L-Asparaginase from *Aspergillus* spp.: production based on kinetics, thermal stability and biochemical characterization

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Abstract

This study describes the production of native L-asparaginases by submerged fermentation from *Aspergillus* strains and provides the biochemical characterization, kinetic and thermodynamic parameters of the three ones that stood out for high L-asparaginase production. For comparison, the commercial fungal L-asparaginase was also studied. Both commercial and L-asparaginase from *Aspergillus oryzae* CCT 3940 showed optimum activity and stability in the pH range from 5 to 8 and the asparaginase from *Aspergillus niger* LBA 02 was stable in a more alkaline pH range. About the kinetic parameters, the denaturation constant increased with the heating temperature for all L-asparaginases, indicating that the L-asparaginase activity decreased at higher temperatures, especially above 60 °C. Moreover, L-asparaginase from *A. oryzae* CCT 3940 remained stable after 60 min at 50 °C. None of the L-asparaginases were inhibited by high NaCl concentrations, which are highly desirable for food industry application. The catalytic activities of all the L-asparaginases were enhanced by the presence of Mn²⁺ and inhibited by p-chloromercuribenzoate and iodoacetamide. The L-asparaginase from the *Aspergillus* strains and the commercial enzyme had similar K_m when L-asparagine was used as substrate. None of the L-asparaginases, except the L-asparaginase from *A. niger* LBA 02, could hydrolyze the substrate L-glutamine, which is of interest for medical proposes, since the glutaminase activity is usually related to adverse reaction during the leukemia treatment. This study showed that these new three non-recombinant L-asparaginases studied have potential application in the food and pharmaceutical industries, especially due to their good thermostability.

Keywords *Aspergillus oryzae* · *Aspergillus niger* · Characterization · L-Asparaginase

Introduction

L-Asparaginase (L-asparagine hydrolase, E.C.3.5.1.1) is an enzyme that selectively hydrolyzes L-asparagine, resulting in the formation of aspartic acid and ammonia (Zuo et al. 2015). L-Asparaginase can be used in the food industry to reduce the

levels of acrylamide (Dias et al. 2017), which is known to be neurotoxic and it is considered as a “potential” carcinogen in humans based on its carcinogenicity in rodents (Pedreschi et al. 2011). The conversion of L-asparagine into aspartic acid and ammonium prevents this amino acid from participating in the Maillard reaction and, therefore, reduces the formation of acrylamide without affecting the sensorial attributes of the final product (Batoool et al. 2016; Hendriksen et al. 2009; Kumar et al. 2014; Swanston 2018). The available commercial L-asparaginase was obtained from the recombinant strains of *A. niger* or *A. oryzae* based on the cloning technology (Claus et al. 2008; Hareyan 2007; Xu et al. 2016). However, despite the L-asparaginase be very promising for reducing acrylamide, its implementation is still expensive compared to other strategies due to the high costs of the commercial enzyme (Claus et al. 2008). Microbial sources are preferred in this case, because besides being more economic to produce, the enzymes are also easier to modify and optimize (Vimal and Kumar 2017). Thus, it becomes necessary to reduce the

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L-asparaginases costs to increase the safety of food produced in countries that are not self-sufficient in this enzyme. Therefore, the achievement of a native L-asparaginase from *Aspergillus* strain, which is allowed in various countries, without use cloning technology, should greatly increase the possibility of reducing import costs of this enzyme providing a safe food for the population.

L-Asparaginases have also pharmaceutical application; it is used to treat lymphoblastic leukemia and inhibit tumor growth (Keating et al. 1993; Krishnapura et al. 2016; Shrivastava et al. 2016). The anti-leukemic effect of L-asparaginase results from the depletion of the circulating L-asparagine, since tumor cells are dependent on an exogenous source of this amino acid. However, normal cells can synthesize L-asparagine and thus are less affected by the treatment with L-asparaginase (Sarquis et al. 1994). However, the bacterial L-asparaginases could cause side effects; the toxic effects of the most L-asparaginases are associated with their L-glutaminase activity, causing a prolonged low-level L-glutamine intake, leading to liver enzyme elevations and pancreatitis (Zuo et al. 2015). Therefore, an L-asparaginase with no L-glutaminase activity would be significant in cancer chemotherapy. The demand for L-asparaginase has increased several folds due to its use in the food industry, in addition to its pharmaceutical applications (Moharam et al. 2010).

