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Original Research Article

Production of L-asparaginase by *Aspergillus niveus* under solid-state fermentation using agroindustrial byproducts

Isabela Victorino da Silva Amatto[1], Luis Henrique Souza Guimarães[2]*

[1]Instituto de Química de Araraquara, Araraquara, São Paulo, Brasil
[2]Department of Biology, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Ribeirão Preto, São Paulo, Brasil

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***Correspondence:**
Dr. Luis Henrique Souza Guimarães,
E-mail: lhguimaraes@ffclrp.usp.br

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ABSTRACT

Background: L-asparaginase, produced mainly by microrganisms, cleaves L-asparagine to aspartic acid and ammonia as products. This enzyme has been applied in the treatment of the leukemia and in food preparation preventing the acrylamide formation.
Methods: *Aspergillus niveus* was grown in different solid substrates (agroindustrial byproducts) moistened with different agents (tap water, distilled water and several salt solutions) for different periods (24-240 h) at 30ºC. The enzyme extract was obtained with the addition of cold distilled water, agitation at 50 rpm for 30 min and filtration. The filtrate was used to determine the L-asparaginase activity through the hydroximate aspartic methodology using L-asparagine as substrate. The influence of temperature (30-75ºC), pH (3-9) and chemical compounds on the enzyme activity was analyzed.
Results: The highest level of enzyme production was obtained using the M1 mixture (wheat bran, crushed soybean, orange peel; 1:1:1, w/w/w) as substrate humidified with Czapeck Dox salt solution (1:0.5, m/v) for 48-120 h, at 30ºC. The best temperature and pH for the enzyme activity were 35ºC and 5.0, respectively. The enzyme activity was increased in the presence of NaCl and some organic solvents (acetonitrile, butanol ethanol, isopropanol and methanol).
Conclusions: *A. niveus* produced L-asparaginase under SSF using a mixture of agroindustrial byproducts as solid substrate in the absence of L-asparagine as inducer. The temperature and pH of activity, as well as the NaCl tolerance, indicate its potential to be applied for different purposes. *A. niveus* can be an interesting source of L-asparaginase gene to be investigated targeting future application.

Keywords: *Aspergillus*, L-asparaginase, Solid-state fermentation

INTRODUCTION

L-aparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) is an enzyme with important pharmaceutical and food industrial application. It can be found in prokaryotic and eukaryotic (yeasts, filamentous fungi, plants and animals) organisms catalyzing the hydrolysis of L-asparagine to aspartic acid and ammonia as products.[1]

In the pharmaceutical industry, L-asparaginase is used as a therapeutic agent to treat leukemia, especially acute lymphoblastic leukemia (ALL), a disease that attacks mainly children and adolescents. This enzyme acts hydrolyzing L-asparagine in the circulatory system, depleting the concentration of this important amino acid for the synthesis of proteins in the tumor cells, blocking their proliferation once that these cells have no L-asparagine synthetase.[2] Nowadays, the main sources of L-

asparaginase for therapeutic application are bacteria, specifically *Escherichia coli* and *Erwinia chrysanthemi*.[2]

Regarding the food industry, L-asparaginase is used to hydrolyze the asparagine content in starch-rich food, impairing the formation of acrylamide, a toxic compound found in cooked and fried foods. On the other hand, neither the quality nor the characteristics of foods are impacted.[2,3] For such purpose, the recombinant L-asparaginase has been obtained from fungal strains, as *Aspergillus oryzae* and *Aspergillus niger*.[4]

The potential of filamentous fungi to produce L-asparaginase has been investigated using both submerged and solid-state fermentations.[5-8] The solid-state fermentation is characterized by the absence of free water in the process facilitating the substrate colonization by microorganism, especially filamentous fungi.[9] In addition, it allows the use of agro industrial byproducts as organic substrates, reduces the possibility of catabolic repression and inhibition by substrate and bacterial contamination.[9,10] For example, the fungus *Cladosporium* sp. was able to produce L-asparaginase through solid-state fermentation using wheat bran as substrate, while for enzyme production by *Aspergillus niger*, a mixture of soybean bran, wheat bran and cotton seed bran was used.[7,8] Considering the ability of production of extracellular enzymes by filamentous fungi and the potential of solid-state fermentation to screen more stable enzymes, this manuscript describes the production of L-asparaginase by filamentous fungus *Aspergillus niveus* under solid-state fermentation using a mixture of low-cost agroindustrial byproducts.

