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Production, purification and characterization of an exo-polygalacturonase from *Penicillium janthinellum* sw09

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ABSTRACT

A soil isolate, *Penicillium janthinellum* sw09 has been found to produce significant amounts of an extracellular pectinase subsequently characterized as exo-polygalacturonase (exo-PG). By optimizing growth conditions, *P. janthinellum* sw09 produced high amount of exo-PG (16.54 units/mL). The crude enzyme was purified by gel filtration chromatography and two exo-PG activity peaks (designated as PGI and PGII) were revealed. On SDS-PAGE analysis, purified PGII using DEAE-Sepharose FF column, was found to be a single band with a molecular mass of 66.2 kDa. The purified PGII exhibited maximal activity at the temperature of 45 °C and pH 5.0. The stability profiles show that PGII is more stable in the pH range of 4.0-8.0 and below 60 °C. The K_m and V_{max} for the enzyme was 1.74 mg/mL and 18.08 $\mu\text{mol}/(\text{mL}\cdot\text{min})$, respectively. Due to this enzymatic characterization, this pectinase is an attractive candidate for applications in degradation of pectin.

Key words: *Penicillium janthinellum*, polygalacturonase, purification, characterization.

INTRODUCTION

Pectinases constitute a unique group of enzymes which catalyze the degradation of pectic polymers present in the plant cell walls (Torres-Favela et al. 2003). Polygalacturonases (PGs) (EC 3.2.1.67) are part of a group of pectinases that possess hydrolytic functions and are able to cleave the α -1,4 glycosidic bonds between the two non-esterified galacturonic acid residues in pectin and pectic substances (Mohamed et al. 2006, Niture 2008). PGs have been classified according to their substrate specificity and the position of the bonds which they hydrolyse.

Endo-PG was defined as hydrolysing the α -1,4-glycosidic bonds in the substrate at random, whilst exo-PG hydrolyses the terminal link (Niture 2008). These enzymes are used in different sectors, such as paper, textile, cellulose, and food industries and have many important industrial applications (Joslyn et al. 1952, Kashyap et al. 2001). Additionally, PGs can promote the clarification of wine and grape juice (Sanderson and Spotts 1995).

PG from microbial source is the most important pectinase used in industries. Although a number of organisms producing pectinases have been reported, the selection of potential isolates still remains a tedious task, especially when physiologically potential strains are obtained to achieve

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maximum yield (Pandey et al. 1999). Most of the commercial PGs produced, are investigated from *Aspergillus* species (Mathew et al. 2008). The purification of PG is an important tool for understanding its properties and it reveals the structure and functional mechanism which are important for knowledge regarding the action of these enzymes in the plant infections process, in industrial application, in addition to their importance in biomass degradation. Fungal PGs are generally monomeric proteins with a carbohydrate content of 5–85% and molecular masses in the range of 20 to 95 kDa (Borin et al. 1996, Devi and Rao 1996, Mathew et al. 2008, Niture 2008, Niture and Rao 2004).

In this paper, the production medium of exo-PG obtained from *P. janthinellum* sw09 by submerged culture was optimized by orthogonal design. Furthermore, purification and physicochemical properties of exo-PG were investigated.

MATERIALS AND METHODS

MICROORGANISM AND GROWTH CONDITIONS

The microorganism used in this study was *Penicillium janthinellum* sw09, isolated from a tobacco field in Xuchang, P.R. China, and maintained as stock culture on potato dextrose agar (PDA) medium containing 0.3% citrus pectin at 4 °C. This strain was identified by Sangon Biotech (Shanghai) Co., Ltd and preserved at the Henan Province Microbiological Culture Collection Center (HPMCC no. 1225853). The liquid culture of mycelia was initiated by transferring the fungal mycelia from the stock culture on a Petri dish into the seed culture medium. The seed culture was propagated in a 250 mL Erlenmeyer flask containing 100 mL of liquid medium (10 g/L yeast extract, 5 g/L citrus pectin, FeSO₄ 0.1 g/L, MgSO₄ 0.5 g/L, KH₂PO₄ 1g/L, and pH 6.0) at 28 °C on a shaking incubator at 160 rpm for 72 h. The exo-PG was produced with the inoculation of 4% (v/v) of the

seed culture by submerged fermentation in a stirred tank bioreactor (Infors, Switzerland, 3.5-L working volume). The fermentations were performed under the following conditions: temperature, 32 °C, aeration, 2 vvm; agitation speed, 160 rpm. All experiments were performed in triplicate to ensure the trends observed were reproducible.

