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CHARACTERIZATION OF AN ASPARTIC PROTEASE PRODUCED BY *Amylomyces rouxii*

CARACTERIZACIÓN DE UNA ASPARTATO PROTEASA PRODUCIDA POR *Amylomyces rouxii*

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Abstract

The zigomycete *Amylomyces rouxii* was isolated, in our laboratory, from effluents of paper industries, it was showed that this fungus produces extracellular tyrosinase. In this work, we show that this fungus produces also an extracellular protease. The extracellular protease was partially purified using two-step purification, by $(NH_4)_2SO_4$ fractionation and DEAE-sepharose anion exchange chromatography with 4.25-fold factor purification. The optimal pH for protease activity, using hemoglobin as substrate, was 3.5. Results from protein purification revealed that this protease is an aspartic protease. Protease activity was inhibited 73% by pepstatin A, a specific inhibitor of acid proteases. Results allow conclude that *A. rouxii* produce an extracellular aspartic protease that has similar characteristics to aspartic proteases produced by fungi as *Mucor* and *Rhizopus*.

Keywords: *Amylomyces rouxii*, aspartic protease, inhibitor protease.

Resumen

Amylomyces rouxii es un zigomiceto que fue aislado, en nuestro laboratorio, a partir de un efluente de la industria del papel, este hongo produce tirosinasa extracelular. En este trabajo demostramos que además de la tirosinasa *A. rouxii* produce una proteasa extracelular. Esta proteasa se purificó parcialmente en dos pasos, por precipitación con $(NH_4)_2SO_4$ y después se pasó a través de una columna de intercambio aniónico, el factor de purificación obtenido fue de 4.25. Su pH óptimo usando hemoglobina como sustrato fue de 3.5. Los resultados de la purificación de la proteína demuestran que se trata de una aspartato proteasa, la actividad proteasa fue inhibida en 73% por pepstatin A, un inhibidor específico para proteasas tipo ácido. Los resultados permiten concluir que *A. rouxii* produce una aspartato proteasa extracelular con características similares a las aspartato proteasas producidas por los hongos del género *Mucor* y *Rhizopus*.

Palabras clave: *Amylomyces rouxii*, aspartato proteasa, inhibidor de proteasa.

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1 Introduction

Amylomyces rouxii is a zigomycete isolated from effluents of paper industries (Tomasini *et al.*, 1996). *A. rouxii* has a high similarity with *Rhizopus oryzae* (Montiel *et al.*, 2004; Kito *et al.*, 2009). This fungus produces tyrosinase (Montiel *et al.*, 2004). Tyrosinase is used in food, cosmetic and pharmaceutical industries (Seo *et al.*, 2003; Halaouli *et al.*, 2006; Selinheimo *et al.*, 2006) and also has been used in bioremediation process of waste and soil containing phenolic compounds (Montiel *et al.*, 2004; Girelli *et al.*, 2006; Kim *et al.*, 2008).

On the other hand, many authors have reported fungal proteases produced by *Mucor* and *Rhizopus*, as it is known proteases are one of groups of enzyme most important in industrial processes. The fungal proteases are mainly proteases type acid and can substitute the role of three important protease involved in food processing, i.e. pepsin, rennin and papain. Acid proteases are the main type of protease secreted by *Mucor* and *Rhizopus* spp. Aspartic protease from *Mucor miehei*, *M. rennin* and *M. pusillus* have been studied (Gray *et al.*, 1986; Etoh *et al.*, 1982; Tonouchi *et al.*, 1986). Also some authors have reported the purification and characterization of aspartic proteases from *Rhizopus microsporus* var *rhizopodiformis*, *R. chinesis*, *R. hangchow*, *R. oryzae*, *Mucor* sp, and Rhizomucor *miehei* (Preetha and Boopathy, 1997; Fernández-Lahore *et al.*, 1999; Kumar *et al.*, 2005; Chen *et al.*, 2009). Aspartic proteases have been used as calf rennet substitutes in cheese production. This is a chief enzyme employed in cheese production, aspartic protease not only clots the milk but also play an important role during cheese maturation, which is a vital and complex process for the balanced development of flavor and texture (Fox *et al.*, 1993; Vioque *et al.*, 2000; Fernández-Lahore *et al.*, 1999; Kumar *et al.*, 2005). An aspartic protease from *Trichoderma harzianum* has been identified as a cell wall-degrading enzyme involved in biocontrol activities (Suárez *et al.*, 2005; Liu and Yang, 2007).

