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Biotechnological Potential of Alternative Carbon Sources for Production of Pectinases by *Rhizopus microsporus* var. *rhizopodiformis*

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ABSTRACT

Fungi collected from Brazilian soil and decomposing plants were screened for pectinase production. R. microsporus *var.* rhizopodiformis *was the best producer and was selected to evaluate the pectic enzyme production under several nutritional and environmental conditions. The pectinase production was studied at 40ºC, under 28 carbon sources-supplemented medium. The inducer effect of several agro-industrial residues such as sugar cane bagasse, wheat flour and corncob on polygalacturonase (PG) activity was 4-, 3- and 2-fold higher than the control (pectin). In glucose-medium, a constitutive pectin lyase (PL) activity was detected. The results demonstrated that* R. microsporus *produced high levels of PG (57.7 U/mg) and PL (88.6 U/mg) in lemon peel-medium. PG had optimum temperature at 65 ºC and was totally stable at 55 ºC for 90 min. Half-life at 70 ºC was 68 min. These results suggested that the versatility of waste carbon sources utilization by* R. microsporus*, produce pectic enzymes, which could be useful to reduce production costs and environmental impacts related to the waste disposal.*

Key words: agro industrial residues, lemon peel, pectin lyase, polygalacturonase, *R. microsporus* var. *rhizopodiformis*

INTRODUCTION

Celluloses, hemicelluloses and pectic substances are the most abundant carbohydrates present in the plant cell walls. Pectic substances such as pectin, protopectin and pectic acids are present in the middle lamella and contribute to firmness and structure to the plant tissues. In the pectic substances, D-galacturonic acid units are linked by α-1,4 – glycosidic linkages and the carbonyl side groups are 60 – 90% esterified with methanol (Gummadi and Panda, 2003).

Pectins are naturally degraded by the pectinolytic enzymes, which have been classified on the basis of their mechanism of attack on the galacturonan backbone. Pectin degrading enzymes can be divided into esterases and depolymerases (Rexová-Benková and Markovic, 1976). The first group is represented by pectin esterase (PE) and the second by polygalacturonase (PG) and pectin lyase (PL).

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PG (EC 3.2.1.15) is a depolymerizing pectinase that catalyzes the hydrolysis of α-(1,4) glycosidic bounds between the non-esterified galacturonic acid units (Mohamed et al., 2006). Pectate lyase (EC 4.2.2.2) and PL (EC 4.2.10.10) cleave α-1,4-glycosidic linkages by trans-elimination, which results in galacturonide with an unsaturated bond between C4 and C5 at the non-reducing end of the galacturonic acid formed.

Pectinases can be used in several industrial processes as fruit juices and wine production, tea and coffee fermentation or olive oil recovery, vegetable oil extraction, functional foods, improvement of cassava starch extraction, textile industry, paper and cellulose industry and animal feeds (Gummadi and Panda, 2003). Pectinases have also been used in the paper and pulp industry in addition to cellulases (Reid and Ricard, 2000), due to its presence in the plant host microorganisms (Hershonhorn et al., 1990; Lang and and Dornenburg, 2000).

Brazil has a prominent role in the production and exportation of coffee, sugar cane, soybean, fruit and other products. However, industrial processing of these items results in the generation of huge amount of other materials. Some applications suggested for these residues are their utilization as substrates for microbial fermentation bioprocesses, to obtain valuable products, such as enzymes, alcohol, proteins, organic acids, amino acids, bioactive and flavour compounds (Medeiros et al., 2000).

The Tahiti lemon is a fruit of tropical origin, with recent economic exploration. In the world context, the main producers are Mexico, USA, Egypt, India, Turkey and Brazil. The “albedo", which corresponds the spongy portion, white and adherent to the peel, is rich in hemicellulose, cellulose, pectic substances, soluble carbohydrate, lignin and phenolic compounds (Ros et al., 1996). The aim of this work was to select the fungi from the soil or decomposing plants, with the components for good production of pectinases and to evaluate the effects of several carbon sources and environmental conditions on fungal pectinase production by the selected strains.

