Brewery-residue utilization of a freshly isolated *A. niger* sp. upt-03 for protease production under solid-state fermentation

Salah Din Mahmud Hasan[1]
Citieli Giongo[2]
Sonia Aparecida Reis Lopes-Shikida[3]
Sérgio Luiz de Lucena[4]
Mônica Lady Fiorese[5]

Abstract: The operation parameters used in solid-state fermentation (SSF) support the growth of filamentous fungi, which grow on solid substrates producing important metabolites such as proteases. The aim of this work is to obtain fungal proteases by SSF using the residues of a local brewery industry (barley bagasse and trub), that have high contents of proteins and soluble matter such as carbohydrates, vitamins, and mineral salts. The methodology includes the preparation of residues, the screening of microorganisms, evaluation of operational conditions for SSF using factorial design, purification and partial characterization of protease. The results indicate that *A. niger* sp. UPT-03 isolated from the residue shows higher yields in terms of enzyme production (0.36 U gdm^{-1} h^{-1}). The purification with DEAE-cellulose resulted in protease recovery with 30-fold of purification with a specific activity of 550 U mg $protein^{-1}$. Higher proteolytic activity of purified enzyme was obtained at pH 5.5 and 55 °C.

Keywords: solid-state fermentation, brewery residues, fungi proteases, trub

INTRODUCTION

In recent years, there has been an increasing trend toward efficient utilization and value addition of agro–industrial residues such as coffee pulp and husk, cassava husk, cassava bagasse, sugarcane bagasse, sugar-beet pulp, apple pomace, and declassified potatoes. There are several recent publications describing bioprocesses that have been developed utilizing these raw materials for the production of bulk chemicals and value-added fine products, such as ethanol, single-cell proteins, mushrooms, enzymes, organic acids, amino acids, and biologically active secondary metabolites. The application of agro–industrial residues in bioprocesses not only provides alternative substrates but also helps in solving pollution problems. Biotechnological processes, especially the solid-state fermentation (SSF) technique, have contributed enormously for such fruitful utilization (Singhania et al., 2010; Soccol & Vandenberghe, 2003).

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively. This group of enzymes represents one of the three largest groups of industrial enzymes and accounts for approximately 60 % of the total enzyme sales in the world. They have numerous applications in the industrial production of different items, viz. detergents, foods, pharmaceutical, leather, diagnostics,

[1] Programa de Pós-graduação *Stricto Sensu* em Engenharia Química da Universidade Estadual do Oeste do Paraná – UNIOESTE. Rua da Faculdade, 645. Jardim Santa Maria. CEP 85903-000. E-mail: salahdmh@gmail.com

[2] Programa de Pós-graduação *Stricto Sensu* em Engenharia Química da Universidade Estadual do Oeste do Paraná – UNIOESTE. Rua da Faculdade, 645. Jardim Santa Maria. CEP 85903-000. E-mail: citifarma@yahoo.com.br

[3] Departamento de Biologia da Universidade Paranaense – UNIPAR, campus de Toledo/PR. Avenida Parigot de Souza, 3636. Jardim Prada. CEP 85903-170. E-mail: perysonia@uol.com.br

[4] Programa de Pós-graduação *Stricto Sensu* em Engenharia Química da Universidade Estadual do Oeste do Paraná – UNIOESTE. Rua da Faculdade, 645. Jardim Santa Maria. CEP 85903-000. E-mail: lucenasergio@yahoo.com.br

[5] Programa de Pós-graduação *Stricto Sensu* em Engenharia Química da Universidade Estadual do Oeste do Paraná – UNIOESTE. Rua da Faculdade, 645. Jardim Santa Maria. CEP 85903-000. E-mail: mlfiorese@gmail.com

including waste management and silver recovery (Zambare et al., 2011). They hydrolyze peptide bonds in aqueous environments and synthesize peptide bonds in microaqueous environments. Proteases are generally produced using the submerged fermentation (SmF) process due to its apparent advantages with respect to consistent enzyme-production characteristics controlled by defined medium-and-process conditions and advantages in downstream processing, in spite of the cost intensiveness of the medium components (Prakasham et al., 2006; Pandey et al., 2000). In this context, SSF has gained renewed interest and fresh attention from researchers owing to its importance in the following developments: recent progress in biomass-energy conservation, solid--waste treatment and its application for the production of secondary metabolites.

Production of these biocatalysts using agro–biotech substrates under SSF conditions provides several advantages such as productivity, cost-effectiveness with respect to labor, time, and medium components, in addition to environmental advantages such as less effluent production and waste minimization (Mukherjee et al., 2008). There are several reports describing the use of agro-industrial residues, such as soybean meal (Zambare et al., 2011), pigeon pea (Johnvesly et al., 2002) and wheat bran (Negi & Banerjee, 2009), for the production of protease. However, these production models would have to offer a competitive advantage over other existing procedures. In general, each microbial strain is unique in its molecular, biochemical, metabolic, and enzyme-production properties. This necessitates a thorough characterization of the isolated individual microbial species to evaluate its potential at the commercial level (Prakasham et al., 2006).