OPTIMIZATION PROCEDURE

Orthogonal design is one of the important statistical methods that use the Taguchi method (Taguchi 1986). Taguchi method is a fast and considerable way of optimization conferring outcome in simultaneous study of many factors, making its mark in quality products supplemented with better process performance, and rendering high yield and better stability through limited experimental trials. This approach has been successfully applied to optimize medium composition, condition of enzyme reaction, and extraction conditions for bioactive compounds (De Oliveira and Alves 2000, Xu et al. 2003). An orthogonal L₉(3)⁴ test design was applied to optimize medium condition of PG by *P. janthinellum* in flask culture. As seen on Table I, the experiment was carried out with 4 factors with 3 levels based on preliminary single experimental results. The exo-PG yield was chosen as the response.

TABLE I
Experimental factors and their levels for orthogonal projects.

Level	Glucose (g/L)	Yeast extract (g/L)	KH ₂ PO ₄ (g/L)	Citrus pectin (g/L)
1	30	3	0.5	2
2	40	4	1	4
3	50	5	1.5	6

ENZYME ACTIVITY MEASUREMENTS

The activity of exo-PG was assayed in a reaction mixture containing 1% citrus pectin solution with a degree of esterification (D.E.) of 67-70% (CAS

no. 9000-69-5, Sigma) in 0.2M sodium acetate buffer (pH 5.0) at 45 °C for 30 min. The number of reducing groups, released by enzymatic action, was measured by the DNS method (Miller 1959) and expressed as galacturonic acid. One unit of enzyme activity (U) was defined as the amount of enzyme releasing one μmol of galacturonic acid per minute under the assay conditions (Martins et al. 2002).

ENZYME PURIFICATION PROCEDURE

After 72 h of fermentation, the culture broth was centrifuged at 9,000 g for 15 min, and the resulting supernatant was filtered through a membrane filter (0.45 μm , Millipore). The resulting culture filtrate was precipitated by ammonium sulfate (20–100%) and the mixture was stirred for 2 h, and centrifuged at 15,000 g for 30 min. The ammonium sulphate fraction was dialyzed against Tris-HCl buffer (50 mM, pH 6.5) and directly loaded on a Sepharose CL-6B gel filtration column (2.5 \times 60 cm) equilibrated with 13 mM Na_2HPO_4 -citric acid buffer (pH 5.0). Protein fractions collected from the column, corresponding to the protein peak, were pooled and further applied to the DEAE-Sepharose FF based anion exchangers column equilibrated with 20 mM Na_2HPO_4 -citric acid buffer (pH 6.5) (Mohamed et al. 2006). Fractions of 4 mL were collected and assayed for exo-PG activity. The objective of this procedure was to estimate the number of isoform of exo-PG presented in the crude enzyme solution. The protein fraction with exo-PG activity was desalted overnight by dialysis at 4 °C.

ANALYTICAL ELECTROPHORESIS

The molecular weight of the purified enzyme was determined by SDS-PAGE in a Mini Protean II apparatus (10 \times 8 cm) (Biorad). Electrophoresis was carried out in a vertical slab gel apparatus (Beijing Liuyi Instrument Factory, DYCZ-24DN) with a 5% (w/v) polyacrylamide stacking gel and 12% (w/v) resolving gel in Tris/glycine buffer (pH 8.3) with the Sigma molecular weight marker MP102 (14.4

kDa-94.0 kDa) in a parallel lane (Laemmli 1970). The protein band was visualized by silver staining. The molecular mass of the isolated subunit was estimated by Ferguson plot analysis (Hedrick and Smith 1968).

