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# Pectinase production by *Aspergillus niger* isolated from decomposed apple skin

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## Abstract

Pectinase activity among twelve different fungal strains, *Aspergillus niger* IM09 was identified as a potential one to produce maximal level 831 U/g at pH 4.0. Media composition, incubation temperature, incubation time, substrate concentration, aeration, inoculum size, assay temperature and nitrogen sources were found to effect pectinase activity. Moisture content did not affect the activity significantly. Media composition was varied to optimize the enzyme production in solid state fermentation. It was observed that the highest pectinase activity of 831.0 U/g was found to produce in presence of yeast extract as a nitrogen source in combination with ammonium sulfate in assay media. Aeration showed positive significant effects on pectinase production 755 U/g at 1000 ml flasks. The highest pectinase production was found at 2 g pectin (521 U/g) used as a substrate. Pectinolytic activity was found to have undergone catabolite repression with higher pectin concentration (205 U/g at 5 g pectin). The incubation period to achieve maximum pectinase activity by the isolated strain *Aspergillus niger* IM09 was 3 days, which is suitable from the commercial point of view.

**Keywords:** Fungi; *Aspergillus niger*; Pectin; Pectinase; Solid-state fermentation

## Introduction

Pectinolytic enzymes, responsible for the degradation of pectic substances are of great technical importance (Spanga *et. al.,* 1995). The principal use of pectinase is to depolymerize and esterify plant pectin in fruits such as apples, lemons, cranberries, oranges, cherries, etc. (Neilsen *et. al.,* 1991; White and White, 1997). The pectinases are also useful for extraction of oils, flavors and pigments from plant materials, preparation of cellulose fibers for linen, jute and hemp production (Castilho *et. al.,* 1999), coffee and tea fermentations (Taragano *et. al.,* 1997) and novel applications in the manufacture of oligogalacturonides as functional food components (Hang and Dornenburg, 2000). It was reported the increase applications for pectinase in textile sector to reduce textile effluent, while improving the quality of the textile substrate (Nikolov, 2003).

Utilization of agricultural and domestic waste is important to keep cleaning of the environment and also to save natural resources. The waste contains considerable amounts of carbohydrates capable of utilization by microorganisms, implying the significance of pectinase (Yoshikawa and Tsuetaki, 1979).

The expense of the production bars the commercialization of novel enzyme-sources although, using high yielding strains, optimal fermentation conditions and efficient enzyme recovery methods can reduce the cost. Therefore, the deep comprehension of various physiological and genetic aspects of pectinase is required for producing thermo-stable and acid stable strains of pectenolytic fungi (Phutela *et. al.,* 2005).

Only a very few studies have been carried out on the microbial pectinase in Bangladesh. Utilization of Industrial waste and save huge amount of foreign currency are the main purpose of this research. Usually pectinases are imported from foreign countries for industrial use in exchange of foreign currency. So, prioritizing the biotechnological importance of fungi in the enzyme industry, the present paper reports the isolation of pectin degrading fungi from various sources.

## Materials and methods

### *Collection of microorganisms*

Twelve different fungal strains were collected from different soil and fruit samples. Soils were collected underneath the

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fruit trees (mango, jackfruit, guava and blackberry) around Jahangirnagar University, Savar and fruits (skins of mango, jackfruit, guava, apple, orange, malta and hog-plum) were collected from different markets (Mohammadpur, New market and Karwan bazaar). One mold was supplied from the Industrial Microbiology Section. For fungal strains, all of these samples are labeled as follows: Mango (Soil) = P1, Jackfruit (Soil) = P2, Guava (Soil) = P3, Blackberry (Soil) = P4, Mango (Skin) = P5, Jackfruit (Skin) = P6, Guava (Skin) = P7, Apple (Skin) = P8, Malta (Skin) = P9, Orange (Skin) = P10, Hog-plum (Soil) = P11, Mold = P12. P is pectinase.