The aim of this work was to know if *A. rouxii* also produces some proteases, as well as to partially purify and characterize one of these proteases produced by *A. rouxii*.

2 Methodology

2.1 Strain, media, and growth conditions

A. rouxii, isolated from effluents of paper industries (Tomasini *et al.*, 1996), was used in this study. The strain was maintained by freezing spores suspension at 40% of glycerol at −20°C.

Erlenmeyer flasks containing (potato agar dextrose) PDA were inoculated and incubated at 30°C for 4 days to obtain spores. Spore suspension was obtained in 20 ml of sterile water containing 0.1% Tween-80 and it was used to inoculate the submerged culture for enzymes production. Cultures were performed in flasks containing 50 mL of modified Melin-Norkrans medium containing (g L^{-1}): glucose 5, malt extract 2, yeast extract 1, KH_2PO_4 0.5, $MgSO_4{\cdot}7H_2O$ 0.15, $(NH_4)_2HPO_4$ 0.5, pH 5.6. In this study, 0.1 g tyrosine L^{-1} and 0.0125 g pentachlorophenol L^{-1} were added to the medium, as reported by Montiel *et al.*, (2004) who showed that these compounds increased tyrosinase activity. Medium was inoculated with 1×10^6 spores mL^{-1}, and incubated at 30°C in a rotary shaker at 150 rpm (Tomasini *et al.*, 2001). The enzymes were harvested after 48 h of incubation.

2.2 Preparation of extracellular extract

Crude enzyme extract was obtained from 500 mL of broth culture from *A. rouxii* at 48 h. The culture medium was separated from the mycelium by filtration through Whatman filter paper 41; an aliquot of this crude extract was used to determine protease activity. The rest of the extract was frozen at −80°C and lyophilized. The lyophilized sample from broth cultures was suspended in 30 mL of 10 mM phosphate buffer pH 6.8. This extract was used for enzymatic activity determination, protein quantification, as well as for the partial purification and characterization of the extracellular protease. To conduct the inhibition studies the following substances were used: 1 mM PMSF, 1 mM EDTA, 3 mM pepstatin A and a commercial cocktail (200 μL concentration, according to sigma) that is a mixture of protease inhibitors, containing 4-2-aminoethyl-benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin and aprotinin (sigma, P8340).

2.3 Partial purification of extracellular protease

Proteins from the crude extract were precipitated with ammonium sulphate to 80% w/v saturation. The crude extract was then centrifuged at 20,000 g, for 30 min, at 4°C. The proteins were dialyzed against milli-Q water for 24 h using a 10-kDa membrane cut-off, with three repeated changes of water. Samples containing proteins were poured into an anion-exchange chromatographic column (DEAE-Sepharose, Amersham) equilibrated beforehand with 25 mmol L^{-1} sodium phosphate buffer, pH 7. Proteins were eluted with a linear gradient of 0-0.5 M NaCl in 25 mmol L^{-1} sodium phosphate buffer, pH 7, at a flow rate of 1 mL min^{-1}. Fractions of 5 mL were collected, and the protein concentration and protease activity was determined in each fraction. Fractions with enzymatic activities were concentrated using Microcon tubes YM-10 (0.5 mL Millipore).

The purity of proteins from fractions showing protease activity was analyzed in denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), performed with 10% acrylamide/gels according to Laemmli (1970). The gels were stained using Coomasie brilliant blue R-205 (Sigma) for 30 min followed by incubation over-night with destaining solution.

The protein of each fraction was monitored by measuring the absorbance at 280 nm. Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

2.4 Protease activity assay

The protease activity was determined according to the methods described by Anson (1938) and Asakura *et al.* (1997). Hemoglobin (2% w/v; Sigma, USA) dissolved in universal buffer, pH 3.5, and azocasein (1% w/v; Sigma, USA) dissolved in 0.2 M citrate buffer, pH 7.5, were used as substrates. Enzyme extract (0.25 mL) was added to 1 mL of substrate and incubated at 37°C in a water bath for 20 min. The reaction was stopped by adding 0.25 mL trichloroacetic acid (10% w/v) and centrifuged at 20,000 *g* for 15 min to remove the precipitate. When azocasein was used, one unit of protease activity was defined as the amount of enzyme required to liberate 1.0 μmol of tyrosine per minute at 37°C. For hemoglobin as substrate, one unit of protease activity was the amount necessary to produce an increase in absorbance of 0.01 at 280 nm after 20 min.