MATERIALS AND METHODS

Organisms and maintenance

Ten fungi were isolated from the soil and plants waste from different areas of São Paulo and Paraná states, Brazil, as previously cited by Guimarães et al. (2006) and these were cultivated on PDA medium at 40ºC. The investigated areas were located at S 21º09.190’ – 45º52.150’W, in the municipality of Ribeirão Preto, SP (*Aspergillus phoenicis, A. ochraceus, Mucor rouxii, Humicola grisea var. thermoidea* and *Scytalidium thermophilum*); S 20º25.896’ – 51º20.681’W, in the municipality of Ilha Solteira, SP (*A. caespitosus* and *P. variotii*); S 21º55.626’ – 47º28.109’W, in the municipality of Pirassununga, SP (*Rhizopus microsporus var. rhizopodiformis*); S 24º57’21’’ – 53º27’19’’W in the municipality of Cascavel, PR (*Penicillium herquei*). The fungi were inoculated in Czapeck medium at 40°C with agitation (100 rpm) for three days to screen pectinases production. *Rhizopus microsporus var. rhizopodiformis* was selected as the best pectinase producer among the isolated fungi. The fungus was identified by André Tosello Foundation, Campinas, SP, (OS 990300) according to Schipper and Stalpers (1984).

Growth conditions

For the production of PG the submerged fermentation (SmF) of approximately $3x10^5$ spores/ml from 15-day-old cultures were inoculated into 125 ml Erlenmeyer flasks containing 25 ml of Adams medium (Adams, 1990) with 1.0% citric pectin (w/v) or other carbon sources, (monogalacturonic acid, polygalacturonic acid, corncob, orange peel, lemon peel, oatmeal, apple peel, sugar cane bagasse, passion fruit peel, gum guar, sucrose, cassava flour, penetrose, raffinose, wheat bran, galactose, rice straw, trehalose, arabinose, glucose and milled corn). The cultures were incubated at 40°C with agitation (100 rpm) for five days. Other liquid media compositions were also tested which included Khanna (Khanna et al., 1995), SR (Rizzatti et al., 2001), Czapeck (Wiseman, 1975) and Vogel (Vogel, 1964).

Preparation of crude enzyme

The culture filtrates were obtained by filtration through Whatman No. 1 paper in a Buchner funnel. The filtrate was saved as a source of crude extracellular PG. The mycelial mass was ground with glass beads at 4ºC.

Enzymatic assays and protein determination

PG activity assay was carried out according to Miller (Miller, 1959). This enzyme was assayed at

65ºC (temperature optimum – see results), in a reaction mixture containing 0.25 ml of enzyme and 0.25 ml of 1.0% sodium polypectate in 100mM sodium acetate buffer, pH 3.5 (pH optimum – see results). One unit was defined as the amount of enzyme that released one μmol of reducing sugar per minute under the assay conditions. PL activity was assayed according to Pitt (1988). One unit was defined as the amount of enzyme that changed the absorbance of 0.01 at 550 nm under the assay conditions. Pectate lyase activity was assayed according to Collmer and Ried, (1988). One unit was defined as the amount of enzyme that released one μmol of unsaturated digalacturonate per minute under the assay conditions. The growth was estimated as protein of the mycelial extract (total mg), according to Lowry et al. (1951) using bovine serum albumin as standard.

Effects of temperature and pH

The crude extract obtained from the lemon peel as carbon source was used to evaluate the PG activity as a function of pH in McIlvaine buffer (pH 3.0-8.0) at 65ºC with 1% sodium polypectate (NaPP) as substrate. The effect of temperature on PG activity was analysed in McIlvaine buffer incubated between 30 and 80ºC at the optimum pH. The thermal stability was investigated by measuring the residual activity of the enzyme after 90 min of incubation at 30-80ºC.

Reproducibility of the results

All data are the mean of at least three independent experiments showing consistent results.

