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Full Length Research Paper

Production and partial characterization of pectinases from forage palm by *Aspergillus niger* URM4645

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The activity of endo-polygalacturonase (endo-PG), exo-polygalacturonase (exo-PG), pectin lyase (PL) and pectinesterase (PE) produced by *Aspergillus niger* URM4645 was studied in solid-state fermentation (SSF) using forage palm as the substrate. The effect of variable substrate amount, inoculum concentration and temperature on the pectinase production was studied using a full factorial design (2^3). The maximum activity obtained was 66.19 U/g for endo-PG, 3.590 U/g for exo-PG and 40,615.62 U/g for PL at 96, 24 and 72 h of fermentation, respectively. PE showed no activity. The production of endo-PG and exo-PG was significantly influenced by varying substrate amount, inoculum concentration and temperature, but these variables had no influence on PL production. The best conditions for production of the three enzymes at the same time were obtained with 10.0 g of substrate, 10^7 spores/g at 28°C. Endo-PG and PL presented optimum activity at pH 5.0 and exo-PG at pH 7.0. The maximal activity of endo-PG and PL was determined at 50°C and exo-PG at 40°C. Endo-PG and exo-PG were stable at a pH range of 3.5 - 11.0 and at 50 and 80°C, respectively. PL showed stability only at pH 5.0 and at 50°C.

Key words: *Aspergillus niger*, pectinolytic activities, forage palm, solid-state fermentation.

INTRODUCTION

Currently, the fundamental exploitation of agricultural and food wastes, which participate in pollution, is the controlled biological degradation of the wastes by microorganisms for the production of valuable compounds such as proteins, polysaccharides, oligosaccharides, vitamins, hormones, enzymes and other raw materials for medicinal and industrial uses (El-Sheekh et al., 2009).

Pectinolytic enzymes are classified according to their cleavage of the galacturonan part of the pectin molecule. They can be distinguished between pectinesterases (PE, E.C 3.1.1.11), which modify pectin esters into low methoxyl pectins or pectic acid and pectin deploymerases, which split the glycosidic linkages between galacturonosyl (methyl ester) residues. Polygalacturonases (PGs) split glycosidic linkage next to free carboxyl groups by hydrolysis, while pectate lyases (PALs) split glycosidic linkages next to free carboxyl groups by β-elimination. Both endo types of PGs and PALs (E.C 3.2.1.15 and E.C 4.2.2.2, respectively) are known to randomly split the pectin chain. Exo-polygalacturonases (exo-PGs, E.C 3.2.1.67) release monomers or dimers from the non-reducing end of the chain, whereas exo-pectate lyases (exo-PALs, E.C 4.2.2.9) release unsaturated dimers from the reducing end. Highly methylated

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Abbreviations: Endo-PG, Endo-polygalacturonase; **exo-PG,** exo-polygalacturonase; **PL,** pectin lyase; **PE,** pectinesterase; **SSF,** solid-state fermentation; **PGs,** polygalacturonases; **PALs,** pectate lyases; **exo-PALs,** exo-pectate lyases; **endo-PL,** endo-pectin lyases; **Sa,** substrate amount; **Ic,** inoculum concentration; **T,** temperature; **EDTA,** ethylenediaminetetraacetic acid; **DNS,** 3,5-dinitrosalicylic acid.

Table 1. Variable levels of the 2^3 experimental design for the production of pectinases by *Aspergillus niger* URM4645.

Variables	Levels		
	Low (-1)	Central (0)	High (+1)
Sa (g)	5.0	7.5	10.0
Ic (spores/g)	10^5	10^6	10^7
T (ºC)	28	32	36

pectins are degraded by endo-pectin lyases (endo-PL, E.C 4.2.2.10) and also by a combination of PE with PG or PAL (Sarkanen, 1991; Pilnik and Voragen, 1993).

Pectinases are widely used in the disintegration of plant tissues in the fruit and vegetable processing industries, for increasing the extraction of juice, for decreasing the viscosity of concentrates and for making pectic complexes soluble to complete sedimentation and clarification of juices (Gummadi and Panda, 2003; Crook and Corredig, 2006) and wines (Pérez-Maganino and González-San José, 2001).