3 Results and discussion

3.1 Partial purification of the protease

Protease activity from extract crude, using azocaseine as substrate, was 0.706 ± 0.77 U mL^{-1} and, with hemoglobin, it was 273.86 ± 7.16 U mL^{-1}. Protease activity was 387-times higher when hemoglobin was used as substrate at pH 3.5 than when using azocasein as substrate at pH 7.0. Then, protease activities reported in the following results are determined only using hemoglobin as substrate.

The crude enzyme extract from the lyophilized sample was precipitated using 80% $(NH_4)_2SO_4$ and the enzyme was further partially purified by anion-exchange chromatography, DEAE-Sepharose, the procedure is summarized in Table 1. A single peak of protease activity was eluted in fraction 10, (Table 1, Fig. 1) with a 4.25-fold purification. This value of purification factor is similar to the value reported by an aspartic protease from *Centaurea calcitrapa*, obtained in the same purification step (Raposo and Domingos 2008). The molecular mass of the purified enzyme was approximately 40 kDa by SDS-PAGE (Fig. 2). These results strongly suggest that the protease produced by *A. rouxii* is an acid protease.

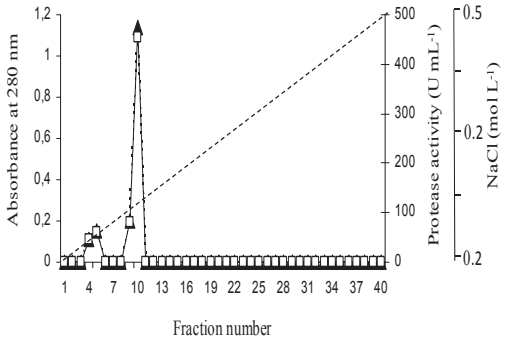

Fig. 1: Elution profile of proteins after anion-exchange chromatography on a DEAE-sepharose column. DEAE-sepharose was equilibrated with 25 mmol L^{-1} sodium phosphate buffer, pH 7. The elution was performed by increasing the NaCl concentration stepwise from 0 to 0.5 M. Fractions of 5 mL each were collected. (▲) O.D. 280 nm, (o) protease activity, (- - -) NaCl gradient.

Table 1. Partial purification of the protease from *A. rouxii*

Purification step	Vol (mL)	Protein Concentration (mg mL^{-1})	Protease Total Activity (U mL^{-1})	Activity Specific Activity (U mg^{-1} protein)	Purification (-fold)
Crude extract*	30	0.232	1542	6646	1
80% $(NH_4)_2SO_4$	10	0.176	1304	7409 1.1	
DEAE-Sepharose	3	0.016	452	28250	4.25

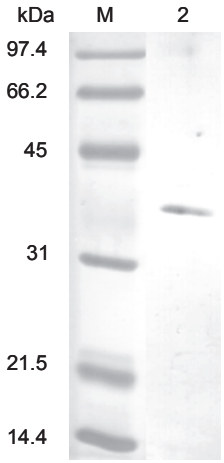

Fig. 2: SDS-PAGE (12% (w/v) gel) analysis for the purified fraction extracted from *Amylomyces rouxii*. Samples were stained with Coomassie brilliant blue. Lane 1 corresponds to a molecular mass marker and lane 2 to a purified protease after anion-exchange DEAE-sepharose column (fraction 10).

3.2 Identification of protein and amino acid sequence

Peptides sequence was attained by electrospray LC-MS/MS (IBT, UNAM). The N-terminal amino acid sequence of the purified protease showed 99% similarity to rhizopuspepsin-2 precursor (aspartate protease) of *Rhizopus oryzae*. These results confirm that the extracellular protease produced by *A. rouxii* is an acid protease, specifically an aspartic protease.

Based on its similitude with other fungal aspartic protease, the protease from *A. rouxii* was expected to contain 391 residues, with a molecular weight of 40 kDa.

