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ORIGINAL ARTICLE

Production and characterization of polygalacturonase from thermophilic *Thermoascus aurantiacus* on submerged fermentation

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Abstract Microbial pectinase production by submerged fermentation (SMF) has been conducted mainly using mesophilic microorganisms. However, the use of thermophilic fungal strains capable of producing thermostable and thermophilic enzymes offers specific alternatives to biotechnology processes that use enzymes with such characteristics. Pectinases produced by thermophilic fungi may exhibit interesting characteristics, such as high thermostability, in terms of industrial application. Polygalacturonase (PG) production by the thermophilic fungus *Thermoascus aurantiacus* on SMF was carried out in culture media containing commercial pectin or agro-industrial by-products as carbon source under different fermentation conditions. The highest enzyme production occurred when the liquid waste from orange juice processing was used as substrate, after 5 days of cultivation. PG activity was highest when the fungus was cultivated at 45°C with an initial pH of 5.5. PG showed an optimum temperature of 60°C and optimum pH of 5.0. The enzyme showed greater stability in acid pH (3.0–4.5) and remained stable when incubated at 55°C for 1 h.

Keywords *Thermoascus aurantiacus* · Polygalacturonase · Submerged fermentation

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Introduction

Pectinases are a group of enzymes that catalyze the degradation of pectic substances present in plant material (Kashyap et al. 2001). The classification of these enzymes is based on their mode of attacking the chain of polygalacturonic acid of pectic polymers, and can be divided into two broad groups: de-polymerizing enzymes that break α-1,4 linkages in the principal pectin chain, such as polygalacturonase (PG; poly-[1,4-α-D-galacturonide] glycanohydrolase, E.C.3.2.1.15), pectin lyase (poly-[1,4-α-D-methoxygalacturonide] lyase, E.C.4.2.2.10) and pectate lyase (poly-[1,4-α-D-galacturonide] lyase, E.C.4.2.2.2); and de-methoxylating enzymes such as pectinesterase (pectin pectylhydrolase, E.C.3.1.1.11), that de-esterify pectin from pectic acid by removing methoxyl residues (Rombouts and Pilnik 1980; Malvessi and da Silveira 2004).

Since the 1960s, with further elucidation of the chemical nature of plant tissues, pectinases have been used in several technological processes (Kashyap et al. 2001). Moreover, the evaluation of fermentative parameters and knowledge of their properties can improve production and increase efficiency of these enzymes (Gummadi and Panda 2003; Martos et al. 2009).

Commercial pectinase preparations most commonly used are of fungal origin—mainly *Aspergillus* and *Penicillium* (Pandey et al. 2000). However, the search for new producer

microorganisms is crucial in order to obtain novel enzymes with characteristics suitable for specific materials or industrial processes (Sathish-Kumar and Palanivelu 1998).

Enzyme production by microorganisms is affected by the fermentative process (submerged or solid state), fermentation time, medium composition and physico-chemical factors such pH and temperature (Aguilar et al. 1991; Malvessi and da Silveira 2004; Gewali et al. 2007). The selection of substrate for submerged fermentation (SMF) depends on several factors, related mainly to cost, availability and efficiency. In this context, the use of agro-industrial by-products becomes attractive for this purpose (Martins et al. 2002; Pandey et al. 2000; Silva et al. 2005).

Literature data about pectinase production, biochemical characterization of enzymes, new strains and genetic modification usually involve mesophilic fungi. However, some data on pectinases from thermophilic fungi have been presented (Martins et al. 2002, 2007, 2010; Puthela et al. 2005).

The objectives of this work were to evaluate PG production by the thermophilic fungus *Thermoascus aurantiacus* in SMF, using commercial pectin and agro-industrial by-products as carbon source, and investigation of the influence of pH and temperature on enzymatic production. Furthermore, the effect of pH and temperature on the activities of the PGs produced was assessed.

Materials and methods

Microorganism

The *Thermoascus aurantiacus* CBMAI756 strain used in this study was isolated from decaying hemicellulosic material collected in the state of Amazonas-Brazil, and was maintained on Sabouraud slopes as a stock culture, at 7°C. The strain is deposited with the Coleção Brasileira de Microrganismos de Indústria e Meio Ambiente-CBMAI, Unicamp, Campinas-SP (Martins et al. 2007).