The ability to synthesise pectinolytic enzymes is very common in groups of microorganisms, but fungus is preferred on an industrial scale. This is because about 90% of the enzymes produced may be secreted in the culture medium (Blandino et al., 2001). *Aspergillus niger* is the most commonly used fungal species for industrial production of pectinolytic enzymes (Naidu and Panda, 1998; Dinu et al., 2007). PGs, the most abundant and extensively studied of the pectinolytic enzymes, typically exist in multi-gene families and may have both endo (Parenicová et al., 2000) and exo activities (Sakamoto et al., 2002). Pectin lyases (PLs) (Vitali et al., 1998) and PEs (Khanh et al., 1991) were also purified and characterized from some *Aspergillus* strains. Secretion of PALs from *Aspergillus* spp. is rarely reported (Benen et al., 1999).

The forage palm (*Opuntia ficus indica* Mill) is considered an excellent energy source, which is rich in non-fibrous carbohydrates (61.79%) (Wanderley et al., 2002), total digestible nutrients (62%) (Melo et al., 2003a) and pectin (23.3%) (Melo et al., 2003b). This high pectin content gave reason for exploring forage palm as a cheap substrate for the microbial pectinase production.

The goal of this study is to evaluate the production of polygalacturonases (endo-polygalacturonase (endo-PG) and exo-PG), PL and PE by *A. niger* URM4645 in solid-state fermentation (SSF) using forage palm as the substrate and to partially characterize the enzymes.

MATERIALS AND METHODS

Microorganism

A. niger URM4645 strain used in this study was obtained from the Culture Collection, Micoteca URM of the Mycology Department, Federal University of Pernambuco, Brazil. The strain was maintained on malt extract agar (Klich, 2002) and kept at 28°C for 7 days.

Inoculum preparation

To fully sporulated (6 – 8 days old) agar slope culture, 10 ml of sterile distilled water was added. Then the spores were scrapped under aseptic conditions. The spore suspension obtained was used as the inoculum.

Solid-state fermentation

For fermentation, forage palm was used as the substrate with a particle size between 3 - 8 mm. The substrate was autoclaved at 120°C for 15 min in Erlenmeyer flasks of 250 ml capacity containing the substrate without the addition of nutrient solution. The initial moisture content of the medium was approximately 85%. The pH of the substrate solution was adjusted to pH 5.5 with 2 M HCl.

Experimental design

The influence of the substrate amount (Sa), inoculum concentration (Ic) and temperature (T) on the four responses was evaluated from the results of experiments performed according to a 2^3 factorial experimental design plus three central points (Table 1). All statistical analyses were carried out using Statistica 8.0 software (Statistica, 2008).

Extraction of the enzyme

The production of pectinase was followed for 120 h. The contents of the flasks were harvested at regular intervals (24 h) by adding 0.2 M acetate buffer, pH 5.5 (1:2.5 - substrate:buffer), incubated in a temperature controlled bath at 32°C for a period of 1 h and filtered with filter paper (Whatman No. 1) under vacuum. The supernatant was used as a crude enzymatic extract and subjected to enzymatic analysis.

Endo-PG activity

Endo-PG activity was measured viscosimetrically by mixing 5.5 ml of 1% (p/v) citric pectin in 0.025 M acetate buffer (pH 5.0 and 1 mM ethylenediaminetetraacetic acid (EDTA)) with 250 µl of the crude enzymatic extract, according to the method of Tuttobello and Mill (1961). The reaction was incubated at 50°C for 10 min and then cooled in an ice bath. A viscosimetric unit (U) was defined as the enzyme quantity required to decrease the initial viscosity per min by 50% under the conditions previously described. Enzyme activity was given in U/g.

Exo-PG activity

Exo-PG activity was determined through reduction of groups from citrus pectin using the 3,5-dinitrosalicylic acid (DNS) reagent assay (Miller, 1959). The reaction mixture, containing 500 µl of 0.5% citric pectin in 0.025 M acetate buffer (pH 5.0), 1 mM EDTA and 500 µl of the crude enzymatic extract, was incubated at 50°C for 10 min and then 500 µl of DNS solution was added to each tube and boiled for 5 min. After cooling, 5 ml of distilled water was added and agitated; then, the absorbance at 575 nm was measured. The reductant sugar values were determined from a standard curve of monogalacturonic acid. One enzymatic unit (U) was defined by the enzyme quantity which liberates 1 mmol of monogalacturonic acid per min under the described experimental conditions. The enzymatic activity was expressed in U/g.

