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SCREENING OF BACTERIAL STRAINS FOR PECTINOLYTIC ACTIVITY: CHARACTERIZATION OF THE POLYGALACTURONASE PRODUCED BY *BACILLUS* SP

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ABSTRACT

One hundred sixty eight bacterial strains, isolated from soil and samples of vegetable in decomposition, were screened for the use of citrus pectin as the sole carbon source. 102 were positive for pectinase depolymerization in assay plates as evidenced by clear hydrolization halos. Among them, 30% presented considerable pectinolytic activity. The cultivation of these strains by submerged and semi-solid fermentation for polygalacturonase production indicated that five strains of *Bacillus* sp produced high quantities of the enzyme. The physico-chemical characteristics, such as optimum pH of 6.0 – 7.0, optimum temperatures between 45ºC and 55ºC, stability at temperatures above 40ºC and in neutral and alkaline pH, were determined.

Key words: *Bacillus* sp, polygalacturonase, pectinolytic activity

INTRODUCTION

Many plant-pathogenic bacteria and fungi are known to produce pectolytic enzymes useful for invading host tissues. Moreover, theses enzymes are essential in the decay of dead plant material by nonpathogenic microorganisms and thus assist in recycling carbon compounds in the biosphere (2).

Pectinases include depolymerizing and demethoxylating enzymes. Depolymerizing enzymes are polygalacturonase (EC 3.2.1), which cleaves the α-1,4 glycosidic bonds between two galacturonic acid residues, and pectin-lyase (EC 4.2.2), which catalyses a β-elimination reaction between two methylated residues (3). De-esterifying enzymes include pectin-esterase (EC 3.1.1), which catalyses the demethoxylation of methylated pectin, producing methanol and pectin (20).

Preparations containing pectin-degrading enzymes have been extensively used to improve the stability of fruit and vegetable nectars and in the clarification of fruit juices and wines (5, 13, 21, 23, 24). Currently, they are widely used in industry for retting of natural fibers and extraction of oils from vegetable and citrus peels (4, 6).

The enzymes preparations used in the food industry are of fungal origin because fungi are potent producers of pectic enzymes and the optimal pH of fungal enzymes is very close to the pH of many fruit juices, which range from pH 3,0 to 5,5 (26). Such preparations are not suited for production of vegetable purées or other preparations in which pH

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values are close to neutral (7). Furthermore, due to the relatively low temperature stability of the fungal enzyme preparations, maceration needs to be carried out at temperatures not exceeding 45ºC, necessitating the incorporation of a pasteurization step to limit the growth of mesophilic microorganisms (22).

The present investigation was on pectinolytic activities of bacteria strains isolated from Brazilian soil and samples of vegetable in decomposition.

MATERIALS AND METHODS

Microorganisms: portions of 5g of samples of soil, from agricultural and vegetable wastes were pooled and homogenized in sterile medium with pH 6.0 containig 1% citrus pectin with 67% of metoxilation, 0.14% $(NH_4)_2SO_4$, 0.20% K_2HPO_4, 0.02% $MgSO_4$ $7H_2O$ and 0.10% nutrient solution (5 mg/L $FeSO_4.7H_2O$; 1.6 mg/L $MnSO_4$. H_2O; 1.4 mg/L $ZnSO_4$. $7H_2O$; 2.0 mg/L $CoCl_2$). A loop of the homogenate was then streaked onto nutrient medium and incubated at 30ºC for 24 to 72h. All morphological contrasting colonies were purified by repeated streaking. Pure cultures were sub-cultured onto slants media and maintained for identification and enzymes studies. Identification of the genus was based on morphological and biochemical characteristics (15).

Plate assay of depolimerized pectin: The medium was the same used for isolation of cultures, supplemented with 2% agar. Pure culture was inoculated by puncture in the medium and incubated for 48h at 30ºC. After the colonies reached around 3 mm, iodine-potassium iodide solution (1.0g iodine, 5.0g potassium iodide and 330ml H_2O) was added to detect clearance zones (11).

Production of pectic enzyme on solid-state (SSF) and submerged (SmF) fermentation: Strains presenting large clearing zones were used for enzyme production assays on liquid and solid medium. The liquid medium containing 1% citrus pectin, 0.14% $(NH_4)_2SO_4$, 0.6% K_2HPO_4, 0.20% KH_2PO_4 and 0.01% $MgSO_4$ $7H_2O$, pH 6.0 was inoculated with a suspension containing 10^6 cells/ml. Cultures were grown in 125ml Erlenmeyers flasks with 25 ml of medium in a rotary shaker (150rpm) at 30ºC. After 48h the biomass was separated by centrifugation at 1000xg for 20 min and the supernatant was used to evaluate polygalacturonase (PG) activity. The SSF was done using a 250 ml Erlenmeyer flask containing 5g of wheat bran and 10 ml of 1% $(NH_4)_2SO_4$ and 0.02% $MgSO_4$ (67% of moisture). 10^6 cells per gram of wheat bran were added to each flask and maintained at 30ºC. After 72h, fermented material was mixed with 40 ml distilled water and filtered at vacuum and centrifuged. The supernatant was used to evaluate PG activity.

