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Assessment of pectinase-producing fungi isolated from soil and the use of orange waste as a substrate for pectinase production

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ABSTRACT

Pectinases are important enzymes for their potential applications in different industries such animal feed, agricultural, textile, beverage, food processing, oil extraction, etc. Ten fungal species were isolated from the soil and screened for production of pectinase enzyme by using the pectin agar medium. Pectinolytic enzymes synthesis were attained at a temperature of 30 °C and activities were determined after a seven-days culture of *Aspergillus* sp. 391 and *Aspergillus* sp. 031, in a basic medium containing 2% citrus pectin and as the sole carbon source. The extract enzymatic showed an optimum activity for exo-polygalacturonase (PG) and pectin lyase (PNL) against galacturonic acid and pectin at pH 4.5 and 5.5, respectively. There were variations in PG and PNL enzymes levels produced in culture filtrates obtained of *Aspergillus* sp. 391 with addition of citrus waste (2.0 and 4.0% w/v) to the medium. Maximum activity for PNL activity was observed in the medium containing 5% pectin or 4% citrus waste, as sole carbon source, after 7 days of growth. The results showed that the isolate *Aspergillus* sp. 391 is a promising for pectinolytic enzymes production at the industrial level.

Keywords: *Aspergillus* sp. Pectinase. Enzyme Production. Activity.

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1. Introduction

Brazil is the world's largest producer of orange juice and consequently enormous quantities of industrial waste residues are generated (Fonseca & Said, 1994). Orange juice industries produce a large amount of waste material in the form of peel, pulp, seeds, etc. This waste material generate disposal problems and ultimately leads to pollution. Citrus waste contains a substantial amount of pectin and thus can be used as a substrate to produce pectinolytic enzymes. Peel waste contains approximately 17% soluble sugar, 9% cellulose, 10% hemicellulose and 42% pectin as the main components (Rivas et al., 2008). The carbon source is the major component in a culture medium for microorganism, and generally plays a dominant role in fermentation productivity due to its direct relationship with the formation of biomass and enzyme production .

Molds of the filamentous ascomycete genus *Aspergillus* can synthesize a variety of pectinases (Fontana et al., 2005; Banakar & Thippeswamy, 2012) and are the most frequently used microorganism in the enzyme industry because at least 90% of enzymes are produced extracellularly (Sandri et al., 2011). *Aspergillus niger* is the specie most commonly used in pectinolytic enzymes production due a number of technical reasons, including been assigned the status of GRAS (Generally Recognized As Safe) (US Food and Drug Administration, 2015).

The activity of these enzymes is affected by physicochemical conditions (Galiotou-Panayotou et al., 1997), such as initial pH, temperature and growth time, or the presence of inhibitors or activators (Leuchtenberger & Mayer, 1992); therefore, their optimization is essential for the success of the process (Fawole & Odunfa, 2003). Industrial production of enzymes is performed, predominantly by submerged fermentation (SmF), due to the ease control of pH,temperature and nutrient gradients in large scale (Leuchtenberger & Mayer, 1992).

The aim of this study was to evaluate the conditions required for growth and production of extracellular pectinases (PG and PNL) by submerged fermentation using filamentous *Aspergillus* sp isolated from Brazilian soil, aimed at industrial food products.

2. Materials and Methods

2.1 Chemicals

The chemicals used were purchased from Synth (São Paulo, Brazil) and all reagents were of analytical grade and were used as received. Waste citrus was crushed and obtained from orange, pear-river type acquired in the local market. Drying was carried out in an oven with air circulation, temperature 50 °C and pectin content was performed according to McCready & McComb (1952), using D-galacturonic acid as standard. The amount of pectin, expressed as D-galacturonic acid, found in the dry residue was 2.49% (w / w).

2.2 Microorganism

2.2.1. Isolation and identification of pectinolytic enzymes from microorganisms from soil samples.

Citrus dump waste soil was used as samples and were collected into sterile flask with a sterile spatula in the UNESP Campus, Araraquara, SP, Brazil, through a systematic screening program for the isolation of pectinolitic fungus (Brazil gov. SisGen – Nº A6273FD), according Gattas et al. (2003).