The solid residues from the brewery industry, such as trub and barley bagasse, contain large amounts of proteins and other soluble substances including carbohydrates, vitamins, and salts. Due to their physical, chemical, and biochemical characteristics, these residues have a great potential for application in SSF processes, for the production of high-priced bio-products such as proteases. The present investigation aims to exploit the locally available, inexpensive agro-substrates, trub and barley bagasse, for fungal protease production under SSF conditions.

MATERIALS AND METHODS

MICROORGANISMS

Aspergillus niger (ATCC 16404), *A. awamori* (NRRL 3112) and *Trichoderma pseudokoningii rifai* were used in the present study. Moreover, different fungal strains secreting protease were screened from the brewery residues, including the fungus *A. niger* sp. UPT-03 and UPT-13. The strains were maintained on potato--dextrose-agar slants and subcultured for growth at 35 °C for 7 days. The spores obtained from a fully sporulated slant were dispersed in 10 mL of 0.1 % Tween 80. The spore suspension was used as inoculum, and the spore concentration was estimated by direct microscopic counting using a Neubauer cell. The final spore concentration used for inoculation was 4 x 10^6 spores per g of solid medium.

SSF PROCESS

The agro-residues, trub and barley bagasse, collected from a local brewery industry, were dried at 50 °C for 3 days and processed using standard sieve sets to obtain mean particle sizes of 0.6–1.18 mm for trub and 1.18 mm for barley bagasse. The barley bagasse, which shows larger size, less protein (27 %), and large fiber content (18 %), was used preferentially as support, for facilitating the easier diffusion of gases and quicker release of heat formed during the fermentation process. The residues were stored till further use. Before fermentation, the residues were mixed in the proportion of 70 % trub and 30 % barley bagasse. In this case, the trub was considered as the main substrate, because it has much higher protein content (70 %) and an appropriate particle size. The mixture was then enriched with a nutrient solution composed of 10 g K_2HPO_4; 1.6 g $NaNO_3$; 2.4 g urea; 0.6 g $MgSO_4$; 0.4 g $ZnSO_4$; 0.4 g $FeSO_4$; 0.15 g $MnSO_4$; and 0.15 g $CuSO_4$, made up to 1 L with sterile water. The final pH was adjusted with 0.5 N HCl and the substrate was moistened with sterile water. The substrate was dispensed in 250-mL Erlenmeyer flasks and autoclaved at 121 ºC for 20 min. After inoculation, the SSF process was carried out by incubation at 32 °C.

ANALYTICAL METHODS

The protease activity, PA, was measured using the modified method described by Germano et al. (2003). One unit of proteolytic enzyme activity (U/gram of dried medium) was defined as the amount of enzyme that produced an absorbance difference (in relation to control) during 1-h-incubation period at 45 °C for one gram of fermented medium (based on dry weight). The unit *gdm* indicates grams of dried medium. It was used water instead the substrate at the control. The productivity, Y (U $gdm^{-1}h^{-1}$), was determined by dividing PA by the fermentation time. Protein content, Prot (mg gdm^{-1}), was estimated by Bradford method (1976), using bovine serum albumin as the protein standard. Specific protease activity, SPA (U/mg of protein), was determined by dividing the PA value by the protein content. Reducing sugars, RS (mg glucose gdm^{-1}), were quantified by the method of Miller (1959). Moisture content in dry basis, M (%), was determined by the gravimetric method.

EXPERIMENTAL DESIGN

The fermenting parameters were evaluated by the statistical design of experiments according to a fractional factorial design of the type 2^{5-2} (Montgomery, 1997). The influence of five variables on the protease activity were evaluated using eight experiments with two levels (-1 and +1) for each factor and a trial of experiments for the central point (level 0) for error estimation. In this study, the five factors analyzed were: initial moisture of fermenting medium (M), initial pH, inoculum concentration (Co), mean diameter of substrate particle (dp), and amount of peptone addition (Pep), levels of which are shown in Table 1. Pep = 0 % (for level -1) implies that no peptone was added, and this consideration does not restrict further evaluation of the data. The results obtained from the factorial design were subjected to a first-order multiple-regression analysis using the least squares regression methodology to obtain the parameters of the response-surface equation and plots.

Table 1: Coded and assigned factors of different levels of the 2^{5-2} design.

