Purification and Characterization of Polygalacturonase Enzyme by *Aspergillus Species* Using Citrus Peel by Solid State Fermentation

Motwani D.R.[1], Meshram V.G. [1]and Jambhulkar V.S.[2]
1 Department of Biochemistry, Nagpur University, Nagpur-440033.
2 SindhuMahavidyalaya ,Panchpaoli Nagpur-440017.
E.mail:daminimotwani@rediffmail.com.

Abstract:

The economic and ecological function of pectinase enzymes in industries is gaining much attention with the need of highly productive strains of fungus to reduce production cost. The present investigation is a comparative evaluation of Aspergillus species. polygalacturonase. The three fungal isolates isolated from agro-waste, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigates* showed polygalacturonase very high polygalacturonase activity with polygalacturonic acid as the substrate. The three isolates also showed varying higest degree activity towards citrus peel as compared to other substrate. The effect of temperature on the polygalacturonase of the three isolates showed that *Aspergillus flavus* had optimum temperature at 40° C while *Aspergillus niger* and *Aspergillus fumigatus* both showed optimum activity at 35°C.On the effect of pH all the Aspergillus species showed optimum polygalacturonase activities at pH 4.5 The enzyme had an approximate molecular weight of 53kDa. Metal ions affects the activity of polygalacturonase produced by the three isolates They were activated by Ca2+ and significantly inhibited by Ba2+, Hg2+.Application of PG in fruit juice clarification was found.

Keywords:
Aspergillus species, polygalacturonase, polygalacturonic acid, agro-waste, citrus peel.

Introduction:

In today's world, degradation of vegetable waste is an important phenomenon. About 72 per cent of the vegetables produced in India go waste because of lack of proper retailing and adequate storage capacity. So in recent years, considerable attention is being paid by the scientific community in order to exploit commercially useful products from these wastes. A perusal of literature indicates that enzymes such as pectinase can be easily extracted from vegetable waste like beet, carrot, cabbage, tomatoes, by simple extraction process.

Enzymes involved in degradation of pectins are wide spread in nature and can be found in many plants, bacteria, and fungi (Rexova Benkova, L., and Markovic, O. 1976). The most important enzymes of a pectinase complex are polygalacturonase (EC 3.2.1.15), pectin lyase (EC 4.2.2.10), pectatelyase (EC 4.2.2.2), and pectinesterase (EC 3.1.1.11) (Gracheva, I. M., andKrivova, A. Yu. (2000).Recently pectinases have become more widely used in food, textile, and other branches of industry. The best known microbial producers of pectinases

are different species of *Aspergillus* fungi exhibiting as a rule a wide spectrum of pectinase activities

(Godfrey, T., and West, S. 1996)Enzyme preparations based on *Aspergillus* have been successfully used in practice, being employed for processing of vegetables and fruits, as well as for clarification of juices and wines. Among the commercial pectinolytic enzymes, preparations obtained by the industrial cultivation of different strains of *Aspergillus niger* and *Aspergillus awamori* are the most popular ones (Godfrey, T., and West, S. 1996).The goal of the present study was to isolate and study the properties of the enzyme polygalacturonase that is produced by *Aspergillus species*, promising producer of pectinases The rationale behind carrying out thepresent investigation was to utilize the wastethat is generated from vegetable market andextract fungal isolates that produce polygalacturonase enzyme at a cheaper rate.

Material and methods:

In view of getting efficient pectin degrading cultures, soils rich in pectic waste and fruit waste samples were scrutinized including fruit processing area, sewage of juice centres of different locations. For isolation of pectinase producing microorganisms pectin containing agar medium was used (Panda et al, 2004). Isolation was carried out in two steps comprising
i) enrichment and ii) isolation as follows;

i) Enrichment- The 1gm of sample was inoculated in 100 ml of pectin broth (5% w/v yeast extract, 1 ml of 0.1% v/v bromothymol blue, 0.6 ml of 10% v/v $CaCl_2 \cdot 2H_2O$, pH 6.0) supplemented with 0.2% w/v pectin and incubated on rotary shaker (120 rpm) at 30 °C for 7 days. A 1ml of previously enriched culture was inoculated in 100 ml of freshly prepared pectin broth supplemented with 0.5% w/v of pectin for next 7 days of enrichment. After several serial transfers in pectin broth, each time with increasing pectin concentration up to 2.0%, the soil sample was finally subjected for isolation of pectinase producers

ii) Isolation- After fifth week of enrichment, 0.1 ml of broth was spread on the surface of pectin gel containing basal agar medium plate and incubated at 30 °C.

