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Pectinolytic complex production by *Aspergillus niger* URM 4645 using yellow passion fruit peels in solid state fermentation

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The activities of endo-polygalacturonase (endo-PG), exo-polygalacturonase (exo-PG), pectin lyase (PL), and pectin methylesterase (PE), produced by *Aspergillus niger* URM 4645, were studied in solid state fermentation (SSF) using yellow passion fruit peels as substrate. The effect of substrate amount, initial moisture content, and temperature on pectinase production was studied using a full factorial design (2^3). Maximum endo-PG, exo-PG, PL, and PE activities were 31.35, 7.98, 551,299.39, and 447.93 U g^{-1} dry substrate, respectively. Optimum activities of the four enzymes were obtained with 5.0 g of the substrate and an initial moisture content of 30% at 34°C with 96 h of fermentation. Optimum endo-PG activity was found at pH 7.5 at an optimum temperature of 40°C; exo-PG and PL at pH 7.0 at an optimum temperature of 80°C; and PE at pH 3.5 at an optimum temperature of 30°C. Endo-PG was stable at pH 7.0 to 8.0 at 40°C, and exo-PG and PL at pH 6.0 to 8.0 and 6.0 to 7.5, respectively at 60 to 70°C. PE was stable at pH 3.5 to 5.0 at 30 to 60°C. The enzyme production optimization clearly demonstrated the impact of process parameters on the yield of pectinolytic enzymes.

Key words: *Aspergillus niger*, residue, pectinolytic activities, solid state fermentation, characterization.

INTRODUCTION

Passiflora edulis f. *flavicarpa*, commonly known as the yellow passion fruit, is cultivated on a large scale in Brazil and is of agronomic importance because of the use of its fruits in nature and in the juice industry. The waste

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resulting from passion fruit processing consists of more than 75% of the raw material. The rind constitutes 90% of the waste product and is a source of pectin (20% of dry weight) (Arvanitoyannis and Varzakas, 2008).

Pectinases or pectinolytic enzymes are naturally produced by plants, filamentous fungi, bacteria, and yeasts (Maciel et al., 2013). Conversion of pectin to soluble sugars is possible through enzymatic reactions catalyzed by pectinolytic enzymes that are common in fungi, such as pectin lyase (PL) (EC 4.2.1.10), pectin methylesterase (PE) (EC 3.2.1.11), and polygalacturonases (PG) (EC 3.2.1.15) (Holck et al., 2011). Pectinases are of great importance for clarification and viscosity reduction in fruit juices, which result in increased filtration efficiency. They improve juice extraction, reduce clarification time, and enhance terpene content when employed in wine production (Uenojo and Pastore, 2007).

Filamentous fungi are the most frequently used microorganisms in the enzyme industry because as much as 90% of the enzymes produced by these fungi are extracellular (Blandino et al., 2001; Souza et al., 2003; Sandri et al., 2013). Pectinases can often be produced at high concentrations by strains of filamentous fungi belonging to *Aspergillus* genus. Recently, certain *Aspergillus* species have been characterized by the types of pectinolytic enzymes they produce (Maciel et al., 2011; Fontana et al., 2012; Heerd et al., 2012; Demir and Tari, 2014). PG, the most abundant and extensively studied of the pectinolytic enzymes, typically exist in multigene families and may have both endo (Parenicová et al., 2000) and exo activities (Sakamoto et al., 2002).

The microbial production of pectinolytic enzymes can be achieved by solid state fermentation (SSF) or submerged fermentation (SMF) (Maciel et al., 2013). Agro-industrial residues or wastes are generally considered suitable substrates for enzyme production by SSF (Pandey, 2003; Patil and Dayanand, 2006). The application of agro-industrial wastes as a carbon source in enzyme production processes reduces the cost of production and helps in solving problems associated with their disposal (Rodriguez-Leon et al., 2008). SSF systems have generated much interest in recent years because they offer several economic benefits (Fang et al., 2010).

The goal of this study was to evaluate the production of the pectinolytic complex comprising PG (endo- and exo-PG), PL, and PE by *A. niger* URM 4645 in SSF using yellow passion fruit peels as substrate and to partially characterize the enzymes.

MATERIALS AND METHODS

Microorganisms

Twenty-five (25) isolates of *A. niger* obtained from the Micoteca URM culture collection (URM, Recife, Brazil) (Micoteca URM, 2013) were inoculated on malt extract agar (MEA: malt 20 g L^{-1}, glucose 20 g L^{-1}, peptone 1 g L^{-1}, and agar 20 g L^{-1}) and maintained at 28°C.

