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Antarctic yeasts as a source of L-asparaginase: Characterization of a glutaminase-activity free L-asparaginase from psychrotolerant yeast *Leucosporidium scottii* L115

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ABSTRACT

Microorganisms from extreme environments, such as the Antarctic ecosystems, have a great potential to produce enzymes with novel characteristics. Within this context, L-asparaginase (ASNase) obtained from yeast species has been poorly studied. In this study, yeasts isolated from samples collected at Admiralty Bay (King George Island, Antarctica) were tested to produce ASNase. From an initial screening of 40 strains, belonging to 13 different species, *Leucosporidium scottii* L115 produced an ASNase activity (LsASNase activity: 6.24 U g^{-1} of dry cell weight) with the lowest glutaminase activity. The LsASNase was purified 227-fold, with a specific activity of 137.01 U mg^{-1} at 37 °C, without glutaminase activity. Moreover, the maximum enzyme activity was observed at pH 7.5 and at a temperature of 55 °C. The enzyme is a multimer of 462 kDa, presenting a single band of 53 kDa molecular mass in reduced conditions; after PGNase F treatment, a single band of 45 kDa was observed. The enzymatic kinetic evaluation revealed an allosteric regulation of the enzyme and the kinetic parameters were determined at 37 °C, pH 7.0 as substrate affinity constant, $K_{0.5}$ = 233 μM, k_{cat} = 54.7 s^{-1} and Hill coefficient, n_H = 1.52, demonstrating positive cooperativity by the enzyme and the substrate. This is the first study to report *L. scottii* as a source of glutaminase-activity free L-asparaginase, an acute lymphoblastic leukemia drug feature suitable for the treatment of asparagine synthetase negative cancer cells.

1. Introduction

The L-asparaginase (ASNase) commonly used in the treatment of acute lymphoblastic leukemia (ALL) is a bacterial type II enzyme (amidohydrolase E.C. 3.5.1.1), isolated from *Escherichia coli* (EcASNase II) [1]. Although it was approved as a biopharmaceutical drug 40 years ago, intrinsic problems still need to be overcome, such as hypersensitivity reactions and the inactivation by antibody production against the enzyme in 60 % of patients with ALL [2,3]. The commercial ASNases used in the treatment of ALL also deamidates glutamine to glutamic acid and ammonia [4] . Several other side effects, like anaphylaxis, thrombosis, hepatic dysfunctions, acute pancreatitis, brain dysfunctional syndrome, coagulopathies and glycaemia, also occur in patients treated with this biopharmaceutical, some of which are attributed to the L-glutaminase (GLNase) side activity [1,5]. In addition, due to the higher concentrations of glutamine in human serum, the GLNase activity

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of ASNases can lead to excessive ammonia production, causing a clinical condition known as hyperammonemia, which has toxic effects on the liver and brain [6,7]. In fact, some studies defend that GLNase activity is not required for anti-cancer activity against asparagine synthetase (ASNS) (EC 6.3.5.4) negative cancer cells . In front of this, ASNases with GLNase activity could be necessary only for cytotoxicity against tumor cells that express significant ASNS enzyme [8]. Therefore, for ASNS-negative tumor cells, which are highly sensitive just to ASNase activity, the finding an ASNase with no affinity to L-glutamine and regimens with a more active enzyme therefore needs to be reconsidered, in order to avoid these drawbacks [8,9]. Furthermore, future therapies like site- directed delivery systems, stents, nanoreactors, based on the separated administration of ASNase and GLNase without side activities could generate the desired therapeutic effect [10,11].

The two ASNase enzymes currently used in ALL treatment, produced by *E. coli* and *Dickeya chrysanthemi* (formerly *Erwinia chrysanthemi*), have the same mechanism of action, but exhibit notably different pharmacokinetic profiles and serum half-life [12,13]. In *E. coli* in particular, the high molecular mass of tetrameic enzyme (~140 kDa) contains immunogenic regions in its amino acid sequence which are responsible for its hypoallergenic effects [14]. For these reasons, the search for novel alternative sources of this enzyme, with improved biochemical characteristics and reduced or non-life-threatening adverse reactions, will resolve the current enzyme toxicity-associated problems and consequently improve ALL treatment [3,12,15]. In this context, eukaryotic microorganisms (yeasts and filamentous fungi) have been used as sources of new ASNases that can lead to fewer adverse effects due to post-translational properties (absent in prokaryotic beings) and without GLNase activity [16–18].

The first purification study of yeast ASNase was carried out using *Saccharomyces cerevisiae* [17,19], when two enzyme forms were isolated: ASNase I, an internal constitutive enzyme, and ASNase II, an extracellular enzyme released in response to nitrogen starvation. This pattern is shared by the ASNase production in *E. coli*, where only the type II extracellular enzyme is effective for the treatment of ALL. It is believed that only type II or extracellular enzymes have antineoplastic activity due to their high affinity for the substrate, unlike type I asparaginases which show a higher K_M value for asparagine (low affinity). Even so, amino-acid sequences analysis of the two types of ASNase enzyme from *S. cerevisiae* (the cytosolic and the cell wall glycoprotein) classifies both as bacterial type II enzymes [20]. This agrees with the findings reported by Costa et al. [19] where the ASNase I (cytosolic) from *S. cerevisiae* presents cytotoxic activity in the MOLT-4 leukemic cells, exhibiting the possible antineoplastic properties of yeast ASNases. Even so, little is known about the properties of yeast ASNases as anti-ALL drugs, and it is expected that new ASNases derived from microorganisms that have not been studied for this purpose can be an alternative source of the biopharmaceutical molecule.

The harsh conditions present in Antarctic soils, such as the limited nutrients, extreme pH, low temperature and high salt concentrations, expose microorganisms to constant stress, generating ecological and evolutionary adaptations, which are expressed at physiological, metabolic and structural levels [21,22]. The common strategy used to maintain cell activity at a permanently low temperature is to produce a cold-adapted enzyme with an enhanced catalytic efficiency (i.e., the k_{cat}/K_M ratio). In fact, there is a compilation of available data to indicate that cold-adapted enzymes optimize their catalytic efficiency by increasing k_{cat}, decreasing K_M or by changes in both kinetic parameters [23,24]. In front of this, psychrophile and psychrotolerant microorganisms are potential sources of biocatalysts of great biotechnological interest for the development of new molecular entities, biosimilars and follow-on-biologics.

The main yeast species isolated from Antarctic ecosystems, and other cold environments, belong to the genera *Cryptococcus, Rhodotorula, Leucosporidiella, Sporobolomyces, Leucosporidium, Candida, Mrakia* and *Meyerozyma* [25,21,26]. The vast majority (about 90%) are basidiomycetous yeasts, which are able to produce valuable metabolites, such as enzymes, terpenoids and carotenoids, among other biomolecules. However, they have been poorly explored for biopharmaceutical applications, including the presence of novel GLNase-activity free yeast ASNases with clinical potential [27,28]. Therefore, this study aimed to evaluate the production of ASNase by psychrotolerant yeasts isolated from Antarctic samples and to report the characterization of a new GLNase-activity free ASNase produced by *Leucosporidium scottii* L115, prospecting its application as a potential biosimilar or biobetter ASNase. Therefore, our results highlight the potential of extreme environments to provide a rich source of microorganisms capable to produce distinct biomolecules such as enzymes with high added value.

2. Materials and methods

2.1. Microorganisms

The yeasts used in the present study (40 strains) were recovered from terrestrial and marine samples collected at Admiralty Bay (King George Island, Antarctica) during a Brazilian expedition to the Antarctic Peninsula in the austral summer of 2009/2010 (Table 1). The strains were isolated and taxonomically identified as described by Duarte et al. [25]. All yeast strains are deposited at the Central of Microbial Resources (CRM-UNESP) of the São Paulo State University (UNESP, Brazil), where they are being maintained by cryopreservation at − 80 °C.

