

2.3.1. ASNase Activity Determination

SNase activity was determined by quantifying the aspartic β-hydroxamate produced by the hydroxylaminolysis reaction in the presence of hydroxylamine, using the whole cell for the enzymatic reaction. For the reaction, 1.6 mL of cell suspension (at 1.0 of optical density—OD_{600}—in 50 mM Tris–HCl buffer, pH 7.0), 0.2 mL of 0.1 M L-asparagine and 0.2 mL of 1.0 M solution of hydroxylamine hydrochloride at pH 7.0 were added to a microtube. The mixture was agitated in a thermomixer at 850 rpm and 37 °C. After 30 min, the enzymatic reaction was stopped by adding 0.25 mL of TCA/$FeCl_3$ reagent (100 g L^{-1} $FeCl_3$, 50 g L^{-1} trichloroacetic acid (TCA) in 0.66 M HCl). The microtube was centrifuged at 3400× *g* for 5 min and then the absorbance of 1.0 mL of the supernatant was measured in a spectrophotometer—the Expectra Max Plus 384—at 500 nm and room temperature. Controls were prepared in the same conditions, but using only the buffer instead of the samples. For the controls, the hydroxylamine and L-asparagine solutions were added after the ferric chloride reagent. β-aspartohydroxamic acid solution was used to construct the standard curve. One unit of ASNase was defined as the amount of enzyme able to produce 1 μmol of β-aspartohydroxamic acid per minute per gram of dried cell weight (U g^{-1}dcw). This value was further expressed as U L^{-1} of medium [5,34].

2.3.2. Sucrose, Glycerol and Proline Quantification

Sucrose was quantified by the glucose oxidase (GOD)/invertase enzymatic method. For the reaction, 85 μL of distilled water, 5 μL of sample and 10 μL of invertase (8000 U mL^{-1} in distilled water) were added per well to a 96-well plate. The plate was then sealed and incubated at 55 °C for 10 min. After this period, the plate was placed at room temperature and 200 μL of GOD (Bioliquid Kit) reagent was added to the wells, with the plate then incubated at 37 °C for 15 min. At this step, GOD catalyzes a reaction producing hydrogen peroxide (H_2O_2) and glucuronic acid from glucose. Then, H_2O_2 reacts with 4-aminoantipyrine and phenol, by the redox-coupled reaction catalyzed by the peroxidase, producing a red antipirilquinonimine. The intensity of the color resulting from this reaction is proportional to the glucose concentration. Thus, the absorbance of the medium was then measured at room temperature in a spectrophotometer—the Expectra Max Plus 384—at 490 nm. A standard curve was constructed using the standard solution from the kit (glucose 100 mg dL^{-1}) diluted in ultrapure water at the concentrations of 0.0, 0.05, 0.10, 0.15, 0.20 and 0.25 (%) (linearity range of the method). Analysis was performed in duplicate.

Glycerol concentration was estimated by a colorimetric method in the presence of glycerolkinase (GK), glycerol-3-phosphate oxidase (GPO) and peroxidase (HP), using an enzyme-based commercial kit (TL-Labtest®, Biosystem). In this method, the glycerol is phosphorylated to glycerol-3-phosphate in a reaction catalyzed by GK. Then, glycerol-3-phosphate is oxidized by GPO, producing dihydroxyacetone phosphate and H_2O_2. HP catalyzes the redox-coupled reaction with 4-aminoantipyrine and 4-chlorophenol, producing quinoneimine. The intensity of the color resulting from this reaction is proportional to the glycerol concentration. For the reaction, 2 μL of sample and 200 μL of reagent (buffer 50 mmol L^{-1}, pH 7; magnesium ions 4 mmol L^{-1}; 4-chlorophenol 2.7 mmol L-1; 4-aminoantipyrine 300 μmol L^{-1}; ATP 1.8 mmol L^{-1}; GK 1000 U L^{-1}; GPO 1500 U L^{-1}; HP 900 U L^{-1} and sodium azide 14.6 mmol L^{-1}) were placed per well in a 96-well plate. The plate was then sealed and incubated at 37 °C for 15 min. Afterwards, the absorbance was measured at room temperature in a spectrophotometer—the Expectra Max Plus 384—at 505 nm. A standard curve was constructed using the standard solution from the kit (glycerol 0.5 g L^{-1}; sodium azide 1.54 mmol L^{-1} and stabilizer). Analysis was performed in duplicate.

For proline quantification, 100 μL of sample was added to 100 μL of sulfosalicylic acid 3% (*w/v*) solution, 200 μL of glacial acetic acid and 200 μL of acidic ninhydrin (1.25 g of ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M orthophosphoric acid) in test tubes. The tubes were then incubated at 96 °C for 60 min. Afterwards, the reaction was

stopped in an ice bath. Then, 1 mL of toluene was added and the sample was vortexed for 20 s. At the end, the organic phase was extracted and the absorbance was measured at room temperature in a spectrophotometer—the Expectra Max Plus 384—at 520 nm.

3. Results and Discussion

3.1. Screening of Medium Components Using the PBD

A PBD was initially used in this study to select the main components to be used in the formulation of the cultivation medium for ASNase production. In the control experiment, using the modified Czapek Dox medium [35], enzyme activity of 6.24 U $gdcw^{-1}$ was observed. The results obtained for the experiments of the PBD are presented in Table 2. For screening and optimization purposes, the ASNase activity in U L^{-1} was considered as the result, since it is more relevant for bioprocess development than the activity in U $gdcw^{-1}$.

Statistical analysis of these results revealed that three of the nitrogen sources, namely proline, ammonium chloride and L-aspartate at 5 g L^{-1}, had a positive effect (significant at $p < 0.1$) on the ASNase production by *L. scottii*, while the corn steep solids (at 2.5 g L^{-1}) were the only complex source that favored enzyme production (Table 3). For the carbon sources, sucrose, glycerol and citric acid (at 10 g L^{-1}) had a positive effect on enzyme production. On the other hand, some of the tested nutrients negatively affected the production of ASNase by *L. scottii*, which included L-asparagine, L-glutamate, urea, L-arginine (at 5 g L^{-1}) and yeast extract (at 2.5 g L^{-1}). The other nutrient sources did not present significant effects on ASNase production. In contrast with our results, ASNase production by *Streptomyces albidoflavus* was favored when maltose was used as a carbon source, followed by starch, glucose, trehalose and glycerol [36]. This fact confirms that different strains have different nutritional requirements; therefore, it is important to select the most suitable components for the cultivation of each microorganism in order to maximize the product formation.