RESULTS

Fungi are interesting enzyme sources for biotechnological application. Among the various filamentous fungi collected (maintained at 40°C in the plates with 4% solid oatmeal medium) (Fig. 1), *Rhizopus microsporus* var. *rhizopodiformis* produced the highest PG levels. There was no report in the literature about this thermotolerant strain as a PG producer. This fungus is a thermotolerant fungus, identified and deposited in the André Tosello Foundation (Brazil). The fungus was cultivated in five different media for pectinase production under agitation (100 rpm) for five days at 40ºC. The enzymes were assayed in the culture filtrate. The best enzymatic yields were obtained in Adams medium (Fig. 2).

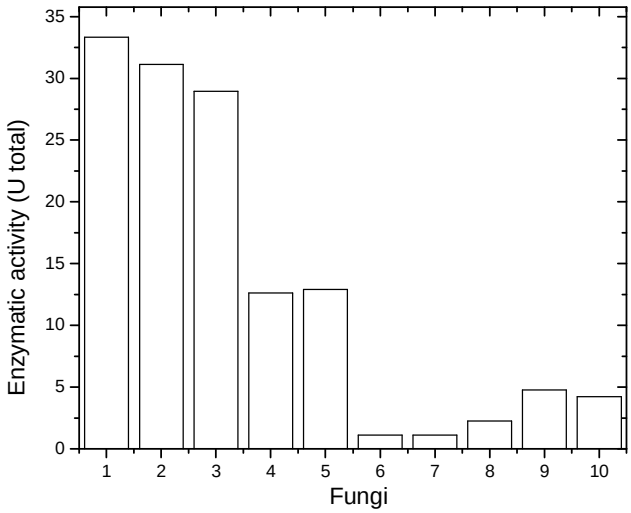

Figure 1 - Pectinases screening. 1) *Rhizopus microsporus var. rhizopodiformis*; 2) *Penicillium herquei*; 3) *Aspergillus caespitosus*; 4) *Aspergillus phoenicis*; 5) *Aspergillus ochraceus*; 6) *Paecilomyces variotii*; 7) *Humicola insolens*; 8) *Humicola grisea var. thermoidea*; 9) *Mucor rouxii*; 10) *Scytalidium thermophilum*. The fungi were inoculated in Czapeck medium, at 40°C, with agitation (100 rpm) for 3 days.

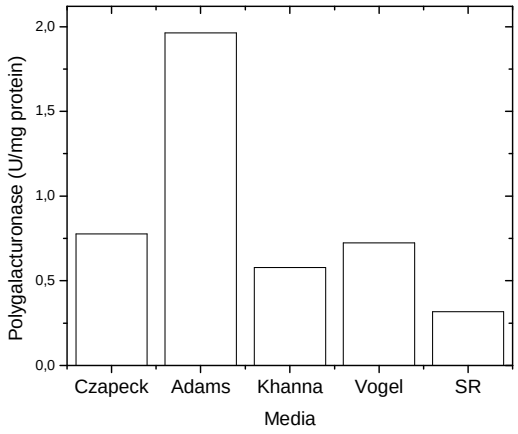

Figure 2 - Production polygalacturonase by *R. microsporus var. rhizopodiformis* in different culture media.

Time-course of pectinases production

The fungus was inoculated in Adams medium supplemented with 1% (w/v) Sigma® citric pectin as carbon source. The maximun PG production was after 72 h (Fig. 3A), (also the highest amount of micelial mass) (Fig. 3B). A decrease in enzymatic synthesis occurred after 72 h, probably due to the inhibition of the enzyme by the end-products. The highest PL production was detected after 48 h of culture. Pectate lyase levels were only detected after 120 h.