In view of the broad application of L-asparaginase, the present enzyme knowledge is not enough and search for new asparaginases is continuously being exploited. In this context, the knowledge about kinetic parameters and thermal stability is important for bioprocesses and it is essential to improve the chemotherapeutic and food application of the L-asparaginase. There is an increasing need to determine the properties of new L-asparaginases for use in existing applications. In general, L-asparaginases exhibited low thermal stability restricting their industrial use, especially for food applications. In this work, eight L-asparaginases from *Aspergillus* spp. were studied and three of them, due to their high activity, were evaluated in terms of pH stability, thermostability and the inactivation kinetics. The effects of ions and inhibitors on L-asparaginase activity and the kinetic parameters (K_m and V_{max}) were also elucidated using L-asparagine and L-glutamine as substrates to obtain information about those L-asparaginases and to compare their characteristics to commercial recombinant L-asparaginase.

Materials and methods

Chemicals

Nessler reagent was purchased from Merck® (Darmstadt, Germany). L-Asparagine was obtained from Sigma-Aldrich® (Missouri, USA). Commercial L-asparaginases from *A.*

oryzae were obtained from Novozymes®; KH_2PO_4 , KCl, $MgSO_4 \cdot 7H_2O$, $CuNO_3 \cdot 3H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ and glucose were obtained from Synth® (São Paulo, Brazil).

Microorganisms

A. oryzae CCT 3279, *A. oryzae* CCT 3940, *A. oryzae* CCT 5321, *A. niger* CCT 4846, *A. niger* CCT 3941 and *A. niger* CCT 4157 were obtained from the Culture Collection of André Tosello Foundation, Campinas-SP, Brazil. The *A. oryzae* LBA 01 and *A. niger* LBA 02 strains were obtained from the culture collection of the Laboratory of Food Biochemistry, Department of Food Science, Faculty of Food Engineering, University of Campinas SP, Brazil. The strains were maintained on inclined tubes containing potato dextrose agar (PDA) medium and incubated at 30 °C for 7 days, stored at 4 °C with subculturing every 3 months.

L-Asparaginase production

Conidial suspensions were prepared from freshly raised 7-day-old cultures on PDA medium with 5 mL of sterile 0.3% Tween 80 solution. The suspension was aseptically transferred to a 250 mL Erlenmeyer flask containing 50 mL of modified Czapeck Dox medium consisting of (g/L): 2.0 glucose, 10.0 asparagine, 1.52 KH_2PO_4 , 0.52 KCl, 0.52 $MgSO_4 \cdot 7H_2O$, 0.01 $CuNO_3 \cdot 3H_2O$, 0.01 $ZnSO_4 \cdot 7H_2O$ and 0.01 $FeSO_4 \cdot 7H_2O$, and the initial pH was adjusted to 6.2 (Gulati et al. 1997). The flasks were incubated at 30 °C and 150 rpm for 5 days. The enzymatic activity was measured every 24 h to determine the effect of the time of fermentation on L-asparaginase production. The fungal cultures were filtered through paper filter and the filtrate was called the enzymatic crude extract. The crude extracts were concentrated by ammonium sulfate 80% precipitation, followed by dialysis and freeze drying.

Screening of fungal L-asparaginase producers using thin-layer chromatography (TLC) and using final pH of the culture medium

For the screening of fungal L-asparaginase producers using TLC, enzymatic extracts and commercial L-asparaginase were incubated with 0.5% w/v asparagine in 100 mM Tris HCl buffer pH 8.0, for 30 min at 37 °C. The samples were applied to 10 × 10 cm silica gel 60 TLC sheets (Merck®, Darmstadt, Germany), and the chromatography was developed with a 70% isopropanol solution with a 1 h run time. For comparison, a 0.5% w/v asparagine solution and 0.25% w/v aspartic acid solution were applied. Control samples were prepared using distilled water instead of asparagine in the reaction medium. The TLC sheets were sprayed with

ninhydrin spray reagent (Sigma-Aldrich®, Missouri, USA) and dried at 100 °C for 10 min.