Table 2. Results of the 2^3 design for the endopolygalacturonase (Endo-PG), exopolygalacturonase (Exo-PG) and pectin lyase (PL) production in solid-state fermentation (SSF) by *A. niger* URM4645.

Runs	Sa[a]	I_C[b]	T[c]	Endo-PG_{96} (U/g)	Exo-PG_{24} (U/g)	Exo-PG_{96} (U/g)	PL_{72} (U/g)	PL_{96} (U/g)
01	5.0	10^5	28	14.08	2.161	2.579	14,814.81	7,682.68
02	10.0	10^5	28	17.23	1.900	2.649	0.00	0.00
03	5.0	10^7	28	29.84	1.778	2.179	9,934.93	10,535.54
04	10.0	10^7	28	66.19	1.621	2.492	25,300.30	35,735.74
05	5.0	10^5	36	17.88	2.632	2.057	27,177.18	16,666.67
06	10.0	10^5	36	39.52	2.213	1.342	14,539.54	9,359.36
07	5.0	10^7	36	19.53	3.590	1.621	40,615.62	19,119.12
08	10.0	10^7	36	57.02	2.335	1.307	11,161.16	28,978.98
09(C)[d]	7.5	10^6	32	25.65	2.144	1.969	19,569.57	13,788.79
10(C)[d]	7.5	10^6	32	35.40	1.882	2.248	12,887.89	18,793.79
11(C)[d]	7.5	10^6	32	36.20	2.283	2.196	10,685.69	12,237.24

For endo-PG, exo-PG and PL, the subscript gives the cultivation time in hours. [a]Sa- Substrate amount (g), [b]I_C- Inoculum concentration (spores/g), [c]T- Temperature (ºC), [d](C)- Central points.

PL activity

PL activity was determined by measuring the increase in absorbance at 235 nm of the substrate solution (1 ml of 0.5% citric pectin in 0.2 M citrate-phosphate buffer, pH 5.5) hydrolysed by 1 ml of the crude enzymatic extract, at 40°C for 1 h. The reaction was halted by adding 3.5 ml HCl 0.5 M. One enzymatic unit (U) was defined as the amount of enzyme which liberates 1 µmol of unsaturated uronide per minute, based on the molar extinction coefficient (ε_{235} = 5550 M^{-1} cm^{-1}) of the unsaturated products (Albershein, 1966; Uenojo and Pastore, 2006). The enzymatic activity was expressed in U/g.

PE activity

This enzyme was evaluated by the pH decrease of the medium and by titration of the carboxylic groups using a modified methodology (Siéssere et al., 1992), wherein 2 ml of 1% citric pectin solution in 0.025 M tris-acetate buffer (pH 6.5) and 1 ml of the crude enzymatic extract were used. The enzymatic reaction was carried out at 50°C for 2 h and then quenched in a boiling water bath for 3 min. Next, the samples were cooled in an ice-bath and titrated with 0.01 M NaOH solution. One enzymatic unit (U) was defined as the quantity of enzyme which liberates 1 microequivalent of carboxylic group in 1 h of reaction under the described conditions. The enzymatic activity was expressed in U/g.

Enzyme characterization

Endo-PG, exo-PG and PL activities from the crude enzymatic extract were measured at different pH values and temperature. PE showed no activity; therefore, it was not characterized.

Optimum pH and temperature for enzyme activity

The effect of pH on enzymes 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 buffer (pH 7.0-8.5) and glycine-NaOH buffer (pH 8.5-11.0). The optimum temperature within the 30 - 80°C range was determined by incubation of the reaction mixture at optimum pH.

pH and temperature stability

Crude enzymatic 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 buffers as above) and maintained at 25°C for 24 h. After incubation, the endo-PG, exo-PG and PL activities were measured for their values of pH and temperature optimum. For the determination of the thermal stability, the crude enzymatic extract was incubated at temperatures from 30 to 70°C for 1 h with endo-PG, exo-PG and PL activity determined.