SMF and enzyme production

Culture media Czapeck (Wiseman 1975), Khanna (Khanna et al. 1995), SR (Rizzatti et al. 2001) and Vogel (Vogel 1964) containing commercial citrus pectin (Braspectina) were tested. After selecting a basal culture medium (with commercial pectin such as carbon source), culture media containing agro-industrial by-products (2%) were used to replace commercial pectin: orange peel, passion fruit peel and wheat bran, purchased in local shops, and the waste from processing of orange juice, called "yellow water". In the first experiment, all media had an initial pH adjusted (with 0.5 M NaOH) to 5.5.

A volume of 25 ml medium was inoculated with 2.0 ml mycelial suspension (approximately 0.6 mg dry mycelium/ml medium).

Cultivation was carried out for 8 days under agitation at 110 rpm, and a sample was collected from each treatment every 24 h, corresponding to an Erlenmeyer flask. The removed material was vacuum filtered and centrifuged at 10,000 *g* for 10 min at 10°C and the supernatant was used as crude enzyme solution.

In each sample taken, enzyme activity, microbial biomass (expressed in dry weight), reducing sugar content of the medium and pH culture medium during the fermentation process were determined.

Influence of initial pH of culture medium and fermentation temperature

After selecting the most suitable culture medium for the production of PG by the fungus *T. aurantiacus* in SMF, the influence of initial pH values and fermentation temperature on the enzyme production was studied. The pH values of 4.5, 5.0, 5.5 and 6.0 and the incubation temperatures of 45°C and 50°C were evaluated, according to an experimental design of 4×2. The media pH was adjusted with 0.5 M NaOH.

Enzyme activity measurements

PG activity was determined in 0.4 ml acetate buffer 0.2 M NaOH pH 5.0, containing 1% low esterification pectin (26 DE-Sigma, St. Louis, MO) and 0.1 ml crude enzyme solution. After incubation of the mixture at 60°C for 10 min, the reducing sugar released (D-galacturonic acid), was quantified by the DNS method (Miller 1959). One unit of PG activity was defined as the quantity of enzyme that liberated 1 μmol reducing sugar, measured as galacturonic acid/ml crude enzyme solution/min.

Enzyme characterization

Optimum pH and temperature for enzyme activity

The optimum pH was determined by measuring activity at 55°C, using as buffers sodium acetate (pH 3.0–5.5), citrate-phosphate (pH 6.0–7.0), Tris-HCl (pH 7.5–8.5) and glycine-NaOH (pH 9.0–11.0). The optimum temperature was assayed by incubating each reaction mixture at 40–85°C for 10 min at the pH determined as optimal.

Thermostability

The enzyme solutions were kept for 1 h in the absence of substrate at temperatures ranging from 10°C to 90°C at pH 5.5. An aliquot was withdrawn and placed on ice before

assaying for residual enzyme activity at the optimum pH and temperature.

pH stability

Crude enzyme was dispersed (1:1) in 0.1 M buffer solutions pH 3.0–5.5 (sodium acetate), pH 6.0–7.0 (citrate-phosphate), pH 7.5–8.5 (Tris-HCl) and pH 9.0–11.0 (glycine-NaOH) and maintained at 25°C for 24 h. An aliquot was used to determine the remaining activity at the optimum pH and temperature.

The stability of the enzyme was expressed as residual activity (percentage of original activity obtained at optimum pH and temperature).

Results and discussion

PG production on SMF using commercial pectin as carbon source

The first experiments were carried out using commercial pectin as a substrate. The culture media used (Czapeck, Khanna, SR and Vogel) have different compositions of the mineral nutrients commonly used in culture media for the production of microbial pectinases (Bailey 2002; Damásio et al. 2011).

In Czapeck medium, the peak of activity occurred after the 3rd day of fermentation (0.5 U/ml) (Fig. 1a). This culture medium yielded the lowest PG activity by the fungus *T. aurantiacus* among the media studied. In Khanna medium, the maximum activity occurred after the 4th day of fermentation (1.3 U/ml) (Fig. 1b). In these substrates, the final values of reducing sugar content and pH of the fermentation were similar (5.0 mg/ml and 4.0, respectively) and the highest enzyme activity was observed near the end of log phase, which occurred after 3 or 4 days of fermentation (Fig. 1a,b).

The highest enzyme activity was obtained in SR (2.0 U/ml) (Fig. 1c) and Vogel (1.9 U/ml) media (Fig. 1d). The highest enzyme activity was also observed in the final stage of log phase, which occurred after 3 or 4 days of fermentation (Fig 1c,d).