Screening of pectinolytic fungi

A. niger isolates were subjected to screening in solid medium for selecting isolates with higher potential for pectin degradation. For selection, Petri dishes (diameter, 6 cm) containing 7 mL of sterilized MEA medium were inoculated with each isolate and incubated for seven days at 30°C. Then, one plug of 6 mm was cut using a sterile cork borer from the colony periphery, transferred to the center of a culture medium containing citric pectin (Sigma), and incubated for seven days at 30°C (Uenojo and Pastore, 2006). The pectinolytic index was determined and expressed by the ratio of the diameter of the degradation halo and the diameter of the colony growth. The plates were flooded with 0.1% (w/v) Congo red solution for approximately 45 min. The plates were then washed with water. Pectin degradation was evidenced by a clear zone around the fungal growth.

Origin of yellow passion fruit peels

The yellow passion fruit peels used in this study were supplied by the Food Supply Center of Pernambuco, located in Recife, Pernambuco, Brazil.

Inoculum standardization

A 6-cm-diameter Petri dish containing 7 mL of sterilized MEA medium was inoculated with the selected isolate and incubated for seven days at 30°C. Then, five plugs of 6 mm were cut using the sterile cork borer from the colony periphery, and spores from these plugs were suspended in 30 mL of Tween 80/water (0.02%). The spore concentration was adjusted to 10^7 spores per gram and inoculated in the substrate used for SSF. The initial moisture content of the substrate was determined in accordance with the standards of Instituto Adolfo Lutz (Zenebon and Pascuet 2005).

Pectinase production by SSF

For pectinase production, the peels were submerged for 1 h in 2% (w/v) sodium hypochlorite solution and then washed in water. The peels were then crushed and incubated at 65°C until they were completely dehydrated. For fermentation, peels with a particle size between 3 and 8 mm were used as substrate in order to provide improved absorption and porosity to facilitate transport of oxygen as well as nutrients during SSF. The substrate was then placed in 250-mL Erlenmeyer flasks and irradiated with ultraviolet light for 2 h in a microbiological cabinet (Spier et al., 2008).

Experimental design and statistical analysis

The influence of substrate amount (Sa), initial moisture content (Im), and temperature (T) on pectinase production was evaluated from the results of the experiments performed according to a 2^3 factorial design (Bruns et al., 2006), plus four central points (Table 1). All statistical analyses were performed using Statistic 8.0 software (Statsoft, 2008).

Extraction of enzymes

Pectinase production was carried out for 96 h. The contents of the flasks were harvested at regular intervals (24 h). A mass of 5.0 g of the fermented mixture was mixed with 30 mL of 0.2 M acetic acid-sodium acetate buffer, pH 5.5 (1 g of the substrate per 2.5 mL of buffer) for 15 min. After maceration, extraction was performed using

Table 1. Variable levels of the 2^3 experimental design for the production of pectinases (Ug^{-1}) in SSF by *Aspergillus niger* URM4645 using yellow passion peels as substrate.

Variable	Level		
	Low (-1)	Central (0)	High (+1)
Sa (g)	5.0	10.0	15.0
Im (%)	30	50	40
T (ºC)	26	34	30

Sa, substrate amount; Im, initial moisture content; T, temperature.

a filter paper (Whatman no. 1) under vacuum. The extract was clarified by filtration and centrifugation at 5000 × *g* for 15 min (Spier et al., 2008). The supernatant was used as a crude enzyme extract and was subjected to enzymatic analysis.

Enzymatic assays

Endo-PG activity

Endo-PG activity was measured viscosimetrically (Tuttobello and Mill, 1961) using a reaction mixture containing 5.5 mL of 0.2% citric pectin in 0.025 M acetate buffer, pH 5.0, 1.0 mM ethylene diamine tetra acetic acid (EDTA), and 250 µL of the enzymatic extract. This mixture was incubated at 50°C for 10 min and then cooled in an ice bath. A viscosimetric unit (U) was defined as the amount of enzyme required to decrease the initial viscosity per min by 50% under the conditions described previously. Enzyme activity was expressed in units per gram ($U\ g^{-1}$).

Exo-PG activity

Exo-PG activity was determined by measuring the release of reducing groups from citric pectin using the 3,5-dinitrosalicylic acid (DNS) reagent assay (Miller, 1959). The reaction mixture containing 0.5 mL of 0.5% citric pectin in 0.025 M acetate buffer, pH 5.0 and 0.5 mL of the enzyme extract was incubated at 50°C for 10 min. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 mmol of galacturonic acid per minute.

PL activity

PL activity was determined by measuring the increase in absorbance at 235 nm of the substrate solution (1.0 mL of 0.5% citric pectin in 0.2 M Tris-HCl buffer, pH 5.5) hydrolyzed by 1.0 mL of the enzyme extract at 40°C for 60 min. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 mmol of unsaturated uronide per minute, based on the molar extinction coefficient (ε_{235} = 5550 $M^{-1}\ cm^{-1}$) of the unsaturated products (Albershein, 1966). Enzyme activity was expressed in $U\ g^{-1}$.

