Brazilian Journal of Microbiology (2006) 37:302-306
ISSN 1517-8382

PRODUCTION AND PARTIAL CHARACTERIZATION OF POLYGALACTURONASES PRODUCED BY THERMOPHILIC *MONASCUS* SP N8 AND BY THERMOTOLERANT *ASPERGILLUS* SP N12 ON SOLID-STATE FERMENTATION

Paula Mendes de Freitas; Natalia Martin; Dênis Silva; Roberto da Silva; Eleni Gomes*

Laboratório de Bioquímica e Microbiologia Aplicada, Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista, São José do Rio Preto, SP, Brasil

Submitted: November 26, 2004; Returned to authors for corrections: April 27, 2005; Approved: May 08, 2006

ABSTRACT

Polygalacturonases production by newly isolated *Monascus* sp N8 and *Aspergillus* sp N12 strains was carried out in solid-state fermentation using mixtures of wheat bran, sugar cane bagasse and orange bagasse as carbon sources. The maximal activity values of exo-polygalacturonases (exo-Pg) from *Monascus* sp and *Aspergillus* sp were obtained using wheat bran/sugar cane bagasse/orange bagasse mixture (6.6 U/mL) and wheat bran/orange bagasse mixture (10 U/mL), respectively. Enzyme production by both strains was higher at 45ºC after 72 h and 1.6 U/mL at 50ºC after 120 h. Endo-polygalacturonase (endo-Pg) production was higher in wheat bran/orange bagasse mixture and was not affected by temperature of incubation for both fungi. Endo-Pg production by *Monascus* was 1.8 U/mL at 45ºC and 50ºC, after 72. Similar values were obtained in *Aspergillus* sp culture, 1.9 U/mL at 45ºC and 1.8 U/mL at 50ºC.Exo-Pg from both strains showed optimum activity at pH 5.5. Maximal activity was determined at 60ºC for enzyme from *Monascus* sp and 50ºC for that produced by *Aspergillus* sp. Exo-Pg from *Monascus* sp was stable at pH range 4.5-6.0 whereas that from *Aspergillus* sp enzyme was stable at pH 4.0. Both enzymes showed stability when incubated at 50ºC for 1 h, in absence of substrate.

Key words: *Monascus* sp, *Aspergillus* sp, thermophilic, polygalacturonase, solid-state fermentation

INTRODUCTION

The increasing energy demand has been focused world-wide attention on the utilization of renewable resources, particularly agricultural and industrial wastes. The agricultural and agro-industrial wastes, such as wheat bran, rice husk, corn straw, corn cob, fruit peels, paper industry wastes and orange and sugarcane bagasses have high organic matter content and their disposal arise both economic and environmental problems. On the other hand, their major components as cellulose, starch, lignin, xylan and pectin can be used by several microorganisms as carbon and energy sources producing enzymes and other products with high commercial value (1,3).

The pectin is degraded by de-esterifiyng enzymes (pectinesterases), depolymerizing enzymes (hydrolases and liases) and protopectinases. Polygalacturonases catalyze the hydrolytic cleavage of the α-1,4-glycosidic bonds between galacturonic acid units by endo and exo action (15). Pectinases are extensively used in the industrial clarification of wine and fruit juices, maceration of fruits and vegetable tissues and coffee and tea processing. In addition, are involved in the retting and degumming of fiber crops, such as jute, flax and ramie (3,9).

Although a number of polygalacturonases have been studied there are few reports about the production of polygalacturonases by thermophilic fungi (7,11,16). The present study was carried out on the production of polygalacturonases from newly isolated thermophilic *Monascus* sp and *Aspergillus* sp in solid-state fermentation and partial characterization of the enzymes.

