Isolation and Characterization of Polygalacturonase Produced by *Tetracoccosporium* sp.

Aminzadeh, Saeed; Naderi-Manesh, Hossein*+; Khajeh, Khosro
Department of Biochemistry and Biophysics, Faculty of Science, Tarbiat Modarres University, P.O. Box 14115-175 Tehran, I.R. IRAN

Soudi, Mohammad Reza
Department of Microbiology, Faculty of Scince, Alzahra University, P.O. Box 19935-644 Tehran, I.R. IRAN

ABSTRACT: *Thirty five fungal strains which isolated from vegetable wastes, were screened for the use of polygalacturonic acid as the sole carbon source. Twenty five isolates were positive for polygalacturonase activity in cup-plate assay, as evidenced by clear hydrolysation zones. The most productive strain was determined by measuring clear zones formed around colonies stained with ruthenium red. The highly pectinolytic fungal strain was tentatively identified as Tetra-occosporium sp. according to morphological characterization. The cultivation of the selected strain (Tetracoccosporium* sp.*) in liquid media resulted in high quantities of polygalacturonase enzyme. Maximum polygalacturonase activity was reached in 48 h of growth in the pectate medium. The collected polygalacturonase had optimum activity at pH 5.0 and maximal activity of the enzyme was determined at 35 °C. Mn^{2+}, Ag^{3+} and surface active detergents such as tween 20 and triton X-100 increased the polygalacturonase activity by 37 % and EDTA enhanced the activity up to 125 %.*

KEY WORDS: *Clear hydrolysation zones, Cup-plate assay, Polygalacturonic acid, Polygalacturonase, Screening, Tetracoccosporium* sp.

INTRODUCTION

The enzyme industry as we know it today is the result of a rapid development seen primarily over the past four decades thanks to evaluation of modern biotechnology [1]. Fungal enzymes are routinely used in many environmentally friendly and economic industrial sectors [2]. The metabolism of pectic substances contained the back bone of α-1,4-linked *D*-galacturonic acid residues is caused in nature by an action of various pectinolytic enzymes such as polygalacturonases, rhamnogalacturonases, methylgalacturonases, esterases, pectate lyases and pectin lyases. Pectinases (polygalacturonases) are well known to be the power tools for elucidation of structural features of pectic polysaccharides [3]. The polygalacturonases (EC 3.2.1.15) catalyze the hydrolytic cleavage of the O-glycosyl bond of α-*D*-(1→4) polygalacturonan. These enzymes are produced by higher

* *To whom correspondence should be addressed.*
+ *E-mail: naderman@modares.ac.ir*
1021-9986/07/1/47 8/$/2.80

and lower plants, bacteria and fungi. The role of the enzyme in microbial pathogenesis, fruit ripening and softening, abscission, and growth, has been well established. Fungal polygalacturonases are also used for industrial applications, and deproteinization of fruit juices, for maceration of fruits and vegetables, and for extraction of vegetable oils [4]. Novel fields of application have also recently been envisaged for polygalacturonase in the production of oligogalacturonides as functional food components. Polygalacturonases from a large number of fungi have been purified and characterized. They show extensive variation in physical and chemical properties [4].

In the present investigation, we report the production and characterization of polygalacturonase from a newly isolated *Tetracoccosporium* sp. with regard to its optimum temperature and pH condition for enzymatic catalysis. To our knowledge, it is the first time that this enzyme is identified in this genus, and there is no previous record in this matter.

MATERIALS AND METHODS

Chemical

Polygalacturonic acid and 3,5-dinitrosalicylic acid (DNS) were obtained from Fluka (Switzerland), and Sigma (St. Louis, MO. USA), respectively. All other chemicals were from Merck (Darmstadt, Germany) and were reagent grade.

Microorganisms

Saprotrophic hyphomycetes isolated from agricultural and vegetable wastes were obtained from culture collection of Applied Microbiology Laboratory at Alzahra University and were cultured on sabouraud dextrose agar (SDA) medium which contained peptone 10 g; glucose 20 g; agar 15 g; streptomycin 100 mg in 1 lit H_2O and incubated at 30 °C for 24 to 72 h. All morphological contrasting colonies were purified by streaking [5,6].