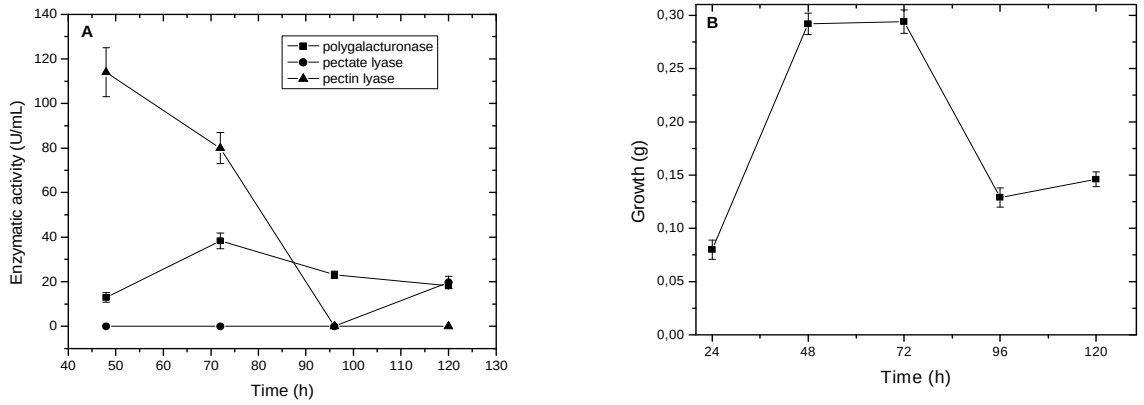

Figure 3 - Time course of pectinases production (A) and growth of the microorganism (B) in cultures under agitation (100 rpm).

Effect of carbon sources on pectinases production

Polygalacturonase

Table 1 shows the influence of carbon sources on the *R. microsporus* var. *rhizopodiformis* PG production, cultivated for 72 h at 40ºC under agitation (100 rpm) in Adams medium. The most effective inducer carbon source was lemon peel, increasing up to 5-fold the PG production compared with 1% Sigma® pectin (control). Under glucose addition, some constitutive PG synthesis was detected, with low activity levels, close to those observed in the absence of additional carbon sources. For the pectins with different methylation degrees, PG production was higher than the control and the extent of enzyme production decreased with an increase in the degree of esterification.

Pectin lyase

Several carbon sources were tested for PL induction (Table 1). The highest activity was

found for polygalacturonic acid and lemon peel-supplemented cultures. In the presence of glucose, a constitutive PL activity was observed. In the absence of a carbon source no enzymatic activity was detected.

Effect of temperature and pH on polygalacturonase activity

The optimum temperature and pH for PG activity were 65ºC and 3.5, respectively (Figs. 4A and C). The thermostability assay (Fig. 4B) was carried out with the extract incubated at 55, 60, 65 and 70ºC, for 90 min under the absence of the substrate and after that the enzymatic assays were carried out. The enzymatic activity decreased about 28 and 39% at 60 and 65ºC, respectively**.** At 70ºC it was possible to calculate the t_{50} (68 min.), which showed a reduction of 50% in the PG activity.

Table 1 - Effect of different carbon sources on the production of polygalacturonase and pectin lyase by *R. microsporus* var. *rhizopodiformis.*

Carbon sources (1%)	Polygalacturonase (U/mg)	Pectin Lyase (U/mg)
None	0.001	0.001
Lemon peel	57.7	88.57
Monogalacturonic acid	40.9	32.89
Polygalacturonic acid	37.0	100.9
Pectin citrus (GM 9%)	30.3	50.75
Pectin citrus (GM 30%)	23.3	43.30
Pectin citrus (GM 70%)	19.1	79.90
Corncob	18.5	18.13
Pectin (Sigma)	11.2	60.32
Orange peel	12.5	0.001
Oatmeal	9.54	11.46
Apple peel	8.04	0,001
Sugar cane bagasse	6.95	35.08
Passion fruit peel	4.85	0.001
Gum guar	4.45	0.001
Sucrose	3.93	0.001
Cassava flour	3.27	24.62
Penetrose	3.18	47.89
Raffinose	3.17	0,001
Wheat bran	2.71	12.18
Galactose	2.50	0.001
Rice straw	2.44	26.01
Trehalose	2.34	34.06
Arabinose	2.31	35.76
Glucose	1.78	62.72
Milled corn	1.75	13.11

[a]None: Adams medium without sugar or agro industrial residues addition..