PE activity

PE activity was determined by the pH decrease of the medium and by titration of carboxylic groups using a modified methodology (Siéssere et al., 1992). The reaction mixture contained 2.0 mL of 1% citric pectin in 0.025 M Tris-acetate buffer, pH 6.5 and 1.0 mL of the enzyme extract. The enzymatic reaction was carried out at 50°C for 2 h and then quenched in a boiling water bath for 3 min. Then, the samples were cooled in an ice bath and the liberated carboxylic groups were titrated with a solution of NaOH (0.01 M), using three drops of the phenolphthalein with pH indicator whose turning point was detected by appearance of a pink color. The volume of NaOH solution spent in each titration was noted. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 microequivalent of a carboxylic group in 60 min of the reaction under the described conditions. The activity was expressed in $U\ g^{-1}$.

Enzyme characterization

Endo-PG, exo-PG, PE, and PL activities of the crude enzyme extract were measured at different pH and temperature values.

Optimum pH and temperature for enzyme activity

The effect of pH on pectinolytic activity was measured using the following buffers: sodium acetate buffer (pH 3.5-5.0), citrate-phosphate buffer (pH 5.0-7.0), Tris-HCl (pH 7.0-8.5), and glycine-NaOH buffer (pH 8.5-11.0). The optimum temperature in the range of 30-80°C was determined by incubating the reaction mixture at the optimum pH.

pH and temperature stability

The crude enzyme extract was diluted (1:1) in different buffers (pH 3.5-11.0, 0.2 M for endo-PG and PL and 0.025 M for exo-PG, for buffers as the cited above) and incubated at 25°C for 24 h. After incubation, endo-PG, exo-PG, PE, and PL activities were measured for the optimum pH and temperature values. The crude enzyme extract was incubated at temperatures ranging from 30°C to 80°C for 60 min to determine temperature stability. Subsequently, endo-PG, exo-PG, PE, and PL activities were determined.

RESULTS AND DISCUSSION

Screening for pectinase production

Fungi are important producers of enzymes, relatively easy to grow in controlled environments, and highly sensitive to genetic alterations, enabling enhanced strains to be obtained in terms of production and quality of enzymes (Santos, 2007). Screening is often the first step in selecting microorganisms with characteristics

Table 2. Pectinolytic Index for 25 isolates of *Aspergillus niger*.

Culture/Access number[a] URM	Øc	Øh	PI[b]
URM 2228	34	-	-
URM 2604	37	-	-
URM 3701	33	17	0.52
URM 3753	52	10	0.19
URM 3806	45	14	0.31
URM 3811	62	14	0.23
URM 3820	52	11	0.21
URM 3856	67	06	0.09
URM 3885	50	07	0.14
URM 4452	40	10	0.25
URM 4645	35	18	0.51
URM 5001	57	06	0.11
URM 5117	47	08	0.17
URM 5149	55	08	0.15
URM 5162	68	08	0.12
URM 5207	64	10	0.16
URM 5437	61	11	0.18
URM 5438	60	10	0.17
URM 5439	56	07	0.13
URM 5555	64	11	0.17
URM 5756	47	11	0.23
URM 5837	54	11	0.20
URM 5838	60	10	0.17
URM 5842	48	12	0.25
URM 5853	52	12	0.23

− = no halo. [a]Access number in the Micoteca URM culture collections (Recife, Pernambuco); [b](PI) pectinolytic index = ratio of the (Øh) ring diameter (mm) to the (Øc) colony diameter (mm).

intended for industrial applications. This allows for the characterization and selection of fungal strains with optimum enzyme production. In addition, the information obtained adds value to these microbial resources preserved in culture collections (Maciel et al., 2013).

Among the 25 *A. niger* isolates tested in the screening on solid medium, 23 (92%) showed potential pectinolytic enzyme production. Of these 23 isolates, 7 had a pectin degradation halo of less than 10 mm in diameter and 16 had halos that varied from 10 mm to 18 mm in diameter (Table 2). Of these 16 isolates, 11 were from plant-derived substrates and soil. Fungi isolated from soil are considered to exhibit high activity in biodegradation and biodeterioration processes through enzyme production, contributing to nutrient cycling and consequently to the maintenance of ecosystems (Allsopp and Seal, 1986). This may explain the reason for *A. niger* URM 4645 presenting a pectinolytic index of 0.51 (Table 2). Bezerra et al. (2012) reported that among 24 endophytic fungal isolates of *Opuntia ficus-indica* (forage cactus), only *A. japonicus* and *Penicillium glandicola* showed pectinolytic activity.