For the screening of fungal L-asparaginase producers using the final pH of the culture medium: after 120 h of fermentation, the pH of the enzymatic extracts was measured.

L-Asparaginase activity assay

The L-asparaginase activity of the crude enzyme solution was assessed with Nesslerization using the method described by Imada et al. (1971) with modifications. The reaction medium consisted of 0.5 mL of 0.04 M L-asparagine, 0.5 mL of 0.1 M Tris–HCl pH 8.0 buffer, 0.1 mL of crude enzyme extract and 0.9 mL of distilled water and was incubated at 40 °C for 30 min. The reaction was stopped with the addition of 0.5 mL of 1.5 M trichloroacetic acid. An aliquot of 125 µL of the reaction mixture was diluted with 1 mL of distilled water, and 125 µL of Nessler's reagent was added to quantify the amount of ammonia. An analytical curve with ammonium sulfate was used to quantify the released ammonia (20–450 µmol ammonia L⁻¹). To assure the accuracy of the results for L-asparaginase activity, three calibration curves were constructed in triplicate on three consecutive days for verification of the linearity, repeatability and limit of detection (LOD) of the method. In addition, for each trial of enzyme activity, a solution with known concentration of ammonium was tested. The estimated limit of detection (LOD) was calculated according to the following equation (Bratinova et al. 2009):

$$\text{LOD} = \frac{\text{SDa} \times 3}{\text{IC}}, \quad (1)$$

where SDa is the estimated standard deviation of the interception (linear coefficient) of the calibration curve and IC is the calibration curve slope.

One unit of enzyme activity (U) was defined as the amount of enzyme that liberates 1.0 µmol of ammonia per minute under standard assay conditions.

Effect of temperature and pH on the L-asparaginase activity

The effect of temperature on the L-asparaginase activity was determined by assaying the activity at different temperatures (10–70 °C, at pH 8.0). The effect of pH on the L-asparaginase activity was determined under standard assay conditions using 50 mM sodium citrate buffer (pH 3.0–6.0), 50 mM phosphate buffer (pH 6.5–7.5), 50 mM Tris–HCl buffer (pH 7.0–9.0), and 50 mM Borax–NaOH buffer (pH 10.0). All assays were made as triplicate. The relative activities were determined using the maximal activity of the enzymes at a specific pH as 100%.

Effect of temperature and pH on the L-asparaginase stability

The thermal stability of L-asparaginases was determined by pre-incubating the enzymes at various temperatures (10–70 °C) for 1 h in the optimum pH and then placing the samples on ice immediately. The remaining activity was then determined after each treatment.

The effect of pH on L-asparaginase stability was determined using the above-mentioned buffer systems over a pH range from 3.0 to 10.0. The enzyme solutions were incubated at the various pH values for 1 h at 25 °C without substrate. The remaining enzyme activity was then measured at the optimum temperature and pH for each strain. The remaining activities were determined using the maximal activity of the enzymes at a specific pH as 100%. The remaining activity was determined according to the following equation:

$$\text{Remaining activity(\%)} = \frac{A_f}{A_0} \times 100, \quad (2)$$

where A_f is the enzyme activity after the 1 h of incubation and A_0 is the enzyme activity before incubation.

Inactivation kinetics of L-asparaginase

The thermal denaturation kinetics of L-asparaginases were described as first-order model (Batista et al. 2014) described by the following equation:

$$\frac{A_t}{A_0} = e^{-k_d t}, \quad (3)$$

where A_0 is the initial enzyme activity, A_t is the residual activity at the time t , t is the treatment time (h), and k_d is the inactivation rate constant at determined temperature.

The half-life ($t_{1/2}$) value for L-asparaginase thermal denaturation was calculated as:

$$t_{1/2} = \frac{\ln(0.5)}{-k_d}. \quad (4)$$

The D value is the time (min) necessary to reduce the initial activity in 90%. It was related to k_d values and mathematically expressed by

$$D = \frac{\ln(10)}{k_d}. \quad (5)$$

Effect of metal ions and inhibitors

The effect of different metal ions and inhibitors was investigated by adding them at final concentrations of 1, 5 and 10 mM to the reaction mixture (1 mL) and incubating at 25 °C for 60 min under standard assay conditions. The

relative activities were determined using the activity of the enzyme in the absence of additives as 100%.