Analysis of the amino acid sequence by SignalP V3.0 identified a cleavage signal-sequence site between positions TEA^{21} and A^{22}. The putative signal peptide corresponding to the first 21 amino acid shows typical features of signal peptides, such as a highly hydrophobic region and alanine residues at −3 and −1 position relative to the cleavage site (Nielsen *et al.*, 1997).

The amino acid sequence found in *A. rouxii* has a consistent degree of similarity with aspartate proteases, according to BlastP analysis (GenBank non-redundant protein sequences). The highest sequence identity was with the aspartic protease from *Rhizopus oryzae* (99%). Lower identity percentages were found with the aspartic protease from *Aspergillus oryzae* (40%), *Fusarium venenatum* (39%), *Hypocrea jecorina* (37%), and *Lentinula edodes* (32%), data shown in Fig. 3. In fungal aspartic proteases, the catalytic residue is contained within the conserved motif DTGS and DTGT as was found in the protease sequence from *A. rouxii* (Fig. 3). The residues involved in substrate specificity were present at positions Y^{144}, G^{145}, and D^{146}, which is conserved in fungal aspartic protease (Fig. 3); hydrophobic motifs responsible for substrate specificity were found also at positions LLD^{191} and IFD^{367}. Another important feature is related to disulfide bonds, the aspartic protease of mammalian origin contains three characteristic loops, the first loop is present in several fungal enzymes, such as *Trichoderma harzianum* (cystein residues C^{99} and C^{105}) (Suárez *et al.*, 2005) and in *R. oryzae* and *A. rouxii* at C^{115} and C^{118} positions. A second loop at C^{319} and C^{352} is also conserved in *A. rouxii* and R. *oryzae*.

3.3 Characterization of the aspartic protease from A. rouxii

The effect of pH on protease activity was studied using hemoglobin as substrate. The aspartic protease from *A. rouxii* was active in the pH range 2-4.5, with an optimal pH of 3.5 (Fig. 4a), which