Qualitative cup-plate assay for polygalacturonase production

To determine the polygalacturonase production incorporating 1 % of polygalacturonic acid was incorporated in a buffered agar solution (agar 1.8 %; sodium acetate buffer 0.1 M, pH 5.6). Gels were cast onto Petri dishes in the normal manner [7]. Culture plates containing 25 ml of polygalacturonic acid medium were inoculated in the center with 10 ml of a suspension containing about 50 spores per micro liters (detected with cell counting Neubauer chamber) and incubated at 30 °C.

After 2 day each plate was stained with 10 ml of a 0.05 % ruthenium red aqueous solution for 1 h as in the polygalacturonase cup plate assay. After 1 h, the plates were rinsed with deionized water and the clear zone (the part of gels not stained completely with ruthenium red aqueous solution) around the fungal colony was measured.

Preparation of spore suspension

Spores, from SDA slants, were washed in 5 ml of 0.01 % tween 80 solution. The spore suspension was adjusted to a final concentration in the culture medium of 10^6 spores per ml [8,9].

Culture media and condition

Several different media were prepared during this study. Sterilization was carried out at 121 °C and 15 psi for 20 min. The initial pH in all cases was 5.6. An appropriate defined medium was chosen to compare the results, in terms of enzyme production. The medium contained the following, per liter: 5 g of polygalacturonic acid, 3.0 g of $(NH_4)_2SO_4$, 10 g of KH_2PO_4, 2 g of $MgSO_4$. H_2O, 0.7 mg of $Na_2B_4O_7$. $10H_2O$, 0.5 mg of $(NH_4)_6Mo_7O_{24}$. $4H_2O$, 10.0 mg of $Fe_2(SO_4)_3$. $6H_2O$, 0.3 mg of $CuSO_4$. $5H_2O$, 0.11 mg of $MnSO_4$. H_2O, 17.6 mg of $ZnSO_4$. $7H_2O$ and 100 mg of streptomycin.

All cultures were incubated at 30 °C in an orbital shaker at 160 r.p.m. and samples were collected every 24 h. The experiments were carried on in 500 ml Erlenmeyer flasks with 100 ml of medium. Then samples were filtered through Whatman No.4 filter paper, and the filtrate was evaluated for enzyme activity.

Enzymatic assay

The polygalacturonase activity was measured by different methods. First, the activity was detected on solid medium through the coloration of Petri dishes with a solution of ruthenium red [10]. Second, polygalacturonase activity was also determined by measuring the increase in production of reducing ends, with polygalacturonic acid as substrate. On the other hand the polygalacturonase

activity was assayed by quantifying reducing groups expressed as galacturonic acid units which had been librated during the incubation of 200 µl of 1 % (w/v) polygalacturonic acid in 10 mM sodium acetate buffer, pH 5.1 (optimum pH) with 200 µl of suitably diluted enzyme at 25 ºC, for 5 min by DNS (dinitrosalicylic acid) method [11]. One unit of polygalacturonase was defined as the amount of enzyme required to release 1 µmol of galacturonic acid (as a standard) from the polygalacturonic acid/ml/min.

Enzyme characterization

1000 ml of cell-free supernatant was saturated with ammonium sulphate to 90 % saturation. The saturated solution was left overnight at 4 ºC, centrifuged at 10000 g for 20 min at 4 ºC, dissolved in minimal amount of 10 mM sodium acetate buffer (pH 5.6) and dialyzed against the same buffer for 24 h at 4 ºC. The dialyzed proteins thus obtained were used for enzyme characterization**.** Enzyme activity was determined at 25 ºC, at different pH values using three buffer solutions with pH values ranging from 2.0 to 11.0. The buffers employed in these measurements were 50 mM glycin-acetate-phosphate buffers. The optimum temperature for hydrolysis of polygalacturonic acid was assayed by incubation of the reaction mixture in temperatures at 20-80 ºC and measuring the polygalacturonase activity at seven different temperatures.