PROTEIN ESTIMATION

Protein concentration was determined in the concentration ranges of 1–10 and 10–100 $\mu\text{g/mL}$ by the Bradford microassay, with bovine serum albumin (BSA) as the standard (Bradford 1976).

PROPERTIES OF PURIFIED ENZYME

All enzyme catalytic properties were assayed with 1% citrus pectin (D.E 67-70%) as substrate using the procedure for enzyme activity determination described above and carried out with three replicates. Exo-PG activity was assayed as a function of pH, in Na_2HPO_4 -citric acid buffer (pH 3.0–8.0), at 45 °C, and temperature, in Na_2HPO_4 -citric acid buffer at the pH optimum, incubated at different temperatures between 35 °C and 60 °C.

The thermal stability was investigated by remeasuring the activity of the purified enzyme solution after it had been kept for 2 h, in the absence of substrate, at different temperatures in the range of 30-60 °C. In these tests, the initial and final exo-PG activities were determined at optimum pH and temperature. The pH stability of the purified enzyme was evaluated by dispersing (1:1, v/v) enzyme solution in Na_2HPO_4 -citric acid buffer (pH 3.0–8.0) and maintaining these solutions at 45 °C for 4 h. An aliquot was taken to determine the remaining activity at the optimum pH and temperature.

The Michaelis constant (K_m) and V_{max} values were determined from Lineweaver-Burk plots of enzyme activity measured with 67-70% D.E. citrus pectin (Sigma) as substrate, at concentrations between 2.0 and 40.0 mg/mL at optimum pH and temperature. The purified enzyme concentration was 0.01 $\mu\text{mol/mL}$. The results were plotted with Excel.

STATISTICAL ANALYSES

Unless otherwise stated, data were expressed as mean \pm SD, and analyzed statistically by ANOVA method. P-values below 0.05 and 0.01 were regarded as statistically significant and highly significant, respectively.

RESULTS AND DISCUSSION

PRODUCTION OF EXO-PG IN FLASK CULTURE

Enzyme production is generally influenced by the composition of the medium, in particular the carbon and nitrogen sources (Gomes et al. 2009). The orthogonal matrix method was employed to optimize the concentration of the medium. The experimental conditions for each project were listed in Table II, and experimental results were also included in the last column of this table. According to the magnitude order of the R value (maximum difference) as shown in Table II, the order of effects of all factors on polygalacturonase production was glucose > citrus pectin > KH_2PO_4 > yeast extract. This result indicated that the effect

of glucose was more important than that of other nutrients. In terms of the maximum K value of each factor based on statistical calculations, optimal composition for polygalacturonase production were 50 g/L glucose, 3 g/L yeast extract, 1 g/L KH_2PO_4 , and 2 g/L citrus pectin. The significance of each factor was evaluated by calculating the F value and the results were summarized in Table III. Both factors, including glucose and citrus pectin had significant effects on the polygalacturonase production ($P < 0.01$). In order to confirm the optimization results, the suggested medium components were confirmed in triplicate. The 16.54 U/mL exo-PG was maximally obtained under the optimum conditions just described. This implied that the selected conditions were really the most suitable. Mathew et al. (2008) found the optimal pectin level as being 1.0 g/L for exo-PG production by a *Penicillium* strain in submerged culture, which suggests that the level of substance requirement for exo-PG production depends on the nature of the specific strain, even though they belong to the same species (i.e. *Penicillium*).

TABLE II
Application of $L_9(3^4)$ orthogonal projects to exo-PG production by *P. janthinellum* after 72 hours of incubation in flask culture.

