



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

Débora N. López^{a,b,c}, Micaela Galante^{a,b,c}, Germán Ruggieri^a, Julia Piaruchi^a, María E. Dib^a, Natalia Montellano Duran^{a,b}, Julia Lombardi^{a,b}, Mariana de Sanctis^a, Valeria Boeris^{a,b,c}, Patricia H. Risso^{a,b,d}, Darío Spelzini^{a,b,c,*}

^a Universidad Nacional de Rosario, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531, Rosario, Argentina

^b Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

^c Universidad Católica Argentina, Facultad de Química e Ingeniería del Rosario, Pellegrini 3314, Rosario, Argentina

^d Universidad Nacional de Rosario, Facultad de Ciencias Veterinarias, Ovidio Lagos y Ruta 33, Casilda, Argentina

ARTICLE INFO

Keywords:

Agroindustrial wastes
Extracellular fungal peptidase
pH stability
Temperature stability

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 3 g 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 4–11. Taking all this into account, we herein describe the production and purification of a serine peptidase from *Aspergillus niger* NRRL3 for the first time.

1. Introduction

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

Among the several species of filamentous fungi suitable for the production of metabolites and industrial enzymes, *Aspergillus niger* is certainly one of the most commonly used in industries. Citric, gluconic

and fumaric acids are well-known value-added compounds produced by *A. niger*. Besides, such fungus produces many types of enzymes, such as α-amylase, cellulase, amyloglucosidase, glucose oxidase, catalase, xylanase, lipase, peptidase and pectinase (Schuster, Dunn-Coleman, Frisvad, & Van Dijck, 2002).

Solid-state fermentation (SSF) consists in the growth of microorganisms on a moistened solid substrate. Although there is no free-moving water, the moisture content is enough to maintain microbial growth and metabolism (Sandhu, Punia, & Kaur, 2016). SSF presents several advantages over 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

* Corresponding author. Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, CONICET, Suipacha 531, S2002RLK, Rosario, Argentina.

E-mail address: dspezlini@fbioyf.unr.edu.ar (D. Spelzini).

<https://doi.org/10.1016/j.lwt.2018.09.013>

Received 17 April 2018; Received in revised form 7 September 2018; Accepted 8 September 2018

Available online 10 September 2018

0023-6438/© 2018 Elsevier Ltd. All rights reserved.

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, & Escalera, 2008). Tari, Göğüs, and 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, and 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 hispanica* (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 MgSO₄; 10 g/L NaCl; 0.08 g/L FeSO₄ and different concentrations of K₂HPO₄ and NaNO₃. 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 μ m 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 3000 g 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 × 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 10000 g 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 10000 g 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 × 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 × 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 30–95 °C. The effect of temperature on PAN stability was determined by incubating PAN in the range of 30 °C–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 μ L 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 μ L of 50 g/L trichloroacetic acid, followed by centrifugation at 15000 g for 10 min. The supernatant (500 μ L) was mixed with 1250 μ L of 0.44 mol/L Na_2CO_3 and 250 μ L 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.

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.

Table 1
Water absorption index of the four assayed solid substrates^a.

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

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

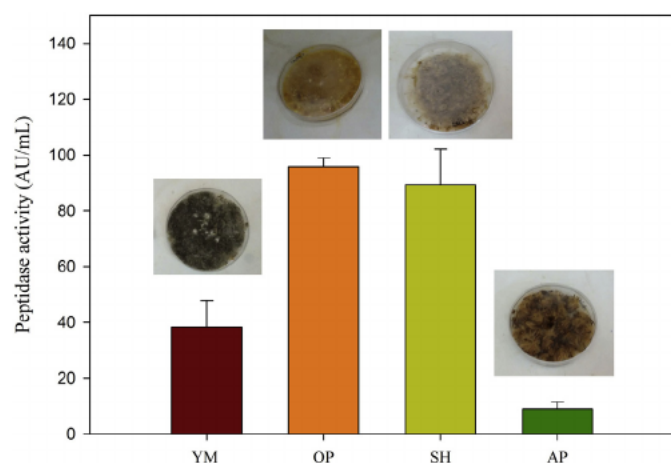


Fig. 1. Peptidase activity after solid-state fermentation of *A. niger* NRRL 3 in different agroindustrial wastes.

Fig. 1 shows the growth of *A. niger* and the peptidase activity obtained after a 7-day incubation in each solid medium.

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.

