

Production of Pectin Lyase by *Aspergillus niveus* under Submerged and Solid State Fermentations Using Agro-Industrial Residues as Carbon Sources

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Agricultural wastes are rich in sugars and other nutrients that can support the growth of microorganisms which may in turn produce a variety of technologically valuable enzymes, such as pectin lyase (PL). This work reports the production of PL *A. niveus* grown under Submerged (SbmF) and Solid State Fermentations (SSF), with agro-industrial wastes or citrus pectin as substrate. *A. niveus* produced high levels of PL under SbmF using Czapeck medium supplemented with citrus pectin, after 4 days at 40 °C, without agitation. Besides citrus pectin, 16 other carbon sources were also tested. Apple peel provided the highest PL levels, followed by orange peel and passion fruit peel. Other agricultural residues were used for SSF, among them wheat bran which showed to be the best PL inducer, followed by apple peel. PL had maximum activity at 55 °C, was stable at 50 °C with the addition of 1% PEG and maintained 50% activity at 70 °C adding 1% sorbitol, during 30 min. These results suggested that the production of PL by *A. niveus* can be obtained at low cost using inexpensive residues resulting from agro-industrial activities. Furthermore, the thermal stability of this enzyme is promissory for eventual exploration in industrial processes.

Keywords: Pectin lyase, *Aspergillus niveus*, Agro-industrial residues, Submerged fermentation, Solid-state fermentation

INTRODUCTION

Pectin is the generic name given to complex structures that have pectic acids as their main component. When interlined with other structural polysaccharides and proteins, these pectic acids form an insoluble compound known as protopectin. Pectin is rich in galaturonic acids (GalA) and three polysaccharide distinct domains are

evident: homogalacturonan (HGA), which can be methyl-, rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II) (Willats et al., 2006). Recently also was described other structure namely xylogalacturonan (XGA) (Round et al., 2010).

Pectinases or pectinolytic enzymes hydrolyze pectic substances, and can be divided into two main groups: (i) pectinesterases which are able to de-esterify pectin by removal of methoxyl residues and (ii) depolymerases which readily split the main chain. The depolymerizing enzymes are divided into polygalacturonase (PG), which cleave the glycosidic bonds by hydrolysis, and into lyases, which break the glycosidic bonds by trans-elimination (Blanco et al., 1999). Regarding PL, it is receiving major attention because of its application in the depectinization of fruit juice, since it is the only enzyme capable of directly depolymerizing pectins. In fact, PL exerts its action directly on highly esterified pectins (i.e. apple pectin) through a trans-elimination cleavage of

ABBREVIATIONS

GalA; Galaturonic Acid, HGA; Homogalacturonan, PDA; Potato Dextrose Agar, PG; Polygalacturonase, PL; Pectin Lyase, RG-I; Rhamnogalacturonan-I, RG-II; Rhamnogalacturonan-II SbmF; Submerged Fermentations, SSF; Solid State Fermentations, XGA; Xylogalacturonan

glycosidic bonds (Busto et al., 2006; Spagna et al., 1995).

Pectic enzymes can be applied in food industry, as in fruit ripening, clarification and diminution of the viscosity in juices, grape treatment in wine manufacturing, tomato pulp extraction, tea fermentation, vegetable waste treatment, textile and paper manufacture, animal feed, and oil extraction (Uenojo and Pastore, 2007). PL decreases the juice viscosity without the need of a preliminary de-esterification, which usually precedes hydrolytic cleavage of pectin. Furthermore, it enables juice clarification without the destruction of volatile ester components, conferring specific fruit fragrance to the product and its use does not generate methanol during the manufacturing process (Sinitsyna et al., 2007).