2.2. Inoculum preparation, culture and enzyme production

The yeast strains were grown in 100 mL Erlenmeyer flasks containing 50 mL of potato dextrose broth at 15 °C for 72 h in a rotatory shaker at

Table 1
Yeast strains isolated from marine and terrestrial samples (collected at Admiralty Bay, King George Island, Antarctica) assayed for asparaginase and glutaminase activity.

Specie	Strain original code (CRM-UNESP code)	Antarctic sample
Phylum Ascomycota		
Candida davisiana	L114 (CRM 673)	Stone with lichen
Candida glaebosa	L79 (CRM 677), L83 (CRM 678), L84 (CRM 679)	Ornithogenic (penguin) soil
Candida sake	L58 (CRM 681)	Ascidian (squirt)
Debaryomyces hansenii	L3 (CRM 653), L6 (CRM 682)	Marine sediment
Debaryomyces macquariensis	L81 (CRM 685), L82 (CRM 686)	Ornithogenic (penguin) soil
Meyerozyma guilliermondii	L4 (CRM 689), L5 (CRM 690), L9 (CRM 691), L10 (CRM 692), L11 (CRM 693), L13, (CRM 694) L17 (CRM 696), L18 (CRM 697), L19 (CRM 698), L20 (CRM 699), L21 (CRM 700)	Isopod
Wickerhamomyces anomalus	L36 (CRM 657), L37a (CRM 701), L55 (CRM 702)	Ascidian (squirt)
Phylum Basidiomycota		
Naganishia albidosimilis[a]	L94 (CRM 660)	Marine star
Papiliotrema laurentii[b]	L35 (CRM 661)	Sea snail (*Nacella concina*)
Vishniacozyma victoriae[c]	L32 (CRM 715), L42 (CRM 662), L43 (CRM 712), L92 (CRM 713), L97 (CRM 716), L122 (CRM 717), L123 (CRM 718), L124 (CRM 714)	Tunicate (*Salpa* sp.)
Tausonia pullulans[d]	L88 (CRM 725)	Lichen
Leucosporidium scottii	L115 (CRM 667), L117 (CRM 728), L118 (CRM 729)	Marine sediment
Rhodotorula mucilaginosa	L7 (CRM 669), L26 (CRM 737), L38 (CRM 740)	Seaweed (algae)

Synonym (formerly known as): [a]*Cryptococcus albidosimilis*; [b]*Cryptococcus laurentii*; [c]*Cryptococcus victoriae*, [d]*Guehomyces pullulans*

150 rpm. Afterwards, the cells were harvested by centrifugation at 3400xg for 15 min and washed with sterile water. For ASNase production, 100 mL flasks containing 50 mL of modified Czapeck Dox's medium were inoculated with the cells at 1 g L^{-1} final concentration, as reported by Gulati et al. [29]. Samples of the culture were collected after 24, 48, 72 and 96 h, the cells and medium were separated by centrifugation, and both were assayed for ASNase and GLNase activities.

2.3. ASNase and GLNase by hydroxylaminolysis reaction assay

The ASNase and GLNase activity were determined by the hydroxylaminolysis reaction, using the whole-cell and culture broth for the enzymatic reaction [30]. This assay is indicated for the screening of non-purified enzymes instead of the Nesslerisation method due to the interference of media components, such as ammonium, in the latter method [1]. In summary, a microtube containing 1.6 mL of cell suspension (optical density = 1.0) 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 hydroxylamine hydrochloride pH 7.0 was agitated on a thermomixer at 850 rpm at 37 °C; after 30 min, the enzymatic reaction was stopped by adding 0.50 mL of trichloroacetic acid (TCA)/$FeCl_3$ reagent (100 g L^{-1} $FeCl_3$, 50 g L^{-1} TCA in 0.66 M HCl). The tube was centrifuged at 3400*xg* for 5 min, and 1.0 mL of the supernatant was measured at 500 nm using a spectrophotometer model Spectramax® plus 384 (Molecular Devices, Sunnyvale, CA, USA). To confirm the extracellular production of the enzyme, the culture broth was used instead of the cell suspension. Controls were prepared in the same way as the samples, but the hydroxylamine and L-asparagine solutions were added after the ferric chloride reagent. The GLNase activity was assayed using the same procedure but using 0.1 M L-glutamine instead of L-asparagine solution. One unit of ASNase and GLNase was defined as the amount of enzyme that produces 1 μmol of β-aspartohydroxamic acid or 1 μmol of γ-glutamohydroxamic acid, respectively, formed per minute by gram of dried cell weight (U g^{-1} DCW) or milliliter of culture broth (U mL^{-1}). The periplasmic activity (P_{ASNase} or P_{GLNase}) was calculated according to Eq. 1 [14]:

$$P_{ASNase} or P_{GLNase}(U/g) = \frac{\mu mol\beta - HA}{30 * DCW} \quad (1)$$

where β-HA is the β-aspartohydroxamate formed by the ASNase or GLNase reaction, the value 30 value corresponds to 30 min of incubation, and DCW is the dry cell weight (in g).

2.4. ASNase and GLNase by hydrolysis reaction assay

The purified enzyme was assayed by quantifying the ammonia released using Nessler's reagent [30]. In summary, a microtube containing a 50 μL sample incubated in 0.5 mL of 50 mM Tris-HCl buffer pH 8.0, 50 μL of 189 mM L-asparagine, and 0.45 mL ultrapure water was agitated on a thermomixer at 850 rpm at 37 °C. The reaction was interrupted after 30 min by the addition of 50 μL of 1.5 M TCA; the solutions were mixed by inversion and centrifuged at 3400×g for 5 min. The release of ammonia was determined by adding 0.25 mL Nessler's reagent to 0.1 mL of the sample diluted in a final volume of 2.5 mL ultrapure water. The control was prepared in the same way as the sample, but the L-asparagine solution was added after the TCA solution. The GLNase activity was assayed using the same procedure, using 189 mM L-glutamine solution instead of the L-asparagine solution. The color that developed was quantified at 436 nm. One unit of ASNase or GLNase activity was defined as the amount of enzyme that produced 1 μmol of ammonia per minute of reaction.

2.5. Thin-layer chromatography (TLC) of reaction products from *Leucosporidium scottii* cells

The reaction of ASNase was performed by the addition of 0.2 mL of 0.1 M L-asparagine, 0.2 mL of 1.0 M hydroxylamine pH 7.0, and 1.6 mL of cells suspension at 1.0 of optical density in 50 mM Tris-HCl buffer pH 7.0. The mixture was agitated on a thermomixer at 850 rpm at 37 °C. After 30 min, the enzymatic reaction was stopped by centrifugation at 3400×g for 5 min, and 1.0 mL of supernatant was stored at 5 °C. The blank reaction was prepared in the same way as the sample, but the yeast cells were inactivated at 80 °C for 15 min. The reaction and controls were run on a silica gel plate according to the method reported by Ramakrishnan and Joseph [31], using phenol/water (4:1) (w/v) as the mobile phase. The plate was revealed by spraying with ninhydrin 0.5 % (w/v) dissolved in acetone and heated using a hairdryer until the points appeared.

2.6. Enzyme extraction and purification

Cultures of *L. scottii* L115 were centrifuged at 3400×g, and the cells were harvested, washed twice with sterile water, and resuspended at 50 % (v/v) in the buffer for lysis (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 10 mM cysteine, 1 mM PMSF - Phenylmethylsulfonyl fluoride). The cell suspension was passed through a French press homogenizer at 1500 psi for 10 cycles, maintaining the suspension in an ice bath between each cycle, to avoid overheating. The homogenized suspension was centrifuged at 18,500×g for 20 min, and the supernatant was collected.

