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**Peptidase from *Aspergillus niger* NRRL 3: Optimization of its production
by solid-state fermentation, purification and characterization**

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Abstract

Orange peels, soybean hulls, *Ilex paraguariensis* and *Platanus x hispanica* were evaluated as solid substrates in order to produce peptidases from *Aspergillus niger* NRRL3 (PAN) under solid-state fermentation. The mixture of soybean hulls and orange peels enabled fungal development and showed the highest peptidase production. The optimal conditions for PAN production were found to be as follows: soybean hulls/orange peels mass ratio, 0.25; initial pH, 7.05; K₂HPO₄ 43.5 g/L and 4.03 g/L NaNO₃; inoculation with 5000 conidia per 3g of solid substrate; incubation conditions, 30°C for 5 days. Under these conditions, the peptidase activity was 1000 ± 100 AU/mL. PAN concentration was performed by adsorption on a DEAE-cellulose matrix. The subsequent purification was carried out by gel filtration on Sephadex G-100, with a global purification factor of about 9. PAN proved to belong to the serine-type of peptidases, its highest peptidase activity being at 65 °C. However, proteolysis at 60 °C proved more suitable due to the differences in the inactivation rate. Besides, PAN showed high stability over a pH range of 4 to 11. Taking all this into account, we herein describe the production and purification of a serine peptidase from *Aspergillus niger* NRRL3 for the first time.

KEYWORDS: Agroindustrial wastes, extracellular fungal peptidase, pH stability, temperature stability

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44 **1. Introduction**

45 Peptidases constitute one of the most important groups of enzymes and
46 are used in detergents as well as in the brewing, meat, leather and dairy
47 industries (Bertucci, Liggieri, Colombo, Vairo Cavalli & Bruno, 2015). In fact,
48 they are one of the most studied groups of enzymes because of their wide
49 applications as beer clarifier, debittering agent, digestive aid and protein
50 modifier in food processing (Dey, Adak, Bhattacharya & Banerjee, 2014; Cimini,
51 De Francesco & Perretti, 2017; Yu et al., 2018). In addition, peptidases are
52 used to produce protein hydrolyzates with improved bioactivities or functional
53 properties that may be of high interest in food science and technology (Segura-
54 Campos, Salazar-Vega, Chel-Guerrero & Betancur-Ancona, 2013; Agrawal,
55 Joshi & Gupta, 2017).

56 Among the several species of filamentous fungi suitable for the
57 production of metabolites and industrial enzymes, *Aspergillus niger* is certainly
58 one of the most commonly used in industries. Citric, gluconic and fumaric acids
59 are well-known value-added compounds produced by *A. niger*. Besides, such
60 fungus produces many types of enzymes, such as α -amylase, cellulase,
61 amyloglucosidase, glucose oxidase, catalase, xylanase, lipase, peptidase and
62 pectinase (Schuster, Dunn-Coleman, Frisvad & Van Dijck, 2002).

63 Solid-state fermentation (SSF) consists in the growth of microorganisms
64 on a moistened solid substrate. Although there is no free-moving water, the
65 moisture content is enough to maintain microbial growth and metabolism
66 (Sandhu, Punia & Kaur, 2016). SSF presents several advantages over
67 submerged fermentations. Firstly, culture conditions are more similar to those of

the natural habitat of the fungus. Secondly, the concentration of extracellular enzyme after extraction is usually higher than that obtained by submerged fermentation (Mazotto, Couri, Damaso & Vermelho, 2013). Finally, the use of agroindustrial wastes in SSF makes SSF more eco-friendly and economic than submerged fermentation (da Luz et al., 2013).

Agricultural, food and forestry industries produce huge amounts of diverse wastes which their improper disposal involves a negative environmental impact. Consequently, they have been increasingly used as raw materials in SSF so as to solve environmental hazards (John, Nampoothiri & Pandey, 2006).

The following biological wastes contain several reusable high-value substances, which makes them suitable for microbial growth and enzyme production: (a) *Platanus x hispanica* (London or Hybrid Plane) is one of the most common trees in some countries such as Argentina, Spain, and Uruguay. After its achenes are released in springtime, they are collected and disposed of together with house wastes (Cedro, 2006). (b) *Ilex paraguariensis* is a widely distributed shrub that grows in Argentina, Brazil, Paraguay and Uruguay. Its leaves and stems are industrialized to prepare “yerba mate”, a commercial product used to prepare “mate”, the most popular beverage in these countries. Once “yerba mate” is used to prepare the infusion, it is discarded together with solid household waste (Valerga, Reta & Lanari, 2012). (c) Orange peel is generated from the extraction of juice in industrial plants (Torrado et al., 2011), the management of which being a major problem for the food industry. (d) Soybean is one of the most extensively cultivated crops in the world and its processing generates about 18–20 million tons of hulls (Liu & Li, 2017).

Apart from the proper selection of the solid substrate, cultivation

conditions such as pH and temperature are of great importance in successful enzyme production. In addition, peptidase production is profoundly influenced by the C/N ratio, inoculum density, incubation time and the presence of metal ions and easily metabolizable sugars (Souza et al., 2015). Optimization techniques are efficient tools to find conditions to produce the best possible response (Kanimozhi, Moorthy, Sivashankar & Sivasubramanian, 2017). Response surface methodology (RSM) is based on the analysis of a response as influenced by several factors and its goal is to determine the optimal condition of the response (Bezerra, Santelli, Oliveira, Villar & Escaleira, 2008). Tari, Göğüs & Tokatli (2007) optimized three parameters by using RSM for the optimal production of α -amylase by *Aspergillus oryzae* NRRL 6270 in SSF. Zhu, Han, Chen & Han (2010) also applied RSM to obtain the optimal level of different factors to optimize the microwave-assisted extraction of astaxanthin from *Phaffia rhodozyma*.

The aim of this work was to determine the optimal conditions for the production of fungal extracellular peptidases from *Aspergillus niger* NRRL3 (PAN) by means of the fermentation of agroindustrial wastes as solid substrates. PAN purification and characterization were also carried out.

2. Materials and methods

2.1. Materials

The fungal strain used in the present study was *Aspergillus niger* NRRL3, which was kindly provided by CEREMIC Rosario. The culture was maintained at 4 °C on slants of malt agar. The conidia from a fully sporulated slant were dispersed in water. Conidia quantification was carried out by serial dilution

followed by plate count.

The non-inert solid substrates used were achenes from *Platanus x hispánica* (AP), orange peels (OP), soybean hulls (SH) and leaves from *Ilex paraguariensis*, commonly named as yerba mate (YM).

