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RESEARCH ARTICLE

Optimization of Process Conditions for the Production of a Prolyl-endopeptidase by *Aspergillus niger* ATCC 11414 in Solid State Fermentation

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Abstract The effect of 8 factors [(with/without) daily mixing and moisture control, incubation time (t), temperature, ratio between dry substrate mass and bed's cross section area (MA), inoculum size (spores/g), wheat germ content (WG), initial pH, and moisture content (M)] in the production of a prolyl endopeptidase (PEP) by *Aspergillus niger* ATCC 11414 in solid state fermentation (SSF) was tested. Contribution of all the factors was significant ($p<0.05$); main effects were those of MA, t, and M. The 4 interactions that presented high interaction severity indexes involved the WG. Under optimized conditions PEP and protease activity were 9.76±0.06 and $3.6\times10^6\pm1.5\times10^5$ U/kg, respectively. The enzyme was partially purified (ammonium sulfate precipitation, dialysis, DEAE-Sepharose ion-exchange); it has a molecular weight of 66 kDa (SDS-PAGE), and maximum activity was exhibited at pH 4 and 50°C. The enzyme is stable in a wide pH range (2.2-10) and at temperatures lower than 70ºC.

Keywords: prolyl endopeptidase, proteases, *Aspergillus niger*, solid state fermentation, protein purification

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Introduction

Prolyl endopeptidase (PEP, EC 3.4.21.26) is a serine protease that hydrolyses peptide bonds at the carboxyl side of a proline residue in low molecular weight proteins (1). Proline is not accepted by most proteases at the potential cleavage site (2). PEPs synthesized by bacterial strains, *Flavobacterium meningosepticum*, *Sphingomonas capsulate*, *Aeromonas hydrophila*, and *Myxococcus xanthus,* are cytosolic and not secreted enzymes with neutral optimum pH. These are characterized by the presence of a unique domain that excludes large-sized peptides at the active site (3). Unlike these, some *Aspergillus* strains (4-6) secrete monomeric proline specific endoproteases. These enzymes belong to the S28 serine peptidase family which includes proline-specific peptidases (5).

PEP and proline specific endoproteases have been studied as potential therapeutic agents in coeliac disease, characterized by the intestinal villi inflammation and atrophy in response to proline rich peptides generated during digestion of gluten in wheat, barley, rye, and probably oat (7-9). In addition, *Aspergillus* PEPs are able to degrade peptides responsible for the bitter taste of casein hydrolysate (6), and their use avoids beer turbidity at low temperatures (10). A possible application for fungal PEPs, that has not been widely explored, is gluten degrading in various food products that are elaborated with cereal.

Few works describe the production of fungal PEP (4,6) and, to our knowledge its production in solid state fermentation (SSF) has not been reported. SSF presents advantages over submerged fermentation (SmF), mainly because low cost substrates are used. This technology is

particularly appropriate for fungi cultivation, because in it the natural conditions in which they grow are simulated. Several products of interest for the food industry (enzymes, flavors, and pigments) can be produced through SSF at higher yields or with better characteristics compared with those produced in SmF (11).

Optimization of environmental and operational conditions for maximizing growth and product yield in SSF is a complex task due to the high number of factors; the traditional optimization process, in which only one factor is tested at a time, is impracticable. In contrast, statistical design of experiments (DOE) allows obtaining results from small sets of experiments. Taguchi's methodology provides a quick and yet accurate way for determining the effect of main factors and their interactions through the use of orthogonal arrays (12). One of the several advantages of this methodology is the capability to deal with discrete and continuous variables at the same time. Several biotechnological applications have developed by means of Taguchi's DOE methodology (13).

In this work the production of a highly specific endoprotease having PEP activity, by *Aspergillus niger* ATCC 11414 in SSF of a low cost substrate is described. Taguchi's DOE methodology was used to test the effects of environmental conditions [temperature (T), initial pH, and initial moisture content (M)], substrate conditions [composition and ratio between dry substrate mass and bed's cross section area (MA)] and operational conditions [inoculum size (S), incubation time (t), substrate mixing, and moisture control (MMC)] on PEP production. The production of the enzyme under the predicted optimal SSF conditions was followed as a function of time to validate the predicted result. Biochemical properties (optimal temperature and pH, and both thermal and pH stabilities) of the enzyme produced in SSF were compared with those reported for related enzymes produced in SmF. To make this comparison, the enzyme was partially purified.