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 pH_0), the culture conditions (time and temperature) and the inoculum, as shown in Table A

Table 2
Chemical composition (%) of the selected solid substrates^a.

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

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

(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 pH_0 and the $NaNO_3$ 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, pH_0 and $NaNO_3$ 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 K_2HPO_3 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:

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

Although both pH_0 and $NaNO_3$ significantly affected peptidase production, there was no interaction between them ($p = 0.2133$), as shown in Table D (Supplementary material). Thus the term $pH_0 * NaNO_3$ 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.

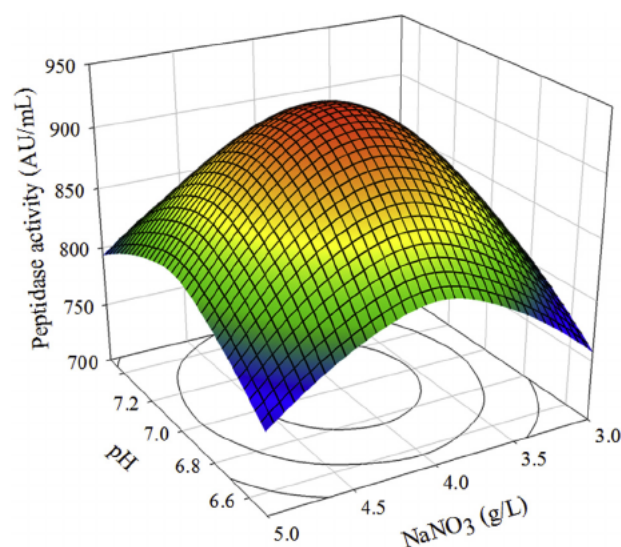


Fig. 2. Response surface curve of peptidase production as a function of $NaNO_3$ concentration and pH_0 .

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 pH₀ and NaNO₃ concentration that maximized peptidase production were determined and turned out to be pH₀ 7.05 and 4.03 g/L NaNO₃. Several authors have also reported the production of peptidases from *A. niger* to be optimum when the pH₀ was 7 (Ahmed, Zia, Iftikhar, & Iqbal, 2011; Paranthaman, Alagusundaram, & Indhumathi, 2009). Regarding the optimal NaNO₃ 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.

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

A wide range of molecular weights of peptidases from *A. niger* has been reported (Ahmed et al., 2011; Coral, Arikian, Unaldi, & Guvenmez, 2003; Devi, Banu, Gnanaprabha, Pradeep, & Palaniswamy, 2008; 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

Table 3
Purification strategy of PAN obtained under SSF^a.

	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

^a Means ± standard deviation of triplicate assays.

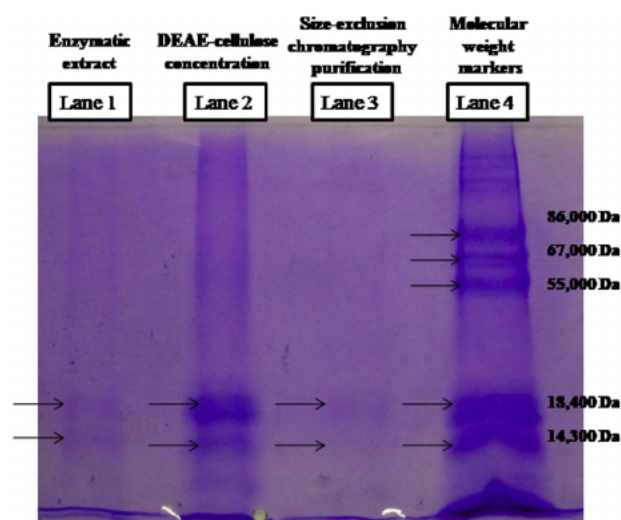


Fig. 3. SDS-PAGE of the crude enzymatic extract (lane 1), the concentrate obtained after adsorption with a DEAE-cellulose matrix (lane 2) and the purified fraction obtained by size-exclusion chromatography (lane 3). Lane 4 corresponds to molecular weight markers.

Table 4
Effect of different peptidase inhibitors on peptidase activity of purified PAN^a.

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

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

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.

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 Fig. 4 A.