Table 3. Effects of the variables and statistical analysis of the Plackett–Burman design on the responses of L-asparaginase (ASNase) and biomass (as dried cell weight, DCW) production.

	ASNase Production				DCW Production			
	Effect	**S.E.**	***t* Student**	***p*-Value**	**Effect**	**S.E.**	***t* Student**	***p*-Value**
Intercept	146.27	5.82	50.21	0.00001	5.20	0.31	33.77	0.00005
Yeast extract	−33.39	5.82	−5.73	0.010 *	−0.07	0.31	−0.21	0.841
Soybean peptone	11.20	5.82	1.92	0.150	0.49	0.31	1.59	0.209
Corn steep solids	19.33	5.82	3.31	0.045 *	0.60	0.31	1.96	0.144
Potato dextrose broth	4.91	5.82	0.84	0.461	0.90	0.31	2.94	0.060 *
L-Glutamine	−8.17	5.82	−1.40	0.255	−0.28	0.31	−0.91	0.429
L-Asparagine	−15.79	5.82	−2.71	0.073 *	−0.02	0.31	−0.08	0.939
Maltose	−1.24	5.82	−0.21	0.845	0.08	0.31	0.26	0.808
Glycerol	58.18	5.82	9.99	0.002 *	2.89	0.31	9.36	0.002 *
L-Glutamate	−14.71	5.82	−2.52	0.085 *	−0.94	0.31	−3.06	0.054
Sucrose	90.78	5.82	15.58	0.001 *	4.02	0.31	13.04	0.001 *
Proline	18.63	5.82	3.20	0.049 *	1.06	0.31	3.46	0.040 *
Urea	−25.31	5.82	−4.34	0.022 *	−2.68	0.31	−8.70	0.003 *
L-Aspartate	14.51	5.82	−2.49	0.088 *	−0.25	0.31	−0.80	0.478
Citric acid	20.33	5.82	3.49	0.039 *	−0.10	0.31	−0.34	0.755
L-Arginine	−21.08	5.82	−3.62	0.036*	0.64	0.31	2.08	0.128
Ammonium chloride	25.76	5.82	4.42	0.021 *	0.19	0.31	0.63	0.570

S.E. = standard error. * effects significant at 90% confidence level. $R^2 = 0.99$.

It is interesting to note that the production of ASNase was affected by the nitrogen source used for cultivation, revealing that the ASNase produced by *L. scottii* is a nitrogen-regulated enzyme. However, the production of ASNase was not favored in the presence of easily assimilated nitrogen sources such as L-asparagine, L-glutamine, L-aspartate and L-glutamate. This is probably due to the fact that these sources do not stimulate the increase in enzymes responsible for the uptake, synthesis and interconversion of amino

acids, such as proteases, amidases (such as ASNase) and ureases [26]. Previous studies reported that the production of ASNase II by *Saccharomyces cerevisiae* was also not induced by L-asparagine, but the use of non-preferred nitrogen sources such as proline gave higher enzyme activity [37]. These data suggest that the ASNase production by *L. scottii* may undergo similar regulation to ASNase II production by *S. cerevisiae*. Moreover, for the yeast *Saccharomyces cerevisiae*, aspartate, arginine, glutamine, asparagine and glutamate support rapid growth because the nitrogen is directly assimilated by the cell metabolism. Moreover, these nitrogen sources are related to nitrogen catabolite repression, which indicates a preferred nitrogen source. Interestingly, non-preferred nitrogen sources such as proline activate nitrogen catabolite repression, the unfolded protein response and proline catabolism genes, while urea activates retrograde control and urea catabolism genes [38].

For biomass formation, the nutrients that contributed significantly included sucrose and glycerol at 10 g L^{-1}, proline at 5 g L^{-1} and potato dextrose broth at 2.5 g L^{-1}. Urea (at 5 g L^{-1}) was the only nutrient that negatively affected the production of biomass, while all the other sources evaluated did not present significant effects ($p < 0.1$) on biomass formation (Table 3).

Among the nitrogen sources, proline was the only one that favored both responses, ASNase and biomass production. Proline is commonly reported as a non-preferred source of nitrogen, and is used in most of the studies on nitrogen catabolic repression in yeasts [27,38]. The production ASP3 by *Saccharomyces cerevisiae* occurs under nitrogen catabolite repression, being strongly repressed by ammonium compounds, but reasonably induced by proline [39]. Nitrogen catabolite repression is a phenomenon that explains why proline usually is more efficient as a nitrogen source for ASNase production than nitrogen-rich sources (e.g., glutamine and ammonium compounds). Similar behavior was noticed by Freire et al. (2020) working with *Leucosporidium muscorum* [5], which suggests that *Leucosporidium* sp. may have a mechanism of nitrogen catabolite repression similar to that found in yeasts such as *S. cerevisiae*. Furthermore, proline is a non-essential amino acid that is involved in stress protection, namely a protein and membrane stabilizer, protein-folding chaperone and reactive oxygen species (ROS) scavenger. Its effect of protein folding in chaperones is important for enzyme activity, ensuring the correct enzyme conformation [39]. This role of proline can explain its positive effect in ASNase activity improvement.

As a carbon source, sucrose and glycerol were the other two variables that presented significant effects on both responses. Both carbon sources are often used to promote microbial growth and have potential to result in increased enzyme production by the strain, which is usually observed in the synthesis of primary metabolites [40]. In addition, glycerol is a solute compatible with the amino acid proline in terms of efficiency of energy metabolism and redox balance in yeast cells, favoring cell growth and enzyme production [41].

According to the results of the PBD, proline, sucrose and glycerol were the nutrient sources with the most significant influence on the production of ASNase and biomass by *L. scottii* and were therefore selected for the subsequent step of this study, which aimed to determine their optimum concentrations for use in the formulation of the cultivation medium. An important aspect to highlight is that no complex sources will then be used for the cultivation of *L. scottii* for ASNase production. This is an important advantage for the industrial production of this biopharmaceutical since the use of defined media avoids variations in the product formation and allows better stability between production batches, being also useful for the modeling of processes [42]. Another important point to highlight is that both selected carbon sources, sucrose and glycerol, are of low cost and widely produced. Glycerol, for example, can be obtained as a by-product from the biodiesel industry and has been considered as a promising carbon source for industrial microbiology applications [43]. Furthermore, low-cost substrates contribute to the economic feasibility of the process, enabling it to be scaled up.

