

Production and characterization of protease from *Penicillium aurantiogriseum* URM 4622

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Abstract

Proteases with new properties are required due to their increasing industrial importance. In this work, the optimal fermentation conditions for the production of a protease from *Penicillium aurantiogriseum* dierchx (URM-4622) are presented together with partial characterization of the protease catalytic properties. The batch fermentation conditions that allow for the highest specific proteolytic activity are 26 °C, pH 7.0, and 25 % saturation dissolved O₂ concentration. The obtained protease is stable over a wide range of pH (5.8 to 9.5) and temperature (25 to 40 °C) values. In the presence of Zn²⁺ a 26 % reduction in the enzyme proteolytic activity occurs and, in contrast, Mn²⁺ enhances its activity by 28.9 %, 96.2 % and 70.8 % of the protease activity are maintained after 90 min incubation in 5 and 10 % (v/v) H₂O₂ aqueous solutions, respectively. PMSF inhibition reveals that this enzyme is a serine protease. Protease is able to hydrolyze different proteins.

Keywords: *Penicillium aurantiogriseum*; production; characterization; protease; batch; bioreactor; detergent.

Introduction

Proteases, also known as peptidyl-peptide hydrolases, are important industrial enzymes, which are responsible for approximately 60% of all enzyme sales, and are extensively used in a variety of industries, including foods, pharmaceuticals, leathers and detergents [1,2].

Microbes represent an excellent source of enzymes, including proteases, because of their broad biochemical diversity [3,4]. Microbial proteases can be produced from bacteria, fungi and yeast using solid-state fermentation as well as submerged fermentation [5]. Fungi as enzymes producers have many advantages, considering that the produced enzymes are normally extracellular, making easier its recuperation from the fermentation broth [6].

Any fermentation process is significantly influenced by physical and chemical parameters and different results can be obtained as we move from shake flask to large bioreactor fermentation as different mixing and mass transfer patterns may occur as scale is increased [7]. It is well known that extracellular protease production by microorganisms in bioreactors is greatly influenced by medium components, physical factors such as aeration, agitation, temperature, inoculum density, dissolved oxygen and incubation time. Industrial fermentation is moving away from traditional and largely empirical operation towards knowledge based and better-controlled process [5]. This work addresses the optimization of the fermentation conditions for protease production by *Penicillium aurantiogriseum* dierchx (URM 4622) and the characterization of the enzyme catalytic properties.

Materials and methods

Microorganism and culture medium

The *Penicillium aurantiogriseum* dierchx (URM 4622) was supplied by the Micoteca of Micology Department of the Universidade Federal de Pernambuco (UFPE). The strain was maintained at 28°C in malt extract agar, consisting of: 2% (w/v) malt extract, 0.1% (w/v) peptona, 2% (w/v) glucose and 1.5% (w/v) agar. Soy flour medium (SM), as described by Porto *et al.* [8], was used for protease production and it is composed of: 1% (w/v) filtered soy flour, 0.1% (w/v) NH₄Cl, 0.06% (w/v) MgSO₄·7H₂O, 0.435% (w/v) K₂HPO₄, 0.01% (w/v) glucose and 0.8 mL mineral solution. The composition of the mineral solution, per 100 mL of distilled water, is: 100 mg of FeSO₄·7H₂O; 100 mg of MnCl₂·4H₂O; 100 mg of ZnSO₄·H₂O; 100 mg of CaCl₂·H₂O. The fermentation medium was sterilized in autoclave at 121 °C, for 20 minutes.

Production of protease

The inoculum was prepared in test tubes, containing 10 mL of a solution of 0.9% (w/v) NaCl and 0.01% (v/v) Tween 80 sterilized previously at 121°C, for 20 minutes. The solution of spores was prepared so that a final concentration of 10⁶ spores.mL⁻¹ was obtained. Fermentations were realized using 18 Erlenmeyer flasks (50 mL) each containing 10 mL of the Soy flour medium with 10⁶ esporos.mL⁻¹ of the inoculum. Flasks were incubated at 28°C, with constant shaking at 150 rpm for 96 hours. Two samples were taken every 12 h for the determination of biomass concentration, pH and protease activity.