About 10 g of sample was transferred to a sterile flask containing 90 mL sterile saline solution (0.85%). The flasks were shaken on rotary shaker for 10 min to settle down the particulate matter. The clarified supernatant obtained by centrifugation (8,130 x g) was diluted with sterile saline solution. These dilutions (10^{-5} and 10^{-6}) were used as inoculum. One mL of each of these dilutions was pipetted out into the medium, plated into 10 cm diameter petri dishes containing: Medium - 20 g/L pectin, 2 g/L ammonium sulfate, 2 g/L magnesium sulfate, 0.5 g/L potassium phosphate, 2 g/L agar.

For selection of the pectinolytic activity fungus red ruthenium dye (0.5%) was employed (McKay, 1988) on the fungus colony grown for 24 and48 hours, at 30 °C. The pectinolytic indices of fungus were determined by measuring the diameter of color zone and colony size of fungus, expressed in millimeter. The experiment was carried out in triplicate and the mean of the readings were taken.

For the identification of selected microorganism were emphatisized the morphological methods including: macroscopic and microscopic characteristics. Comparative morphological characteristic of *Aspergillus* species was realized of standard *Aspergillus niger.* Moreover, *Aspergillus niger* (industrial use) was used as standard.

2.2 Growth of cultures

The basic culture medium contained 2 g of KH_2PO_4, 1.4 g of $(NH_4)_2SO_4$, 0.3 g of $MgSO_4$ and 0.01% of $CaCl_2$ in 1 liter of water supplemented with 1 mL of a solution containing 1.6 mg of $MnSO_4$ and 1.4 mg of $ZnSO_4$. The culture was carried out with a medium containing citrus pectin (2% w/v) as the sole carbon source.

All experiments were carried out in duplicate. The media were adjusted to appropriate pH value at 30 °C. After 7 days, samples were aseptically withdrawn and estimated for pectinase activity on submerged fermentation.

2.3 System for PNL production

Batch experiments were performed in 250 mL Erlenmeyer flasks holding 50 mL of medium. The initial spore density was 1 x 10^7 units/mL of medium. The flasks were incubated without shaking at 30 ºC for 7 days. Different concentrations (1-5% w/v) of pectin citrus were added to the medium in order to study the effects on pectin lyase production by cells of *Aspergillus* sp. 391 and *Aspergillus* niger (industrial use).

2.4 System for Exo-PG production

Batch experiments were performed in 250 mL Erlenmeyer flasks holding 50 mL of medium. The initial spore density was 1 x 10^8 units/mL of medium. The flasks were incubated on an orbital shaker (120 rpm) at 30 °C for 48 h.

In order to measure the effects of waste citrus on exo-PG production two different concentrations (2 and 4% w/v) of waste were added to the medium in order to study the effects on exo-polygalacturonase and pectin lyase productions by *Aspergillus* sp. 391 at 30 °C for 7 days.

2.5 Determination of total protein

Protein was determined in the culture filtrate using method of Bradford (1976), with bovine serum albumin as the standard.

2.6 Enzyme assay

Pectinase activity was assayed on supernatants from the incubated shake flasks at 24 and 48 h were used as crude extract pectinases for determination of activity using method described by Okafor et al. (2010). One unit of pectinase activity is defined as the amount of enzyme which liberates 1 µmol of reducing sugar per mL, per minute under assay conditions.

PNL activity was assayed as described by Gattas et al. (1999). The reaction mixture contained 1.75 mL of 0.1 M citrate buffer (pH 5.0), 1.75 mL of 1% (w/v) pectin citrus (Sigma Chemical Co., St Louis, MO, USA) and 0.7 mL of culture filtrate. The reaction was incubated at 30 ºC for 10 min and started by adding the culture filtrate. One unit of PNL activity was expressed as the amount of enzyme that catalyzes the release of 1 µmol 4,5-unsaturated oligogalacturonides per unit volume of culture filtrate per min under standard assay conditions. The absorbance was read in 235 nm, on spectrophotometer. For determination of activity, it was used Equation 1:

$$Activity(\mu mol / mL.\min) = \frac{ABS.V_1.10^3}{OC.V_2.\varepsilon.time} \quad (1)$$

where: ABS was calculated as the difference between sample absorbance and white values; V_1 = 3.5 mL (solution volume added to the sample volume); V_2 = 0.7 mL (volume of enzyme used); Optical path is 1cm; ε is the molar extinction coefficient of 5500 $M^{-1}cm^{-1}$ (Yadav et al., 2009); time is 10 minutes.