3.2. Optimization of Medium Composition

The three nutrient sources selected from the sixteen initially evaluated, namely proline, sucrose and glycerol, were then further investigated in this part of the study with the aim of defining their ideal concentrations to be used for medium formulation in order to obtain efficient production of ASNase by *L. scottii*. These three variables were tested in different concentrations, which were combined through a 2^3 CCD. The conditions tested in these experiments, as well as the results obtained for ASNase production, are presented in Table 4. Statistical analysis of these results confirmed the high significance ($p < 0.1$) of the three variables in enzyme production, since great variation in the results was observed according to the condition used for each variable.

Table 4. Conditions used for the 2^3 central composite design and results (experimentally obtained and predicted by the model) obtained for the response of L-asparaginase (ASNase) production.

Run	Proline (g L^{-1})	Sucrose (g L^{-1})	Glycerol (g L^{-1})	ASNase (U L^{-1})	
				Experimental	Predicted
1	4	10	10	2489.46	2283.89
2	4	10	40	2563.13	2254.16
3	4	40	10	3012.48	2955.93
4	4	40	40	2328.63	2367.27
5	10	10	10	2110.09	1879.50
6	10	10	40	1939.43	1812.33
7	10	40	10	2048.70	2169.23
8	10	40	40	1535.50	1543.12
9	2	25	25	2081.85	2290.97
10	12	25	25	1259.26	1267.19
11	7	0	25	1456.93	1856.54
12	7	50	25	2374.05	2192.24
13	7	25	0	3108.25	3212.95
14	7	25	50	2556.99	2666.41
15	7	25	25	3202.20	3366.92
16	7	25	25	3382.46	3366.92
17	7	25	25	3251.87	3366.92

A regression analysis was then performed and, after eliminating the non-significant effects, the data were fitted to a second-order polynomial equation (Equation (3)), where the concentrations of proline, sucrose and glycerol were described as $X1$, $X2$ and $X3$, respectively. Analysis of variance of the model obtained for ASNase production revealed that both linear and quadratic effects of all the three variables were significant at a 90% confidence level, while only one interaction (sucrose × glycerol) showed a significant effect on the response (Table 5). In fact, the magnitude of the coefficients presented in Equation (3) clearly confirms that the individual effect of the variables was higher than the interaction effects observed between them.

$$SNase\ (U\ L^{-1}) = -799.51 + 732.54\ X1\ -\ 59.6\ X1^2 + 121.99\ X2 - 1.99\ X2^2 + 31.034\ X3 - 0.53\ X3^2 - 0.62\ X2 \times X3 \quad (3)$$

The established model is able to predict the variations in enzyme production according to the variations in the concentrations of proline, sucrose and glycerol used for the formulation of the cultivation medium, being useful for scale-up experiments. It is important to highlight that the model presented a high correlation coefficient ($R^2 = 0.93$), and the lack of fit was not significant, implying that the model is able to efficiently predict the results. Indeed, as can be seen in Table 4, there is good agreement between the results obtained experimentally and those predicted by the model.

Table 5. Analysis of variance of the model describing the production of L-asparaginase by *L. scottii*.

Source	S0053\A	d.f.	MS	*F-Value*	*p-Value*
Proline (L)	1,251,591	1	1,251,591	144.37	0.0068 *
Proline (Q)	3,141,439	1	3,141,439	362.37	0.0027 *
Sucrose (L)	137,444	1	137,444	15.85	0.0576 *
Sucrose (Q)	2,195,471	1	2,195,471	253.25	0.0039 *
Glycerol (L)	360,911	1	360,911	41.63	0.0231 *
Glycerol (Q)	154,271	1	154,271	17.79	0.0518 *
Proline × Sucrose	70,724	1	70,724	8.15	0.1038
Proline × Glycerol	679	1	679	0.07	0.8059
Sucrose × Glycerol	151,267	1	15,267	17.44	0.0528 *
Lack of fit	452,698	5	90,540	10.44	0.1087
Pure error	17,338	2	8669		
Total SS	6,734,472	16			

SS = sum of squares; d.f. = degrees of freedom; MS = mean square; L = linear; Q = quadratic. * significant at 90% confidence level. $R^2 = 0.93$.

In order to define the concentrations of the variables able to maximize the enzyme production, the response surface methodology was employed. The response surfaces presented in Figure 1 show the existence of a region where the ASNase production is maximum (dark red region). From this region, further changes (increase or decrease) in the level of the variables result in a decrease in the production of ASNase. This result demonstrates that it is possible to optimize the values of the variables in the studied region. The statistical program indicated an optimal condition within this region able to result in the maximum production of ASNase, which corresponded to the use of 6.15 g L^{-1} proline, 28.34 g L^{-1} sucrose and 15.61 g L^{-1} glycerol. Assays to validate this condition were then performed and resulted in enzyme production of 2850 U L^{-1} (productivity of 23.75 U L^{-1} h^{-1} and 178.1 U $gdcw^{-1}$).

The profile of substrate consumption and ASNase production by *L. scottii* under the optimum medium conditions is shown in Figure 2. As can be seen, sucrose was the preferred carbon source consumed by the yeast, being exhausted after approximately 72 h of cultivation, while glycerol was exhausted after approximately 144 h. Proline was consumed simultaneously with the carbon sources. Although ASNase production was observed from the initial hours of cultivation, the enzyme production was significantly increased after proline starvation. Similar behavior (high enzyme production under nitrogen starvation) was reported for ASNase II production by *S. cerevisiae* [44].