Kinetic parameters

The kinetic parameters were determined using the experimental system described above with varying amounts of the substrate, L-asparagine (0–30 mM) and L-glutamine (0–40 mM). The Michaelis–Menten constant (K_m) and maximum velocity (V_{max}) were determined as the reciprocal absolute values of the intercepts on the x and y axes, respectively, of the linear regression curve (Lineweaver and Burk 1934). The values of V_{max} can be deduced from the reciprocal of the intercept of the straight line on the ordinate and the values of K_m from the negative reciprocal of the interception, using the following equation:

$$\frac{1}{V} = \frac{K_m}{V_{max} \times [S]} + \frac{1}{V_{max}}, \quad (6)$$

where V is the initial reaction rate, $[S]$ is the initial substrate concentration, V_{max} is the maximum reaction rate substrate concentration and K_m is the Michaelis–Menten constant.

Protein determination

The amount of protein was determined by the Lowry method (Lowry et al. 1951) with bovine serum albumin as the standard.

Statistical analysis

All the values reported for the biochemical characterization of the enzymes represent the mean of three replicates. Significant differences ($p < 0.05$) between enzyme biochemical properties were determined by ANOVA and the Tukey test. Statistical analyses were performed using the Statistica® 7.0 Statsoft, Inc., software (Tulsa, OK, USA).

Results and discussion

Preliminary selection of fungi producing L-asparaginase

All the L-asparaginases from the eight *Aspergillus* tested exhibited L-asparaginase activity as indicated by the formation of spots corresponding to the retention factor of L-aspartic acid in the TLC assay (Fig. 1). Hendriksen et al. (2009) reported that strains of *A. oryzae* that showed L-asparaginase activity in the test on TLC plates also showed the ability to reduce acrylamide levels in foods (Hareyan 2007).

The increase in the culture medium pH within the fermentation time is an indicative of L-asparaginase production (Gulati et al. 1997). All the culture medium presented an increase in the pH in the relation to the initial pH (6.2) of the medium, except the *A. niger* CCT 3941, which indicates the production of L-asparaginase at least by seven of the eight strains (Fig. 2).

Confirmation of the veracity of the determination of L-asparaginase activity

To ensure that all values determined were based on a solid method, some parameters were determined. Table 1 shows that the actual values found for the concentration of ammonia changed little compared to the theoretical values, indicating good recovery for the method used. Also, worth noting that the detection limit values for the method were very low ($8.6 \mu\text{mol ammonia L}^{-1}$). The limit of quantification was considered the lowest point of the curve ($\mu\text{mol ammonia L}^{-1}$). The applied method also showed good recovery (99.6% on average). The values for the correlation coefficients (r) and r^2 were 0.9999 and 0.0998, respectively, which is highly satisfactory, indicating good linearity of the method. With these parameters, it is possible to confirm the

Fig. 1 Thin-layer chromatography for qualitative assay for L-asparaginase activity. S1=L-asparagine 0.5% (m/v); S2=aspartic acid 0.25% (m/v); A1–A9=reaction medium of commercial, and L-asparaginases

from *A. oryzae* CCT 3940, *A. oryzae* CCT 3279, *A. oryzae* CCT 5321, *A. niger* CCT 4846, *A. niger* CCT 3941, *A. niger* CCT 4157, *A. niger* LBA 02 and *A. oryzae* LBA 01, respectively

Fig. 2 Final pH of the culture medium after 120 h of fermentation in agitated flasks

Table 1 Analytical curve made with ammonium sulfate parameters to ensure the veracity of analytical response

