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Effects of Different Carbon Sources on Pectinase Production by *Penicillium oxalicum*

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Abstract. Currently, obtaining enzymatic preparations with low production costs is the ultimate challenge for researchers worldwide. This study compares the productivity of pectinolytic enzymes using *Penicillium oxalicum* strain in submerged and solid state fermentation. Agro-industrial residues used as carbon sources were beet pulp, wheat bran and rapeseed cake. Enzyme production was higher in both fermentation types using wheat bran. Comparing the two types of fermentation, it was observed that enzyme activity is higher in solid state fermentation. The maximal activity was reached after seven days by solid state fermentation. Pectinase activity decreased progressively after 96 days, in solid state fermentation when was used beet pulp and rapeseed cake. Optimum pH and temperature for the crude enzyme activity was obtained by wheat bran in solid state fermentation at 5 and 60°C, respectively. The crude enzyme lost 50% of its activity after 40 minutes, when was heated at 60°C.

Keywords: enzymes, submerged fermentation, solid state fermentation, *Penicillium oxalicum*.

INTRODUCTION

Pectinolytic enzymes are mainly obtained from fungal sources (Alkorta *et al.*, 1997). Polygalacturonase, pectinlyase, pectate lyase and pectinesterase are the main pectinolytic enzyme complexes. These enzymes catalyze the hydrolysis of pectin into sugars (Debing *et al.*, 2006).

Pectinolytic enzymes are acidic and occur as polysaccharides with high molecular weight and negatively charged (Jayani *et al.*, 2005). Chemically speaking, pectic substances are complex colloidal polysaccharide, which is the core of structural polysaccharide chain composed of galacturonic acid residues, residues, connected by α-1, 4 (Pedrolli *et al.*, 2009).

Pectinases can be produced either by submerged or solid-state fermentation (SSF) procedures (Solis-Pereira *et al.*, 1992).The nature of solid substrate is the most important factor in solid state fermentation. This, not only supplies the nutrient to the culture but also serve as anchorage for the growth of microbial cell. The selection of substrate SSF depends upon several factors mainly with the cost of availability and this may involve the screening of several agro-industrial residue. An optimum substrate provides all the necessary nutrients to the microorganism for optimum function (Bhardwaj and Garg, 2010).

Most enzymes used in industry have fungal origin (Piccoli-Valle *et al.*, 2001). The genus *Penicillium* is worldwide known for production of secondary metabolites and extracellular enzymes of commercial value, including pectinases. A new challenge for this area is the production of enzymes with low production cost and desirable characteristics (Banu *et al.*, 2010).

Pectinolytic enzymes are increasingly used in biotechnological processes such as: improved extraction of plant bio-compounds from tomato (Zuorro *et al.*, 2011; Zuorro and Lavecchia, 2010; Choudhari and Ananthanarayan, 2007), raspberry residues (Laroze *et al.*,

2010), grape skin (Pinelo *et al.*, 2006), apple skin (Pinelo *et al.*, 2008), processing of cotton fabric (Solbak *et al.*, 2005)

Selection of a carbon source that allows the production of pectinolytic enzymes, with higher enzymatic activity in a shorter fermentation time using submerged and solid state fermentation, was the objective of the present study.

MATERIALS AND METHODS

Strain characterization. The strain of *Penicillium oxalicum MT7* was provided by The Walloon Centre of Industrial Biology (Gembloux Agro-Bio Tech, Bio-industries Unit , Belgium). The characteristic of this strain are typical for *Penicillium*. This strain belongs at *P. oxalicum species*, and is characterized by an abundant sporulation and a big speed to grow at 25 and 30°C. The cultures were maintained on potato dextrose agar at 4°C by periodic sub culturing. The inoculum was prepared in peptonate water harvesting the spores from 96 h old cultures at 30°C. The conidia were dispersed in peptonate water for successive dilution.

Media and fermentation condition for submerged and solid state fermentation. Media for submerged fermentation (SmF) contained (%) $(NH_4)_2SO_4$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 0.5 (Solis-Pereira *et al.*, 1996) and 7 g carbon source. Beet pulp, wheat bran and rapeseed cake with particle size > 100 mm was used as carbon source. Medium was autoclaved for 20 min at 121°C. Inoculum size and pH were $1.4x10^7$ spores/ml and 5, respectively.