Table 6 compares the medium composition optimized for ASNase production by *L. scottii* (present study) with the medium composition reported for ASNase production by other yeasts. As can be noted, most of these media are composed of glucose as a single carbon source, L-asparagine as a nitrogen source and also contain complex sources, being then quite different from the medium composition established for *L. scottii*. Although some authors report that the use of L-asparagine can improve the production of ASNase [45,46], the use of a non-preferable nitrogen source and its starvation led to more efficient production of ASNase by *L. scottii*. Moreover, a mixture of carbon sources, sucrose and glycerol, was the most appropriate to result in high enzyme production by this yeast, and no complex nutrients were required to improve the enzyme production efficiency. The differences in enzyme production are justified not only by the medium used, but also by the producer strain. Therefore, wide variation in ASNase activity can be observed in Table 6.

Finally, the results obtained in the present study highlight the potential of the new yeast strain, *L. scottii* L115, as an ASNase producer. The enzyme production obtained under the optimum medium cultivation conditions, 2850 U L^{-1} (with productivity of 23.75 U L^{-1} h^{-1}), represented a 28-fold increase in the enzyme production per gram of cell (178.1 U $gdcw^{-1}$) when compared to the control (modified Czapek Dox—non-optimized medium), and a 50-fold increase when compared to the results obtained from a reference medium used for ASNase production (glucose 2.0 g L^{-1}; L-asparagine 10.0 g L^{-1}; KH_2PO_4 1.52 g L^{-1}; KCl 0.52 g L^{-1}; $MgSO_4 \cdot 7H_2O$ 0·52 g L^{-1}; trace elements: $CuNO_3 \cdot 3H_2O$;

$ZnSO4{\cdot}7H_2O$ and $FeSO_4{\cdot}7H_2O$) [35]. This medium was chosen as a reference because it was used in classical references for L-asparaginase-producing fungi [35,47]. In addition, according to a systematic review of the literature, Czapek Dox medium is the most widely used medium for the production of asparaginase from fungi [48].

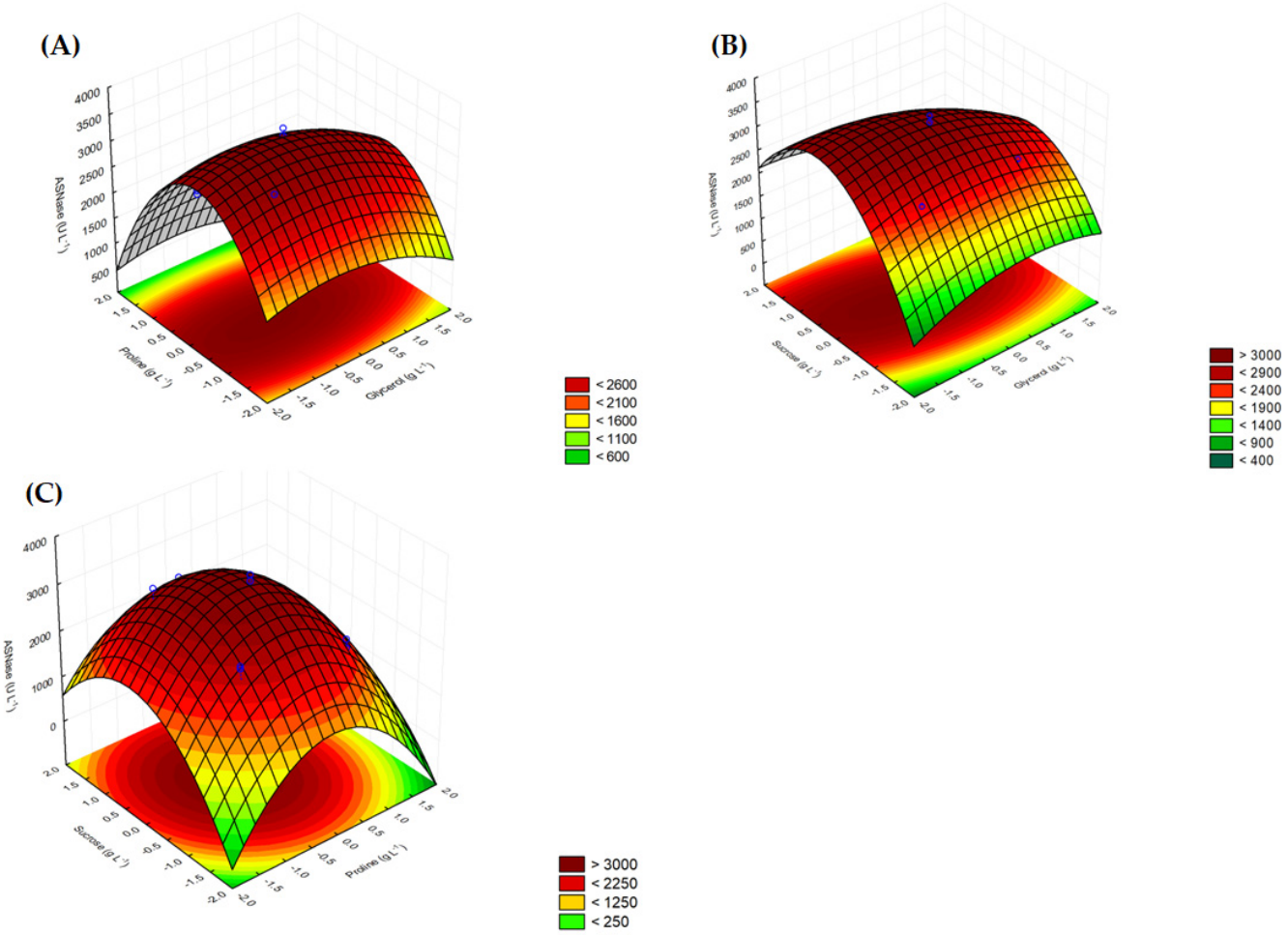

Figure 1. Response surfaces representing the production of L-asparaginase (ASNase UL^{-1}: *y*-axis) by *L. scottii* as a function of the (**A**) proline (*x*-axis) and glycerol (*z*-axis) concentrations, (**B**) sucrose (*x*-axis) and glycerol (*z*-axis) concentrations and (**C**) sucrose (*x*-axis) and proline (*z*-axis) concentrations used to formulate the cultivation medium. Dots represent the experimental data obtained.