Bioreactor studies

Protease production in bioreactor was evaluated using a statistical design. A 2³ full design was carried out to verify the effects and interactions of pH, temperature and aeration rate on the protease production. In this design, a set of 11 experiments, with three replicates at the central points, was performed. The range and levels of the components (factors or independent variables) under study are given in Table 1. The production of protease of *Penicillium aurantiogriseum* was carried out in a stirred tank reactor (Fermenter RALF 2.0 L) with 1.5L working volume, equipped with temperature, pH and dissolved oxygen measurement and control. The pre-inoculum was prepared in Erlenmeyer flasks (250 mL) each containing 50 mL of the Soy flour medium with 10⁶ esporos.mL⁻¹ and incubated at 28°C, 150 rpm for 24 h. After this, the culture was added in bioreactor containing 1.45 L of the Soy flour medium previously sterilized. The pH of the medium was adjusted with NH₃ (6 % v/v) and/or H₃PO₄ (21% v/v). Samples were collected at regular intervals of 12 h for determinations of the biomass, protease activity and total protein concentration.

Analytical methods

The growth curve of the *Penicillium aurantiogriseum* was accompanied by measuring biomass dry weight. The fermented broth was filtered through previously weighted 0.45 µm porosity membranes (after drying at 80°C, for 1 hour). The membranes, after the filtration of the fermented broth, were again dried at 80°C for 2 hours. The dry weight of the samples was obtained by the difference of the weight of the membranes, before and after filtration.

The protein concentration was determined according to Bradford [9], using bovine serum albumin (BSA) as standard. The protease activity was determined according to Leighton *et al.* [10] using 1% (w/v) azocasein as substrate in a 0.1 M Tris-HCl pH 7.2 buffer. One unit of protease activity was defined as the amount of enzyme required to produce an absorbance variation of 1 for 1 hour and was expressed in U/mL.

Effect of pH and temperature on the activity and stability of the protease

The optimum pH of protease activity was determined with 1% (w/v) azocasein as substrate dissolved in different buffers. The buffers used were: 0.1 M sodium-phosphate (pH 5.8, 6.2 and 7.2); 0.1 M Tris-HCl (pH 7.2, 8.6 and 9.0) and 0.1 M glycine-NaOH (pH 8.6, 9.0 and 9.5). The pH stability of the protease was determined by incubating the enzyme solution with the different buffers mentioned in a proportion of 1:1 (v/v) during 120 min at 25°C. Samples were collected each 30 min, for the determination of the protease activity.

The protease activity was assayed at various temperatures (25 - 70°C) to determine the optimum temperature, using 1% (w/v) azocasein as substrate in a 0.1 M Tris-HCl pH 9.0 buffer. For determination of thermostability, the enzyme was pre-incubated at different temperatures for 120 min, being samples taken every 30 min for determination of the protease activity. The shelf life at low temperatures (-20°C and 4°C) was determined after 30 days of incubation.

Table 1. Factor levels used in the 23 full design used for studying of protease production by *P. aurantiogriseum*.

Variables	Levels		
	Lower (-1)	Center (0)	Higher (+1)
Temperature (°C)	22	26	30
pH	5.0	7.0	9.0
O ₂ (%)	10	25	40

Effect of metal ions, inhibitors and oxidizing agent on activity

The effects of metal ions were investigated using: CaCl₂, AlCl₃, LiCl, ZnCl₂, MnCl₂, KCl, and NaCl in the concentration 1mM. The substrate 1% (w/v) azocasein was prepared with different metal ions and dissolved in 0.1 M Tris-HCl pH 9.0. Enzyme activities in the presence of metal ions were compared with the control (without metal ions).

To determine the type of the protease, enzyme was pre-incubated for 15 min at 37°C with different specific protease inhibitors at a 0.1 M concentration and protease activity was determined. Tested inhibitors were: PMSF, EDTA and iodoacetic acid. Activity in the presence of inhibitors was compared with the control (without inhibitors).

The stability of enzyme activity in the presence of hydrogen peroxide was studied by incubating the protease for 120 min at 40°C with different concentrations of hydrogen peroxide from 5 to 15% (v/v). Samples were collected each 30 min for determination of the protease activity.