The saline basal medium contained 10 g/L MgSO_4 ; 10 g/L NaCl ; 0.08 g/L FeSO_4 and different concentrations of K_2HPO_4 and NaNO_3 . Whey, kindly provided by Cooperativa de Trabajadores Rurales Unidos Rosario, was also evaluated as a basal medium. Soybean hulls were kindly donated by Molinos Río de la Plata S.A. Whey protein isolate (WPI) and casein were purchased from Sigma Aldrich (St. Louis, USA).

All other chemicals used were of analytical grade.

2.2. Solid substrate preparation

AP were washed, dried, and sieved. OP were washed, dried and ground and the particles retained by a mesh of 297 μm were selected. SH were washed and ground in a mechanical crushing mill. YM were leached with water at 80°C and then dried.

2.3. Water absorption index (WAI) determination

The WAI of solid substrates was determined according to Orzua et al. (2009). The solid substrates (1.25 g) were suspended in 10 mL of distilled water. The slurry was stirred for 1 min at room temperature and centrifuged at 3000g for 10 min. The supernatant was discarded, and the WAI was calculated from the weight of the wet substrate (g) and expressed as grams of water per gram of dry substrate.

2.4.PAN production by SSF using agroindustrial wastes

2.4.1. Screening of solid substrates and basal medium

All the solid substrates studied were first analyzed regarding their ability to enable fungi development and to produce peptidases. The experimental conditions were set as follows: incubation time, 7 days; temperature, 35 °C; inoculation with 50000 conidia per 3 g of solid substrate. Both whey and the saline basal medium (containing 10 g/L MgSO₄, 10 g/L NaCl, 0.08 g/L FeSO₄, 43.5 g/L K₂HPO₄ and 5 g/L NaNO₃) were assayed to hydrate the solid substrates. The amount of liquid added to each substrate was calculated as 3 g substrate * WAI (g liquid/g substrate). The results of these preliminary tests allowed the subsequent experimental design, in which only the most suitable basal medium and solid substrates were used.

2.4.2. Experimental design

2.4.2.1.Screening of the significant factors affecting PAN production

Significant factors affecting peptidase production were selected and identified by the Plackett–Burman experimental design. The response variable assayed was peptidase activity. The fermentation factors and their levels chosen were the following: 1) incubation time, 5 or 8 days; 2) incubation temperature, 30 or 40°C; 3) initial pH (pH₀), 6.00 or 7.00; 4) inoculation with 5000 or 50000 conidia by 3 g of solid substrate; 5) NaNO₃ concentration, 0 or 2.5 g/L; 6) K₂HPO₃ concentration, 43.5 or 87.0 g/L; and 7) SH to OP mass ratio, 0.25 or 0.40 g/g.

2.4.2.2. Optimization of PAN production by response surface methodology

A central composite design was applied in order to determine the optimal levels of the factors that maximize peptidase production. The two significant factors selected from the previous screening were assayed at five coded levels (-1.42, -1, 0, 1, 1.42) and five replicates at the center of the design were used to estimate the error sum of squares. A total of 13 experiments were performed.

2.4.3. Solid-state fermentation

In order to produce PAN by SSF, solid substrates, basal medium and whey were autoclaved at 121 °C for 15 min. Then, 3 g of each substrate, or a mixture of them, were placed in a Petri dish (90 x 15 mm). The solid substrates were hydrated -according to the determined WAI- by using saline basal medium or whey, and were then allowed to stand for 24 h at room temperature. After this, the media were inoculated with a conidia suspension. Fermentation was carried out at a given temperature and for a selected incubation time, according to the experimental conditions.

2.5. Recovery, concentration, and purification of PAN from the extracellular enzymatic extract

The enzymatic extract was recovered by adding 10 mL of distilled water to each Petri dish and by magnetically stirring at 300 rpm for 10 min. This slurry was centrifuged at 10000g for 15 min at 4 °C and the aqueous phase containing extracellular peptidases was recovered.

Two different methodologies were applied to evaluate the best PAN concentration strategy. The first one consisted in salt precipitation followed by

dialysis. Ammonium sulfate was added with continuous stirring to the extracellular enzymatic extract to achieve 70 % saturation. After overnight precipitation at 4 °C, the mixture was centrifuged at 10000g for 15 min at the same temperature in order to recover the precipitated enzymes. The precipitate was redissolved in a minimum volume of buffer phosphate 50 mmol/L pH 7 and dialyzed against the same buffer solution for 8 h at 4 °C. The dialyzed sample was assayed for peptidase activity and protein content (see Section 2.9. Analytical methods). The second concentration strategy consisted in adsorption on an ion-exchange matrix. Adsorption was carried out with a DEAE-cellulose matrix (Sigma-Aldrich; St. Louis, USA) pre-equilibrated with buffer acetate 10 mmol/L pH 5. Twenty-five milliliters of crude extract were passed through the column (10 cm x 0.7 cm) and fractions of 1 mL exhibiting peptidase activity were collected and pooled together.

Size-exclusion chromatography was applied in order to purify PAN. The concentrated samples were then passed through a Sephadex-G100 (Sigma-Aldrich; St. Louis, USA) column (10 cm x 0.7 cm) pre-equilibrated with buffer phosphate 50 mmol/L pH 7. The same buffer solution was used for elution. Fractions of 1 mL were collected and those which exhibited the highest peptidase activity were pooled together. Samples were freeze-dried and the recovery yields of the purified fractions were evaluated by means of peptidase activity and protein content determinations (see Section 2.9. Analytical methods).

2.6. Gel electrophoresis

The purification of the crude extracellular enzymatic extract was

evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using β -mercaptoethanol as the reducing agent. Gel electrophoresis was carried out in an SDS-Tris-Glycine discontinuous buffer system (10 % stacking gel, 13 % resolving gel) in a mini-PROTEAN 3 Cell system (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. Electrophoresis was performed at a constant intensity of 40 mA. Proteins were visualized by staining with Coomassie brilliant blue (0.025% (w/v)). A solution of 1 g/L of whey proteins was used as the molecular weight standard. Such proteins consists of lactoferrin (86,000 Da), bovine serum albumin (67,000 Da), immunoglobulin (55,000 Da), β -lactoglobulin (18,400 Da), and α -lactalbumin (14,300 Da).

2.7. Inhibition assays

Inhibition assays were carried out in order to classify PAN according to its catalytic type. Chemicals were added to PAN solutions and incubated for 15 min before being tested for peptidase activity. Phenylmethylsulfonyl fluoride (PMSF), a serine peptidase inhibitor; EDTA, a metallopeptidase inhibitor; and pepstatin, an aspartyl peptidase inhibitor, were used at the following working concentrations: 1 mmol/L, 5 mmol/L and 1 mmol/L, respectively.