Medium optimization was also a successful strategy reported by other authors to increase ASNase production. Freire et al. (2020) improved nine-fold the enzyme production by *Leucosporidium muscorum* [5], achieving 4582.5 U L^{-1} and 63.64 U L^{-1} h^{-1} for enzyme activity and productivity, respectively. However, in their work, a more complex medium composition was used (besides sucrose and proline, seawater and yeast extract were also added to the modified Czapek Dox medium). In the present study, the optimum medium composition for the cultivation of *L. scottii* L115, which does not require the addition of complex nutritional sources, is an important advantage for the scaling up of the process. It is also worth highlighting that, similarly to the enzyme produced by *L. muscorum*, the enzyme produced by *L. scottii* is a new enzyme that presents lower glutaminase activity [49].

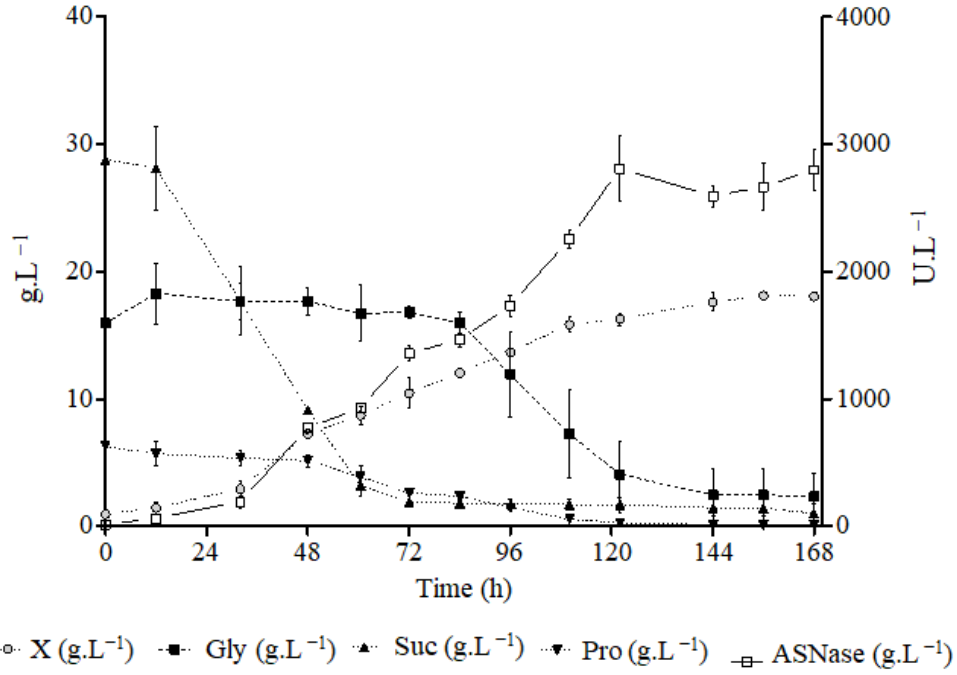

Figure 2. Kinetic profile for substrate consumption, L-asparaginase (ASNase) and biomass (X) production during the cultivation of *L. scottii* under the optimum medium conditions. Gly = glycerol; Suc = sucrose; Pro = proline.

Table 6. Medium composition used for L-asparaginase production by different yeasts.

Yeast Strain	Medium Composition (g L^{-1})	ASNase (U L^{-1})	Reference
Different species including *Rhodotorula*, *Candida utilis*, *Saccharomyces*, *Cryptococcus* and *Pichia* (*Hansenula)*	Glucose, 20.0; L-asparagine, 5.0; K_2HPO_4, 1.0; KCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.01; yeast extract, 1.0; pH 6.0	from 5000 to 53,000 (depending on the strain)	[50]
Different species including *Pichia* (*Hansenula), Cryptococcus*, *Sporobolomyces* and *Rhodotorula*	Sucrose, 30.0; polypepton, 5.0; meat extract 5.0; yeast extract, 2.0; malt extract, 2.0; KH_2PO_4, 5.0; K_2HPO_4, 1.5; $MgSO_4{\cdot}7H_2O$, 0.5	from 111 to 1320 (depending on the strain)	[51]
S. cerevisiae X-2180-A2	Growth medium: glucose, 20.0; yeast nitrogen base (without amino acids and ammonium sulfate), 2.0; and ammonium sulfate, 1.32 Production medium: glucose, 30.0; 20 mM potassium phosphate buffer, pH 7.0	3506	[44]
Candida utilis	Glucose, 20.0; yeast nitrogen base (without amino acids and ammonium sulfate), 2.0; and peptone, 2.0	800	[52]
Trichosporon asahii IBBLA1	Glucose, 2; L-asparagine or glutamine or filter-sterilized urea, 10; KH_2PO_4, 1.52; KCl, 0.52; $MgSO_4{\cdot}7H_2O$, 0.52; trace amounts of $FeSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}7H_2O$ and $CuNO_3{\cdot}3H_2O$	20,570	[6]
Rhodosporidium toruloides CBS14, ATCC10788, Rhodotorula glutinis NCYC59, ATCC90950, Rhodotorula rubra MTCC248	Mannitol, 20.0; L-asparagine, 5.0; K_2HPO_4, 1.2; KH_2PO_4, 6.0; KCl, 0.5; $MgSO_4{\cdot}7H_2O$, 0.01; yeast extract, 1.0; pH 6.0	583,000	[53]
Leucosporidium scottii L 115	Sucrose, 28.34; glycerol, 15.61; proline, 6.15; KCl, 0.52; $MgSO_4{\cdot}7H_2O$, 0.52; $CuNO_3{\cdot}3H_2O$, 0.001; $ZnSO_4{\cdot}7H_2O$, 0.001; and $FeSO_4{\cdot}7H_2O$, 0.001; pH 5.0	2850	Present study

A detailed study on the properties of the purified ASNase produced by *L. scottii* L115, including the complete biochemical characterization, kinetic parameters and glutaminase activity, among others, is required for a better evaluation of this enzyme's use for pharmaceutical applications. Previous results demonstrated that the enzyme produced by *L. scottii* L 115 is a multimer of 462 kDa, and its maximum enzyme activity was observed at pH 7.5 and 55 °C [49]. These characteristics are different from those reported for ASNase II from *E. coli*, which is a tetrameric enzyme (~140 kDa) with optimum pH 6.0 and temperature of 37 °C [54], and for ASNase II from *Saccharomyces cerevisiae*, which has a molecular size of 136 kDa, optimum temperature of 46 °C and pH 7.2 [55]. Considering that circulatory fluids in the human body have a pH value of 7.4, the pH profile of the produced enzyme is a therapeutically remarkable feature of the enzyme for potential use in ALL treatment, despite its size. Although *L. scottii* is a cold-adapted yeast, the maximum activity of its enzyme was observed at a higher temperature than other mesophilic microorganisms. According to the literature, cold-adapted enzymes generally have an optimum temperature above their physiological temperature, suggesting that evolutionary pressure has acted to allow the enzyme to be active at low temperatures, but not to optimize its structure to present maximal activity at low temperatures [56].