Protease activity on different substrates

The proteolytic activity assay was applied to other protein substrates: bovine serum albumin (BSA), hemoglobin, and ovalbumin according to a modified method described by Pokorný *et al* [11], azocasein according to Leighton *et al.* [10] and azocoll, according to Chavira *et al.* [12].

Results and discussion

The time variation of biomass concentration, pH and protease activity in shake flask culture by *P. aurantiogriseum* is shown in Fig. 1. Maximum biomass production (2.7 g/L) occurred at 48 h, while maximum

enzyme production was observed at 84 h when the medium pH was 8.0. The growth curve shows a clear exponential phase, with a 48 h lag phase. After 60 h (end of exponential phase) the biomass starts to decrease, probably as a consequence of the depletion of nutrients into the culture medium. Studies on the production of alkaline protease by *Aspergillus tamarii* concluded that protease production occurred at 100 h of growth, when the medium pH was 9.0 [13].

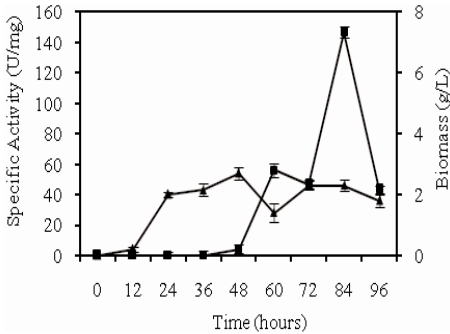


Fig. 1. Growth curve (▲) and protease production (■) of *P. aurantiogriseum*.

The results obtained with the 2³ full factorial design are show that the central point of the experimental design (26°C, pH 7 and 25% saturation dissolved oxygen concentration) was the best condition for the protease production, showing the highest values of specific activity 44.48, 41.39 and 44.55 (U/mg). The analysis of the Pareto chart (Fig. 2), demonstrates that only the pH value was significant for enzyme production and cell growth. A pH significant negative effect was observed, suggesting (inside the appraised conditions) that the decrease of the pH value improves the protease production and cell growth, observing that the pH 7 was the best condition. Similar results were obtained by Çalik *et al.* [14] when investigating the influence of controlled-pH and uncontrolled-pH conditions together with the initial pH in serine alkaline protease production by recombinant *Bacillus licheniformis*.

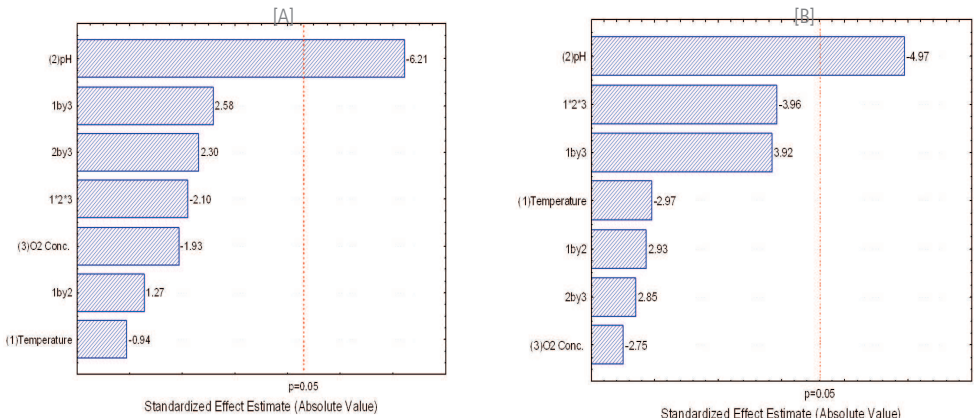


Fig. 2. Pareto charts for the effects of the variables temperature (1), pH (2) and O₂ concentration (3) on the (a) Biomass (mg/L) and (b) specific activity (U/mg).