RESULTS AND DISCUSSION

Microorganisms

Thirty five strains of filamentous fungi were isolated from the cultures. Pure cultures were sub-cultured onto slants media and maintained for further identification and enzymatic studies. The highly pectinolytic fungal strain was tentatively identified as *Tetracoccosporium* sp. according to morphological characterization [6]. Fig. 1A shows the microscopic view of the fruiting body. Biometric assays indicated vegetative mycelia with the average diameter of 3 µm and short conidiophores. The mature conidia have a sphere shape of 10 µm in diameter which is composed of four cells. The macroscopic features of the colony on SDA showed cream to gray mycelia at surface, and brown-to blue-black view at the back of Petri plates (Fig. 1B and 1C).

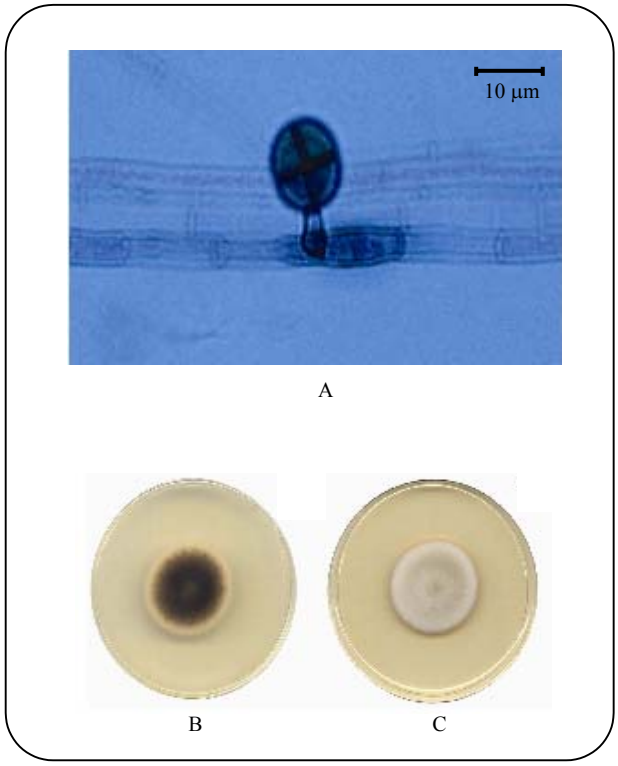

Fig. 1: Microscopic view of Tetracoccosporium sp. fruiting body (A). The macroscopic features of the colony on SDA. Brown-black to blue-black view at the back of Petri plates (B). Cream to gray mycelia at surface (C). For more details please see materials and methods section.

Fig. 2: Clear zone reactions produced by Tetracoccosporium polygalacturonase. Clear zone (arrow) indicates poly-galacturonase activity after 2 days of incubation at 30 °C. For more details please see materials and methods section.

Selection of the strain

Twenty five strains which were able to grow on medium containing polygalacturonic acid as the sole carbon source were isolated. These strains were tested for polygalacturonic acid hydrolysis by plate assay, at pH 5.6. Clear zone against a red background indicated the presence of polygalacturonase activity (Fig. 2).

Isolates varied widely in their reaction to this test. Clear zones did not form around colonies of 10 of the 35 assayed isolates, indicating that even if polygalacturonase was produced they were at a lower than the sensitivity of our assay. The strains were classified as very good producers of pectin depolymerizing enzymes when presented clear zones around colonies of at least 20 mm, good producers when the zones were at least 14 mm, weak producers when zones were at least 8 mm and poor producers when no polygalacturonase activity and no clear zones were observed (Fig. 3).The strain with the largest zone (about 20 mm) was used for enzyme production in liquid media.