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

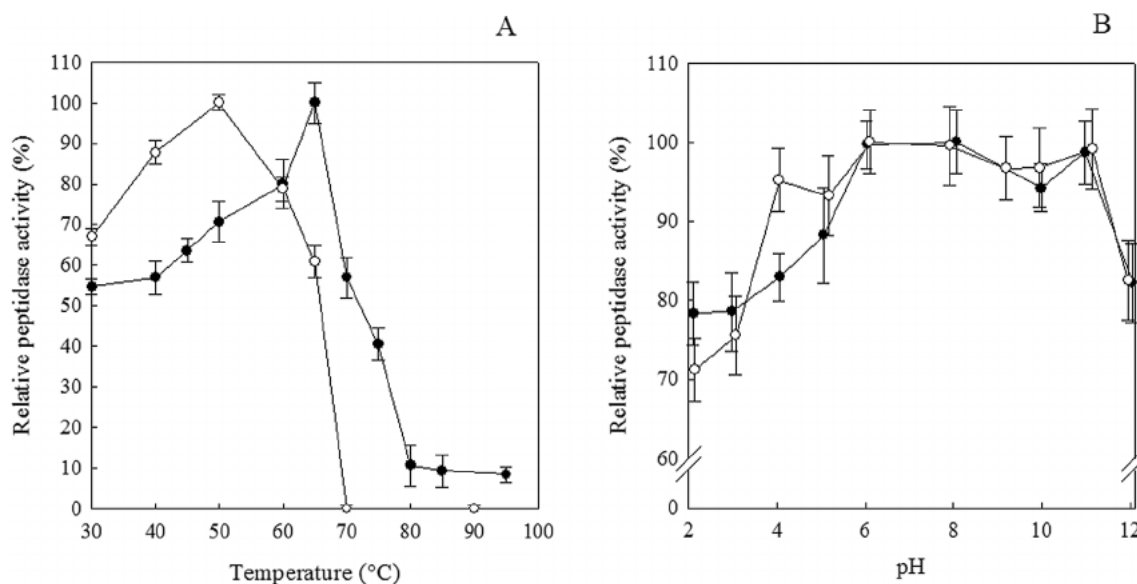


Fig. 4. Effect of temperature (A) and pH (B) on activity and stability of PAN obtained under SSF. Filled circles: determination of optimal pH and temperature conditions for peptidase activity. Empty circles: determination of the stability range of pH and temperature.

A. niger but also to culture conditions (Novelli, Barros, & Fleuri, 2016; Tsang, Butler, Powlowski, Panisko, & Baker, 2009). 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, Cabral, Gomes, and Da-Silva (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 aquerifolia*, 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: 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.

Acknowledgments

Authors would like to thank those who provide financial support: Universidad Nacional de Rosario (1BIO585), Agencia Nacional de Promoción Científica y Tecnológica (PICT 2017-0937), Secretaría de Políticas Universitarias (VT12-UNR4183), Universidad Católica Argentina and Fundación Nuevo Banco de Santa Fe. The authors would like to thank the English Department of Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR, for the language correction of the manuscript. Débora N. López, Micaela Galante, Julia Lombardi and Natalia Montellano Duran would also like to thank CONICET and Germán Ruggieri would like to thanks to Fundación Nuevo Banco de Santa Fe for the fellowship they were granted.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.lwt.2018.09.013>.