*Isolation of fungi*

After successive dilution of the different samples were inoculated on potato dextrose agar (PDA) media and incubated at 30°C for 1-2 days. The growth of organism from PDA plate was transferred to PDA slant by inoculation with a sterile loop and incubated at 30°C for 3-5 days. For identification of specific strain for the selected fungal isolate, microscopic examination was done by tease mount technique (Baneke and Rogers, 1970).

*Medium and Culture Conditions*

The solid-state cultivation was carried out in medium composed of following composition in gram, (pectin, 1.0; urea, 0.3; saccharose, 3.1; $(NH_4)_2SO_4$, 1.3; $KH_2PO_4$, 0.65; starch, 1.0; $FeSO_4.7H_2O$, 0.30; wheat bran, 16.0; rice husk, 6.0; pH, 4.5-5.0) added in 500 ml conical flask. The final moisture was adjusted to 70% by adding tap water. The flasks were inoculated with concentrated spore suspension and incubated at 30°C for 3 days. After incubation 100 ml distilled water was added to each flask and allowed then to shake at 150 rpm for two hours at 30°C in an orbital shaker. The culture media was then filtered and centrifuged at 4000 rpm for 5 minutes and the supernatants were preserved for determination of the enzyme activity.

*Assay of enzyme activity*

For pectinase assay suitably diluted 1 ml enzyme solution was incubated with 5 ml of pectin solution and 1ml of 0.05 M citrate buffer (pH 5.5) was incubated at 40°C for 10 min. The reducing sugar formed was estimated by Stiles *et. al.* (1926).

One unit of pectinase activity was defined as the amount of enzyme which liberated 1 micromole of galacturonic acid per minute under the above condition.

The selected strain which showed maximum enzyme production was used for the optimization of pectinase production. The effect of pectin composition (1-5 g), incubation period (2-8 days), temperature (30-50°C), moisture adjustment (50%-70%), assay temperature (40-80°C), aeration (250-1000 ml conical flasks), amount of seed (inoculum) 25-100 mg and various nitrogen sources for optimum pectinase production were studied.

**Results and discussion**

*Screening of pectinase producing fungi*

In this study a total of twelve fungi were screened for the pectinase production. Fig.1 indicates that the isolate P-8 produced high pectinase activities (405 U/g), followed by P-10 (392 U/g), P-12 (377 U/g), P-5 (364 U/g), P-4 (343 U/g) and P-2 (324 U/g), respectively. This isolate P-8 was identified as *Aspergillus niger* IM09 by microscopic study. This culture was used for the optimization of pectinase production using solid substrate culturing in different conditions. Due to the constraint of time we focused solely on the study of P-8 in our subsequent investigations.

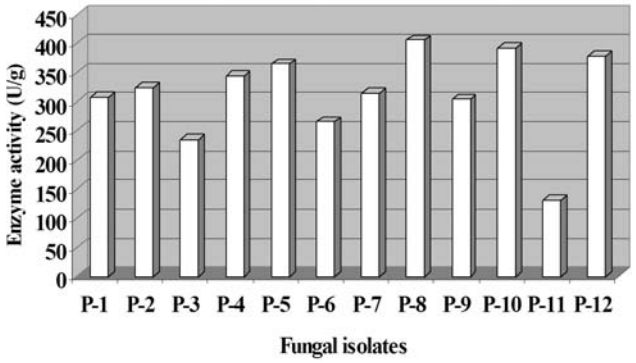


**Fig. 1. Pectinase activity of different fungal isolates from solid state culture**

*Optimization of the selected fungal strain*

*Concentration of Pectin (as the soul source of carbon) optima for the enzyme*

Addition of different amount of commercial pectin (1-5 g) in the production media on the pectinase production was studied. The addition of 2 g pectin to the production media resulted in marked increase in pectinolytic activities producing maximum 521 U/g of pectinase. The enzyme production however was strongly repressed by the addition of 3, 4 and 5 g pectin, showing 424 U/g, 372 U/g and only 205 U/g of pectinase activities, respectively (Fig. 2). The further inves-

tigation was carried out at 2 g pectin level, varying the other parameters such as temperature, incubation period, etc.