RESULTS AND DISCUSSION

Production of pectinolytic enzymes

The fungus *A. niger* URM4645, when cultured in the forage palm without any addition of nutrient solution, produced endo-PG, exo-PG and PL, but was not able to produce PE in these conditions. The enzymes produced in SSF were analysed in 120 h, and the experiments were carried out in 11 assays.

The maximum endo-PG activity was 66.19 U/g at 96 h of fermentation, using 10.0 g of substrate with 10^7 spores/g at 28°C. Using 5.0 g of substrate with 10^7 spores/g at 36°C the maximum activity for exo-PG and PL was 3.590 and 40615.62 U/g obtained with 24 and 72 h of fermentation, respectively (Table 2).

Using wheat bran with the substrate, Freitas et al. (2006) working with *Monascus* sp and *Aspergillus* sp obtained 1.6 and 1.9 U/ml for endo-PG activity in 20 and 72 h of SSF, respectively. The value obtained by Fontana et al. (2005) was 152 U/g at 72 h of SSF using wheat bran and the citric pectin substrates. This value is higher

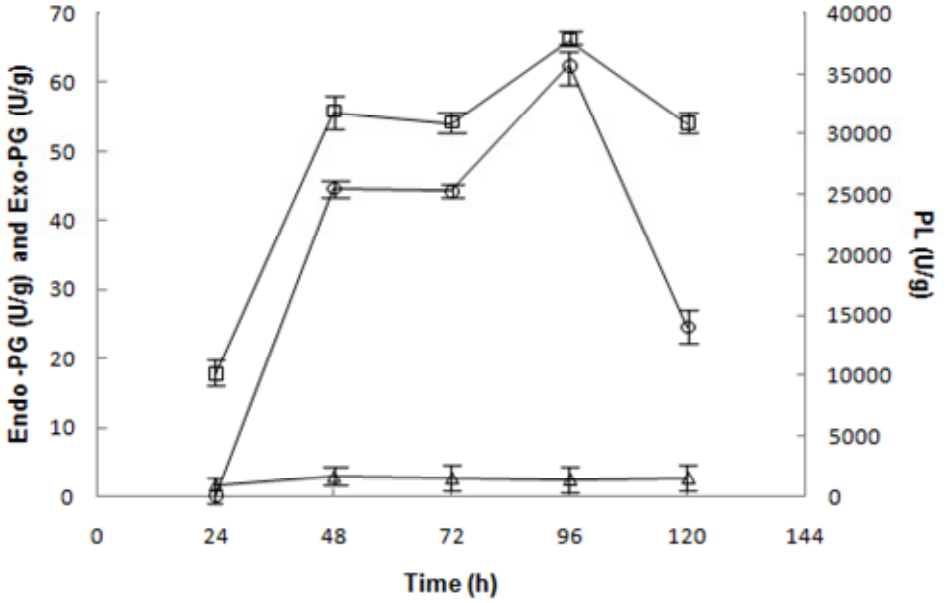

Figure 1. Activity of endopolygalacturonase (□ - endo-PG), exopolygalacturonase (Δ - exo-PG) and pectin lyase (○ - PL) in the best production conditions for the three enzymes (10.0 g of substrate, 10^7 spores/g and 28°C with 96 h of fermentation).

than that of our results, as no not carbon source or pectin was added in an attempt to increase the pectinase production.

The value of the exo-PG activity obtained with forage palm was low compared with the results obtained by Silva et al. (2007) and Patil and Dayanand (2006). They worked with *Penicillium viridicatum* RFC3 (16.0 U/g) and *A. niger* (17.1 U/g), using a mixture of wheat bran and orange bagasse (1:1) and de-seeded sunflower head as substrate in 14 days and 96 h of SSF, respectively.

The PL production was observed in the genus *Moniliella* sp. and *Penicillium* sp. when cultured in medium with substrates containing orange bagasse, sugar cane bagasse and wheat bran (1:1:1). The PL activity was detected in 144 and 96 h of SSF for *Moniliella* sp. (19.40 U/g) and *Penicillium* sp. (11.0 U/g), respectively (Martin et al., 2004). According to the research of Silva et al. (2002), the PL production by *P. viridicatum* using orange bagasse as substrate presented a value of 2.0 U/g. observing the influence of the composition of the medium in the production of the enzyme to the mix of orange bagasse and wheat bran; they brought production to 3.54 U/g of substrate. The amount of PL obtained in this study was high compared to those reported for pectinolytic strains cultivated on solid substrates. However, the comparison of enzyme levels produced by different organisms is not straightforward, since distinct culture conditions and enzyme activity determinations have been used. These results showed that SSF was suitable for pectinases production by *A. niger* URM4645 using forage palm as the substrate.