References

- Abidi, F., Chobert, J.-M., Haertlé, T., & Marzouki, M. N. (2011). Purification and biochemical characterization of stable alkaline protease Prot-2 from *Botrytis cinerea*. *Process Biochemistry*, 46, 2301–2310. <https://doi.org/10.1016/j.procbio.2011.09.010>.
- Agrawal, H., Joshi, R., & Gupta, M. (2017). Isolation and characterisation of enzymatic hydrolysed peptides with antioxidant activities from green tender sorghum. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, 84, 608–616. <https://doi.org/10.1016/j.lwt.2017.06.036>.
- Ahamed, A., Singh, A., & Ward, O. P. (2007). Chymostatin can combine with pepstatin to eliminate extracellular protease activity in cultures of *Aspergillus Niger* NRRL-3. *Journal of Industrial Microbiology & Biotechnology*, 34, 165–169. <https://doi.org/10.1007/s10295-006-0183-3>.
- Ahmed, I., Zia, M. A., Iftikhar, T., & Iqbal, H. M. (2011). Characterization and detergent compatibility of purified protease produced from *Aspergillus Niger* by utilizing agro wastes. *BioResources*, 6, 4505–4522.
- AOAC (1996). *Official methods of analysis of AOAC International* (16th ed.). Washington, DC, USA: Association of Official Analytical Chemists.
- Beg, Q. K., & Gupta, R. (2003). Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme and Microbial Technology*, 32, 294–304. [https://doi.org/10.1016/S0141-0229\(02\)00293-4](https://doi.org/10.1016/S0141-0229(02)00293-4).
- Bertucci, J. I., Liggieri, C. S., Colombo, M. L., Vairo Cavalli, S. E., & Bruno, M. A. (2015). Application of peptidases from *Maclura pomifera* fruit for the production of active biopeptides from whey protein. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, 64, 157–163. <https://doi.org/10.1016/j.lwt.2015.05.041>.
- Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escalera, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta*, 76, 965–977. <https://doi.org/10.1016/j.talanta.2008.05.019>.
- Braaksma, M., Smilde, A. K., van der Werf, M. J., & Punt, P. J. (2009). The effect of environmental conditions on extracellular protease activity in controlled fermentations of *Aspergillus Niger*. *Microbiology*, 155, 3430–3439. <https://doi.org/10.1099/mic.0.031062-0>.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3).
- de Castro, R. J. S., Nishide, T. G., & Sato, H. H. (2014). Production and biochemical properties of proteases secreted by *Aspergillus Niger* under solid state fermentation in response to different agroindustrial substrates. *Biocatalysis and Agricultural Biotechnology*, 3, 236–245. <https://doi.org/10.1016/j.cbab.2014.06.001>.
- de Castro, R. J. S., Ohara, A., Nishide, T. G., Bagagli, M. P., Dias, F. F. G., & Sato, H. H. (2015). A versatile system based on substrate formulation using agroindustrial wastes for protease production by *Aspergillus Niger* under solid state fermentation. *Biocatalysis and Agricultural Biotechnology*, 4, 678–684. <https://doi.org/10.1016/j.cbab.2015.08.010>.
- Cedro, A., N. G. (2006). Effects of climatic conditions on annual tree ring growth of the *Platanus x hispanica* "Acerifolia" under urban conditions of Szczecin. *Dendrobiology*, 55, 11–17.
- Chauhan, K., Trivedi, U., & Patel, K. C. (2007). Statistical screening of medium components by Plackett–Burman design for lactic acid production by *Lactobacillus* sp. KCP01 using date juice. *Bioresource Technology*, 98, 98–103. <https://doi.org/10.1016/j.biortech.2005.11.017>.
- Cimini, A., De Francesco, G., & Perretti, G. (2017). Effect of crossflow microfiltration on the clarification and stability of beer from 100% low- β -glucan barley or malt. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, 86, 55–61. <https://doi.org/10.1016/j.lwt.2017.07.033>.
- Coral, G., Arkan, B., Unaldi, M., & Guvenmez, H. (2003). Thermostable alkaline protease produced by an *Aspergillus Niger* strain. *Annals of Microbiology*, 53, 491–498.
- Cupp-Enyard, C. (2008). Sigma's non-specific protease activity assay-casein as a substrate. *Journal of Visualized Experiments: Journal of Visualized Experiments*, 19, 1–2.
- Deng, A., Wu, J., Zhang, Y., Zhang, G., & Wen, T. (2010). Purification and characterization of a surfactant-stable high-alkaline protease from *Bacillus* sp. B001. *Bioresource Technology*, 101, 7100–7106. <https://doi.org/10.1016/j.biortech.2010.03.130>.
