Partial characterization of pectinase produced by ***Aspergillus niger*** grown on wheat bran

Motwani D.R[1] , Meshram V.G [1] Jambhulkar V.S[2]

Institutions: 1)Department of Biochemistry, RTM Nagpur university,Nagpur.440033,India.

2)Sindhu Mahavidyalaya , Panchpaoli Nagpur.440017,India

Email:daminimotwani7@Gmail.com

***Corresponding Author:** Damini Rakesh Motwani.

1. Department of Biochemistry, RTM Nagpur university,Nagpur.440033,India.

Mobile no:9970999562

Email:daminimotwani7@Gmail.com

Abstract

Four filamentous fungi isolated from soil, were found to depolymerise pectin.Best pectinolytic activity ,as indicated by the diameter of clear ,hydrolyzed zones on the medium containing commercial citrus pectin as sole carbon sources,was obtained with *Aspergillus niger*. *Aspergillus niger* also produce pectinase with different agrowastes(rice bran,sugarcane bagasse,lemon peel and banana peel) as sole carbon source.The highest pectinase activity was produced with wheat bran as sole carbon source.The optimum pectinase activity of 368u/ml was obtained by solid state fermentation(SSF) at 4th day by *Aspergillus niger*.The isolated strain of *Aspergillus niger* have good prospect for pectinase production. Wheat bran is a good low cost fermentation substrate for pectinase production by investigated fungi. The specific activity went up to 35.72 U/mg protein with a purification fold 6 and 68.64% recovery. The enzyme showed a molecular weight of 35.022 kDa by SDS polyacrylamide gel

electrophoresis..The pectinase was found to be stable at pH4.5 and temperature 47°C respectively.

Key words: *Aspergillus niger*, agrowastes , solid state fermentation(SSF), wheat bran, pectinase.

Introduction:

Solid-state fermentation is traditionally defined as those processes in which microbial growth and products formation occur on the surfaces of solid substrates in the near absence of free water. Due to this low amount of water available in solid-state bioprocessing, the class of microorganisms that are most commonly used are fungi (Zheng et al, 2000; Pandey et al., 2001). Several agro-industrial wastes and by-products such as orange bagasse (Martins et al, 2002), sugar cane bagasse (Silva et al., 2002) wheat bran (Cavalitto et al., 1996) and other food processing waste (Zheng and Shetty,2000) are effective substrates for depolymerizing enzyme production by solid-state fermentation. Recently, a large number of microorganisms, isolated from different materials, have been screened for their ability to degrade polysaccharide present in vegetable biomass producing pectinases on solid-state culture (Soares et al, 2001; Gomes et al., 2001).Pectinolytic enzymes are classified according to their way of attack on the galacturonan part of the pectin molecule. They can be distinguished between pectin methylesterases (EC 3.1.11.1) that deesterify pectins to low methoxyl pectins or pectic acid, and pectin depolymerases, which split the glycosidic linkages between galacturonosyl (methyl ester) residues. Polygalacturonases split glycosidic linkage next to free carboxyl groups by hydrolysis while pectate lyase split glycosidic linkages next to free carboxyl groups by β-elimination. Both endo types of PGs and PALs (EC 3.2.1.15 and EC 4.2.2.2, respectively) are known by spliting randomly the pectin chain. Exo-PGs (EC 3.2.1.67) release monomers or

dimmers from the non-reducing end of the chain, whereas exo-PALs (EC 4.2.2.9) release unsaturated dimmers from the reducing end. Highly methylated pectins are degraded by endo-pectin lyases (PL; EC 4.2.2.10) and also by acombination of PE with PG or PAL (Sarkanen et al, 1991, Pilnik et al, 1993). The aim of this work was to study pectinolytic enzymes production by newly isolated strains of fungi by solid-state fermentation using wheat bran as a substrate. Wheat bran was obtained commercially, dried and used untreated.

Materials and Methods:

Sample Collection:

Agriculture and vegetable waste dump soil samples were collected from Sausar, Chhindwara ,district, M.P, India. The soil sample were collected from Sausar fruit market in sterile plastic bags ..

