

Factors Affecting Pectinase Enzymes Activity Produced by Three Fungi

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ABSTRACT

Three fungi (*Trichoderma viride*, *Asperigillus niger* and *Penicillum digitatum*) are used for production of pectinase enzymes. The present study was investigated the effects of some factors on the activities of these pectinase enzyme systems of above three fungi, under laboratory conditions. Two methods (reducing sugar and viscometry) were used for measuring enzymes activities. The addition of Mg^{++} , Ba^{++} and Ca^{++} to the enzyme assay caused a higher increase in the activity of the pectinase enzyme of the three fungi. However, less increase was found by the addition of Cu^{++} , Fe^{++} , Zn^{++} and Mn^{++} . On the other hand, the addition of Ag^{++} , Cd^{++} and Hg^{+} caused a decrease in the activity of this enzyme. The activity of the pectinase enzymes produced by *A. niger* and *P. digitatum* was increasing with increasing temperature with the maximum at 40°C. However, the pectinase enzyme of *T. viride* was highly active at temperature 50 °C. It was found that the maximum activity of the pectinase enzymes produced by *A. niger* was at pH 9. While the filtrates of both *T. viride* and *P. digitatum* showed two peaks at pH 6 and pH 9.

Key words: Fungi, Pectinase enzymes, Factors affecting Enzyme activity

INTRODUCTION

The effect of cofactors (activators and inhibitors):

One important class of chemicals that can influence enzyme action is the cofactors. Cofactors are enzyme specific and highly diverse, ranging from simple ions to large molecules. They usually associated with the enzyme at or near the active site and can greatly enhance the catalytic properties of the enzyme (Cruikshank and Perrin, 1964). Certain ions like Ca^{++} are absolutely necessary for the activity of some enzymes, while other ions like (Ag^+ , Hg^+ , Pb^{++}) are highly toxic to nearly all enzymes. Some may inhibit an enzyme at one concentration and be activators of the same enzyme at another concentration (Dixon and Webb, 1964). Dong *et al.*, (2001) reported that Cu^{++} and Hg^{++} are generally inhibitory agents. On the other hand, Ca^{++} and Mg^{++} have been found to be either stimulatory or at least required for the enzyme stability. Alana *et al.*, (1990) reported that Ca^{++} , Mg^{++} , Zn^{++} and Mn^{++} did not affect the pectinlyase activity of *P. italicum*, while Cu^{++} and Fe^{++} at the same concentration produced complete inhibition. Odeniyi *et al.* (2009) reported that the enzyme polygalacturonase produced by *Bacillus coagulans* strain were fully inhibited by Hg^+ ion at 1.0 mM concentration.

Effect of temperature:

Mrudula and Anithara (2011) reported that the pectinase enzymes produced by *A. niger* have maximum activity at a temperature of 50°C . Pectinesterase of *A. Japonicas* have best activity at 50°C (Hasunuma *et al.*, 2003). Pectinmethylesterase reported from *Erwinia chrysanthemi* shows best activity at 50°C (Laurent *et al.*, 2000). The pectinase activity of *Penicillium chrsogenum* was found to be highest at 50°C (Rasheedha *et al.*, 2010). An extracellular pectinase was purified to apparent homogeneity from liquid state culture of the thermophilic fungus *Acrophialophora nainiana* by ultra-filtration and a combination of gel filtration and ion exchange chromatographic procedures. It was found that the enzyme was more active at 60°C (Maria *et al.*, 2006). In study presented by Baladhand and Thangavelu (2010) the optimum temperature was found to be 40°C using *Asperigillus niger* in solid state fermentation.

Semenova *et al.* (2003) reported that the pectinlyase and pectinmethylesterases of a mutant strain of *Asperigillus japonics* were stable at $40\text{-}50^{\circ}\text{C}$. Joshi *et al.*, (2011) reported that the partially purified enzymes produced from apple pomace fermented by *A. niger* and *P. dierckii* were remained stable up to a temperature of 50°C , but after this temperature, there was a continues decline in the activity of the enzyme. After 50°C , it started declining slowly up to 65°C , then, a sharp decrease in Pectinmethylesterase activity took place up to 80°C . The optimum temperature for the polygalacturonases of *A. niger* and *P. dierckii* were shown to be 50 and 60°C , respectively. However, the optimal temperature for the different pectinases has been reported by other researcher. The optimum temperature for polygalacturonase and pectinlyase was found to be 65°C , while the Pectinmethylesterase activity remained unaffected up to 40°C but was completely lost its activity after 1 minute of incubation at 90°C (Martin *et al.*, 2002). Denis *et al.*, (2002) reported that the maximum activity of pectinlyase and polygalacturonase was determined