METHODS

Culture conditions

The solid-state fermentations were performed in 125-ml Erlenmeyer flasks containing 6 g of different agro-industrial byproducts (wheat bran, rice bran, rye flour, soybean flour, crushed soybean, orange peel, cassava husk and oat flakes) both isolated and combined (1:1:1, w/w/w) (M1: wheat bran, crushed soybean, orange peel; M2: rice bran, orange peel, crushed soybean; M3: cassava husk, wheat bran, crushed soybean; M4: soybean flour, oat flakes, wheat bran; M5: oat flour, oat flakes, cassava husk). The media were humidified with 3 ml of tap water or 100 mmol L^{-1} L-asparagine solution.

The influence of the proportion of the substrates in the M1 mixture upon enzyme production was analyzed. 6 g of the substrate mixture was used according to the proportions (w/w/w, wheat bran/crushed soybean/orange peel) 1:1:1, 0.5:1:1, 1:0.5:1, 1:1:0.5, 2:1:1, 1:2:1 and 1:1:2.

The substrates were sterilized at 121ºC, 1.5 atm for 30 minutes and later inoculated with a spore suspension (10^7 spores/ml). The cultures were maintained at 30ºC for different periods (24-240 h) as defined for each experiment.

Influence of the moistening agent on enzyme production

After the selection of the best substrate for enzyme production, the influence of different moistening agents (100 mmol L^{-1} asparagine solution, tap water, distilled water, Czapeck Dox salt solution, SR salt solution, Khana salt solution, Vogel salt solution and 10 mmol L^{-1} phosphate buffer) on enzyme production was analyzed. The influence of the proportion (w/v - 1:0.085, 1:0.167, 1:0.333, 1: 0.5, 1:0.667, 1:0.833, 1:1, 1:1.133, 1:1.5, 1:1.667, 1:1.833, 1:2) of the agent that allowed best enzyme recovery was also verified.

Obtaining of the extracellular extract

After cultivation, the cultures were added with 50 ml of cold distilled water and maintained under agitation at 140 rpm, for 20 minutes at 4ºC. Them, the cultures were harvested using a vacuum pump and the cell-free filtrate containing L-asparaginase was dialyzed against distilled water for 24 h at 4ºC, with 3 water replacements. The dialyzed filtrate was used for the determination of the L-asparaginase activity.

Determination of the L-asparaginase activity

The L-asparaginase activity was determined according to methodology described by Drainas et al through the formation of aspartic β-hydroxamate from L-asparagine and hydroxylamine.[11] The reaction was constituted by 300 µl 20 mmol L^{-1} Tris-HCl buffer, 100 µl of 100 mmol L^{-1} L-asparagine as substrate, 100 µl of 1 M hydroxylamine and 500 µl of enzymatic sample. The reaction was conducted at 37ºC for 15 minutes and stopped by the addition of 250 µl of a solution composed by 2.4% HCl, 10 % ferrous chloride and 5% TCA. The β-hydroxamate formed was determined at 500 nm and one unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 µmol of aspartic β-hydroximate per minute under the assay conditions.

Influence of temperature and pH on enzyme activity

The enzymatic reaction was conducted at different temperatures (30-80°C) and pH values (3-10), using 20 mmol L^{-1} citric acid buffer (pH 3-5), sodium acetate buffer (pH 5-7), Tris-HCl buffer (7-9) and glycine buffer (9-10).