Run	Glucose	Yeast extract	KH_2PO_4	Citrus pectin	Enzymatic activity (U/mL)
1	1	1	1	1	9.51 \pm 1.07
2	1	2	2	2	9.69 \pm 0.27
3	1	3	3	3	8.18 \pm 0.22
4	2	1	2	3	11.56 \pm 0.01
5	2	2	3	1	12.16 \pm 0.43
6	2	3	1	2	11.10 \pm 0.82
7	3	1	3	2	15.96 \pm 0.20
8	3	2	1	3	14.74 \pm 0.08
9	3	3	2	1	16.27 \pm 0.15
k1	9.12	12.34	11.78	12.65	
k2	11.61	12.19	12.51	12.25	
k3	15.66	11.85	12.10	11.49	
R	6.53	0.49	0.72	1.16	
Optimal level	3	1	2	1	
Order	glucose > citrus pectin > KH_2PO_4 > yeast extract				

TABLE III
Variance analysis of $L_9(3^4)$ orthogonal experiment on the optimization of exo-PG production.

Variance source	Sum of squares	Degree of freedom	Mean square	F-ratio	Significance
A:glucose	201.50	2	100.75	267.85	$p < 0.01$
B:yeast extract	1.16	2	0.58	1.54	
C:KH ₂ PO ₄	1.89	2	0.94	2.51	
D:Citrus pectin	5.02	2	2.51	6.67	$p < 0.01$
Errors	6.77	18	0.38		
Sum	216.32	26			

PURIFICATION OF EXO-PG

The crude enzyme solution separated on a Sepharose CL-6B column, afforded two peaks of exo-PG activity (designated as PGI and PGII) suggesting two isoforms (Fig. 1a). Because the PGII had higher amount and was stronger activity than PGI, PGII was selected for further purification. When the PGII solution (tube no. 38 to 64 as shown in Fig. 1a) was combined, concentrated and loaded on a DEAE-Sepharose FF column, one peak was eluted (Fig. 1b). The results were in agreement with the reports of Mohamed et al. (2006), who observed two polygalacturonases PGI and PGII separated from *Trichoderma harzianum* by two chromatography steps using DEAE-Sepharose and Sephacryl S-200.

CHARACTERIZATION OF EXO-PG

The homogeneity of the purified PGII was demonstrated by the presence of one single protein band on polyacrylamide gel and its molar mass was estimated to be 66.2 kDa as a single subunit (Fig. 2). This observation was in the range reported for PGs from several fungi, which have molecular masses ranging from 20 to 95 kDa (Gomes et al. 2009, Silva et al. 2007). In comparison with other *Penicillium* sp., lower molecular weights (24.1 kDa) were detected for PG from *P. viridicatum* (Mohamed et al. 2003).

The effect of pH on the *P. janthinellum* PGII activity toward polygalacturonic acid was examined at 45 °C. As shown in Figure 3a, the