Materials and Methods

Microorganism *Aspergillus niger* ATCC 11414 (American Type Culture Collection, Rockville, MD, USA) was used in the fermentation assays. The fungus was seeded on potato dextrose agar (BD Diagnostic Systems, Spark, MD, USA); the incubation was carried out at 25ºC until spore formation. Spores were recovered with sterile 1%(v/v) Tween 80 (Merck, Hohenbrunn, Germany) and the suspension was maintained at 4ºC until use.

Design of experiments (DOEs) To study the effect of 8 factors (with/without daily MMC, t, MA, S, WG, initial pH, M, and T) on PEP production in SSF an orthogonal array L_{18} ($2^1 \times 3^7$) [18 experimental runs, 7 factors tested at 3 levels, and 1 factor tested at 2 levels] was used. Levels of the factors were determined in preliminary experiments (not shown). The conditions for the 18 experimental runs are shown in Table 1. Each run was carried out with 3 replicates and the results are presented as the average ± standard deviation (SD).

Solid state fermentation The substrate (30 g) was moisturized with Czapek-dox solution ($NaNO_3$ 2.5 g/L, KH_2PO_4 1 g/L, $MgSO_47H_2O$ 0.5 g/L, KCl 0.5 g/L). A volume corresponding to 70% of that required for obtaining the desired initial moisture content was added. The moisturized substrate was placed in previously weighed closed flasks (10 cm height, 7 cm diameter) for sterilization (121ºC, 15 min). Once cooled at room temperature, known aliquots of 0.2 M NaOH or 0.1 M HCl, were added for pH adjustment. The substrate was mixed with a spoon and inoculated with the spore solution diluted in Czapek-dox solution (30% of the volume required for obtaining the desired initial moisture). The flasks were covered with filter paper (Whatman No.1) to enable the gas exchange. Incubation was carried out in a constant temperature water bath. Each of the experiments carried out with MMC were performed in duplicate; the content in the duplicate flask was sampled for measurement of moisture content using a thermo-balance (Ohaus MB200; Florham Park, NJ, USA). The production of proteases and PEP was followed as a time function in the SSF carried out under the optimal conditions for PEP production predicted by DOEs; for this purpose, 24 flasks were inoculated and incubated. Three flasks were taken at 0 h and every 24 h for analysis during 168 h.

Partial purification of the PEP All the purification steps were carried out at 4ºC. The fermented substrate was extracted with distilled water (substrate mass to water volume ratio of 1:3) during 1 h in orbital shaker (250 rpm). The suspension was centrifuged (7,000×*g*, 10 min, 4ºC) and the precipitates were discarded. The crude extract was supplemented with ammonium sulfate to attain 50% saturation; precipitation was achieved overnight. The suspension was centrifuged and the precipitates were discarded. Ammonium sulfate was added to the supernatant to attain 80% saturation. After 1 night, the precipitates recovered by centrifuging, were dissolved in 0.5 M acetate buffer (pH 5.0), and dialyzed using membrane cut-off 6,000-8,000 Da (Spectra Por, Spectrum Laboratories, Inc., Rancho Dominguez, AC, USA) in acetate buffer (0.5 M, pH 5.0) for 2 days; the buffer was changed twice a day. The dialyzed solution was separated in a DEAE-Sepharose Fast Flow column (1 mL, 0.7×2.5 cm, HiTrap; Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with

acetate buffer (0.5 M, pH 5.0). Elution was carried out discontinuously with 0.1 to 1.0 M NaCl prepared in the same buffer at a rate of 0.5 mL/min. Fractions with PEP activity were freeze-dried.

Electrophoresis SDS-PAGE was carried out according to Laemmli (14), using 8% polyacrylamide gels. The sample was diluted in loading buffer and heated in boiling water for 5 min. Electrophoresis was run at 200 V. Gels were stained with R 250 Coomassie brilliant blue. Unstained protein molecular weight marker (Fermentas, Burlington, Canada) containing a mixture of 7 proteins (14.4-116 kDa) was used.

Protease zymogram The methodology described by Aoki *et al*. (15) was used with some modifications. The protein sample was diluted in loading buffer without mercaptoethanol. SDS-PAGE was performed at 4ºC using 8% polyacrylamide gels containing 0.1% gelatin or 0.1% BSA (Calbiochem, Darmstadt, Germany) at 200 V. After electrophoresis, SDS was removed by washing twice with 2.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) changing the washing solution every 30 min for 2 h; the washed gel was rinsed with deionized water. Gel was incubated in citrate-phosphate buffer (0.1 M, pH 4.0) at 37ºC overnight. Gel staining was made with R 250 Coomassie brilliant blue and distained with acetic acid 10%(v/v) until pale bands were visible. Gel spots were excised to recover protein in citrate-phosphate buffer and examined for PEP activity.