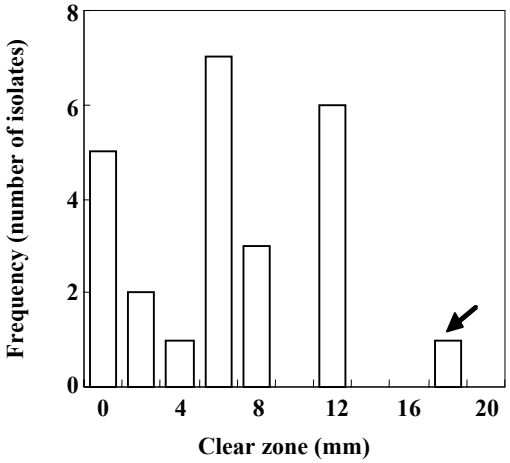

Fig. 3 : Polygalacturonase production by fungal isolates. The width of the clear zone around fungal colonies in the polygalacturonase culture assay indicates the relative level of polygalacturonase produced. The most effective isolate (Tetracoccosporium* sp.*) which indicated by arrow was used for enzyme production in liquid media. For more details please see materials and methods section.

Production of polygalacturonase from Tetracoccosporium sp.

The polygalacturonase production by *Tetracoccosporium* sp. was high after 2 day of cultivation. The enzyme production was increased by stirring at 160 r.p.m. as compared to the static conditions (data not shown). The supernatant was concentrated by ammonium sulphate precipitation (Fig. 4).

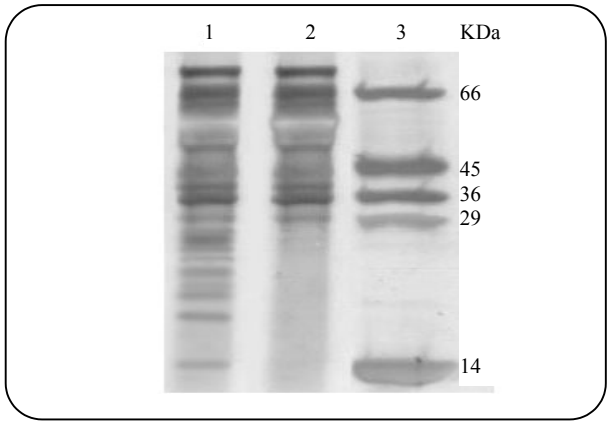

Fig. 4: Silver staining SDS-PAGE illustrates the polygalacturonase production. Lane 1, the crude enzyme; Lane 2, after precipitated with $(NH_4)_2SO_4$; and Lane 3, molecular size markers.

Effect of incubation time on polygalacturonase production

Polygalacturonase production was high after 2 day cultivation. The polygalacturonase production started to decline, thereafter (Fig. 5). This decline is perhaps due to the lack of nutrients in the medium as proposed by *Darcota* [7].

Influence of the carbon and nitrogen sources on polygalacturonase synthesis

Biosynthesis of polygalacturonase is induced by polygalacturonic acid and inhibited in the presence of easy metabolized monosaccharides (glucose, fructose etc.) and some other compounds. Glucose and fructose inhibited polygalacturonase biosynthesis, and synthesis of the enzyme in relatively small amounts with low enzymatic activity occurred in the presence of galactose and mannose. Maltose, lactose and sucrose decreased polygalacturonase production about 65 %, whereas starch and cellulose decrease polygalacturonase production about 55 % (table 1). Inhibition of polygalacturonase production in the presence of glucose and other simple sugars might be due to catabolite repression. Similar results showing a higher polygalacturonase yield with polygalacturonic acid polymer compared with simple sugars such as arabinose, glucose and galactose have been reported earlier, which is in accordance with the present study [12].

For elucidation of influence of nitrogen sources on polygalacturonase biosynthesis, organic (casein, peptone) and inorganic (NH_4HCO_3, $(NH_4)H_2PO_4$, $(NH_4)_2SO_4$, NH_4Cl) sources of nitrogen were added to the medium containing the polygalacturonic acid (5 g/l) as the sole

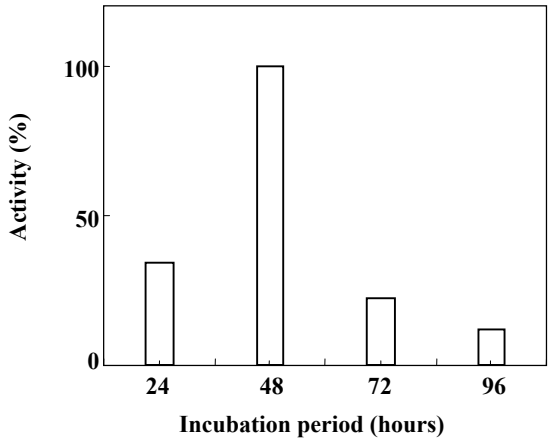

Fig. 5 : Effect of growth time on enzyme production. Polygalacturonase production by the Tetracoccosporium sp. was high after 2 days in liquid media at 30 °C (pH 5.1) under shaking conditions (160 r.p.m).

