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Production and partial characterization of proteases from *Mucor hiemalis* URM3773

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ABSTRACT. The current study evaluated the proteases production from 11 fungal species belonging to the genera *Mucor*, *Rhizomucor* and *Absidia*. The species were obtained from the Collection of Cultures URM at the Mycology Department-UFPE, Brazil. The best producing species was *Mucor hiemalis* URM 3773 (1.689 U mL^{-1}). Plackett-Burman design methodology was employed to select the most effective parameter for protease production out of 11 medium components, including: concentration of filtrate soybean, glucose, incubation period, yeast extract, tryptone, pH, aeration, rotation, NH_4Cl, $MgSO_4$ and K_2HPO_4. Filtrated soybean concentration was the significant variable over the response variable, which was the specific protease activity. The crude enzyme extract showed optimal activity in pH 7.5 and at 50ºC. The enzyme was stable within a wide pH range from 5.8 to 8.0, in the phosphate buffer 0.1M and in stable temperature variation of 40-70ºC, for 180 minutes. The ions $FeSO_4$, NaCl, $MnCl_2$, $MgCl_2$ and KCl stimulated the protease activity, whereas $ZnCl_2$ ion inhibited the activity in 2.27%. Iodoacetic acid at 1mM was the proteases inhibitor that presented greater action.The results indicate that the studied enzyme have great potential for industrial application.

Keywords: proteolytic activity, substrate fermentation liquid, Plackett-Burman design, biochemical characterization.

Produção e caracterização parcial de proteases a partir de *Mucor hiemalis* URM3773

RESUMO. Foi avaliada a produção de proteases por 11 espécies fúngicas pertencentes aos gêneros *Mucor*, *Rhizomucor* e *Absidia*, obtidas da Coleção de Culturas URM do Departamento de Micologia-UFPE, Brasil. A melhor espécie produtora foi *Mucor hiemalis* URM3773 (1,689 U mL^{-1}). A metodologia de planejamento Plackett-Burman foi empregada para selecionar o parâmetro mais efetivo para a produção de proteases através de 11 componentes do meio, incluindo: concentração do filtrado de soja, glicose, período de incubação, extrato de levedura, triptona, pH, aeração, rotação, NH_4Cl, $MgSO_4$ e K_2HPO_4. A variável significante sobre a variável-resposta, atividade proteásica específica, foi a concentração do filtrado de soja. O extrato enzimático bruto apresentou atividade ótima ao pH 7,5 a 50ºC. A enzima foi estável em uma ampla variação de pH de 5,8–8,0 em tampão fosfato 0,1M e termicamente estável a uma variação de 40-70°C, durante 180 minutos. Os íons $FeSO_4$, NaCl, $MnCl_2$, $MgCl_2$ e KCl estimularam a atividade proteásica, enquanto que o íon $ZnCl_2$ inibiu 2,27% da atividade. O inibidor de proteases que teve maior ação foi o ácido iodoacético a 1mM. Os resultados obtidos indicam que a enzima estudada tem grande potencial de aplicação industrial.

Palavras-chave: atividade proteolítica, fermentação em substrato líquido, planejamento Plackett-Burman, caracterização bioquímica.

Introduction

In the recent years, the world has witnessed a significant increase in the use of enzymes as industrial catalysts. Proteases, in particular, represent an important group of industrially produced enzymes and they account for 60% of the worldwide sales value of the total industrial enzymes. Proteases are very useful in several industrial sectors, such as detergents, leather, food, feed, textile, organic synthesis, pharmaceutical products and silk, for recovery of silver from used X-ray films and for wastewater treatment. Proteases have also clinical and medical applications such as that in the reduction of tissue inflammation (ABIDI et al., 2011).

The biggest sector in the biotechnological industry relies on the production and use of microbial origin enzymes (BON et al., 2008). Due

to their great variety, possibility of genetic manipulation and their rapid microorganism growth in inexpensive medium, the microbial enzymes are often more useful than those from animals and vegetables (HASAN et al., 2006).