Production of pectinolytic enzymes

A. niger URM 4645 was the isolate selected for evaluating enzyme production at different substrate amounts, initial moisture contents, and temperatures by SSF using yellow passion fruit peels. High activities were obtained using this substrate, demonstrating that this agro-industrial residue is a good substrate for pectinase production by *A. niger* URM 4645. This substrate contains considerable amounts of valuable substances such as sugars, oils, fibers, polyphenols, and pectin (Arvanitoyannis and Varzakas, 2008), which contribute to pectinase production by filamentous fungi. Mrudulab and Anitharaj (2001) tested rice bran, wheat bran, sugarcane bagasse, orange peels, lemon peels, and banana peel for pectinase production. In their study, orange peel was the substrate that allowed highest pectinase production by *A. niger*. Moreover, Maller et al. (2011) concluded that agro-industrial residues, such as orange and lemon peels, induced production of high levels of PG by *A. niveus*. The use of orange peels can be highly economical at the industrial scale for PG production (Maciel et al., 2013).

Table 3. Results of the 2^3 design for endo-PG, exo-PG, PL, and PE production in SSF by *Aspergillus niger* URM 4645.

Run	Sa	Im	T	Endo-PG_{96} (Ug^{-1})	Exo-PG_{72} (Ug^{-1})	PL_{96} (Ug^{-1})	PE_{72} (Ug^{-1})
01	5.0	30	26	29.02	6.14	520,867.96	320.48
02	15.0	30	26	15.02	**7.98**	373,563.73	348.47
03	5.0	50	26	13.26	5.89	271,052.60	245.07
04	15.0	50	26	17.37	3.67	213,617.93	245.87
05	5.0	30	34	**31.25**	5.67	**551,299.39**	320.90
06	15.0	30	34	5.39	7.93	304,155.37	**447.93**
07	5.0	50	34	17.12	5.09	359,785.98	243.36
08	15.0	50	34	18.64	5.08	262,886.63	275.66
09(C)	10.0	40	30	16.87	5.26	284,650.96	301.29
10(C)	10.0	40	30	21.24	5.56	317,871.66	302.39
11(C)	10.0	40	30	22.22	6.20	315,238.53	300.22
12(C)	10.0	40	30	22.14	5.06	299,374.14	340.70

For endo-PG, exo-PG, PL, and PE, the subscript gives the cultivation time in hours. Sa, substrate amount; Im, initial moisture content; T, temperature.

A. niger URM 4645, when cultured using yellow passion fruit peels without the addition of nutrient solution, produced endo-PG, exo-PG, PL, and PE. Maximum endo-PG activity observed using 5.0 g of the substrate with an initial moisture content of 30% at 34°C was 31.35 U g^{-1} at 96 h of fermentation; maximum exo-PG activity observed using 15.0 g of the substrate with an initial moisture content of 30% at 26°C was 7.98 U g^{-1} at 72 h of fermentation; maximum PL activity observed using 5.0 g of the substrate with an initial moisture content of 30% at 26°C was 551, 299.39 U g^{-1} at 96 h of fermentation; and maximum PE activity observed using 15.0 g of the substrate with an initial moisture content of 30% at 34°C was 447.93 U g^{-1} at 72 h of fermentation (Table 3).

Endo-PG activity obtained using yellow passion fruit peels was lower than that obtained by Fontana et al. (2005) and Maciel et al. (2011). Endo-PG activity obtained by Fontana et al. (2005) was 152 U g^{-1} using wheat bran and citrus pectin as substrates with 72 h of SSF. Using forage palm as substrates, Maciel et al. (2011) obtained endo-PG activity of 66.19 U g^{-1} after 96 h of SSF at 28°C.

Using the mixture of wheat bran and orange bagasse (1:1) as substrate, Silva et al. (2007) obtained exo-PG activity of 16.0 U g^{-1} with *P. viridicatum* RFC3 in SSF for 14 days. Patil and Dayanand (2006) obtained exo-PG activity of 17.1 U g^{-1} with *A. niger* after 96 h of SSF using sunflower head as substrate. Exo-PG activity obtained by these authors is higher than those obtained in the present study; however, in this study, exo-PG activity was approximately 8 U g^{-1} after 24 h of fermentation. Maciel et al. (2011) also obtained exo-PG activity of 3.59 U g^{-1} with *A. niger* URM 4645 after 24 h of SSF at 36°C using forage palm as substrate. Higher enzyme production during the first hours of fermentation allows a reduction in the cost of production compared to higher production toward the end of the fermentation process (Maciel et al., 2013).