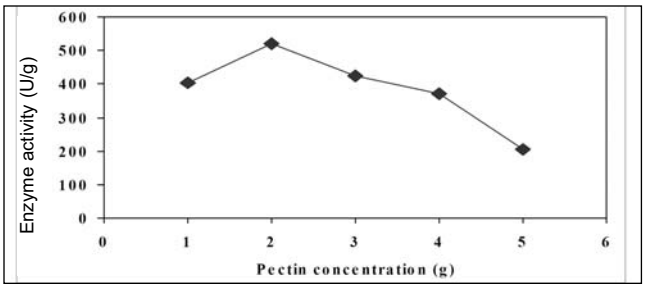


**Fig. 2. Effect of pectin concentration on pectinase production**

*Effect of incubation period on enzyme activity*

The results (Fig. 3) showed that three days incubation was most suitable for maximum pectinase activity (524 U/g) followed by two days incubation (403 U/g). With the increase in incubation period, a sharp decrease in enzyme activity was observed i.e. in 4th, 5th, 6th, 7th and 8th days showing 337 U/g, 248 U/g, 164 U/g, 147 U/g and 123 U/g of pectinase activity.

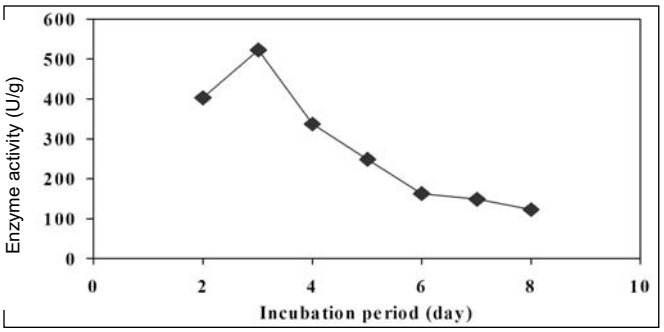


**Fig. 3. Effect of incubation period on pectinase production**

*Effect of temperature on enzyme activity*

The results in Fig. 4 showed that the flasks containing assay media after cultivation supported maximal pectinase (647 U/g) activity at 40°C, followed by 583 U/g (at 35°C), 516 U/g (at 30°C), 515 U/g (at 45°C) and 392 U/g (at 50°C), respectively.

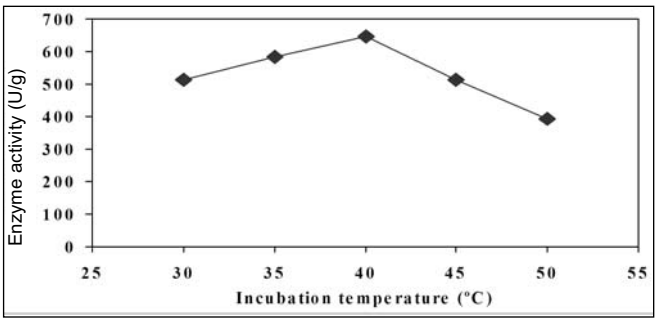


**Fig. 4. Effect of temperature on pectinase production**

*Effect of Moisture for enzyme production*

The results (Fig. 5) showed that the adjustment of 70% moisture to the production media resulted in a maximum pectinolytic activity of 648 U/g of, followed by 614 U/g (at 80%), 595 U/g (at 90%), 593 U/g (at 60%) and 527 U/g (at 50%), respectively. Pectinase activity gradually increased till 70% of moisture adjustment and then decreased till 90%.