Analysis The fermented substrate was extracted with 1 M NaCl, in a mass to volume ratio of 1:10, during 1 h at 4ºC in orbital shaker (250 rpm). The suspension was centrifuged (7,000×*g*, 10 min, 4ºC) and precipitates were discarded. The supernatant was used as crude extract for measuring enzyme activities. Soluble protein (SP) content was determined by the method of Lowry *et al*. (16) using BSA as standard. Total sugar (TS) content was determined using the phenol-sulfuric acid method (17) with glucose as standard.

Enzymatic activities Protease activity was determined using the method reported by Brock *et al*. (18); briefly, the sample (250 μL, appropriately diluted) was incubated (37ºC, 20 min) with an equal volume of 2.5 g/L azocasein (Sigma-Aldrich). In sample's blank, substrate volume was replaced by distilled water. Assays corresponding to 0 and 100% protease activity were prepared replacing the sample volume by distilled water. The reaction was stopped with 1 mL of 2 M trichloroacetic acid. Absorbance was measured at 400 nm after centrifuging (10,000×*g*, 10 min). One unit of protease activity was defined as the quantity of enzyme that hydrolyses 1 μg of azocasein/min under the specified conditions. Protease activity is reported/kg of dry solids used in the initial extraction.

PEP activity was determined using the methodology described by Edens *et al*. (6) with some modifications. The substrate (benzyloxycarbonyl-glycine-proline-*p*-nitroanilide, Z-Gly-Pro-pNA, Sigma-Aldrich), was dissolved in 1,4-dioxane (40%, v/v in water) to prepare a 250 μM solution. Reaction mixture contained the sample (300 μL), 300 μL of acetate buffer (0.5 M, pH 5.0) and 200 μL of substrate solution. The blank for the sample was prepared replacing the substrate volume by 1,4-dioxane solution. Reaction carried out at 50ºC for 1 h was stopped adding 400 μL of 30% acetic acid. Sample was centrifuged (10,000×*g*, 5 min) and its absorbance was measured at 410 nm. Released *p*-nitroanilide was calculated using an extinction coefficient of 8,800/M·cm (19). One unit of PEP activity was defined as the quantity of enzyme that releases 1 μmol of *p*-nitroanilide/min under the specified conditions. PEP activity is reported in U/kg of dry solids used in the initial extraction.

Properties of the PEP produced in SSF PEP activity dependence in terms of pH was determined using 0.1 M citrate-phosphate buffers (pH of 3, 4, 5, 6, and 7). Enzyme stability against pH was determined after incubating the enzyme for 1 h at room temperature (20ºC) with the following buffers: 0.1 M KCl/HCl (pH 1.2 and 2.2), 0.1 M citrate-phosphate (pH 3, 4, 5, and 7), and 0.05 M boric acid-NaOH (pH 10 and 10.5). Thermal dependence of PEP activity was determined incubating the reaction mixture (enzyme in 0.1 M citrate-phosphate buffer pH 4 and the substrate) at temperatures between 20 and 60ºC. To evaluate thermal stability 100 μL of the enzyme solution and 600 μL of 0.1 M citrate-phosphate buffer pH 4 were incubated for 1 h at different temperatures (4, 20, 30, 40, 50, 60, 70, and 80ºC), before reaction with the substrate.

Analysis of experimental data and performance prediction For data analysis, Qualitek-4 software (Nutek Inc., Bloomfield Hills, MI, USA) for automatic experiment design using Taguchi's methodology was used. Experimental data was processed with bigger is better quality characteristic for determining the optimal SSF conditions for the enzyme production. The significance and relative influence of individual factors on the enzyme production was determined from analysis of variance (ANOVA). Significance of the factors was determined at 5% confidence level. For each factor, main effect analysis was carried out. To do this the difference between averages obtained at 2 levels was computed; the higher the difference, in absolute value, the higher is the influence. Interaction severity indexes [SI] were computed to establish the magnitude of interactions