DISCUSSION

This study evaluated the environmental and nutritional influence on pectinase production by *R. microsporus* var. *rhizopodiformis*, cultivated in submerged culture. Adams medium showed the best composition for the fungus growth and enzyme production. Other media also supported significant enzymatic yield. For example, potato-dextrose liquid medium plus 0.1% citric pectin for *Trichoderma harzianum* (Mohamed *et al.*, 2006), or Kebede medium for *Kluyveromyces wickerhamii* (Moyo et al., 2003).

Pectinolytic enzyme production has been reported as carbon source dependents (Gummadi and Panda, 2003; Blandino *et al.*, 2001), as well as pH (Malvessi and Silveira, 2004), and temperature, culture time, aeration, and other parameters (Souza et al., 2003). For the growth time-course and pectinolytic enzyme production, *R. microsporus*

var. *rhizopodiformis* showed a good adaptation in Adams medium. The logarithmic phase was well-defined; it started around 24 h and finished at around 48 h. *Talaromyces flavus* pectic enzyme production was also maximum in 48 h (Crotti et al., 1999) in Manachini medium (Manachini et al., 1988) supplemented with 0.8% (w/v) pectin at 30ºC under constant agitation.

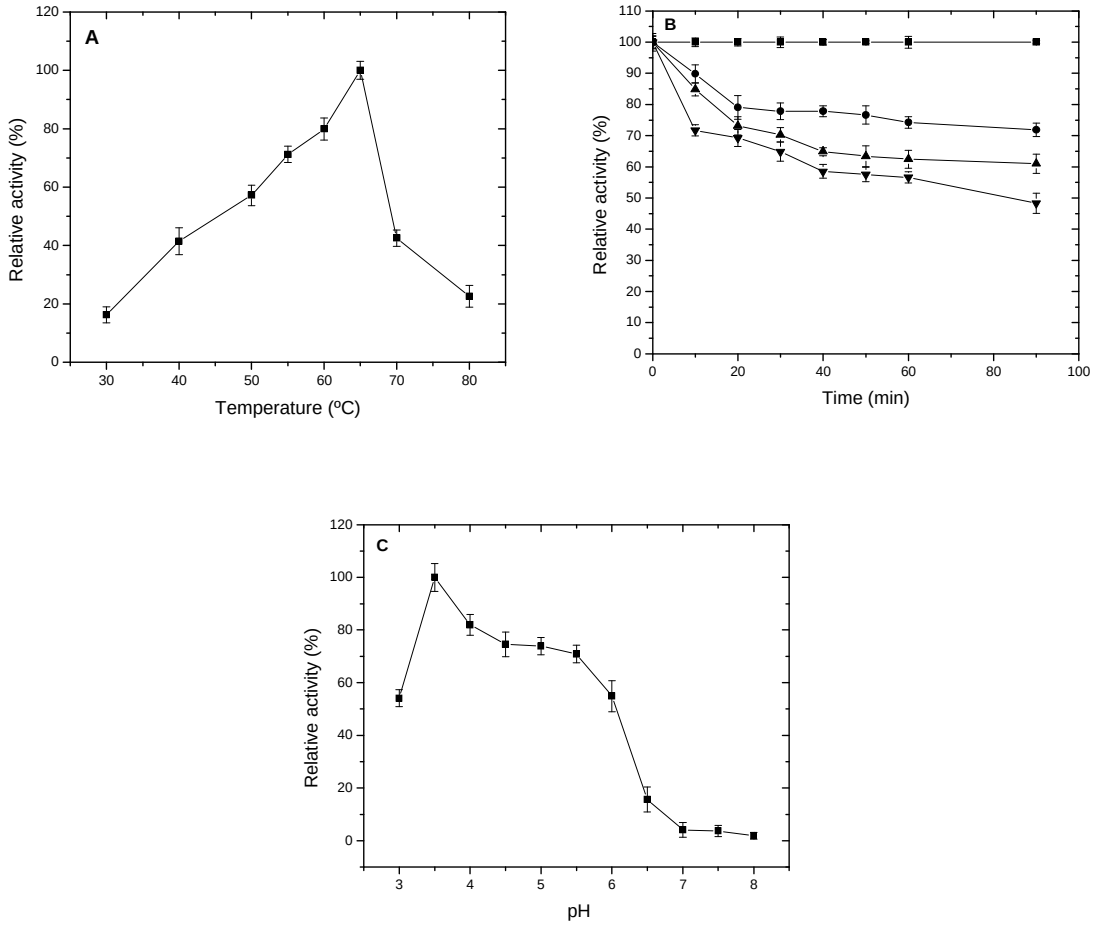

Figure 4 - Influence of temperature (A and B) and pH (C) on polygalacturonase activity. Determination of optimum temperature (A) was carried out at pH 3.5. Thermal inactivation (B) was carried out at 55ºC (■), 60°C (●), 65°C (▲) or at 70°C (▼). The assay of determination of optimum pH (C) was carried out at 65ºC.The polygalacturonase activity was estimated by the DNS method using sodium pectate as substrate.

The agro industrial residues used for the enzyme production represent an economic alternative for the carbon sources and also contribute to decrease the pollution concern about waste accumulation in the environment, therefore they have been frequently studied (Martin et al., 2004). Lemon peel was an interesting carbon source for PG production. Ros et al. (1996) worked with different variety of pectic substances from the lemon "albedo" cell walls, where alcohol insoluble solids were extracted to obtain a chelating agent soluble pectin fraction (ChSS), a diluted sodium hydroxide soluble pectin fraction (ASS) which was separated into a water soluble and water insoluble part (ASSws and ASSwi), and an insoluble residue (Residue). ChSS, ASSws, ASSwi and residue represented 64.3, 10.4, 0.4 and 8.1% of the galacturonic acid residues present in the lemon "albedo", respectively.

