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# Purification, characterization and antiproliferative activity of L-asparaginase from *Aspergillus oryzae* CCT 3940 with no glutaminase activity

Fernanda Furlan Gonçalves Dias[1*], Ana Lúcia Tasca Gois Ruiz[2], Adriana Della Torre[2], Helia Harumi Sato[1]

[1]*Department of Food Science, School of Food Engineering, University of Campinas, P.O. Box 6121, 13083-862 Campinas, SP, Brazil*

[2]*Division of Pharmacology and Toxicology, Multidisciplinary Center for Chemical, Biological and Agricultural Research, University of Campinas, P.O. Box 6171, 13148-218 Campinas, SP, Brazil*

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ABSTRACT

**Objective:** To explore the anti-proliferative activity of purified L-asparaginase from *Aspergillus oryzae* CCT 3940 (*A. oryzae*).

**Methods:** L-asparaginase was produced by submerged fermentation and purified to electrophoresis homogeneity by ionic exchanged chromatography in a fast protein liquid chromatographic system. The purified enzyme was characterized and used for the anti-proliferative assay against nine tumor cell lines and one non-tumor cell line.

**Results:** The free glutaminase L-asparaginase was purified 28.6 fold. L-asparaginase showed high stability under physiological condition, remaining stable in the pH range 7.0–8.0 after 1 h incubation at temperature range 30–45 °C. The Km and Vmax values of purified L-asparaginase were estimated as 0.66 mmol/L and 313 IU/mL, respectively. The purified enzyme could inhibit the growth of a broad range of human tumor cell lines at the concentrations studied. Also, the enzyme from *A. oryzae* CCT 3940 could inhibit tumor growth of leukemia cell line (K562) with a total growth inhibition value of (3.2 ± 2.5) IU/mL and did not inhibit the non-carcinogenic human cell line growth at the concentrations studied.

**Conclusions:** The sensitivity of the cells lines to purified L-asparaginase from *A. oryzae* CCT 3940 appeared to be concentration dependent affording a more significant decrease in cell growth than that observed for the commercial L-asparaginase from *Escherichia coli*. The L-asparaginase from *A. oryzae* CCT 3940 has a high potential for pharmaceutical exploitation in the treatment of leukemia.

## 1. Introduction

L-asparaginase is an important chemotherapeutic agent used to treat a variety of diseases of the lymphatic system, and lymphomas such as acute lymphoblastic leukemia [1]. The enzyme has also been applied in the treatment of Hodgkin's disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphoblastic leukemia, lymphosarcoma, reticulosarcoma and melanosarcoma [2]. In the normal animal cell, L-asparagine is not an essential amino acid for the maintenance of cell viability since cell supplies the absence of amino acid through the action of asparagine synthetase [3]. Asparagine synthetase is an intracellular enzyme responsible for the *de novo* synthesis of asparagine. However, the neoplastic cells are not able to induce the synthesis of asparagine synthetase, and they are dependent on the extracellular level of L-asparagine to protein synthesis. As L-asparagine is withdrawn from plasma by L-asparaginase, the resulting small concentration of this amino acid leads to disrupting protein synthesis and consequently cell growth inhibition in neoplastic cells [4]. Thus, L-asparaginase is used as an antitumor agent injecting the enzyme intravenously for lowering the concentration of L-asparagine, selectively affecting neoplastic cells dependent on this amino acid [5].

*Corresponding author: Fernanda Furlan Gonçalves Dias, Department of Food Science, School of Food Engineering, University of Campinas, P.O. Box 6121, 13083-862 Campinas, SP, Brazil.
Tel: +55 19 35212175
Fax: +55 19 35212153
E-mail: fernandafgd@gmail.com

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There are three formulations of asparaginase widely used against acute lymphoblastic leukemia, the native L-asparaginase from *Escherichia coli* (*E. coli*) (Elspar®), its pegylated form (Oncaspar®) and L-asparaginase from *Erwinia chrysanthemi* (Erwinase®). The ability to substantially reduce the plasma levels of L-asparagine and keep it low for an extended period is the main feature responsible for the anti-neoplastic activity of L-asparaginase [6]. Despite significant progress in developing formulations of L-asparaginase, they are not free of adverse effects. There are some limiting factors for the use of L-asparaginase in chemotherapy, such as low catalytic activity that requires the use of high concentrations of each application [1], toxic effects, such as hyperglycemia, decreased serum albumin, lipoproteins, and fibrinogen, increased fat in the liver and some mild brain dysfunction [7]. The primary limiting factor is the development of hypersensitivity to the processing, which occurs in 50% of treated patients during the therapy with L-asparaginase from *E. coli* [8]. Even the pegylated form of L-asparaginase from *E. coli* also shows hypersensitivity reactions, thus requiring a shift to another form of L-asparaginase [9]. The hypersensitivity is associated with the production of antibodies, which may reduce the activity of L-asparaginase, causing the increase of the amino acid asparagine and possible development of drug resistance [10]. L-asparaginase also contributes to the activity of glutaminase, rapidly reducing the circulating glutamine concentrations in the plasma of patients, since this is converted into glutamic acid and ammonia [11]. The toxicity of asparaginase is partly attributable to the glutaminase activity of these proteins, and the cytotoxicity of L-asparaginase is determined primarily by its glutaminase activity [12]. Therefore, the activity of glutaminase in therapeutic preparations of L-asparaginases has been implicated in the cause of some side effects [1].

The extensive research is, therefore, going on worldwide with eukaryotic L-asparaginases looking for less adverse effects than those observed for bacterial enzymes [2,13]. However, few reports are available for the extracellular secretion of L-asparaginases by fungi, their characterization, and purification. The search for an L-asparaginase producing organism with no glutaminase activity, relevant biochemical characteristics, and high purification yields is a continuous exercise.

This paper deals with the purification and biochemical characterization of a glutaminase-free L-asparaginase from *Aspergillus oryzae* CCT 3940 (*A. oryzae*) and its anti-proliferative activity against human cancer cell lines in comparison with L-asparaginase from *E. coli* and the cytostatic antibiotic doxorubicin.

## 2. Materials and methods

### 2.1. Chemicals

Commercial L-asparaginase from *E. coli* was purchased from Megazyme (Wicklow, Ireland). Nessler's reagent was purchased from Merck® (Darmstadt, Germany); L-asparagine and L-glutamine were purchased from Sigma–Aldrich® (Missouri, USA). Other reagents were obtained from Synth® (São Paulo, Brazil).