Isolation of fungi:

One gram of soil samples from each collection site was pooled and homogenized in sterile distilled water and 10-fold serial dilutions were prepared. One ml from each dilution was inoculated by spread plate method on to the sterile petriplates containing yeast extract pectin (YEP) medium with pH 4.5 containing pectin 2.5g, and yeast extract 5.0g at 37°C. Pure cultures were sub cultured onto solid media and maintained for identification and enzyme studies.

Identification of isolated fungi:

The identification of isolated fungi was done by studying morphological characteristics as observed under the microscope (Onions *et al*. 1981)

Screening Of Pectinase:

The YEP medium was used for isolation of cultures supplemented with 2% agar. Pure culture was inoculated by puncture in the medium and incubated for 48hrs at 30°C. After incubation, conidial suspension was poured 50ul into well. After 6 days of incubation period, the plates were flooded with 0.12% congo red solution for one hour and then washed with tap water. The diameter of colonies and clear zones were measured. The relative clear zones were calculated with the following formula

$$\text{Relative clear zone} = \frac{\text{Diameter of clear zone}}{\text{Diameter of fungal colony}}$$

Preperation of spore suspension: *Aspregillus niger* was subcultured on PDA slants and used to prepare the spore suspention. The spore crop from each slant was scrapped into 5ml of sterile distilled water using glass rod..

Solid state fermentation (SSF):

10g wheat bran powder was weighed (10 g/flask) and distributed into 250 ml Erlenmeyer flasks (without carbon source) to a desired solid-liquid ratio (up to 20% solid) and 0.2M sodium acetate buffer (pH = 4.5), followed by sterilization for 15 min. at 15 psi (121°C) in an autoclave. To the production medium 10^8 spores of *Aspergillus niger* were inoculated asceptically and the flasks were then covered with cotton to allow CO_2 produced during fermentation to escape. The flasks were incubated in a rotary shaker (200 rpm) at 30°C for 144 h. Samples were withdrawn periodically (24 h interval) and were analyzed for total pectinase enzyme activity.

Preparation of crude enzyme fraction:

The crude pectinase was extracted by mixing 10 g of fermented materials with distilled water, stirred for 20 min. in the shaker, filtered and then centrifuged for 20 min. The supernatant was used as the crude enzyme fraction..

Total pectinase assay

A suitably diluted sample of 0.5 ml was added to a solution containing 2 ml of 1% citrus pectin in acetate buffer (pH 4.8) in a test tube. Samples are kept at 45°C for 30 min. in a water bath, cooled, added with 2.5 ml of DNS reagent, heated for 5 min.(Miller GL.1959). Finally the contents were cooled and 10 ml of distilled water was added to it and optical density was measured at 540 nm using UV/Vis Biospectrophotometer (ELICO BL 198). The concentration of β-galacturonic acid was determined from the standard β-galacturonic calibration curve

Enzyme unit:

One unit of enzyme activity is defined as the amount of enzyme required to liberate 1µ mole of reducing groups per minute with galacturonic acid as standared under the assay condition.

Protein determination*:*

Protein was determined by the Lowry *et al*., (1951) method using bovin serum albumin as a standard

Ammonium sulphate precipitation: The supernatant was brought to 60% saturation by mixing ammonium sulphate (pH 4.5) slowly with gentle agitation and allowed to stand for 24hrs at 4°C in the cold room. After the equilibration, the precipitate was removed by centrifugation (10,000 rpm at 4°C for 20 min). The precipitate obtained was dissolved in 10ml of 0.2M sodium acetate Buffer (4.5)

Desalting by dialysis: The precipitate was desalted by dialysis following the standard protocol .The 10 c. m pretreated dialysis bag was taken and activated by rinsing in double distilled water. One end of the dialysis bag was tightly tied and the precipitate recovered was taken inside the bag. The other end of the dialysis bag was tightly tied to prevent the leakage. After that, dialysis bag was suspended in a beaker containing sodium acetate buffer (pH 4.5)

Determination of molecular weight:

The molecular weight of the pectinase was determined by sodium dodecyl sulphate-ployacrylamide gel electrophoresis (SDS-PAGE) . SDS-PAGE was preformed as described earlier by Laemmli. (Laemmli 1970)

Results and Discussion:

The main objectives of this research is to utilize of worthless agro industrial waste in the field of industries for making fermentation economic and cost effective . About seven isolate were obtained from decompost soil and grown on PDA. Out of the seven isolates, four strains were able to grow on pectin- congo red agar medium . The decolourisation around the colonies observed against a white background were accepted as pectinolytic activity . The strains having diameter of relative clear zone below 1.386 were discarded, and the strains showing diameter above 1.386 were selected for final screening by using optimized enzyme assay methods. Out of the four isolates , only one strain was selected based on maximum concentric ring surrounding the colonies . The high pectinase producing strain was identified as *Aspergillus niger* .