2.6.1. Extract clarification

A polyethylene glycol (PEG 4000) solution at 66% w/v was added to the supernatant obtained previously, to give a final concentration of 6.6 % w/v PEG. The mixture was left in an ice bath for 30 min and later centrifuged at 18500×g for 15 min. The supernatant was filtered through a 0.45 μm membrane, and the solution was stored at 4 °C and used for subsequent analysis.

2.6.2. Ion exchange chromatography

The clarified enzyme extract was passed through a DEAE-HiTrap® GE 5 mL column (DEAE-sepharose) (Cytiva, São Paulo, Brazil) using a FPLC purifier system model AKTA® pure (GE Healthcare Life Science, Marlborough, USA). The column was previously equilibrated with 20 mM Tris-HCl (pH 8.0) buffer, and the protein was eluted with a step gradient of NaCl (50, 100, 150, 200, 250, and 500 mM) in 20 mM Tris-HCl pH 8.0 buffer with 100 mM glycerol; fractions of 1 mL were collected. Protein quantification was measured using BCA reagent, and the ASNase activity was assayed. The fractions with greater activity were pooled and concentrated using an ultrafiltration membrane cartridge with a 10 kDa molecular weight cut-off.

2.6.3. Size exclusion chromatography (SEC)

The concentrated protein fraction from the previous step was passed through a Superdex® 200 Increase 10/300 GL column (cross-linked agarose-dextran resin) (Cytiva, São Paulo, Brazil) using a FPLC purifier system model AKTA® pure (GE Healthcare Life Science, Marlborough, USA). The column was equilibrated with 20 mM Tris-HCl (pH 8.0) buffer and 100 mM glycerol and eluted at a flow rate of 0.50 mL min^{-1}; fractions of 0.5 mL were collected and assayed for ASNase activity. SDS-PAGE was performed to check the protein purity in each fraction that was collected with ASNase activity.

2.7. Molecular weight determination by size exclusion chromatography

The molecular weight of the enzyme was determined using the elution volume (V_e) presented in the Superdex® 200 Increase 10/300 GL column (Cytiva, São Paulo, Brazil) for the fraction that presents the enzyme activity. The column was calibrated by the protein molecular weight size-markers thyroglobulin 669 kDa, ferritin 440 kDa, aldolase 232 kDa, ribonuclease 137 kDa, conalbumin 75 kDa, and ovalbumin 45 kDa; the void volume (V_0) was determined using blue dextran. The standard proteins were run at the same assay conditions as the enzyme

fraction, 20 mM Tris-HCl pH 8.0 buffer with 100 mM glycerol, at a flow rate of 0.75 mL min^{-1}. The molecular mass of the enzyme was determined by the linear relationship obtained by plotting the K_{av} value of the proteins vs the logarithms of the molecular weight (MW) [32], as presented in Fig. 3.

2.8. Determination of molecular weight in reduced conditions and purity of the fractions obtained

The samples obtained from the different steps of purification were analysed by the SDS-PAGE technique in a 12 % polyacrylamide gel under reducing conditions, in accordance with the method reported by Laemmli [33]. Gels were stained with Coomassie blue R-250 and subsequently with silver staining. The approximate enzyme molecular weight was estimated by determining the relative migration distance (R_f) of the Precision Plus Protein™ Standards molecular weight protein marker (Bio RAD, Hercules, CA, USA) and interpolating the value from the linear relation of Log_{Mw} vs R_f. The purity of the enzyme was confirmed by the presence of a single band in the fraction analysed.

2.9. Enzyme glycosylation analysis by SDS-PAGE

First of all, 1 μL of SDS 5 % (w/v) and 1 μL of 1 M DDT were added to 10 μL purified enzyme (0.5 mg mL^{-1}), and the sample was then heated at 95 °C for 5 min and cooled for 5 min at room temperature. The deglycosylation reaction was performed by adding 2 μL of 0.5 M sodium phosphate buffer pH 7.5, 2 μL of Triton X-100 10 % (w/v), and finally 2 μL of peptide N-glycosidase F (PNGase F, Promega™), and the reaction was incubated at 37 °C for 3 h. The sample was treated and visualized by SDS-PAGE.

2.10. Protein quantification

The protein concentration in the samples was determined using the Non-Interfering Protein Assay™ Calbiochem® kit (Sigma-Aldrich, St. Louis, MO, USA), based on the specific interaction of copper ions with the peptide backbone of the protein, allowing protein-to protein variations to be to overcome.

2.11. Determination of kinetic enzymatic parameters

The kinetic behavior of the enzyme was followed by the oxidation of β-NADH in the presence of glutamate dehydrogenase (GDH) and ammonia, a product of L-asparagine hydrolysis by the action of ASNase. The depletion of β-NADH was monitored spectrophotometrically at 340 nm, as the change in absorbance over time is proportional to the ASNase reaction rate [34]. The reaction quantities were modified to use a microtiter plate. In each well, the enzyme reaction was prepared by adding 20 μL of the purified *Leucosporidium scottii* ASNase (LsASNase, ≈ 2.7×10^{-8} M), 87.5 μL of 200 mM Tris-HCl buffer pH 7.5, 53.75 μL distilled water, 3.5 μL of 110 μM α-ketoglutarate (dissolved in 100 mM Tris-HCl buffer pH 8.0), 8.75 μL of 128 μM β-NADH, and 17.5 μL of 0.148 μM GDH (dissolved in phosphate buffer pH 7.5 with glycerol at 50 % (v/v)) and 159 μL of the corresponding L-asparagine solution to get the concentration needed (2.0, 1.0, 0.75, 0.5, 0.25, 0.1, 0.075, 0.05, 0.025, 0.0125, and 0 mM). The molar extinction coefficient of β-NADH was experimentally determined as 6100 mol^{-1} cm^{-1}. The substrate affinity and turnover number were estimated using non-linear regression fitting in the GraphPad Prism software version 5.0 (La Jolla, California, USA).

2.12. Effect of pH and temperature

The following buffers were used to determine the optimum pH for LsASNase, at 50 mM concentration: acetate (pH 4.0–5.5), phosphate (pH 6.0–7.0), Tris-HCl buffer (pH 7.5–9.0), glycine-sodium hydroxide (pH 9.5–10.5), and sodium bicarbonate (11.0). The enzyme was incubated in the corresponding buffer for 24 h at 4 ºC. To determine the optimum temperature, the enzymatic reaction was performed at different temperatures, from 5° to 65 °C for 30 min.

2.13. Effect of metal ions on enzyme activity

The effect of different metal ions, including K^+ (50 mM), Mg^{2+} (50 mM), Na^+ (50 mM, 1 M), Ca^{2+} (10 mM), Ni^{2+} (10 mM), Cu^{+2} (10 mM), Zn^{2+} (10 mM), Mn^{2+} (10 mM), and EDTA (1 mM), on the partially purified ASNase was studied. The enzyme was incubated for 30 min at 37 °C in phosphate buffer with the corresponding metal ion and 20 mM L-asparagine added; the residual activity was assayed and compared with the control, which was considered to have 100 % activity.

2.14. Circular dichroism for secondary structure comparison of LsASNase and commercial E. coli ASNase II

Circular dichroism (CD) spectra of the samples were obtained in a Jasco J-815 Spectropolarimeter (Jasco, Tokyo, Japan). The final spectra were the average of 6 scans, following subtraction of the spectrum of the 20 mM Tris-HCl pH 8.0 buffer obtained under the same conditions. CD spectra were obtained in the far-UV range (190–260 nm). Samples were placed in 5.00 mm optical length quartz cells with a concentration of 0.28 μM LsASNase I and 1.92 μM EcASNase II (control – commercially obtained from ProSpec Tany (Ness-Ziona, Central District, ISR)). The intensities of the spectra (θ, mdeg) were converted to residual molar ellipticity ([θ], deg.cm^2.$dmol^{-1}$) using Eq. 2.

$$[\theta] = \frac{\theta}{10cln} \tag{2}$$

where c is the protein concentration in mol L^{-1}, $\boldsymbol{l}$ is the optical length in cm, and n is the estimated number of residues in the protein.