### 2.2. Microorganisms

The filamentous fungi *A. oryzae* CCT 3940 was previously selected as an L-asparaginase producer strain from the Culture Collection of André Tosello Foundation, Campinas, SP, Brazil. The strain was periodically subcultured and maintained on potato dextrose agar slants.

### 2.3. L-asparaginase production

Conidial suspension was prepared from freshly raised seven-day-old culture on potato dextrose agar medium with a 5 mL of sterile 0.3% Tween 80 solution. The suspension was aseptically transferred to 250 mL Erlenmeyer flask containing 50 mL of modified and previously optimized Czapek Dox medium [14] composed of 5.0 g glucose, 10.0 g proline, 2.0 g L-asparagine, 5 g yeast extract, 1.52 g $KH_2PO_4$, 0.52 g KCl, 0.52 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CuNO_3 \cdot 3H_2O$, 0.01 g $ZnSO_4 \cdot 7H_2O$ and 0.01 g of $FeSO_4 \cdot 7H_2O$ per liter and initial pH adjusted to 7.0. The flasks were incubated at 30 °C and 150 r/min for 72 h. The fungi cultures were filtered through a paper filter, and the filtrate was called crude extract.

### 2.4. Purification of L-asparaginase

Crude extract filtrate was precipitated by adding ammonium sulfate with constant stirring until 80% saturation and incubated overnight at 5 °C to assure that the precipitation had come to completion. The precipitate was separated by centrifugation at 7 722 r/min for 30 min at 5 °C. The protein precipitate was resuspended in 0.01 mol/L Tris–HCl buffer (pH 8.0) and dialyzed against distilled water. The chromatographic purification steps were performed in a fast protein liquid chromatography (GE Healthcare, Uppsala, Sweden) with a flow rate of 1 mL/min, temperature of 20 °C and volume fraction of 0.5 mL. The elution of protein was estimated by measuring the absorbance at 280 nm (Beckman DU-640, CA, USA). The dialyzed enzyme was filtered through a 0.45 μm membrane filter (Millipore, Billerica, MA, USA) and loaded onto a Q Sepharose™ Fast Flow 1 mL column (GE Healthcare, Uppsala, Sweden) that was pre-equilibrated with 10 mmol/L Tris–HCl (pH 7.0). The bound enzyme was eluted with the NaCl gradient: 0–1.0 mol/L, in the same buffer. Fractions containing L-asparaginase activity were pooled, dialyzed against distilled water and concentrated by lyophilization. The lyophilized fraction was resuspended in Tris–HCl buffer (pH 7.0) and then it was applied to an SP Sepharose™ Fast Flow 1 mL column (GE Healthcare, Uppsala, Sweden) that was pre-equilibrated with 10 mmol/L Tris–HCl (pH 7.0). The protein elution was done with the same buffer, and the bound enzyme was eluted with a linear NaCl gradient: 0–1.0 mol/L. Eluted proteins were pooled, collected, dialyzed against distilled water and lyophilized. After resuspension in Tris–HCl buffer (pH 7.0), the enzyme was applied to the CM Sepharose™ Fast Flow 1 mL column (GE healthcare, Uppsala, Sweden) previously equilibrated with 10 mmol/L Tris–HCl (pH 7.0). The proteins were eluted with a linear NaCl gradient from 0.0 to 1.0 mol/L and collected in 0.5 mL fractions. The active fractions were pooled, dialyzed against distilled water concentrated by lyophilization and used for the characterization studies.

### 2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for purified L-asparaginase

The lyophilized L-asparaginase obtained from the CM Sepharose™ Fast Flow column was subjected to SDS-PAGE to

ascertain the homogeneity and estimate the molecular weight of the protein. SDS-PAGE was performed on a Mini Protean® TGX™ precast gel 10% Bio-Rad (Hercules, California, U.S.) which was employed to achieve separation of proteins [15]. All gels were run at 200 V for 35 min. Molecular mass markers (GE Healthcare, Uppsala, Sweden) ranging from 53 to 220 kDa were used, and molecular mass of the purified enzyme was determined by plotting the logarithm of protein molecular mass markers *vs*. the relative mobility of the protein. After run time, the gel was directly placed in the Coomassie brilliant blue R-250 staining solution for 1 h, destained three times, photographed while wet, dried and kept for the determination of relative mobility of the purified L-asparaginase.

### 2.6. L-asparaginase and L-glutaminase activity assay

L-asparaginase and L-glutaminase activity of the crude enzyme solution were assessed by Nesslerization [16]. The reaction medium was composed of 0.5 mL of 0.04 mol/L L-asparagine or of 0.04 mol/L L-glutamine, 0.5 mL of 0.1 mol/L pH 8.0 Tris–HCl buffer, 0.1 mL of enzyme extract and 0.9 mL of distilled water and was incubated at 40 °C for 30 min. The reaction was stopped by addition of 0.5 mL of 1.5 mol/L trichloroacetic acid. A 125 µL aliquot 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 for the quantification of ammonia released (20–450 µmol ammonia/L). The enzyme activity was expressed in IU/mL of the substrate, and one unit of enzyme activity (IU) was defined as the amount of enzyme that liberated 1.0 µmol of ammonia per minute under standard assay conditions.

### 2.7. Protein determination

The protein concentration was determined using Lowry method [17]. A calibration curve at 0.02–20.00 mg/mL ($r^2$ = 0.998 8) was made using bovine serum albumin as standard.

### 2.8. Biochemical characterization of L-asparaginase

#### 2.8.1. Effects of pH and temperature on the activity and stability of L-asparaginase determined using an experimental design

Univariate studies were previously performed to determine the central composite design (CCD) levels. The temperature was investigated over the range from 30 °C to 70 °C, and the pH was studied over the range from 3.0 to 10.0.