at 55°C and 50°C, respectively. Abdul-Sattar *et al.* (2012) reported that the maximum temperature of pectinase produced by *Bacillus subtilis* is clearly at 45°C. However, Sharma and Saayyanarayana (2006) reported that it was 50°C for *B. pumilus*. However, it was 45-50°C for pectinase production by *Thermomucor indicae-seudaticae* N31 (Martin *et al.*, 2010). But it was 50°C for exo-polygalacturonase from *Streptomyces eurmpens* MTCC 7317 (Shaktimay and Ramesh, 2011). The growth medium of citrus peel was fermented by *T. harizianum* in solid state fermentation at pH 5.5 and 28°C for 72hours. Pectinlyase produced in solid state fermentation was partially purified by ammonium sulphate precipitation. Then, the purified enzyme showed maximum activity at 40°C temperature (Nazia *et al.*, 2003).

Variation in the reaction temperature as a small as 1 or 2 degrees may introduce changes of 10 to 20 % in the results. This is complicated by the fact that many enzymes are adversely affected by high temperatures. The reaction rate increase with temperature to a maximum level, then abruptly declines with further increase of temperature. This is because most enzymes are rapidly become denatured at temperatures above 40°C (Cheetham, 1985).

However, a few enzymes can be heated to above 100°C and still retain activity; for instance a denylatekinase can retain activity even after being maintained at a temperature of 100°C. Storage of enzymes at 5°C or below is generally the most suitable, although, some enzymes lose their activity when frozen (Bennett and Frieden, 1969).

Effect of pH:

Pectinmethylesterase reported from *Erwinia chrysanthemi* shows best activity at alkaline pH (Laurent *et al.*, 2000). Pectinmethylesterase of *A. Japonicas* have a maximum activity at pH of 3.8 (Hasunuma *et al.*, 2003). The pectinase activity of *Penicillium chrsogenum* was found to be highest at pH 6.5(Rasheedha *et al.*, 2010). The highest pectinase and polygalacturonase activity of *Asperigillus fumigatus* isolated from wheat bran and orange peel were obtained at pH 4.0 and 5.0, respectively (Urmila *et al.*, 2005). Kashyap *et al.* (2001) reported that the maximum activity of pectinlyase of *Bacillus* species observed at a pH 8. Joao *et al.* (2003) reported that forty- six fungal strains isolated from soil and cacao jelly were screened for pectinase production. All strains were positive for pectinase activity in a cup plate assay, as evidenced by clear hydrolysatation halos. It was notice that *A. niger* (thermotolerant), *Penicillium canescents* (acidotolerant) and *T. viride* (alkaliotolerant) gave the highest pectinase activity at pH 4.0, pH 4.0 and pH 7.5, respectively. An extracellular pectinase was purified to apparent homogeneity from liquid state culture of the thermophilic fungus *Acrophialophora nainiana* by ultra filtration and a combination of gel filtration and ion exchange chromatographic procedures. The results showed that the pectinase enzyme was more active at pH 8.0 (Maria *et al.*, 2006). In a study presented by Baladhand and Thangavelu (2010), the optimum pH was found to be 5.0 for maximum production of pectinase, using *Asperigillus niger* in solid state fermentation. The lower pH (3-4) was reported for polygalacturonase production on complex substrates like citrus pectin and the wheat bran (Galiotuo *et al.*, 1997). However, optimum pH for different

pectinases has been reported to vary from 3.8– 9.5 depending upon the type of enzyme and the source of enzyme (Acuna *et al.*, 1995); optimal pH of PME of *A. niger* was found to be 4.0. While, PGs were shown to possess maximal catalytic activity at pH 5.0 (Shubakov and Elkina, 2002).

Denis *et al.*, (2002) reported that pectinlyase and polygalacturonase production by a newly isolated *Penicillium viridicatum* strain RFC3 were more active at pH 5.0 and 10.5, respectively. Polygalacturonase was stable in neutral pH range and at 40°C, whereas, pectinlyase was stable in acidic pH at 35°C for 1 hour.

In general, enzymes are active only over a limited pH range. A definite optimum pH for an enzyme activity is usually important because, like other proteins, enzymes possess many ionizable groups so that pH changes may alter the conformation of the enzyme, the binding of the substrate, and the catalytic activity of the groups in the active site of the enzymes.