Assay of polygalacturonase (PG) activity: PG activity was determined by measuring the release of reducing groups using the dinitrosalicylic acid reagent DNS assay (18). The reaction mixture containing 0.8 ml of 1% citric pectin with 67% of metoxilation in 0.2M citrate-phosphate, pH 6.0 buffer and 0.2 ml of culture supernatant, was incubated at 40ºC for 10 min. One unit of enzymatic activity (U) was defined as 1 µmol of galacturonic acid released per minute.

RESULTS

1- Selection of strains with pectinolytic activity:

168 bacterial strains able to grow on medium containing citrus pectin as the only carbon source were isolated. 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 (14), good producers when the halos were of at least 1 cm (36), weak producers when halos were at least 0.5 cm (52) and poor producers when no pectinolytic activity and no clear lysis zones were observed (66).

2- Production of poligalacturonase from *Bacillus* sp isolated on liquid and semi-solid medium

Five *Bacillus* sp demonstrated high pectinolytic activity when assayed by the plate method. These strains were cultivated for polygalacturonase production using SSF and SmF. The data obtained are presented in Table 1.

Table 1. Production of polygalacturonase (U / ml) by *Bacillus* sp in solid-state (SSF) and submerged (SmF) fermentation

Microorganism	SSF	SmF
Bacillus sp Ar1.2	0.8	3.5
Bacillus sp B1.3	0.4	3.0
Bacillus sp M2.1	0.7	4.0
Bacillus sp P4.3	0.3	4.0
Bacillus sp P6.1	0.4	2.7

Extracts from SSF cultures showed higher pectionolytic activities than those obtained by SmF. Maximal polygalacturonase activity was obtained for strains M2.1 and P4.3.

Physico-chemical characteristics of PG

The effect of pH on the activity of PG produced by the *Bacillus* sp strains in SSF was determined by using reaction mixtures at pH values ranging between 3.0 and 10.0 (Fig.1). Pectinolytic activity was the highest at pH 6.0 for all strains except for *Bacillus* P4.3, which presented optimal pH between 6.5 and 7.0.

Assay for determination of the optimal temperature for PG activity indicated maximal activities at 40-50ºC for AR1.2, B1.3 and P4.3 strains and 50-55ºC for M2.1 and P6.1 strains (Fig 2). PG from AR1.2 showed the largest spectrum of activity as a function of temperature.

To determine the effect of temperature on the stability of PG, enzyme solutions were incubated for 60 min at temperatures between 10ºC and 70ºC and the residual activity was assayed after cooling. Practically no changes in pectinolytic activities were observed when the incubation temperature was lower than 50ºC. The PG of P4.3 was stable for 1h at 60ºC (Fig. 3), and the results indicated that the PG activity was similar to the previously obtained values.

Results of the incubation of enzyme solutions at pH values between 3.0 and 10.0 for up to 24 h at 25ºC indicated that these PG were stable in the pH range 6-8. PG P4.3 was stable between pH 7 and 9 (Fig. 4).

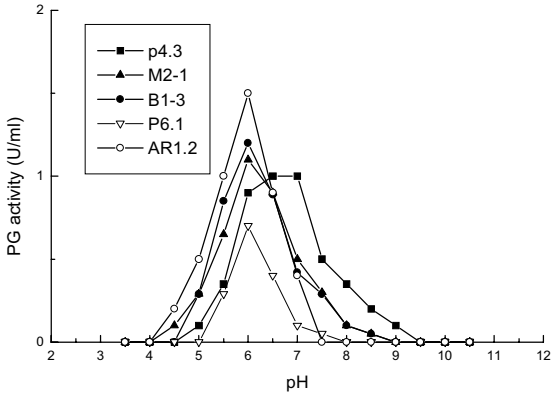

Figure 1: Effect of pH on the polygalacturonase activity produced by *Bacillus* sp on wheat bran. The buffers used were sodium acetate (pH 3.5-5.5), citrate-phosphate (pH 5.5-7.0), tris-HCl (pH 7.0-8.5) and Gly-NaOH (pH 8.5-10.5).