For thousands of years, fungi have been used for food making as well as for medicinal and biotechnological purposes (GOMES-DA-COSTA et al., 2008). Fungi have been extensively used in the production of different substances of economic interest such as enzymes, antibiotics, vitamins, amino acids and steroids (ABIDI et al., 2008).

It has been reported that approximately ninety percent of the industrial enzymes are produced by means of submerged fermentation (JOO; CHANG, 2006). Submerged fermentation has been defined as the fermentation that occurs in the presence of water excess, thus presenting a better monitoring and easy handling (SINGHANIA et al., 2010).

Proteases production by microorganisms is greatly influenced by medium components, especially carbon and nitrogen sources, and physical factors such as temperature, pH, incubation time, agitation and inoculum density. For potential industrial applications, hyper-productive organisms growing in economical substrates are required (ABIDI et al., 2008).

It is essential to optimize the fermentation medium for proteases growth and production, in order to obtain high and commercially viable yields (ESPÓSITO et al., 2009).

Many studies have investigated the optimization of culture medium in the fermentative process for producing certain substance of interest (AÇIKEL et al., 2010; RIGO et al., 2010). The statistical methods are believed to be effective and powerful approaches for rapidly screening key factors from a multivariable system, optimizing culture conditions and they have been extensively used recently (KONG et al., 2012).

It is essential to know the enzyme properties, such as its stability, optimal working conditions and mechanistic class. Such knowledge is essential before considering its potential industrial uses (BALQIS; ROSMA, 2011).

The present study aimed to quantitatively select the best filamentous fungus proteases producing species that does it by submerged fermentation in order to obtain crude proteases preparation. It also aimed to evaluate the proteolytic potential and to quantify the proteins in the used crude enzyme extract. Plackett-Burman experimental design was used to select the best fermentative condition for optimizing the proteases production. The study characterized the proteases obtained from pH and temperature optimum performance, pH and temperature stability as well as the effects of the metal ions and of proteases inhibitors.

Material and methods

Microorganisms and culture medium

Cultures of filamentous fungus of the genera *Mucor*, *Rhizomucor* and *Absidia* were obtained from the Collection of Cultures URM of the Mycology Department at Federal University of Pernambuco, both for the screening and production of proteases. The studied species were: *Mucor hiemalis* URM3773, *M. subtilissimus* URM4133, *M. racemosus* URM4143, *M. circinelloides* URM4148, *M. ramosissimus* URM4424, *M. circinelloides* URM4425, *M. pusillus* URM4567, *M. hiemalis* URM5063, *Absidia blakesleeana* URM5604, *Rhizomucor pusillus* URM5605 and *M. hiemalis* URM5687.

The culture medium used for fungal strains sporulation was the PDA (potato dextrose agar). The sterilization was held in autoclave at 121°C, for 20 minutes. The samples were incubated at 30°C for seven days in test tubes and subcultured every 30 days.

As for the metabolites production, a modified version of the fermentation medium described by Porto et al. (1996) was used. It held the following components: soy flour filtrate (1.0% w v^{-1}); K_2HPO_4 (0.158% w v^{-1}); NH_4Cl (0.0083% w v^{-1}); $MgSO_4.7H_2O$ (0.005% w v^{-1}); glucose (0.083% w v^{-1}) and 0.03 mL mineral solution (100 mg of $FeSO_4.7H_2O$, $MnCl_2.4H_2O$ and $ZnSO_4.7H_2O$ in 1000 mL distilled water).

Submerged fermentation

Erlenmeyer flasks of 125 mL capacity were used. They were properly sterilized and added with 25 mL of proteases production medium. The inoculum was prepared by adding a solution of NaCl (0.9%) and Tween 80 (0.3%) in the test tubes containing the fungus, in order to form a solution with the maximum of spores. An aliquot was removed from this solution aiming at counting the spores in a Neubauer's camera. The medium received the inoculum, in aseptic conditions, at a concentration of 10^5 spores mL^{-1}.

Culture was grown for 96 hours at 30°C in an orbital shaker (Tecnal, TE-421) at 120 rpm. After the incubation period, the mycelium formed during the cultivation was separated by filtration in qualitative filter paper. The cell-free filtrate, contained the crude preparation of proteases to be analyzed.