The optimum pH and temperature for activity and stability were determined using a CCD with three replicates at the central point and four axial points (a total of 11 runs). The enzyme was incubated for 1 h at various pH values (7.0–9.0 for optimum activity and 6.0–10.0 for stability studies) and temperature values (40–60 °C for optimum activity and 30–70 °C for stability studies) to investigate the L-asparaginase stability. The experiments were randomized to maximize the variability in the observed responses caused by exogenous factors. A second-order model equation was used for this model, represented by equation (1):

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} x_i j_i \quad (1)$$

where Y is the estimated response, i and j are values from one to the number of variables (n), $\beta_0$ is the intercept term. $\beta_i$ values are the linear coefficients, $\beta_{ij}$ values are the quadratic coefficients, and $x_i$ and $x_j$ are the coded independent variables. The coefficient of determination R and the *F* test (ANOVA) were used to verify the quality of the fit of the second-order model equation. The relationships between the responses and the variables were determined using the Statistica® 7.0 software package from StatSoft Inc.

#### 2.8.2. Effects of metal ions and inhibitors

The impact of different metal ions and inhibitors on L-asparaginase activity was investigated at a final concentration of 0.05, 0.5 and 5.0 mmol/L, incubating at 25 °C for 60 min under the standard assay conditions. The relative activities were determined by considering 100% activity of the enzyme in the absence of additives. Each experiment was performed in triplicate.

#### 2.8.3. Kinetic parameters

Kinetic parameters were determined using the substrate L-asparagine (0–3 mmol/L) and L-glutamine (0–40 mmol/L), separately. The Michaelis–Menten constant (Km) and maximum velocity (Vmax) were determined as the absolute reciprocal values of the intercepts on the x and y-axes, respectively, of the linear regression curve [18]. The measurements were made at the optimum temperature and pH as previously determined.

### 2.9. Antiproliferative activity

#### 2.9.1. Cell lines

Human tumor cell lines [U251 (glioma), UACC-62 (melanoma), NCI-ADR/RES (ovary with the multidrug resistance phenotype), 786-0 (kidney), NCI-H40 (lung, non-small cell type), PC-3 (prostate), OVCAR-03 (ovarian), HT29 (colon), K562 (leukemia)] were kindly provided by National Cancer Institute (Frederick, MA, USA). Non-tumor cell line HaCat (human keratinocytes) was donated by Prof. Dr. Ricardo Della Coletta, FOP/UNICAMP. Stock cultures were grown in medium RPMI 1640 (GIBCO) supplemented with 5% fetal bovine serum (GIBCO) and 10 IU/mL penicillin, 10 µg/mL streptomycin at 37 °C in 5% $CO_2$.

#### 2.9.2. Antiproliferative assay

Cells in 96-well plates (100 µL cells/well) were exposed to L-asparaginase (isolated from *A. oryzae* and the commercial one, final concentration 2.0–20.0 IU/mL) at 37 °C, 5% of $CO_2$ in air for 48 h. Doxorubicin was used as standard (0.025, 0.25, 2.5 and 25 µg/mL). Before (T0 plate) and after sample addition (T1 plate), cells were fixed with 50% trichloroacetic acid and cell growth was determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay [19]. The concentration that produces total growth inhibition (TGI) was determined through non-linear regression analysis using the concentration–response curve for each cell line in the software Origin 8.0® (OriginLab Corporation) [20].

### 2.10. Statistical analysis

All values reported in biochemical characterization of the enzymes represent the mean from three replicates. Significant differences ($P < 0.05$) between enzymes biochemical properties were determined by ANOVA. Statistical analyses were performed using the Statistica® 7.0 StatSoft Inc software (Tulsa, OK, USA).

## 3. Results

### 3.1. Purification of L-asparaginase and molecular mass estimation by SDS-PAGE electrophoresis

The L-asparaginase from *A. oryzae* CCT 3940 was purified 28.6 folds, with a yield of 6% (Table 1) after sulfate fractionation, Q Sepharose™ Fast Flow, SP Sepharose™ Fast Flow and CM Sepharose™ Fast Flow column chromatography (Figure 1). The purified L-asparaginase showed a specific activity of 282 IU/mL and the molecular mass was estimated as 115 KDa by SDS-PAGE.

### 3.2. Biochemical characterization of L-asparaginase

#### 3.2.1. Effects of pH and temperature on the activity and stability of L-asparaginase determined using an experimental design

The purified L-asparaginase from *A. oryzae* CCT 3940 exhibited optimum activity at pH 8.0 and 50 °C using univariate study (Figure 2). Based on these results, the levels of the experimental design were defined, and the CCD was used to verify possible interactions between the independent variables. The highest value obtained for the L-asparaginase activity was in run 10 (552.2 IU/mL), and the lowest was in run 4 (133.8 IU/mL) (Table 2). The relative standard deviation (%) of L-asparaginase activity values at the central point of CCD was 13%, indicating good repeatability for the method developed.

The linear (L) and quadratic (Q) terms for the pH and the quadratic term for temperature demonstrated a significant effect on L-asparaginase activity, with *P*-values below 0.05 (Table 3). An ANOVA showed that the model explained 89.12% of the total variation (Table 4). The *F* value calculated (8.19) for the regression was 1.37 folds higher than the *F* tabulated (3.45), reflecting a statistical significance of the model equation (Table 4).

The response surface and the contour plot were generated from the model (Figure 3a, b). The purified L-asparaginase from *A. oryzae* CCT 3940 was more active in the pH range 7.5–8.0 and the temperature range 45–50 °C. The temperature had a positive effect on L-asparaginase activity. Conversely, the pH had a negative effect.