Exo-PG activity was assayed by the determination of reducing sugars released by the dinitro salicylic acid method (Miller , 1959). The reaction mixture contained 2.50 mL of

0.2 M citrate buffer (pH 5.0), 2.50 mL of 1% (w/v) galacturonic acid (Sigma Chemical Co., St Louis, MO, USA) and 0.05 mL of culture filtrate. The reaction was incubated at 30 °C for 15 min and started by adding the culture filtrate. One unit of PG activity was expressed as the amount of enzyme that catalyzes the release of 1 μmol galacturonic acid per unit volume of culture filtrate per min under standard assay conditions.

3. Results and discussion

From the morphological characters of the isolates, fungal species were identified as *Aspergillus*. Ten fungal isolates obtained from soil showed different diameters of pectin degradation in solid medium. These strains were tested for pectin hydrolysis by plate assay, at pH 6.0. The strains were classified as very good producers (+++) of pectin depolymerizing enzymes when presented clear halos around colonies of at least 1.5 cm, good producers (++) when the halos were of at least 1.0 cm, weak producers (+) halos were at least 0.5 cm and poor producers when no pectinolytic activity and no clear lysis zones were observed, after 24 and 48 h of growth. Selection of strains with pectinolytic activity – fungus strains able to grow on medium containing 2% citrus pectin as the only carbon source were isolated (Table 1). Reddy & Screeramulu (2012) determined the pectinases production by *Aspergillus flavus* by measuring the clear zones formed around the colonies stained with ruthenium red. Sandhya & Kurup (2013) isolated pectinolytic fungi from same source and showed a comparative study on the production of pectinase under solid state and submerged fermentation system.

Table 1. Isolated fungal species and *Aspergillus niger* showed different rate of pectinolytic activity (halo diameter/colony diameter) on pectin agar using red ruthenium dye test.

Strain	Color zone/colony size		Pectinase activity[1] (%)
	After 24 h	After 48 h	
Aspergillus sp. 492	++	++	70.9
Aspergillus sp. 391	+++	+++	100
Aspergillus sp. 190	No	+	25.7
Aspergillus sp. 099	++	+++	93.2
Aspergillus sp. 084	+	++	75.0
Aspergillus sp.207	++	++	84.4
Aspergillus sp.046	+++	++	92.2
Aspergillus sp.054	+	+	52.9
Aspergillus sp.043	+++	+	49.9
Aspergillus sp.031	++	+++	97.2
Aspergillus niger (Industrial use)	+++	+++	100

[1] medium containing pectin 2% after 7 days of growth.

According to results, two effective pectinolytic fungal strains were selected among the others, like, *Aspergillus* sp 391 and *Aspergillus* sp 031 and they were used for extracellular exo-polygalacturonase and pectin lyase production under submerged fermentation.

Solís-Pereira et al. (1993) reported that exo and endo pectinase activities of *Aspergillus niger* increased with increase in the concentration of different carbon source in submerged and solid fermentation.

In the previous work Gattas et al. (2003) reported and enhanced pectinase when 2% (w/v) pectin medium by tree fungi studied for 144 hours and the maximum pectinolytic activity was obtained in 15 hours. The free cells produced different types of pectinolytic enzyme activity (Pashova et al., 1999) and the production of pectinolytic enzymes from many moulds is known to be enhanced by presence of pectic substrates in the medium (Aguilar & Huitrón, 1987).