2.8. Effects of temperature and pH on enzymatic activity and stability

The effect of temperature on peptidase activity was determined by measuring casein hydrolysis in the range of 30 to 95 °C. The effect of temperature on PAN stability was determined by incubating PAN in the range of 30°C to 90°C for 1 h. In order to determine the time dependence of PAN stability,

the residual peptidase activity was determined at 60 and 65 °C incubating PAN during 180 min. The buffer medium was Tris-HCl 50 mmol/L pH 9.00 in all cases.

The effect of pH was determined by measuring peptidase activity of PAN in the range of pH 2 to 12 in buffer acetate-phosphate-Tris 50 mmol/L using WPI as substrate. The pH-stability profile was determined by measuring the residual activity at pH 9 after incubation for 8 h at various pH values, in the range of 2-12.

2.9. Analytical methods

The composition of both OP and SP were determined by following AOAC official procedures (1996).

Unless other conditions were specified, peptidase activity was determined at 37°C by Folin–Ciocalteu method using casein as substrate (Cupp-Enyard, 2008). Briefly, an aliquot of PAN suspension was added to 600 µL of 2.5 g/L casein solution in 50 mmol/L acetate-phosphate-Tris buffer pH 9. After a 15-min incubation, the reaction was stopped by the addition of 400 µL of 50 g/L trichloroacetic acid, followed by centrifugation at 15000g for 10 min. The supernatant (500 µL) was mixed with 1250 µL of 0.44 mol/L Na₂CO₃ and 250 µL of Folin–Ciocalteu reagent. The absorbance was measured at 600 nm after incubation at 37°C for 30 min. One arbitrary unit (AU) of enzymatic activity was defined as the amount of enzyme that caused a change in absorbance of 0.01 units.

Protein quantification was carried out by the Bradford method (Bradford, 1976).

2.10. Statistical analysis

Optimal conditions of PAN production were evaluated by means of a Plackett-Burman design for the exploratory phase and a central composite design for the optimization phase. The significance of factors was determined by means of ANOVA analysis or t-Student tests. Significant differences were analyzed by means of p-values ($p < 0.05$). Normality of the residues was evaluated through Shapiro-Wilk test. The software used for the experimental design, for the analysis of variance and for testing the normal distribution of residues was Design Expert 6 and Sigma Plot 11, both in a trial version.

3. Results and discussion

3.1. Solid substrate screening

WAI was determined for the four materials that can be potentially used as solid substrates. The results obtained are shown in Table 1.

Table 1

WAI is the quantity of water that can be absorbed by a material. Substrates with high WAI are preferred since their moisture content can be modified during solid-state culture. In fact, *A. niger* strains in solid-state cultures do need the modification of the moisture content of the absorbing media (Robledo et al., 2008). Taking this into account, OP could be proposed as a suitable solid substrate, as it recorded the highest WAI.

Several medium conditions affect both the growth of *A. niger* and the production of peptidase. Among them, the composition of the solid substrate and of the liquid used to hydrate the solid substrate should be particularly considered due to their great importance. In preliminary experiments, a 7-day

incubation proved to be appropriate for the growth of *A. niger* and the development of peptidase activity. In addition, it was observed that peptidase production was negligible when the final pH of the enzymatic extract was lower than 5.10. In order to prevent a sharp decrease in pH, K_2HPO_3 was added to the basal medium so as to adjust the pH_0 and to prevent a fast acidification of the culture. Fig. 1 shows the growth of *A. niger* and the peptidase activity obtained after a 7-day incubation in each solid medium.

Figure 1

It is to be noted that AP was not appropriate to support *A. niger* cultures. On the other hand, OP and YM showed the highest growth, whereas OP and SH showed the highest activity. The presence of whey improved neither the growth nor the peptidase activity ($p = 0.4509$); thus, the subsequent experiments were carried out using a saline medium to hydrate the solid substrates.

The combination of different solid substrates has been previously studied and both antagonistic and synergistic effects have been found among them (de Castro et al., 2015). Therefore, the peptidase activity were determined after fermentation in solid media containing binary mixtures of OP and SH. SSF carried out using OP-SH mixtures caused higher peptidase activity than in the other media assayed.

Peptidase production by SSF can be affected by the composition of the substrates and by several cultivation factors. Once the two components of the solid substrate were selected, their chemical composition was determined and is shown in Table 2.

Table 2

The presence of protein sources is believed to induce protease secretion by the microorganism (de Castro, Nishide & Sato, 2014). According to our results, mixtures of OP and SH were appropriate to be used as complex sources of carbon and nitrogen for the fungal cultures in order to produce extracellular peptidase.

3.2.Screening of the appropriate conditions for peptidase production from *A niger* NRRL 3

In order to select the most significant factors that affect peptidase production during the SSF of *A. niger*, a Plackett–Burman experimental design was carried out (Chauhan, Trivedi & Patel, 2007), assaying different factors, such as the composition of the culture media (solid substrate mass ratio, salt concentration and pH₀), the culture conditions (time and temperature) and the inoculum, as shown in Table A (Supplementary material).

A multiple regression analysis of the data was carried out to determine the significant factors affecting peptidase production. Although peptidase production was observed in almost all the assayed conditions, which confirms that the combinations of the selected components for the solid substrate were appropriate, the activity was extremely low in the cases in which the final pH of the media dropped below 5.10 (Table A of Supplementary material), as previously stated.

In order to increase the symmetry of the analyzed data, a square root transformation was applied to the peptidase activity before the analysis of variance. According to the ANOVA shown in Table B (Supplementary material), the most significant factors affecting peptidase production were the pH₀ and the

NaNO₃ concentration. The normality of the residues was confirmed by the Shapiro-Wilk test ($p = 0.104$), which null hypothesis is that the sample comes from a normally distributed population.

3.3.Optimization of peptidase production

A central composite design was used to analyze the interactive effect of the two significant factors, pH₀ and NaNO₃ concentration, and to achieve an optimal condition for peptidase production. Most of the non-significant factors – the incubation time, the temperature, the inoculum, and the K₂HPO₃ concentration- were adjusted in such a way as to minimize them. The remaining non-significant factor -the OP/SH ratio- was maximized since OP is more economically convenient than SH.