The inevitable population and economic growth generates an increasing alimentary demand and, consequently, production of residues. Thus, its environmental effect constitutes one of the problems of food security/safety. It is believed that half of the food produced is discarded before and after it reaches consumers and one third to half of waste comes from the food sector. Then, there is an increasing interest in recycling these residues in order to add value to the product and to diminish environmental contamination (Lin et al., 2009). Yearly, 45 million tons of vegetables, fruits, and grain products are lost to waste in the United States, with costs approximately \$1 billion. Also, the United Kingdom produces 20 million tons of food waste annually (Kosseva, 2009). According to the data of Brazil's Ministry of Agriculture and Food Supply, wheat production in 2005 was 4,659 thousand tons. The production of apple, orange, and passion fruit was 19,184 thousand tons, where the citric fruit production was greater, with 17,868 thousand tons (Lin et al., 2009). It is believed that up to 96% of orange production is consumed and 50% of this is formatted residue (Alexandrino et al., 2007), used mostly in animal feed.

Agricultural wastes are rich in sugars which can be easily assimilated by microorganisms. This means that residues, like sugarcane bagasse, apple pomace, fruit peels and cereal straws, can be used as carbon sources in SSF to obtain industrially useful products, and can help reduce pollution problems (Couto, 2008; Soccol and Vandenberghe, 2003). Fungi have the ability to degrade lignocellulosic materials due to their efficient enzymatic systems (Sánchez, 2009). In this sense, *Aspergillus niveus*, showed great capacity to produce large amounts of hydrolytic enzymes, like xylanases (Peixoto-Nogueira et al., 2009) and amylases (Silva et al., 2009) when cultivated in media supplemented with agricultural wastes. The aim of this study was to define the conditions for PL production by *A. niveus* under SbmF and SSF using fruit peels and other agro-industrial residues as carbon sources.

MATERIALS AND METHODS

Organism and growth conditions in SbmF

Aspergillus niveus Saito strain was isolated from *Mangifera indica* in our laboratory. The microorganism was identified and deposited in the culture collection of Pernambuco Federal University (PE, Brazil). The organism was maintained on slants of Potato Dextrose Agar (PDA) medium at 30 °C. A quantity of 5×10^6 conidia from these cultures was inoculated into 125-mL Erlenmeyer flasks containing 25 mL of liquid Czapeck medium (Wiseman, 1975) with 1.0% citric pectin Sigma-Aldrich (w/v) or with different agro-industrial residues, as described in Results. The fungus was incubated at 40 °C, under static conditions, for 4 days. After that, the cultures were filtered through Whatman # 1 in a Buchner funnel. The filtrate was saved as a source of crude extracellular PL. Mycelial pads were grounded with glass beads (425 – 600 microns), at 4 °C with 10 vol. of cold 100 mM sodium acetate buffer, pH 6.0. After centrifugation (15,000 x g, 15 min, 4 °C) the supernatant fraction was the source of crude intracellular PL.

Culture condition on SSF

The fungus was inoculated (5×10^6 conidia) on SSF medium, composed by 4 g of different agro-industrial residues plus 2 mL sterile distilled water. The cultures were incubated at 40 °C, for 7 days, at 76 % relative humidity. After that, the medium was extracted with 50 mL of distilled water, for 30 min. at 100 rpm, at 4 °C, followed by vacuum filtration. The filtrate was centrifuged at 15,000 x g, 4 °C, to remove solid residues and the supernatant was used as crude enzyme source.

Enzymatic assays and protein determination

PL was assayed according to Pitt (1988). The reaction mixture consisted of 5.0 mL of 1.0 % pectin solution in 0.05 M Tris-HCl buffer, pH 8.5; 1.0 mL of 0.01 M CaCl_2 ; 1.0 mL of crude enzyme and 3.0 mL of distilled water. After incubation for 2 hours at 30 °C in water bath, 0.6 mL of 9 % $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.6 mL of 0.5 M NaOH were added. The precipitated proteins were removed by centrifugation at 3,000 g, for 10 min. A volume of 1.0 mL of supernatant was removed and added into a mixture composed by 3.0 mL of 0.04 M thiobarbituric acid; 1.5 mL of 0.1 M HCl and 0.5 mL of distilled water. After boiling for 30 min. and posterior cooling, the absorbance was measured at 550 nm. A unit was defined as the amount of enzyme that changes 0.1 absorbance unit, under the assay conditions. Protein was assayed according to

Table 1. Time-course of growth and PL produced by *A. niveus*.