MATERIALS AND METHODS

The effect of cofactors (activators and inhibitors):

Different salts were used, these included Ca^{++} , Ba^{++} , Zn^{++} , Mg^{++} , Mn^{++} , Cu^{++} , Cd^{++} , Ag^+ , Hg^+ and Fe^{++} . 1.0 mg/ml of each salt were added to enzymes extract prepared from *T. viride*, *A. niger* and *P. digitatum*. The salt was shaken manually till it completely dissolved in the enzyme extract. Immediately after adding the appropriate salt, the enzyme was assayed spectrophotometric ally using CMC as substrate for cellulase and pectin as substrate for pectinase. A more detailed study was carried out for Mn^{++} , Ca^{++} , Ba^{++} , these salts were added in different concentrations (0.1– 0.9 mg / ml) to the cellulase preparation from three isolates, before enzyme assayed.

Effect of temperature:

Enzyme preparations were incubated in the presence of substrates (CMC 1% for cellulase and pectin 1% for pectinase) at temperatures ranging from 20°C- 60°C for different period of time (from 4 minutes up to 24 minutes in viscometric method and for 15 minutes in spectrophotometric method). The reactions temperatures were maintained separately using different water – baths thermostatically controlled. The cultures used for enzymes filtrate preparations 7-15 days old.

Effect of pH:

Preparations of cellulase and pectinase enzymes obtained from cultures of the three fungi were assayed by incubation in buffers of different pH values ranging from 4- 10. The following buffers were used: acetic acid - sodium acetate (pH 4.0 – 5.0), phosphate buffer (pH 6.0 – 7.0), Tris-HCL (pH 8.0 – 9.0) or glycine-NaOH (pH 9.0 – 10.0) (Appendix, 1).

RESULTS

The effect of cofactors (activators and inhibitors):

The effect of different metal ions (Zn^{++} , Ca^{++} , Ba^{++} , Mn^{++} , Mg^{++} , Cu^{++} , Ag^+ , Hg^+ , Cd^{++} and Fe^{++}) on the pectinase activity of the three fungi (*A. niger*, *P. digitatum* and *T. viride*) were made using the reducing sugar method. The results in Table (1) showed that the addition of Mg^{++} , Ba^{++} and Ca^{++} to the enzyme assay caused a higher increase in the activity of the pectinase enzyme of the three fungi. However, less increase was found by the addition of Cu^{++} , Fe^{++} , Zn^{++} and Mn^{++} . On the other hand, the addition of Ag^{++} , Cd^{++} and Hg^+ caused a decrease in the activity of this enzyme. Although the different methods were used, the same results were obtained. The analysis of data gave significant difference at rows and the columns.

Table. (1). Effect of different ions (mg/ml) on pectinase activity produced by the three fungi

Ions	<i>A. niger</i>	<i>T. viride</i>	<i>P. digitatum</i>
Ca^{++}	110.8	108.5	118.1
Ba^{++}	113.8	114.1	88.1
Mn^{++}	27.5	16	38.6
Mg^{++}	130	118	86.6
Zn^{++}	80	25.3	35.6
Cu^{++}	6.8	19	30.8
Ag^+	5.0	0.5	83
Hg^+	31.1	4.16	34.6
Cd^{++}	37.5	17.8	21.0
Fe^{++}	56	58.5	43.1

Effect of temperature:

The filtrates of the three fungi (*A. niger*, *P. digitatum* and *T. viride*) were incubated in the presence of pectin as a substrate, at temperatures varying from 10°C to 60°C, then the reaction mixtures were assayed by using the reducing sugar method. The results (Fig. 1) showed that the activity of the pectinase enzymes produced by *A. niger* and *P. digitatum* was increasing with increasing temperature with the maximum at 40°C. However, the pectinase enzyme of *T. viride* was highly active at temperature 50°C.