The results (Table C of Supplementary material) were modeled with the following equation ($R^2 = 0.8516$), which includes the significant as well as the hierarchical terms:

$$\text{Peptidase activity (AU/mL)} = -1.39 \cdot 10^4 + 3.8 \cdot 10^3 \text{ pH}_0 + 7.14 \cdot 10^2 [\text{NaNO}_3] \text{ (g/L)} - 2.7 \cdot 10^2 \text{ pH}_0^2 - 88.65 [\text{NaNO}_3]^2 \quad (\text{equation 1})$$

Although both pH₀ and NaNO₃ significantly affected peptidase production, there was no interaction between them ($p = 0.2133$), as shown in Table D (Supplementary material). Thus the term pH₀ * NaNO₃ was not included in equation 1. The analysis of variance showed that there is only a 0.37 % of probability that the total variation was due to noise and that there is no lack of fit ($p = 0.0666$). The normality of the residues was confirmed by the Shapiro-Wilk test ($p = 0.798$).

The data obtained and the response surface generated from the model

are shown in Fig. 2

Figure 2

The convex surface plotted in Fig 2 suggests that a well-defined optimal operating condition was found. In order to validate the above mentioned predictive model, the values of pH_0 and NaNO_3 concentration that maximized peptidase production were determined and turned out to be pH_0 7.05 and 4.03 g/L NaNO_3 . Several authors have also reported the production of peptidases from *A. niger* to be optimum when the pH_0 was 7 (Paranthaman, Alagusundaram & Indhumathi, 2009; Ahmed, Zia, Iftikhar & Iqbal, 2011). Regarding the optimal NaNO_3 concentration, there is no coincidence with other studies, probably due to the different solid substrates used and their varied nitrogen content (Braaksma, Smilde, van der Werf & Punt, 2009).

According to equation 1, a fermentation carried out under optimal conditions would yield a maximum enzymatic activity of 931.5 AU/mL. In a separate set of validation experiments performed in quintuplicate to verify the predictions, peptidase activity (AU/mL) was 1000 ± 100 , which is within the predicted value. The enzymatic activity for solid-state production is commonly expressed per gram of solid substrate. In this case, each gram of solid substrate mixture produced 3300 ± 300 AU.

3.4. Concentration and purification of PAN

There are many studies reporting the successful concentration of fungal peptidases by means of the fractionation with ammonium sulfate and dialysis (Krishnan & Vijayalakshmi, 1986). However, this method was not appropriate for this enzyme since peptidase activity abruptly dropped (from 1005 to 255 AU/mL)

after dialysis. The negative effect of ammonium sulfate fractionation on enzymatic activity had been previously reported on a milk-clotting enzyme obtained from *A. niger* (Osman, Abdel-Fattah, Abdel-Samie & Mabrouk, 1969).

The best procedure for concentrating and purifying PAN was the adsorption onto a DEAE-cellulose matrix followed by gel filtration on Sephadex G-100. These methods have also been reported to be useful for purifying a wide variety of fungal peptidases (Esparza et al., 2011). The concentration of PAN by adsorption onto a DEAE-cellulose matrix allowed increasing specific activity almost twice due to the decrease in the protein content. Purification with size-exclusion chromatography allowed reducing even more the protein concentration, resulting in a global purification factor of 9 and a recovery yield of 36 %. The results are shown in Table 3.

Table 3

In order to evaluate the purification strategies, electrophoresis gels under denaturing and reducing conditions were carried out (Fig. 3).

Figure 3

A wide range of molecular weights of peptidases from *A. niger* has been reported (Coral, Arikan, Unaldi & Guvenmez, 2003; Devi, Banu, Gnanaprabha, Pradeep & Palaniswamy, 2008; Ahmed et al., 2011; Esparza et al., 2011; Mazotto et al., 2013). As can be seen in the electrophoresis gel, the crude enzymatic extract showed two bands, corresponding to polypeptides of molecular weights of 14 and 16 kDa (lane 1). The intensity of these bands was enhanced after adsorption with a DEAE-cellulose matrix (lane 2). By contrast, these bands were less intense after the purification by size-exclusion chromatography, due to sample dilution (lane 3).

3.5.Characterization of PAN

Inhibition assays were carried out in order to classify PAN according to its catalytic type. The results are shown in Table 4. PMSF fully inhibited PAN, whereas EDTA and pepstatin caused no effect on peptidase activity ($p = 0.533$). These results suggest that PAN belong to the serine peptidase group. Although part of the peptidases from *A. niger* NRRL 3 was reported to have been classified as serine peptidases (Ahamed, Singh & Ward, 2007), it is the first time they have been produced from the mixture of OP and SH.

Table 4

Since the most frequently studied peptidases from *A. niger* are classified as aspartyl peptidases, the characterization of serine peptidase production from *A. niger* may provide valuable information for the food industry. In fact, Alcalase and Novozym are commercial serine peptidases with wide application in food science (Tavano, 2013).

The dependence of peptidase activity on temperature is shown in Figure 4 A.

Figure 4

The optimal temperature of PAN was 65 °C and at least 40% of peptidase activity was retained when it was measured in the temperature range 30-75 °C. Although there are previous reports showing that some peptidases from *A. niger* were thermostable (Devi et al., 2008), a great variety of optimal temperatures was found in the reported optimum temperature, probably due not only to variations in the strain of *A. niger* but also to culture conditions (Tsang, Butler, Powlowski, Panisko & Baker, 2009; Novelli, Barros & Fleuri, 2016).

Nevertheless, the optimum temperature of PAN is similar to that found for peptidases from *Bacillus* sp (Beg & Gupta, 2003; Deng, Wu, Zhang, Zhang & Wen, 2010) but higher than the optimum temperature reported for several peptidases from *Aspergillus* sp (Tunga, Shrivastava & Banerjee, 2003; Wang, Chen & Yen, 2005). In addition, PAN optimum temperature was similar to that of the crude enzymatic extract of the thermophilic fungi *Thermoascus aurantiacus*, studied by Merheb et al. (2007), and *Thermomyces lanuginosus*, studied by Li, Yang, and Shen (1997), which exhibited optimal proteolytic activity at high temperatures (60 °C and 70 °C, respectively).

Regarding temperature stability, PAN was stable in the range from 30 to 65°C. The highest peptidase activity was measured when PAN was incubated at 50 °C, in agreement with what was found for a peptidase from *A. niger* produced by wheat bran fermentation (Ahmed et al., 2011). It is to be noted that the maximum peptidase activity was reached at 65°C, which is, in fact, the limit of PAN stability range. In order to determine the kinetics of inactivation at 60 and 65 °C, PAN was incubated at these temperatures for 180 min and the residual peptidase activity was periodically measured. At 60° C, peptidase activity was not altered during the time assayed. However, at 65 °C, peptidase activity was lowered to 40 % after 80 min of incubation and decreased to 20% after 180 min. These data are encouraging as the time required for the inactivation of PAN was higher than the time required for the inactivation of both Flavourzyme 500 MG[®] and a proteolytic extract obtained from *A. oryzae* (García-Gómez, Huerta-Ochoa, Loera-Corral & Prado-Barragán, 2009). Considering these results, the most appropriate temperature to carry out the proteolysis with PAN is proposed to be 60°C. Similar results were reported by

Esparza et al (2011), who produced a prolyl-endopeptidase from *A. niger* that was stable at temperatures below 70°C.