The findings of this study are promising for the higher production of this new enzyme, being also the first step to achieve a feasible production process aiming to produce a new enzyme with different characteristics, and with potential to result in an enzyme with less side effects, produced by yeast instead of bacteria, with lower glutaminase activity.

4. Conclusions

This study demonstrated that *L. scottii* L115, a psychrotolerant yeast isolated from the Antarctic ecosystem, is a good producer of ASNase, a biological molecule of great interest and with important applications in the pharmaceutical and food industries. Additionally, *L. scottii* L115 is undemanding in terms of nutritional needs and can grow well in a simple cultivation medium, without the addition of complex nutrient sources. The best medium formulation to obtain efficient production of ASNase by this yeast was composed of sucrose and glycerol as carbon sources and proline as the sole nitrogen source, with salts added for a micronutrient supply.

By combining knowledge in bioprocesses with statistical analysis and modeling, it was possible to select the medium composition able to maximize the production of ASNase by this yeast, and such conditions resulted in a 28-fold increase in the enzyme activity per gram of cell (178.1 U $gdcw^{-1}$) when compared to the control (non-optimized medium), and in a 50-fold increase when compared to the results obtained from a reference medium reported in the literature for ASNase production. The significance of this work is further strengthened by the fact that the three main medium components that led to the high production of ASNase by *L. scottii* L115, namely proline, sucrose and glycerol, are relatively cheap and easily available at an industrial scale, which contributes to the economic feasibility and future scale-up of the process. Finally, since there are few studies on the production of ASNase by yeasts, the results obtained in the present study open up new perspectives for the development of a new method for the production of this enzyme, with potential to result in an ASNase with better properties than those currently available in the market, produced by bacteria.

Author Contributions: Conceptualization, A.P.J. and S.I.M.; methodology, I.S.M. and C.K.Y.; investigation, I.S.M. and C.K.Y.; resources, S.I.M.; data curation, I.S.M. and C.K.Y.; writing—original draft preparation, C.K.Y. and L.P.B.; writing—review and editing, S.I.M. and L.P.B.; visualization, C.K.Y., L.P.B. and S.I.M.; supervision, S.I.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Novo Nordisk Foundation, Denmark (grant number NNF20SA0066233), the State of São Paulo Research Foundation, FAPESP, Brazil (grant numbers 2013/08617-7 and 2019/03981-9) and the Mexican Council for Science and Technology, CONACYT, Mexico (grant number 298596).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Robinson, C.H. Cold adaptation in Arctic and Antarctic fungi. *New Phytol.* **2001**, *151*, 341–353. [CrossRef]
2. Zhang, C.; Kim, S.-K. Research and Application of Marine Microbial Enzymes: Status and Prospects. *Mar. Drugs* **2010**, *8*, 1920–1934. [CrossRef] [PubMed]
3. Zaky, A.S.; Tucker, G.A.; Daw, Z.Y.; Du, C. Marine yeast isolation and industrial application. *FEMS Yeast Res.* **2014**, *14*, 813–825. [CrossRef]
4. Duarte, A.; Dayo-Owoyemi, I.; Nobre, F.S.; Pagnocca, F.C.; Chaud, L.C.S.; Pessoa, A.; Felipe, M.D.G.A.; Sette, L.D. Taxonomic assessment and enzymes production by yeasts isolated from marine and terrestrial Antarctic samples. *Extremophiles* **2013**, *17*, 1023–1035. [CrossRef] [PubMed]
5. Freire, R.K.B.; Mendonça, C.M.N.; Ferraro, R.B.; Moguel, I.S.; Tonso, A.; Lourenço, F.R.; Santos, J.H.P.L.; Sette, L.D.; Pessoa Jr, A. Glutaminase-free L-asparaginase production by *Leucosporidium muscorum* isolated from Antarctic marine-sediment. *Prep. Biochem. Biotechnol.* **2020**, *51*, 277–288. [CrossRef] [PubMed]
6. Ashok, A.; Doriya, K.; Rao, J.V.; Qureshi, A.; Tiwari, A.K.; Kumar, D.S. Microbes Producing L-Asparaginase free of Glutaminase and Urease isolated from Extreme Locations of Antarctic Soil and Moss. *Sci. Rep.* **2019**, *9*, 1423. [CrossRef]
7. Muneer, F.; Siddique, M.H.; Azeem, F.; Rasul, I.; Muzammil, S.; Zubair, M.; Afzal, M.; Nadeem, H. Microbial l-asparaginase: Purification, characterization and applications. *Arch. Microbiol.* **2020**, *202*, 967–981. [CrossRef]
8. Xu, F.; Oruna-Concha, M.-J.; Elmore, J.S. The use of asparaginase to reduce acrylamide levels in cooked food. *Food Chem.* **2016**, *210*, 163–171. [CrossRef]
9. Zuo, S.; Zhang, T.; Jiang, B.; Mu, W. Reduction of acrylamide level through blanching with treatment by an extremely thermostable l-asparaginase during French fries processing. *Extremophiles* **2015**, *19*, 841–851. [CrossRef]
10. World Health Organization. *Model List of Essential Medicines, 21st List*; World Health Organization: Geneva, Switzerland, 2019.
11. Brumano, L.P.; da Silva, F.V.S.; Costa-Silva, T.A.; Apolinário, A.C.; Santos, J.H.P.M.; Kleingesinds, E.K.; Monteiro, G.; Rangel-Yagui, C.D.O.; Benyahia, B.; Junior, A.P. Development of L-Asparaginase Biobetters: Current Research Status and Review of the Desirable Quality Profiles. *Front. Bioeng. Biotechnol.* **2019**, *6*, 212. [CrossRef]
12. Merck. *Elspar Asparaginase*; Ovation Pharmaceuticals: Deerfield, IL, USA, 2000. Available online: https://www.accessdata.fda.gov/drugsatfda_docs/label/2002/aspamer080102LB.pdf (accessed on 27 July 2022).
13. European Medicines Agency. *Assessment Report Spectrila*; European Medicines Agency: London, UK, 2015. Available online: https://www.ema.europa.eu/en/documents/assessment-report/spectrila-epar-public-assessment-report_en.pdf (accessed on 8 August 2022).
14. European Medicines Agency. *Assessment Report Oncaspar*; European Medicines Agency: London, UK, 2016. Available online: https://www.ema.europa.eu/en/documents/assessment-report/oncaspar-epar-public-assessment-report_en.pdf (accessed on 8 August 2022).
15. Medicines Evaluation Board. *Public Assessmen Report Crisantaspase*; European Medicines Agency: London, UK, 2015; Available online: https://file.wuxuwang.com/hma/NL_H_3194_001_PARSummary.pdf (accessed on 8 August 2022).
16. Asparaginase (*Erwinia chrysanthemi*) (Recombinant)-rywn. *Am. J. Health Pharm.* **2021**, *78*, 1919–1921. [CrossRef] [PubMed]
17. El-Naggar, N.E.-A.; Moawad, H.; El-Shweihy, N.M.; El-Ewasy, S.M. Optimization of Culture Conditions for Production of the Anti-Leukemic Glutaminase Free L-Asparaginase by Newly Isolated *Streptomyces olivaceus* NEAE-119 Using Response Surface Methodology. *BioMed Res. Int.* **2015**, *2015*, 627031. [CrossRef] [PubMed]
18. Narta, U.K.; Kanwar, S.S.; Azmi, W. Pharmacological and clinical evaluation of l-asparaginase in the treatment of leukemia. *Crit. Rev. Oncol.* **2007**, *61*, 208–221. [CrossRef]
19. Doriya, K.; Kumar, D.S. Isolation and screening of l-asparaginase free of glutaminase and urease from fungal sp. *3 Biotech* **2016**, *6*, 239. [CrossRef] [PubMed]
20. Muso-Cachumba, J.J.; Antunes, F.A.F.; Peres, G.F.D.; Brumano, L.; Santos, J.; Da Silva, S.S. Current applications and different approaches for microbial l-asparaginase production. *Braz. J. Microbiol.* **2016**, *47*, 77–85. [CrossRef]
21. Liu, Z.; Feist, A.M.; Dragone, G.; Mussatto, S.I. Lipid and carotenoid production from wheat straw hydrolysates by different oleaginous yeasts. *J. Clean. Prod.* **2020**, *249*, 119308. [CrossRef]