Factor	Level		
	-1	0	+1
pH = initial pH	3.0	4.5	6.0
M = initial moisture of fermenting medium (%)	40.0	55.0	70.0
Co = inoculum concentration (spores g^{-1})	4.10^5	4.10^6	4.10^7
dp = mean diameter of substrate particle (mm)	0.748	0.844	0.940
Pep = initial peptone concentration (%)	0.00	1.67	3.33

PEPTONE ADDITION

Batch and fed-batch systems were tested for the addition of peptone, used as a protease inducer in SSF of brewery residues by the fungus *A. niger* sp. UPT-03. Both SSF processes were carried out in 250-mL Erlenmeyer flasks at 32 °C. Samples were collected after every 12-h period for the characterization of the fungal growth curve. For the fed-batch process, 1 % peptone was added to the system every 12 h (Rao et al., 2006; Sanzo et al., 2001; Bertolin et al., 2001).

ENZYME PURIFICATION

The protease enzyme was extracted by the simple contact method of extraction, using distilled water as solvent (Chellappan et al., 2006; Hasan et al., 2003). Ten volumes of distilled water were added per gram of fermented medium (based on dry weight), and enzyme extraction was carried out by agitation at room temperature for 1 h. The suspension was filtered under vacuum, and the filtrate was used as the crude enzyme extract.

Protease produced under SSF was partially purified employing ammonium sulphate precipitation, followed by dialyzis and ion-exchange chromatography. Ammonium sulphate at a 70 % final concentration was used to precipitate the protease enzyme from the crude extract. The precipitated protein was resuspended in 0.1 M phosphate buffer (pH 6), dialyzed against 0.01 M solution of the same buffer for 36 h, at

5 ºC, with six changes of buffer. Further an ion--exchange chromatography with DEAE cellulose packing column was carried out. Dialyzed sample was applied to DEAE-cellulose column that had been pre-equilibrated with 0.01 M phosphate buffer pH 6. Elution was done at a flow rate of 120 $ml.h^{-1}$ with 0.2 M NaCl in the same buffer and 5 ml fractions were collected. Peak fractions from the column were assayed for protease activity and protein content.

The molecular weight of active fractions of protease collected after ion-exchange chromatography was electrophoresed by reductive conditions of SDS-PAGE in a 15 % polyacrylamide gel according to Laemmlli (1970). Protein bands were visualized by silver staining. Low molecular weight markers was used as molecular mass standard and molecular weight of protease was estimated.

PARTIAL CHARACTERIZATION

The pH for maximal protease activity was determined with purified enzyme by checking the enzyme activity in different buffer systems of pH 2–10 including, 0.2 M HCl–KCl (pH 2), 0.1 M citrate (pH 3–6), 0.2 M phosphate (pH 7), Tris–HCl (pH 8), glycine–NaOH (pH 9-10). The temperature ranged from 30 to 70 ºC.

RESULTS AND DISCUSSION

FUNGI SCREENING

For the screening of microorganisms, five Erlenmeyer flasks were prepared for SSF, and each flask, including the initial sample (t = 0 h), was removed after every 24 h, until the 96 h. Each run was duplicated, totaling 10 samples. The yield profiles of the protease obtained by SSF were evaluated for the five microorganisms tested in this work. A higher yield was obtained for the fungus *A. niger* UPT-03 after 24 h of fermentation (Fig. 1). This fungus, which was isolated from the trub residue and identified as *A. niger* sp. UPT-03 that produced the extracellular protease enzyme within 24 h, was screened for subsequent studies.

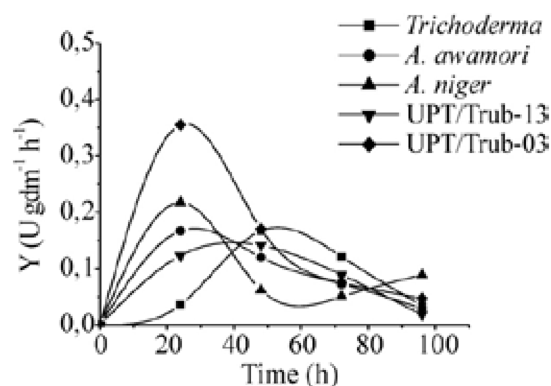

Figure 1. Yield (Y) profiles of the protease obtained by SSF for the five microorganisms.

The strain *Trichoderma pseudoko-ningii rifai* had a smaller yield of protease until 24 h, but at 48 h it showed a higher value, together with the fungus *A. niger* sp. UPT-03. The microorganisms *A. niger* (ATCC 16404), *A. awamori* (NRRL 3112), *Trichoderma pseudokoningii rifai,* and UPT-Trub-13 showed less visual proliferation and virulence in the solid culture medium and had smaller final yield values at 96 h. In this work, fungal growth alone was investigated.