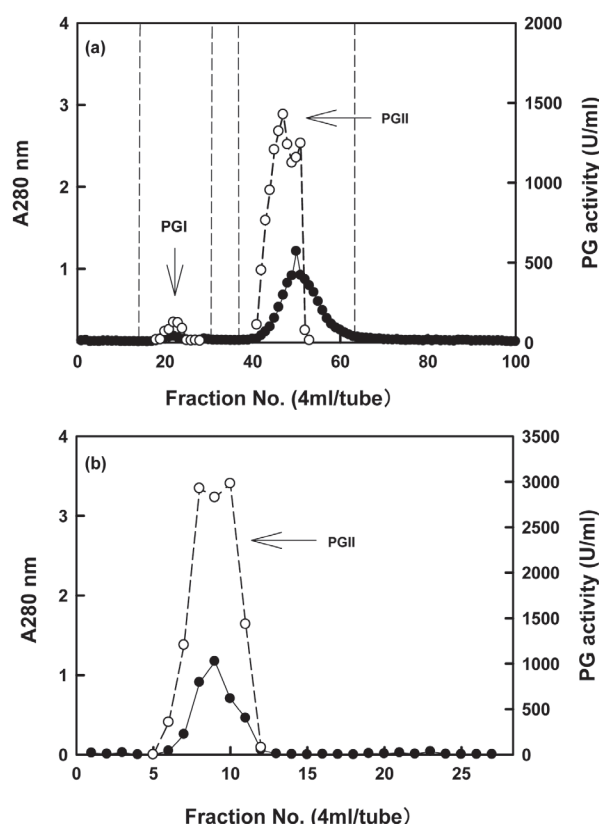


Figure 1 - Elution of exo-PG from Sepharose CL-6B gel filtration column previously equilibrated with 13 mM Na₂HPO₄-citric acid buffer (pH 5.0) (a) and DEAE-Sepharose FF column based anion exchangers column equilibrated with 20 mM Na₂HPO₄-citric acid buffer (pH 6.5) (b). Protein (●); exo-PG activity (○).

enzyme showed hydrolase activity from pH 3.0 to 8.0, and maximum enzymatic activity (15.72 U/mL) at pH 5.0. The same pH optimum was reported for PGs from *Aspergillus niger* (Behere et al. 1993), *T. harzianum* (Mohamed et al. 2006). The effect of pH on the stability of *P. janthinellum* PGII was

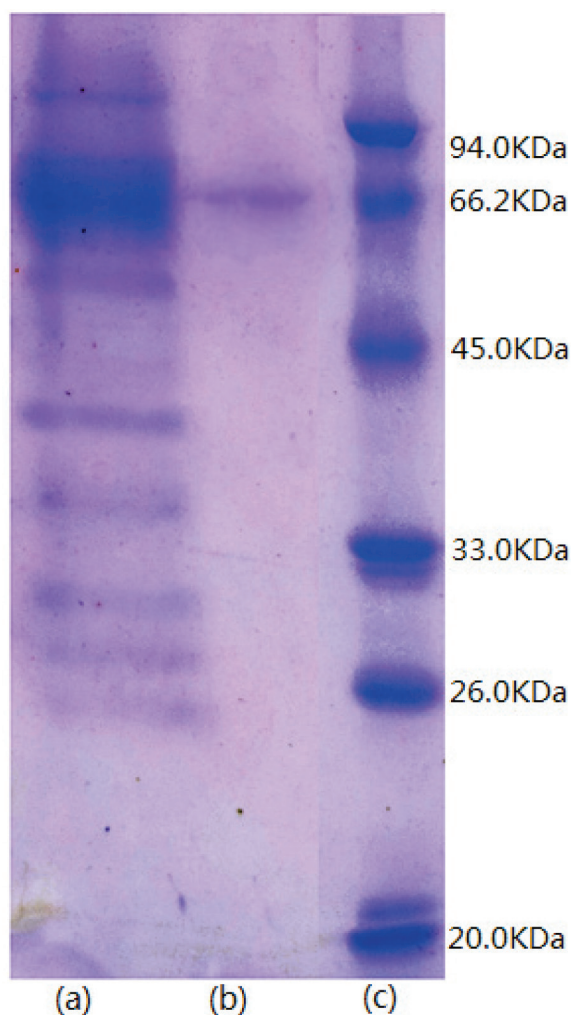


Figure 2 - SDS-PAGE of exo-PG (a), purified PGII (b) and standard proteins (c) from DEAE-Sepharose FF chromatography.

investigated by incubating the enzyme at 45 °C at different pH's for 4 h. The results showed that the enzyme was the most stable in pH of 6.0, with 90–100% of the full activity in a broader pH range of 4.0–8.0 (Fig. 3b). This is in agreement with the results reported by Kobayashi et al. (2001) for *Bacillus* sp. PG. They reported that PG activity was very stable in various 50 mM buffers between pH 6 and 12 when incubated at 30 °C for 1 h.