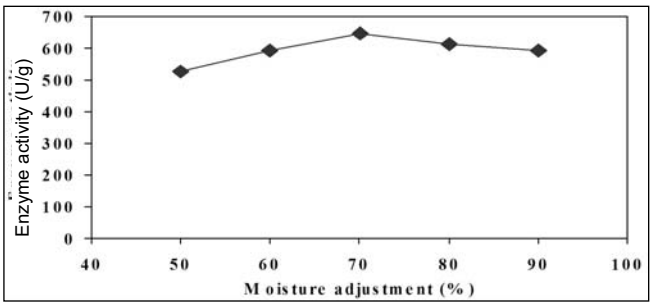


**Fig. 5. Effect of moisture adjustment on pectinase production**

*Effect of assay temperature on enzyme activity*

The results (Fig. 6) showed that the enzymatic activity was most suitable at assay temperature of 40°C for maximum pectinase activity (651 U/g) followed by 50°C (572 U/g). With the increase in temperature the decrease was pronounced. This is evident from the fact that incubating the enzyme at 80°C for 10 min resulted in 45% loss of activity.

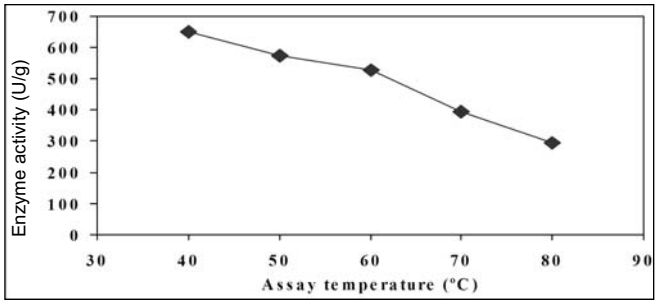


**Fig. 6. Effect of assay temperature on pectinase production**

*Effect of aeration for the enzyme production*

The results in Fig. 7 showed that the flasks containing assay media after cultivation supported maximal pectinase (755 U/g) activity in 1000 ml conical flask, followed by 677 U/g (750 ml conical flask), 638 U/g (500 ml conical flask), 578 U/g (250 ml conical flask), respectively.

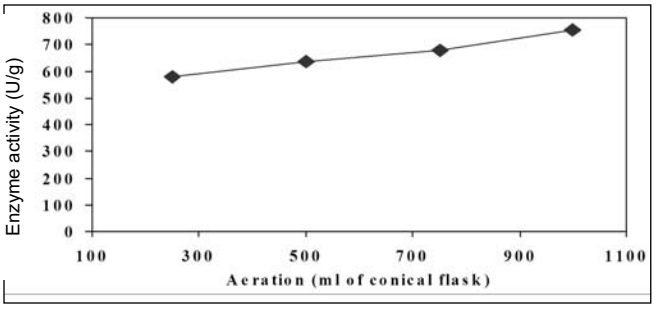


**Fig. 7. Effect of aeration on pectinase production**

*Optimum amount of seed for the enzyme production*

The results in Fig. 8 showed that the flasks containing 50 mg of seed inoculated in assay media after cultivation supported maximal pectinase (819 U/g) activity, followed by 739 U/g (25 mg seed), 697 U/g (75 mg), 466 U/g (100 mg), respectively.

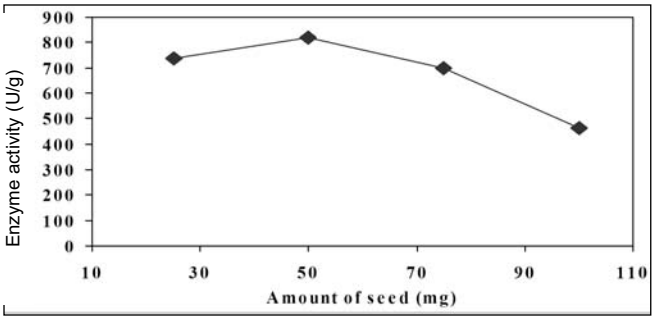


**Fig. 8. Effect of amount of seed on pectinase production**

*Effect of nitrogen sources on enzyme activity*

Various nitrogen sources used in the assay media (Fig. 9), yeast extract in combination with $(NH_4)_2SO_4$ was found to support maximal production of pectinase (831 U/g) followed by urea and $(NH_4)_2SO_4$ (761 U/g), $(NH_4)_2NO_3$ and $(NH_4)_2SO_4$ (532 U/g) and peptone and $(NH_4)_2SO_4$ (417 U/g), respectively. Here, AS = $(NH4)_2SO_4$, AN = $(NH_4)_2NO_3$, YE=Yeast extract, U= Urea and P= Peptone. All (i.e. AS, AN, YE & U) were used as 1.3 g in the assay media.