Media for the solid state fermentation (SSF) contained the same ingredients, and the initial moisture value was 60%. In this case medium constituents were dissolved in 150 ml distilled water. The solution was heated at 70°C for 15 min and the carbon source was separately sterilized for 15 min at 121°C. After cooling, both fractions were mixed and inoculated with *Penicillium oxalicum* spore suspension in aseptic conditions. Inoculum size and pH were $1.4x10^7$ spores/g and 5, respectively. Fermentation was carried in 250 ml Erlenmeyer flask for both fermentation types at 30°C on static condition. Fermentation duration was examined from 24 to 144 h. The selection of the substrate for the process of enzyme biosynthesis in solid state fermentation had three main objectives: to be the cheapest agro-industrial product, to be available at any time of the year and to be bought whit out storage problem. They were purchased from shops specialized in selling agro - industrial products.

Enzymatic activity measurements and characterisation. The crude enzyme (CE) was extracted from a known quantity of fermented material which was mixed with an equivalent amount of distilled water and then on a magnetic stirrer for 20 min and centrifuged at 4000 RPM for 20 min. The supernatant containing CE was stored at 4°C for enzymatic assay.

Enzymatic activity was determined by measuring the release of reducing groups from apple pectin using a 3, 5-dinitrosalicylic acid (DNS) reagent assay. The number of reducing groups, expressed as galacturonic acid released by the enzymatic action, was quantified by the dinitrosalicylic acid (DNS) method. 3, 5-dinitrosalicylic acid is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at 540 nm. Enzymatic methods are usually preferred to DNS due to their specificity (Miller G.L., 1959)

The reaction mixture containing 1 ml 1% apple pectin (Sigma-Aldrich, Darmstadt, Germany) in 0.02 M acetate buffer, pH 5.0 and 0.2 ml of crude enzyme solution, was

incubated at 50°C for 10 min. After 1 ml of DNS reagent was added, the mixture was boiled for 5 min in test tubes. Boiled test tubes were passed directly in cold water bath and supplemented with 10 ml of distillate H_2O. After gentle but thorough mixing, was measured the absorbance at 550 nm with a spectrophotometer (PerkinElmer LAMBDA 25 UV/Vis, Shelton,WA, USA). The activity of the enzyme is calculated according to the galacturonic acid release, calculated using the calibration curve (R^2=0.998) and expressed in mg. One unit of enzyme activity (U) was defined as the amount of enzyme which released one mmol of galacturonic acid per minute.

Optimal pH. In order to determine the optimum pH value for the CE, the enzymatic activity was assayed at 50°C, at different pH values between 3.0 and 8.0. Different buffers were used: citrate (pH 3), acetate (pH 4-6) and phosphate (pH 7 and 8).

Optimal temperature. The crude enzyme was incubated with the substrates at different temperatures ranging from 20°C to 70°C, at pH 5. The reaction mixtures were analyzed for pectinase activity.

Thermostability. The CE was allowed to stand at 60°C for different time (10 min until 60 min), and the activity was assayed by the usual procedure at optimum pH and temperature.

RESULTS AND DISCUSSIONS

Enzymatic activity monitorization of pectinase obtained from SmF and SSF on different substrates.

The effect of the carbon source on pectinase production in SmF is shown in Table 1. Enzymatic activities are reported as units per millilitre of crude enzymes for the CE obtained in SmF and units per gram for SSF. In both cases production begins from the first day of incubation. For both fermentation types using three carbon sources, the production of pectinolytic enzymes was evaluated up to 144 h.

In order to study the production of pectinase in SmF on theses three substrates it was observed a progressive increase until the last day of fermentation. Using wheat bran substrate the maximal activity 1.57 U/ml was obtained in the last day of fermentation. These have been followed by beet pulp and rapeseed cake with maximal activities at 1.39 U/ml and 1.23 U/ml, respectively. The results are in agreement with those reported by Solis-Pereira *et. al.*, (1993).