However, in many researches, during the screening procedures for the microorganism, some bacterial species were also observed to grow in the substrates. Bacterial cultures, in general, were considered nonsuitable for SSF due to their greater water-activity requirement. However, bacterial cultures can be maintained and manipulated for SSF processes (Mahanta et al., 2008; Pandey, 2003).

KINETICS OF SSF PROCESS

The production of proteases is related to the metabolic demands spanning many different aspects, including the type of fermentation (SSF or SmF), substrate used, microorganism considered (different species of bacteria or fungi), nutrients added to the medium, and operating parameters (pH, inoculum concentration, temperature, water activity, fermentation time, etc.). The metabolic changes occurring inside the fermentation medium due to the growth of the microorganism, substrate and nutrient consumption, respiration, and metabolite production were evaluated. For growth-curve calibration, five Erlenmeyer flasks

were prepared for SSF, and each one, including the initial sample (t=0h), was removed after every 24 h, until the 96 h. Each run was duplicated, totaling 10 samples.

The curves obtained from the analysis of all fermented samples are shown in Fig. 2. The profiles of reducing sugars (RS), moisture (M), protease activity (PA), specific protease activity (SPA), and protein (Prot) during SSF were obtained for the fungus *A. niger* sp. UPT-03. A comparison of the protease activity (U gdm^{-1}) in Fig. 2 with the yield values (U gdm^{-1} h^{-1}) in Fig. 1 with respect to the time of incubation (h) showed a lag phase between 0 and 24 h due to a small variation of RS, M, PA, and Prot. After 24 h, RS decreased and PA and M increased indicating a probable exponential phase of growth, evidenced by the increase of protein content. After 48 h of culture, an increase in the protein content occurred, probably mainly due to the cellular mass increase, because an additional increase in protease production was not observed (almost constant values of protease activity and a decrease of SPA values).

After 72 h, the protein content decreased indicating decay of the fungus, as evidenced by the almost-constant final values of PA, RS, and M. Hence, protease production could be achieved, without yield prejudice, within 48 h of fermentation of the fungus *A. niger* sp. UPT-03 on trub and barley-bagasse substrates.

The increase in moisture content may be due to the fact that no flux of air was added to the medium during the SSF process, rendering moisture control, release of gases, and heat dissipation more difficult. In studies with *Penicillium* sp., some authors observed a peak in protease activity after 3 days of incubation in wheat bran containing 50 % moisture.

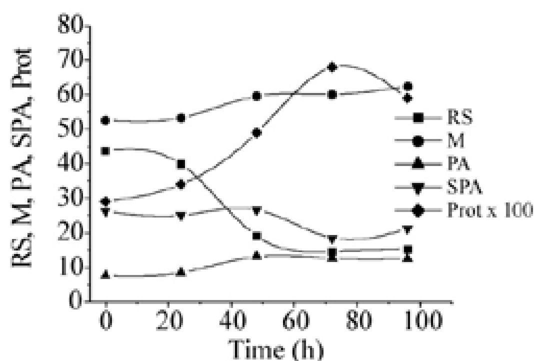

Figure 2. Profiles of reducing sugars RS (mg glucose gdm^{-1}), moisture (M, %), protease activity (PA, U gdm^{-1}), specific protease activity (SPA, U mg $protein^{-1}$) and protein (Prot, mg gdm^{-1}x100) obtained for SSF of the fungus *A. niger* sp. UPT 03.

Other reports indicated the requirement of 55, 63, and 140 % initial moisture content for maximum protease production by *Penicillium* LPB-9 (Germano et al., 2003; Agrawal et al., 2004), *Aspergillus flavus* IMI 327634 (Pandey, 2003), and *Rhizopus oryzae* (Tunga et al., 1998), respectively.

A. niger was also shown to secrete maximum levels of alkaline and acid proteases after 2–3 days of incubation at 52–72 % moisture levels (Mahanta et al., 2008), which supports the present findings with *A. niger* sp. UPT-03.

Various agro–industrial residues have been tested as substrates for protease production by *A. Oryzae,* including spent brewing grain, wheat bran, rice husk, rice bran, coconut-oil cake, palm-kernel cake, sesame-oil cake, jackfruit-seed powder, and olive-oil cake. An almost two-fold increase in enzyme activities was observed in wheat bran-based SSF medium (11.2 U gdm^{-1}) when compared to the yield in the next most effective substrate, coconut-oil cake (5.9 U gdm^{-1}).

Almost similar patterns were obtained in the SmF process (Sandhya et al., 2005). It was interesting to note that all the substrates tested for SSF showed measurable enzyme production. Working with spent brewing grain under SSF and SmF, approximately 0.9 U gdm^{-1} and 4.8 U gdm^{-1} of protease activity were obtained, respectively. In the present work, approximately 12.5 U gdm^{-1} was obtained for trub and barley bagasse (used as substrate for SSF) at the end of the same period of incubation, which indicates a promising use for this barley residue on a large scale.