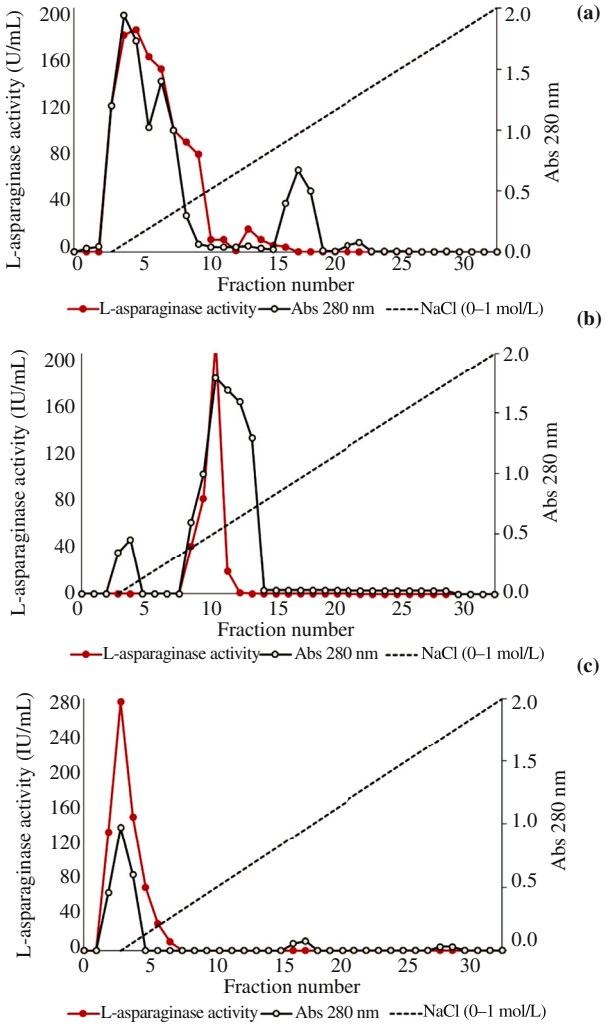


**Figure 1.** Chromatographic separation of L-asparaginase produced by *A. oryzae* CCT 3940.
a: Q Sepharose™ Fast Flow; b: SP Sepharose™ Fast Flow; c: CM Sepharose™ Fast Flow.

In the experimental design used to determine the pH and temperature stability for the L-asparaginase from *A. oryzae* CCT 3940, the highest L-asparaginase activity (539.4 IU/mL) was achieved in run 7, and the lowest value was 31.6 IU/mL in run 3 (Table 2). The central points (runs 9–11) showed little variation (relative standard deviation of 18%) indicating good repeatability for the method developed.

For the estimated regression coefficients for the L-asparaginase stability, only the intercept and the linear term of the temperature showed statistical significance with a *P* value < 0.05. The pH and temperature linear terms showed adverse effects on the L-asparaginase stability (Table 3).

**Table 1**
Summary of purification procedure of L-asparaginase from *A. oryzae* CCT 3940.

| Stages of purification process | Volume (mL) | Enzyme activity (IU/mL) | Protein (mg/mL) | Total activity (IU) | Total protein (mg) | Specific activity (IU/mg) | Yield (%) | Purification factor |
|---|---|---|---|---|---|---|---|---|
| Crude extract | 1 500 | 60.1 | 6.10 | 90 150 | 9 150 | 10 | 100 | 1.0 |
| Ammonium sulfate precipitation | 200 | 175.9 | 2.70 | 35 180 | 540 | 65 | 39 | 6.6 |
| Q Sepharose™ column | 60 | 182.3 | 2.00 | 10 938 | 120 | 91 | 12 | 9.3 |
| SP Sepharose™ column | 30 | 209.8 | 1.80 | 6 294 | 54 | 117 | 7 | 11.8 |
| CM Sepharose™ column | 20 | 276.1 | 0.98 | 5 522 | 20 | 282 | 6 | 28.6 |

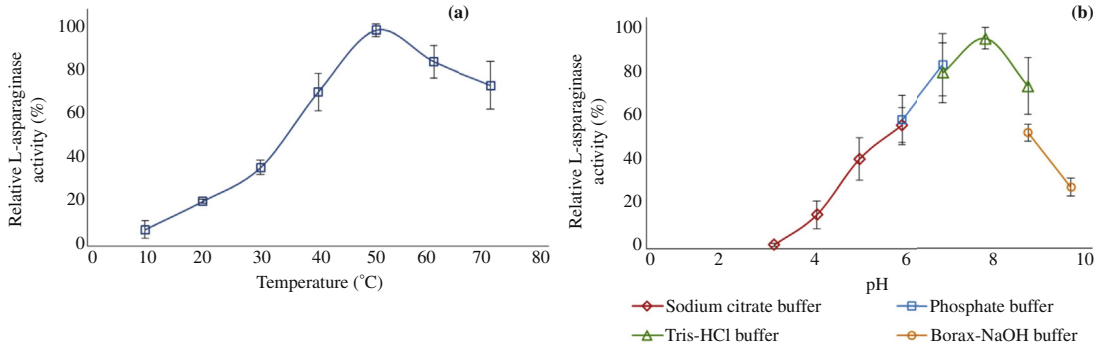


**Figure 2.** Effects of temperature (a) and pH (b) on the L-asparaginase activity from *A. oryzae* CCT 3940.

**Table 2**

CCD matrix for the determination of the optimum pH and temperature for activity and stability for the L-asparaginase from *A. oryzae* CCT 3940, with the coded and real values for the variables and responses.

| Run | Optimum pH and temperature for activity | | | Optimum pH and temperature for stability | | |
|---|---|---|---|---|---|---|
| | pH* | Temperature (°C)* | L-asparaginase activity (IU/mL) | pH* | Temperature (°C)* | L-asparaginase activity (IU/mL) |
| 1 | (7.3) −1 | (34.4) −1 | 177.7 | (6.6) −1 | (36) −1 | 490.3 |
| 2 | (8.7) +1 | (34.4) −1 | 164.3 | (9.4) +1 | (36) −1 | 528.0 |
| 3 | (7.3) −1 | (55.6) +1 | 378.1 | (6.6) −1 | (64) +1 | 31.6 |
| 4 | (8.7) +1 | (55.6) +1 | 133.8 | (9.4) +1 | (64) +1 | 58.9 |
| 5 | (7.0) −1.41 | (45) 0 | 368.6 | (6.0) −1.41 | (50) 0 | 405.1 |
| 6 | (9.0) +1.41 | (45) 0 | 167.6 | (10.0) +1.41 | (50) 0 | 175.0 |
| 7 | (8.0) 0 | (30) −1.41 | 227.4 | (8.0) 0 | (30) −1.41 | 539.4 |
| 8 | (8.0) 0 | (60) 1.41 | 443.3 | (8.0) 0 | (70) 1.41 | 112.5 |
| 9 | (8.0) 0 | (45) 0 | 428.0 | (8.0) 0 | (50) 0 | 268.0 |
| 10 | (8.0) 0 | (45) 0 | 552.2 | (8.0) 0 | (50) 0 | 310.8 |
| 11 | (8.0) 0 | (45) 0 | 515.3 | (8.0) 0 | (50) 0 | 213.6 |

*: Real values are in the parenthesis followed by the coded values of the independent variables (pH and temperature) for the determination of the optimum pH and temperature for the activity and stability of the L-asparaginase from *A. oryzae* CCT 3940.