Influence of different compounds on L-asparaginase activity

The influence of different salts at 1 mmol L^{-1} ($BaCl_2$, $CaCl_2$, $CuCl_2$, $FeCl_3$, $MgCl_2$, $MnCl_2$, NaCl and NH_4Cl), detergents (1 mmol L^{-1} SDS and 0.01% (v/v) Tween-20), organic solvents at 1% (v/v) (acetone, acetonitrile,

butanol ethanol, isopropanol and methanol), chelant (1 mmol L^{-1} EDTA) and denaturing agents at 1 mmol L^{-1} (urea and β-mercaptoethanol) on L-asparaginase activity was analyzed.

RESULTS

Production of L-asparaginase using SSF

The production of L-asparaginase by filamentous fungus *A. niveus* under SSF was influenced by the substrate used and by the moistening agent (Table 1). The best production was achieved using cassava husk (0.16 U/g of substrate) and the M1 mixture (wheat bran, crushed soybean, orange peel) (0.15 U/g of substrate) as substrates in the presence of tap water as moistening. The enzyme production in the presence of asparagine solution as moistening was reduced for all substrates used when compared to the tap water. The enzyme was not produced in the presence of oat flocks and M4 mixture.

Table 1: Influence of different solid substrates on the production of L-asparaginase under SSF, moistened with tap water or asparagine, by *A. niveus*.

Solid substrate	L-asparaginase activity (U/g of substrate)	
	Tap water	Asparagine
Crusehd soybean	0.02±0.01	0.03±0.01
Oat floks	0	0
Cassava husk	0.16±0.01	0.15±0.01
Wheat bran	0.04±0.01	0.02±0.01
Soybean flour	0.06±0.01	0.02±0.01
Rice bran	0.05±0.01	0.06±0.01
Orange peel	0.13±0.02	0.02±0.01
Rye flour	0	0.02±0.01
M1 mixture	0.15±0.01	0.12±0.02
M2 mixture	0.13±0.02	0
M5 mixture	0.05 ±0.01	0.03±0.01
M4 mixture	0	0
M5 mixture	0.06±0.01	0.04±0.01

Mixtures - M1: wheat bran, crushed soybean, orange peel; M2: rice bran, orange peel, crushed soybean; M3: manioc peel, wheat bran, crushed soybean; M4: soybean flour, oat flakes, wheat bran; M5: oat flour, oat flakes, cassava husk.

Table 2. Influence of different moistening agents on the production of L-asparaginase by *A. niveus* under SSF using the mixtures M1 and M2, and cassava husk as solid substrates.

Moistening	L-asparaginase activity (U/g of substrate)		
	M1	M2	Cassava husk
Asparagine solution	0.16±0.02	0	0.08±0.02
Tap water	0.18±0.02	0.17±0.01	0.08±0.01
Czapeck salts	0.29±0.01	0.18±0.02	0.09±0.01
SR salts	0.22±0.01	0.18±0.02	0.08±0.01
Khanna salts	0.22±0.01	0.21±0.02	0.08±0.01
Vogel salts	0.14±0.02	0.05±0.02	0.07±0.01
Phosphate buffer	0.21±0.02	0.16±0.02	0.09±0.01
Distilled water	0.22±0.04	0.08±0.03	0.09±0.03

Mixtures - M1: wheat bran, crushed soybean, orange peel; M2: rice bran, orange peel, crushed soybean.

Considering that the best results for the production of L-asparaginase by *A. niveus* were obtained using cassava husk, and the M1 and M2 mixtures as substrates, the influence of different moistening agents on enzyme production was analyzed (Table 2). When cassava husk was used, the production of L-asparaginase was similar for all moistening agents used. However, for the M1 mixture as substrate, the best enzyme production was obtained using Czapeck Dox salt solution (0.29 U/ g of substrate) while for the use of M2 mixture, Khanna (0.21 U/g of substrate), Czapeck Dox and SR salt solutions allowed best L-asparaginase production (0.18 U/ g of substrate). The L-asparaginase production by *A. niveus* was also influenced by the proportion of the components of the M1 mixture and by the proportion of the Czapeck Dox salt solution as moistening agent, with maximal production obtained at 1:0.5:1 (wheat bran: crushed soybean: orange peel; w/w/w) and 1:0.5 (m/v), respectively (Figure 1).