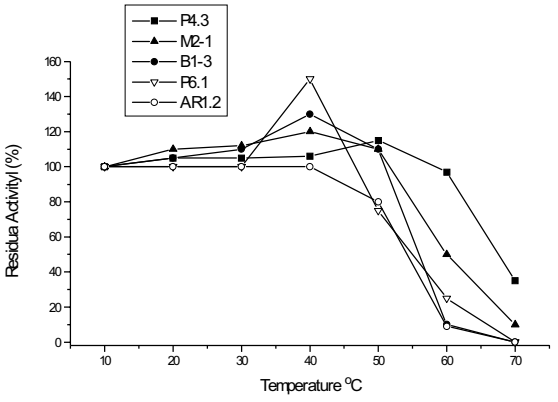

Figure 3: Stability of PG against temperature. The enzymatic solutions were maintained in the temperature range of 10 to 70ºC, for 1h. 110% of activity (U/ml): P4.3=3.5; M2.1=2.5; B1.3=3.2; P6.1= 2.3; Ar1.2=3.3

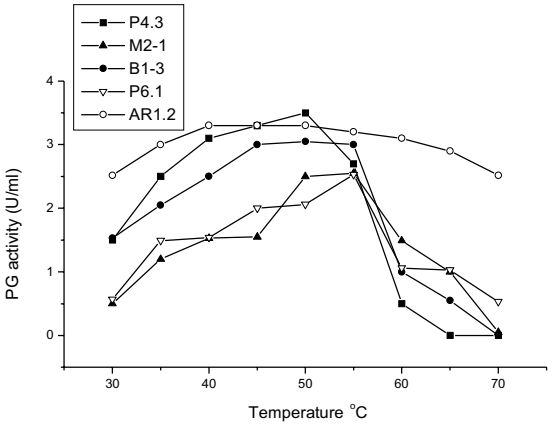

Figure 2: Effect of temperature on the polygalacturonase activity produced by *Bacillus* sp on wheat bran.

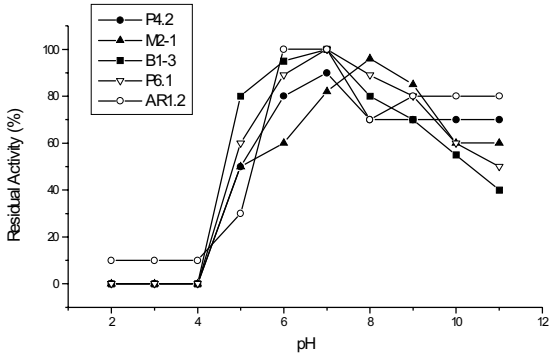

Figura 4: Stability of the PG against pH. The enzymatic solutions were dispersed on 0.1M buffers solutions at various pH values and maintained at 25°C for 24 h. An aliquot was assayed for the residual activity as described in the text. 110% of activity (U/ml): P4.3=3.5; M2.1=2.5; B1.3=3.2; P6.1= 2.3; Ar1.2=3.3

DISCUSSION

Production of pectinases in solid medium was higher than that produced in liquid medium. These results are similar to those obtained with endoglucanases and β- glucosidades (14), xylanases (8), α-amilases (17) and pectinases produced by *A. niger* (1, 25).

Activities of PG produced by *Bacillus* sp strains were higher than those produced by *Aspergillus niger* (19), *Aspergillus sp* and *A. niger* ATCC20107 (16), *Aureobasidium pullulans* (10) and *Tubercularia vulgaris* (12). However, they were lower than those reported using *Bacillus* GK-8 (9).

The pectinolytic activity of the crude solution has specific properties which may offer advantages over currently available pectinase preparations. The enzyme solution can be applied directly to vegetables without the need for pH modification. Furthermore, because of the temperature stability of the enzyme, it can be possibly used at processing temperature of 50ºC, which is sufficient to limit the growth mesophilic contaminants (7) in the process.

RESUMO

Seleção de linhagens bacterianas para atividade pectinolítica: caracterização das poligalacturonases produzidas por *Bacillus* sp

A partir de amostras de solo e de material vegetal em decomposição, foram isoladas 168 linhagens bacterianas capazes de utilizar pectina de citrus como única fonte de carbono. Destas, 102 foram positivas para a despolimerização da pectina, através de ensaios em placa de Petri nos quais foram detectados halos claros ao redor das colônias. Entre essas, 30% (50) foram consideradas ótimas ou boas produtoras de poligalacturonase. O cultivo dessas linhagens através de fermentação submersa e semi-sólida para a quantificação das poligalacturonases (PG) produzidas indicou que 6 linhagens de *Bacillus* sp foram as melhores produtoras da enzima. As características físico-químicas, como pH ótimos entre 6,0 e 7,0 e temperaturas ótimas entre 45 e 55ºC, estabilidade à temperaturas acima de 40ºC e a pH neutros e alcalinos foram determinadas.

Palavras-chave: *Bacillus* sp, poligalacturonase, atividade pectinolítica

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