The results shown in this study for endo-PG, exo-PG and PL had maximum production in different conditions and times of cultivation. As such, it was necessary to choose a common condition for the complex pectinolitic production, since this enzymatic complex is applied in the food industry for the processing of fruit juices. The condition of 10.0 g of substrate, 10^7 spores/g at 28°C with 96 h of fermentation was chosen using statistical analysis, which is a common condition for the production of endo-PG, exo-PG and PL (Figure 1). The results of the statistical analysis with the effects of each variable studied in the experimental design on endo-PG, exo-PG and PL activity, at 96 h of SSF are shown in Table 3.

The temperature was not an important variable at a 95% confidence level in the endo-PG activity. The variable substrate amount and inoculum concentration had a positive effect on the activity of endo-PG during the 96 h of fermentation (Table 3). Thus, the use of 10.0 g of the substrate and 10^7spores/g showed maximum activity of this enzyme. The runs 4 and 8 showed similar behaviour to endo-PG production. This could be explained by the fact that these runs were performed at the same level of the substrate amount and inoculum concentration (Table 2).

The variable temperature showed significant negative effects, which indicated that an increase in exo-PG activity was obtained by reducing the temperature. The substrate amount and inoculum concentration did not significantly influence the production of exo-PG. None of the variables showed significant effect on the PL production (Table 3).

Table 3. Effects calculated from the responses of the 2^3 design for the production of the endopolygalacturonase (Endo-PG), exopolygalacturonase (Exo-PG) and pectin lyase (PL) with 96 h of solid-state fermentation (SSF) by *A. niger* URM4645.

Variables/Interactions	Endo-PG_{96}	Exo-PG_{96}	PL_{96}
Substrate amount (1)	4.72[a]	-0.57	0.89
Inoculum concentration (2)	4.01[a]	-0.92	2.69
Temperature (3)	0.32	-3.18[a]	0.89
1x2	2.35	0.57	2.22
1x3	0.94	-1.26	-0.66
2x3	-2.19	0.08	-0.73
1x2x3	-0.83	0.14	-0.70

[a]Statistically significant values (at the 95% confidence level).