Determination of the total proteins and protease activity

Total proteins were determined according to a modified version of Bradford (1976) method, by using Coomassie Brilliant Blue that detects minimum quantities of protein in biological liquids. The calibration curve was generated from stock solutions of bovine serum albumin (BSA) in a concentration range of 0 - 600 μg mL^{-1}. Solutions were subjected to an absorbance reading at 595 nm in a spectrophotometer. The protein concentration was expressed in $mg.mL^{-1}$ of the sample.

The proteolytic activity was developed according to the method described by Alencar et al. (2003), by adding 100 μL substrate (1.0% azocasein solution in 0.1 M Tris-HCl buffer, pH 7.2) to 60 μL enzyme extract. The reaction mixture was incubated for one hour at room temperature in the dark. After incubation, the reaction was stopped by adding 480 μL trichloroacetic acid (TCA) 10% (w v^{-1}) to the reaction mixture. It was followed by centrifugation for 10 minutes at 8000 *g* and at the temperature of 4°C. After centrifugation, 320 μL was removed from the supernatant and added to 560 μL sodium hydroxide (1 M). One unit of protease activity was defined as the quantity of enzyme required to produce an absorbance variation equal to 0.01 in one hour, and it was expressed in U mL^{-1}. The specific protease activity was calculated by the ratio between the total protease activity and the total proteins contained in 1 mL of the sample, and it was expressed in U mg^{-1}.

Screening of proteases producing species

The above mentioned species were inoculated in the proteases production culture medium and cultivated in submerged fermentation at 30°C, 120 rpm, for 96 hours. The fermentation of each species was prepared in double. The evaluation of the best proteases producing species was quantitatively conducted by determining the total protease activity of each filtrate obtained from different fermentations. The metabolites produced in each cultivation were compared in order to select the most promising strains, thus discarding the other ones.

Selection of significant variables by Plackett-Burman

Plackett-Burman design is an efficient technique for optimizing the cultivation factors and it was used for selecting *k* variables in exactly *k+1* number of experiments. The Plackett-Burman design was then used for evaluating the significance of the effects of 11 variables on proteases production in submerged fermentation at 30°C.

All variables were denoted as numerical factors and investigated at two levels, low (-1) and high (+1). The studied variables were: A. soy flour concentration (%), B. glucose (%), C. incubation period (hours), D. NH_4Cl (%), E. $MgSO_4$ (%), F. K_2HPO_4 (%), G. yeast extract (%), H. tryptone (%), I. pH, J. aeration (vvm) and K. rotation (rpm) (Table 1).

The specific protease activity was executed in triplicate and the average value was considered as the response variable. After the determination of the best culture conditions, the enzyme extract obtained under such conditions was partially characterized.

pH effect on enzyme activity and stability

The optimum pH was studied with several buffers at different pH values, containing 1% azocasein as substrate. The buffers used at 0.1 M were: phosphate buffer (pH 5.8; 6.0; 7.0; 8.0), Tris-HCl buffer (pH 7.2; 7.5; 8.0; 9.0) and carbonate-bicarbonate buffer (pH 9.2; 10.0; 10.7).

Regarding pH stability, the enzyme extract was diluted (1:1) in the following buffer solutions at 0.1 M: phosphate (pH 5.8; 6.0; 7.0; 8.0) and Tris-HCl (pH 7.2; 7.5; 8.0; 9.0). The enzyme remained in contact with each one of these buffers for 180 minutes, at room temperature, whereas one aliquot was removed for protease activity determination every 60 minutes. The assays were conducted in triplicate, taking the averages under consideration.

Table 1. Matrix of Plackett-Burman statistical design. The signal (-) represents the low level and the signal (+) represents the high level.