Exo-poligalacturonase produced by SmF with *Aspergillus* sp. was induced by 4% of pectin and its derivatives on the medium. Exo-poligalacturonase enzyme synthesis was attained at a temperature of 30 °C and activities were determined after two-days culture of *Aspergillus* sp. 391 and *Aspergillus* sp. 031 in a basic medium containing 2% and 4% citrus pectin and as the sole carbon source. Galactose and Polygacturonic acid were added to the medium containing 2% of pectin and the production of exo-PG enzyme were stimulated by *Aspergillus* sp. 391 and *Aspergillus* sp. 031 (Table 2). The synthesis of enzyme was significantly higher than pectin 4% was addition in the medium.

Fungal extracellular crude enzyme preparations were assayed for PNL activity at different time of incubation. The two investigated species showed very high PNL activities at 7th day of incubation (data not show).

PNL production by *Penicilluim italicum* CECT 2294 was influenced by the type of medium (natural and synthetic) used its production (Alana et al., 1990).

Optimal conditions for fungal growth and pectin liase production were chosen based on pectin concentration of medium. As shown in Figure 1, the PNL activity in the culture media increased from the medium containing 1% pectin to reach a maximum at 5% concentration by *Aspergillus sp 391* strains, while PNL activity decreased with pectin concentration for the *Aspergillus niger* (industrial use).

The use of complex substances like citrus peel is substrates for pectinolytic enzymes (Aguilar & Huitrón, 1987). Pectin substance and their degradation products are inducers for the enzyme's synthesis by microorganisms (Tahara et al., 1972; McMillan et al., 1992). Camargo et al. (2005) stated that by using *Aspergillus* sp. 0492, pectin lyase with an activity of 11.3 U/mL after 96 hours of culture can

Table 2. Effect of carbon source on exo-polygalacturonase relative activity (%) by *Aspergillus* sp. 391 and *Aspergillus* sp. 031*.

Aspergillus sp	Pectin 2%	Pectin 4%	Pectin 2% Galactose 2%	Pectin 2% Polygacturonic acid 2%
391	50.6	**100**	80.8	92.3
031	48.9	**100**	95.5	63.7

*These experiments were carried out at 30 °C for 2 days, pH 4.5.

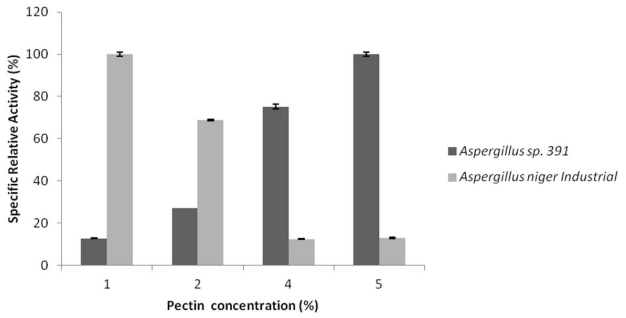

Fig. 1. Effect of the concentration of carbon source on pectin lyase (PGL) synthesis by *Aspergillus* sp. 391 and *Aspergillus niger* (industrial use) growing with 1% to 5% pectin as carbon source.

Table 3. Effect of citrus waste concentration on pectin lyase and polygalacturonase production by *Aspegillus* sp. 391*.

Carbon source	*PNL*	*PG*
	Relative Activity (%)*	Relative Activity (%)*
Pectin 2%	18.2	66.1
Citrus waste 0.5%	11.9	27.5
Citrus waste 2.0%	55.9	71.4
Citrus waste 4.0%	100	100

*These experiments were carried out at 30 °C for 7 days.

be obtained from 4% (w/v) citrus waste supplement with mineral salts.

Effect of various concentration of citrus waste on the production of polygalacturonase and pectin lyase by *Aspergillus* sp 391 was studied in submerged fermentation (Table 3). The PNL and PG production from *Aspergillus* sp 391 in a medium containing 4% (w/w) citrus waste presented maximum values after 7 days of fungal growth. Highest relative enzymes activities of for pectin lyase and for polygalacturonase were obtained in medium contained 4% of citrus waste and at pH 5.5 and 4.5, respectively.