With regard to temperature, optimal *P. janthinellum* exo-PG activity was observed at 45 °C (Fig. 4a). Similarly, temperature optima for PGs from *T. reesei* (Mohamed et al. 2003), *Mucor flavus* (Grade et al. 2003), *Bacillus* sp. (Kobayashi et al. 2001) and *T. harzianum* (Mohamed et al. 2006) were around 40 and 50 °C. The effect of temperature on thermal stability of *P. janthinellum* PGII was investigated by incubation the enzyme for 2 h in 13 mM Na₂HPO₄-citric acid buffer, pH 5.0 at different temperature ranging from 30 to 60 °C prior to substrate addition (Fig. 4b). In the absence of substrate for 1 h, PGII showed 83–100% of the original activity at 30–60 °C. After 2 h, PGII showed 68–88% of the original activity at 30–50 °C, while at 60 °C, the enzyme lost 58% of its initial activity. Similarly, Kobayashi et al. (2001) found that *Bacillus* sp. PG was stable up to 50–55 °C in the presence of CaCl₂.

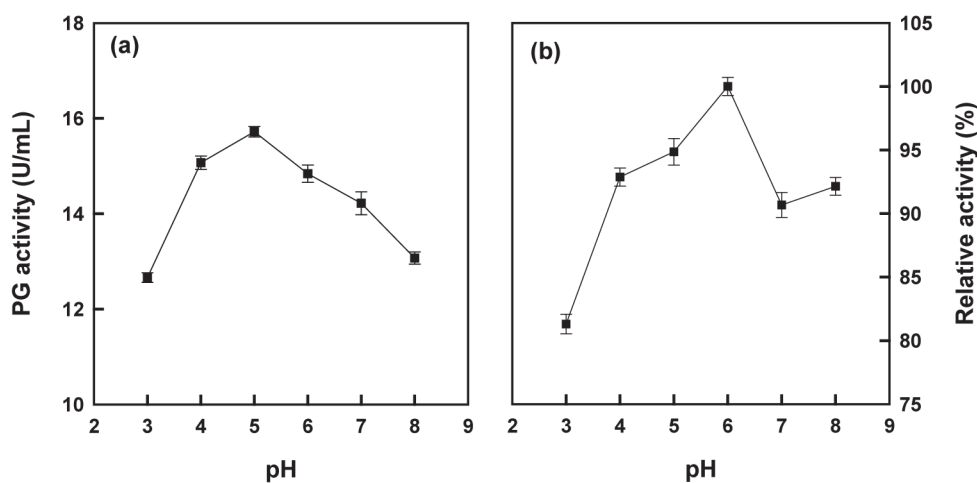


Figure 3 - Effect of pH on PGII activity (a) and PGII stability (b).

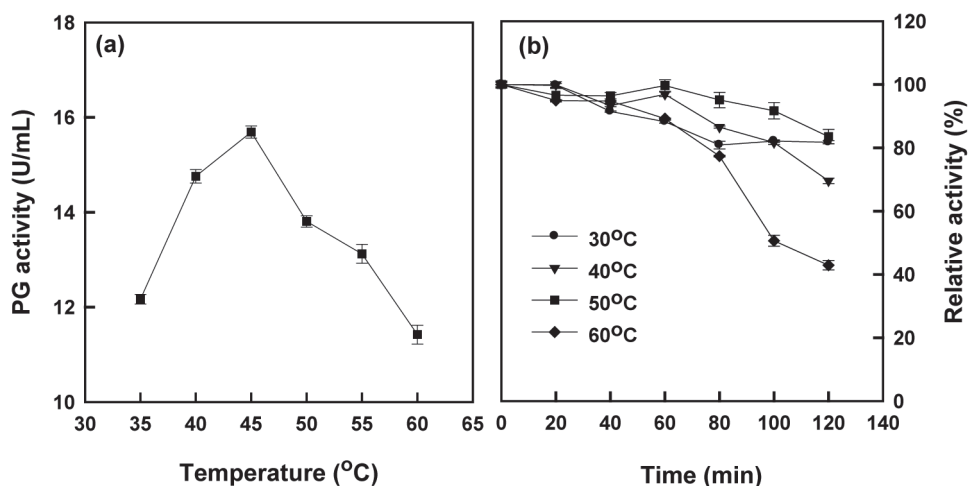


Figure 4 - Effect of temperature on PGII activity (a) and PGII stability (b).