22. Dinarvand, M.; Rezaee, M.; Masomian, M.; Jazayeri, S.D.; Zareian, M.; Abbasi, S.; Ariff, A.B. Effect of C/N Ratio and Media Optimization through Response Surface Methodology on Simultaneous Productions of Intra- and Extracellular Inulinase and Invertase from *Aspergillus niger* ATCC 20611. *BioMed Res. Int.* **2013**, *2013*, 508968. [CrossRef]
23. Mussatto, S.I.; Roberto, I.C. Establishment of the optimum initial xylose concentration and nutritional supplementation of brewer's spent grain hydrolysate for xylitol production by *Candida guilliermondii*. *Process. Biochem.* **2008**, *43*, 540–546. [CrossRef]
24. Mussatto, S.I.; Fernandes, M.; Dragone, G.; Mancilha, I.M.; Roberto, I.C. Brewer's spent grain as raw material for lactic acid production by *Lactobacillus delbrueckii*. *Biotechnol. Lett.* **2007**, *29*, 1973–1976. [CrossRef]
25. Gancedo, J.M. Yeast Carbon Catabolite Repression. *Microbiol. Mol. Biol. Rev.* **1998**, *62*, 334–361. [CrossRef]
26. Sanchez, S.; Demain, A.L. Metabolic regulation and overproduction of primary metabolites. *Microb. Biotechnol.* **2008**, *1*, 283–319. [CrossRef] [PubMed]
27. Magasanik, B.; Kaiser, C.A. Nitrogen regulation in Saccharomyces cerevisiae. *Gene* **2002**, *290*, 1–18. [CrossRef]
28. Margesin, R.; Schinner, F. Properties of cold-adapted microorganisms and their potential role in biotechnology. *J. Biotechnol.* **1994**, *33*, 1–14. [CrossRef]
29. Segal-Kischinevzky, C.; Romero-Aguilar, L.; Alcaraz, L.D.; López-Ortiz, G.; Martínez-Castillo, B.; Torres-Ramírez, N.; Sandoval, G.; González, J. Yeasts Inhabiting Extreme Environments and Their Biotechnological Applications. *Microorganisms* **2022**, *10*, 794. [CrossRef]
30. Moguel, I.S.; Yamakawa, C.K.; Pessoa, A., Jr.; Mussatto, S.I. L-asparaginase Production by *Leucosporidium scottii* in a Bench-Scale Bioreactor with Co-production of Lipids. *Front. Bioeng. Biotechnol.* **2020**, *8*, 576511. [CrossRef]
31. Granato, D.; Calado, V.M.D.A. The use and importance of design of experiments (DOE) in process modelling in food science and technology. In *Mathematical and Statistical Methods in Food Science and Technology*; John Wiley & Sons, Ltd.: Chichester, UK, 2013; pp. 1–18. [CrossRef]
32. Hymavathi, M.; Sathish, T.; Brahmaiah, P.; Prakasham, R.S. Impact of carbon and nitrogen sources on L-asparaginase pro-duction by isolated *Bacillus circulans* (MTCC 8574): Application of saturated Plackett-Burman Design. *Chem. Biochem. Eng. Q.* **2010**, *24*, 473–480. [CrossRef]
33. Analytical Methods Committee. Experimental design and optimisation (4): Plackett–Burman designs. *Anal. Methods* **2013**, *5*, 1901–1903. [CrossRef]
34. Freitas, M.; Souza, P.; Cardoso, S.; Cruvinel, K.; Abrunhosa, L.S.; Filho, E.X.F.; Inácio, J.; Pinho, D.B.; Pessoa, A.; Magalhães, P.O. Filamentous Fungi Producing L-Asparaginase with Low Glutaminase Activity Isolated from Brazilian Savanna Soil. *Pharmaceutics* **2021**, *13*, 1268. [CrossRef]
35. Gulati, R.; Saxena, R.K.; Gupta, R. A rapid plate assay for screening l -asparaginase producing micro-organisms. *Lett. Appl. Microbiol.* **1997**, *24*, 23–26. [CrossRef]
36. Narayana, K.J.P.; Kumar, K.G.; Vijayalakshmi, M. L-asparaginase production by *Streptomyces albidoflavus*. *Indian J. Microbiol.* **2008**, *48*, 331–336. [CrossRef]
37. Bon, E.P.S.; Carvajal, E.; Stanbrough, M.; Rowen, D.; Magasanik, B. Asparaginase II of *Saccharomyces cerevisiae*. In *Biotechnology for Fuels and Chemicals*; Applied Biochemistry and Biotechnology Book Series; Davison, B.H., Wyman, C.E., Finkelstein, M., Eds.; Humana Press: Totowa, NJ, USA, 1997; Volume 63–65, pp. 203–212. [CrossRef]
38. Godard, P.; Urrestarazu, A.; Vissers, S.; Kontos, K.; Bontempi, G.; van Helden, J.; André, B. Effect of 21 Different Nitrogen Sources on Global Gene Expression in the Yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **2007**, *27*, 3065–3086. [CrossRef] [PubMed]
39. Kaul, S.; Sharma, S.S.; Mehta, I.K. Free radical scavenging potential of L-proline: Evidence from in vitro assays. *Amino Acids* **2008**, *34*, 315–320. [CrossRef]
40. Chen, X.-C.; Bai, J.-X.; Cao, J.-M.; Li, Z.-J.; Xiong, J.; Zhang, L.; Hong, Y.; Ying, H.-J. Medium optimization for the production of cyclic adenosine 3′,5′-monophosphate by *Microbacterium* sp. no. 205 using response surface methodology. *Bioresour. Technol.* **2009**, *100*, 919–924. [CrossRef] [PubMed]
41. Takagi, H. Proline as a stress protectant in yeast: Physiological functions, metabolic regulations, and biotechnological applications. *Appl. Microbiol. Biotechnol.* **2008**, *81*, 211–223. [CrossRef] [PubMed]
42. Posch, A.E.; Spadiut, O.; Herwig, C. Switching industrial production processes from complex to defined media: Method development and case study using the example of *Penicillium chrysogenum*. *Microb. Cell Fact.* **2012**, *11*, 88. [CrossRef]
43. da Silva, G.P.; Mack, M.; Contiero, J. Glycerol: A promising and abundant carbon source for industrial microbiology. *Biotechnol. Adv.* **2009**, *27*, 30–39. [CrossRef]
44. Dunlop, P.; Meyer, G.; Ban, D.; Roon, R. Characterization of two forms of asparaginase in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **1978**, *253*, 1297–1304. Available online: https://www.jbc.org/content/253/4/1297.full.pdf (accessed on 8 August 2022). [CrossRef]
45. Baskar, G.; Renganathan, S. Statistical and evolutionary optimisation of operating conditions for enhanced production of fungal l-asparaginase. *Chem. Pap.* **2011**, *65*, 798–804. [CrossRef]
46. Farag, A.M.; Hassan, S.W.; Beltagy, E.A.; El-Shenawy, M.A. Optimization of production of anti-tumor l-asparaginase by free and immobilized marine *Aspergillus terreus*. *Egypt. J. Aquat. Res.* **2015**, *41*, 295–302. [CrossRef]
47. Saxena, R.K.; Sinha, U. L-Asparaginase and glutaminase activities in the culture filtrates of *Aspergillus nidulans*. *Curr. Sci.* **1981**, *50*, 218–219.