Assays	A	B	C	D	E	F	G	H	I	J	K
1	4 (+)	2 (+)	48 (-)	0.25 (+)	0 (-)	0.2 (-)	0.25 (-)	2 (+)	8 (+)	10 (+)	100 (-)
2	4 (+)	2 (+)	48 (-)	0.25 (+)	0.1 (+)	0.2 (-)	2 (+)	0.25 (-)	6 (-)	5 (-)	150 (+)
3	0.5 (-)	2 (+)	96 (+)	0 (-)	0.1 (+)	0.2 (-)	0.25 (-)	0.25 (-)	8 (+)	10 (+)	150 (+)
4	0.5 (-)	0.5 (-)	96 (+)	0.25 (+)	0.1 (+)	0.2 (-)	2 (+)	2 (+)	6 (-)	10 (+)	100 (-)
5	4 (+)	2 (+)	96 (+)	0 (-)	0.1 (+)	0.6 (+)	0.25 (-)	2 (+)	6 (-)	5 (-)	100 (-)
6	0.5 (-)	0.5 (-)	48 (-)	0 (-)	0 (-)	0.2 (-)	0.25 (-)	0.25 (-)	6 (-)	5 (-)	100 (-)
7	4 (+)	0.5 (-)	96 (+)	0 (-)	0 (-)	0.2 (-)	2 (+)	2 (+)	8 (+)	5 (-)	150 (+)
8	0.5 (-)	0.5 (-)	48 (-)	0.25 (+)	0.1 (+)	0.6 (+)	0.25 (-)	2 (+)	8 (+)	5 (-)	150 (+)
9	4 (+)	0.5 (-)	96 (+)	0.25 (+)	0 (-)	0.6 (+)	0.25 (-)	0.25 (-)	6 (-)	10 (+)	150 (+)
10	0.5 (-)	2 (+)	96 (+)	0.25 (+)	0 (-)	0.6 (+)	2 (+)	0.25 (-)	8 (+)	5 (-)	100 (-)
11	0.5 (-)	2 (+)	48 (-)	0 (-)	0 (-)	0.6 (+)	2 (+)	2 (+)	6 (-)	10 (+)	150 (+)
12	4 (+)	0.5 (-)	48 (-)	0 (-)	0.1 (+)	0.6 (+)	2 (+)	0.25 (-)	8 (+)	10 (+)	100 (-)

Temperature effect on enzyme activity and stability

The optimum temperature was verified by adding 60 µL of the enzyme extract to 100 µL of the solution containing Tris-HCl buffer 0.1 M, pH 7.5 and 1% azocasein. The reaction mixture incubation lasted one hour under reaction temperatures that ranged from 30 to 80°C, with interval of 10°C.

Temperature stability was determined by incubating the enzyme extract at different temperatures ranging from 30 to 80°C, with interval of 10°C, for 180 minutes. Aliquots were removed each 60 minutes for testing the remaining protease activity. The experiments were conducted in triplicate and the average values were analyzed.

Metal ions effect on protease activity

The metal ions action was determined aiming to identify ions with potential for inhibiting or activating the protease activity. Different ions were added to a 1% azocasein solution in Tris-HCl buffer 0.1M, pH 8.0: $FeSO_4$, NaCl, $MnCl_2$, $MgCl_2$, $ZnCl_2$ e KCl. The assays were conducted in triplicate, and the average values were analyzed.

Protease inhibitors effect on protease activity

Different protease inhibitors were evaluated: metalloproteases inhibitor (ethylenediamine-tetraacetic acid – EDTA), at 1mM and 10mM; serine proteases inhibitor (fluoride fenilmetilsulfonila – PMSF), at 1mM and 10mM; cysteine proteases inhibitor (iodoacetic acid) at 1mM and 10mM; and the aspartate proteases inhibitor (pepstatin A) at 0.1 mM.

The enzyme extract was preincubated with each of these inhibitors at room temperature, during 30 minutes, in the ratio of 1:1. Hereafter, the residual protease activity was determined. The assays were conducted in triplicate and the averages were analyzed.

Results and discussion

Figure 1 represents the results from the studied species selection for proteases production. The best proteases producing species was *Mucor hiemalis* URM3773.

Figure 2 shows the submerged fermentations performed at 30°C with *M. hiemalis* URM3773 strain, according to Plackett-Burman design. The macroscopic morphology of the fungal cell was obviously different among the 12 tested media.

As for the selection of the significant cultivation variable for proteases production, Table 2 represents the matrix of the Plackett-Burman experimental design.