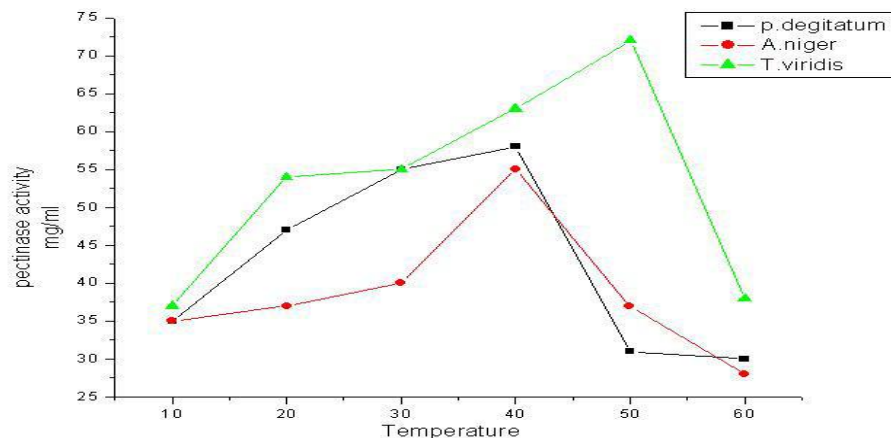


Fig. (1). Effect of different temperatures on pectinase activity produced by the three fungi

Effect of pH:

The three fungi (*A. niger*, *P. digitatum* and *T. viride*) were cultured on the medium for the enzyme production as above. Each culture was incubated for the require incubation time found in the above experiment to give the maximum yield of the enzyme. Preparations of the pectinase enzymes obtained from cultures of the three fungi were assayed by incubation in buffers of different pH values ranging from 4- 10.

The following buffers were used: acetic acid - sodium acetate (pH 4.0 –5.0), phosphate buffer (pH 6.0 –7.0), Tris-HCL (pH 8.0–9.0) or glycine-NaOH (pH 9.0–10.0). Then the reaction mixtures were assayed by using the reducing sugar method at different pH values ranging from 3 to 10. Results on Fig. (2), showed that the maximum activity of the pectinase enzyme produced by *A. niger* was at pH 9. While the filtrates of both *T. viride* and *P. digitatum* were showed two peaks at pH 6 and pH 9.

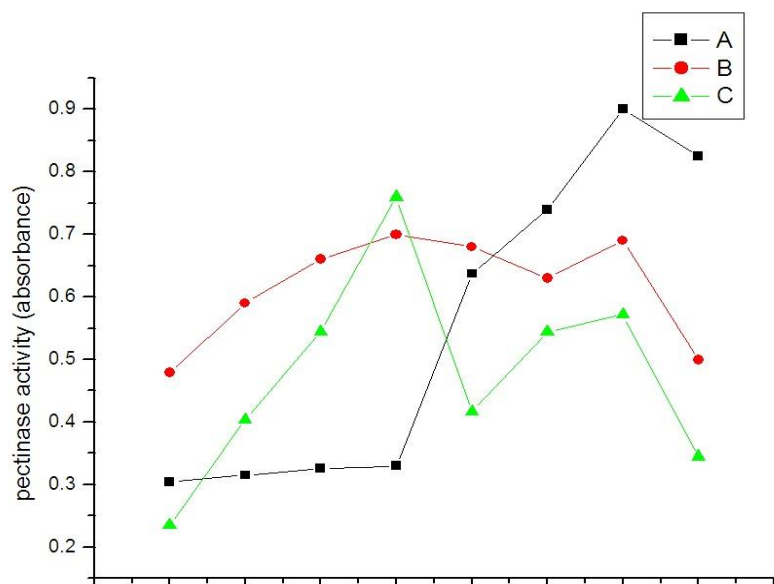


Fig (2): Effect of pH level on pectinase enzyme activity produced by Three fungi (A) *A. niger*, (B) *P. digitatum*, (C) *T.*

DISCUSSION

The optimum temperature of the pectinase activity produced by *A. niger* and *P. digitatum* was found at 40 °C. This result was in agreement with Sangeeta and Shastri (2005) who reported that pectinlyase of *Pecticillum oxalicum* was highly active at 40 °C. Semenova *et al.* (2003) also reported that the five pectic enzymes isolated from *A. japonics*, were stable at 40-50 °C. However, Joshi *et al.* (2011) reported that the optimum temperature for polygalacturonases of *A. niger* and *P. direckii* were shown to be at 50 and 60 °C, respectively. On the other hand, the results showed that the optimum activity of the pectinase produced by *T. viride* was obtained at 50°C, although, Nazia *et al.* (2003) reported that the maximum activity of pectinase produced by *T. harizianum* was at 40°C.