Table 1. Orthogonal array used to test the effect of the factors daily mixing and moisture control (MMC), incubation time (t), ratio between the substrate mass and bed cross section area (MA), inoculum size (S), wheat germ content (WG), pH, initial moisture of the substrate (M), and temperature on PEP production by *A. niger* ATCC 11414 in the SSF

Run	Level of the factors								Activity±SD[3] (U/kg)
	MMC[1]	t (h)	MA (g/cm^2)	S[2] (%, w/w)	WG (%, w/w)	pH	M (%, v/w)	T (ºC)	
1	N	72	0.88	1	50	5.5	50	25	0.17±0.02
2	N	72	1.76	2	75	6.5	60	30	3.26±0.12
3	N	72	2.64	3	100	7.5	70	35	1.46±0.12
4	N	120	0.88	2	50	6.5	70	35	3.52±0.01
5	N	120	1.76	3	75	7.5	50	25	2.59±0.02
6	N	120	2.64	1	100	5.5	60	30	3.41±0.01
7	N	168	0.88	1	75	7.5	60	35	1.27±0.04
8	N	168	1.76	2	100	5.5	70	25	4.96±0.07
9	N	168	2.64	3	50	6.5	50	30	3.27±0.11
10	Y	72	0.88	3	100	6.5	60	25	2.41±0.03
11	Y	72	1.76	1	50	7.5	70	30	3.49±0.04
12	Y	72	2.64	2	75	5.5	50	35	2.50±0.07
13	Y	120	0.88	3	75	5.5	70	30	4.33±0.04
14	Y	120	1.76	1	100	6.5	50	35	3.87±0.03
15	Y	120	2.64	2	50	7.5	60	25	4.85±0.04
16	Y	168	0.88	2	100	7.5	50	30	1.43±0.01
17	Y	168	1.76	3	50	5.5	60	35	2.90±0.07
18	Y	168	2.64	1	75	6.5	70	25	5.78±0.03

[1]N: no, Y: yes
[2]1, 5×10^4; 2, 5×10^5; 3, 5×10^6 spores/g
[3]Corresponds to the mean PEP activity of 3 replicates

between the factors.

Results and Discussion

Effect of SSF conditions on PEP production by *A. niger* ATCC 11414 PEP activity in the 18 SSF experiments, carried out with factors at the levels specified by the L_{18} ($2^1\times3^7$) orthogonal array are shown in Table 1. The highest activity (5.78±0.03 U/kg) was obtained in experiment 18 in which conditions were: with daily MMC, incubation for 168 h at 25ºC, MA equal to 2.64 g/cm^2, inoculated with 5×10^5 spores/g dry substrate, 50%(w/w) WG, initial pH 6.5, and initial moisture 70%. The lowest PEP activity (0.17±0.02 U/kg) was obtained in experiment 1 in which all the factors were tested at level 1.

From the main effect analysis higher PEP activities were obtained in the SSF in which the factors t (120 h), S (5×10^5 spore/g), WG (75%), and initial pH (6.5) were tested at level 2. For the factors MA (2.64 g/cm^2) and M (70%), higher PEP activities were obtained at level 3. The increase of T from 25 to 35ºC showed a negative effect on the enzyme production while the effect of daily MMC was positive.

All the factors significantly affected ($p<0.05$) PEP production (Table 2); differences in PEP activity were explained by changes in M (22.6%), t (21.5%), and MA (20.6%). The effects of initial pH, daily MMC, T, and WG on PEP production had a lower impact, contributing to the response variability in percentages of 11.8, 9.4, 6.9, and 3.2%, respectively. The changes in these 7 factors explain 96% of the observed differences in PEP production. Inoculum size had the lowest influence on the response variability (1.2%), and both experimental error and non controlled factors influenced the variability by 2.8%.

Interactions between the different operational and environmental conditions in a fermentation process can also affect microbial growth and thus the production of enzymes and metabolites. Magnitude of these interactions can be more relevant at specific levels of the factors. In order to establish the relative significance of the interactions between the examined factors on PEP production, the estimated interaction severity indexes [SI] were software-generated (Table 3). WG, a factor that showed a relatively low impact on the enzyme production (3.2%) was found in the 4 main interactions ([SI]>50%). Although wheat bran is considered a suitable substrate for protease production through SSF (20), in some cases better results are obtained when a protein source is added (21,22). The highest [SI] (67.81%) corresponded to the interaction between the factor MMC (level 2, Yes) and WG (level 1, 50%). This behavior can be explained because of the improvement of