2.15. Circular dichroism for the thermal stability of LsASNase

Thermal stability studies of the enzyme samples were performed in a Jasco J-815 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Peltier-type temperature controller (working range 10–90 °C). The temperature was scanned from 30 °C to 95 °C and back to 30 °C, at a rate of 1 °C/minute, to study the unfolding and refolding processes, respectively. Samples with a concentration of 0.28 μM LsASNase I or 1.92 μM EcASNase II (control) were placed in a 5.00 mm optical length quartz cells, and the intensities of the ellipticity at 222 nm (θ_{222}, mdeg) were registered throughout the experiment. The intensities of the ellipticity at 222 nm were converted to residual molar ellipticity ($[\theta]_{222}$, deg.cm^2.$dmol^{-1}$) using Eq. 3.

$$[\theta]_{222} = \frac{\theta_{222}}{10cln} \tag{3}$$

where c is the protein concentration in mol L^{-1}, l is the optical length in cm, and n is the estimated number of residues in the protein.

2.16. Effect of temperature on the hydrolysis reaction

The temperature dependence of the catalytic activity of purified LsASNase was investigated based on the production of ammonium by the L-asparagine hydrolysis [30]. The assays were performed in duplicate independently, with the temperature increasing from 20 °C to 45 °C. The activity data were analysed by plotting Ln Vmax/Z against 1/T and fitting to the Arrhenius equation (Eq. 4) [35]:

$$LnV_{max} = LnZ - \frac{Ea}{RT} \tag{4}$$

$$K = \frac{V_{max}}{Z} \quad (5)$$

$$K(s^{-1}) = V_{máx}/mgEnzyme * MW(mg/mmol) * 10^{-3} mmol/\mu mol * 1\text{min}/60sec \quad (6)$$

where *Ea* is the energy of activation, *R* is the gas constant, *Z* is the pre-exponential factor, and *MW* is molecular mass of the enzyme.

3. Results and discussion

3.1. ASNase and GLNase activity screening

Results from the screening step showed that ASNase and GLNase extracellular activities were not detected in all of the culture broths of the 40 Antarctic-derived yeast strains studied. Most of the microbial ASNases in nature are intracellular [36], and the secretion of proteolytic enzymes by yeasts is not a common property [37,38]. For example, *Candida utilis* and *Rhodosporidium toruloides* seem to produce a periplasmatic ASNase [27,39,31].

When intact cells, present in the suspensions, were used to evaluate the ASNase activity, 16 strains showed positive results (the formation of the corresponding hydroxamic acid from one or both substrates used), 15 of which were from the phylum Basidiomycota (Table 1). The ASNase and GLNase activities are shown in Fig. 1. The yeast *R. mucilaginosa* strain L38 showed the highest ASNase activity, with 13.72 U g^{-1} DCW, but this yeast also showed a higher GLNase activity (30.50 U g^{-1} DCW). However, the highest GLNase activity, 33.19 U g^{-1} DCW, was produced by *R. mucilaginosa* strain L26, although the ASNase activity of this strain was low (2.63 U g^{-1} DCW). The following species of Antarctic yeasts showed ASNase activities: *Vishniacozyma victoriae* (formerly *Cryptococcus victoriae*) (L42, L43, L94, L97, L122, L123, L124), *Rhodotorula mucilaginosa* (L7, L26, L38), *Leucosporidium scottii* (L115, L118), *Naganishia albidosimilis* (formerly *Cryptococcus albidosimilis*) (L92), *Candida glaebosa* (L79) and *Tausonia pullulans* (formerly *Guehomyces pullulans*) (L88). The results obtained from our screening coincide with those reported in other studies, wherein the positive strains were from the genus *Hansenula, Cryptococcus,* and *Rhodotorula,* with ASNase activity, GLNase activity, or both activities [30,40]. Previous studies also reported that GLNase activity was observed without ASNase in a strain of the yeast *L. scottii* (formerly *Candida scottii*) [30].

Considering that *L. scottii* L115 showed 6.24 U g^{-1} DCW of ASNase activity with a very low GLNase activity (0.41 U g^{-1} DCW), this strain was selected for the production, purification, and characterization of the ASNase enzyme. Species of Leucosporidiales have been isolated predominantly from cold environments and are identified as psychrotolerant yeasts (high growth rate between 4 °C and 22 °C), with some species reported as potential sources of extracellular cold-adapted enzymes and antifreeze proteins [26,38].

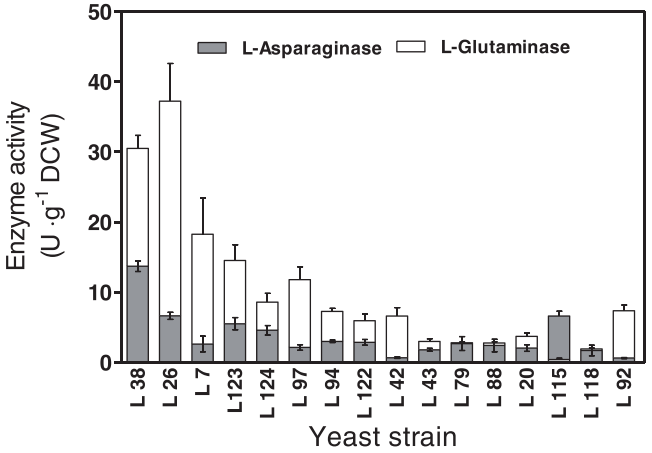

Fig. 1. Enzyme activity of asparaginase and glutaminase (U g^{-1} DCW) of yeast strains isolated from marine-sediment samples collected in the Antarctic Peninsula. *Rhodotorula mucilaginosa* (L38, L26, L7), *Cryptococcus victoriae* (L123, L124, L97, L94, L122, L42, L43), *Candida glaebosa* (L79), *Guehomyces pullulans* (L88), *Meyerozyma guillermondii* (L20), *Leucosporidium scottii*, (L115, L118), and *Cryptococcus albidosimilis* (L92).

The whole cell enzyme activity determination by hydroxylaminolysis was the most convenient method for enzyme screening expression. GLNase activity was not observed after the purification process of *L. scottii* L115 ASNase, this indicated that the activity was not intrinsic to the purified enzyme but perhaps to another enzyme produced by the strain. It is important to reinforce that all commercial ASNases used in the treatment of ALL exhibit GLNase side activity (hydrolyzing L-glutamine up to 9 % of the total hydrolysis activity), which can be responsible for innumerable sides effects, mainly hyperammonemia and neurotoxicity [7,10]. Therefore, any approach to obtain ASNases with lower GLNase activity should be encouraged. A previous study reported the production of ASNase free of GLNase by other species of the genus *Leucosporidium* (*L. muscorum* CRM 1648), isolated from Antarctic marine sediment [27]. Herein, the yeast *L. scottii* strain L115, isolated from Antarctic marine sediment, produced a ASNase showing GLNase-activity free that could be applied particularly against asparagine synthetase negative cancer cells. These findings highlight the potential of *Leucosporidium* yeasts from marine Antarctic samples as sources of GLNase-activity free ASNase.