As it can be observed in Fig. 4 B, peptidase activity reached a maximum at pH 10 and was higher than 80% between pH 7 and 12. The same pH effect on peptidase activity and stability was found on an alkaline protease from *A. niger* isolated from Indian soil (Devi et al., 2008). However, that enzyme was reported to be inhibited by EDTA. Other proteases, such as VQ-VII, which is a cysteine protease secreted by *Vasconcelle aquercifolia*, showed activity in a wide pH range (6.0 - 9.5) (Torres, Trejo, Natalucci & López, 2013). An extracellular alkaline protease secreted by *Botrytis cinerea* proved active in a wide pH range (4 - 11) (Abidi, Chobert, Haertlé & Marzouki, 2011). Commercial peptidases from microorganisms have maximum activity in the pH range from 8 to 12 (Yadav, Bisht, Tiwari & Darmwal, 2015). The pH stability profile of PAN showed that it is highly stable in the pH range 4-11, retaining over 80% of residual activity.

4. Conclusion

The production of a serine peptidase from *Aspergillus niger* NRRL3 was carried out by solid fermentation of a mixture of SH and OP at a ratio of 0.25:1. The mixture was inoculated with 5000 conidia per 3 g solid substrate and incubated at 30 °C for 5 days. The saline basal medium was optimized, the optimal conditions being at pH₀ 7.05 and 4.03 g/L NaNO₃.

Although fractionation with ammonium sulfate was not suitable for PAN, the enzymatic extract was properly concentrated and purified 9-fold by means of adsorption onto a DEAE-cellulose matrix, followed by gel filtration on

Sephadex G-100. PAN showed high stability over a wide pH range. Although the highest peptidase activity was reached at 65 °C, a higher stability was achieved at 60 °C after 180 min of incubation.

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710 Figure legends

711 Figure 1: Peptidase activity after solid-state fermentation of *A. niger* NRRL 3 in
712 different agroindustrial wastes.

713 Figure 2: Response surface curve of peptidase production as a function of
714 NaNO_3 concentration and pH_0 .

715 Figure 3: SDS-PAGE of the crude enzymatic extract (lane 1), the concentrate
716 obtained after adsorption with a DEAE-cellulose matrix (lane 2) and the purified
717 fraction obtained by size-exclusion chromatography (lane 3). Lane 4
718 corresponds to molecular weight markers.

719 Figure 4: Effect of temperature (A) and pH (B) on activity and stability of PAN
720 obtained under SSF. Filled circles: determination of optimal pH and temperature
721 conditions for peptidase activity. Empty circles: determination of the stability
722 range of pH and temperature.

723

Table 1: Water absorption index of the four assayed solid substrates*

Solid substrate	WAI (mL H ₂ O / g solid substrate)
AP	3.8 ± 0.4 ^b
OP	6.9 ± 0.2 ^a
SH	3.6 ± 0.7 ^b
YM	4.0 ± 0.4 ^b

*Means ± standard deviation of triplicate assays. Mean values in the same column followed by the same letter are not significantly different ($p > 0.05$).

Table 2: Chemical composition (%) of the selected solid substrates*

Chemical components (%)	OP	SH
Moisture (AOAC 925.10)	9.0 ± 0.2 ^a	8.4 ± 0.3 ^b
Lipids (AOAC 920.85)	1.1 ± 0.5 ^a	0.6 ± 0.3 ^b
Protein (AOAC 920.87, Factor 5.70)	4.2 ± 0.1 ^a	8.3 ± 0.2 ^b
Ash (AOAC 923.30)	3.1 ± 0.2 ^a	4.9 ± 0.2 ^b
Carbohydrates	54 ± 1 ^a	17 ± 1 ^b
Fiber (AOAC 973.18)	28.7 ± 0.4 ^a	60.9 ± 0.3 ^b

*Means ± standard deviation of duplicate assays. Mean values in the same row followed by the same letter are not significantly different ($p > 0.05$).

Table 3: Purification strategy of PAN obtained under SSF*

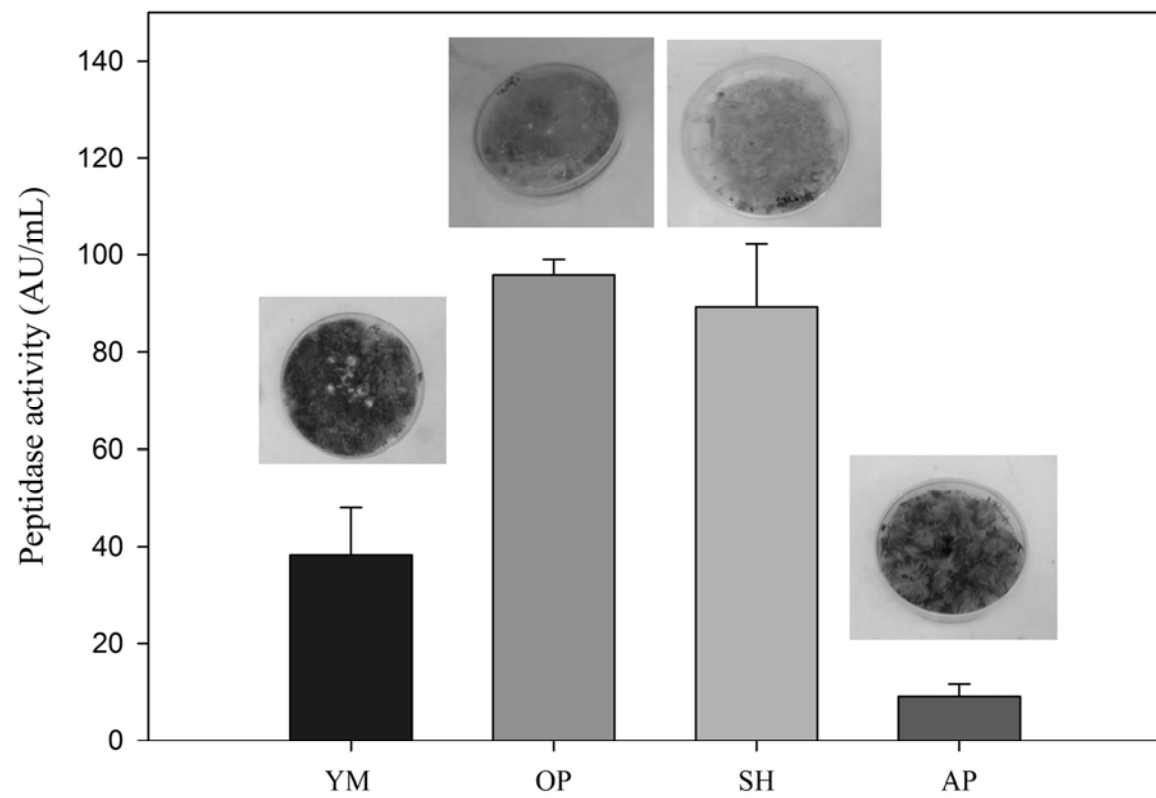
	Peptidase activity (AU/mL)	Protein concentration (mg/mL)	Specific activity (U/mg)	Purification	Yield (%)
Crude enzymatic extract	1000 ± 40	0.88 ± 0.03	1100 ± 80	1.00	100
DEAE-cellulose	900 ± 40	0.42 ± 0.02	2200 ± 200	1.9 ± 0.3	90 ± 7
Sephadex G-100	360 ± 20	0.034 ± 0.001	11000 ± 900	9 ± 1	36 ± 3