Time (h)	PL Intracellular (total U)	PL Extracellular (total U)	Mycelial extract Protein (total mg)*
24	70.0 (± 1.0)	0.1 (± 1.0)	3.5 (± 0.221)
48	30.0 (± 3.0)	30.0 (± 1.0)	3.2 (± 0.122)
72	120.0 (± 1.0)	70.0 (± 1.0)	3.1 (± 0.179)
96	140.0 (± 3.0)	150.0 (± 1.0)	1.8 (± 0.170)

* Growth expressed by intracellular protein.

Lowry *et al.* (1951) using bovine serum albumin as the standard. Total activity and total protein are represented by U/mL and mg/mL, respectively, multiplied by the total volume of enzyme extract.

Preparation of agro-industrial residues

Several residues were used, as orange peel, lemon peel, passion fruit peel, apple peel, commercial mate herb (*Ilex paraguariensis*), sugarcane bagasse, corncob, rice straw, and wheat bran. The first stage of the treatment consisted in drying the residues for 3 days at 50 °C. Afterwards, the fruit peels and corncob were crushed with a pestle in a porcelain mortar until the fragments to reach 0.1 to 0.3 mm.

Reproducibility of the results

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

RESULTS

Time course of PL production under SbmF

A. niveus was incubated without agitation for up to 96 h at 40 °C in Czapeck medium supplemented with 1 % citric pectin (w/v) as carbon source. The maximum growth occurred at 24 h, corresponding at 3.5 mg total intracellular protein (Table 1). On the other hand, PL maximum secretion occurred after 96 h, totaling 150 Units.

Effect of carbon source concentration and agro-industrial residues on growth and enzymatic production under SbmF in *A. niveus*

In order to optimize the enzymatic production and growth of *A. niveus*, the Czapeck medium was supplemented with citrus pectin in different quantities and the cultures were performed without agitation. Growth was expressed as total intracellular proteins. The best citric pectin concentration for growth was 3.5 % (Figure1A), and for PL production was 2.0 % (Figure1B). Therefore, PL production was analyzed supplementing Czapeck medium with 16 different agro-industrial residues in same growth conditions. Apple peel was the most effective waste inducer carbon source, increasing up to 5.21-fold the PG production compared with Sigma-Aldrich citrus pectin as control, followed by orange peel and passion fruit peel (Table 2).

Effect of agro-industrial residues on growth and enzymatic production under SSF in *A. niveus*

In this experiment several agro-industrial residues of low economic value were tested, including Sigma-Aldrich citrus pectin, which was used as a control. Wheat bran showed to be the best substrate, inducing more PL production than the control, followed by apple peel and Sigma-Aldrich citrus pectin. Other residues, as citrus fruit peels, had low inducing effect (Table 3).

Effect of temperature on PL activity

The optimal temperature for extracellular PL activity was 55 °C (Figure 2A), but the enzyme was not stable at 50