The relative activities at various pH values (5.8 to 9.5) are shown in Fig. 3a. Regarding the effect of the buffers themselves, differences in the activity were observed with different buffers. The protease exhibited a maximum activity at pH 9.0 in buffer Tris-HCl 0.1 M. More than 90% of the maximum activity was detected for the protease between pH 7.2-9.5, which is a typical characteristic of alkaline proteases. The results are in accordance with several earlier reports showing a pH optimum of 9.0 and 8.5 for protease from *Penicillium* sp. and *Aspergillus tamari* [15,13]. Protease was very stable in a broad pH range from acid to basic, maintaining over 90% of its initial activity between pH 5.8 and 9.5, after incubation for 2h (Fig. 3b), indicating its potential for practical use in industrial purposes which require stability over wide pH ranges. Similar results were obtained with protease produced by *Penicillium* sp. [6].

The optimum temperature for the protease was found to be 50°C (Fig. 4a). The protease was stable between 25 and 40°C after 2 h incubation retaining above 100% of the activity, which allows lower wash temperatures when added to detergents. More than 25% of maximal activity was maintained after 30 min pre-incubation at 50°C but total inactivation occurred above 60°C (Fig. 4b). These results are in accordance with those obtained by Tunga *et al.* [16] and Azeredo *et al.* [17] for a protease produced by *Aspergillus parasiticus* and *Streptomyces* sp. At low temperatures (-20 and 4°C), the protease retained 54.5% of its activity after 1 month storage. The protease from *Streptomyces* sp. was retained 75% of its activity after 2 months at low temperatures (-20 and 4°C) [17].

The effect of metal ions on the activities of the protease is shown in Table 2. The proteolytic activity decreased about 26% in the presence of Zn²⁺ ion and increased 28.9% in the presence of Mn²⁺. The presence of Li⁺, Ca²⁺ and K⁺, resulted in a discrete increase in the proteolytic activity. Similar effects of Mn²⁺ on the activity of protease were found by Agrawal *et al.* [15], when studying the production of alkaline protease by *Penicillium* sp.

The ability to hydrolyze several protein substrates is a criterion for the potential application of the enzyme [18]. In this study, the enzyme was examined for the ability to hydrolyze several proteins (Table 2). Protease activity on bovine serum albumin (BSA) and hemoglobin was 26 and 29%, respectively, as compared to its activity on azocasein. The protease showed the best activity against azocool (144%). The broader specificity of the protease from *Penicillium aurantiogriseum* may be advantageous for its use in detergents against a wide variety of stains.

The nature of the protease, enzyme activity was measured in the presence of different protease inhibitors (Table 2). No inhibition was detected when the cysteine type inhibitor, i.e. iodoacetamide was added. Almost no inhibition was observed with EDTA. In contrast, protease was strongly inhibited by the serine protease inhibitor PMSF (0.1 M). This finding was similar to those of Germano *et al.* [6] and Anandan, *et al.* [13], for proteases from *Penicillium* sp. and *Aspergillus tamarii*, respectively. This result shows that *P. aurantiogriseum* secreted a serine type protease during submerged fermentation.

The results obtained in the stability of the protease in the presence of hydrogen peroxide (Fig. 5) showed that at concentrations of 5, 10 and 15% (v/v) the protease retained 96.7, 81.2 and 67.35% of its activity, respectively, after 60 minutes of incubation. After 120 minutes incubation, the protease retained 77.3, 46.5 and 27.9% of its activity for hydrogen peroxide concentrations of 5, 10 and 15%, respectively. In the detergent industry, several oxidizing agents like sodium perborate used as detergent compositions, which may release hydrogen peroxide and hence bleach stable enzymes are prepared for detergent industry [19]. The protease from *Aspergillus parasiticus* [16] was not influenced by hydrogen peroxide, while the protease from *Penicillium* sp. [6] showed a good stability for a concentration of 5% (v/v) for 1 h of incubation.

Fig. 3. Effect of pH on the activity (a) and stability after incubation for 120 min (b) of the protease production by *P. aurantiogriseum*. Buffers: (■) 0.1M Sodium-phosphate; (▲) 0.1M Tris-HCl; (●) 0.1M Glycine-NaOH.