Concentration ($\mu\text{mol ammonium L}^{-1}$)	Abs 450 nm ^a	RSD (%) ^b	Real concentration ($\mu\text{mol ammonium L}^{-1}$)	Recovery (%)
20	0.039 ± 0.006	14.2	23.3	116.6
40	0.065 ± 0.004	6.2	39.5	98.8
75	0.121 ± 0.006	4.6	73.1	97.5
150	0.247 ± 0.012	4.7	149.6	99.7
225	0.369 ± 0.020	5.3	224.1	99.6
300	0.491 ± 0.023	4.7	298.1	99.4
375	0.620 ± 0.037	5.9	376.4	100.4
450	0.743 ± 0.024	3.2	450.9	100.2

^aResults are presented as the mean $\pm$ standard deviation of nine determinations (3 per day)

^bRSD: relative standard deviation

quality of the method employed for the determination of L-asparaginase activity.

Influence of fermentation time on L-asparaginase activity

The effect of time on the production of L-asparaginase was tested in all strains. The L-asparaginases from *A. niger* LBA 02, *A. oryzae* LBA 01 and *A. oryzae* CCT 3940 showed remarkable values of activity, standing out from the other L-asparaginases (Fig. 3). *A. niger* LBA 02 presented the maximal production of L-asparaginase (26.0 U mL^{-1}) after 72 h of fermentation at 30°C . *A. oryzae* LBA 01 showed maximum activity (20.6 U mL^{-1}) after 96 h at 30°C , whereas the *A. oryzae* CCT 3940 showed high activity (19.1 U mL^{-1}) after 72 h at 30°C (Fig. 3). At 120 h of fermentation, a decrease in L-asparaginase

activity with the time of fermentation was observed. These extracts were concentrated by ammonium sulfate 80% precipitation and the results are presented in Table S1.

Dorya and Kumar (2018) produced L-asparaginase from a newly isolated strain of *Aspergillus* sp. After 6 days of fermentation at 35°C and pH 8, a maximum activity of 12.57 U mL^{-1} was obtained. Siddalingeshwara and Lingappa (2011) reported a maximum activity of 6.05 U mL^{-1} at 72 h of fermentation for L-asparaginase from *Aspergillus terreus* KLS2, and the authors reported a decrease in activity after 72 h of fermentation. Baskar and Renganathan (2009) observed a decrease in the activity of L-asparaginase from *A. terreus* MTCC 1782 after 48 h of fermentation; 13.67 U mL^{-1} . The authors associated the reduction of L-asparaginase activity with the rapid growth and maturity of fungal spores.

Fig. 3 Effect of the fermentation time on the L-asparaginase activity

Effect of temperature and pH on L-asparaginase activity and stability

All procedures were performed under the same conditions for all samples of L-asparaginases from *Aspergillus* as well as the commercial enzyme. To compare the results obtained for each enzyme sample, the total concentration of protein present in each reaction was constant (0.5 mg mL^{-1}).

L-Asparaginase from *A. niger* LBA 02 and the commercial enzyme exhibited optimal activity at 50°C . L-Asparaginase from *A. oryzae* CCT 3940 showed optimum activity between 40 and 50°C , whereas L-asparaginase from *A. oryzae* LBA 01 showed optimal activity at 40°C (Fig. 4a). The L-asparaginases from *A. oryzae* LBA 01, *A. oryzae* CCT 3940 and *A. niger* LBA 02 showed higher activity at pH 7.0, 7.0–8.0 and 9.0, respectively (Fig. 4b). Commercial L-asparaginase presented optimum activity at pH 6.0–7.0.

Concerning stability, L-asparaginases from *A. niger* LBA 02, *A. oryzae* CCT 3940 and the commercial L-asparaginase at 50°C retained approximately 100% of their activity after

60 min (Fig. 5a). L-Asparaginase from *A. oryzae* LBA 01 retained only 60% of its activity after 60 min at 50°C . The L-asparaginases from these *Aspergillus* strains exhibited a 40 and 10% reduction of activities after 1 h at 60 and 70°C , respectively. The pH stability tests revealed that the enzymes from *A. oryzae* CCT 3940 and the commercial L-asparaginase were stable from pH 5.0 to 8.0, retaining over 70% of their activity in this range (Fig. 5b). The L-asparaginases from *A. oryzae* LBA 01 and *A. niger* LBA 02 were stable from pH 7.0 to 9.0. In general, the optimal pH range for L-asparaginase was pH 6.5–9.0 (Kumar et al. 2014), which is consistent with this study. L-asparaginases with the greatest activity in the pH range from 5.5 to 8.0 are recommended for application in starch products (Hendriksen et al. 2009). This indicates that L-asparaginases from *A. oryzae* LBA 01 and *A. oryzae* CCT 3940 are highly suitable for acrylamide mitigation in starch products and could promote cost reduction in the application of this enzyme.