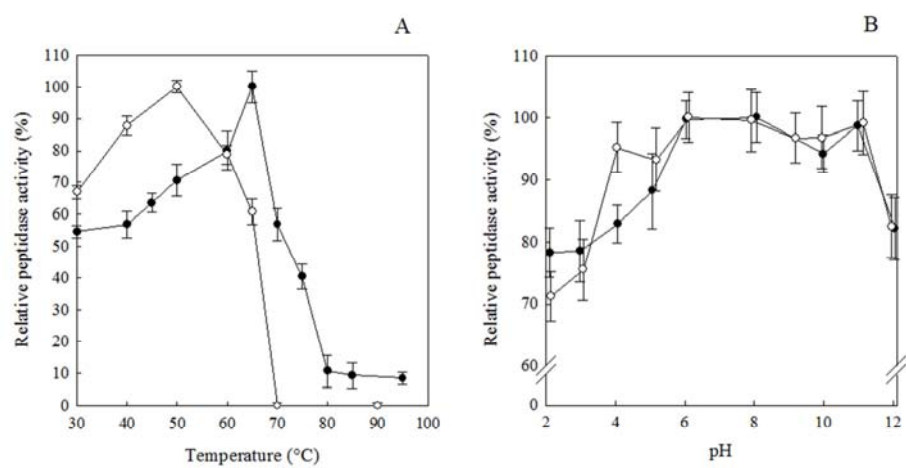
*Means ± standard deviation of triplicate assays.

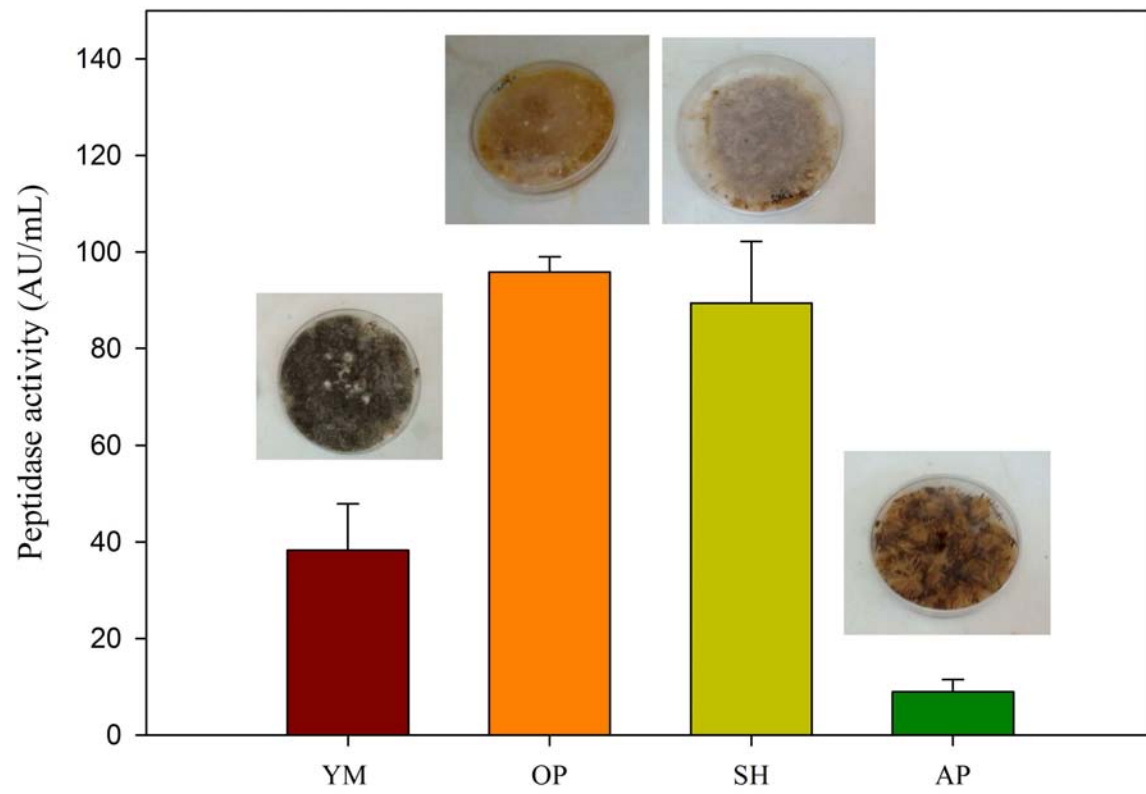
Table 4: Effect of different peptidase inhibitors on peptidase activity of purified PAN*