Some studies showed the toxicity potential of some ASNases showing GLNase activity. ASNase from *Acinetobacter glutaminasificans* showed a low therapeutic index due to its high high-GLNase that yielded considerable toxicity during clinical evaluation and the ASNase from *Wolinella succinogenes* also was evaluated clinically and showed toxicity to the patients since it was found to possess GLNase activity too [10]. Again, this clinical evaluation results highlight the importance to search new ASNase without GLNase activity.

Representatives of some marine yeasts, such as *Cryptococcus, Leucosporidium, Rhodosporidium, Rhodotorula, Sporidiobolus,* and *Sporobolomyces*, can produce iron chelators, such as rhodoturolic acid and hydroxamic siderophores, under certain metabolic conditions [41,42]. To confirm enzyme production and rule out a false positive by an iron chelator molecule produced by *L. scottii* strain L115, the products formed during the enzyme reaction were evaluated qualitatively by TLC. In Fig. 2B, the first and second track correspond to the L-asparagine (ASN, 20 mM) and β-aspartohydroxamic acid (β-AHA, 20 mM) standards, respectively. Only the presence of L-asparagine was detected in the blank reaction (BR) track, while a depletion of L-asparagine was observed in the reaction products (RP) track after 30 min of reaction, along with the presence of β-AHA, formed from the reaction of L-asparagine with hydroxylamine by the action of the ASNase enzyme, proving that the ASNase enzyme was produced by the *L. scottii* L115 yeast cells.

This screening implied that the amidohydrolase activity was more common in the basidiomycetous yeasts isolated from the Antarctic samples. Some yeast ASNases present an affinity for both substrates and are denominated as GLNase-ASNases (EC. 3.5.1.38) [20,43]. The clinical application of GLNase-ASNase enzymes has been poorly studied, but earlier studies performed *In vitro* [44] and *In vivo* with humans [45,46] showed anti-leukemia effects. Recently, new therapeutic proposals were addressed for this kind of enzyme [9,47]. From these results, we can say that psychrotolerant basidiomycetous yeasts may be an interesting source for the production and study of new ASNases.

3.2. Enzyme purification

The enzyme purified and concentrated is required for study of thermodynamic and kinetic properties, structural conformation, physiological activity and stability . The purification of ASNase from *L. scottii*

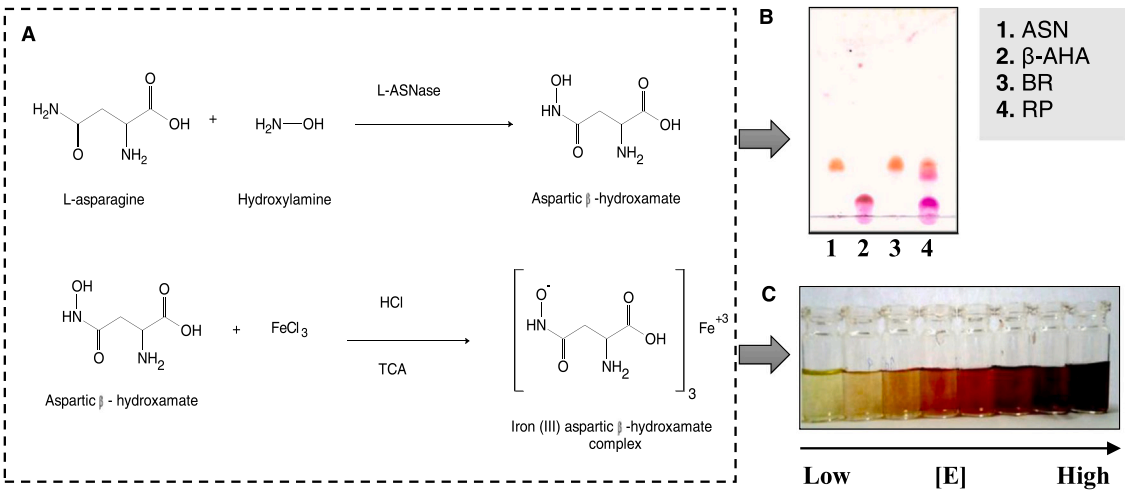

Fig. 2. A Hydroxyl aminolysis reaction steps of asparaginase in cell suspension of *Leucosporidium scottii* L115; B Molecule identification involved in hydroxyl aminolysis by TLC. ASN: asparagine 0.1 M, β-AHA: aspartic β-hydroxamate acid, BR: blank reaction, RP: asparaginase reaction products; C Final mixed coloration of reaction at different enzyme concentrations.

L115 was carried out using a combination of steps, starting from the cellular extract (obtained by mechanical disruption in a high-pressure homogenizer) and followed by extract clarification, anionic exchange chromatography, and size exclusion chromatography (SEC). Table 2 shows the results after each purification step. Clarification with PEG 4000 at 6.6 % (w/v) removed some unwanted macromolecules and lipids, clarifying the homogenized extract prior to further processing using chromatography methods. The PEG can be easily removed during the subsequent ion-exchange chromatography step since it does not interact with the column resin, due to its neutral charge [14,48,49]. After clarification, ASNase showed a total activity of 7.23 U.mL^{-1} and a specific activity of 0.60 U mg^{-1}. The clarified extract was subjected to anionic exchange chromatography by passing through a DEAE column and eluting using a step gradient at 10, 50, 100, 250, and 500 mM NaCl. All fractions were eluted with 50 mM NaCl, showed ASNase activity and were collected and concentrated. The ASNase activity was estimated to be 11.02 U.mL^{-1} and the specific activity was 14.65 U mg^{-1}. For further purification, concentrated fractions were loaded into a pre-equilibrated Superdex® 200 Increase 10/300 GL column, and all fractions presenting ASNase activity were collected and concentrated to a final volume of 9.9 mL. GLNase-activity free ASNase was purified to homogeneity, with a total activity of 6.85 U mL^{-1}, a specific activity of 137.01 U mg^{-1}, and 5.26 % of yield (Table 2).

3.3. Determination of molecular mass and glycosylation analysis by SDS-PAGE

The analysis of purified ASNase exhibited a protein band of around 50 kDa, corresponding with a monomeric structure. SEC analysis allowed estimate a molecular weight of 440 kDa for LsASNase, indicating that the enzyme has a multimeric structure (Fig. 3). This result agrees with other multimeric ASNases, especially with the intracellular ASNase I reported from *S. cerevisiae*, which is reported as a decameric enzyme, with a molecular weight of 400 kDa determined by SEC, and a molecular weight of 41.4 kDa using SDS-PAGE [19,50].

The best-characterized yeast ASNase, the ScASNase type II, is an external enzyme mannan-glycoprotein located in the cell wall. Glycosylation is a post-translational modification that is found on about 50 % of all secreted proteins and transmembrane proteins. This process shows a special role in protein stability since it can prevent some destabilizing events such as aggregation, thermal denaturation, and proteolytic degradation [51]. In front of this, several important commercial biopharmaceuticals have effectively been stabilized by this post-translational modification [52]. To check if LsASNase is a glycosylated enzyme form, it was treated with the PNGase F enzyme, which removes the N-glycosylation moieties, reducing their apparent molecular weight. The results indicate that treatment with PNGase F influenced the mobility of the treated enzyme, presenting a new band with 45 kDa (Fig. 3A). This result showed that LsASNase is a glycosylated enzyme, which suggests that it is an external ASNase, located in the cell wall. Glycosylation is one of the most important post-translation modifications, and the presence of this process in ASNase could improve the pharmacokinetic and pharmacodynamic properties of the drug. N-glycosylation of recombinant human erythropoietin and recombinant human alpha interferon enhanced the *In vivo* activity and prolonged the half-life of these bioproducts [53,54]. For example, a new ASNase from *E. coli* with human-like glycosylation was created by *Pichia pastoris* expression and site-directed mutagenesis, which showed an increase in biological activity and stability, and decreased immunogenic epitopes [55,56].