Identification of fungal isolate

The isolated fungal culture was identified as *Aspergillus* ssp. based on its morphological andmicroscopic characteristics and these values matched with values in standard referencebook compendium of soil fungi.

Screening for pectinase producers

The isolates were screened for pectinase activity. These was done by inoculating the organisms on the pectinase screening agar medium (PSAM) plates and incubated at 37°C for 7 days. Then the plates were flooded with iodinesolution and incubated for 15min at room temperature. Aclear zone

around the growth of microorganisms indicated pectinase activity (E. Venkata Nagaraju, G. Divakar (2013). The efficiency to solubilize pectin wasassessed in terms of solubilization index calculated using theformula:

Solubiization index = Colony + Halozone diameter/Colony diameter

Composition of the media used for isolation (g/l, wt/vol.): 1g pectin, 0.3g ammonium chloride, 0.2g KH_2PO_4, 0.3gK_2HPO_4, 0.01g $MgSO_4$, 2.5gm agar in 100ml.

Confirmation of polygalacturonase enzyme

The polygalacturonase (PG) type of enzyme was determined by using DNSA method (Miller, 1959)

Viscosity

The reduction of the viscosity of the peach juice after the enzymatic treatment using the crude extract was evaluated in viscometer "Falling Ball" (Ustok et al., 2007).

Partial optimization of culture conditions for PG production

The operating variables for fermentation were tried to optimize which included pH, temperature and time of incubation. Optimal temperature and pH were obtained by varying the temperature range from 10 °C to 50 °C and pH 2.0 to pH 8.0. In addition, PG enzyme production was monitored from 2 to 10 days of incubation. The optimized culture conditions were used for *in vitro* PG enzyme production.

Precipitation and dialysis of enzyme

The crude enzyme was subjected to 20-80 % Ammonium Sulphate precipitation (Olutiola and Akintunde, 1979), saturated overnight at 4°C, then centrifuged at 10,000 ×g for 30 minutes (Coelho *et al.,* 1995) and thereby dialyzed using dialysis bag.

SDS–Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE was performed to check the homogeneity of the enzyme on a 12% gel (Laemmli, 1970) and molecular weight determined by standard protein marker. The protein bands on gel were visualized by staining it with Coomassie brilliant blue G- 250.

Effect of metal cations:

The effect of various metal cations at a concentration of 10 mM on *Aspergillus species* PGase was examined. Aspergillus species PGase are activated by Ca2+ metal ion. The divalent cations Hg2+ and Ba2+ strongly inhibited *Aspergillus species* PGase.

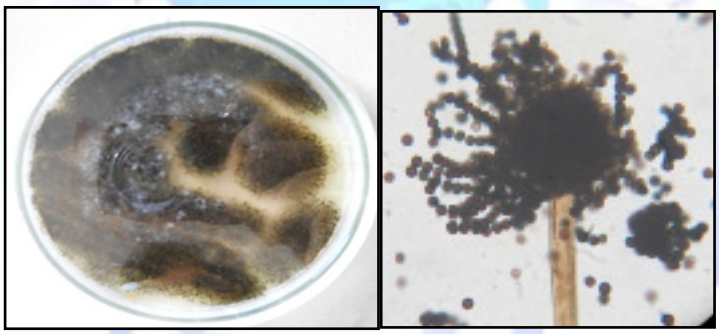
Results and discussion:

As the main objective of this study was to isolate pectinolytic enzymes from the cheap sources, spoiled fruits, vegetables and soil were selected. The strains which produced maximally was cultured by changing the various parameters for the better output. *Aspergillu* sp. was cultured under solid state

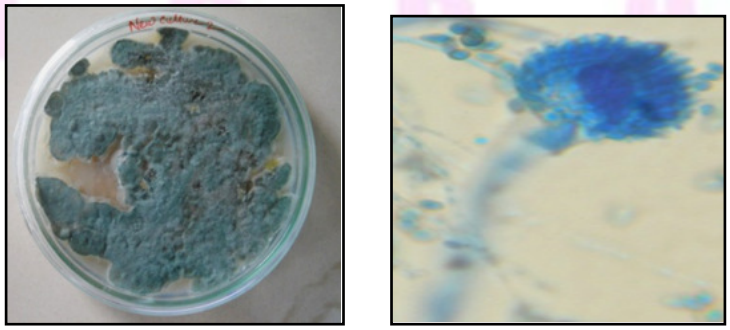
fermentation for PG enzyme production. Solid state fermentation was done by using citrus peel as a solid substrate. Total three fungi were selectively isolated by enrichment method. The morphological studies identified the strains as *Aspergillus species*. On the basis of prominent zone of clearance on pectin agar, plate strains were selected for further study (Figure 1 and 2).