**Table 3**

Estimation of the coefficients obtained from the regression model in the CCD for the optimum pH and temperature for activity and stability for the L-asparaginase from *A. oryzae* CCT 3940.

| Factors | Optimum pH and temperature for activity | | | | Optimum pH and temperature for stability | | | |
|---|---|---|---|---|---|---|---|---|
| | Coefficients | Standard error | *t*-value | *P*-value | Coefficients | Standard error | *t*-value | *P*-value |
| Intercept | 498.84 | 41.33 | 12.07 | < 0.001 | 264.27 | 50.79 | 5.20 | 0.003 |
| pH (L) | −135.69 | 50.69 | −2.68 | 0.044 | −65.07 | 62.30 | −1.04 | 0.344 |
| pH (Q) | −275.92 | 60.49 | −4.56 | 0.006 | 10.34 | 74.34 | 0.14 | 0.895 |
| Temperature (°C) (L) | 118.94 | 50.69 | 2.35 | 0.066 | −383.58 | 62.30 | −6.16 | 0.002 |
| Temperature (°C) (Q) | −208.23 | 60.49 | −3.44 | 0.018 | 46.44 | 74.34 | 0.62 | 0.560 |
| pH × temperature | −115.45 | 71.58 | −1.61 | 0.168 | −5.21 | 87.97 | −0.06 | 0.955 |

L: Linear terms; Q: Quadratic terms.

**Table 4**

ANOVA of a second-order polynomial model for the optimum pH and temperature activity and the stability for L-asparaginase from *A. oryzae* CCT 3940.

| Source of variation | Optimum pH and temperature for activity | | | | | Optimum pH and temperature for stability | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Sum of squares | Degrees of freedom | Mean of squares | *F* test | *F* tabulated | Sum of squares | Degrees of freedom | Mean of squares | *F* test | *F* tabulated |
| Regression | 209 882.9 | 5 | 41 976.6 | 8.19 | 3.45 | 304 899.6 | 5 | 60 979.93 | 11.9 | 3.45 |
| Residue | 25 621.7 | 5 | 5 124.3 | | | 38 693.5 | 5 | 5 124.3 | | |
| Total | 235 504.6 | 10 | | | | 343 593.1 | 10 | | | |

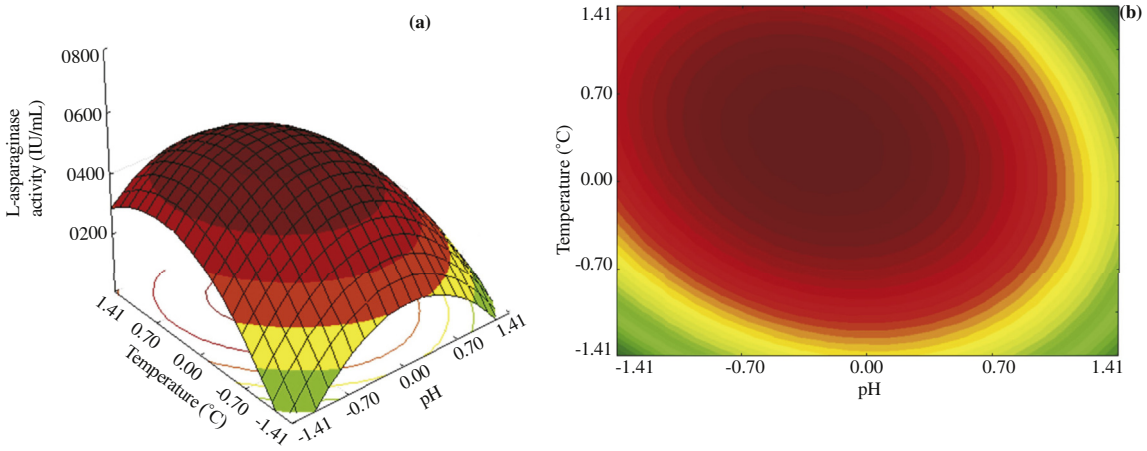


**Figure 3.** Response surface (a) and contour plots (b) for L-asparaginase optimum activity as a function of pH and temperature.

The model developed could explain 88.7% of the variation in the results (Table 4). The value obtained in the *F* test (11.9) for the regression was 2.45 folds higher than the critical value, indicating great significance (Table 4).

The response surface and the contour plot of the second-order polynomial model were generated as a function of the independent variables (pH and temperature) (Figure 4a, b). An evaluation of the contour plot showed that the L-asparaginase was more stable in the pH range 6.0–7.0 after 1 h incubation at temperature range 30–40 °C, under the assay conditions (Figure 4). Moreover, the enzyme still showed 112.5 IU/mL (21% of the highest activity) of activity when incubated at 70 °C, at pH 8.0 for 1 h.

### 3.2.2. Effect of different inhibitors and metal ions on L-asparaginase activity

The purified L-asparaginase from *A. oryzae* CCT 3940 was not significantly affected by the presence of $MgSO_4$ and $MnSO_4$ (0.05 and 0.5 mmol/L). However, at 5 mmol/L those salts acted as activators for L-asparaginase activity, increasing the activity in 33% and 49%, respectively (Table 5). The presence of $NaSO_4$, $(NH_4)_2SO_4$ and NaCl (0.05, 0.5 and 5 mmol/L) did not affect the purified L-asparaginase activity; the same occurred in the presence of $CaCl_2$ (0.05 and 0.5 mmol/L), KCl and $CaCO_3$ (0.05 mmol/L). A slight decrease in L-asparaginase activity was observed within the presence of $FeSO_4$ (0.05, 0.5 and 5 mmol/L), $CuSO_4$, KCl, $CaCO_3$ (0.05 and 0.5 mmol/L) and $ZnSO_4$ (0.05 mmol/L) (Table 5). Moreover, the presence of $ZnSO_4$, $CuSO_4$ and $CaCl_2$ (5 mmol/L) acted as inhibitors of L-asparaginase activity reducing its activity to around 60%.