*Corresponding Author. Mailing address: Laboratório de Bioquímica e Microbiologia Aplicada, Instituto de Biociências, Letras e Ciências Exatas, IBILCE, UNESP. 15054-000, São José do Rio Preto, SP, Brasil. Tel.: (+5517) 3221-2393, Fax: (+5517) 3221-2390. E-mail: eleni@ibilce.unesp.br

MATERIALS AND METHODS

Microorganisms

The *Monascus* sp N8 and *Aspergillus* sp N12 strains used in this study were recently isolated from compost samples, at Constroeste Construtora e Participações Ltda in São José do Rio Preto, São Paulo state, Brazil. The strains were sub-cultured on to agar slants (pH 5.5), containing: 1% citrus pectin; 0.2% meat peptone; 0.2% yeast extract; 0.2% $(NH_4)_2SO_4.7H_2O$; 0.1% $MgSO_4.7H_2O$; 0.5% element trace solution (2.2g $ZnSO_4.7H_2O$; 1.1g H_3BO_3; 0.5g $MnCl_2.4H_2O$; 0.5g $FeSO_4.7H_2O$; 0.16g $CoCl_2.5H_2O$; 0.16g $CuSO_4.5H_2O$; 0.11g $(NH_4)_6Mo_7O_{24}.4H_2O$; 5g EDTA) and 3% agar.

Enzyme production in solid-state fermentation (SSF)

The solid substrates were prepared as follows:

(a) Orange bagasse: The pellet of orange bagasse (pressed mixture of pulp and peel) was provided by Citrovita Agro-Industrial Ltda, Catanduva, SP, Brazil. Chemical analysis revealed that the dry material was composed of 11.8% fibre, 6.4% protein, 6.3% nitrogen, 6.7% ash, 19% total sugar (9% reducing sugar) and 0.1% pectin. The material was ground and particles sieved by a Bender USS 230 strainer and dried at 80ºC.

(b) Sugar cane bagasse: The sugar cane bagasse was provided by Usina de Açúcar e Álcool São Domingos, Catanduva, SP, Brazil. Chemical analysis revealed that the dry material was composed of 75% fibre, 5% nitrogen, 3.4% ash and 10.3% total sugar (3.5% reducing sugar). The material was washed in tap water and the same procedure described above was followed.

(c) Wheat bran: This material was obtained commercially, dried and used untreated. Chemical analysis revealed that the dry material was composed of 8.1% fiber, 7.5% nitrogen, 4.6% ash and 16.7% total sugar (5.2% reducing sugar).

The substrate mixtures was prepared in proportions of (w/w): 1-Wheat bran; 2-wheat bran / sugar cane bagasse (1:1); 3-wheat bran / orange bagasse (1:1); 4-wheat bran / sugar cane bagasse / orange bagasse (1:1:1).

Solid-state fermentation (SSF) by *Monascus* sp N8 was carried out using 5 g of sterilized substrate inoculated with micelial suspension (approximately 3.0 mg dry micelial mass per g dry substrate), which was obtained from a 3-day agar slant culture. After inoculation, 10 mL of nutrient solution, composed of 0.1% $(NH_4)_2SO_4$ and 0.1% $MgSO_4.7H_2O$ were added. The final moisture content of the medium was 70%. SSF by *Aspergillus* sp N12 was carried out in the same way except and the inoculum was approx. 10^7 spores per g dry substrate.

Cultures were incubated at 45ºC and 50ºC for 6 days. After 48 h and at intervals of 24 h, 40 mL distilled water were added to the cultures, stirred for 40 min, filtered under vacuum and centrifuged. The supernatant was used as enzyme solution. The shown results are the mean of 3 experiments.

Enzyme activity measurements

Exo-polygalacturonase (Exo-Pg) activity was determined by measuring the release of reducing groups from citrus pectin by the dinitrosalicylic acid method (12). The reaction mixture containing 0.8 mL 1% citric pectin 67% methoxylated (CPKelco) in 0.2 M acetate buffer, pH 5.5 and 0.2 mL of enzyme solution, was incubated at 50ºC for 10 min. A control containing water instead of enzyme solution was performed. One activity unit (U) was defined as the amount of enzyme which releases 1 µmol of galacturonic acid in one minute.

Endo-polygalacturonase (endo-PG) was measured viscosimetrically by adding 2 mL of crude enzyme to 6mL of citrate-NaOH buffer (pH 5.5) containing 3% of 67% methoxylated citric pectin. The reaction mixture was incubated at 50ºC for 15 minutes, after which its viscosity was determined with a Basic viscosimeter (Fungilab).The blank contained thermally-inactivated crude enzyme. One unit of enzyme activity (U) was defined as the amount of enzyme that reduced the initial viscosity by 50% per minute.