The amount of reducing sugars present initially in the commercial media containing pectin as carbon source ranged from 0.8 to 1.7 mg/ml, and at the end of the fermentation their amount was higher. This result suggests that substrate polysaccharides were hydrolyzed by the enzyme, increasing the concentration of reducing sugars in the medium. It was not possible to indicate if *T. aurantiacus* was able to utilize the residues produced by its PG activity, because no pectin derivatives assimilation test was done.

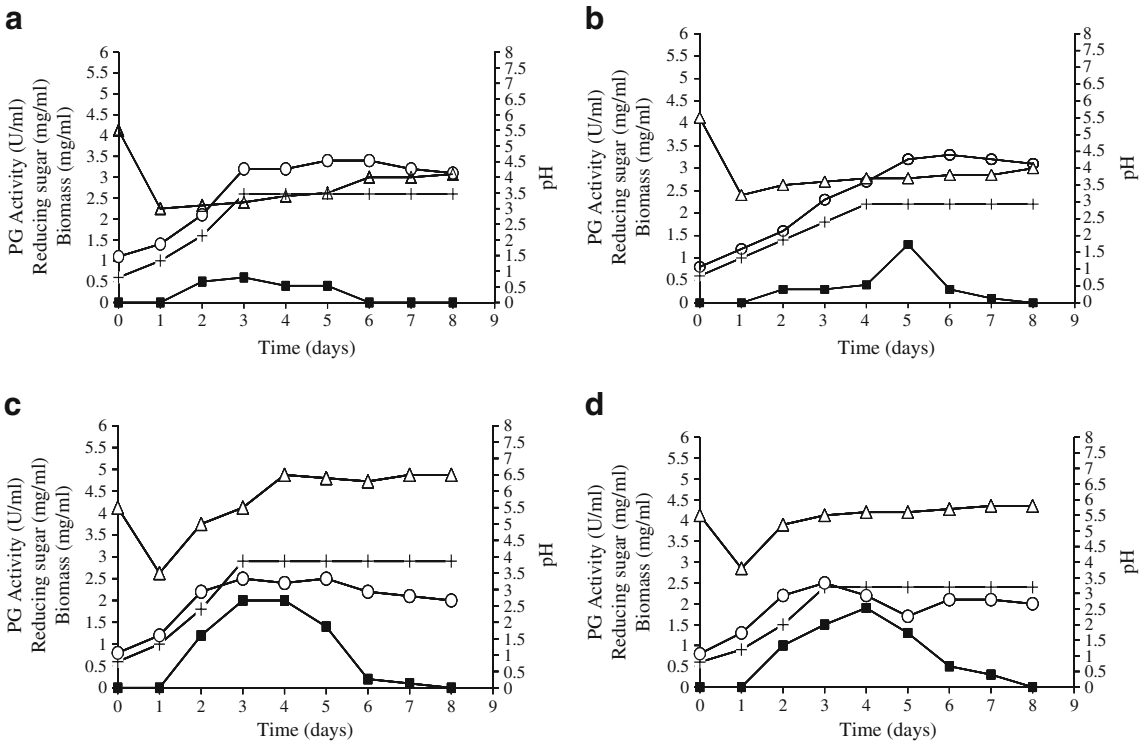

Fig. 1a–d Polygalaturonase (PG) production by *Thermoascus aurantiacus* on submerged fermentation (SMF) in various culture media containing commercial citrus pectin. **a** Czapek, **b** Khanna, **c** SR; **d** Vogel. ■ PG activity; ○ reducing sugar, Δ pH of culture medium + fungal biomass

With respect to pH variation, there was a decrease in pH after the 1st day of fermentation, decreasing to 3.0, 3.2, 3.5 and 3.8 in the Czapek (Fig. 1a), Khanna (Fig. 1b), SR (Fig. 1c) and Vogel medium (Fig. 1d), respectively. At the end of fermentation, pH values differed depending on the medium used, and were around 4.0 at the end of the process in the Czapek (Fig. 1a) and Khanna (Fig. 1b), between 6.5 in SR (Fig. 1c) and 5.8 in Vogel medium (Fig 1d). There was no correlation between pH of the culture medium and enzyme activity, since the peak of activity occurred at different pH values depending on the medium used. In Czapeck and Khanna medium, greater activity occurred at pH values between 3.0 and 4.0 (Fig. 1a,b). In SR and Vogel medium, the peak of enzyme activity occurred at higher pH values, between 5.5 and 6.5 (Fig. 1c, d).