A slight decrease, around 30%, on L-asparaginase activity was observed in the presence of ethylenediaminetetraacetic acid, L-cysteine, *N*-bromosuccinimide, iodoacetamide, glutathione and urea (0.05, 0.5 and 5 mmol/L). The presence of *p*-chloromercuribenzoate (0.5 and 5 mmol/L) acted as a potent inhibitor, reducing about 53% and 45%, respectively of the L-asparaginase activity. The presence of sodium azide (0.5 and 5 mmol/L) also acted as an inhibitor, reducing about 50% of the L-asparaginase activity.

### 3.2.3. Determination of the kinetic parameters Km and Vmax

In the enzyme kinetic studies, the purified L-asparaginase from *A. oryzae* CCT 3940 demonstrated high affinity for the substrate L-asparagine with Km and Vmax values estimated in 0.66 mmol/L and 313 IU/mL, respectively (Figure 5). Conversely, for the substrate L-glutamine no activity was detected even when the glutamine concentration was increased to 40 mmol/L.

### 3.3. Antiproliferative activity

The cell growth data (%) were expressed in relation to controls cells ($T_0$ and $T_1$), and the doxorubicin profile is shown in Figure 6a. The assessment of *in vitro* antiproliferative activity

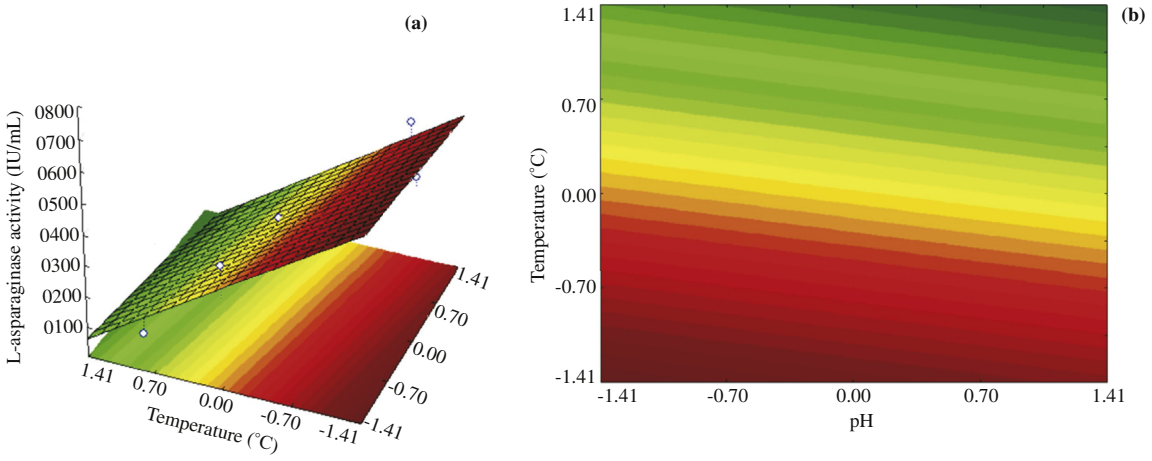


**Figure 4.** Response surface (a) and contour plots (b) for L-asparaginase stability as a function of the pH and the temperature.

**Table 5**

The effects of various salts and compounds on the activity of L-asparaginase from *A. oryzae* CCT 3940.

| Ions and inhibitors | Residual activity (%) | | |
|---|---|---|---|
| | 0.05 mmol/L | 0.5 mmol/L | 5 mmol/L |
| $MgSO_4$ | 99.42 ± 1.78 | 98.94 ± 1.25 | 133.64 ± 2.67 |
| $MnSO_4$ | 103.32 ± 2.06 | 101.17 ± 3.03 | 149.58 ± 2.99 |
| $CuSO_4$ | 87.75 ± 1.44 | 85.24 ± 2.55 | 57.50 ± 1.72 |
| $NaSO_4$ | 99.35 ± 1.78 | 105.17 ± 3.23 | 97.36 ± 2.62 |
| $ZnSO_4$ | 86.25 ± 2.58 | 58.75 ± 1.76 | 53.07 ± 1.79 |
| $(NH_4)_2SO_4$ | 92.24 ± 1.86 | 105.16 ± 2.14 | 99.125 ± 1.17 |
| $FeSO_4$ | 83.29 ± 1.89 | 77.13 ± 1.54 | 77.13 ± 1.54 |
| KCl | 98.08 ± 2.64 | 83.50 ± 1.67 | 66.04 ± 1.32 |
| NaCl | 97.31 ± 1.62 | 97.24 ± 1.46 | 95.38 ± 2.58 |
| $CaCl_2$ | 97.67 ± 1.51 | 95.48 ± 3.02 | 46.36 ± 1.21 |
| $CaCO_3$ | 96.38 ± 1.65 | 86.22 ± 0.89 | 80.14 ± 2.31 |
| EDTA | 90.54 ± 2.31 | 93.19 ± 1.78 | 92.10 ± 3.02 |
| L-cysteine | 78.81 ± 3.21 | 87.58 ± 2.21 | 85.28 ± 2.33 |
| *N*-bromosuccinimide | 91.24 ± 1.82 | 88.49 ± 1.92 | 89.84 ± 1.79 |
| *p*-Chloromercuribenzoate | 64.73 ± 1.29 | 46.87 ± 0.79 | 54.50 ± 1.09 |
| Iodoacetamide | 89.34 ± 1.78 | 86.36 ± 1.29 | 93.67 ± 2.27 |
| Sodium azide | 73.06 ± 2.12 | 49.02 ± 2.19 | 49.02 ± 1.89 |
| Urea | 82.49 ± 2.47 | 73.31 ± 1.46 | 73.32 ± 1.67 |
| Glutathione | 92.68 ± 2.23 | 95.33 ± 1.89 | 83.26 ± 3.45 |
| Control | 100.00 ± 1.20 | 101.00 ± 0.67 | 102.00 ± 1.44 |

EDTA: Ethylenediaminetetraacetic acid. Results are presented as the mean ± SD ($n$ = 3).