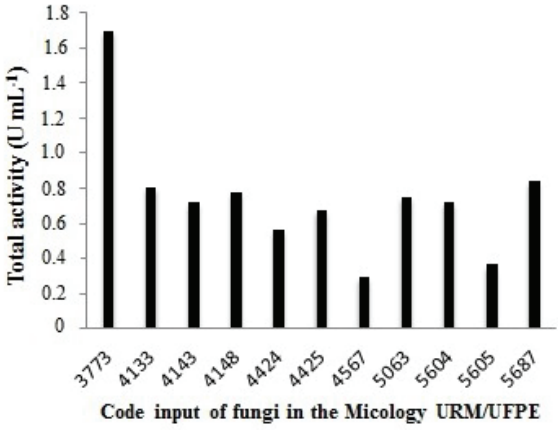

Figure 1. Screening of the best proteases producing species by the evaluation of total or volumetric activity (U mL^{-1}).

According to the results in Table 2, it is noticed that assay 3 obtained the highest value of response variable, specific protease activity, with 16.694 U mg^{-1}.

Through the statistical analysis of the results from Plackett-Burman experimental design, the following independent variables were identified: A. filtrate soybean concentration (%), D. NH_4Cl (%) and J. aeration (vvm), as shown in Table 3.

Aiming to determine the relation between the significant variables and the response variable, statistical analysis of variance (ANOVA) was performed with the independents variables (Figure 3). The factors were considered as significant to the proteases production at a 95% level ($p < 0.05$).

According to Figure 3, it is observed that the only significant cultivation factor on the proteases production was (1) the filtrate soybean concentration (%), thus showing negative effect. It means that the increase in filtrate soybean concentration results in a decreased response variable.

This result is in accordance with the literature. According to Abidi et al. (2008), the source of nitrogen in the culture medium affects both microorganism growth and proteases production. Using the statistical design was of fundamental importance, because it reduced the number of required experiments.