Table 2 shows the coded factors and the results obtained for protease activity after 48 h of fermentation during each experiment. Trub, used as the substrate, was mixed with enough quantity of barley bagasse to give the desired mean size of the particles (dp), as specified in Table 1.

The protease activity varied markedly in the range of 0.9–20 $Ugdm^{-1}$ (Table 2). Runs 9 to 11 represents a triplicate at the central point of the variables and is important for the error term calculation, with a higher degree of freedom.

The results showed that higher protease activities (20 U gdm^{-1}) were obtained for run 6 with the following parameters: pH = 6.0; M = 40 %; Co = 4 x 10^7 spores g^{-1}; dp = 0.748 mm, and Pep = 0 %.

However, according to the Pareto chart of effects (Fig. 3), only the following factors – pH, mean diameter of substrate particle (dp), and peptone concentration (Pep) – significantly influenced the protease activity results, with 95 % confidence interval, as evidenced by the horizontal bands extending beyond the dotted line.

Table 2. Matrix of fractional factorial design 2^{5-2}, with coded variables and results of PA obtained for SSF of *A. niger* sp. UPT 03.

Run	pH	M	Co	dp	Pep	PA (U gdm^{-1})
1	- 1	- 1	- 1	- 1	+ 1	5.7
2	+ 1	- 1	- 1	+ 1	- 1	11.6
3	- 1	+ 1	- 1	+ 1	- 1	4.5
4	+ 1	+ 1	- 1	- 1	+ 1	7.9
5	- 1	- 1	+ 1	+ 1	+ 1	0.9
6	+ 1	- 1	+ 1	- 1	- 1	20.0
7	- 1	+ 1	+ 1	- 1	- 1	12.0
8	+ 1	+ 1	+ 1	+ 1	+ 1	1.5
9	0	0	0	0	0	5.7
10	0	0	0	0	0	5.7
11	0	0	0	0	0	3.5

An exponential increase in the enzyme activity of *Penicillium* sp. with an increase in the size of inoculum has already been observed (Agrawal et al., 2004), and the optimum spore concentration required for obtaining maximum protease activity from *Rhizopus oryzae* NRRL 21498 was ~2 x 10^3 spores g^{-1} wheat bran; moreover, a further increase in the size of inoculum did not show increased protease activity. However, in the case of *Penicillium* sp., no such optimum inoculum size could be observed up to a very high spore count of 10 billion spores g^{-1} wheat bran (Tunga et al., 1998). The negative values for the effects of dp and Pep in this study indicate that PA tends to increase for their lower values, whereas the positive effect of pH is indicated by the increase in protease activity with pH increase.

These results are evidenced by the response surfaces shown in Fig. 4. The lowest values of protease activity were achieved when an acidic medium was used (pH 3.0), indicating a neutral nature of the protease. The differential sensitivity of protease-producing fungal strains toward pH was also pointed earlier (Agrawal et al., 2004). Moreover, the larger mean diameters of substrate particles (dp) proved to be prejudicial to the cultivation of the fungus. Particle size has a profound effect on enzyme production.

Small particles have more surface area for growth but reduced porosity, leading to a lowering of both gas diffusion and heat transfer, whereas the larger particles absorb less moisture, swell less, and, by drying rapidly, support only a suboptimal growth of fungi (Chellappan et al., 2006). Wheat-bran particles of average size <425 μm enhanced protease production and enzyme production was considerably reduced with increasing particle size. In addition, wheat bran, without sieving, supported the production of considerable levels of enzyme and increased the specific activity.

Moreover, the addition of peptone (Pep), used as a protease inducer, might compact the solid medium because of its chemical and physical constitution; thus, combined with the negative effect of moisture increase, peptone might inhibit fungal growth due to the negligible diffusion of air and nutrients and, consequently, may result in less enzyme production.

Earlier reports, however, indicated that complex carbon and nitrogen sources were better substrates for protease production than simple sugars, such as glucose, which caused catabolic repression (Rao et al., 2006). Conflicting reports are available on the protease activity of fungi grown on protein-supplemented wheat bran.

Aspergillus niger var. *tieghem* showed about 1.4-fold increase in acid protease activity in the presence of casein and trader's protein that were added to wheat bran.