48. Souza, P.M.; de Freitas, M.M.; Cardoso, S.L.; Pessoa, A.; Guerra, E.N.S.; Magalhães, P.O. Optimization and purification of l-asparaginase from fungi: A systematic review. *Crit. Rev. Oncol. Hematol.* **2017**, *120*, 194–202. [CrossRef]
49. Moguel, I.S. Production of L-Asparaginase of Pharmaceutical Nterest from Yeasts Isolated from the *Antarctic continent*. Ph.D. Thesis, University of São Paulo, São Paulo, Brazil, 2018. [CrossRef]
50. Arima, K.; Sakamoto, T.; Araki, C.; Tamura, G. Production of Extracellularl-Asparaginases by Microorganisms. *Agric. Biol. Chem.* **1972**, *36*, 356–361. [CrossRef]
51. Imada, A.; Igarasi, S.; Nakahama, K.; Isono, M. Asparaginase and Glutaminase Activities of Micro-organisms. *J. Gen. Microbiol.* **1973**, *76*, 85–99. [CrossRef]
52. Kil, J.-O.; Kim, G.-N.; Park, I. Extraction of Extracellular L-Asparaginase from *Candida utilis*. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 749–750. [CrossRef]
53. Ramakrishnan, M.S.; Joseph, R. Characterization of an extracellular asparaginase of *Rhodosporidium toruloides* CBS14 exhibiting unique physicochemical properties. *Can. J. Microbiol.* **1996**, *42*, 316–325. [CrossRef]
54. Vidya, J.; Vasudevan, U.M.; Soccol, C.R.; Pandey, A. Cloning, functional expression and characterization of L-asparaginase II from *E. coli* MTCC 739. *Food Technol. Biotechnol.* **2011**, *49*, 286–290.
55. Girão, L.F.D.C.; da Rocha, S.L.G.; Sobral, R.S.; Bom, A.P.D.A.; Sampaio, A.L.F.; da Silva, J.G., Jr.; Ferrara, M.A.; Bon, E.P.D.S.; Perales, J. Saccharomyces cerevisiae asparaginase II, a potential antileukemic drug: Purification and characterization of the enzyme expressed in *Pichia pastoris*. *Protein Expr. Purif.* **2016**, *120*, 118–125. [CrossRef]
56. Bjelic, S.; Brandsdal, B.O.; Åqvist, J. Cold Adaptation of Enzyme Reaction Rates. *Biochemistry* **2008**, *47*, 10049–10057. [CrossRef] [PubMed]