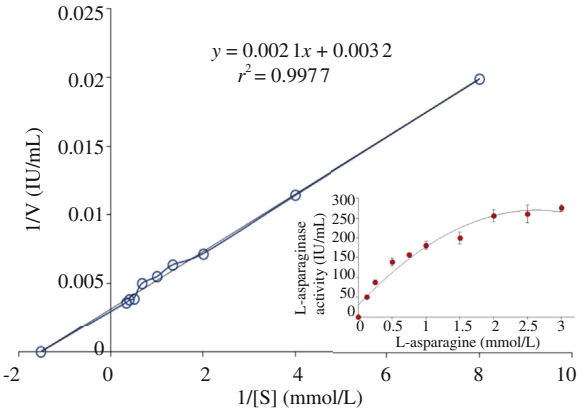


**Figure 5.** Lineweaver-Burk plot for L-asparaginase from *A. oryzae* CCT 3940.

was performed by 48 h of continuous exposure to *A. oryzae* CCT 3940 L-asparaginase and commercial enzyme (Figure 6b, c). Treatment of different human tumor cell lines with increasing concentrations of purified L-asparaginase from *A. oryzae* CCT 3940 resulted in significant cell growth inhibition (Figure 6c). The sensitivity of the cells lines to purified L-asparaginase from *A. oryzae* CCT 3940 appeared to be concentration dependent affording a more significant decrease in cell growth than that observed for the commercial L-asparaginase from *E. coli* (Table 6). The purified L-asparaginase from *A. oryzae* CCT 3940 completely inhibited the cell proliferation of UACC-62 (melanoma), 786-0 (kidney), NCI-H40 (lung, non-small cell type), PC-3 (prostate), NCI-ADR/RES (ovary with the multidrug resistance phenotype) and K562 (leukemia) cell lines with TGI values ranging of 3.20–10.00 IU/mL (Table 6). Otherwise, commercial L-asparaginase only inhibited the growth of UACC-62 [melanoma, TGI = (7.90 ± 4.00) IU/mL].

**Table 6**

Concentration required to completely inhibit cell proliferation (TGI) for doxorubicin, commercial L-asparaginase from *E. coli* and L-asparaginase from *A. oryzae* CCT 3940.

| Human tumor cell lines | Commercial L-asparaginase (IU/mL) | Purified L-asparaginase (IU/mL) | Doxorubicin (μg/mL) |
|---|---|---|---|
| U251 (glioma) | > 20 | > 20 | 0.05 ± 0.06 |
| UACC-62 (melanoma) | 7.90 ± 4.00 | 5.59 ± 0.06 | 0.04 ± 0.01 |
| NCI-ADR/RES (ovary*) | > 20 | 3.40 ± 4.10 | > 25 |
| 786-0 (kidney) | > 20 | 5.80 ± 2.40 | 0.70 ± 0.20 |
| NCI-H40 (lung, non-small cell type) | > 20 | 10.00 ± 1.80 | 0.20 ± 0.10 |
| PC-3 (prostate) | > 20 | 3.20 ± 4.10 | 0.55 ± 0.04 |
| OVCAR-03 (ovarian) | > 20 | > 20 | 1.70 ± 0.50 |
| HT29 (colon) | > 20 | > 20 | 2.70 ± 1.30 |
| K562 (leukemia) | > 20 | 3.20 ± 2.50 | 0.31 ± 0.06 |
| HaCat (immortalized keratinocytes) | > 20 | > 20 | 0.20 ± 0.20 |

Results are presented as the mean ± SD ($n$ = 3). *: With the multidrug resistance phenotype.

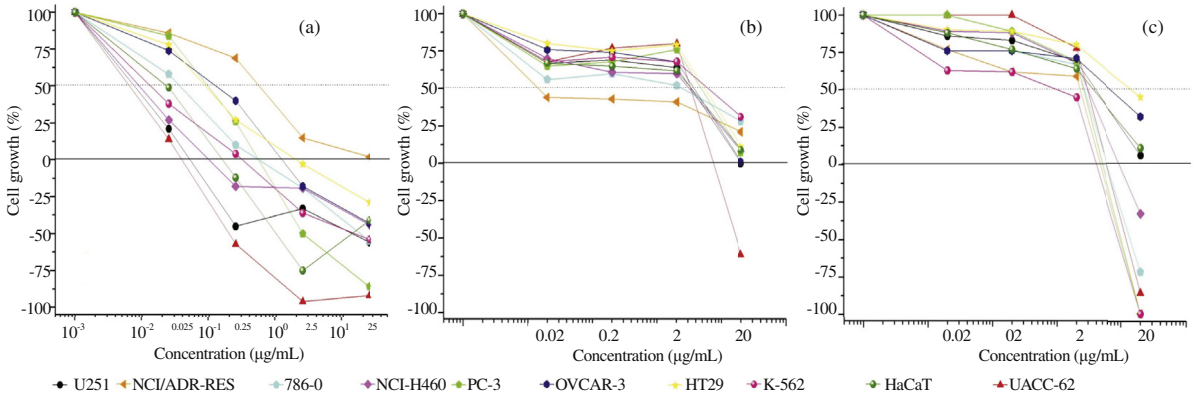


**Figure 6.** Antiproliferative activity of doxorubicin (a), L-asparaginase from *E. coli* (b) and purified L-asparaginase from *A. oryzae* CCT 3940 (c) against human tumor cells.

The cytostatic antibiotic doxorubicin could not inhibit cell growth of the ovary cell line with resistant phenotype (NCI-ADR/RES). However, the enzyme from *A. oryzae* CCT 3940 had greater efficacy against NCI-ADR/RES cell line with a TGI value (3.40 ± 4.10) IU/mL. Also, the enzyme from *A. oryzae* CCT 3940 could inhibit tumor growth of leukemia cell line (K562) with a TGI value of (3.2 ± 2.5) IU/mL, a value that corresponds to almost ten times the TGI value for doxorubicin. The doxorubicin inhibits the proliferation of human keratinocytes cell line (HaCat) with a small TGI. However, L-asparaginase from *A. oryzae* CCT 3940 did not inhibit these cell line growths (TGI > 20) at the concentrations studied. The enzyme purified from *A. oryzae* CCT 3940 could inhibit the growth of a broad range of human tumor cell lines at the concentrations studied.

## 4. Discussion

### 4.1. Purification of L-asparaginase and molecular mass estimation by SDS-PAGE

The molecular weight reported in the literature for the L-asparaginase from *Aspergillus terreus* (*A. terreus*) [21] and *A. terreus* PC-1.7 [22] was 94 kDa and 136 kDa, and the L-asparaginase from *Penicillium brevicompactum* NRC 829 inhibited molecular weight of 94 kDa [23]. Therefore, the value found in this work (115 kDa) is in accordance with those reported in the literature. L-asparaginase from *A. oryzae* CCT 3940 showed only one protein band on SDS-PAGE suggesting that it contained a single peptide chain.