3.4. Effect of pH, temperature and metal ions on LsASNase

The pH activity of LsASNase enzyme profile is presented in Fig. 4A.

Table 2
Summary of purification steps of ASNase from *Leucosporidium scottii* L115 (LsASNase).

Step	Activity[a] (U mL^{-1})	Protein (mg mL^{-1})	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification fold	Recovery (%)
Clarification PEG 4000	7.23	12.01	1082.8	650.8	0.60	1.0	100
Anion exchange chromatography DEAE	11.02	0.75	11.3	165.3	14.65	24.38	25.40
Size Exclusion chromatography (SEC)	6.85	0.05	0.25	34.2	137.0	227.95	5.26

[a] One unit of asparaginase (U) is defined as the amount of enzyme that liberates 1 μmol of ammonia by min^{-1} at 37 °C.

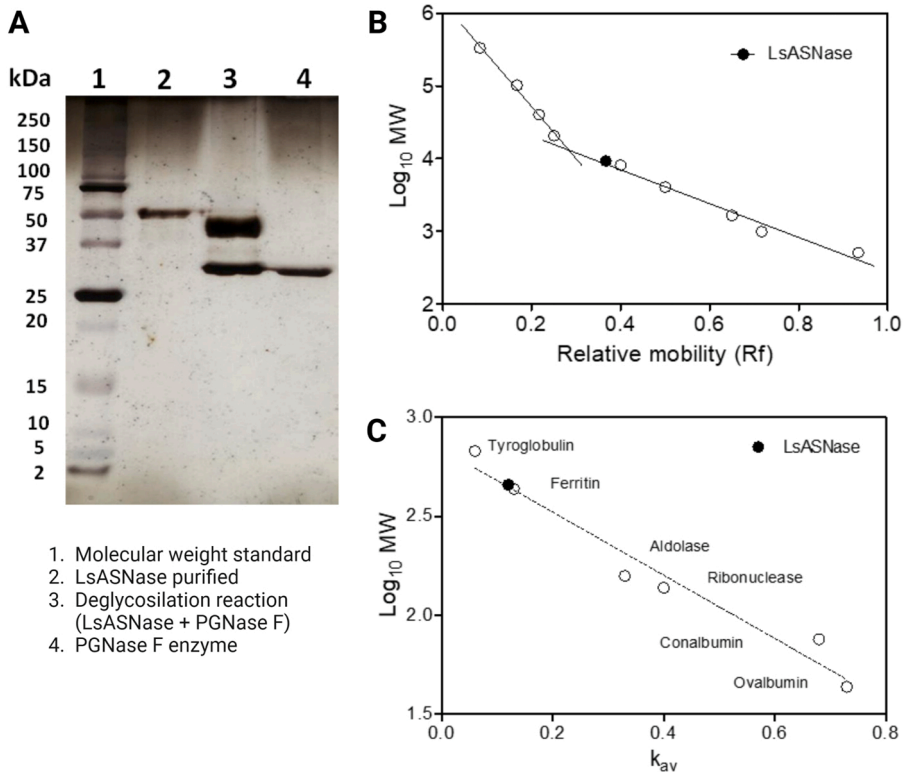

Fig. 3. Molecular weight analysis of purified enzyme asparaginase from *Leucosporidium scottii* L115 A Silver staining SDS PAGE on 12.5% gel; B Relative mobility (R_f) with standard protein comparison under reducing conditions; C Calibration curve for a Superdex® 200 Increase GL column, *Leucosporidium scottii* asparaginase I (K_{av} = 0.1481).

The results indicate that low (4.0–6.0) and high (9.5–11.0) pH values induce a conformationally unstable native state. However, our results were good at pH 6.5–9.0, with more than 80% total activity, including an enzyme that showed 100 % activity at pH 7.5. Considering that circulatory fluids in the human body have a pH value of 7.4, this pH profile is a therapeutically remarkable feature of the enzyme for potential use in ALL treatment [57].

Reviewing the optimal temperature, it was expected that the maximum enzyme activity would be at the optimal physiological temperature of microorganism growth (15 °C). Instead, the optimum temperature of LsASNase was 55 °C, followed by an abrupt decrease at temperatures > 60 °C (Fig. 4B). According to previous reports, *Leucosporidium* species will not grow at temperatures above 25 °C [58], although a steady increase in enzymatic activity was observed over the temperature range from 25 °C to 55 °C. It has been reported that cold-adapted enzymes generally have their optimum temperature above their physiological temperature, suggesting that evolutionary pressure acted to allow the enzyme to be active at low temperatures but not to optimize its structure to present a maximal activity at low temperatures. Different studies indicate that these changes in the enzyme structure are not related to the catalytic site, but to other regions of the protein that increase the flexibility of the structure [59].

Besides the temperature and pH, other factors such as ionic metal presence can also affect the enzyme activity. Some monovalent and divalent cations are essential cofactors for many catalytic reactions, and their presence may positively impact these reactions. However, as shown in Fig. 4C, our results had a non-positive effect on ASNase activity, while Mn^{2+}, Ca^{2+}, Mg^{2+} and K^{+} did not change the activity, Co^{2+}, Fe^{3+} and Na^{+} showed a slightly negative impact, and Zn^{2+}, Ni^{2+} and Cu^{2+} were detrimental to enzymatic activity (< 40 % total activity). The inhibition of activity caused by Zn^{2+} is indicative of the presence of essential vicinal sulphydryl group(s) [60]. The null positive effect of ionic metal obtained for ASNase indicates that this enzyme does not correspond to a metalloprotein group [37].

3.5. Secondary structure and thermal stability assays

As LsASNase production has been previously reported in the literature [28], it is relevant to provide further information about this novel enzyme. Thermal stability assays were performed to establish the denaturation temperature of the protein and to understand the refolding process. Fig. 5B and C present the CD spectra of EcASNase II and LsASNase, respectively. The melting temperature (T_m) obtained for EcASNase II (~60 °C) was higher than for LsASNase (~55 °C). The T_m of LsASNase is in agreement with the kinetic thermal evaluation where the enzyme presents the higher activity at 55 °C. Even so, both enzymes failed to refold after the thermal denaturation. The quaternary structure of all known type II ASNases is reported as a tetramer, stabilized by a tight dimer-dimer arrangement [17], the irreversible loss of structure is the result of the deprivation of this interaction that cannot be recovered by reducing the temperature. The LsASNase shares this behavior in which the protomer interaction is essential for ASNase activity, and follows an all-or-none unfolding process typical of cold-adapted enzymes. This unfolding process consists of stabilization of the oligomeric structure by weaker interactions within the subunits, being observed that the disruption of fewer interactions strongly influences the whole structure, triggering the unfolding of all molecules (i.e., for that the refolding process is not possible [61]); for this reason, a sharp loss in enzyme activity can be observed after the melting point is reached. Instead of the thermophilic enzymes, where higher oligomerization improves its thermostability by strengthening the subunits bonds [62].