Enzyme characterization

Apparent optimun pH and temperature for enzyme activity: The optimum pH was determined by measuring activity at 50ºC for enzyme from *Aspergillus* sp and 60ºC for enzyme from *Monascus* sp, using as buffers sodium acetate (pH 3.0-5.0), citrate-phosphate (pH 5.0-7.0), tris-HCl (pH 7.0-8.5) and glycine-NaOH (pH 8.5-11.0). The optimum temperature was assayed by incubating each reaction mixture at 45-85ºC.

Thermostability: a thin layer of mineral oil prevented evaporation of the crude enzyme solution which was incubated at various temperatures (10 -90ºC) for 1 h 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 5.5-7.0 (citrate-phosphate), pH 7.0-8.5 (tris-HCl) and pH 8.5-11.0 (glycine-NaOH) and maintained at 27ºC for 24 h. An aliquot was used to determine the remaining activity at the optimum pH and temperature.

RESULTS AND DISCUSSION

Polygalacturonases production in SSF

Thermophilic *Monascus* sp and thermotolerant *Aspergillus* sp, when cultivated in medium with wheat bran (W); wheat bran and sugar cane bagasse (W/C); wheat bran and orange bagasse (W/O); wheat bran, sugar cane bagasse and orange bagasse (W/C/O), produced exo-Poligalacturonase (exo-Pg) and endo- Poligalacturonase (endo-Pg) (Figs. 1, 2).

For both strains, the higher exo-Pg production was observed at 45ºC than at 50ºC. The growth of the fungi at 50ºC was negligible with possible hyphae death and low enzyme production (Fig. 1). It was also observed that the enzymatic

production was low for both strains when solely wheat bran was used as carbon source. Exo-Pg production using mixture of wheat bran and orange bagasse at 45ºC peaked after 96h of fermentation with 10 U/mL and 5 U/mL in culture media of *Aspergillus* sp and *Monascus* sp, respectively (Figs. 1A and C). The highest exo-Pg production by *Monascus* sp (6.6 U/mL) was observed after 96h growing in mixture of the wheat bran, sugar cane and orange bagasse (Fig. 1A).The consistence and size of particles in media composed only of wheat bran could with-stand a change in the packing of the substrate particle during the fermentation, since the bed remained static (nonmixed system), playing a role in affecting heat and mass transfer within the system (13). These results are in agreement with the literature, since is known that addition of fibrous material to solid fermentation medium, increase the inter-particle spacing possibly increasing the aeration and nutrient and enzyme diffusion (14,16). However, for *Aspergillus* sp, the presence of sugar cane bagasse reduced of enzyme production (Fig. 1C).

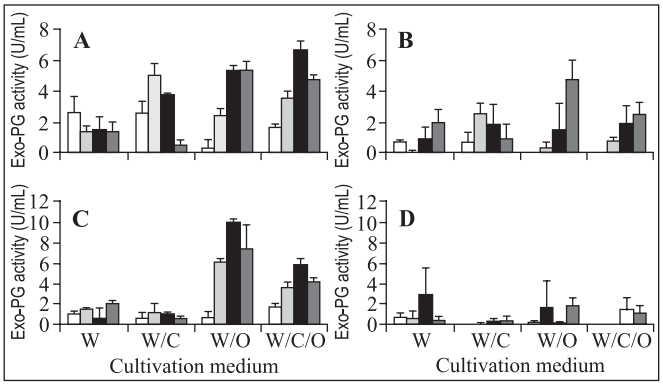

Figure 1. Production of exo-polygalacturonase by *Monascus* sp N8 and *Aspergillus* sp N12 strains in solid-state fermentation. A, B = *Monascus* sp; C, D = *Aspergillus* sp; A, C = 45ºC; B, D = 50ºC. SSF was carried out on various substrates, as indicated in figure, and different time intervals. □ = 48 h, ▒ = 72 h, ■ = 96 h, ▓ = 120 h.