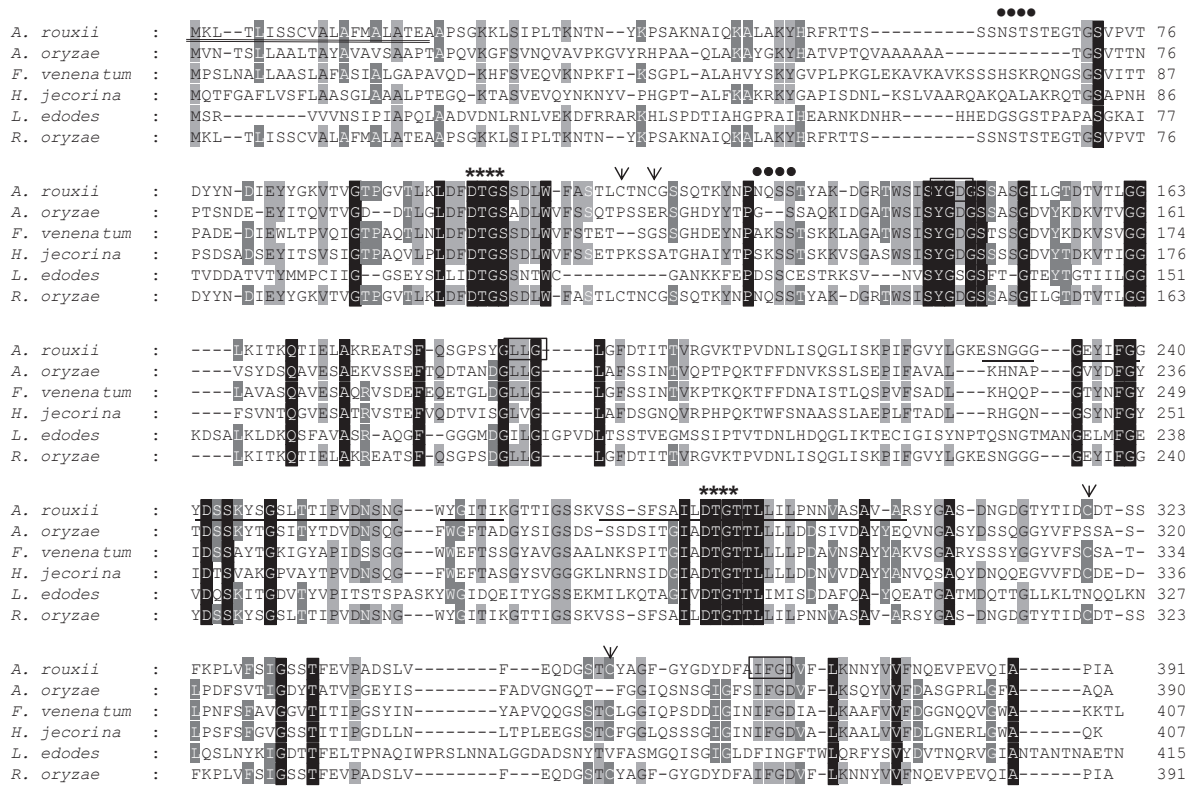

Fig. 3: Comparison of predicted amino acids sequence for the aspartic protease identified from *A. rouxii* with sequences of aspartic proteases from *A. oryzae* (GenBank Accession No. AAB35849.1), *F. venenatum* (GenBank Accession No. AF462062.1), *H. jeroquina* (GenBank Accession No. AM168137.1), *L. edodes* (GenBank Accession No. AB446454.1), *R. oryzae* (GenBank Accession No. FJ539004.1). Identical amino acids in all proteins are shaded in black. Those that are present in nearly all the proteins are shaded in grey. Peptide sequences obtained after LC/MS-MS analysis are underlined. The putative signal peptide is doubled-underlined. Asterisks indicate the catalytic motif. Residues involved in substrate specificity in members of the aspartic proteases family are boxed. Black dotted lines mark N-glycosylation sites. Cysteine residues conserved in *A. rouxii* are marked by a black square. Alignments were performed by the CLUSTALX V method using the MegAlign program of the informatics package DNASTAR (Lasergene).

is similar to the acid protease from *Aspergillus oryzae* MTCC 5341 (Vishwanatha *et al.*, 2009) and to the rhizopuspepsin from *Rhizopus oryzae* NBRC 4749 (Chen *et al.*, 2009). Maximal protease activity from *A. rouxii* was found in the temperature range from 37 to 50°C (Fig. 4b). The optimal temperature reported for the acid protease from *Aspergillus oryzae* MTCC 5341 is 55°C (Vishwanatha *et al.*, 2009) and for the rhizopuspepsin from *Rhizopus oryzae* NBRC 4749 is 50°C (Chen *et al.*, 2009). The activation energy of aspartic protease was 55.82 kcal mol^{-1}; this value was calculated from the data obtained of Fig. 4b using Arrhenius equation.

The thermal stability assays showed that the aspartic protease at 37°C retained 70% of the initial activity after 1 h incubation, and this activity was similar after 1 h, while 60 and 30% of the initial activity remained within 1 h of incubation at 40 and 50°C (Fig. 5). Similar thermal stability has been reported for *Rhizopus oryzae* and *Aspergillus oryzae* (Chen *et al.*, 2009, Vishwanatha *et al.*, 2009).

The Km and Vmax values for the partial purified protease were determined using 0 - 20 mg mL^{-1} hemoglobin as substrate. Km value was

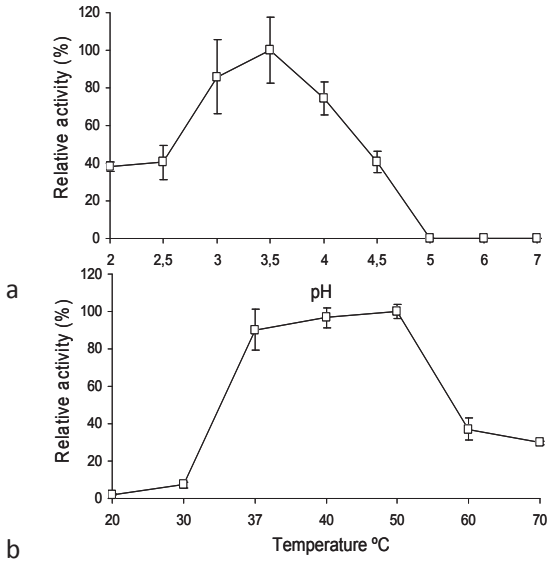

Fig. 4: Effect of pH (a) and temperature (b) on the acid protease activity from *A. rouxii*. (a) Protease activity was measured at 37°C, with universal buffer. (b) Protease activity was measured at pH 3.5. Data are means of three samples.