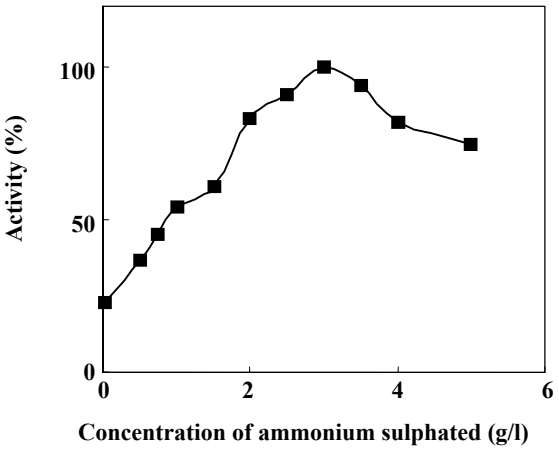

Fig. 6 : Influence of ammonium sulphate concentration on polygalacturonase synthesis after 2 days in liquid media at 30 °C (pH 5.1) under shaking conditions (160 r.p.m). There was consistent increase in enzyme production with increase in ammonium sulphate concentration up to 3 g/l.

carbon source. The medium without the nitrogen source was used as a control (table 2). The high values of polygalacturonase activity in a comparison with a control were observed in the medium with peptone water and ammonium sulphate. Typical nitrogen sources in *Tetra-coccosporium* sp. cultivations are ammonium sulphate or peptone water solution. Ammonium sulphate appeared to be the most optimal nitrogen source for polygalacturonase production which caused also a stabilizing effect on the enzymes. The optimal concentration of ammonium sulphate in the medium proved to be 3 g/l (Fig.6).

Table 1: Growth and polygalacturonase activity on various carbon sources.

The carbon sources [a]	Growth [b]	% Activity
Glucose	+ + +	8
Galactose	+ + +	23
Mannose	+ + +	25
Fructose	+ + +	11
Disaccharides		
Lactose	+ + +	38
Sucrose	+ + +	31
Maltose	+ + +	29
Polysaccharides		
Starch	+ + +	41
Cellulose	+ +	47
Polygalacturonic acid + glucose	+ + +	9
Polygalacturonic acid (control)	+ +	100

a)Growth in liquid culture on minimal culture containing 1 % w/v carbon source for 48 hr.
b) + + + = good, + + = moderate.

Effect of the initial pH of the medium on polygalacturonase synthesis

Enzymatic biosynthesis is influenced not only by the composition of the nutrient medium but also by the other conditions of cultivation including pH of the medium which will limit the growth of the culture or exert influence upon catalytic activity of the enzyme. The effect of medium pH on polygalacturonase biosynthesis was studied at seven pH-values in a range of 2.0-8.0 (Fig. 7). The polygalacturonic acid (5 g/l) was used as the carbon source in this case. The maximal

Table 2: An influence of nitrogen source on polygalacturonase synthesis.

The nitrogen sources	% Activity
Control	100
Peptone water	178
Casein	146
Beef extract	139
Yeast extract	141
$(NH_4)H_2PO_4$	137
NH_4HCO_3	143
$(NH_4)_2SO_4$	181
NH_4Cl	129