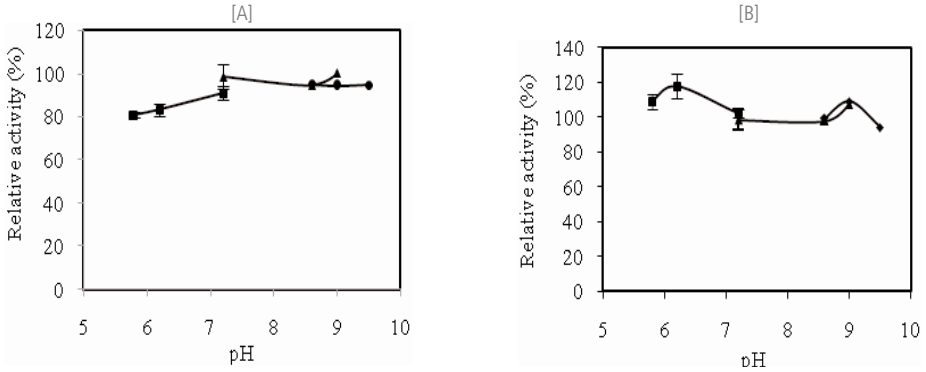


Fig. 4. Effect of temperature on the activity (a) and stability (b) of the protease production by *P. aurantiogriseum*. Temperatures: 25°C (◆); 30°C (■); 40°C (▲); 50°C (○); 60°C (□) and 70°C (△).

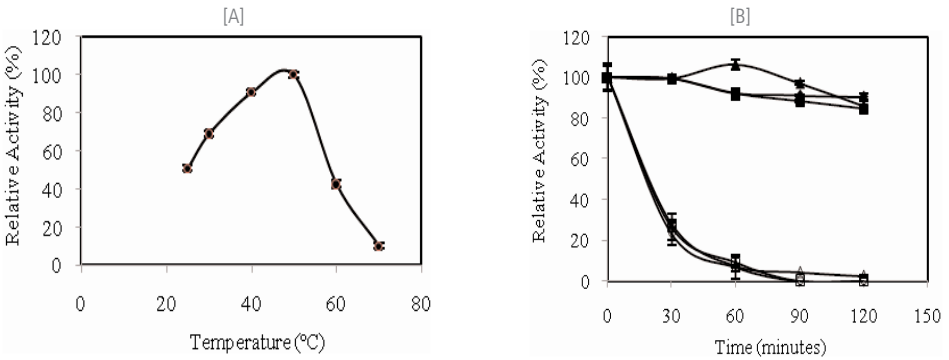


Fig. 5. Effect of oxidant agent H_2O_2 on the stability of the protease. Concentrations: (○) 5 %; (■) 10 % and (▲) 15%.

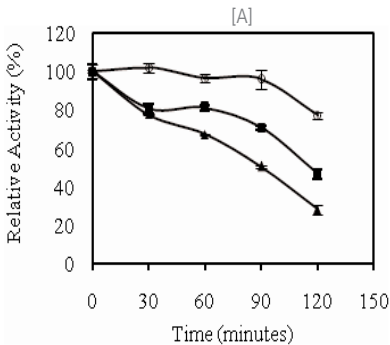


Table 2. Effect of various reagents on activity of protease

Agent	Concentration	Relative Activity (%)
AlCl ₃	0.1 M	99.4
MnCl ₂	0.1 M	128.9
LiCl	0.1 M	104.5
CaCl ₂	0.1 M	104.8
KCl	0.1 M	102.9
NaCl	0.1 M	100.0
ZnCl ₂	0.1 M	74.0
BSA	1 %	25.9
Hemoglobin	1 %	29.4
Ovoalbumin	1 %	12.1
Azocoll	0.2 %	143.8
Azocasein	1 %	100.0
EDTA	0.1 M	94.8
PMSF	0.1 M	7.0
Iodoacetic acid	0.1 M	94.9

Conclusions

The alkaline protease produced by *P. aurantiogriseum* dierchx (URM 4622) is a serine type peptidase, stable over a wide range of pH (5.8 to 9.5) and temperature (25 to 40 °C) values. Furthermore, the presence of Mn²⁺ enhances its activity, it is not inhibited by high concentrations of H₂O₂ and is able to hydrolyze different proteins. These properties make this protease useful for application in detergent industries.