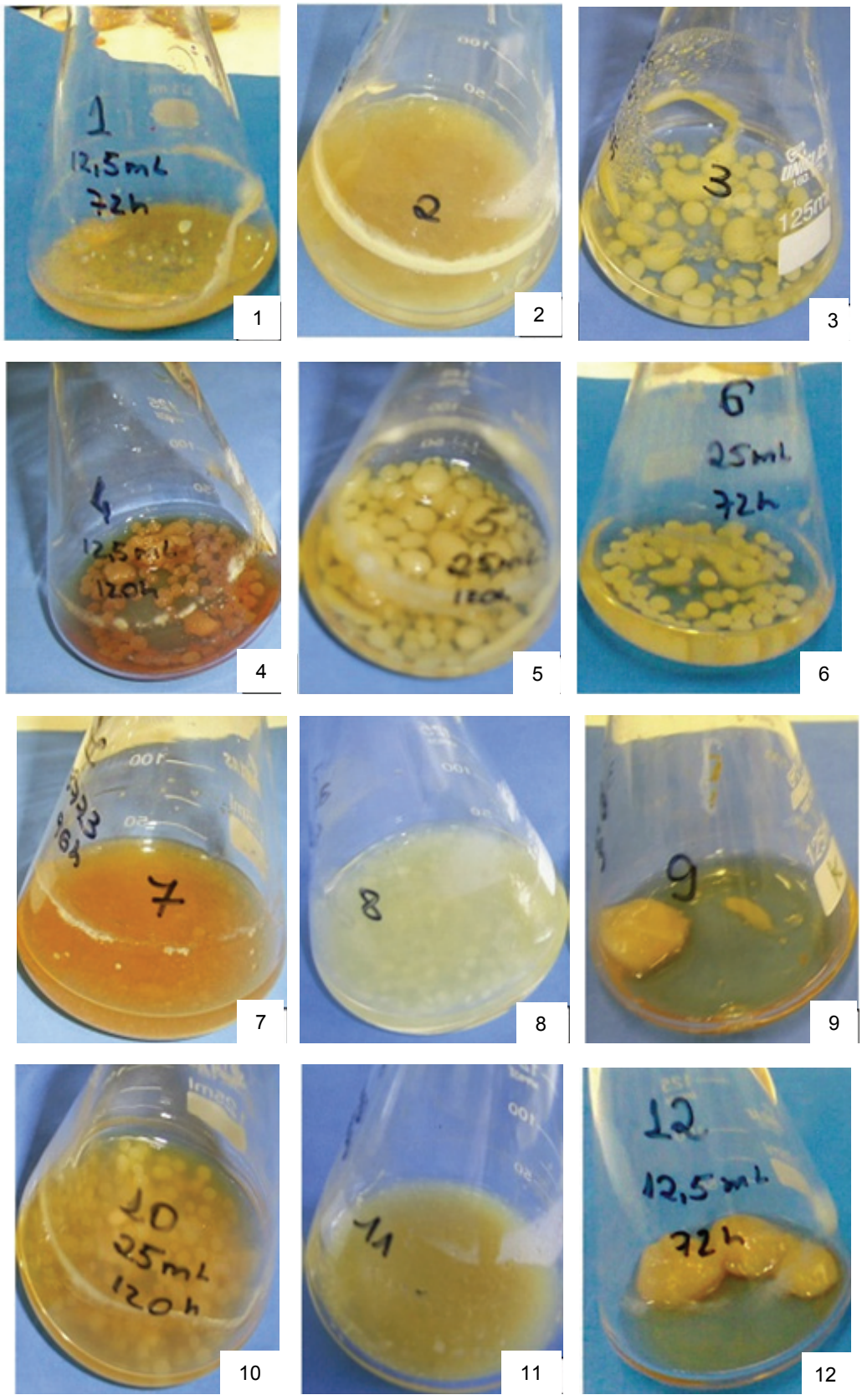

Figure 2. Images of 12 assays of submerged fermentations conducted with *M. hiemalis* URM3773 species at 30°C, according to Plackett-Burman experimental design.

Table 2. Symbols of the analyzed cultivation variables, their actual levels and the results from the response variable, specific protease activity.

Assay	A	B	C	D	E	F	G	H	I	J	K	SA⋆
1	4	2	48	0.25	0	0.2	0.25	2	8	10	100	0.622
2	4	2	48	0.25	0.1	0.2	2	0.25	6	5	150	0.808
3	0.5	2	96	0	0.1	0.2	0.25	0.25	8	10	150	16.694
4	0.5	0.5	96	0.25	0.1	0.2	2	2	6	10	100	7.229
5	4	2	96	0	0.1	0.6	0.25	2	6	5	100	1.252
6	0.5	0.5	48	0	0	0.2	0.25	0.25	6	5	100	8.358
7	4	0.5	96	0	0	0.2	2	2	8	5	150	0.875
8	0.5	0.5	48	0.25	0.1	0.6	0.25	2	8	5	150	4.831
9	4	0.5	96	0.25	0	0.6	0.25	0.25	6	10	150	4.229
10	0.5	2	96	0.25	0	0.6	2	0.25	8	5	100	5.685
11	0.5	2	48	0	0	0.6	2	2	6	10	150	10.966
12	4	0.5	48	0	0.1	0.6	2	0.25	8	10	100	3.054

⋆ SA – Specific protease activity (U mg^{-1}).

Source: personal archive.

Table 3. Effects and coefficients of the cultivation variables according to Plackett-Burman statistical design.

Variables	Effects	Coefficients
(A) Filtrate soybean concentration (%)	-7.15393	-3.57696
(B) Glucose (%)	1.24182	0.62091
(C) Incubationperiod (hours)	1.22101	0.61050
(D) NH_4Cl	-2.96585	-1.48293
(E) $MgSO_4$	0.52230	0.26115
(F) K_2HPO_4	-0.76158	-0.38079
(G) Yeastextract	-1.22805	-0.61403
(H) Tryptone	-2.17531	-1.08765
(I) pH	-0.18032	-0.09016
(J) Aeration (vvm)	3.49755	1.74878
(K) Rotation (rpm)	2.03389	1.01695

Source: personal archive.