Martin et al. (2004) tested a culture medium containing orange peel, sugar cane bagasse, and wheat bran (1:1:1) as substrate and detected PL activities of 19.40 and 11.0 U g^{-1} at 144 and 96 h of SSF for *Moniliella* sp. and *Penicillium* sp., respectively. Using orange peels as substrate, Silva et al. (2002) observed PL production by *P. viridicatum*. The results show PL activity of 2.0 U g^{-1}, and when wheat bran was added to orange peels the PL activity rose to 3.54 U g^{-1}, showing the influence of medium composition on enzyme production. Maciel et al. (2011) detected PL activity of 40,615.62 U g^{-1} by *A. niger* URM 4645 at 72 h of SSF using only forage palm as substrate. Joshi et al. (2006) tested apple pulp for PE production and detected a maximum activity of 5.5 U g^{-1} by *A. niger* after 25 h of fermentation. These PL and PE activities were lower than those obtained in the present study, which found more satisfactory results without the addition of a carbon source or nutrient solution to increase pectinase production.

Comparing enzyme production by different microorganisms can be difficult because growing conditions and the methods of determination of enzyme activities are different. The present results show that SSF was suitable for pectinase production by *A. niger* URM 4645 using yellow passion fruit peels as substrate, facilitating and minimizing costs for the industry.

Results show that endo-PG, exo-PG, PL, and PE activities were obtained at different conditions and times of fermentation. Therefore, it was necessary to choose a common condition for pectinase production because the food industry uses enzyme complex in the preparation of the product. The substrate amount of 5.0 g and initial moisture content of 30% at 34°C with 96 h of fermentation was chosen using statistical analysis and is a common

Table 4. Effects calculated from the responses of the 2^3 design for endo-PG, exo-PG, PL, and PE production with 96 h of SSF by *Aspergillus niger* URM 4645.

Variable/Interaction	Endo-PG_{96}	Exo-PG_{96}	PL_{96}	PE_{96}
(1)	-4.94[a]	-0.46	-9.31[a]	1.03
(2)	-2.00	-3.96[a]	-14.72[a]	-4.50[a]
(3)	-0.32	1.16	2.27	0.46
1x2	6.34[a]	-1.36	5.50[a]	-0.99
1x3	-2.02	0.89	-3.19	0.10
2x3	1.75	0.93	4.06[a]	0.64
1x2x3	1.29	0.28	1.38	0.27

[a]Statistically significant values (at the 95% confidence level, $p<0.05$). (1) Substrate amount, (2) Initial moisture content and (3) Temperature.

condition for the production of endo-PG, exo-PG, PL, and PE. This condition contributes to pectinase production on a large scale, facilitating and minimizing costs and production times for the industry.

The results of the statistical analysis, including the effects of each variable studied in the experimental design in order to produce endo-PG, exo-PG, PL, and PE at 96 h of SSF, are shown in Table 4. The initial moisture content and temperature were not significant variables at a 95% confidence level for endo-PG activity. Substrate amount and temperature were not significant variables at a 95% confidence level for exo-PG and PE activities. Temperature was not a significant variable at a 95% confidence level for PL activity (Table 4). The variable substrate amount had a negative effect on endo-PG and PL activities, indicating that smaller substrate amounts increase the enzyme activities. However, our results were different from those obtained by Maciel et al., (2011). These authors studied pectinase production by *A. niger* URM 4645, and highest enzyme activities were obtained in that study when a higher substrate amount was used (10.0 g).

The initial moisture content also showed a negative effect during 96 h of fermentation for exo-PG, PL, and PE activities, which indicated that an increase in these enzyme activities was obtained by reducing the initial moisture content (Table 4). This means that pectinase production was enhanced when the fermentation conditions were as follows: 5.0 g of substrate and 30% initial moisture content. This could be explained by the fact that under such fermentation conditions, the fungus showed good growth and was capable of producing pectinases to hydrolyze the pectin in the carbon sources. Substrate amount and temperature play an important role in influencing the process parameters and thus the yield of enzymes in SSF (Ustok et al., 2007). However, in the present study, the variable temperature was not shown to play an important role in pectinase production.

Some interaction effects were significant (Table 4), demonstrating a dependent relationship between them. For endo-PG and PL activities, the substrate amount and initial moisture content (1 × 2) interacted positively, demonstrating that an increase in the values of these variables results in an increase in these activities. For PL activity, the initial moisture content and temperature (2 × 3) interacted positively, such that an increase in the values of these variables resulted in an increase in the activity. The cultivation of microbial cells in an excess of water can lead to sticking of particles, limited gas exchange, and higher vulnerability to bacterial contamination, whereas that in low levels of water is correlated with reduced microbial growth, reduced enzyme stability, substrate swelling, and diffusion of nutrients (Silva et al., 2007).

Enzyme characterization

For the characterization of endo-PG, exo-PG, PL and PE produced by *A. niger* URM 4645, the crude enzyme extract obtained under the following conditions was used: 5.0 g of the substrate with an initial moisture content of 30% at 34°C (Figure 1 and Table 3, Run 05).