CD spectra allow elucidation of the secondary protein structure and distinguish four classes: all-α, all-β, α + β, and α/β [63,64]. We performed a comparative analysis of the LsASNase and EcASNase II (control) structures: the analysis of the secondary structure is shown in Fig. 5A. The CD spectra exhibit two negative bands (208 and 222 nm)

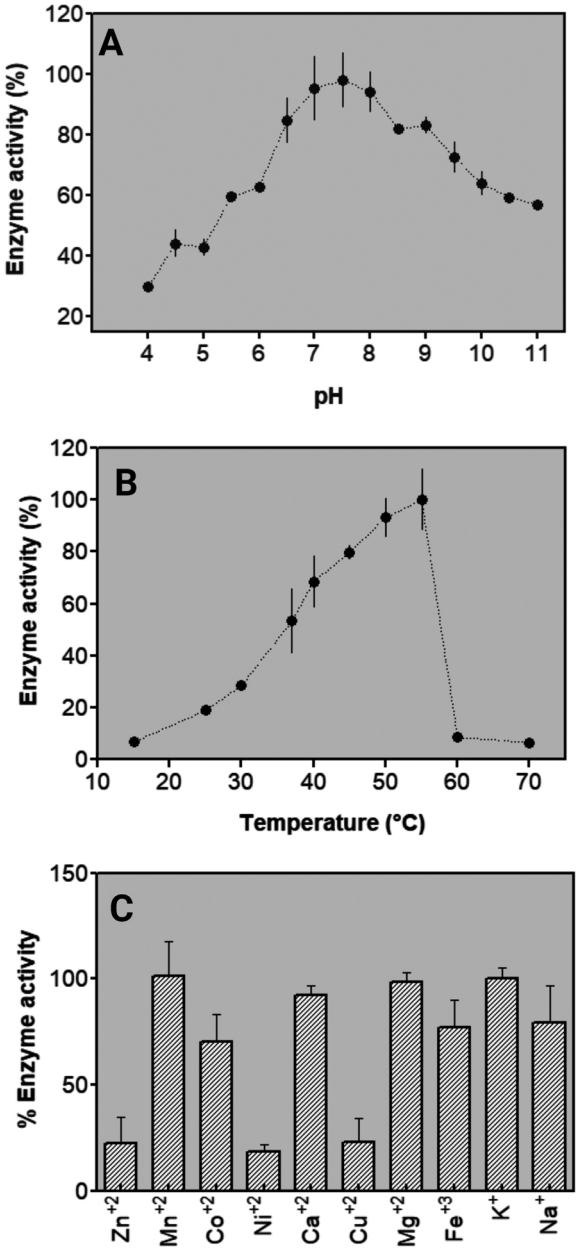

Fig. 4. Enzymatic activity (%) evaluation of purified *Leucosporidium scottii* asparaginase I under different physical conditions. A pH influence carried out in the following buffers: acetate (pH 4.0–5.5), phosphate (pH 6.0–7.0), Tris-HCl (pH 7.5–9.0), glycine-sodium hydroxide (pH 9.5–10.5) and sodium bicarbonate (pH 11.0); B Temperature influence; C Presence of metal ions. The error bars represent the SD of three experiments.

and a positive band in the 190–195 nm region, with the 208 nm band being the more prominent. In some cases, a minimum shallow is observed around 220 nm [63]. The spectral profiles of the two enzymes were similar and allow the classification of their secondary structure as an α + β protein.

Previous studies have reported that native ASNase II from *E. coli* presents an α-helix content of 31 % [65]. The α-helix structure contributed to the enzyme thermostability [66]. Our results suggest a minor content of α-helix in LsASNase compared to EcASNase II, according to its reduced thermostability. Even so, the similarity of CD spectra from EcASNase II and LsASNase enzymes confirm the preserved ASNase secondary structure as a tetrameric and multimeric enzyme, and the differences in oligomerization do not affect this structure, suggesting that the LsASNase evolved through an oligomerization process and not from radical structural changes.

3.6. Kinetic parameters determination

Initially, the concentration of purified LsASNase was evaluated, and a linear increase in activity was observed up to 1.5 μg of enzyme at a defined L-asparagine concentration of 0.1 M (Fig. 6A). Our results showed that the kinetics of LsASNase, with respect to the substrate concentration, was typically hyperbolic, with a high affinity for the substrate. The maximum specific activity (V_{max}) for the enzyme was 0.376 UI/mg, while the apparent value of the affinity constant ($K_{0.5}$) was 207 μM (Fig. 6B). Previous studies reported K_M values between 74 μM and 3500 μM for ASNases purified from different microbial sources [67]. The K_M value from LsASNase is similar to that of other GLNase-free enzymes isolated from diverse microorganisms that proved antineoplastic activity in cell cultures, like *Enterobacter cloacae* (1.58×10^{-3} M) [68], *Bacillus licheniformis* (1.4×10^{-5} M) [67], *Bacillus velezensis* (3.6×10^{-5} M) [69] and close to that the ASNase from *Erwinia chrysanthemi* (ErATM2) modified to reduces its GLNase activity (4.8×10^{-6} M) [70]. Therefore, is possible that LsASNase may have anti-ALL activity.

Intracellular ASNases presents allosteric regulation by the L-asparagine at milimolar levels [19,37], LsASNase presented this regulation at 50 μM. Also, the heterotrophic allosteric regulation by the L-glutamine was important for L-asparagine depletion at micromolar levels in EcASNase II[71]. However, the purified LsASNase showed no affinity to the L-glutamine substrate, the kinetic parameters obtained at 37 °C were $K_{0.5} = 230$ μM and a turnover number $k_{cat} = 54\ s^{-1}$, resulting in a catalytic efficiency ($k_{cat}/K_{0.5}$) of $7.5 \times 10^5\ M^{-1}\ s^{-1}$. The sigmoidal curve was fitted using he Hill equation, the Hill coefficient $n_H = 1.52$ (Fig. 6C) indicates a positive level of cooperativity ($n_H > 1$). Allostery is a consequence of re-distributions of protein conformational ensembles in a response to a structural perturbation as could be the binding of inducer or inhibitors, covalent modification as phosphorylation, change in pH, ionic strength or temperature [72,73]. Being the LsASNase a cold-adapted enzyme the increase of temperature induces changes in the inter-subunits interactions favoring the homotropic cooperativity presented by the oligomeric structure resulting in the deviation from the Michaelian behavior that we observe. The cooperativity is a result of the capacity to maintain a good activity performance in adverse environmental conditions, such as low temperature or drastic substrate concentration changes [74–76,57]. In this sense, the evolutionary pressure tends to maximize the catalytic efficiency of oligomeric enzymes, also is related with the enzyme flexibility to allow new catalytic functions [71,76]. The further structural characterization of LsASNase can provide sequences to describe allosteric components to design *de novo* enzymes with tunable activity [74,76].

The activation energy was calculated according to the Arrhenius plot, based on the determination of enzyme activities at five temperatures in a range between 20 and 45 °C (Fig. 6D). The activation energy value obtained (Ea = 1.8 kcal.mol^{-1}) was minor compared to the range of other enzyme-catalyzed reactions, being 3 times lower than recombinant ASNase from *E. chrysanthemi* (4.9 kcal.mol^{-1}) [35] and 6 times lower than ASNase type I from *S. cerevisiae* (11 kcal.mol^{-1}) [17]. In this sense, the cooperativity in a high flexible oligomeric structure can be responsible for the low values of activation energy found in LsASNase.

Finally, the production of GLNase-activity free ASNase is considered to be a mechanism for inhospitable regions, i.e., extremophile yeasts from the Antarctic region could produce GLNase-activity free and urease-activity free ASNase [27]. It is important to consider that the result of evolution is the acquired ability for uni-functional proteins to perform multiple functions (allowing the effective use of amino acids and energy). Interestingly, the differential functional behavior of many of these proteins depends on the structural allostery and cellular location [77]. (Table 3).