The influence of the time of cultivation on L-asparaginase production by *A. niveus* was also analyzed

(Figure 2). Two peaks of enzyme production can be observed, one from 48-120 h and another at 192 h.

Influence of temperature and pH on L-asparaginase activity

The Figure 3 depicts the influence of temperature and pH on L-asparaginase activity. The highest enzyme activity was obtained at 35ºC and pH 5.0, respectively. When the enzymatic assay was conducted at 40ºC and 45ºC, the enzyme activity was maintained at about 85%, lowering with high temperatures.

Influence of different compounds on L-asparaginase activity

The results on the influence of different compounds on L-asparaginase activity in the crude filtrate previously dialyzed are presented in the Table 3. The L-asparaginase activity was positively influenced mainly by Na^{+} (+31%), and Mn^{2+} (+10%). On the other hand, Cu^{2+} reduced the enzyme activity about 23% and Fe^{3+} about 12%. The influence of the other salts used was not significant. Considering the effect of solvents, the presence of acetone promoted reduction of 22% of the L-asparaginase activity, while increment ranging from 41-46% was observed in the presence of butanol, ethanol, isopropanol and methanol. With acetonitrile, increment of 19% was observed in the enzyme activity. The use of SDS inhibited drastically the enzyme activity, while an increment was observed with Tween-20 (+34%). Increment of the L-asparaginase activity was also observed in the presence of EDTA (+19%) and urea (34%). In the presence of β-mercaptoethanol, the activity was reduced (-19%).

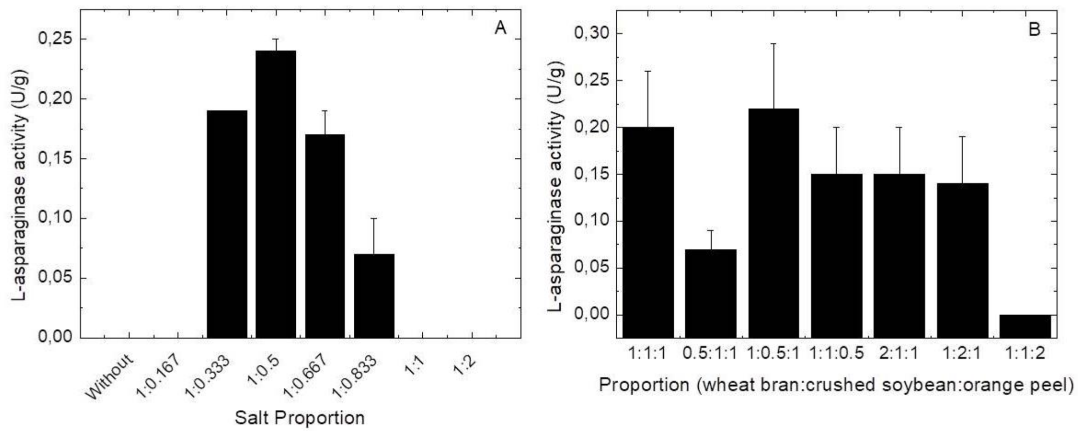

Figure 1: Influence of the proportion of the Czapeck Dox salt solution (A) and of the proportion of the components of the mixture M1 used as solid substrate (B) on the production of L-asparaginase by *A. niveus* under SSF.