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References

- [1] Chellappan S, Jasmin C, Basheer SM, Elyas KK, Bhat SG, Chandrasekaran M. Production, purification and partial characterization of a novel protease from marine *Engyodontium album* BTMFS10 under solid state fermentation. *Process Biochem*, 41:956–961, 2006.
- [2] Rao YK, Lu S, Liu B, Tzeng Y. Enhanced production of an extracellular protease from *Beauveria bassiana* by optimization of cultivation processes. *Biochem Engin Jour*, 28:57–66, 2006.
- [3] Bhaskar NA, Sudeepa ES, Rashmi HN, Tamil AS. Partial Purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. *Bioresource Tech*, 98:2758-2764, 2007.

- [4] Najafi MF, Deobagkar D, Deobagkar D. Potencial application of protease isolated from *Pseudomonas aeruginosa* PD100. *Electronic Journal of Biotech*,8:198-207,2005.
- [5] Portumarthi R, Ch S, Jetty A. Alkaline protease production by submerged fermentation in stirred tank reactor using *Bacillus licheniformis* NCIM-2042: Effect of aeration and agitation regimes. *Biochem Engin Jour*, 34:185–192, 2007.
- [6] Germano S, Pandey A, Osaku CA, Rocha,SN, Soccol CR. Characterization and stability of protease from *Penicillium* sp. produced by solid-state fermentation. *Enz Microb Tech*, 32:246–251, 2003.
- [7] Dasu VV, Panda T, Chidambaram M. Determination of significant parameters for improved griseofulvin production in a batch bioreactor by Taguchi's method *Process Biochem*, v. 38, p. 877-880, 2003.
- [8] Porto ALF, Campos-Takaki GM, Lima-Filho J L. Effects of cultura conditions on protease production by *Streptomyces clavuligerus* growing soy bean flour medium. *Appl Biochem of Biotech*, 60:115-122, 1996.
- [9] Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochem*,72:248-254, 1976.
- [10] Leighton TJ, Doi RH, Warren RAJ, Lelen RA. The relationship of serine proteaseactivity to RNA polymerase modification and sporulation in *Bacillus subtilis*. *Jour Mol Biol*, 76:103-122, 1973.
- [11] Pokorny M, Vitale LJ, Turk V, Renko M, Zuvanic J. *Streptomyces rimosus* extracellular proteases. *Eur Jour Appl Microb*, 1979;8:81-90.
- [12] Chavira RJ, Burnett TJ, Hageman JH. Assaying proteinases with Azocoll, *Anal Biochem*.136:4446-4450, 1984.
- [13] Anandan D, Marmer WN, Dudley RL. Isolation, characterization and optimization of culture parameters for production of an alkaline protease isolated from *Aspergillus tamarii*. *Jour Ind Microb Biotech*, 34:339–347, 2007.
- [14] Çalik P, Bilir E, Çalik G, Özdamar TH. Bioreactor operation parameters as tools for metabolic regulations in fermentation processes: influence of pH conditions. *Chemical Engineering Science*, 58:759-766, 2003.
- [15] Agrawal D, Patidar P, Banerjee T, Patil S. Production of alkaline portease by *Penicillium* sp. under conditions and its application to soy protein hydrolysis. *Process Biochem*, 39:977-981, 2004.
- [16] Tunga R, Shrivastava B, Banerjee R. Purification and characterization of protease from solid state culture of *Aspergillus parasiticus*. *Process Biochem*, 38:1553–1558, 2003.
- [17] Azeredo LA, Freire DMG, Soares RMA, Leite SGF, Coelho RRR. Production and partial characterization of thermophilic proteases from *Streptomyces* sp. isolated from Brazilian cerrado soil. *Enz Microb Tech*, 34:354-358, 2004.
- [18] Grebeshova RN, Saldeco-Torres LE, Hidalgo MA. Serine protease of *Bacillus subtilis* R. *Appl Biochem Microbiol* 1999;35:131-134.
- [19] Johnvesly B, Naik, GR. Studies on production of thermostable alkaline protease from thermophilic and *Bacillus* sp. JB-99 in a chemically defined medium. *Process Biochemistry*, 37:139-144, 2001.