Tab. 1.

Evaluation of fermentation period for the production of pectinase by *P. oxalicum* in SmF using beet pulp, wheat bran and rapeseed cake as substrate.

Time (hours)	Activity (U/ml)		
	Beet pulp	Wheat bran	Rapeseed cake
24	0.54±0.02	0.28±0.00	0.32±0.01
48	0.65±0.00	0.64±0.02	0.37±0.08
72	0.77±0.01	0.88±0.03	0.98±0.02
96	0.96±0.00	1.04±0.01	1.03±0.01
120	1.25±0.01	1.29±0.02	1.15±0.00
144	1.39±0.02	1.57±0.01	1.23±0.04

*Triplicates data were expressed as mean ± standard deviation.

Using the same agro – industrial wastes in solid state fermentation (Table 2), as was expected the enzymatic activity was higher than in submerged fermentation. Like in SmF in the media that used wheat bran as carbon sources was found the higher activity.

A gradual decrease in the production of pectinase over a period up to 96 h was observed in the medium that used beet pulp and rapeseed cake carbon source in SSF. In case of wheat bran the activity increased gradually until last day of fermentation, when was obtained 7.10 U/g. The obtained results are similar to those reported by Silva *et. al.*, (2005).

Tab. 2.

Evaluation of fermentation period for the production of pectinase by *P. oxalicum* in SSF, using beet pulp, wheat bran and rapeseed cake as substrate.

Time (hours)	Activity (U/g)		
	Beet pulp	Wheat bran	Rapeseed cake
24	2.16±0.56	1.12±0.17	1.29±0.38
48	2.62±0.38	2.56±0.49	1.49±0.26
72	4.64±1.02	3.52±0.51	3.94±0.34
96	5.86±0.79	4.97±0.29	4.94±0.98
120	4.77±0.28	5.18±0.94	4.79±0.68
144	4.14±0.42	7.12±1.17	4.65±0.89

*Triplicates data were expressed as mean ± standard deviation

As was expected the maximal activity in SmF for wheat bran was obtained at 144 h, in contrast with a shorter time in SSF (96 h). Solis-Pereira (1993) observed the same differences in pectinases production between SmF and SSF. Also the enzymatic activity was higher in SSF 7.12 U/g, compared with SmF where was obtained only 1.57 U/ml for the same carbon source.

Optimal pH. For enzyme characterization was used crude enzyme obtained from SSF of wheat bran in 144 hours. Determination of optimal pH (Figure 1) was made for six pH values, from pH 3 until 8. The pH of 5 was found to be the optimum obtaining an enzymatic activity of 7.98 U/g.

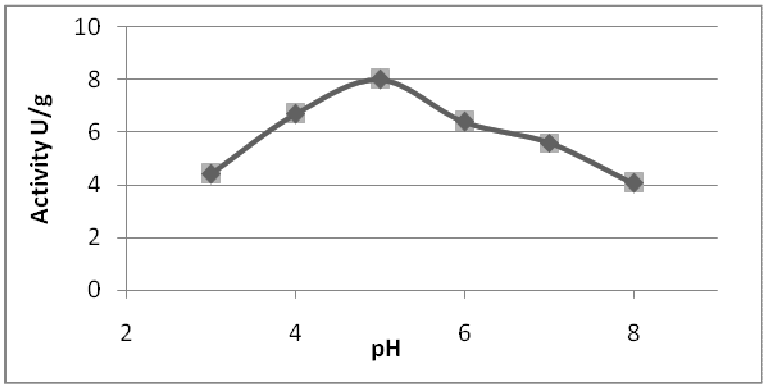

Fig. 1. Enzymatic activity dependent on the pH values (range 3-8)

As shown in Figure 1 the best enzymatic activity was at a pH of 5.0. This finding is similar to that reported in China by Zhang C. (2009) for the three endo-polygalacturonases obtained from *Penicillium oxalicum* (CGMCC 0907). Fermentation preparation of the best

enzymatic activities at an optimal pH of 4.5, 5.0 and 5.5was reported for polygalacturonases from *Sclerotium rolfsii* (PG1 pH 5.0, PG2 pH 4.5) (Schnitzhofer *et al.*, 2007).