With respect to the biochemical characterization of the crude enzyme extract, Figure 4 presents its pH optimum performance and its stability pH. The crude enzyme extract was active in wide pH range (5.8 – 10.7), with optimum pH 7.5 in the 1% azocasein solution in Tris-HCl buffer 0.1 M. Under acidic conditions, at pH 5.8 in phosphate buffer 0.1 M, the enzyme activity showed approximately 64% of activity in relation to optimum pH. At pH 9.2 in carbonate-bicarbonate buffer 0.1 M, the relative protease activity decreased approximately by half.

The enzyme extract was stable on a wide pH range from 5.8 to 8.0 in phosphate buffer 0.1 M, for 180 minutes of incubation. It was completely stable, with 100% residual activity, at pH 5.8 in phosphate buffer. At pH 6.0 in phosphate buffer, enzyme activation occurred during the incubation period of 180 minutes, in which the residual activity reached 133%. At pH 9.0 in Tris-HCl buffer 0.1 M, the residual enzyme activity decreased to 27% of the maximum activity.

The optimum pH of the studied enzyme extract is in accordance with that from previous reports. According to Hajji et al. (2007), the fungal proteases present an optimum pH at 7.5 – 8.5. Abidi et al. (2011), by studying the production of an alkaline protease Prot-2 by *Botrytis cinerea*, found an optimum pH of 8.0.

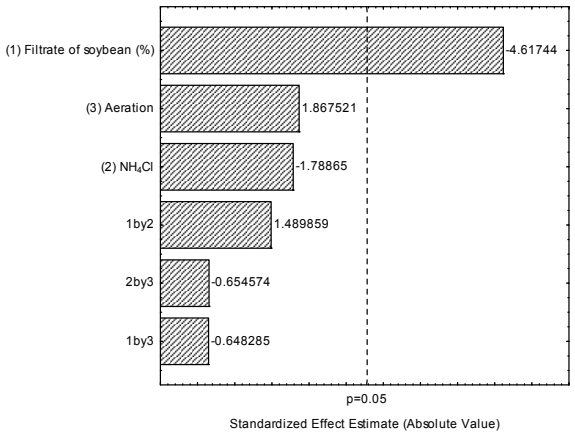

Figure 3. Pareto chart of the independent variables of Plackett-Burman statistical design taking the specific protease activity (U mg^{-1}) as the response variable). MS Pure Error: 5.836474.
Source: personal archive.

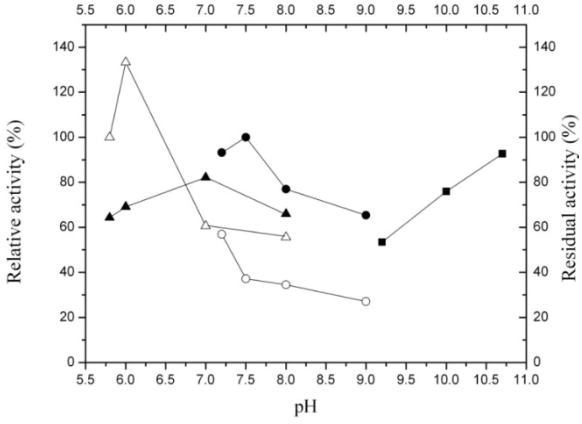

Figure 4. pH effect on protease activity (▲,●,■) and in stability (△,○). Phosphate buffer 0.1 M (▲, △), Tris-HCl buffer 0.1 M (●,○) and carbonate-bicarbonate buffer 0.1 M (■).
Source: personal archive.

The evaluated crude enzyme extract was more stable than the milk clotting protease of *Thermomucor indicae-seudaticae* N31, which was stable only at pH 5.0 – 7.0, with approximately 65 – 70% of residual activity (MERHEB-DINI et al., 2010).

Figure 5 represents the optimum temperature and the thermal stability of the studied enzyme extract. The protease was active between 30 and 80°C. The maximum protease activity was achieved at 50°C. The relative activities at 40 and 60°C were approximately 89 and 91%, respectively. These results are identical to those found by Abidi et al. (2011). Even at high temperatures, 70 and 80°C, the relative protease activity was of about 60%, which indicated that the enzyme was active in high temperatures, thus being a thermo-resistant enzyme. This protein characteristic is probably associated with the presence of disulfide bonds.

