PRODUCTION OF FUNGAL L-ASPARAGINASE FOR BIOTECHNOLOGICAL APPLICATIONS

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ABSTRACT

The study investigated the production of L-asparaginase by 25 filamentous fungi and highlighted *C. subuliforme* as the most promising. After characterization and standardization of the enzymatic assay, the *C. subuliforme* L-asparaginase activity in the crude was 1.048 U/mL. The enzyme was purified 10.62 times with a yield of approximately 40% and a specific activity of 13.64 U/mg. The SDS-PAGE analysis showed a 56 kDa protein band relative to L-asparaginase. The L-asparaginase exhibited maximum performance at pH 8.0 and 50-60 °C, showing considerable thermostability, with half-life times of 49.51 h; 46.21 h and 7.29 h at 37 °C, 50 °C, and 60 °C, respectively. The enzyme showed low sensitivity to inhibitors SDS, DTT, and urea. These enzyme properties, especially thermostability at 37 °C and 50 °C, which are the temperatures for application of this enzyme, indicate its potential for biotechnological proposals. The fungus *C. subuliforme* emerges as a promising source of L-asparaginase for pharmaceutical and food applications.

Keywords: *Cladosporium subuliforme*. L-asparaginase. Purification. Characterization. Thermostability.

1 INTRODUCTION

L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) catalyzes the hydrolysis reaction of L-asparagine residue into ammonia and aspartic acid. It can be found in bacterias, fungi, yeasts, actinomycetes, algae, plants, and animals.[1] In the food industry, L-asparaginase (L-ASNase) is employed in the pretreatment of foods containing L-asparagine and reducing sugars in their composition[2], which when thermally processed above 120 °C are susceptible to the Maillard reaction, resulting in the formation of acrylamide, that is a probable human carcinogen.[3] The use of L-ASNase has been shown to be efficient in reducing acrylamide levels, since it acts by reducing the amount of L-asparagine and does not alter the sensory and nutritional food properties.[4] In the pharmaceutical field, this enzyme acts as the main antitumor agent against acute lymphoblastic leukemia. When injected into the bloodstream, L-asparaginase hydrolyzes L-asparagine, making it unavailable to cancer cells; thus, tumor cells undergo apoptosis due to amino acid deprivation.[5]

The commercial L-asparaginases are derived from bacterial (*Escherichia coli* and *Erwinia chrysanthemi*) and fungal sources (*Aspergillus niger* and *Aspergillus oryzae*). However, these commercial enzymes are not sufficient to meet market demand, and those of bacterial origin are reported to cause side effects in human health.[6] Fungi are interesting alternative sources for L-asparaginase production, since fungal enzymes are extracellular, which facilitates extraction and purification processes, and cause less damage to health.[7] The objective of this work was to produce a fungal L-asparaginase with suitable properties for biotechnological application.

2 MATERIAL & METHODS

Fungus selection. The total of 25 fungal isolates were subcultured on plates containing potato dextrose agar (PDA) for 7 - 15 days at 28 °C and subjected to screening on plates containing modified Czapek Dox agar (MCDA) with 0.009% phenol red indicator.[8] The fungi that tested positive were subjected to submerged cultivation. Fungal mycelium discs were transferred to flasks containing 50 mL of modified Czapek Dox broth (MCDB), without indicator and maintained at 28 °C, 150 rpm for 7 days. The cultures were filtered, centrifuged and the supernatant was used as crude enzymatic extract. The L-ASNase activity was determined following previous methodologies with slight modifications.[9] The enzymatic assay contained 0.04 M L-asparagine, appropriate volume of crude extract and 0.05 M Tris-HCl buffer pH 7.2 and was carried out at 37 °C. After 1 hour, 1.5 M trichloroacetic acid was added to stop the reaction. The volume of 20 µL of the supernatant was collected and mixed with Nessler's reagent, followed by incubation for 20 minutes at room temperature. The released ammonia was measured spectrophotometrically at 450 nm. One unit of enzyme (U) was defined as the amount of enzyme capable of generating 1 µmol of ammonia per minute under the assay conditions.

Standardization of the L-ASNase reaction assay and protein quantification. The L-ASNase assay with the crude extract of the fungus *Cladosporium subuliforme* was standardized by analyzing variations in L-asparagine concentration (0.04 M - 0.4 M), temperature (28 ºC – 80 ºC), pH (2.2 - 10.0) and reaction time (15 min - 120 min). Protein quantification was performed using the Coomassie Brilliant Blue reagent.[10]

Purification of L-ASNase and SDS-PAGE. The crude extract was initially concentrated 10 times by ultrafiltration (Amicon® Ultra-15 30 kDa Millipore) and subjected to fast protein liquid chromatography (FPLC) analysis using an ion exchange column (HiTrapTM DEAE FF 5 mL). The fractions eluted with gradient from 0 – 1 M NaCl were analyzed for protein concentration and L-ASNase activity. The active samples were analyzed by SDS-PAGE.[11]

Characterization of purified L-ASNase. The purified enzyme was characterized for the effects of pH (2.2 to 13.0), temperature (28 ºC to 80 ºC.) and the presence of several compounds on enzymatic activity using the L-ASNase standard assay. For thermostability analysis, purified L-ASNase was pre-incubated at 37 ºC, 50 ºC and 60 ºC and the residual activity was determined. To investigate the pH stability, the enzyme was kept for 1 hour in an ice bath containing different buffers at pH values from 2.2 to 13.0 and residual activity was determined. The effects of $FeSO_4$, $CaCl_2$, $MnCl_2$, $MgSO_4$, $CoCl_2$, $CuSO_4$, KI, $ZnSO_4$, $AlCl_3$, NaCl, EDTA, DTT, SDS, and urea at 5 mM on enzyme activity and the specificity of the enzyme to the substrates L-asparagine and L-glutamine at 0.08 M were determined. The K_{Mapp} and V_{max} values of the enzyme against L- asparagine (0.04 M - 0.2 M) were calculated using SigmaPlot 15 software.