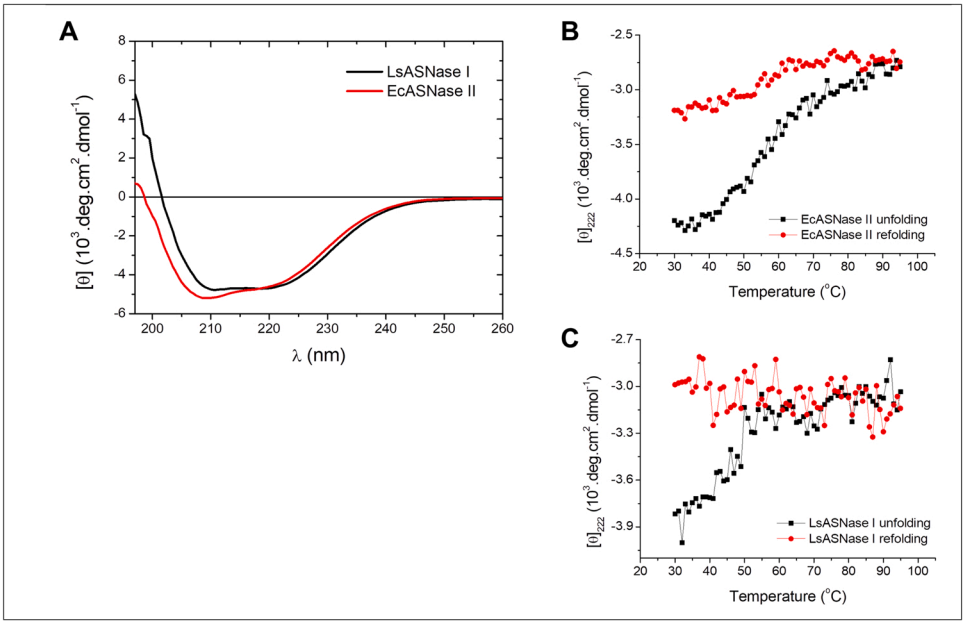

Fig. 5. Structural analysis by circular dichroism spectra of *Leucosporidium scottii* asparaginase I and *E. coli* asparaginase II (control). The protein concentration used in these experiments was 0.28 μM and 1.92 μM, respectively, in Tris-HCl (20 mM) buffer pH 8.0; A Spectra evaluated using a scan range of 190–260 nm and corrected against the Tris-HCl (20 mM) buffer pH 8.0, graphical representations are the average of six consecutive scans; B Thermal melting profiles for *E. coli* asparaginase II (control), recorded at 222 nm; the temperature was evaluated in the range 20–95 °C. The melting temperature obtained was around 60 °C; C Thermal melting profiles for *Leucosporidium scottii* asparaginase I, recorded at 222 nm; the temperature was evaluated in the range 20–95 °C. The melting temperature obtained was around 55 °C.

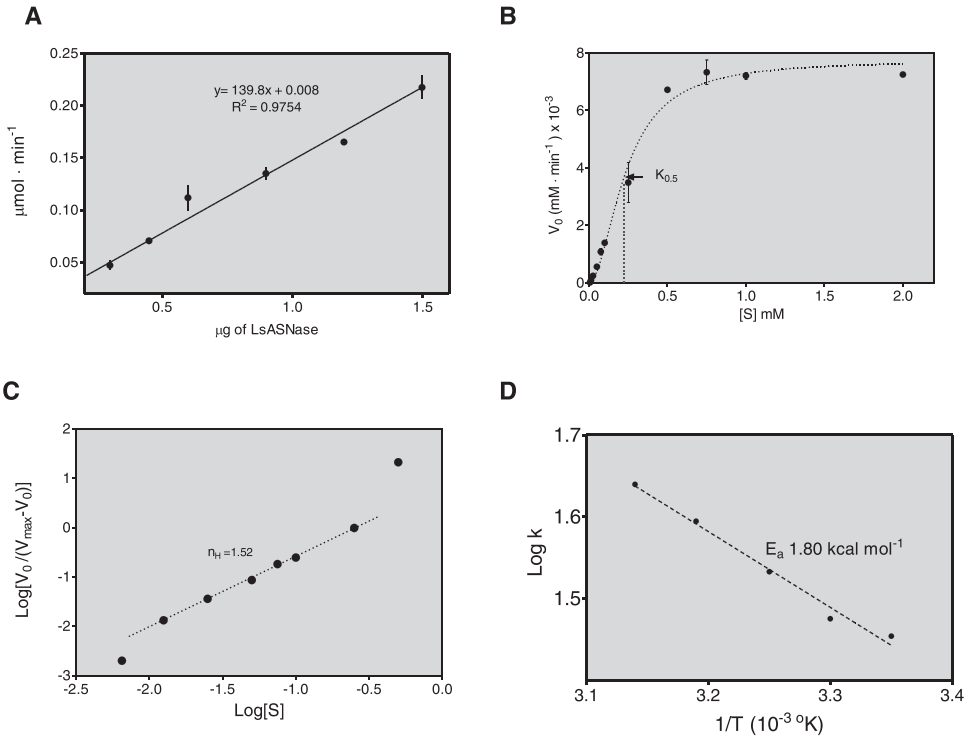

Fig. 6. Influence of asparagine on the parameters of *Leucosporidium scottii* asparaginase I; A Product formation under different purified *Leucosporidium scottii* asparaginase I concentrations in Tris-HCl buffer pH 8.0 at 37 °C; B *Leucosporidium scottii* asparaginase I kinetic parameters, activity dependence on a substrate concentration plot (Michaelis-Menten, $K_{0.5}$ = 207 μM and k_{cat} = 54 s^{-1}); C Hill plot of the data (n_H = 1.52); D Arrhenius plot of the data. The line represents the best least-square fit of the activity (Ea = 1.8 Kcal.mol^{-1}). The error bars represent the SD of three experiments.

4. Conclusion

The bioprospecting of ASNase from marine and terrestrial psychrotolerant Antarctic yeasts was successfully applied, revealing the potential to obtain novel enzymes with distinct characteristics from those produced by other microorganisms. Amongst the 40 yeast strains analyzed, 16 were able to produce ASNase, and the enzyme produced by *L. scottii* L115 (LsASNase) presented novel characteristics, revealing the potential to obtain novel enzymes with distinct characteristics from those produced by other microorganisms. Data from enzymatic kinetics showed an allosteric regulation with positive cooperativity by the substrate L-asparagine, GLNase-activity free, and glycosylation post-translational modification. All of these aspects highlight the potential of the L. scottii L115 ASNase as an antileukemic follow-on biologic with improved characteristics, leading to fewer side effects. Being an enzyme with particular properties to those already reported, the future amino acid sequencing of LsASNase may contribute to the structural knowledge of ASNases. Sequence analysis could reveal crucial amino acids for loss of glutamine affinity and describe how enzyme oligomerization aids its thermal stability and allosteric regulation, contributing to the identification of structural fingerprints for de novo design.

Table 3
Catalytic constants and specific activity in hydrolysis from different ASNases reported that presents allosteric regulation.

Enzyme	K_M, $K_{0.5}$*(μM)	k_{cat} (s^{-1})	V_{max} U.min^{-1}	n_H	Specific activity (U mg^{-1})	k_{cat} /$K_{0.5}$ (M^{-1} s^{-1})	Reference
EcASNase II[a]	18*	60	NR	1.5	NR	3.3×10^6	[71]
EcASNase I[b]	1200*	NR	0.016	2.6	173	NR	[77]
rScASNase I[c]	75*	217	0.042	2.2	196.2	1.6×10^4	[19]
ScASNase I[d]	740	NR	0.06	NR	5.4	NR	[17]
ScASNase II[e]	240	NR	57.8	NR	30.5	NR	[50][4]
hASNase I[f]	11,500*	6.7	NR	3.9	NR	5.8×10^2	[78][80]
LsASNase[g]	233*	54	0.002	1.5	130	7.5×10^5	This article

NR: not reported
[a] Asparaginase II from *E. coli*
[b] Asparaginase I from *E. coli*
[c] Recombinant asparaginase I from *S. cerevisiae*
[d] Asparaginase I from *S. cerevisiae*
[e] Asparaginase II from *S. cerevisiae*
[f] Asparaginase I from human
[g] Asparaginase from *L. scottii*

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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