Optimal temperature. The effect of different temperatures on CE activity is shown in Figure 2. 60°C was found to be the optimal temperature for the pectinolytic enzymes obtained in SSF, which was close to that reported by Zhang C. (2009). Fermentation preparation of the best enzymatic activities was found to be at an optimal temperature of 65, 55 and 50°C, respectively.

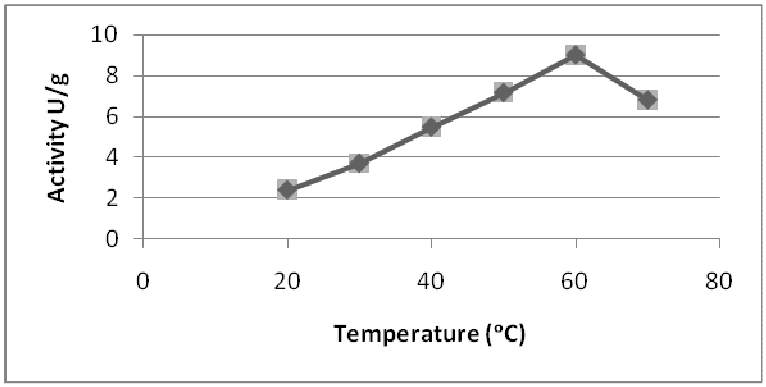

Fig. 2. Enzymatic activity dependent on the temperature (range 20-70°C)

Close to obtained results was polygalacturonases from *Aspergillus kawachii* (50°C) obtained by Esquivel and Voget (2004), but quite different from those reported for endo-polygalacturonase from *Mucor flavus* (45°C) (Gadre *et al.*, 2003).

Termostability. Stability of the enzymatic activity after thermal treatment at 60°C, from 10 to 60 minutes, is shown in Figure 3. In 40 minutes at 60°C the enzymatic activity decreased below 50%.

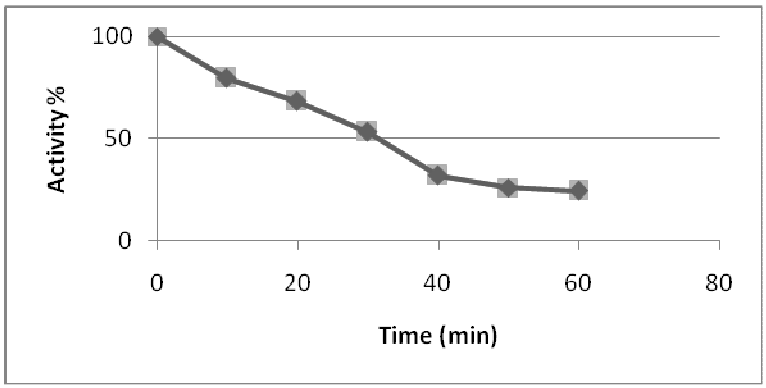

Fig. 3. Evolution of enzyme activity after thermal treatment at 60°C, different time (from 10 to 60 minutes)

The obtained results are different from that reported by Martins *et al.*, (2002), when the polygalacturonase from *T. aurantiacus*, had a 30% residual activity after 4 hours of incubation at 60°C.

CONCLUSIONS

The present study indicates that agro-industrial residues were feasible for pectinase production in submerged and solid state fermentation. Wheat bran is an appropriate carbon source for preparation of pectinolytic enzymes using *Penicillium oxalicum*. Referring to the fermentation, it can be observed that the solid state fermentation had a higher efficiency compared to that of submerged fermentation, and the incubation time to obtain was shorter. The maximal activity was obtained using wheat bran in solid state fermentation followed by rapeseed cake and beet pulp. Pectinase obtained from *P. oxalicum* in solid state fermentation, using wheat bran as substrate, had a high optimum temperature (60°C) and optimum pH was 5. This crude pectinase can be an alternative for the industrial area, where hydrolytic enzymes are used.

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