Bhagat et al. (2016) described L-asparaginase from *Pseudomonas oryzae* active from pH 8.0 to 10.0 showing

Fig. 4 The effect of **a** temperature and **b** pH on L-asparaginase activity of L-asparaginases

Fig. 5 The effect of **a** temperature and **b** pH on L-asparaginase stability of L-asparaginases

maximum activity at pH 8.0. Loureiro et al. (2012) reported that the optimum pH for L-asparaginase from *A. terreus* was 9.0 and, at physiological pH, the enzyme retained 70% of its maximum activity. Siddalingeshwara and Lingappa (2011) reported that the L-asparaginase from *A. terreus* KLS2 was stable at alkaline pH and retained 100% activity after incubation for 30 and 60 min at pH 8.0. In this study, the enzymes were also more stable at alkaline pH values.

Inactivation kinetics of L-asparaginase

A detailed kinetic study of thermal inactivation for the L-asparaginases was performed at different temperatures (10–70 °C). The denaturation constants (k_d) were calculated and are given in Table 2. The rate constant increased with the heating temperature for all the L-asparaginases, indicating that the L-asparaginase activity decreases at higher temperatures, especially above 60 °C. The half-life ($t_{1/2}$) and the decimal reduction time (D) are other important parameters commonly used in the characterization of enzyme stability. Increasing the temperature from 20 to 70 °C resulted in a decrease in $t_{1/2}$ and D values for all the L-asparaginases tested (Table 2).

A linear relationship was observed in the plot of $\log D$ versus temperature. From this plot, the z value was calculated as 21.8 °C for the L-asparaginase from *A. oryzae* CCT 3940 ($r^2=0.85$), 23.6 °C for the L-asparaginase from *A. oryzae* LBA 02 ($r^2=0.99$), 22.7 °C for the L-asparaginase from *A. niger* LBA 01 ($r^2=0.88$) and 20.6 °C for the commercial L-asparaginase ($r^2=0.96$). In general, low z values are thought to indicate the sensitivity to heat. In this sense, considering the z values, all L-asparaginases can be considered as slightly thermostable.

Akilandeswari et al. (2012) reported that the L-asparaginase from *A. niger* showed an optimum activity at 35 °C. However, L-asparaginase from *A. terreus* produced in Czapeck Dox medium exhibited the highest levels of

activity at 40 °C and was stable at this temperature for 120 min (Loureiro et al. 2012). Siddalingeshwara and Lingappa (2011) observed that L-asparaginase from *A. terreus* KLS2 retained 80% and 50% of its activity when incubated at 50 °C for 30 and 60 min, respectively. The commercial L-asparaginase from a recombinant strain of *A. oryzae* had rapidly reduced its activity at temperatures above 60 °C. L-asparaginases from *A. niger* LBA 02 and *A. oryzae* CCT 3940 were more thermostable than that described by Siddalingeshwara and Lingappa (2011) because, after 60 min at 50 °C, it retained 100% of the initial activity, which is highly desirable for further applications.

Effect of metal ions and other compounds on L-asparaginase activity

The presence of NaCl, KCl and CaCl_2 slightly affected the L-asparaginase activity for all the L-asparaginases tested. L-asparaginase activity was not inhibited by NaCl, and this characteristic is important for application in the food industry to reduce the acrylamide levels (Table S2). The presence of ZnCl_2 , MgCl_2 and FeCl_3 acted as activators only for the L-asparaginase from *A. oryzae* LBA 01 and did not cause any major changes in the other enzymes. However, MnCl_2 acted as an activator for all the L-asparaginases studied and increased the commercial L-asparaginase activity by 70% at a 1 mM concentration. Thakur et al. (2014) and Huang et al. (2014) also reported that Mn^{2+} is an activator for L-asparaginase from *Mucor hiemalis* and *Rhizomucor miehei*, respectively. On the other hand, Vala et al. (2018) described that L-asparaginase of *A. niger* AKV-MKBU was inhibited by Mn^{2+} at the concentration of 10 mM.