In SSF fermentation the PG production reached maximum at 6th(Figure 3) day of incubation period and the exact temperature was found to be 35 °C. Arpita *et al.*, 2011, also stated that, when the temperature is altered below or above the optimum the activity is decreased or becomes denatured. The maximum production of PG enzyme was obtained at 37 °C by the *Aspergillus specie*(Figure 4).The maximum production of PG enzyme was obtained at pH 4.5(Figure 5)and hence, considered as optimum. Increase in pH further decreased the production of enzyme.

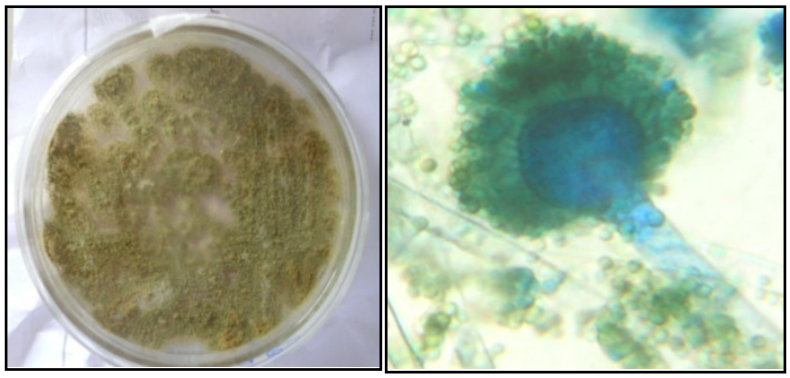
According to this study, the molecular weight of the pectinase was known by the formation of single band in gel adjacent to marker and was found to be 53kDa(Figure 7). In this case of polygalacturonase, activity was enhanced by $CaCl_2$ only. $Mg^{2+}$was the activator of polygalacturonase, and $BaCl_2$, $FeCl_3$ and $ZnCl_2$ were the potent inhibitors of polygalacturonase (Figure 6).



Aspergillus niger

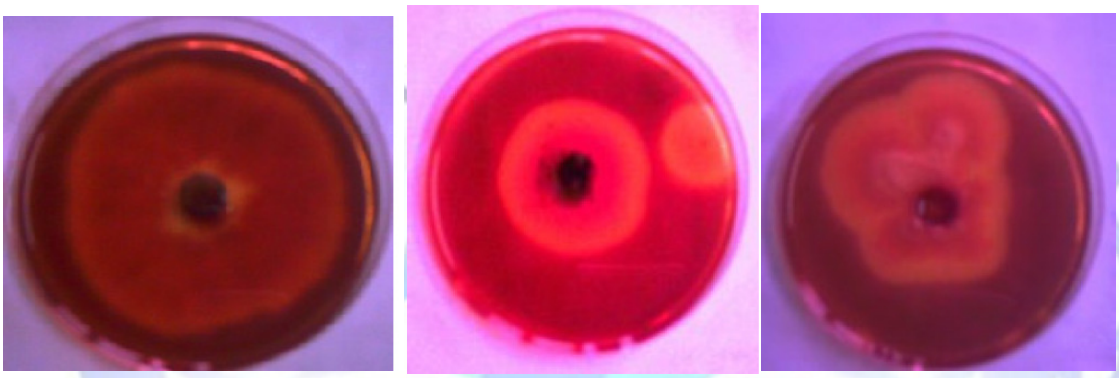


Aspergillus fumigatus



Aspergillus flavus

Figure. 1: Morphological and Microscopic characteristics of *Aspergillus species.*