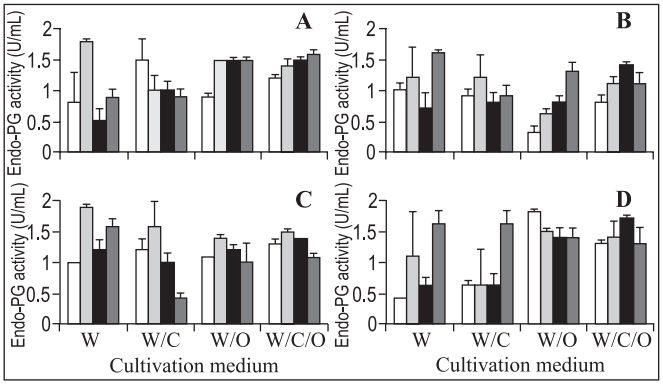

Figure 2. Production of endo-polygalacturonase by *Monascus* sp N8 and *Aspergillus* sp N12 strains by solid-state fermentation. A, B = *Monascus* sp; C, D = *Aspergillus* sp; A, C = 45ºC; B, D = 50ºC. SSF was carried out on various substrates, as indicated in figure, and different time intervals. □ = 48 h, ▒ = 72 h, ■ = 96 h, ▓ = 120 h.

The effect of different carbon sources on pectinase synthesis by fungi in SSF have been studied and it is generally agreed that the optimum medium for the enhanced production of extracellular pectinase is that containing pectic materials as an inducer (5,8,17). According to this, the present results corroborate that the culture medium with high levels of pectin, such as orange bagasse, raised the highest exo-Pg activity (11).

Data of Fig. 2 showed that, on the contrary of obtained for exo-Pg, the production of endo-Pg was not significantly affected by temperature and composition of the culture medium (Figs. 2B and D). The enzyme production by *Monascus* sp at 45ºC was 1.8 U/mL after 72 h, when wheat bran was the carbon source (Fig. 2A). At 50ºC, the maximum activity value was 1.6 U/mL in 20 h in the same conditions (Fig. 2B). In *Aspergillus* sp culture, at 45ºC, activity of 1.9 U/mL was detected after 72 h with wheat bran as substrate (Fig. 2C). The maximum activity obtained in fermentation at 50ºC was 1.8 U/mL when a mixture of wheat bran and orange bagasse was the substrate (Fig. 2D).

The present results indicate that the agro-industrial wastes such as wheat bran, orange bagasse and sugar cane bagasse are suitable for polygalacturonase production by newly isolated *Monascus* sp N8 and *Aspergillus* sp N12 at 45ºC in SSF.

The pH culture medium didn't show large variation in any medium (Fig. 3). In all of them the pH increased a little after 96h of cultivation.

Enzyme characterization

For characterization of exo-Pg from *Monascus* sp N8 was used the enzyme obtained after 96 h of fermentation at 45ºC, in mixture of wheat bran, orange bagasse and sugar cane bagasse as substrate. For exo-Pg form *Aspergillus* sp N12 it was used the enzyme obtained in the same incubation conditions but using mixture of wheat bran and orange bagasse.

The maximal activity was observed at pH 5.5 for exo-Pg from both strains (Fig. 4). The optimum pH found for the exo-Pg is comparable to those from other fungal strains (6). Exo-Pg from *Monascus* sp was stable at pH 4.5-6.0 while that from *Aspergillus* sp, was stable at pH 4.0 (Figs. 6A and B). The acid tolerance property of these enzymes is a great advantage in fruit and vegetable processing applications, since most fruit and vegetable tissues and juices have acidic pH.

Exo-Pg from *Monascus* sp and *Aspergillus* sp exhibited maximal activity at 60ºC and 50ºC, respectively (Fig. 5). Both enzymes showed stability at temperatures up to 50ºC (Fig. 7)

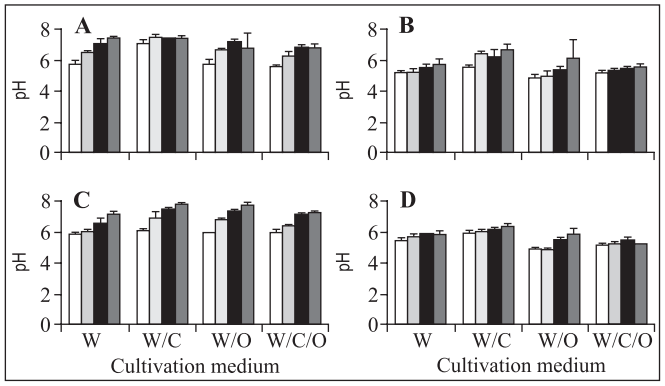

Figure 3. pH values in the cultured medium after solid-state fermentation of *Monascus* sp N8 and *Aspergillus* sp N12 strains. A, B = *Monascus* sp; C, D = *Aspergillus* sp; A, C = 45ºC; B, D = 50ºC. SSF was carried out on various substrates, as indicated in figure, and different time intervals. □ = 48 h, ▒ = 72 h, ■ = 96 h, ▓ = 120 h.