A marked feature of the lemon "albedo" is the exceptionally high level of pectin throughout the cell walls from early on fruit development (Bain, 1958). They found that over 25% of the CWM (Cell Wall Material) of the immature lemon "albedo" consisted of galacturonic acid, the main constituent of pectin. Although the yield of CWM decreased during the maturation this level of galacturonic acid is maintained until the fruit is ripe. The non-cellulosic monosaccharide composition of cell wall material isolated from lemon "albedo" was 3.4 mol % rhamnose, 1.1 mol % fucose, 29.2 mol % arabinose, 5.2 mol % xilose, 2.1 mol % mannose, 16.5 mol % galactose, 4.1 mol % (glucose) and 38.5 mol % galacturonic acid (Ros et al., 1988).

R. microsporus var. *rhizopodiformis* produced PG, which exhibited an optimum temperature (65ºC) suitable for future biotechnological applications. Mohamed et al. (2006), reported a *T. harzianum* PG with optimum temperature at 40ºC, which retained 30% of its activity at 60ºC. The optimum temperature for PG from *Bacillus sp.* (Kobayashi et al., 2001) and *T. reesei* (Mohamed et al., 2003) were around 40 and 50ºC, respectively. Channe and Shewal (1995) studied a PG from *Sclerotium rolfsii* with optimum temperature at 55ºC. The enzymatic activity decreased 28, 39 and 51% after incubation for 90 min at 60, 65 and 70ºC, respectively. The thermal inactivation of the *T. harzianum* (Mohamed et al., 2006) PG was investigated by incubating the enzyme for 15, 30 and 60 min, at 10 to 100ºC in the absence of substrate. The PG from T. *reesei* was stable at 40 and 50ºC (Mohamed et al., 2003).

The present work showed that agro industrial wastes, especially, lemon peel, improved the PG and PL production by the thermotolerant fungus *R. microsporus* var. *rhizopodiformis*. The agro industrial residues used for the enzyme production represent alternative carbon sources, and also contribute to decrease the pollution concern about waste accumulation in the environment. These alternative carbon sources might reduce the enzyme production costs too.

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