- Devi, M. K., Banu, A. R., Gnanaprabha, G., Pradeep, B., & Palaniswamy, M. (2008). Purification, characterization of alkaline protease enzyme from native isolate *Aspergillus Niger* and its compatibility with commercial detergents. *Indian Journal of Science and Technology*, 1, 1–6.
- Dey, T. B., Adak, S., Bhattacharya, P., & Banerjee, R. (2014). Purification of polygalacturonase from *Aspergillus awamori* Nakazawa MTCC 6652 and its application in apple juice clarification. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, 59, 591–595. <https://doi.org/10.1016/j.lwt.2014.04.064>.
- Esparza, Y., Huaiquil, A., Neira, L., Leyton, A., Rubilar, M., Salazar, L., et al. (2011). Optimization of process conditions for the production of a polylenoipeptidase by *Aspergillus Niger* ATCC 11414 in solid state fermentation. *Food Science and Biotechnology*, 20, 1323–1330. <https://doi.org/10.1007/s10068-011-0182-7>.
- García-Gómez, M., Huerta-Ochoa, S., Loera-Corral, O., & Prado-Barragán, L. (2009). Advantages of a proteolytic extract by *Aspergillus oryzae* from fish flour over a commercial proteolytic preparation. *Food Chemistry*, 112, 604–608. <https://doi.org/10.1016/j.foodchem.2008.06.016>.
- John, R. P., Nampoothiri, K. M., & Pandey, A. (2006). Solid-state fermentation for L-lactic acid production from agro wastes using *Lactobacillus delbrueckii*. *Process Biochemistry*, 41, 759–763. <https://doi.org/10.1016/j.procbio.2005.09.013>.
- Kanimozhi, J., Moorthy, I. G., Sivashankar, R., & Sivasubramanian, V. (2017). Optimization of dextran production by *Weissella cibaria* NITCSK4 using Response Surface Methodology-Genetic Algorithm based technology. *Carbohydrate Polymers*, 174, 103–110. <https://doi.org/10.1016/j.carbpol.2017.06.021>.
- Krishnan, S., & Vijayalakshmi, M. (1986). Purification and some properties of three serine carboxypeptidases from *Aspergillus Niger*. *Journal of Chromatography A*, 370, 315–326. [https://doi.org/10.1016/S0021-9673\(00\)94702-2](https://doi.org/10.1016/S0021-9673(00)94702-2).
- Liu, H.-M., & Li, H.-Y. (2017). *Application and conversion of soybean hulls*. London: InTech (Chapter 7).
- Li, D.-C., Yang, Y.-J., & Shen, C.-Y. (1997). Protease production by the thermophilic fungus *Thermomyces lanuginosus*. *Mycological Research*, 101, 18–22.
- da Luz, J. M. R., Paes, S. A., Torres, D. P., Nunes, M. D., da Silva, J. S., Mantovani, H. C., et al. (2013). Production of edible mushroom and degradation of antinutritional factors in jatropha biodiesel residues. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, 50, 575–580. <https://doi.org/10.1016/j.lwt.2012.08.006>.
- Mazotto, A. M., Couri, S., Damaso, M. C., & Vermeelho, A. B. (2013). Degradation of feather waste by *Aspergillus Niger* keratinases: Comparison of submerged and solid-state fermentation. *International Biodeterioration & Biodegradation*, 85, 189–195. <https://doi.org/10.1016/j.ibiod.2013.07.003>.
- Merheb, C. W., Cabral, H., Gomes, E., & Da-Silva, R. (2007). Partial characterization of protease from a thermophilic fungus, *Thermoascus aurantiacus*, and its hydrolytic activity on bovine casein. *Food Chemistry*, 104, 127–131. <https://doi.org/10.1016/j.foodchem.2006.11.010>.
- Novelli, P. K., Barros, M. M., & Fleuri, L. F. (2016). Novel inexpensive fungi proteases: Production by solid state fermentation and characterization. *Food Chemistry*, 198, 119–124. <https://doi.org/10.1016/j.foodchem.2015.11.089>.
- Orzua, M. C., Mussatto, S. I., Contreras-Esquivel, J. C., Rodriguez, R., de la Garza, H., Teixeira, J. A., et al. (2009). Exploitation of agro industrial wastes as immobilization carrier for solid-state fermentation. *Industrial Crops and Products*, 30, 24–27. <https://doi.org/10.1016/j.indcrop.2009.02.001>.
- Osman, H., Abdel-Fattah, A., Abdel-Samie, M., & Mabrouk, S. (1969). Production of a milk-clotting enzyme preparation by *Aspergillus Niger* and the effect of various factors on its activity. *Microbiology*, 59, 125–129. <https://doi.org/10.1099/00221287-59-1-125>.
- Paranthaman, R., Alagusundaram, K., & Indhumathi, J. (2009). Production of protease from rice mill wastes by *Aspergillus Niger* in solid state fermentation. *World Journal of Agricultural Sciences*, 5, 308–312.