As for thermal stability, the enzyme extract was stable in a wide temperature range (40 – 70°C), and it was completely stable at 70°C. At 60°C, there was enzyme activation in the incubation period of 180 minutes, in which the residual activity reached 189%. At 80°C, the enzyme residual activity decreased to 26% of the maximum activity.

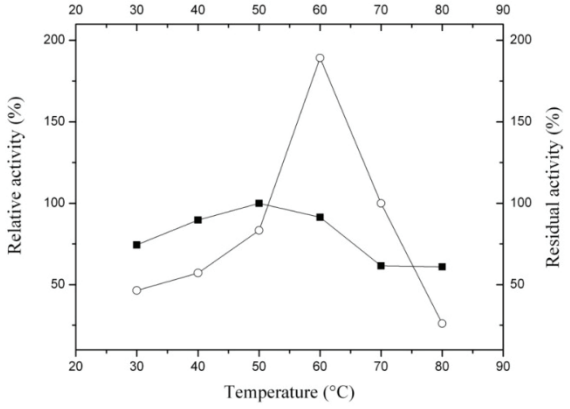

Figure 5. Temperature effect on crude enzyme extract activity (■) and stability (○). The stability was determined after 180 minutes of incubation.
Source: personal archive.

The enzyme extract optimum temperature was similar to that of the protease of *Aspergillus clavatus* ES1 (HAJJI et al., 2007), as well as to the results obtained by Silva et al. (2011). Savitha et al. (2011) identified the optimum temperature or the performance of serine proteases from *Graphium putredinis* at 50°C for application in the detergent industry.

The thermal stability of the evaluated enzyme extract was higher when compared with other reported proteases, such as: *Hirsutella rhossiliensis*, in which the enzyme was stable for 2 hours between 40 and 50°C (WANG et al., 2009) and *Monacrosporium microscaphoides* that were incubated at 70°C for 10 minutes and resulted in a reduction of 90% in the proteolytic activity (MIAO et al., 2006).

Table 4 shows the analyzed ions effects on the protease activity. It is observed that the ions $FeSO_4$, NaCl, $MnCl_2$, $MgCl_2$ and KCl stimulated the protease activity, whereas the ion $ZnCl_2$ inhibited only 2.27% of the enzyme activity.

Similar results are reported in the literature. Zanphorlin et al. (2011), when evaluating the metallic ions effects on the alkaline serine protease activity of the *Myceliophthora* sp. thermophilic fungus, identified that adding $MgCl_2$ increased the enzyme activity in about 22% when compared with the control, whereas the cation Zn^{2+} had influence on enzyme activity by decreasing it.

The residual protease activity was calculated in the presence of different proteases inhibitors, in order to set the type of studied enzyme extract (Table 5).

Table 4. Effects of metallic ions at 10 mM on protease activity.

Metallic ions	Relative activity (%)
Control	100
$FeSO_4$	115.909
NaCl	134.091
$MnCl_2$	156.818
$MgCl_2$	129.545
$ZnCl_2$	97.727
KCl	122.727

Source: personal archive.

Table 5. Effects of proteases inhibitors on enzyme activity.

Protease inhibitors	Concentration (mM)	Residual activity (%)
Control	--	100
EDTA	1	100
	10	79.310
PMSF	1	75.862
	10	65.517
Iodoacetic acid	1	48.276
	10	86.207
Pepstatin A	0.1	72.414

Source: personal archive.

Iodoacetic acid at 1 mM was the inhibitor that had higher action on the enzyme thus indicating that the evaluated proteases can be a cysteine protease. EDTA at 1 mM was the inhibitor that did not show influence on protease activity. This result meet those found by Hajji et al. (2007) who verified that the protease was slightly inhibited by EDTA.

Conclusion

The proteases produced by *Mucor hiemalis* URM3773 have great potential use as industrial catalysts, since the studied enzyme presented activity within a wide range of pH and temperature. *Mucor hiemalis* also had an important role as protease producer so it is a great source of industrial enzymes.

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