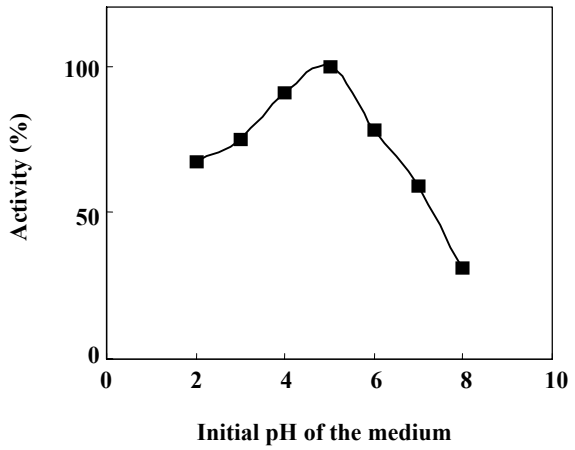

Fig. 7 : Influence of the initial pH-values of the medium on polygalacturonase synthesis in liquid media at 30 °C (pH 5.1) under shaking conditions (160 r.p.m). The polygalacturonic acid (5 g/l) was used as the carbon source in this case.

polygalacturonase activity was observed in the medium with the acidic initial pH-values within a range of 4.0-6.0 (optimum 5.0).

Effect of assay temperature and pH on polygalacturonase activity

Since any biotechnological process is likely to be based on the use of crude or partially purified enzymes, it is important to determine the optimal temperature and pH for enzyme activity under these conditions. The influence of temperature and pH on the activity of enzyme is shown in Fig. 8. The optimum temperature for enzyme activity was from 30 to 40 ºC (Fig. 8.A). This enzyme was active in a broad acidic pH range (3-6) at room temperature with maximum activity being at pH 5.0 (Fig. 8.B). Other polygalacturonase with pH optima in the acidic range have been reported from fungal strains [12] such as polygalacturonase from *P. capsulatum* with 4.7 [13], *R. stolonifer* with 5 [14], and *A. pullulans* with 4.5 [15].

The reported polygalacturonase produced by several fungal sources have a variety of temperature and pH profiles. For example the optimum temperature activity of *S. sclerotiorum* is 45 ºC and polygalacturonase activity is mostly inactive at 65 ºC. In this organism polygalacturonase has a narrow pH activity curve [16]. In *A. kawachii* polygalacturonase did not show any activity at pH 5.0 [17]. Polygalacturonase from *S. chevalieri* and *C. albidus*, were found to have optimum temperature of 25 and 37 ºC, respectively [18]. In *T. aurantiacus* polygalacturonase exhibit maximal activity at pH 5.0 and optimum temperature is determined to be 65 ºC [19]. *A. alliaceus* showed a maximum of activity at pH 5.5 and temperature 35 ºC [3] and the pH optimum of *P. expansum* polygalacturonase is 5.5 [3] which is similar to our results.

Effect of metal ions, metal chelators and chemical compounds on polygalacturonase activity

The polygalacturonase activity was measured at pH 5.0 in the presence of various metal ions (1 mM). Metal ions such as Mn^{2+} and Ag^{3+} increased polygalacturonase activity by up to 37 %. Iodoacetamide and idoacetic acid didn't inhibit the enzyme activity at 1 mM concentration which indicated that cycteine residues are not part of catalytic site of polygalacturonase (table 3). It is also interesting to note that all the surface-active detergents i.e. tweens (20 and 80), triton X-100 and SDS, stimulated the polygalacturonase activity by up to 29 %. Perhaps this is due to the fact that the surface-active reagents might have increased the turnover number of polygalacturonase by increasing the contact frequency between the active site of the enzyme and the substrate by lowering the surface tension of the aqueous medium [12]. Ca^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Ba^{2+}, Fe^{3+}, Al^{3+}, Cr^{+3} and K^{+} partially inhibited activity and EDTA stimulated the activity up to 125 % (table 3).

Table 3: Effect of metal ions (1 mM), metal chelators and chemical compounds on polygalacturonase activity.