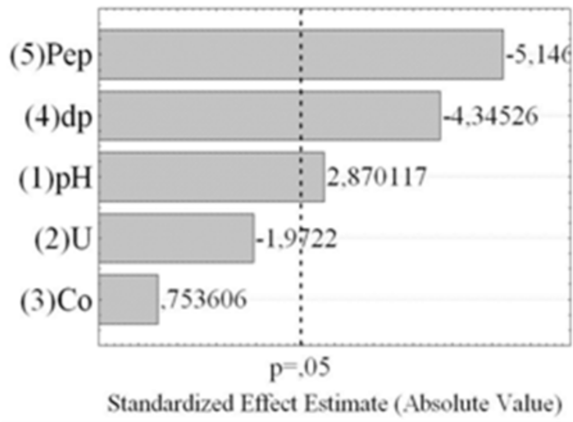

Figure 3. Pareto chart of effects, 95 % of confidence interval, obtained for the fractional factorial design 2^{5-2}.

On the contrary, 6, 14, and 55 % reduction in alkaline protease activity of *Aspergillus flavus* IMI 327634 was recorded after supplementing wheat bran with casein, traders' protein, and gelatin, respectively; this result leads to the conclusion that induction of protease activity by supplementing wheat bran with protein appears to be dependent on the fungal strain used for the experiment (Agrawal et al., 2005; Chakraborty et al., 2005).

Therefore, a parallel study was carried out for establishing whether a fed-batch regime is necessary for peptone addition. Although the fractional factorial design 2^{5-2} caused some partial confounding in the interpretation of the mean effects and two-way interaction effects due to its small resolution, it was possible to verify the influence of the main parameters on protease activity at 95 % confidence intervals.

The equation below represents the dependence of the protease activity on the variables.

$$PA(Ugdm^{-1}) = 7.18 + 2.24.pH - 3.39.dp - 4.01.Pep$$

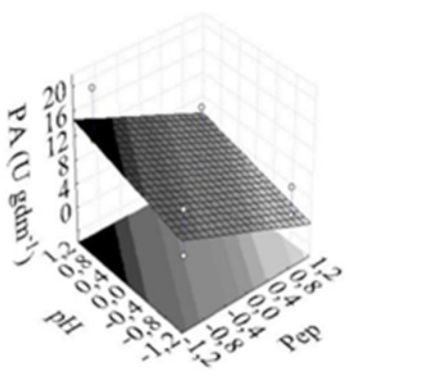

Figure 4. Response surface of PA (real values), affected by Pep and pH (coded values), obtained for the fractional factorial design 2^{5-2}.

This linear regression was carried out to a determination coefficient R^2=0.921, and it was considered valid at 95 % confidence interval through the analysis of variance (ANOVA) (Table 3), wherein F_{CALC} is higher than the F_{TAB}.

The result of the F-test indicated the fitness and validated the use of the model in response--surface construction.

Table 3. ANOVA obtained for linear regression of the factorial design

Variation Source	Sum of squares	Degrees of freedom	Mean squares	FCALC*
Model regression	282.33	5	56.47	11.61
Residues	24.31	5	4.86	
Total	306.64	10		

* F_{TAB} (0.95, 5, 5) = 5.05

There is a growing acceptance among scientists for the use of statistical experimental designs in biotechnology. Many scientists have reported a satisfactory optimization of protease production from microbial sources using the statistical approach. The application of the statistical design for screening and optimization of culture conditions facilitates the quick identification of the important factors in the process and characterization of the interactions between them. Almost two-fold enhancement in protease production was achieved using the statistical design of experiments. Subsequent designs of experiments, such as the central composite rotary designs for the significant factors–pH, mean diameter of substrate particle (dp), and peptone concentration (Pep)–will prove to be helpful for a statistical optimization of the process in terms of protease activity.

FED-BATCH PROCESS

When using discontinuous systems, nutrients are added right at the start of cultivation and productivity might hence be impaired by substrate repression. Fed-batch fermentation, where all or some of the nutrients are added during the cultivation process, aims to avoid such reductions in product yields (Bertolin et al., 2001; Tunga et al;. 1998).

However, with respect to fed-batch SSF, not much information is available in literature, which poses hurdles in the investigation of scale-up strategies for such modes of cultivation (Lonsane et al., 1992).

Several studies have shown peptone, casein, soy protein, gelatin, and yeast extract as nitrogen sources and as typical inducers of protease production during submerged and SSF processes (Rao et al., 2006; Mahanta et al., 2008; Chauhan & Gupta, 2004; Joo & Chang, 2005). In the present work, the effect of peptone on batch and fed-batch SSF for protease production was determined.

Figures 5 and 6 illustrate the kinetic behavior of fungal growth in SSF carried out by batch and fed-batch processes, respectively. Considering the protein profile as an indirect estimation of fungal biomass, the gradual addition of peptone into the fed-batch system decreased the lag-phase by almost 12 h. However, after 24 h of fed-batch cultivation, a probable inhibition by the substrate occurred, characterized by the lack of fungal growth (constant values of reducing sugars and protein content) and by the lower values of protease activity.