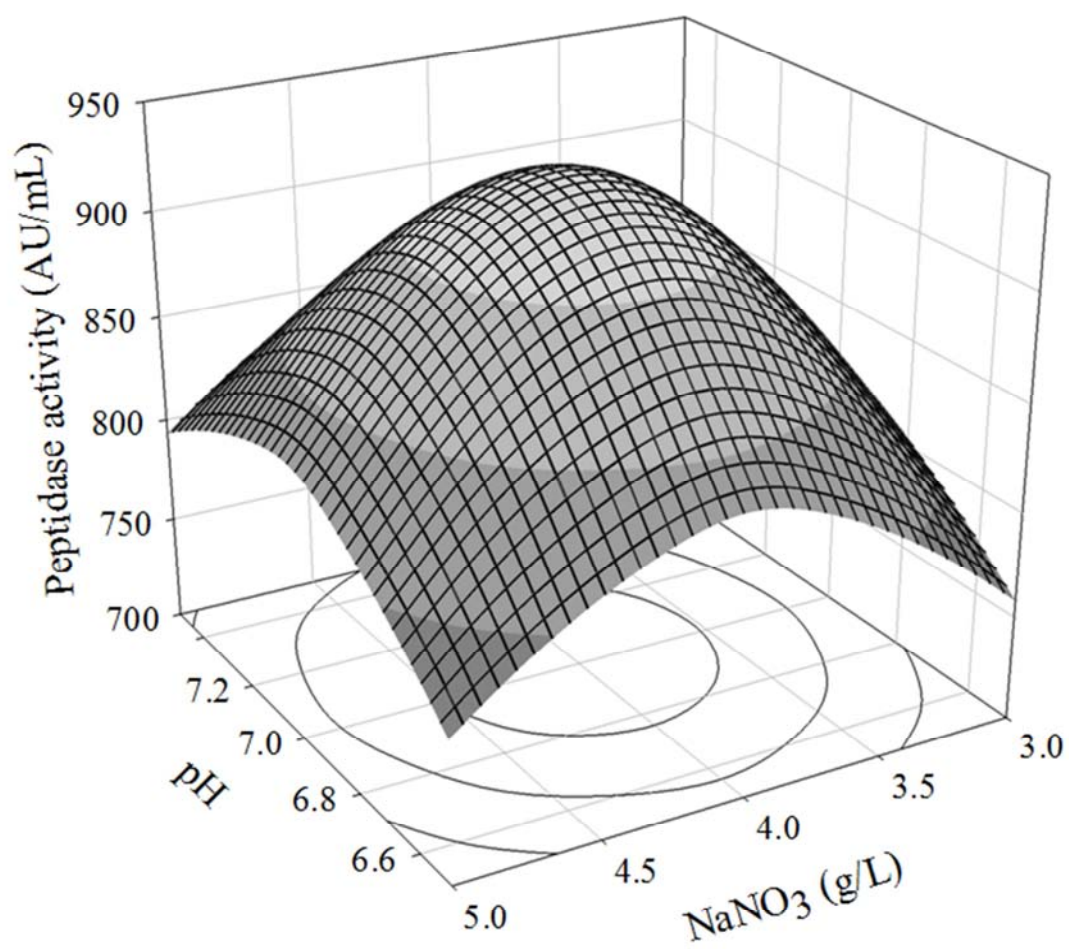
Conditions	Peptidase activity (AU/mL)
buffer	360 ± 10^b
1 mmol/L PMSF	0 ± 9^a
1 mmol/L pepstatin	360 ± 10^b
5 mmol/L EDTA	350 ± 20^b

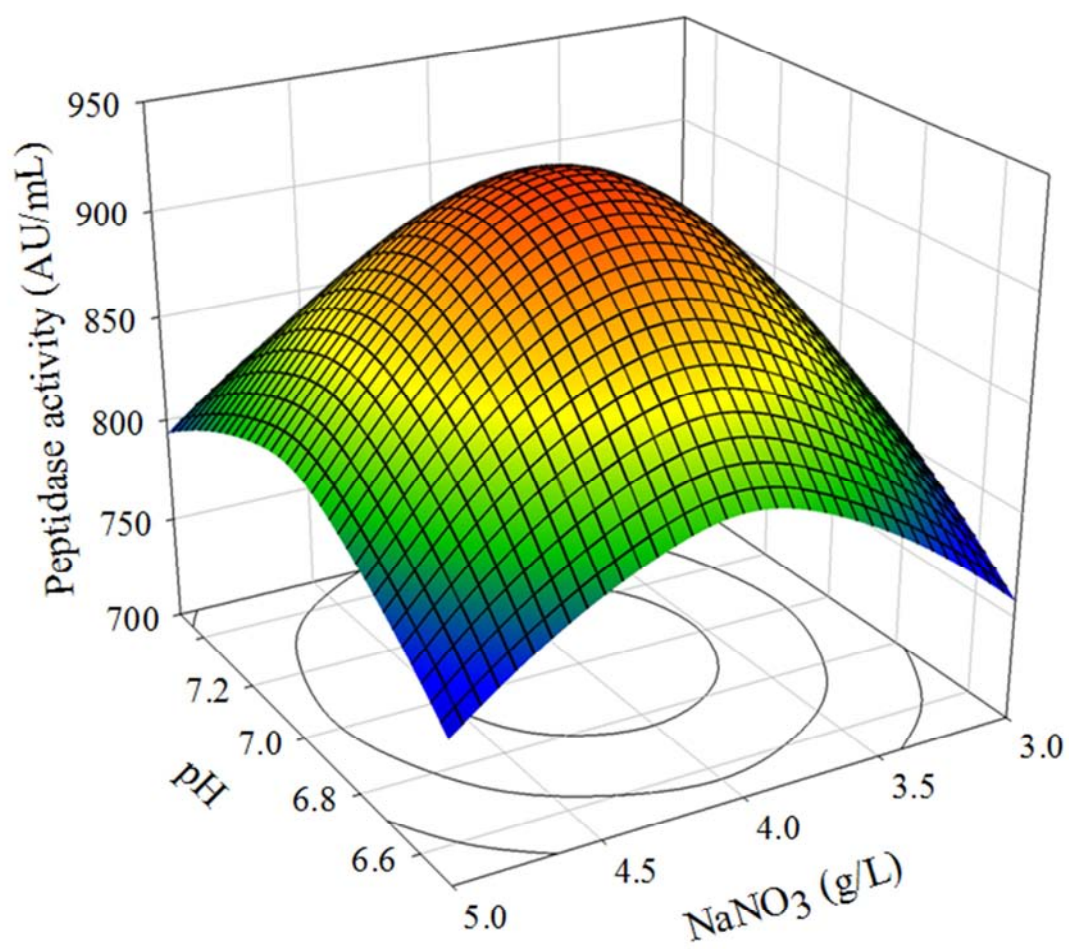
*Means \pm standard deviation of triplicate assays. Mean values in the same column followed by the same letter are not significantly different ($p > 0.05$).