Table 2. Analysis of variance of main effects on PEP production by *A. niger* ATCC 11414 in SSF

Factor[1)]	Degree of freedom	Sums of squares	Variance	F-ratio	Pure Sum	%
MMC	1	9.79	9.79	128.10	9.71	9.4
Incubation time (t)	2	22.51	11.25	147.29	22.43	21.5
MA	2	21.55	10.77	141.01	21.47	20.6
Inoculum size (S)	2	1.26	0.63	8.23	1.18	1.2
Wheat germ content (WG)	2	3.36	1.68	21.99	3.28	3.2
pH	2	12.37	6.18	80.93	12.29	11.8
Moisture content (M)	2	23.70	11.85	155.09	23.62	22.6
Tenperature (T)	2	7.22	3.16	47.27	7.15	6.9
Other/Error	38	2.90	0.08			2.8
Total	53	104.66				100

[1)]MMC, substrate mixing and moisture control; MA, ratio between substrate mass and bed cross section area

gas exchange due to high substrate porosity (high bran content) and mixing. On the other hand, moisture control during fermentation decreases the risk of growth inhibition due to substrate dehydration. Even though the experiments were conducted at a constant temperature by incubating the flasks in a water bath for better removing of the heat generated during microbial growth, the resistance to heat transfer in the solid develops a temperature gradient that increases the evaporation rate. This water loss was compensated in the experiments carried out with periodic moisture control.

Another interaction with high [SI] (63.73%) was that between S (level 1, 5×10^4 spores/g) and WG (level 2, 75%). Although these 2 factors showed a low impact on the response (Table 2), the magnitude of [SI] suggests that their combined effect affects PEP production. Wheat germ is a rich nutrient source that would improve fungus growth when it is inoculated at a low concentration. Interaction [SI] between WG (level 2, 75%) and T (level 1, 25°C) resulted to be 55.72% and occurred at the optimal levels of these 2 factors. Interaction between WG (level 1, 50%) and initial pH (level 2, 6.5) also showed a high [SI] (55.63%). The combined effect of WG, and pH could be attributed to a buffer effect due to the highest bran content in the solid substrate; pH has a significant effect on the activity of enzymes involved in nutrient degradation and uptake, and also on transport mechanisms of several components through cell membrane (23).

Optimal conditions for PEP production in SSF

Contributions of the factors MMC, t, MA, S, WG, pH, M, and T at their optimal levels on the enzyme production resulted to be 0.43, 0.68, 0.46, 0.21, 0.34, 0.60, 0.84, and 0.38 U/kg, respectively. Thus, total contribution of all the factors (3.94 U/kg) resulted to be higher than the grand average of performance (3.08 U/kg) (Table 1). A SSF carried out under these conditions would allow obtaining 7.01 U/kg of the PEP.

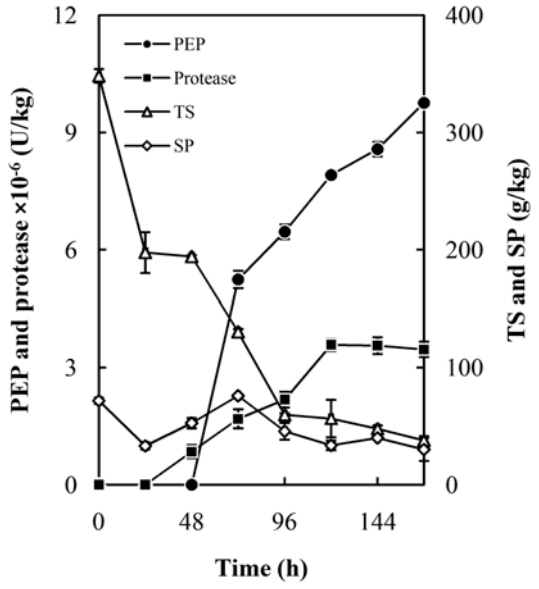

Fig. 1. PEP and protease production by *A. niger* ATCC 11414 in SSF of wheat germ and bran under the optimal conditions suggested by Taguchi's DOEs methodology, and contents of soluble protein (SP) and total sugars (TS) in the solid substrate.