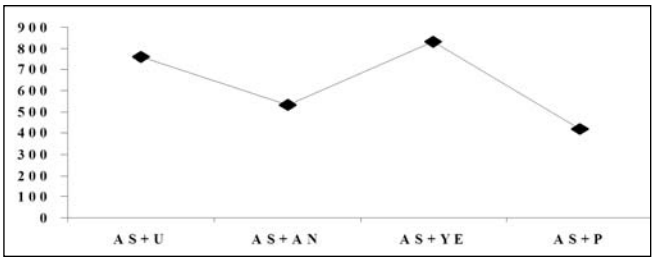


**Fig. 9. Effect of nitrogen sources on pectinase activity**

In the present study, the isolate P-8 was found to be a potential source of pectinolytic enzymes and this isolate was identified as *Aspergillus niger* IM09 by microscopic study and selected for further experiment.

In the investigation, pectin is used as an inducer and the sole carbon source, similar to that reported by Fontana *et. al.* (2005) and Maldonado and Strasser de Saad (1998) with *A. niger.* The result showed that pectinase activity was increased (521 U/g) up to 2 g addition of pectin markedly from 1 g addition (405 U/g). But the enzyme activity decreased continuously till 5 gm addition, showing activity of 424, 372 and 205 (U/g) for 3, 4 and 5 g, respectively, similar to Galiotou *et. al.*, (1996) who reported that higher pectin concentrations caused retardation of fungus growth and enzyme production. These results also might be indicative of an enzymatic repression, probably due to the presence of some pectin degradation products which act as enzyme inhibitors or different molecular forms of the pectinase sensible to these compounds, as observed by Maria *et. al.*, (2000); Leone and Van der Heuvel (1987) and Malvessi and Silveira (2004).

The period of incubation depends upon the nature of the medium, fermenting organism, concentration of nutrients and the process physiological conditions (Sarvamangala and Dayanand, 2006). The present study showed that three days (72 h) incubation was most suitable for maximum pectinase activity (524 U/g) supported by Acuna-Argulles *et. al,.* (1995); Linde *et. al*., (2007); Baracat *et. al.,* (1989) and Castilho *et al*. (2000) and better than that found by Friedrich *et. al*., 1989 (95 hour). Sarvamangala and Dayanand (2006) observed that the period of fermentation in synthetic medium by pectinolytic fungi vary from 48 to 72 h and this observation is supported by Shivakumar and Krishnanand (1995), Solis-Pereira *et. al.*, (1996) and Pandey (1991). So, the max-

imum pectinase enzyme production at 3 days (72 h) in the present study indicates that it is suitable from the commercial point of view (Phutela *et. al.*, 2005). Incubation temperature has been found to be a significant factor for enzyme production (Kitpreechavanich *et. al.*, 1984). The results in the present study showed that the flasks containing assay media after cultivation supported maximal pectinase (647 U/g) activity at 40ºC followed by 583 U/g (at 35ºC), 516 U/g (at 30ºC), 515 U/g (at 45ºC) and 392 U/g (at 50ºC), respectively. The results showed that this pectinase producing fungal strain is more thermo-tolerant (40ºC) than other findings by Tuttobello and Mill (1961); Ueda *et. al.* (1982); Maldonado and Callieri (1989) who showed their pectinase producing fungi grow at 30ºC and similar to the results of Larios *et al.* (1989), Bailey (1990), Baracat *et. al.* (1991) and Angayarkanni *et. al.*, (2002) (at 40ºC).

Moisture adjustment varied at different cultural conditions. Excess moisture may contaminate the media by supporting the growth of bacteria and virus and low moisture content suppress the growth of pectinase producing fungal strain. The results showed that the adjustment of 70% moisture to the production media resulted in increase in pectinolytic activities producing maximum 648 U/g of pectinase activity which is supported Silva *et. al*. (2005) and Krishna and Chandrasekaran (1996) and Kashyap *et. al.* (2002) but not similar to the result observed Linde *et. al.,* (2007).