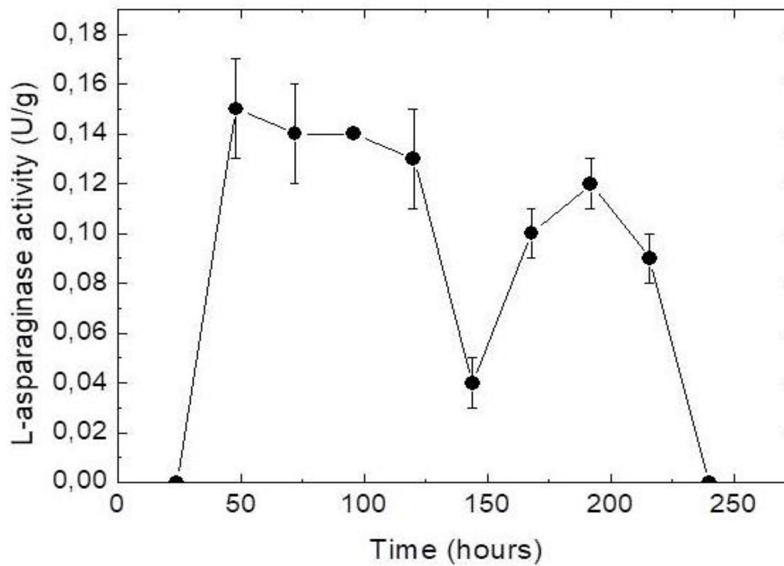

Figure 2: Influence of the period of cultivation on the production of L-asparaginase by *A. niveus* under SSF using the mixture M1 as solid substrate humidified with Czapeck Dox salt solution (1:0.5, w/v).

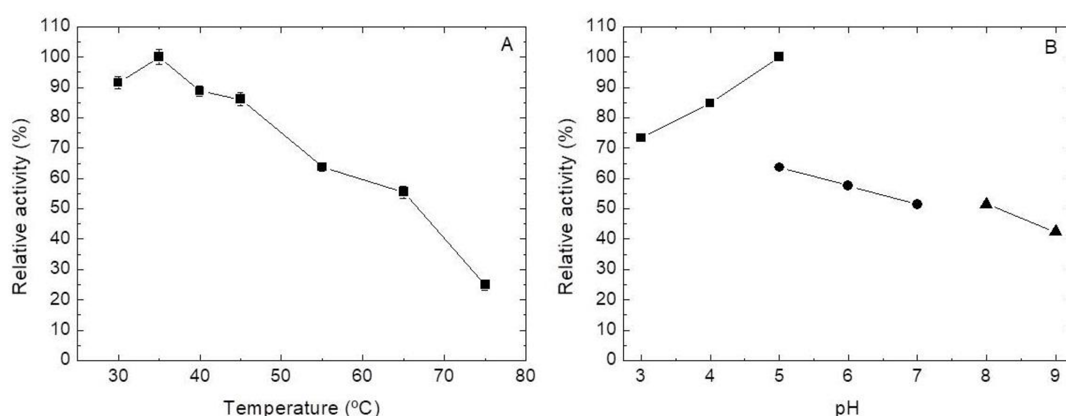

Figure 3: Influence of temperature (A) and pH (B) using citric acid buffer pH 3-5 (■), sodium acetate buffer pH 5-7 (●) and Tris-HCl buffer pH 8-9 (▲) on the L-asparaginase activity from *A. niveus*.

Table 3: Influence of different compounds on the L-asparaginase activity from *A. niveus*.

Compound	Relative activity (%)
Without	100
Salts (1 mmol L^{-1})	
$BaCl_2$	104.3±6.2
$CaCl_2$	92.4±7.6
$CuCl_2$	66.8±4.8
$FeCl_3$	88±3.8
$MgCl_2$	103.2±5.9
$MnCl_2$	110.4±6.9
NaCl	131.3±6.5
NH_4Cl	117±1.8
Chelant (1 mmol L^{-1})	
EDTA	119.8±9.2
Detergents	
SDS (1 mmol L^{-1})	0
Tween-20	134.8±6
Solvents (1%)	
Acetone	78.1±10.2
Acetonitrile	119.2±6.9
Butanol	141.8±7.5
Ethanol	141.8±6.7
Isopropanol	145.7±4.1
Methanol	146±5.3
Others (1 mmol L^{-1})	
Urea	134.9±6.2
Mecaptoethanol	81.7±1.1