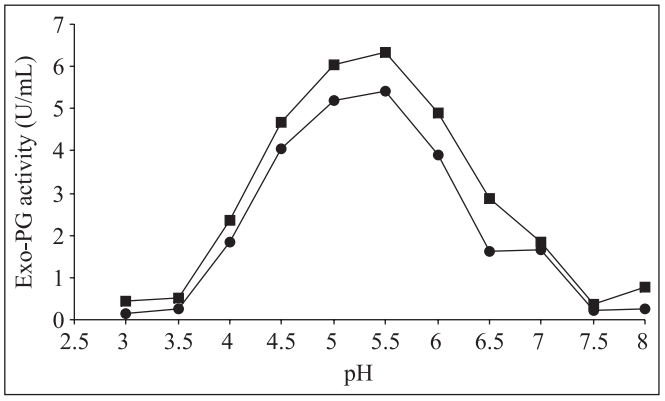

Figure 4. Effect of pH on exo-polygalacturonases activities. ● = Exo-Pg from *Monascus* sp N8, ■ = Exo-Pg from *Aspergillus* sp N12.

when incubated for one hour in absence of substrate. Pectinases from *Aspergillus* strains have been described as susceptible to denaturation in temperatures above 50ºC (2,4,18). Juice enzymatic clarification may be carried out at 15ºC for 12 h or at 54ºC for 1-2 h for to prevent yeast growth (10). An enzyme that maintaining its stability in these condition is desirable.

CONCLUSIONS

The present work shows that it is feasible to use agro-industrial wastes for polygalacturonase production by newly isolated thermophilic *Monascus* sp N8 and thermotolerant *Aspergillus* sp N12. These fungi are able to produce high levels of polygalacturonases during the solid-state fermentation using

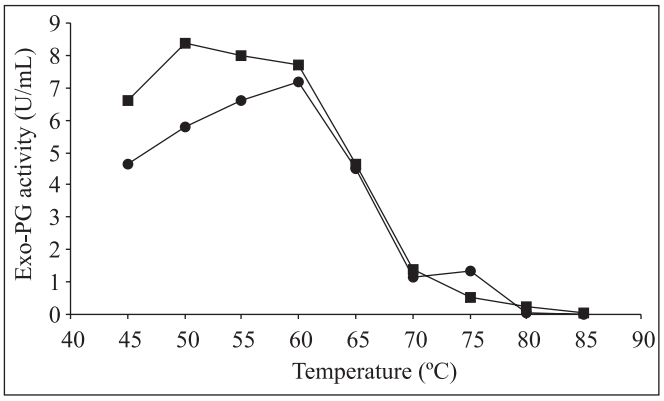

Figure 5. Effect of temperature on the exo-activity of exo-polygalacturonases activities. ● = exo-Pg from *Monascus* sp N8, ■ = exo-Pg from *Aspergillus* sp N12.

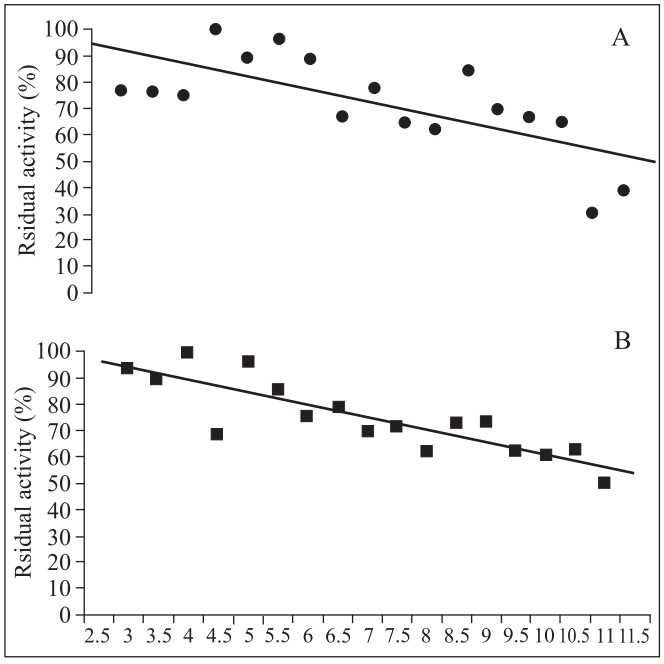

Figure 6. Stability of the exo-polygalacturonases against pH. A = exo-Pg from *Monascus* sp N8, B = exo-Pg from *Aspergillus* sp N12.

a mixture of wheat bran, orange bagasse and sugar cane bagasse as substrate.