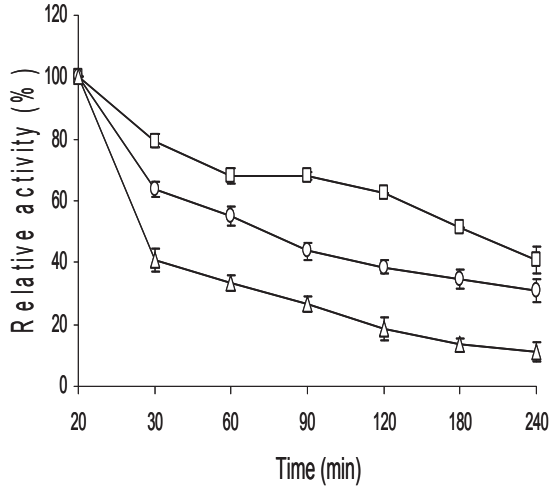

Fig. 5: Stability studies on the aspartic protease from *A. rouxii*, during 3 h. Proteolytic activity was measured using hemoglobin as substrate. Blanks were done for each time and temperature. At 37°C (□), at 40°C (o), 50°C (△). The points are the means of three samples.

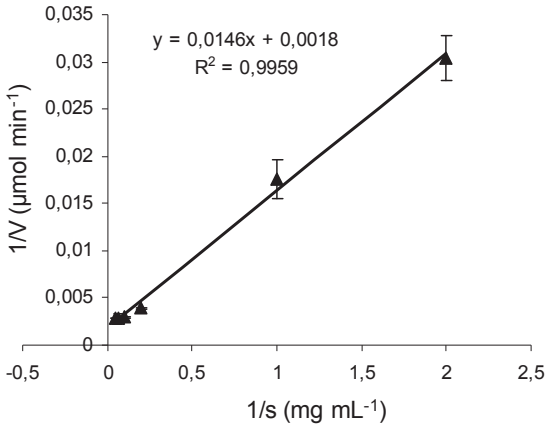

Fig. 6: Linewear-Burk plot to obtain kinetics parameters.

8.03 mg mL^{-1} and Vmax was 551.7 μmol min^{-1}, calculated from Linewear-Burk plot (Fig. 6).

3.4 Effect of inhibitors

Various protease inhibitors were assayed and results showed that pepstatin A (3 mM) inhibited 73% the aspartic protease activity. It has been reported that pepstatin A is specific inhibitor for acid proteases, binding to the active aspartate site (Palmieri *et al.*, 2001; Kudryavtseva *et al.*, 2008). Kumar *et al.*, (2005) reported an acid protease from *R. oryzae* and the inhibition of its activity was of 73 and 93% using 0.01 and 0.02 mM of pepstatin A, respectively. Vishwasnatha *et al.* (2009) observed 100% inhibition of acid protease from *A. oryzae* using 0.018 mM of pepstatin A. Both authors reported that PMSF did not inhibit acid protease activity, similar results were obtained in this work, PMSF inhibited only 13% of the aspartic protease activity (Table 2).

Table 2. Relative inhibition of the aspartic protease from *A. rouxii* by various protease inhibitors

Inhibitor	Concentration (mM)	Relative Activity (%)
Control	0	100
Commercial cocktail	200 μl (Sigma)	46.7
Pepstatin A	3	26.6
PMSF	1	86.6
EDTA	1	80

Conclusion

The results obtained indicate that *A. rouxii* besides produce tyrosinase, as has been reported, produce also an aspartic protease. This protease showed higher activity when hemoglobin as used as substrate that when azocasein was used. Protease produced by *A. rouxii* was identified as an aspartic protease. The protease from *A. rouxii* showed similar characteristics with other aspartic protease from *Rhizopus*. This study allows proposing *A. rouxii* as a fungus producer extracellular aspartic protease. The culture medium used was proposed to produce tyrosinase, it must be assayed other culture media in order to increase protease production. Also, it could be study the potential of this protease to be used in cheese production or whatever use.

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