The kinetic parameters of *P. janthinellum* PGII affinity for citrus pectin and different pectins at pH 5.0 and 45 °C were determined by a typical double reciprocal Lineweaver-Burk plot (Fig. 5). The K_m and V_{max} for the enzyme was 1.74 mg/mL and 18.08 $\mu\text{mol}/(\text{mL}\cdot\text{min})$, respectively. The K_m values of *P. janthinellum* PGII were lower than K_m s ranged from 2.5 to 14.08 mg/mL of PGs from *A. niger* (Parenicova et al. 1998), *Neurospora crassa* (Polizeli et al. 1991), *T. harzianum* (Mohamed et al. 2006) and *Saccharomyces cerevisiae* (Blanco et al. 1997). Low K_m indicated that *P. janthinellum* PGII had high affinity using citrus pectin as substrate. V_{max} of *P. janthinellum* PGII was in the range of V_{max} s, i.e. 13.0 to 2600 $\mu\text{mol}/(\text{mL}\cdot\text{min})$ from above organisms. The K_{cat} for the enzyme was calculated as 301.33/s. The K_{cat} values of *P. janthinellum* PGII were in the range of K_{cat} s, i.e. 21.0 to 592/s from above organisms.

CONCLUSIONS

The fungal strain *Penicillium janthinellum* sw09 produced an exo-polygalacturonase able to release galacturonic acid as a hydrolysis product from natural D.E. pectin. This enzyme exhibited the pH and temperature profiles for optimum activity and stability that differed from other fungal PGs, which

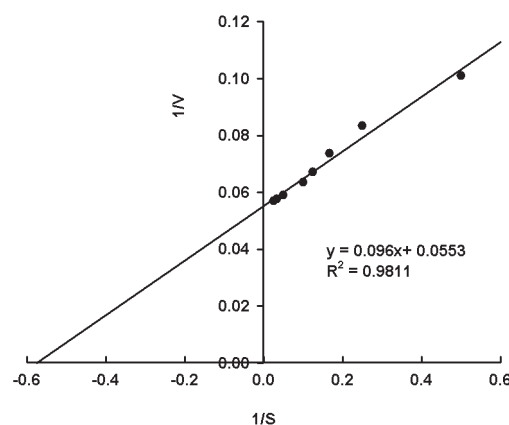


Figure 5 - Kinetics parameters of purified PGII.

kept the stability of the enzyme in a pH range of 4.0-8.0 and at a temperature range of 30-60 °C. A thermostable nature with a high pH range for activity makes it possible to have wide range of industrial applications. Further works on scale-up fermentation optimization (i.e. aeration rate and agitation speed) in bioreactor and industrial application are in progress in our laboratory.

RESUMO

Penicillium janthinellum sw09, isolado do solo, produz quantidades significativas de uma pectinase extracelular que foi posteriormente caracterizada como exo-poligalacturonase (exo-PG). Ao otimizar as condições de crescimento, *P. janthinellum* sw09 produz quantidades

elevadas de exo-PG (16,54 unidades/ml). A enzima impura foi purificada por cromatografia de filtração em gel e dois picos com atividade elevada de exo-PG (designados como PGI e PGII) foram revelados. Por análise em SDS-PAGE, PGII foi purificada utilizando uma coluna de DEAE-Sepharose FF, observou ser formada por uma única banda com uma massa molecular de 66,2 kDa. PGII purificada exibiu uma atividade máxima a uma temperatura de 45 °C e pH 5,0. Os perfis de estabilidade mostram que PGII é mais estável numa faixa de pH de 4,0-8,0 e abaixo de 60 °C. O Km e Vmax para a enzima foi de 1,74 mg / ml e 18,08 µmol / (mL • min), respectivamente. Devido a esta caracterização enzimática, pectinase é um candidato atrativo para ser aplicado na degradação da pectina.

Palavras-chave: *Penicillium janthinellum*, poligalacturonase, purificação, caracterização.

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