- Robledo, A., Aguilera-Carbó, A., Rodríguez, R., Martínez, J. L., Garza, Y., & Aguilar, C. N. (2008). Ellagic acid production by *Aspergillus Niger* in solid state fermentation of pomegranate residues. *Journal of Industrial Microbiology & Biotechnology*, 35, 507–513. <https://doi.org/10.1007/s10295-008-0309-x>.
- Sandhu, K. S., Punia, S., & Kaur, M. (2016). Effect of duration of solid state fermentation by *Aspergillus awamori* nakazawa on antioxidant properties of wheat cultivars. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, 71, 323–328. <https://doi.org/10.1016/j.lwt.2016.04.008>.
- Schuster, E., Dunn-Coleman, N., Frisvad, J., & Van Dijk, P. (2002). On the safety of *Aspergillus Niger*—a review. *Applied Microbiology and Biotechnology*, 59, 426–435. <https://doi.org/10.1007/s00253-002-1032-6>.
- Segura-Campos, M. R., Salazar-Vega, I. M., Chel-Guerrero, L. A., & Betancur-Ancona, D. A. (2013). Biological potential of chia (*Salvia hispanica* L.) protein hydrolysates and their incorporation into functional foods. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, 50, 723–731. <https://doi.org/10.1016/j.lwt.2012.07.017>.
- Souza, P. M. d., Bittencourt, M. L. d. A., Caprara, C. C., Freitas, M. d., Almeida, R. P. C. d., Silveira, D., et al. (2015). A biotechnology perspective of fungal proteases. *Brazilian Journal of Microbiology*, 46, 337–346. <https://doi.org/10.1590/S1517-838246220140359>.
- Tari, C., Gögs, N., & Tokatli, F. (2007). Optimization of biomass, pellet size and polygalacturonase production by *Aspergillus sojae* ATCC 20235 using response surface methodology. *Enzyme and Microbial Technology*, 40, 1108–1116. <https://doi.org/10.1016/j.enzmictec.2006.08.016>.
- Tavano, O. L. (2013). Protein hydrolysis using proteases: An important tool for food biotechnology. *Journal of Molecular Catalysis B: Enzymatic*, 90, 1–11. <https://doi.org/10.1016/j.molcatb.2013.01.011>.
- Torrado, A. M., Cortés, S., Salgado, J. M., Max, B., Rodríguez, N., Bibbins, B. P., et al. (2011). Citric acid production from orange peel wastes by solid-state fermentation. *Brazilian Journal of Microbiology*, 42, 394–409. <https://doi.org/10.1590/S1517-83822011000100049>.
- Torres, M. J., Trejo, S. A., Natalucci, C. L., & López, L. M. I. (2013). Biochemical characterization of VQ-VII, a cysteine peptidase with broad specificity, isolated from *Vasconella quercifolia* latex. *Planta*, 237, 1651–1659. <https://doi.org/10.1007/s00425-013-1872-6>.
- Tsang, A., Butler, G., Powlowski, J., Panisko, E. A., & Baker, S. E. (2009). Analytical and computational approaches to define the *Aspergillus Niger* secretome. *Fungal Genetics and Biology*, 46, S153–S160. <https://doi.org/10.1016/j.fgb.2008.07.014>.
- Tunga, R., Shrivastava, B., & Banerjee, R. (2003). Purification and characterization of a protease from solid state cultures of *Aspergillus parasiticus*. *Process Biochemistry*, 38, 1553–1558. [https://doi.org/10.1016/S0032-9592\(03\)00048-7](https://doi.org/10.1016/S0032-9592(03)00048-7).
- Valerga, J., Meta, M., & Lanari, M. C. (2012). Polyphenol input to the antioxidant activity of yerba mate (*Ilex paraguariensis*) extracts. *LWT-Food Science and Technology*, 45, 28–35. <https://doi.org/10.1016/j.lwt.2011.07.022>.
- Wang, S.-L., Chen, Y.-H., & Yen, Y.-H. (2005). Purification and characterization of a serine protease extracellularly produced by *Aspergillus fumigatus* in a shrimp and crab shell powder medium. *Enzyme and Microbial Technology*, 36, 660–665. <https://doi.org/10.1016/j.enzmictec.2004.10.006>.
- Yadav, S. K., Bisht, D., Tiwari, S., & Darmwal, N. S. (2015). Purification, biochemical characterization and performance evaluation of an alkaline serine protease from *Aspergillus flavus* MTCC 9952 mutant. *Biocatalysis and Agricultural Biotechnology*, 4, 667–677. <https://doi.org/10.1016/j.cbab.2015.08.007>.
- Yu, J., Wang, G., Wang, X., Xu, Y., Chen, S., Wang, X., et al. (2018). Improving the freeze-thaw stability of soy protein emulsions via combing limited hydrolysis and Maillard-induced glycation. *LWT-Food Science and Technology*, 91, 63–69. <https://doi.org/10.1016/j.lwt.2018.01.031>.
- Zhu, C., Han, W., Chen, Z., & Han, Z. (2010, October). Statistical optimization of microwave-assisted astaxanthin extraction from *Phaffia rhodozym*. *Conference at the meeting of Biomedical engineering and informatics (BMEI)*, Yantai, China.