3 RESULTS & DISCUSSION

From the 25 isolates tested, 13 provided positive results from the qualitative plaque assay. After cultivation of the selected fungi under submerged fermentation for 7 days, the L-ASNase activity was detect in crude extract of 9 fungi. *Cladosporium subuliforme* showed the highest enzymatic activity, 0.11 U/mL (Figure 1).

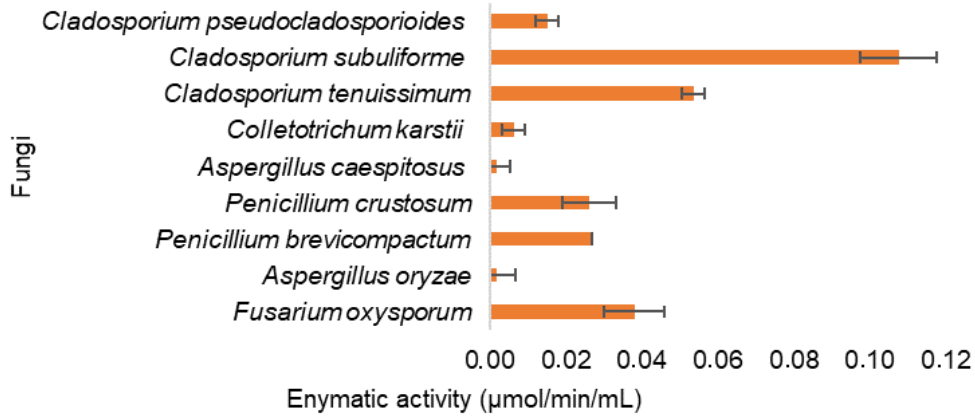

Figure 1 Quantitative assay of L-ASNase activity detected in crude extracts of fungi cultivated under submerged fermentation.

After standardizing the enzymatic assay, the parameters that promoted the best enzyme performance were: pH 6.0, 50 ºC, 0.08 M L-asparagine and a reaction time of 30 minutes. Using the standard enzyme assay, the L-ASNase activity of the *C. subuliforme* crude extract was 1.048 U/mL.

In addition, through a single purification step, the L-ASNase was purified 11 times, with a yield of 38.23% of activity. The SDS-PAGE analysis of the purified sample allowed the visualization of one more evident protein band with molecular weight of 56 kDa, relative to L-ASNase (Figure 2).

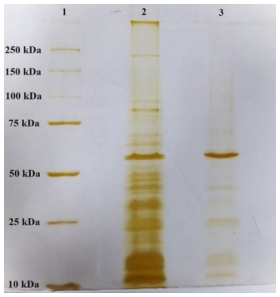

Figure 2 SDS-PAGE gel electrophoresis (12%) of samples containing L-ASNase from *C. subuliforme*. 1 – Molecular weight marker; 2 – Concentrated crude extract; 3 – Sample after the purification step by ion exchange chromatography, DEAE.

As shown in Table 1, the purified L-ASNase presented its highest enzymatic performance at 50 and 60 ºC and showed good thermostability, with a half-life of 49.51 h at 37 ºC, 46.21 h at 50 ºC and 7.29 h at 60 ºC. The enzyme had greater activity at pH 8.0 and maintained more than 80% activity between pH 7.0 and 12.0. The higher thermostability of the enzyme at 50 ºC and 37 ºC, which are the temperatures used for its application in food processing and for medicinal purposes, respectively, suggest that the L-ASNase has potential for biotechnological application.

Table 1 Results obtained from the biochemical characterization of L-asparaginase from *Cladosporium subuliforme*.

Purified L-asparaginase from *Cladosporium subuliforme*	
Optimal temperature	Between 50 ºC and 60 ºC
Half life at 37 ºC	49.15 hours
Half life at 50 ºC	46.21 hours
Half life at 60 ºC	7.29 hours
Optimal pH	pH 8.0
pH stability	Maintains more than 80% activity at pH 7.0 – 12.0

The enzymatic activity was increased in the presence of $MnCl_2$, $FeSO_4$ and $MgSO_4$ and significantly inhibited by $CoCl_2$ and $CuSO_4$. About 80 % of residual activity was maintained in the presence of $ZnSO_4$, $CaCl_2$, KI and $AlCl_3$ and NaCl did not affect the enzyme activity. The enzyme activity was inhibited by EDTA at 5 mM, and SDS, urea and TDT in this concentration. However, at at 20 mM SDS and urea reduced the activity by approximately 30% and 40%, respectively, while DTT completely inhibited the enzyme. The K_{Mapp} and V_{max} of the enzyme with the substrate L-asparagine were 0.025 M and 0.98 µmol/mL/min. The enzyme specificity was 5 times higher against L-Asn, compared to L-Gln, which is a characteristic interesting for the pharmaceutical sector, since L-glutaminase activity is generally accompanied by undesirable side effects for patients undergoing cancer treatment.[12]

4 CONCLUSION

The evaluation of filamentous fungi as alternative sources for L-ASNase production showed that *C. subuliforme* stood out with the higher capacity for producing the enzyme within the established conditions. *C. subuliforme* is poorly reported regarding its ability to produce extracellular enzymes and it is one of the first studies that showed its asparaginolytic activity. The analysis of thermostability, pH stability and effect of several compounds on enzyme activity indicated significant enzyme stability, that is an attractive characteristic for the industrial sector. The results suggest that *C. subuliforme* is a good candidate for production of L-ASNase with suitable properties for biotechnological applications.

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