PG production on SMF using agro-industrial by-products as carbon source

In the medium with orange peel, there were two peaks of production: the first occurred after the 3rd day of fermentation, and the second at the end of the fermentation process, with a similar activity (2.1 U/ml) (Fig. 2a). On this substrate, the reducing sugar content was higher than that found in cultures that used passion fruit peel or wheat bran as substrate (Fig. 2b, c). But a correlation between the initial amount of reducing sugar and enzyme activity cannot be established, since activity on orange peel (Fig. 2a) was similar to that found on wheat bran, in which the amount of initial sugar is small (Fig. 2c).

Similar results were observed when passion fruit peel was used as carbon source. On this substrate, the first peak occurred at the 3rd day of fermentation (2.0 U/ml) and the second was observed after the 8th day, with maximum activity (3.0 U/ml) (Fig. 2b). In this medium, the initial content of reducing sugar was about 1.3 mg/ml. The data indicate that passion fruit peel could be a suitable substrate for PG production by the fungus *T. aurantiacus*. As this material is rich in pectin, it may have induced expression of the enzyme.

In medium with wheat bran, there were two peaks of production. However, these occurred after the 3rd day (2.0 U/ml) and the other after the 7th (1.8 U/ml). On the 8th day, the activity decreased (1.6 U/ml) (Fig. 2c).

The enzyme activity and growth of the fungus were higher when we used the yellow water as substrate. In this case, the maximum activity was 3.2 U/ml after the 5th day of fermentation. The initial content of reducing sugar in this substrate was high (4.5 mg/ml), with reduction at the end of fermentation (about 0.6 mg/ml). However, the data does not allow a direct correlation between reducing sugar content and enzyme activity, because substrates such as passion fruit peel, for example, showed a low content of reducing sugar and high enzyme activity (Fig. 2d).

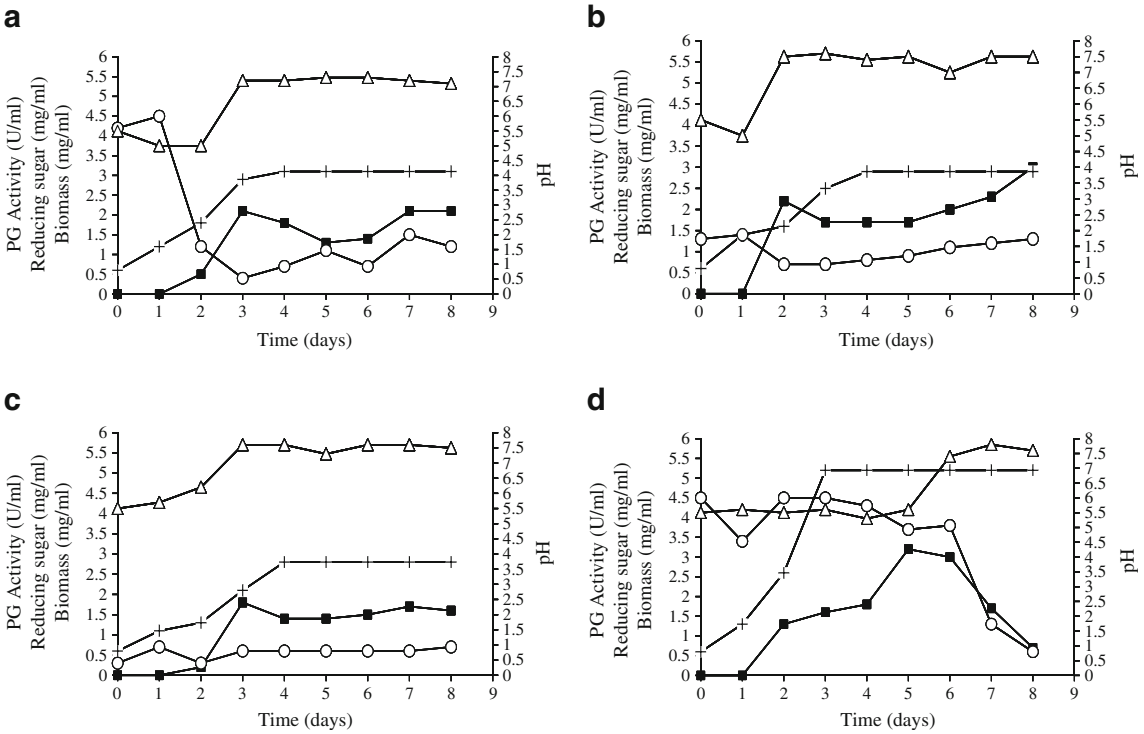

Fig. 2 PG production by *T. aurantiacus* on SMF in culture media containing agro-industrial by-products as carbon source. **a** orange peel, **b** passion fruit peel, **c** wheat bran, **d** Yellow water. ■ PG activity, ○ reducing sugar, Δ pH of culture medium, + fungal biomass