4. Conclusion

In the present work, we isolated ten strains of *Aspergillus* sp. of soil with potential application in the production of pectinolytic enzymes and identified as *Aspergillus*. The production of PG and PNL enzymes were realized by SmF cultivation. The highest PNL activity was obtained in the assay with 5% (w/v) pectin by *Aspergillus* sp 391 strain without aeration after seven days of fermentation, while the highest exo-PG activities were obtained in the assay with 4% of pectin or citrus waste. Further investigations will be required to achieve a maximum amount of PG and PNL enzymes by optimization of medium contained different wastes and enzymes kinetics studies.

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RESUMO

Avaliação de fungos produtores de pectinase isolados do solo e uso de resíduos de laranja como substrato para a produção de pectinase

As pectinases são enzimas importantes por suas aplicações potenciais em diferentes indústrias, como ração animal, agricultura, têxtil, bebidas, processamento de alimentos, extração de óleo, etc. Dez espécies de fungos foram isoladas do solo e examinadas para produção da enzima pectinase usando a pectina meio de ágar. A produção das enzimas pectinolíticas foi realizada a uma temperatura de 30 ° C e as atividades foram determinadas após uma cultura de *Aspergillus* sp. 391 e *Aspergillus* sp. 031, por sete dias, em meio básico contendo 2% de pectina cítrica e como única fonte de carbono. O extrato enzimático mostrou uma atividade ótima para exo-poligalacturonase (PG) e pectina-liase (PNL) contra ácido galacturônico e pectina a pH 4,5 e 5,5, respectivamente. Houve variações nos níveis de enzimas PG e PNL produzidas em filtrados de cultura obtidos de *Aspergillus* sp. 391 com adição de resíduos cítricos (2,0 e 4,0% p / v) ao meio. A atividade máxima para a atividade do PNL foi observada no meio contendo 5% de pectina ou 4% de resíduos cítricos, como única fonte de carbono, após 7 dias de crescimento. Os resultados mostraram que o isolado *Aspergillus* sp. 391 é promissor para a produção de enzimas pectinolíticas em nível industrial.

Palavras-chave: *Aspergillus* sp. Pectinase. Produção de Enzimas. Atividade.

5. REFERENCES

Aguilar G, Huitron C. Stimulation of the extracellular production of pectinolytic activities of *Aspergillus* sp. by galacturonic acid and glucose addition. Enzyme Microb Technol. 1987;9(11):690-6. http://dx.doi.org/10.1016/0141-0229(87)90129-3.

Alaña A, Alkorta I, Domínguez JB, Llama MJ, Serra JL. Pectin lyase activity in a *Penicillium italicum* strain. Appl Environ Microbiol. 1990;56(12):3755-9. http://dx.doi.org/10.1128/AEM.56.12.3755-3759.1990. PMid:16348377.

Banakar SP, Thippeswamy B. Isolation, production and partial purification of fungal extracellular pectinolitic enzymes from the forest soil of Bhadra Wildlife Sanctuary, Western Ghats of Southern India. J Biochem Technol. 2012;3:138-43.

Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of the protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72(1-2):248-54. http://dx.doi.org/10.1016/0003-2697(76)90527-3. PMid:942051.

Camargo LA, Dentillo DB, Cardello L, Gattas EAL. Utilização de bagaço de laranja na produção de pectinases de *Aspergillus* sp. Aliment Nutr. 2005;16:153-6.

Fawole OB, Odunfa SA. Some factors affecting production of pectic enzymes by *Aspergillus niger*. Inter Biodeter Biodegr. 2003;52(4):223-7. http://dx.doi.org/10.1016/S0964-8305(03)00094-5.

Fonseca MJV, Said S. The pectinase produced by *Tubercularia vulgaris* in submerged culture using pectin or orange-pulp pellets as inducer. Appl Microbiol Biotechnol. 1994;42(1):32-5. http://dx.doi.org/10.1007/BF00170220.

Fontana RC, Salvador S, Silveira MM. Influence of pectin and glucose on growth and polygacturonase production by *Aspergillus niger* in solid-state cultivation. J Ind Microbiol Biotechnol. 2005;32(8):371-7. http://dx.doi.org/10.1007/s10295-005-0004-0. PMid:16059783.