DISCUSSION

Production of L-asparagimase by A. niveus under SSF

The best solid substrates for the L-asparaginase production by *A. niveus* under SSF were cassava husk and the mixture M1, composed of wheat bran, crushed soybean and orange peel. Cassava husk, the main byproduct of the cassava industry, when dehydrated, is composed of 58.1% of starch, 3.4% of crude protein and 28.6% of fiber.[12] Analyzing each of the constituents of the mixture M1, it is possible to verify their composition. Orange peel is constituted by phenolic compounds, flavonoids and soluble and insoluble fibers characterized by a mixture of carbohydrate polymers (oligosaccharides and polysaccharides), pectins, gums and some non-carbohydrates moiety.[13] Another component of the M1 mixture, soybean, is constituted mainly by protein (40-41%; dry seed basis), lipids (8.1-24%; dry seed basis), carbohydrates (35% dry seed basis) and minor constituents as phytic acid and isoflavones.[14] The last component, wheat bran, is composed of fibers (33.4-63% dry basis), ash (3.9-8.1% dry basis), protein (9.6-18.6% dry basis) and carbohydrates (60-75% dry basis), mainly starch (9.1-38.9% dry basis), and micronutrients such as phosphorus, zinc, manganese, magnesium, iron and vitamins.[15] All these components mentioned for both cassava husk and M1 mixture are important for fungal development and, consequently, enzyme production. The difference between the mixtures M1 and M2 is the presence of rice bran in the latter, while in mixture M1 wheat bran was used. Compared to wheat bran, rice bran presents minor proteins (16.6% dry basis) and carbohydrates (33% dry basis). Calcium, iron, sodium, zinc and potassium are also found in rice bran.[16] Apparently, orange peel is the main component of the mixtures M1 and M2 that allows better enzymatic production. Production of L-asparaginase by other filamentous fungi using SSF has been investigated. The fungus *Aspergillus niger* produced L-asparaginase using a mixture of wheat bran, crushed soybean and crushed cotton seed as substrate[8], while only wheat bran was used for the enzyme production by *Cladosporium* sp.[7]

Despite the nutrients available in the solid substrates, the fungal development can be improved by the addition of other micronutrients through the moistening agents. In general, Czapeck Dox salt was the moistening agent that

promoted the best enzymatic production for all solid substrates analyzed, especially the M1 mixture. This solution contains magnesium, cooper, zinc, iron, phosphate, sulfate and chloride, which complements the nutritional characteristics of the substrate for an efficient fungal development and enzyme production. According to Soccol et al, according to the substrate used in SSF, salt supplementation is necessary to dispose all nutrients for microbial growth.[17] Interestingly, the asparagine solution was not essential for L-asparaginase production by *A. niveus*. Under this condition, the absence of some specific nutrients impacted negatively upon enzyme production by the fungus.

The period of the cultivation of the microorganism is another important factor to be considered in the enzyme production. Two peaks of L-asparaginase production by *A. niveus* were obtained in SSF using the M1 mixture as solid substrate humidified with Czapeck Dox salt solution, what can be explained by the presence of two L-asparaginase isoforms with distinct periods for their maximal production. Production of L-asparaginase isoforms was also reported by Ahmed et al. (2015)[18] for the fungus *Aspergillus* sp. ALAA-2000. The period of L-asparaginase production using SSF can vary according to the fungal source. For example, the maximal production of L-asparaginase by *Fusarium culmorum* (ASP-87) was obtained with 144 h of cultivation, while for the enzyme produced by *A. niger*, 48 h of cultivation allowed the best production.[19,20]

Influence of the temperature and pH on L-asparaginase activity

According to Souza et al, most of L-asparaginase presents pronounced activity with temperature ranging from 30 to 50ºC.[21] The enzymes produced by the fungi *Mucor hiemalis*, *Penicillium brevicompactum* and *Tichoderma viride* presented best activity at 37ºC, while for the enzyme from *Aspergillus niger*, the best temperature was 40ºC.[6,22-24]