In the present findings, maximum pectinase activity of 651 U/g was observed at assay temperature of 40ºC followed by 50ºC (572 U/g) 60ºC (527 U/g), 70ºC (392 U/g) and 80ºC (293 U/g). This highest enzyme activity at 40ºC supports the result observed by Galiotou *et al.,* (1996). But after 40ºC, enzyme activity was decreased up to 80ºC. So, with the increase in temperature the decrease of enzyme activity was pronounced and this result supports Acuna-Argulles *et. al.* (1995) who reported that at higher temperatures (50ºC or more) all enzymatic activities decreased after the incubation time in solid-state fermentation (SSF) . Moreover, this is evident from the fact that incubating the enzyme at 80ºC resulted in 45% loss of activity compared to the enzyme activity at 40ºC and is quite similar to the observation of Phutela *et. al.* (2005) who reported only 25% loss of enzyme activity at 80ºC incubation did not support the result in the case of optimum temperature for optimum enzyme activity compared to the present study.

Aeration affected fungal growth (Dekker and Barbarosa, 2001). The results showed that the flasks containing assay media after cultivation supported maximal pectinase (755 U/g) activity in 1000 ml conical flask Enzyme activity was increased 24% at 1000 ml conical flasks (755 U/g) compared to 250 ml conical flasks (578 U/g). The present study clearly indicates that pectinase activity of the fungus was quite actively increased with increasing rate of aeration and supports the results obtained by Linde *et. al*., (2007); Mitchell *et. al.*, (2003) and Santos *et. al*., (2004). This result also indicates that growth and activity of the fungi (Aspergillus niger) is quite effective in optimum aerobic condition supported by Venugopal *et. al.*, (2007).

In the present study, the results showed that the flasks containing 50 mg of seed inoculated in assay media after cultivation supported maximal pectinase (819 U/g) activity, followed by 739 U/g (25 mg seed), 697 U/g (75 mg), 466 U/g (100 mg), respectively. The enzymatic activity increased up to 50 mg inoculum addition in the solid-state culture but then decreased continuously showing the activity of 697 U/g and 466 U/g by the inoculation of 75 mg and 100 mg inoculum, respectively in the assay media. The reason of decreasing activity would be due to the substrate of enzyme and initial amount of spores used as inoculum which is observed and supported by Alana *et. al.*, (1989) the substrate (16 g wheat bran and 6 g rice husk) in the assay media was not sufficient for greater amount of inoculum (for 75 and 100 mg) as substrate.

In the present study, $(NH_4)_2SO_4$ with Yeast extract showed the highest enzyme activity (831 U/g) followed by $(NH_4)_2SO_4$ with Urea (761 U/g), $(NH_4)_2SO_4$ with $(NH_4)_2NO_3$ (532 U/g) and $(NH_4)_2SO_4$ with peptone (418 U/g) in the given order. The important findings in this study is that the yeast extract is the most useful nitrogen supplement for optimum productivity of the pectinase enzyme comparing to urea, $(NH_4)_2NO_3$ and peptone. The result of the highest enzymatic activity (532 U/g) by addition of yeast extract + $(NH_4)_2SO_4$ is similar to the results investigated by Boccas *et. al.,* (1994), Aguilar and Huitron (1990); Galiotou *et. al.,* (1996) and Larios *et. al.,* (1989) and specially supported by Phutela *et. al.*, (2005).

## Conclusion

The study has highlighted that the novel *Aspergillus niger* IM09 isolate is a good source for producing pectinases on cheap carbon source in short incubation period during solid-state of cultivation. The produced pectinases are catalytically active at rather high temperature and low pH. Further study will be carried out on the production, characterization and purification of pectinases for the industrial use of this highly demanding enzyme in Bangladesh as well as all over the world. This fungal strain could be considered under genetic modification for enormous production of pectinolytic enzymes, pectinase.

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