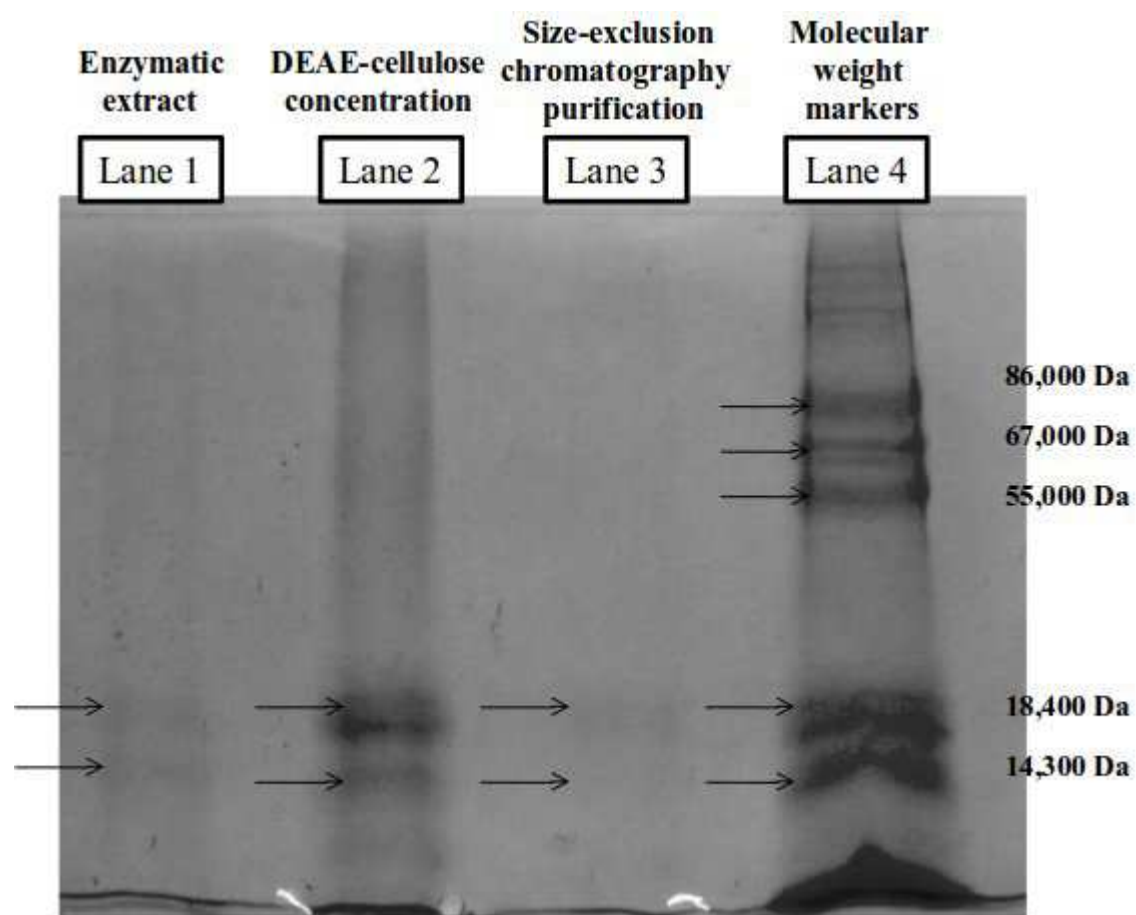


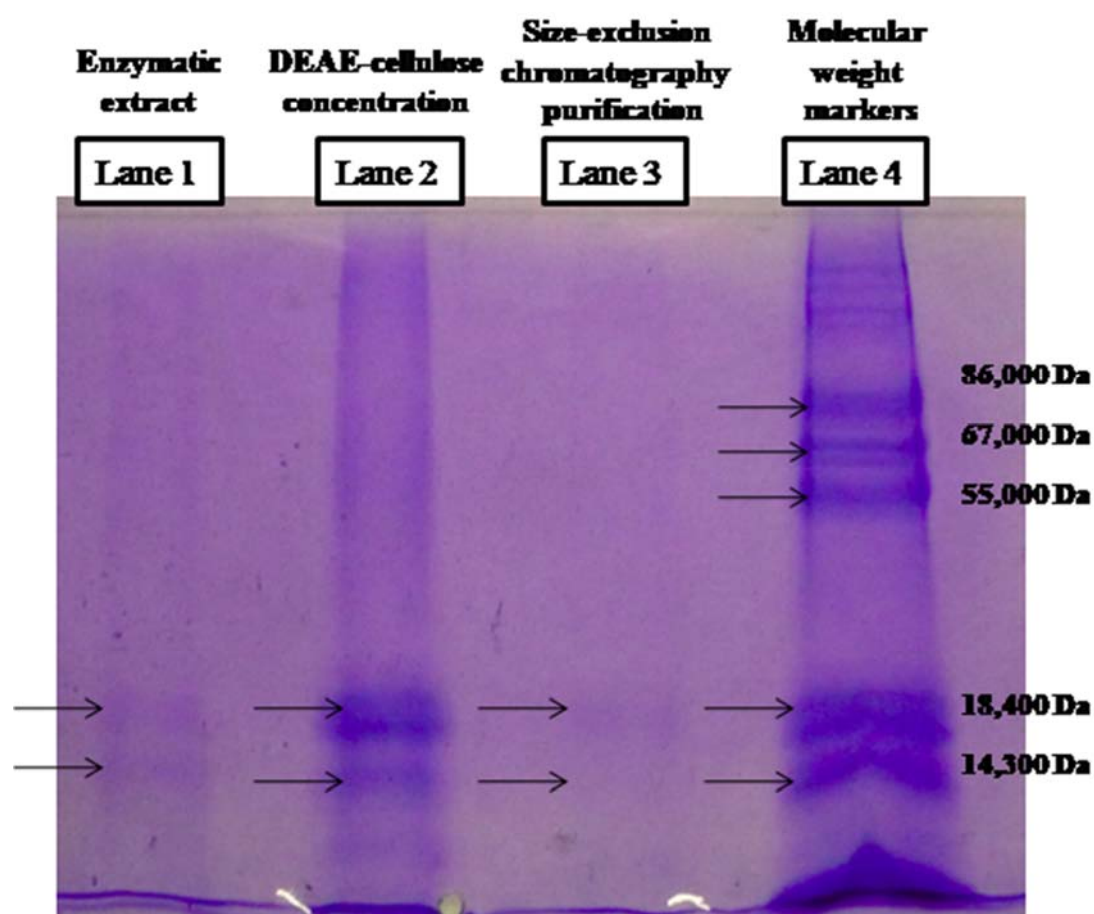












Highlights

Peptidase of *Aspergillus niger* (PAN) was produced by solid state fermentation

The use of different agroindustrial wastes as solid substrates was evaluated

The highest PAN production was obtained from a soybean hulls and orange peels mixture

Adsorption and size exclusion strategies were carried out to purify PAN

PAN showed adequate pH and temperature stability