Waste processing from the citrus juice industry has been used as a substrate for the production of fungal pectinases. Fonseca and Said (1994) compared the production of endo-PG on SMF, using orange pulp and pectin as inducers of the enzyme, and observed that the orange pulp was more efficient for enzyme production (0.44 U/ml) than pectin (0.18 U/ml). Similar results were found by Larios et al. (1989), who reported high production of endo-PG by *A. niger* when grown in liquid medium containing lemon peel as inducer of the enzyme. Enzyme activity was 26.17 U/ml and 6.08 U/ml in media containing lemon peel and commercial pectin, respectively.

The final pH in all culture media containing agro-industrial by-products increased to about 7.0–7.6 on the 8th day (Fig. 2d). Increasing the pH of the culture medium containing by-products corroborates results reported by Solís-Pereira et al. (1993) in a study of the production of pectinases by *A. niger* in SMF. The initial pH of the culture medium, which was around 4.5, increased to pH 7.0 after 120 h of cultivation in medium that contained only pectin as carbon source. Alkalinization of the culture medium in fermentation processes has been generally associated with the release of ammonia resulting from protein metabolism. Other possible metabolic reactions leading to alkalinization are uptake of nitrate from the medium or metabolism of organic acids (Silva et al. 2005). Whereas protein and amino acids are not major sources of energy in this fermentation, the latter mechanism was probably responsible for the alkalinization.

The majority of works on pectinase production by fungi refer to mesophilic strains. Our present results demonstrate the possibility of using of agro-industrial by-products for the production by a thermophilic fungus of PG with characteristics suitable for industrial application.

Effect of initial pH and temperature of fermentation on PG production in yellow water

A factorial experiment with cultivation of the fungus on yellow water at an initial pH of 4.5, 5.0, 5.5 and 6.0, and

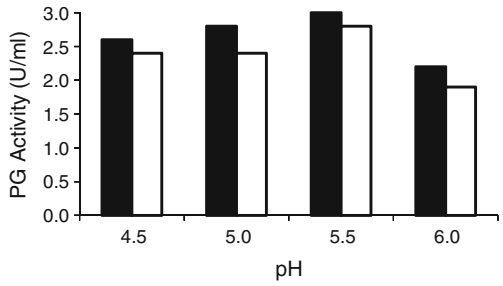

Fig. 3 PG production by *T. aurantiacus* on SMF of waste processing from citrus juices industries (yellow water) at different initial values of pH and temperature fermentation. ■ 45°C, □ 50°C

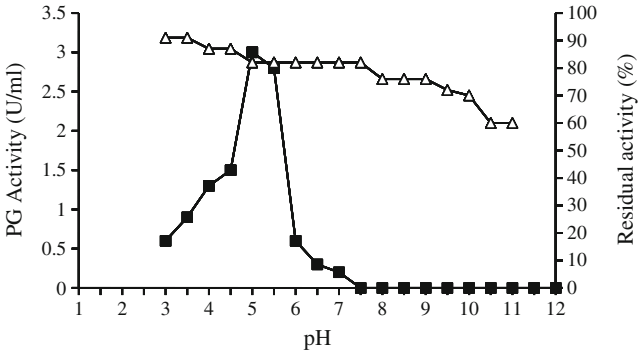

Fig. 4 Effect of pH on the activity of PG produced by *Thermoascus aurantiacus* on yellow water (■) and pH stability (Δ). Remaining activity was assayed at the optimum pH

temperatures of 45°C and 50°C was conducted. The most appropriate conditions for enzyme production were pH 5.5 and an initial fermentation temperature of 45°C (Fig. 3). Temperatures between 45°C and 50°C are the most commonly used for the growth of thermophilic microorganisms in studies of production of pectinases (Sathish-Kumar and Palanivelu 1998; Puchart et al. 1999; Kaur et al. 2004).