PEP production was followed by 168 h in the SSF carried out under optimal conditions (Fig. 1) for validating the DOEs prediction. Protease production and the contents of SP and TS were also determined. PEP activity was only detected after 72 h; at 120 h PEP activity was 7.91±0.11 U/kg, a 13% higher than the expected result. A further increase in PEP activity (39%) was obtained after 168 h, reaching 9.76±0.06 U/kg. The fact that the highest PEP activity was obtained after 168 h is not surprising because among the experiments in the DOEs (Table 1) the one with the highest activity was that incubated for the same period (experiment 18). The difference between the predicted optimum incubation time and the one found in the validating experiment could be attributed to the contribution of the main interactions among the factors. Incubation time at

Table 3. Estimated interaction severity index [SI] of the factors whose effect on PEP production by *A. niger* ATCC11414 in SSF was examined

No.	Interaction	Column[1)]	[SI][2)] (%)	Col[3)]	Optimal level[4)]
1	MMC×WG	1×5	67.81	4	[2,1]
2	S×WG	4×5	63.73	1	[1,2]
3	WG×T	5×8	55.72	13	[2,1]
4	WG×pH	5×6	55.63	3	[1,2]
5	MMC×T	1×8	44.26	9	[2,1]
6	t×MA	2×3	42.97	1	[3,3]
7	pH×T	6×8	39.15	14	[2,1]
8	pH×M	6×7	38.58	1	[2,3]
9	t×S	2×4	37.69	6	[2,1]
10	S×M	4×7	36.51	3	[2,3]
11	WG×M	5×7	33.05	2	[1,3]
12	S×T	4×8	27.47	12	[2,1]
13	t×pH	2×6	26.23	4	[3,2]
14	MA×T	3×8	24.73	11	[3,1]
15	MA×S	3×4	23.95	7	[2,3]
16	t×T	2×8	23.66	10	[3,1]
17	MMC×MA	1×3	22.96	2	[2,3]
18	MA×M	3×7	17.39	4	[2,3]
19	MA×WG	3×5	17.15	6	[3,1]
20	MA×pH	3×6	17.08	5	[3,2]
21	M×T	7×8	15.92	15	[3,1]
22	S×pH	4×6	12.35	2	[2,2]
23	MMC×S	1×4	10.74	5	[2,2]
24	t×WG	2×5	10.71	7	[2,2]
25	t×M	2×7	7.39	5	[3,3]
26	MMC×pH	1×6	6.07	7	[2,2]
27	MMC×M	1×7	2.93	6	[2,3]
28	MMC×t	1×2	0.06	3	[2,2]

[1)]Represents the column locations to which the interacting factors are assigned.
[2)]100% for 90° angle between the lines, 0% for parallel lines
[3)]Column that should be reserved if this interaction effect were to be studied (2-L factors only).
[4)]Indicates the factor levels desirable for the optimum condition (based strictly on the first 2 levels)

level 3 (168 h) was found in 4 interactions (with MA, pH, M, and T) although these [SI] values were lower than 50% (Table 3). PEP titres obtained in the optimized SSF process could not be compared to any other report since in the few works in which the enzyme has been produced this information is not presented.

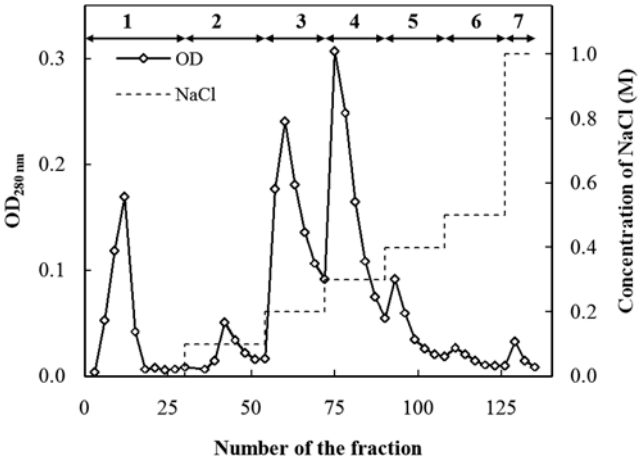

Fig. 2. Elution curve of the soluble protein extracted from the solid substrate fermented by *A. niger* ATCC 11414 obtained using DEAE-Sepharose Fast Flow anion-exchange chromatography. PEP specific activity of the pooled fractions in U/mg (1) 0, (2) 0.168, (3) 0.020, (4) 0.084, (5) 0.452, (6) 0.994, and (7) 0.146