The presence of CuSO_4 , particularly at 10 mM, was an inhibitor for the L-asparaginases from *A. oryzae* CCT 3940 and *A. oryzae* LBA 01. This may be because of the precipitation of the protein because metals, such Cu^{2+} , at higher concentrations (> 1 mM) are general protein precipitants

Table 2 Kinetic parameters for the thermal inactivation for L-asparaginase at different temperatures

Temperature (°C)	k_d (h ⁻¹)	$t_{1/2}$ (h)	D (h)
<i>A. oryzae</i> CCT 3940			
10	0.007 ± 0.000 ^c	276.93 ± 0.02 ^a	919.94 ± 22.15 ^a
20	0.007 ± 0.002 ^c	276.92 ± 6.66 ^a	919.85 ± 54.74 ^a
30	0.008 ± 0.004 ^c	156.14 ± 6.67 ^b	518.43 ± 47.13 ^b
40	0.011 ± 0.003 ^c	64.92 ± 14.13 ^c	215.74 ± 62.23 ^c
50	0.013 ± 0.006 ^c	55.31 ± 18.45 ^c	183.84 ± 4.56 ^c
60	0.382 ± 0.018 ^b	1.82 ± 0.08 ^d	6.08 ± 0.29 ^d
70	1.172 ± 0.033 ^a	0.62 ± 0.03 ^e	2.01 ± 0.05 ^d
<i>A. oryzae</i> LBA 01			
10	0.01 ± 0.000 ^f	53.93 ± 0.21 ^b	229.80 ± 0.45 ^a
20	0.02 ± 0.014 ^f	82.62 ± 18.87 ^a	101.06 ± 6.22 ^b
30	0.13 ± 0.007 ^e	6.30 ± 1.25 ^c	21.03 ± 3.15 ^c
40	0.21 ± 0.004 ^d	3.45 ± 0.09 ^c	11.29 ± 2.22 ^d
50	0.46 ± 0.009 ^c	1.56 ± 0.21 ^c	5.15 ± 0.12 ^e
60	1.14 ± 0.037 ^b	0.62 ± 0.03 ^c	2.02 ± 0.07 ^e
70	1.69 ± 0.041 ^a	0.42 ± 0.01 ^c	1.43 ± 0.03 ^e
<i>A. niger</i> LBA 02			
10	0.007 ± 0.000 ^c	276.9 ± 4.45 ^{a,b}	919.83 ± 14.77 ^b
20	0.004 ± 0.001 ^c	311.6 ± 6.67 ^a	1035.08 ± 22.15 ^a
30	0.012 ± 0.002 ^c	59.4 ± 8.25 ^{b,c}	197.42 ± 27.43 ^c
40	0.011 ± 0.001 ^c	64.8 ± 3.63 ^{b,c}	215.25 ± 12.08 ^c
50	0.024 ± 0.009 ^c	38.1 ± 6.68 ^c	126.86 ± 8.85 ^d
60	0.284 ± 0.062 ^b	2.5 ± 0.48 ^c	8.33 ± 1.62 ^e
70	1.131 ± 0.063 ^a	0.6 ± 0.03 ^c	2.00 ± 0.11 ^e
Commercial enzyme			
10	0.007 ± 0.001 ^c	276.92 ± 36.17 ^a	919.88 ± 26.46 ^a
20	0.010 ± 0.001 ^c	265.43 ± 37.58 ^a	881.50 ± 23.04 ^b
30	0.014 ± 0.002 ^c	252.81 ± 38.09 ^a	839.63 ± 24.96 ^c
40	0.065 ± 0.011 ^c	11.73 ± 4.64 ^b	38.70 ± 9.44 ^d
50	0.094 ± 0.021 ^c	7.58 ± 1.71 ^b	25.15 ± 5.70 ^d
60	0.534 ± 0.181 ^b	1.31 ± 0.06 ^b	4.38 ± 0.21 ^d
70	2.412 ± 0.111 ^a	0.30 ± 0.01 ^b	1.05 ± 0.05 ^d