An initial culture medium pH of 5.5 was also nominated as the most suitable for PG production by thermophilic fungus *Thermomyces lanuginosus* (Sathish-Kumar and Palanivelu 1998). Ueda et al. (1982) reported that production of pectinesterase, PG and pectin lyase by *A. oryzae* A-3 varied with the initial pH of the medium. According to these latter authors, differences in production are related to the stability of enzymes at different pH values.

Enzyme characterization

PG production was highest at pH 5.0, but this value was very close to that obtained at pH 5.5. Above this pH, there was a sharp decline, while at pH 6.0 it was only 24% of maximum activity at pH 7.5, and this was no longer detected.

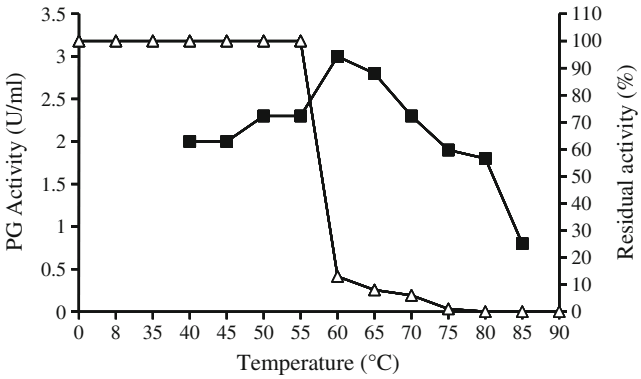

Fig. 5 Effect of temperature on activity of PG produced by *Thermoascus aurantiacus* on yellow water, in reaction conditions (■) and thermostability (Δ) in the absence of substrate. Remaining activity was assayed at the optimum pH and temperature

Similar results were found by Acuña-Argüelles et al. (1995) in a study on the production of PG produced by *A. niger* in SMF, which was most active at pH 4.0–5.0. At values above 6.0, enzymatic activity was practically no longer detected. Zheng and Shetty (2000) reported that the optimum pH of the exo-PG produced by *Lentinus edodes* was 5.0, and barely detected at values greater than 7.0.

Regarding pH stability, the enzyme from *T. aurantiacus* retained about 91% of the original activity at pH 3.0–3.5. At higher pH values, there was a gradual decrease in its stability. However, it still retained about 60% activity at high pH values (10.5–11.0) (Fig. 4). Similar results were found by Martin et al. (2010), who reported that the crude PG produced by the thermophilic fungus *Thermomucor indicae-seudaticae* N31 from SMF was more stable in acidic pH.

The optimum temperature for PG activity was around 60–65°C. The enzyme showed an increase in activity up to a temperature of 65°C, showing a gradual reduction of activity at temperatures above this value. At 85°C, the activity was reduced to only 17% of maximum activity. It can also be observed that the enzyme produced showed thermostability up to 55°C. This feature becomes more evident when the enzyme was exposed to 60°C, at which temperature it retained only 13% of its original activity. In addition, it preserved only 6% at 70°C and, when kept at 80°C, activity was no longer detected (Fig. 5).

The influence of temperature on pectinase activity from mesophilic microorganisms has been reported by several authors. An optimum temperature of 50°C was found for the PGs generated by *L. edodes*, growing in strawberry pulp in the same fermentation process (Zheng and Shetty 2000). Martin et al. (2004) reported that the optimum temperature of PG produced by the mesophilic fungus *Moniliella* SB9 was 55°C, while for *Penicillium* sp. EGC5, the optimum was was 40°C.

However, little information exists regarding crude pectinase of thermophilic fungi. Data on exo-PG from *T. lanuginosus*, presented by Sathish-Kumar and Palanivelu (1998) indicate a temperature of 70°C for optimal activity of the enzyme, although the same fungus has also produced another PG, with optimal activity at 60°C. Martin et al. (2010) reported that the crude PG produced by the thermophilic fungus *Thermomucor indicae-seudaticae* N31 from SMF was more thermostable than that from solid-state fermentation.

Conclusion

The use of agro-industrial by-products in the production of PG by the thermophilic fungus *Thermoascus aurantiacus* on SMF was feasible since the enzyme activity was higher in these substrates compared with a commercial pectin. The crude enzymatic extract showed thermostability up to 55°C and stability over a wide pH range. Enzyme clarification of juice may be carried out at 15°C for 12 h or at 54°C for 1–2 h (Lea 1998). Thus, the thermostability of enzymes produced by *Thermoascus aurantiacus* is a desirable feature, since these can be used in industrial processes that require high temperatures.

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