As observed before, there was a negative influence of higher values of dp, Pep, and M over PA, indicating a damage or destruction of the favorable substrate conditions for fungus growth, probably due to material compacting, which was visible at the final stages of the process.

In addition, the overall yield of protease was reduced by 21.7 % in the fed-batch system. Better results were obtained for the batch process of SSF, with the unique addition of 0.53 % peptone to the nutrient solution at t = 0 h.

In earlier studies, the production of proteases under batch and fed-batch systems of SmF for *Bacillus sphaericus*, an obligate alkalophile that overproduces extracellular alkaline proteases, the initial concentration of glucose and nitrogen sources was shown to significantly affect the cell growth and enzyme secretion.

Fed-batch cultures were conducted to maximize protease production in a bioreactor. By using suitable feeding strategies, the protease activity and productivity in a fed-batch process were increased by 44 % compared to that in a batch process, primarily due to the longer maintenance of increased rates of growth and enzyme production by providing a continuous and controlled supply of additional substrate and nutrients (Singh et al., 2004).

This is the first study showing the effect of peptone on protease synthesis during a fed-batch SSF process. Complementary studies should be designed to minimize the effect of homogenization (shaking) in the fed-batch mode and thus improve the advantages of SSF for producing bioactive compounds.

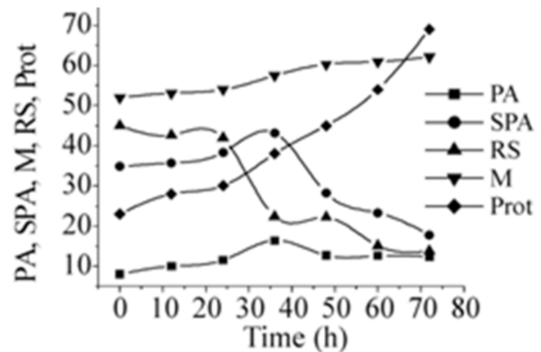

Figure 5. Profiles of protease activity (PA, U gdm^{-1}), specific protease activity (SPA, U mg protein^{-1}), reducing sugars (RS, mg glucose gdm^{-1}), moisture (M, %) and protein (Prot, mg gdm^{-1}x100) obtained for SSF of the fungus *A. niger* sp. UPT 03 in batch system.

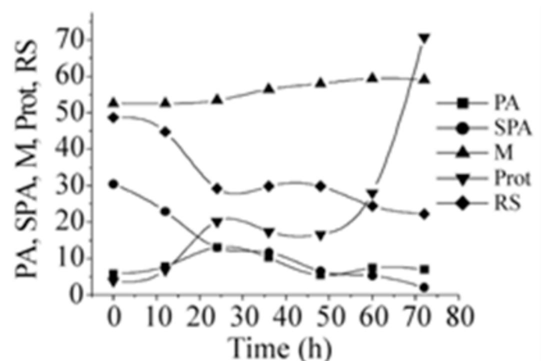

Figure 6. Profiles of protease activity (PA, U gdm^{-1}), specific protease activity (SPA, U mg protein^{-1}), reducing sugars (RS, mg glucose gdm^{-1}), moisture (M, %), protein (Prot, mg gdm^{-1}x100), obtained for SSF of the fungus *A. niger* sp. UPT 03 in fed-batch system.

ENZYME PURIFICATION

The purification level of crude enzyme is summarized in Table 4. The precipitate formed at 70 % saturation of ammonium sulphate, which showed a 4.5-fold increase in specific activity compared to the crude sample, was used for further pu-

rification employing ion-exchange chromatography. Elution at the DEAE-cellulose column gave a peak with protease activity, which was eluted with buffer containing 0.1M NaCl. This step resulted in protease recovery with 30-fold purification with specific activity of 550 U mg protein^{-1}.

SDS-PAGE under reducing conditions yielded a single band (Fig. 7) with the molecular mass of protease estimated by comparing the electrophoretic mobility of marker protein, showing that the homogeneous protease obtained has an apparent molecular mass of 37 kDa.

EFFECT OF PH AND TEMPERATURE

Protease from *A. niger* UPT-03 was active in the pH range 5–6, with an optimum pH of 5.5 (Fig. 8-a). Enzyme activity decreased under very acidic and alkaline conditions, indicating slightly acid characteristic. The enzyme was stable in the temperature range 30–60 °C with optimum temperature for activity of 55 °C (Fig. 8-b). More than 80 % of the activity was conserved between 35 and 55 °C. The enzyme was stable at pH 5.5 and more than 90 % activity was retained even after incubation (55 °C) for 1h at pH between 5 and 6.