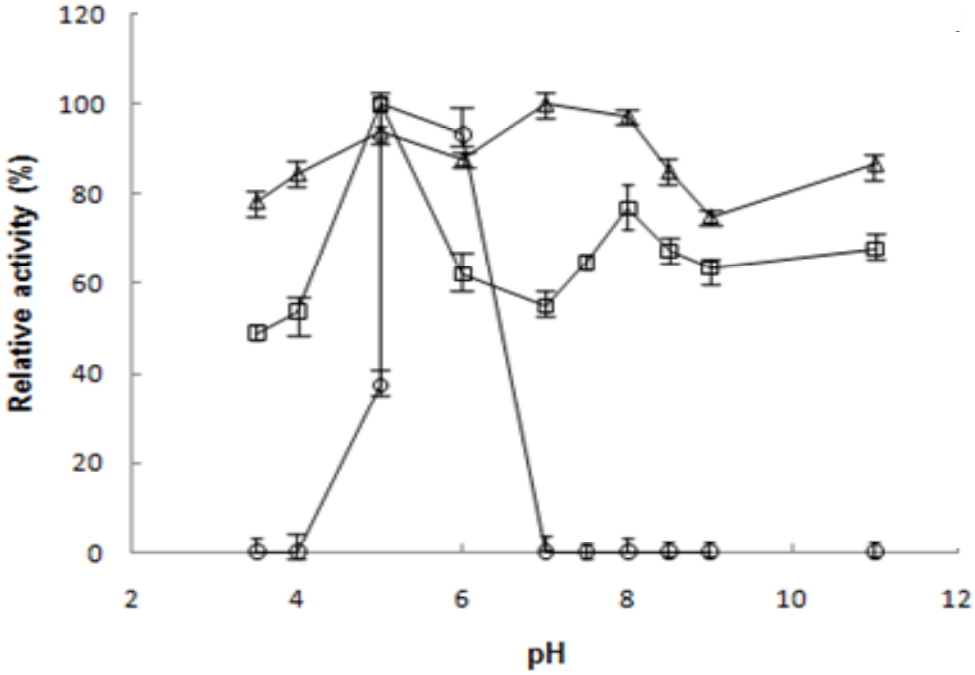

Figure 2. Effect of pH on endopolygalacturonase (□ - endo-PG), exopolygalacturonase (Δ - exo-PG) and pectin lyase (○ - PL) activity from *A. niger* URM4645.

Enzyme characterization

For characterization of endo-PG, exo-PG and PL from *A. niger* URM4645, the crude enzymatic extract was used and it provided the best production of three enzymes (Table 2, Run 04).

The effect of pH on endo-PG, exo-PG and PL activities is shown in Figure 2. The optimum activity for endo-PG and exo-PG was pH 5.0 and 7.0, respectively. For these PGs at pH 8.0, 77 and 97% of the maximum enzyme activity was obtained, respectively. The optimum pH for exo-PG was higher than the majority of fungal PGs described, and they are acidic enzymes (Favela-Torres et al., 2006). *P. viridicatum* RFC3 showed an optimum 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) for maximum activity of exo-PG. Freitas et al. (2006) working with *Monascus* sp. and *Aspergillus* sp. obtained the maximum activity for exo-PG at pH 5.5.

The optimum activity for PL was pH 5.0. At pH 6.0, PL activity decreased by 93% but was inactivated in values of neutral and basic pH. The pH optima of the previously reported PL have been found to be acidic for *Penicillium canescens* (5.5) (Sinitsyna et al., 2007), neutral for *Penicillium expansum* (Silva et al., 1993) and basic for *Aspergillus flavus* (8.0) (Yadav et al., 2008) and *Aspergillus terricola* (8.0) (Yadav et al., 2009). The acid tolerance property of these enzymes is of great advan-tage in the fruit and vegetable processing applications, since most fruit and vegetable tissues and juices have acidic pH

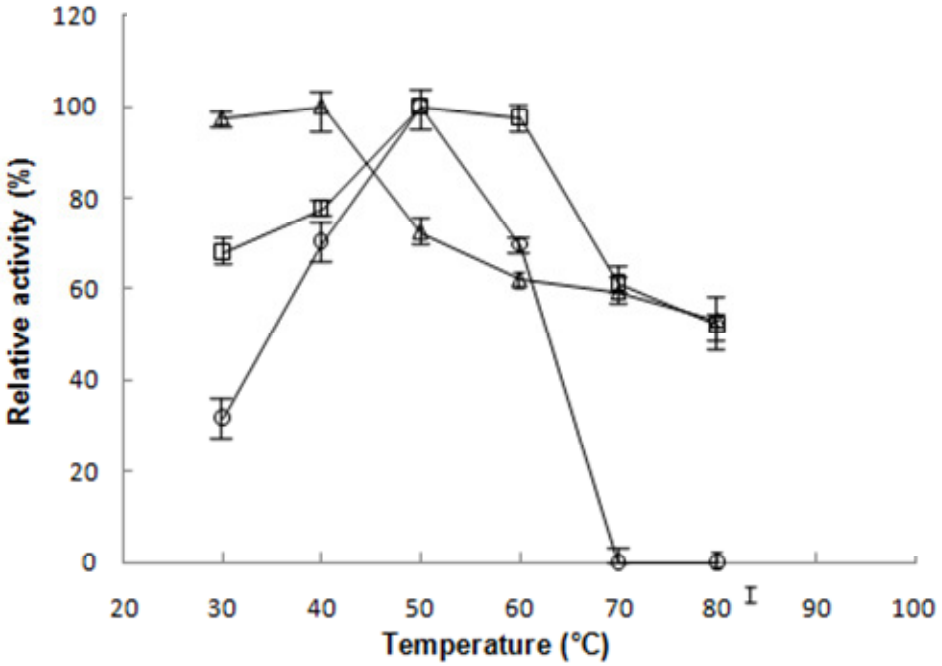

Figure 3. Effect of temperature on the endopolygalacturonase (□ - endo-PG), exopolygalacturonase (Δ - exo-PG) and pectin lyase (○ - PL) activity from *A. niger* URM4645.