Protease activity in the fermented substrate increased to a maximum of $3.6\times10^6\pm1.5\times10^5$ U/kg at 120 h; after this time, changes in protease activity were not significant ($p>0.05$). In the optimized SSF, PEP activity was detected after protease production (Fig. 1). Besides, the increases in PEP activity after 120 h did not correlate with changes in protease activity that remained almost constant; the lack of correlation suggests that conditions for PEP production are different from the ones that induce protease production. Such conditions could be related to the concentration of certain peptides generated by protease activity that stimulate PEP secretion; it is also possible that PEP production takes place after the microorganism reaches a certain physiological state. Related to this, it is worth mentioning that mycelium growth decreased (visual inspection) after 96 h, probably due to nutrient depleting. After 168 h, reductions in the contents of SP and TS were equal to 57.9 and 89.1%, respectively. Because PEP activity showed a constant increase towards the end of fermentation period, its synthesis is not growth-related, a behavior reported in proteases production (24,25).

Partial purification of the PEP produced in SSF The results obtained in the partial purification of the PEP produced in SSF are summarized in Table 4. The stages of

Table 4. Partial purification of the PEP from *A. niger* ATCC 11414 produced in SSF of wheat germ and wheat bran mixture

Step[1)]	Protein (g/L)	Activity (U/L)	Specific activity (U/g)	Fold purification
Crude extract	0.63	0.20	0.32	1
Ammonium sulfate (50%-80%) fraction	0.80	0.71	0.89	3
DEAE-Sepharose Fast Flow chromatography	0.01	0.17	30.42	34

[1)]Initial total activity in the crude extract was 0.06 U.

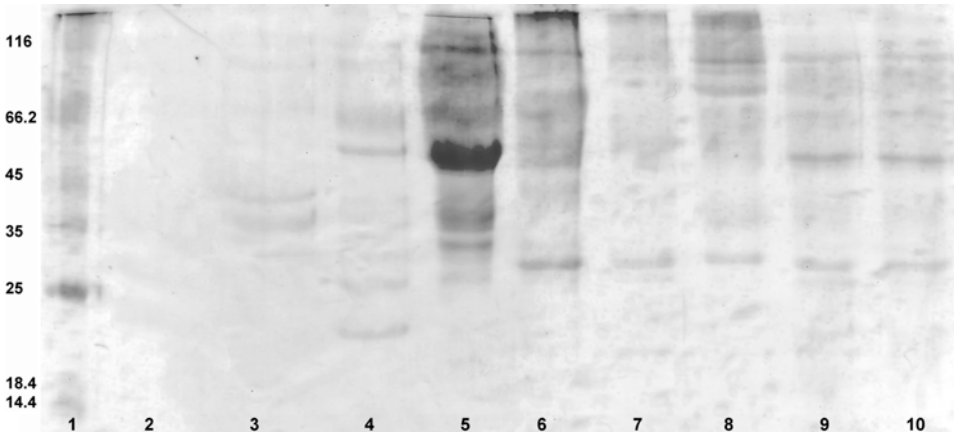

Fig. 3. SDS-PAGE of the protein fractions (PF) in the partial purification of the PEP produced by *A. niger* ATCC 11414 in SSF. lane 1, Mw marker; lane 2 to 6, PF from DEAE-Sepharose Fast Flow anion-exchange chromatography [PF eluted with NaCl 1 M (2), 0.7 M (3), 0.5 M (4), 0.3 M (5), 0.1 M (6)], unbound PF removed during the initial washing (lane 7 and 8). Protein precipitated with ammonium sulfate after and before dialysis in lane 9 and 10, respectively.

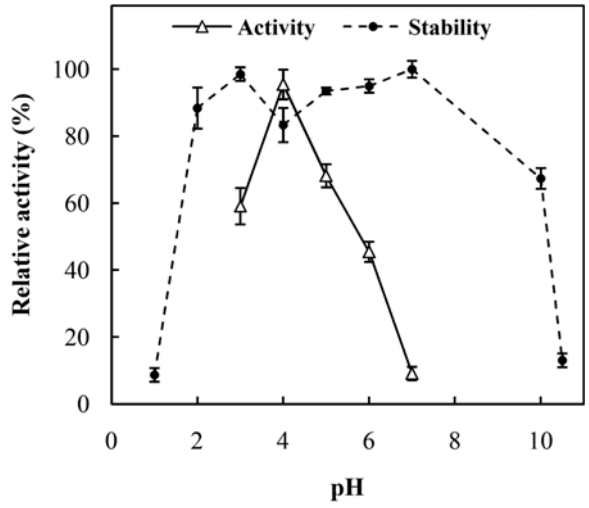

Fig. 4. Effect of pH on activity and stability of the PEP produced by *A. niger* ATCC 11414 in SSF of wheat germ and bran.