CONCLUSIONS

There is no general defined medium that can be used for protease production by different microbial strains. Every microorganism has its own peculiar physicochemical and nutritional requirements for protease production. In view of the commercial utility of the enzyme, devising a cost-effective medium formulation is a primary concern. This study indicated the possibility of producing fungal protease from an easily available industry residue, namely the trub and the barley bagasse derived from the brewery industry.

The fungus *A. niger* sp. UPT-03, a new strain isolated from the trub residue, was appropriated for protease production. The main factors affecting protease activity were found to be pH, mean diameter of substrate particle, and peptone concentration. Different levels of these parameters are proposed to be tested in a subsequent optimization study of protease production. The unique addition of 0.53 % peptone at the start of the conventional batch process showed better results of protease yield.

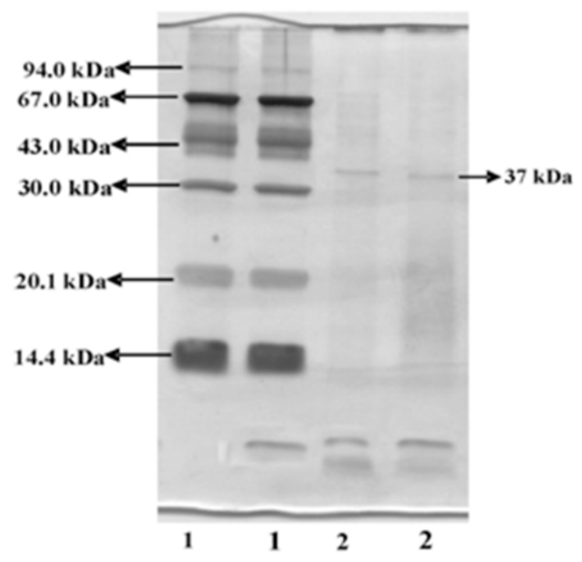

Figure 7. Reductive SDS-PAGE of purified sample conducted on 15 % polyacrylamide gel. Lane 1: molecular weight marker (MW), lane 2: fraction after ion-exchange chromatography. MW and purified sample were injected twice.

Table 4. Purification level

Sample	Protein (mg gdm^{-1})	PA (U gdm^{-1})	SPA (U mg protein^{-1})	Fold of purification
Crude	0.65	12.0	18.5	1.0
Sam precipitation	0.12	10.0	83.3	4.5
DEAE-cellulose	0.01	5.5	550.0	30

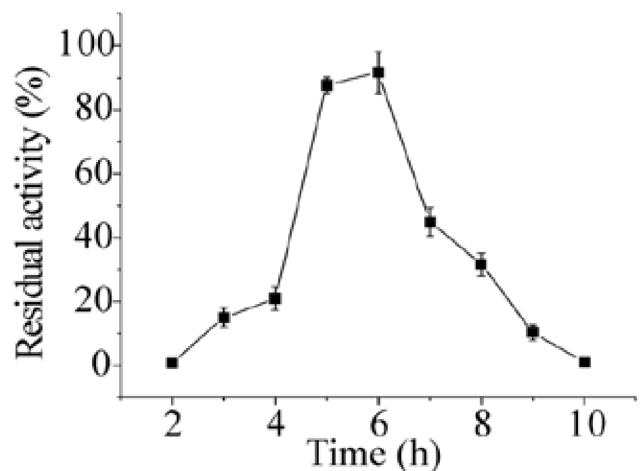

Figure 8-a. Activity at different pH: enzyme assay was conducted in different buffer systems of pH 2–10 at 45 °C.

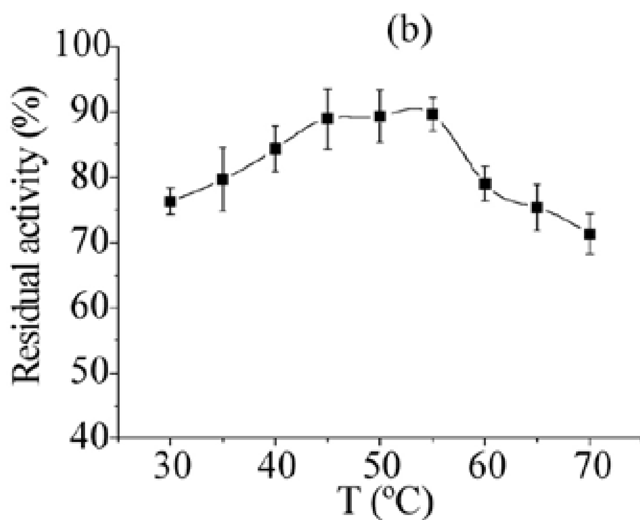

Figure 8-b. Activity at different temperature: enzyme assay was carried out at pH 5.5 at different temperatures.

ACKNOWLEDGMENT

The research was supported by Cnpq, Brazil.

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