The effect of pH on endo-PG, exo-PG, PL, and PE activities is shown in Figure 2. Optimum endo- and exo-PG activities were observed at pH 7.5 and 7.0, respectively. Endo- and exo-PG showed a second peak of activity at pH 9.0 and 10.0 with 91 and 98.5% of maximum activity, respectively. The optimum pH for endo- and exo-PG was higher than that for most fungal PG already described, which show higher activities at an acidic pH (Favela-Torres et al., 2006). Maciel et al. (2011) obtained the maximum endo- and exo-PG activities of *A. niger* URM 4645 at pH 5.0 and 7.0, respectively. The maximum activity of exo-PG from *P. viridicatum* RFC3 was observed at pH 6.0 (Silva et al., 2007), *Moniliella* sp. SB9 at pH 4.5, and *Penicillium* sp. EGC5 at pH 4.5-5.0 (Martin et al., 2004). Freitas et al. (2005) obtained the maximum exo-PG activity of *Monascus* sp. and *Aspergillus* sp. at pH 5.5, and Phutela et al. (2005) obtained the maximum PG activity of *A. fumigatus* at pH 5.0.

Optimum PL activity was obtained at pH 7.0. PL showed a second peak of activity at pH 6.0 with 93% of

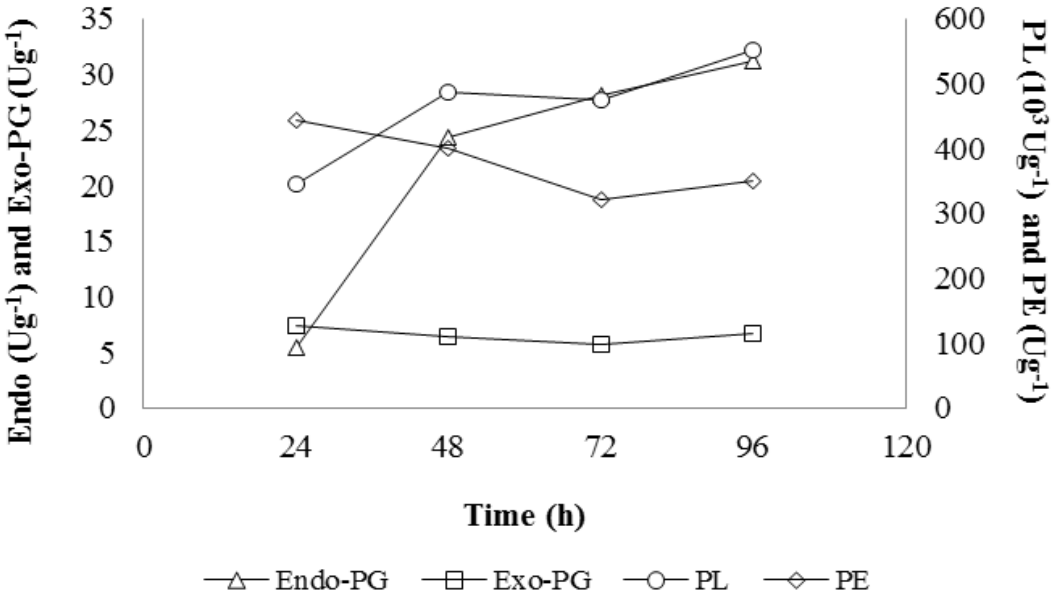

Figure 1. Endopolygalacturonase (Δ- endo-PG), exopolygalacturonase (□ - exo-PG), pectin lyase (○ - PL), and pectin methylesterase (◊ - PE) activities under the optimum conditions used to produce these four enzymes (5.0 g substrate and 30% moisture at 24°C with 96 h of solid state fermentation (SSF) - Run 05).