Polygalacturonases produced by *Monascus* sp N8 and *Aspergillus* sp N12 have a relatively higher optima temperature (60 and 50ºC, respectively), and a low optima pH (5.5), with good thermal stability (50ºC for both) and a high tolerance to low pH (4.5-6.0 and 4.0, respectively). These properties could lead to their becoming acceptable in food industry for fruit juices processing.

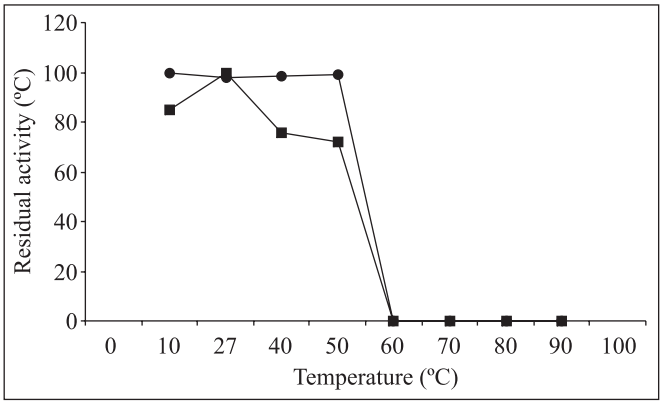

Figure 7. Stability of the exo-polygalacturonases produced by *Monascus* sp N8 and *Aspergillus* sp N12 against temperature. ● = exo-Pg from *Monascus* sp N8, ■ = exo-Pg from *Aspergillus* sp N12.

ACKNOWLEDGEMENTS

We thank Fundação de Amparo a Pesquisa do Estado de São Paulo- FAPESP- and to Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq- for financial support.

RESUMO

Produção e caracterização parcial de poligalacturonases produzidas pelo fungo termofílico *Monascus* sp N8 e pelo termotolerante *Aspergillus* sp N12 em fermentação em estado sólido

A produção de poligalacturonases pelas linhagens fúngicas recentemente isoladas, *Monascus* sp N8 e *Aspergillus* sp N12, foi estudada através de fermentação em estado sólido usando como substratos misturas de farelo de trigo, bagaço da cana-de-açúcar e bagaço de laranja. A atividade máxima de exo-Pg produzida por *Monascus* sp (6,6 U/mL) foi obtida quando o meio de cultivo utilizado continha mistura de farelo de trigo, bagaço da cana-de-açúcar e bagaço de laranja (1:1:1), enquanto que *Aspergillus* sp produziu maior quantidade da enzima (10 U/mL) em meio de farelo de trigo e bagaço de laranja. A maior produção de exo-Pg foi obtida através de incubação das culturas a 45ºC quando comparadas àquelas incubadas a 50ºC. A produção de endo-poligalacturonase (endo-Pg) pelas duas linhagens não foi afetada pela temperatura de incubação. A atividade de endo-Pg em meio de cultura *Monascus* sp foi 1.8 U/mL a 45ºC em 72 hs de fermentação e 1,6 U/mL a 50ºC em 120 hs de fermentação nas mesmas condições. Valores semelhantes foram obtidos pelo cultivo de *Aspergillus* sp com 1.9 U/mL a 45ºC a 1.8 U/mL at 50ºC. As exo-poligalacturonases produzidas por ambas as linhagens mostraram maiores atividades em pH 5,5. Enzimas de *Monascus* sp foi mais ativa a 60ºC e a de *Aspergillus* sp, a 50ºC. A exo-Pg produzida por *Monascus* sp foi estável em valores de pH entre 4,5-6,0, enquanto a de *Aspergillus* sp foi estável somente em pH 4,0. Ambas as enzimas mostraram-se estáveis por 1 hora a 50ºC, quando incubadas em ausência de substrato.

Palavras-chave: *Monascus* sp, *Aspergillus* sp, termofílico, poligalacturonase, fermentação em estado sólido

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