(Freitas et al., 2006).

Endo-PG and exo-PG were stable at a pH range of 3.5 - 11.0, while PL was sensitive to pH variation, showing stability only at pH 5.0. After incubation for 1 h at a pH range of 3.5 - 11.0, more than 40% activity was retained for endo-PG and PL and 70% for exo-PG. The results are in accordance with those obtained by Silva et al. (2002), which showed PG from *P. viridicatum* RFC3 to be stable at pH 5.0 - 8.0 and maintaining 80% of its activity at pH 9.0. PL was more sensitive to pH variation, presenting maximum stability at pH 3.5 - 4.5 which declined to 80% at pH 5.0. PL produced by *Moniliella* sp SB9 and *Penicillium* sp EGC5, and was stable in acidic to neutral pH (4.0-7.0) (Martin et al., 2004). However, the results of Yadav et al. (2008, 2009) reported stability of PL in a pH range of 4.0 - 10.0 and 4.0 - 9.0 with *A. flavus* and *A. terricola*, respectively. Freitas et al. (2006) observed 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. Silva et al. (2007) also working with *P. viridicatum* RFC3 reported stability of exo-PG in a pH of range 7.0 - 10.0.

The optimum temperature was 50°C for endo-PG and PL and 40°C for exo-PG (Figure 3). The results are in accordance with those obtained by Phutela et al. (2005), studying pectinase from *Aspergillus fumigatus* TF3, and Yadav et al. (2009), working with PL from *A. terricola* MTCC 7588. These authors obtained maximum enzyme activity at 50°C. Silva et al. (2002) also observed that the maximum activity of PG and PL was set at 55 and 50°C, respectively. Exo-PG of *Monascus* sp and *Aspergillus* sp exhibited maximal activity at 60 and 50°C, respectively (Freitas et al., 2006). The results obtained by Dinu et al. (2007) and Silva et al. (2007) showed an optimum activity at 40°C for PG of *A. niger* MIUG 16 and 60°C for exo-PG of *P. viridicatum* RFC3, respectively.

Endo-PG and PL were stable at 50°C after 1 h but were inactivated at higher temperatures, and exo-PG was stable at 80°C and showed 60% activity remaining after 1 h at this temperature. Silva et al. (2002) showed that at 40°C for 1 h, the activities of PG and PL were 100 and 80% retained, and while at 50°C, PG and PL retained 55 and 60% of their original activity, respectively. Yadav et al. (2008) showed 98% stability at 50°C for PL from *A. flavus* with decrease at a temperature above 50°C. Exo-PG of *Monascus* sp. and *Aspergillus* sp. showed stability at temperatures up to 50°C (Freitas et al., 2006). Juice enzymatic clarification may be carried out at 15°C for 12 h or at 54°C for 1 - 2 h to prevent yeast growth (Dinu et al., 2007). The stability temperature showed by the enzymes (endo-PG, exo-PG and PL) is sufficiently acceptable for application in the food industry for the processing of fruit juices.

Conclusion

The feasibility of producing endo-PG, exo-PG and PL, using *A. niger* URM4645 and forage palm as the substrate in solid-state fermentation, was demonstrated by the results presented in this work. Forage palm is a

cheap and available raw material. The condition of 10.0 g of substrate, 10^7 spores/g at 28°C with 96 h of fermentation was defined as the best condition for the simultaneous production of endo-PG, exo-PG and PL. The variables analysed (substrate amount, inoculum concentration and temperature) showed significant effects on endo-PG, exo-PG and PL production. Endo-PG and PL from *A. niger* URM4645 had optimal activity at pH 5.0 and 50°C, while exo-PG had optimal activity at pH 7.0 and 40°C. Endo-PG and exo-PG were stable in a pH range of 3.5 - 11.0 and at temperatures of 50 and 80°C, respectively. PL showed stability at pH 5.0 and at only 50°C. These properties could lead to their inclusion by the food industry for the processing of fruit juices.

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