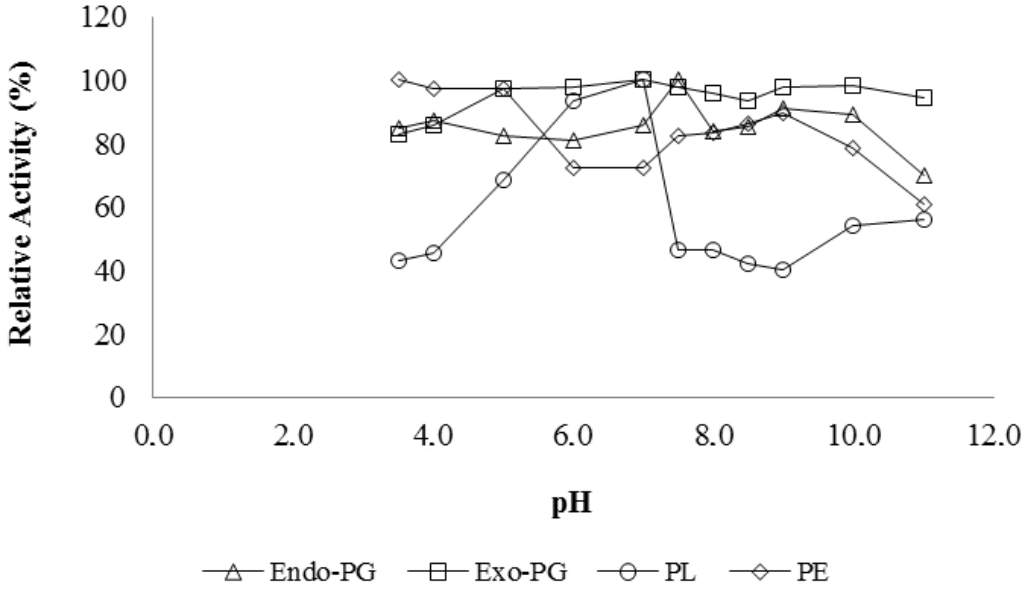

Figure 2. Effect of pH on the activities of endopolygalacturonase (Δ - endo-PG), exopolygalacturonase (□ - exo-PG), pectin lyase (○ - PL), and pectin methylesterase (◊ - PE) produced by *Aspergillus niger* URM 4645.

maximum activity. Optimum pH reported for PL was acidic (5.0) for *A. niger* (Maciel et al., 2011), neutral (7.0) for *P. expansum* (Silva et al., 1993), and basic (8.0) for *A. flavus* (Yadav et al., 2008) and *A. terricola* (Yadav et al., 2009). Piccoli-Valle et al. (2001) obtained the maximum PL activity of *P. griseoroseum* at a pH close to neutral (pH 5.0-7.0).

Optimum PE activity was obtained at pH 3.5, but PE showed a second peak of activity with 97% of maximum activity at pH 4.0 and 5.0. Dinu et al. (2007) obtained the

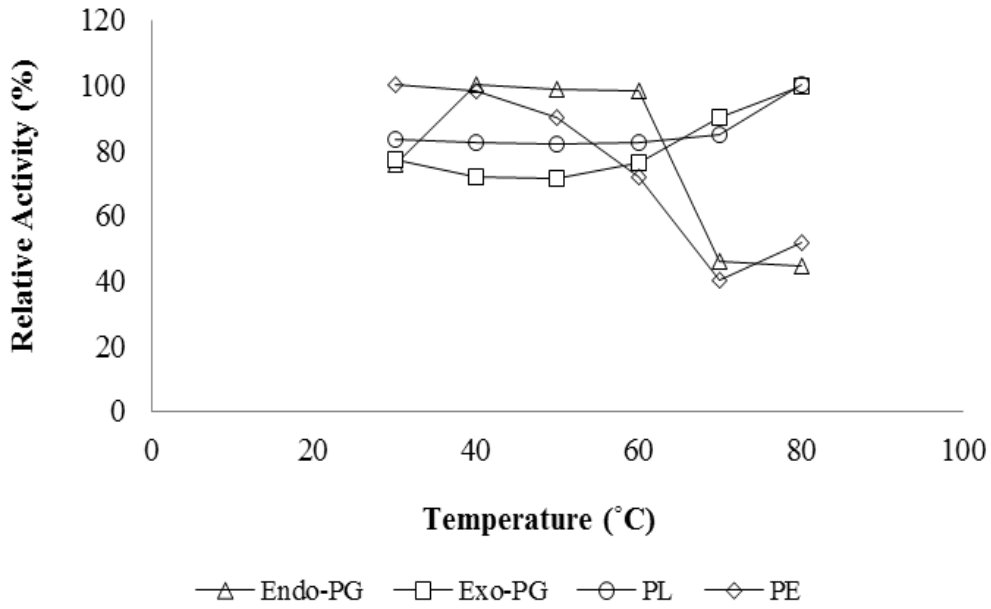

Figure 3. Effect of temperature on the activities of endopolygalacturonase (Δ - endo-PG), exopolygalacturonase (□ - exo-PG), pectin lyase (○ - PL), and pectin methylesterase (◊- PE) produced by *Aspergillus niger* URM 4645.

maximum PE activity of *A*. *niger* MIUG 16 at pH 4.2-4.4. Optimum PE activity of *A*. *niger* and *P*. *griseoroseum* was observed at pH 4.0 and pH 4.5 to 5.0, respectively (Joshi et al., 2006; Piccoli-Valle et al., 2001). Each microorganism possesses a pH range for its growth and activity with an optimum value within the range. Filamentous fungi show reasonably good growth over a broad range of pH (2.0 to 9.0), with an optimum range of pH 3.8 to 6.0 (Gowthaman et al., 2001).