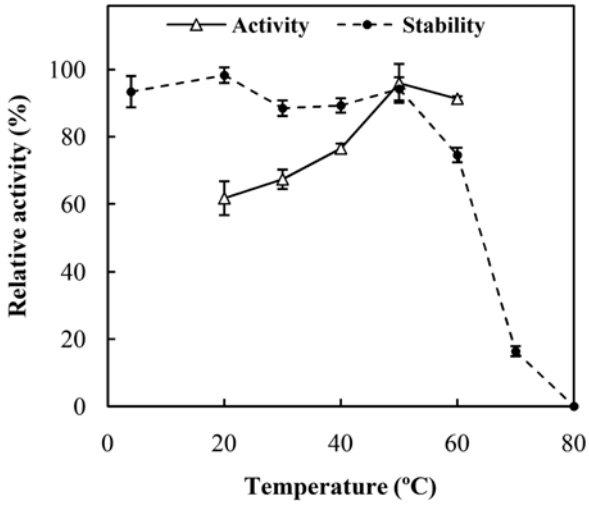

Fig. 5. Effect of temperature on activity and stability of the PEP produced by *A. niger* ATCC 11414 in SSF of wheat germ and bran.

ammonium sulfate precipitation, dialysis, and anionic exchange chromatography increased 34 times the specific PEP activity related to that in the crude extract. In the chromatographic separation whose elution curve is shown in Fig. 2, the fraction with the highest specific PEP activity was eluted with 0.5 M NaCl. SDS-PAGE of the protein fractions in the partial purification of the PEP produced by *A. niger* ATCC 11414 are shown in Fig. 3. Zymogram of the final enzyme preparation detected 2 proteases (25 and 66 kDa) able to hydrolyze gelatin (not shown); nevertheless, only the 66 kDa protein showed PEP activity. PEP enzyme produced by *A. niger* var. *macrospores*, that is also able to hydrolyze gelatin, has a molecular weight of 55 kDa (5). Unlike this enzyme, that is also able to hydrolyze BSA and collagen, the one produced by *A. niger* ATCC 11414 in SSF did not show pale zones in the zymogram containing BSA. The resistance of BSA to proteolysis by *Aspergillus* proteases has already been reported (26).

The partially purified PEP preparation was used to determine optimal pH (Fig. 4) and temperature (Fig. 5), and both thermal (Fig. 5) and pH (Fig. 4) stabilities. The highest PEP activity was obtained at pH 4 and the enzyme presented activities corresponding to 60 and 70% of the maximum activity at pH of 3 and 5, respectively. The enzyme, as others produced by different *Aspergillus* strains, has an optimum acidic pH a distinctive characteristic that differentiates fungal PEP from those produced by bacteria (27,28). PEP produced by *A. niger* ATCC 11414 showed stability in a wide pH range (2-10) (Fig. 4); after the incubation at pH 10 for 1 h, the enzyme retained 68% of the maximum activity, whereas 90% of the activity was retained after the incubation at pH 2 for the same period.

Incubation for 1 h at pH 4 showed a lower stability compared to the values obtained at pH 3 and 5. This behavior can be explained if the isoelectric point of the enzyme is close to the value reported for the enzyme purified by Edens *et al*. (6) that is around pH 4.2. At this condition a prolonged incubation would result in a lower enzyme concentration due to aggregation and precipitation as the protein charges are neutralized.

The PEP produced in SSF presented its maximum activity at 50ºC (Fig. 5); this value was not significantly ($p>0.05$) different from that obtained at 60ºC (91% of the maximum activity). The effect of temperature on the activity of the PEP produced in SSF is similar to that reported by other authors (5,6). Unlike these, optimal temperature for PEP enzymes from *Aspergillus oryzae* FS1-32 and *Pyrococcus furiosus* are 37ºC (4) and between 85 and 90ºC (29), respectively. PEP produced by *A. niger* ATCC 11414 showed thermal stability between 4 and 60ºC (Fig. 5). The incubation at 70ºC for 1 h reduced the activity of the enzyme (84% of the maximum activity) and total loss of activity was obtained after the incubation at 80ºC for the same period.

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