Galiotou-Panayotou M, Kapantai M, Kalantzi O. Growth conditions of *Aspergillus* sp ATHUM-3482 for polygalacturonase production. Appl Microbiol Biotechnol. 1997;47(4):425-9. http://dx.doi.org/10.1007/s002530050951. PMid:9163956.

Gattas EAL, Canguçu UM, Ramos WS. Isolamento de fungos produtores de enzimas pectinolíticas. Rev Ciênc Farm. 2003;24:33-7.

Gattas EAL, Teresawa T, Ramos WS. Producion de pectina liasa utilizando resíduos de la industria cítrica. Alimentacion y Cultura. 1999;2:920-9.

Leuchtenberger A, Mayer G. Changed pectinase synthesis by aggregated mycelium of some *Aspergilllus niger* mutants. Enzyme Microb Technol. 1992;14(1):18-22. http://dx.doi.org/10.1016/0141-0229(92)90020-O.

McCready RM, McComb EA. Extraction and determination of total pectic materials. Anal Chem. 1952;24(12):1986-8. http://dx.doi.org/10.1021/ac60072a033.

McKay AM. A plate assay method for the detection of fungal poligalacturonase secretion. FEMS Microbiol Lett. 1988;56(3):355-8. http://dx.doi.org/10.1111/j.1574-6968.1988.tb03206.x.

McMillan GP, Johnston DJ, Pérombelon MCM. Purification of homogeneity extracellular polygalacturonase and isoenzymes of pectate lyase of *Erwinia carotovora sub* sp. *Atroseptica* by column chromatography. J Appl Bacteriol. 1992;73(1):83-6. http://dx.doi.org/10.1111/j.1365-2672.1992.tb04974.x.

Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem. 1959;31(3):426-8. http://dx.doi.org/10.1021/ac60147a030.

Okafor UA, Okochi VI, Chinedu SN, Ebuehi OAT, Onygene-Okerrenta BM. Pectinolytic activity of wild-type filamentous fungi fermented on agro-wastes. Afr J Microbiol Res. 2010;4:2729-34.

Pashova S, Slokoska L, Krumova E, Angelova M. Induction of polymethylgalacturonase biosynthesis by imobilized cells of *Aspergillus niger*. Enzyme Microb Technol. 1999;24(8-9):535-40. http://dx.doi.org/10.1016/S0141-0229(98)00152-5.

Reddy PL, Screeramulu A. Isolation, purification and screening of pectinolytic fungi from different soil samples of chittoor district. Inst J Life Sc Pharm Res. 2012;1:186-93.

Rivas B, Torrado A, Torre P, Converti A, Domínguez JM. Submerged citric acid fermentation on orange peel autohydrolysate. J Agric Food Chem. 2008;56(7):2380-7. http://dx.doi.org/10.1021/jf073388r. PMid:18321055.

Sandhya R, Kurup G. Screening and isolation of pectinase from fruit and vegetable wastes and use of orange waste as a substrate for pectinase production. Int Res J Biol Sci. 2013;2:34-9.

Sandri IG, Fontana RC, Barfknecht DM, Silveira MM. Clarification of fruit juices by fungal pectinases. Lebensm Wiss Technol. 2011;44(10):2217-22. http://dx.doi.org/10.1016/j.lwt.2011.02.008.

Solís-Pereira S, Favela-Torres E, Viniegra-González G, Gutiérrez-Rojas M. Effects of different carbon sources on the synthesis of pectinase by *Aspergillus niger* on the submerged and solid fermentations. Appl Microbiol Biotechnol. 1993;39(1):36-41. http://dx.doi.org/10.1007/BF00166845.

Tahara T, Doi S, Shinmyo A, Terni G. Translational repression in the preferential synthesis of some mould enzymes. Ind J Ferment Technol. 1972;50:655-61.

US Food and Drug Administration – FDA. Microorganisms & microbial-derived ingredients used in food (Partial list). Silver Spring: FDA; 2015.

Yadav S, Yadav PK, Yadav D, Yadav KDS. Pectin lyase: a review. Process Biochem. 2009;44(1):1-10. http://dx.doi.org/10.1016/j.procbio.2008.09.012.

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