Regarding the effect of the pH on the pectic enzymes activity, the maximum activity of the pectinase produced by *A. niger* was detected at pH 9. While two peaks were recorded for the activity of pectinase of both fungi *T. viride* and *P. digitatum* one at pH 6 and the other at pH 9. However, the higher activity of pectinase produced by *Penicillium chrysogenum* was detected at pH 6.5 (Rasheedha *et al.*, 2010). Denis *et al.* (2002) also found two peaks for the polygalacturonase and pectinlyase production by *Penicillium viridicatum* one at pH 5 and the other at pH 10.5, other authors reported that the optimal pH for pectinases from some fungi (*Asperigillus niger*, *Penicillium canescents* and *Trichoderma viride*) were detected at pH 4, 4 and 7.5, respectively. Pectinase of the thermophilic fungus *Acrophialophora nainiana* was found to be more active at pH 8 (Maria *et al.*, 2006). Amid *et al.* (2012) also reported that the pectinase, which has been extracted and purified from mango (*Mangifera indica*) had its highest enzyme activity at pH 8. The optimal pH for different pectinases has been reported to vary from

separated level ranges 3.8-9.5 depending upon the type of enzyme and the source of enzyme (Acuna *et al.*, 1995). Sangeeta and Shastri (2005) reported that pectinlyase of *Pecticillum oxalicum* was highly active at pH 8. Peciulyte (2007) reported that optimum pH for cellulase activity produced by *A. niger* and *Pecnicillum funiculosum*

was 4.5 and 6, respectively. Soni *et al.* (2008) reported that the optimum pH of cellulase activity of *Asperigillus* sp. was shown at pH 6.

In the present study, it was found that the addition of Ca^{++} , Ba^{++} , K^+ and Mg^{++} to the enzyme assay caused a higher increase in the activity of the pectinase enzyme of the three fungi. On the other hand, the addition of Mn^{++} , Zn^{++} , Cu^{++} and Fe^{++} caused a decrease in the activity. Also Amid *et al.* (2012) reported that Ca^{++} has an important effect on pectinase activity, but Li^+ , Na^+ and K^+ had no effect on its activity, and the reduction in the activity was observed in the presence of Fe^{++} , Cu^{++} , Mn^{++} , Zn^{++} and Al^{+++} . Christina *et al.* (1992) reported that the activity of exo-polygalacturonase of *Scelerotinia scelerotiorum* was inhibited by Hg^{++} , Zn^{++} and Cu^{++} , but Mg^{++} and Ca^{++} were stimulated the activity. However, Alana *et al.* (1990) found that the addition of Ca^{++} , Mg^{++} , Zn^{++} and Mn^{++} have no effect on the pectinlyase activity of *Penicillum italicum*, while, Cu^{++} and Fe^{++} at the same concentration produced complete inhibition.

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العوامل التي تؤثر على نشاط إنزيمات البكتينيز المنتجة بواسطة ثلاثة فطريات

الملخص

استخدمت في هذه الدراسة ثلاثة فطريات لإنتاج إنزيمات البكتينيز وهي الفطريات (*Trichoderma viride*, *Asperigillus niger* and *Penicillum digitatum*) الهيدروجيني) على نشاط الإنزيمات المنتجة بواسطة الفطريات أعلاه. وقد استخدمت طريقة السكريات المختزلة لقياس تلك الإنزيمات. أثبتت النتائج أن إضافة أيونات المغنيسيوم والكالسيوم والبوروم قد أدت إلى زيادة كبيرة في نشاط الإنزيمات المنتجة بواسطة الثلاثة فطريات، في حين أن إضافة أيونات النحاس والحديد والزنك قد أعطت نشاط أقل. ومن ناحية أخرى وجد أن إضافة أيونات الكاديوم والفضة والزيق قد أدى إلى تخفيض نشاط تلك الإنزيمات. ووجد كذلك في هذه الدراسة أن نشاط الإنزيمات البكتينية المنتجة بواسطة الثلاثة فطريات قد زاد مع ارتفاع درجات الحرارة، حيث كانت الدرجة 40°C هي المثلى للإنزيمات المنتجة بواسطة الفطريات *A. niger* و *P. digitatum*. وفي حين كانت الدرجة 50°C هي المثلى للإنزيم المنتج بواسطة الفطر *T. viride*. أما بخصوص درجات الأس الهيدروجيني فقد كانت الدرجة المثلى 9.0 للإنزيم المنتج بواسطة الفطر *T. viride* في حين كانت هنالك درجتان مثلى هما 6.0 و 9.0 لكل من الإنزيمات المنتجة بواسطة الفطريان *A.* و *P. digitatum niger*.