Aspergillus niger ***Aspergillus fumigates*** ***Aspergillus flavus***

Figure. 2: Prelimiary screening of *Aspergillus species* for PG production

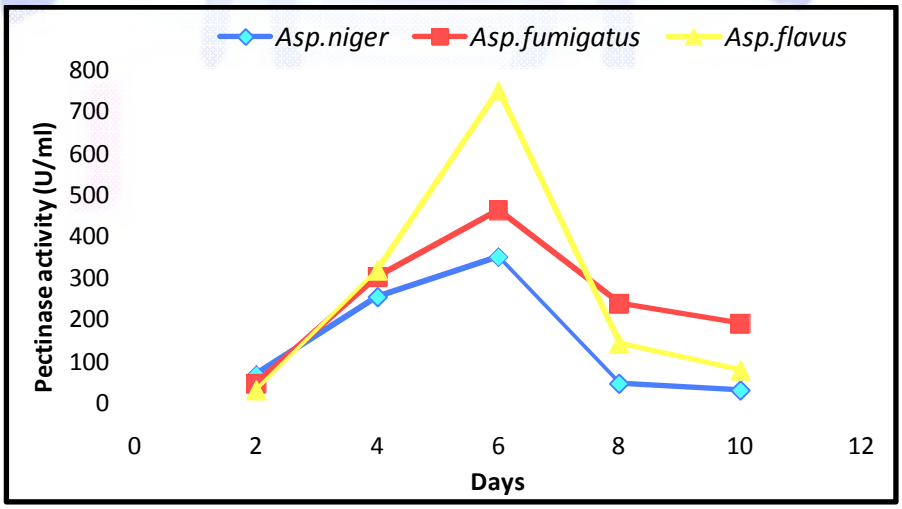


Figure. 3: Effect of incubation period on PG production

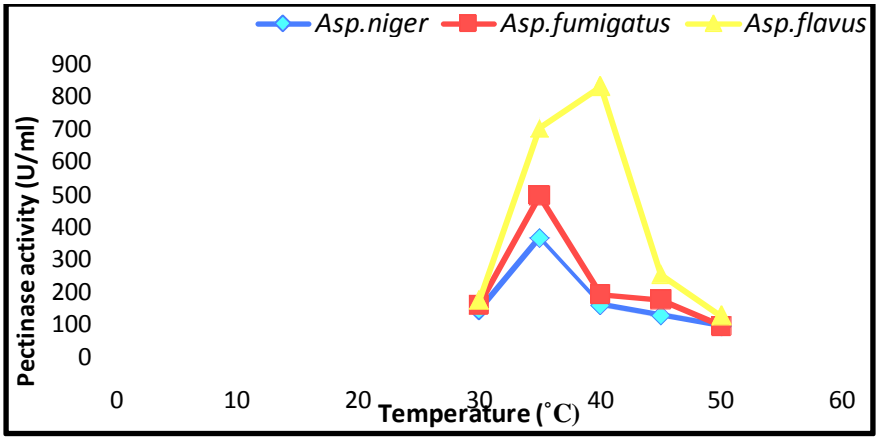


Figure 4: Effect of temperature on PG production

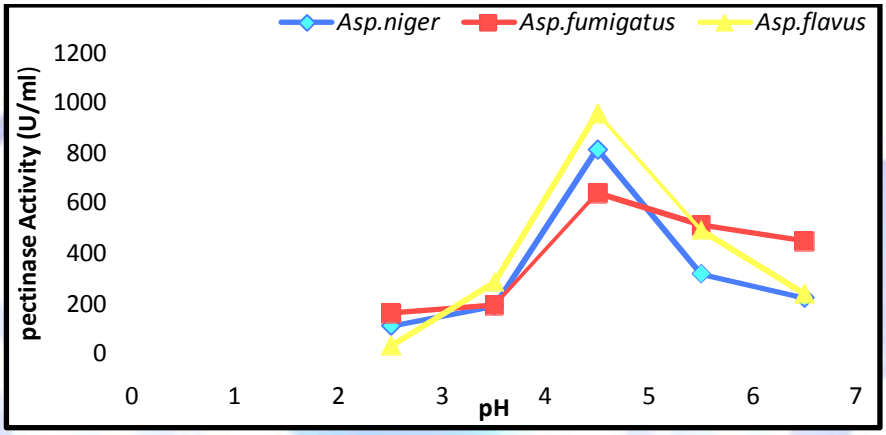


Figure 5: Effect of pH on PG production

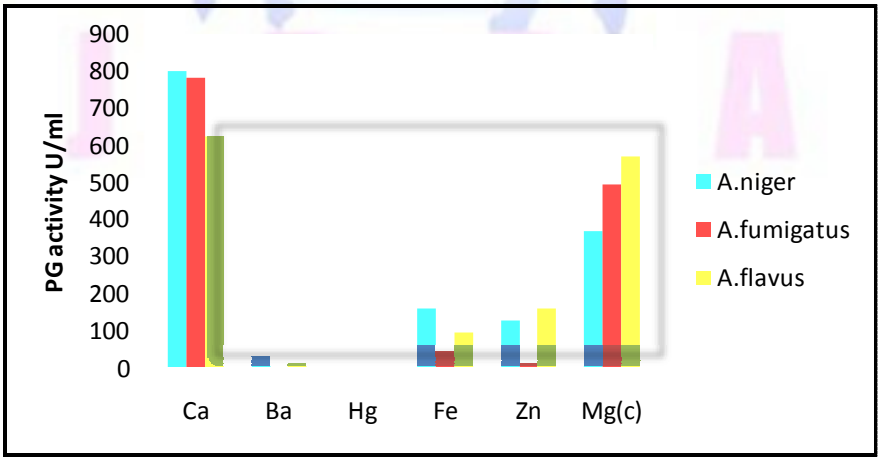


Figure. 6: Effect of metal ions on PG production

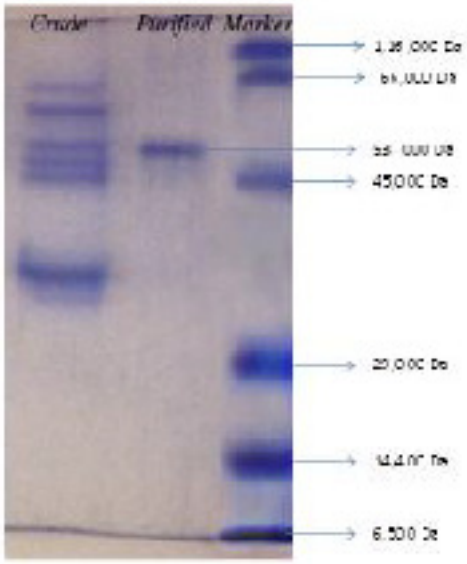


Figure 7: Analysis of Polygalacturonase purification by SDS-PAGE

Figure 8: Fruit juice clarification with the help of extracted PG

References:

ArpitaDey, Moumita Karmakar andRina Rani Ray (2011). *Der Pharm. Lett.*, , 3(2), 358–367.

Coelho M. A. Z., Medronho R. C., Leite S. G. F. and Couri S. (1995). Partial purification of a polygalacturonase produced by solid state cultures of *Aspergillusniger*3T5b. *Rev. Microbiol.*, 26(4):318-322.

Domsch K. H., Gams W. and Trevte-Teidi Anderson (1980) Compendium of soil fungi, Academic Press, London,