Endo-PG was stable at pH 7.0 to 8.0, exo-PG at pH 6.0 to 8.0, PL at pH 6.0-7.5, and PE at pH 3.5-5.0. After incubation for 24 h at pH 3.5-11.0, more than 40% of the pectinase activity was maintained. The results are in agreement with those obtained by Silva et al. (2002) with PG from *P*. *viridicatum* RFC3 that was stable at pH 5.0 to 8.0, retaining 80% of its activity at pH 9.0. Furthermore, Silva et al. (2007) reported the stability of exo-PG from *P*. *viridicatum* RFC3 at pH 7.0 to 10.0. Maciel et al. (2011) observed that endo-PG and exo-PG from *A*. *niger* URM 4645 were stable at pH 3.5 to 11.0, and more than 40 and 70% of the endo- and exo-PG activities were maintained, respectively. Freitas et al. (2005) found that exo-PG from *Monascus* sp. was stable at pH 4.5-6.0, while that from *Aspergillus* sp. was stable at pH 4.0.

In the present study, PL was stable at acidic to neutral pH (4.0-7.0). However, Yadav et al. (2008, 2009) reported the stability of PL from *A*. *flavus* and *A*. *terricola* at pH 4.0-10.0 and 4.0-9.0, respectively. PE from *A*. *japonicus* was stable at pH 3.5 to 5.5 (Semenova et al., 2003) and that from *Aureobasidium pullulans* was stable at pH 4.0 to 6.5 (Manachini et al., 1988).

Regarding the optimum temperature, maximum endo-PG and PE activities were obtained at 40 and 30°C, respectively. The maximum for exo-PG and PL activities were obtained at 80°C (Figure 3). Phutela et al. (2005), studying pectinases from *A*. *fumigatus* TF3 and Yadav et al. (2009) working with *A*. *terricola* MTCC 7588, obtained maximum PL activity at 50°C. Silva et al., (2002) observed that maximum PG and PL activities were obtained at 55 and 50°C, respectively. *Monascus* sp. and *Aspergillus* sp. exhibited maximum exo-PG activity at 60 and 50°C, respectively (Freitas et al., 2005). Dinu et al. (2007) and Silva et al. (2007) showed an optimum activity of PG produced by *A*. *niger* MIUG 16 at 40°C and that of exo-PG produced by *P*. *viridicatum* RFC3 at 60°C.

Regarding temperature stability, endo-PG was stable at 40°C after 60 min of incubation, but the activity decreased with an increase in temperature, and 64% of its activity was maintained after incubation at 40°C. Exo-PG and PL were stable at 60 to 80°C, maintaining 99 and 128% of their activities after 60 min at 80°C, respectively. PE was stable at 30 to 60°C, but the activity decreased with an increase in temperature. Maciel et al. (2011) showed that endo-PG and PL were stable at 50 and 80°C, respectively, and maintained 60% of their original activities. Silva et al. (2002) showed that at 40°C for 60 min, PG and PL activities were maintained at 100 and 80% of their original activities, and at 50°C, they were maintained at 55 and 60% of their original activities, respectively. Yadav et al. (2008) showed that 98% of stability was maintained at 50°C for PL produced by *A*. *flavus*, and the stability decreased at temperatures above

50°C. Exo-PG produced by *Monascus* sp. and *Aspergillus* sp. was stable at temperatures up to 50°C (Fontana et al., 2005). PL and PE produced by *A. japonicus* showed stability between 40 and 50°C (Semenova et al., 2003). The most important factor among all the physical variables affecting the production of enzymes and metabolites is probably incubation temperature because enzymatic activities are sensitive to temperature (Krishna, 2005). The high stability at a certain temperature suggested that the pectinases (endo-PG, exo-PG, PL and PE) are sufficiently acceptable for commercial application.

Conclusions

The results presented demonstrate the feasibility to produce pectinases using *A*. *niger* URM 4645 and yellow passion fruit peels as substrate in SSF. Using 5.0 g of substrate and an initial moisture content of 30% at 34°C with 96 h of incubation was determined to be the best condition for the pectinases production at the same time. Variable substrate amounts and initial moisture contents showed significant effects on pectinase production. Optimum endo-PG activity was obtained at pH 7.5 at an optimum temperature of 40°C. Optimum exo-PG and PL activities were obtained at pH 7.0 at an optimum temperature of 80°C. Optimum PE activity was obtained at pH 3.5 at an optimum temperature of 30°C. Endo-PG was stable at pH 7.0 to 8.0 at 40°C; exo-PG and PL at pH 6.0-8.0 and 6.0-7.5, respectively, and temperature 60 to 80°C; and PE at pH 3.5 to 5.0 and temperature 30-60°C. Enzyme production optimization clearly demonstrated the impact of process parameters on the yield of pectinolytic enzymes for use in commercial applications.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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