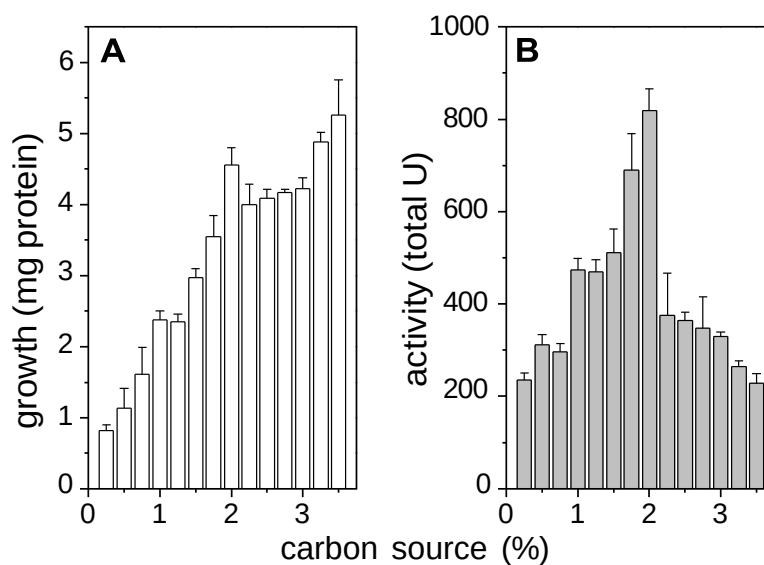


Figure 1. Effect of carbon source concentration on growth and enzyme production of *A. niveus*. (A) Growth; (B) PL production

Table 2. Effect of different carbon sources on the PL production in SbmF from *A. niveus*.

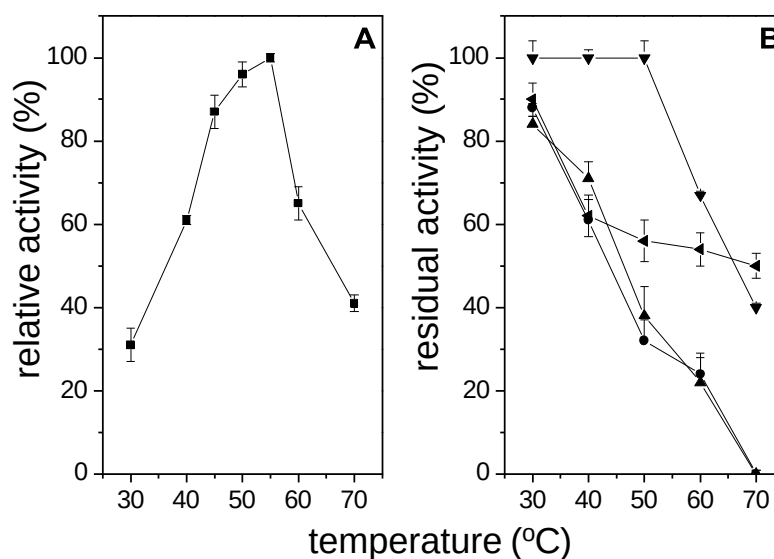
Carbon Source	Growth (mg protein)	Activity (Total U)
Glucose	0.1	150
Monogalacturonic acid	0.1	175
Polygalacturonic acid	0.2	225
Pectin SIGMA	1.9	350
Pectin 7128*	2.5	425
Pectin USP-B*	2.5	350
Pectin JMH6*	2.5	150
Pectin GENU® 8001*	2.2	325
Pectin GENU® 8003*	2.6	175
Apple peel	4.5	1825
Orange peel	2.4	1275
Lemon peel	3.1	450
Passion fruit peel	3.6	1075
Commercial mate herb**	3.2	125
Sugarcane bagasse	1.6	150
Corn cob	1.2	100

*8001/8003: From CPKelco Brasil S/A

***Ilex paraguariensis* milled

Table 3. Effect of different carbon sources on the PL production in SSF from *A. niveus*.

Carbon Source	Growth (mg protein)	Activity (total U)
Pectin SIGMA	0.592 (± 0.03)	90 (± 1)
Pectin Vetec	0.627 (± 0.02)	66 (± 19)
Pectin GENU	1.117 (± 0.06)	38 (± 15)
Orange peel	0.971 (± 0.06)	38 (± 13)
Lemon peel	0.751 (± 0.04)	29 (± 4)
Passion fruit peel	0.835 (± 0.18)	63 (± 18)
Apple peel	0.954 (± 0.06)	90 (± 3)
Rice straw	0.105 (± 0.01)	52 (± 22)
Wheat bran	0.484 (± 0.06)	111 (± 20)
Corn cob	0.260 (± 0.04)	49 (± 11)
Sugarcane bagasse	0.124 (± 0.03)	55 (± 9)

**Figure 2.** Effect of the temperature for PL (A) and thermal stability of PL in function of time of incubation for 30 min. in the substrate absence (B). Symbols: (●) enzyme without additives, (▲) glycerol, (▼) PEG and (◄) sorbitol

°C, 30 min., showing about 30% of residual activity (Figure 2B). However, when 1% PEG was added, the enzyme activity was 100% stable at this temperature. With the addition of sorbitol, PL still maintained 50% activity at 70 °C.