Metal ion/Chemical compound	Polygalacturonase residual activity
Control	100
Ag^{+}	137
Al^{3+}	60
Ba^{2+}	55
Ca^{2+}	64
Co^{2+}	86
Cr^{3+}	36
Cu^{2+}	29
Fe^{3+}	79
K^{+}	73
Ni^{2+}	54
Mg^{2+}	96
Mn^{2+}	110
Zn^{2+}	54
Citric acid	141
EDTA	225
Iodoacetamide	120
Iodoacetic acid	110
Tween 20	122
Tween 80	129
Triton X-100 (0.1%v/v)	108
SDS (0.1%w/v)	100

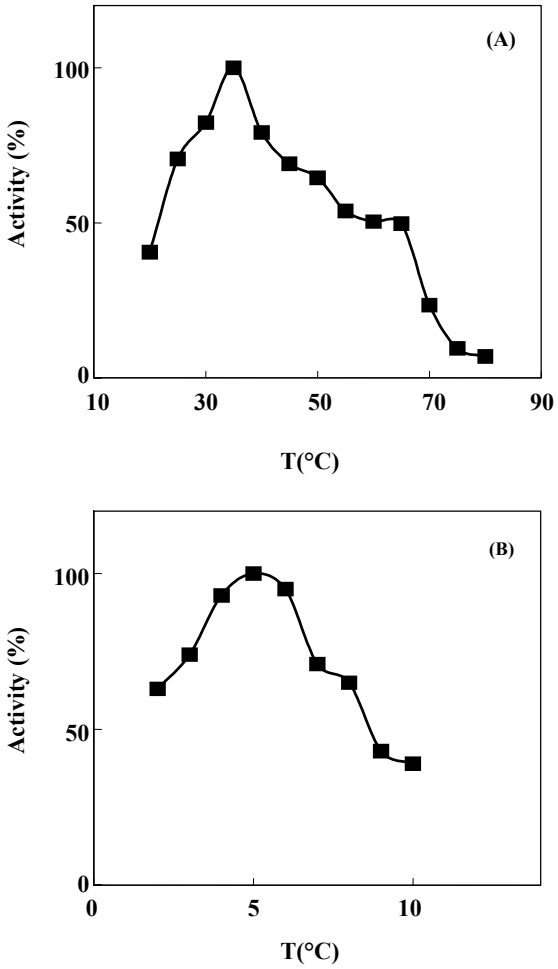

Fig. 8: (A) Effect of temperature on polygalacturonase activity at pH 5.0 at different temperatures between 20 and 80 °C. (B) Effect of pH on the enzyme activity at 25 °C. A mixture of glycine, acetate, phosphate, buffers at a concentration of 10 mM was used.

The cause of the inhibition with Ca^{2+} is that this ion enhances the gelling of the pectin molecules, thereby producing interlinking and making the substrate inaccessible to the enzyme. On the contrary Ca^{2+} inhibited polygalacturonase, probably by interfering with the substrate-forming insoluble complexes [16]. The inhibitory effect of the divalent cations was probably a result of physical interactions with the pectin and not a direct effect on the enzyme [20].

The properties of the enzyme are similar to those reported for other fungal polygalacturonases [21-24]. Ca^{2+}, Mg^{2+}, and SDS inhibit the enzyme activity in *S. sclerotiorum* [16,25].

CONCLUSIONS

Enzymes are currently used in several different industrial products and processes and new areas of application are constantly being added. Thanks to the advances in the modern biotechnology, enzymes can be developed today for processes where no one would have expected an enzyme to be applicable just a decade ago. In a world with a rapidly increasing population and approaching exhaustion of many natural resources, enzyme technology offers a great potential to help many industries to meet the challenges they will face in years to come [1]. This work reveals that production of poly-galacturonase is feasible by newly isolated *Tetracocco-*

sporium sp. This fungus is able to produce extra-cellular polygalacturonase in liquid medium. The pectinolytic activity of the crude solution has specific properties which may offer advantages over currently available polygalacturonase preparations. It is the first time that this enzyme is identified in this genus, and there is no record in this matter before. Polygalacturonase produced from *Tetracoccosporium* has a broad range of optimum temperature, pH and good tolerance to surface-active agents. These results offer an attractive alternative source of enzymes for industrial supply. Further work on the scale-up production and application of this enzyme, and its economic/commercial feasibility is currently underway.

Acknowledgments

The authors express their gratitude to the research council of Tarbiat Modares University and Ministry of Sciences, Researches, and Technology for financial support during the course of this project.

Received : 14th January 2006 ; Accepted : 15th May 2006

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