E.Venkata Naga Raju and G. Divakar (2013). 'Screening and Isolation Of Pectinase Producing Bacteria from Various Regions in Banglore'. International Journal of Research in Pharmaceutical and Biomedical Sciences.Vol. 4(1). *Journal of Analytical Chemistry,* 31: 426 - 428.

Godfrey T. and West S. (1996). Industrial Enzymology ,Second Edition , The Macmillan Press Ltd., London.

Gracheva I. M. and Krivova A. Yu. (2000) Engineering of Enzyme Preparations [in Russian], Elevar, Moscow, pp. 227-255.

Laemmli U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4.*Nature*, 227(5259):680-685.

Miller G. L. (1959). Use of dinitrosalycylic acid reagent for determination of reducing sugar,

Olutiola P. O. and Akintunde and O. A. (1979). Pectinlyases pectinmethylesterase and production by *Pennicilliumcitrinum. Transaction of British Mycological Society*, 72:49-55 (10).

Panda T., Nair S. R., and Kumar P. M., Regulation of synthesis of pectinolytic enzymes of *Aspergillus niger*. Enz. Microbial. Technol. 34, 466–473

Rexova Benkova, L. and Markovic O. (1976). Adv.Carbohydr. Chem. Biochem.33, 323-385.

Ustok F. I., Tari C. andGogus N. (2007). Solid-state production of polygalacturonase by *Aspergillussojae* ATCC 20235. J. Biotechnol.127:322-334