DISCUSSION

A. niveus showed to be a good pectinase producer, having high enzymatic and growth levels. Patil and Dayanand (2006) and Friedrich et al. (1994) showed

similar values for pectinases production cultivating a different strain of *A. niger* under SbmF and SSF. Therefore, *A. giganteus* (Pedrolli and Carmona, 2009) under SbmF showed highest activity levels with 11 day old standing cultures. This result demonstrates the capacity of *A. niveus* in faster PL production, thus reducing fermentation time and consequently, reducing cost for the industry.

The microorganism studied showed parallelism between growth and enzyme production up to 2.0 % carbon source, but at higher pectin concentrations there was an accentuated drop in the enzyme production,

probably due to end-product inhibition. The use of agro-industrial residues as substrate for microbial fermentations in industrial scale may be convenient, once their utilization reduces costs and aggregate value to the fermentation products, and additionally reducing pollution problems. Peel cultures of *A. niveus* have higher levels of PL compared with those of pectin. This occurs probably due to the presence of soluble polysaccharides, such as glucose, fructose, and sucrose, which stimulate fungal growth. The PL produced by *A. giganteus* with orange waste (Pedrolli and Carmona, 2009), also showed the highest activity in cultures that were 192 h old. In this sense, fruit peels, especially apple peels, considerably stimulated PL production by *A. niveus*, reaching higher levels than with citrus pectin. This result showed the high potential of fruit peels to induce PL production, decreasing costs and remaking residues.

Several fungi show high levels of enzymatic production under SSF; however, this fermentation requires a larger amount of substrates. Therefore, strategies have been developed aiming to utilize low cost substrates to induce enzymes production by *A. niveus*. This fungus had excellent PL production in this condition, up to 111 U/mL. Martin et al. (2004) reported the use of cane bagasse and wheat bran mixture to PL production by *Moniliella* sp SB9 and *Penicillium* sp EGC5. Also, Silva et al. (2005) described a PL production of 41.30 U/mL growing *Penicillium viridicatum* RFC3 in the orange bagasse and wheat bran mixture. Further, *A. niveus* showed more enzymatic production under SbmF. On the other hand, Acuña-Argüelles et al. (1995) described that *A. niger* produced more enzyme amounts under SSF than under SbmF. Wheat bran is an agricultural sub product, with less aggregated value and generally used as a supplement in alimentary diets or animal feed, as a source of natural fibers (Kuan and Liong, 2008). So, the biotechnological application of this residue could be interesting for enzyme industrial production

A. niveus is a thermotolerant fungus (Taj-Aldeen and Alkenany, 1996) as previously confirmed in our experiments (Betini et al., 2009). The PL secreted by it presented activity in relatively high temperatures but had relatively little thermo-stability. However, the PEG and sorbitol addition increased this stability. It is believed that these compounds dislocate the water molecules of the region of solvation, favoring the hydrophobic interactions between non-polar amino acids, stabilizing the three-dimensional structure of protein and protecting the catalytic site of the enzyme.

CONCLUSION

In this context, the results of this work suggest that high

levels of PL can be produced by *Aspergillus niveus* with low cost carbon sources, as apple peel and wheat bran, using SbmF or SSF. PL could have a good industrial applicability in relatively high temperatures. Furthermore, the use of these agro-industrial residues would be beneficial both to the agricultural producer for providing value at a low cost, and to nature, for giving a biotechnological application to waste, decreasing environmental contamination.

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