Considering the influence of pH on the L-asparaginase activity, different values have been reported as the best according to the enzyme source. For example, the highest L-asparaginase activity was observed at pH 5.8 for the enzyme produced by *Aspergillus terreus* and at pH 9.0 for the L-asparaginase from *Aspergillus niger*.[24,25] In addition, for the L-asparaginases produced by *Cladosporium* sp., *Mucor hiemalis* and *Talaromyces pinophilus*, the maximal activity was obtained at pH values of 6.3, 7.0 and 8.0, respectively.[7,22,26] L-asparaginases with potential of application in food industries should maintain their activity at a wide pH range. In spite of the better action at pH 5.0, the *A. niveus* enzyme was able to act in other pH values, maintaining its relative activity ranging from 50-70%, indicating its application potential. L-asparaginase was influenced not only by the pH value, but also by the buffer composition. Considering that the pH 5.0 was the best, there is a drastic difference between the activities in the presence of the sodium acetate buffer and citric acid buffer.

Effect of different compounds on L-asparaginase activity

The presence of ions can affect the enzyme activity positively or negatively, as observed for the L-asparaginase produced by *A. niveus*. The ions can interact either with the active site of the enzyme affecting the linkage of the substrate or with amino acid residues outside the active site affecting the enzyme conformation increasing or reducing the enzymatic activity. The increase of the enzyme activity in the presence of NaCl can be considered as an interesting characteristic for a possible pharmaceutical application of the *A. niveus* L-asparaginase because it would not be affected by the 0.9% saline concentration in the blood.[26] On the other hand, reduction of the enzyme activity in the presence of Cu^{2+} was also observed for the L-asparaginases produced by *Rhizomucor miehei*, *A. oryzae* and *A. terreus*.[25,27,28] The positive effect of the EDTA, a chelant, indicates that the *A. niveus* L-asparaginase is not a metalloenzyme, in spite of the activation observed in the presence of some ions. Differently, the L-asparaginase activity was reduced in the presence of EDTA for the enzymes produced by *A. niger* AKV-MKBU and by *R. miehei*.[27,29]

The *A. niveus* L-asparaginase was drastically reduced in the presence of SDS, as also observed by Vala et al, for the enzyme produced by *A. niger* AKV-MKBU.[29] The *Talaromyces pinophilus* enzyme was also inhibited in the presence of SDS.[26] The SDS is a denaturing agent that can interact with hydrophobic regions of the enzyme promoting denaturation or aggregation.[30] Considering other compounds, the *A. niveus* L-asparaginase was reduced in the presence of β-mercaptoethanol, indicating the presence of disulfide linkages important for the enzyme structure and maintenance of the catalytic activity. Reduction of the L-asparaginase activity was also observed for the enzymes from *A. niger* AKV-MKBU, *R. miehei* and *Cladosporium* sp.[27,29,31] In the presence of organic solvents, the enzyme activity was inhibited only in the presence of acetone. Interestingly, the activation in the presence of other solvents is not dependent on the hydrophobicity of the compound. The use of organic solvents can increase or reduce enzyme activity, affecting hydration of the enzyme. Some solvents also facilitate the solubility of the substrate, allowing its better interaction with the active site.[32]

CONCLUSION

The use of solid-state fermentation to find fungal sources of L-asparaginase, an enzyme with biotechnological importance, is an important procedure to discover fungi containing L-asparaginase genes that codify enzymes with distinctive characteristics to be used in heterologous expression aiming at future application. In this context, the fungus *A. niveus* is an interesting producer to be

investigated under molecular view. It was able to produce L-asparaginase using agro industrial by-products as solid substrate in the absence of L-asparagine as inducer. In addition, the enzyme presented characteristics (temperature and pH of activity, and tolerance to NaCl) that indicate its potential to be applied in different areas as pharmaceutical and food industries.

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