Results are presented as the mean ± standard deviation of three determinations. In the columns, the values followed by the same letter are not statistically different by ANOVA and Tukey test ($p > 0.05$)

(Whitaker 1993). The presence of ethylenediaminetetraacetic acid (EDTA) slightly increased the activity of L-asparaginase from *A. oryzae* CCT 3940 and *A. niger* LBA 02 (Table S2). Thakur et al. (2014) reported that EDTA displayed no effect on L-asparaginase from *Mucor hiemalis*, confirming the non-metalloprotein nature of the enzyme. The presence of sodium azide only affected the commercial enzyme and reduced its activity to approximately 60%. The presence of L-cysteine did not affect the L-asparaginase activities of the three strains tested. Kumar et al. (2011) found that L-cysteine and L-histidine increased the enzymatic activity of L-asparaginase from *Pectobacterium carotovorum*

MTCC 1428. The presence of p-chloromercuribenzoate (p-CMB) and IA (iodoacetamide) acted as inhibitors for all four L-asparaginases; however, the reduction in the activity was less significant for the commercial enzyme.

Determination of the kinetic parameters K_m and V_{max}

For the enzyme kinetic studies, all the L-asparaginases studied demonstrated high affinity for the substrate L-asparagine. The K_m and V_{max} values for the L-asparaginase from *A. oryzae* CCT 3940 were estimated as 2.10 mM and 35.8 U mL⁻¹, respectively (Table S3). L-Asparaginase from *A. oryzae* LBA 01 had K_m and V_{max} values estimated at 5.07 mM and 57.14 U mL⁻¹, respectively. The K_m and V_{max} for L-asparaginase from *A. niger* LBA 02 were 1.41 mM and 39.22 U mL⁻¹, respectively, and for the commercial L-asparaginase, the K_m and V_m values were 5.06 mM and 588.24 U mL⁻¹, respectively (Table S3). Notably, the K_m values for the three L-asparaginases from the *Aspergillus* strains were similar to the K_m value of the commercial enzyme. However, for the substrate L-glutamine only the L-asparaginase from *A. niger* LBA 02 exhibited low specific activity and the other ones showed no activity against the L-glutamine substrate (Table S3).

Loureiro et al. (2012) reported that the L-asparaginase from *A. terreus* has a K_m value of 2.42 mM using L-asparagine as substrate, and similar results were obtained in this study. Similarly, a K_m value of 4.3 mM was found for the L-asparaginase from *Mucor hiemalis* (Thakur et al. 2014). The relatively low K_m values for the L-asparaginases in this study indicate high affinity for the substrate and possible uses in the food and pharmaceutical industries.

Conclusion

In this work, native L-asparaginases from *Aspergillus* strains were characterized. The main difference between the studied L-asparaginases and other asparaginases reported is the fact that they are native from Brazil and can be used in the food industry in addition to having suitable characteristics for this purpose and, thus, they could reduce the costs of application of this enzyme, especially importation costs, aiming to increase food security. L-asparaginase from *A. oryzae* CCT 3940 presented optimum activity in the pH range from 5 to 8, and remained stable after 60 min at 50 °C, which is highly desirable for use in starch products for acrylamide mitigation. L-Asparaginases from *A. oryzae* CCT 3940 and *A. oryzae* LBA 01 have a pH response similar to the commercially available enzyme, which is appropriate for food processing. *A. niger* LBA 02 was stable in a more alkaline pH range and also presented great thermal stability. No

enzymes were inhibited by a high NaCl concentration, which is an attractive property for the food industry. The presence of Mn^{2+} acted as an activator for all the L-asparaginases. These desirable traits, such as the stability at physiological pH, great thermal stability and high substrate specificity for L-asparagine of the L-asparaginases, suggest that they can be used in future investigations in the food industry and for pharmaceutical applications and also should greatly increase the possibility of reducing import costs of this enzyme permitting its use.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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