The L-asparaginase from *A. oryzae* CCT 3940 presented a low yield value (6%) probably due to the four-step purification. The specific activity was higher than those reported for the L-asparaginase from *Mucor hiemalis* (*M. hiemalis*) (69.43 IU/mg) and for L-asparaginase from *Penicillium* sp (13.97 IU/mg) [24,25].

### 4.2. Biochemical characterization of L-asparaginase

L-asparaginase from *A. oryzae* CCT 3940 presented a higher optimum temperature and an intermediary optimum pH when compared with the values reported for L-asparaginase from *A. terreus* (25 °C, pH 6.0) [21] and for L-asparaginase from *Aspergillus niger* AK-10 (35 °C, pH 9.0) [26]. Also, the L-asparaginase from *A. oryzae* CCT 3940 showed high stability in the range from 30 to 45 °C and pH range 7.0–8.0 after 1 h of incubation meaning that the enzyme is stable under physiological conditions, which can be useful for further medical applications. Moreover, the enzyme still showed 112.5 IU/mL of activity (21% of the highest activity) when incubated at 70 °C and pH 8.0, indicating high thermal stability.

The $Mn^{2+}$ (2 mmol/L) acted as an activator for L-asparaginase from *M. hiemalis* and *Rhizomucor miehei* [24,27]. An increase in the L-asparaginase activity was also observed for the L-asparaginase from *A. oryzae* CCT 3940 in the presence of $Mn^{2+}$ (5 mmol/L). In this study, the L-asparaginase was strongly inhibited (50% of reduction) in the presence of *p*-chloromercuribenzoate and also inhibited (30% of reduction) in the presence of iodoacetamide (0.5 and 5 mmol/L). Inhibition in the presence of thiol group blocking reagents provided an indication of the presence of sulfhydryl groups in the catalytic activity of the enzyme [28].

The Km value (0.66 mmol/L) found for the purified L-asparaginase from *A. oryzae* CCT 3940 was lower than those reported for L-asparaginase from *M. hiemalis* (4.3 mmol/L) [24], for L-asparaginase from *A. terreus* (2.42 mmol/L) [22] and for L-asparaginase from *Penicillium brevicompactum* NRC 829 (1.05 mmol/L) [23]. The data indicated that the enzyme extracted from *A. oryzae* CCT 3940 is very specific to its natural substrate L-asparagine. Also, L-asparaginase from *A. oryzae* CCT 3940 exhibited no glutaminase activity even in an elevated concentration of glutamine. L-asparaginase preparations that do not show glutaminase activity have an excellent potential for medical applications [29].

### 4.3. Antiproliferative activity

The enzyme purified from *A. oryzae* CCT 3940 could inhibit the growth of a broad range of human tumor cell lines at the concentrations studied.

Gui *et al.* reported that commercial L-asparaginase from bacteria was effective in the treatment of primary lung lymphoma (NKT line) [30]. Shanmugaprakash *et al.* assessed the antiproliferative effect of L-asparaginase purified from pepper (*Capsicum annuum* L.) against three human tumor cell lines HeLa (cervical adenocarcinoma), A549 (lung) and KB (oral cell carcinoma) using a cell viability assay and concluded that treatment with L-asparaginase decreases the viability of all three tumor lines significantly with a dose-dependent response [31]. Lorenzi *et al.* studied the correlation of production of L-asparagine synthetase with the L-asparaginase activity and they reported that the human tumor cell lines OVACAR-03 (ovary) and NCI/ADR (ovary with resistant phenotype) required concentrations of 0.86 IU/mL and 0.20 IU/mL, respectively, for inhibiting the growth by 50% [32]. The enzyme purified from *A. oryzae* CCT 3940 reported here could completely inhibit the growth of human tumor cell line NCI/ADR (resistant phenotype with ovary) at a concentration of 3.5 IU/mL.

In conclusion, the purified glutaminase-free-L-asparaginase from *A. oryzae* CCT 3940 presented a molecular mass estimated in 115 kDa. L-asparaginase showed optimum activity in the pH range 7.0–8.0 and temperature range 40–50 °C. L-asparaginase showed high stability under physiological condition, remaining stable in the pH range 7.0–8.0 after 1 h incubation at temperature range 30–45 °C. The enzyme showed high affinity to L-asparagine with Km and Vmax values of 0.66 mmol/L and 313 IU/mL, respectively. The L-asparaginase was activated by the presence of $MgSO_4$ and $MnSO_4$, at a concentration of 5 mmol/L. Treatment of different human tumor cell lines with increasing concentrations of purified L-asparaginase from *A. oryzae* CCT 3940 results in significant cell growth inhibition. The purified L-asparaginase showed greater antiproliferative activity than the commercial enzyme, completely inhibiting the growth of UACC-62 (melanoma), NCI-ADR/RES (ovary with the multidrug resistance phenotype), 786-0 (kidney), NCI-H40 (lung, non-small cell type), PC-3 (prostate) and K562 (leukemia) cell lines. The cytostatic antibiotic doxorubicin could not inhibit cell growth of the ovary with the multidrug resistance phenotype (NCI-ADR/RES). The enzyme from *A. oryzae* CCT 3940 had greater efficacy with a TGI value of 3.5 IU/mL. Also, the enzyme from *A. oryzae* CCT 3940 could inhibit tumor growth of leukemia cell line (K562) with a TGI value of (3.2 ± 2.5) IU/mL.

The doxorubicin inhibits the proliferation of human keratinocytes cell line (HaCat) with a small TGI. However, L-asparaginase from *A. oryzae* CCT 3940 did not inhibit these cell line growths (TGI > 20) at the concentrations studied. The antiproliferative effect of the enzyme seems to be dose-dependent. Therefore, the new purified L-asparaginase from *A. oryzae* CCT 3940 has high value for future application in the clinical investigation. The production of the free-glutaminase L-asparaginase stable at physiological temperature suggests that this enzyme could be used in the formulations for the therapy of acute lymphoblastic leukemia and could be less susceptible to cause side effects, which makes it advantageous for future clinical investigation.

## Conflict of interest statement

We declare that we have no conflict of interest.

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