Selection of a suitable solid substrate for a fermentation process is a critical factor in SSF and this involves the screening of number of agro industrial materials for microbial growth and product formation . In the present study , among five substrates screened , wheat bran was found to be most significant for pectinase production using *Aspergillus niger* (Table1 and

Figure1). Silva and Martin reported that the mixture of orange bagasse and wheat bran showed to be the best substrate for pectinase production in SSF using *penicillium* species .

Wheat bran with with concentration was taken into 250 ml Erlenmeyer flasks to find at which concentration pectinase production is maximum.The results in (Table2 and Figure2) showed that pectinase production was highest with 10g of wheat bran. Wheat bran which is cheap and readily available carbon source.

To determine optimum incubation period for the highest pectinase production, *Aspergillus niger* was incubated upto 10 days . The maximum pectinase activity was found at fourth day of incubation (Table3 and Figure3) . It indicate that pectinase production activity is correlated with incubation time , which was also suggested by other investigators (Venugopal C. et al 2007) .

The result in Table4 and Figure4 showed that the flask containing medium with wheat bran supported maximum pectinase activity at 47◦C . The production of these enzymes however was drastically affected at incubation temperature of 35 to 55 ◦C.

The pH regulates the growth and synthesis of extra cellular enzymes by several microorganisms particullarly fungal strain . The ideal pH for pectinase production by *Aspergillus niger* was found to be 4.5 (Table5 and Figure5). The result were comparable with finding of Torres etal for pectinase production by *Aspergillus niger* .

The results of partial purification of the crude pectinase enzyme produced by *Aspergillus niger* showed that the highest specific activity (35.72 U/mg protein) and best recovery (68.64%) was achieved by using a fraction of 60% Ammonium salt precipitation followed by desalting and dialysis (Table6).

On comparing electrophoretic mobilities of standard molecular weight markers,with partillaly purified pectinase, it showed molecular weight of 35.022 kDa as shown in (Figure 6)

References

1)Cavalitto, S. F.; Arcas, J. A. and Hours, R.A, (1996), Pectinase production profile of *Aspergillus foedidus* in solid state cultures at different acidities. *Biotechnology Letter*, 18, 251-256.

2)Gomes, E.; Iembo, T. and Silva, R. (2001), Production, characterization and properties of depolymerising enzymes from a *Curvularia inaequalis* strains, *Folia Microbiologica*, 46, 303-308.

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

4)Lowry, O.H., N.G. Rosebrough, A.L. Farr and R.J.Randall. Protein measurement with the folin phenol reagent J. Bio. Chem., 1951;193: 265-275.

5)Martins, E. S.; Silva, R. and Gomes, E. (2000), Solid state production of thermostable pectinases from thermophilic *Thermoascus aurantiacus*. *ProcessBiochem.*, 37, 949-954

6)Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 1959;31:426-8.

7)Onions A. H. S., Allsop D. and Eggins H. O. W. (1981). Smith′s introduction to industrial mycology . 7th ed., Edward Arnold London

8)Pandey, A.; Soccol, C. R.; Nigam, P. and Soccol, V. T. (2000), Biotechnological potential of agro-industrial residues. I: sugar cane bagasse. *BioresourceTechnol.*, 74,69-80.

9)Pilnik, W. and Voragen, A. G. J. (1993), Pectic enzymes in fruit and vegetable juice manufature. In: Nagodawithama, T. and Reed, G. (Eds.). *Enzymes in Food Processing*. New York : Academic Press. pp 363-399.

10)Sarkanen, S. (1991), Enzymatic lignin degradation: an extracellular view. In: Leathem, G. F. and Himmel, M. E. (Eds.). *Enzymes in Biomass Conversion*, ACS Symp. Series 460, *American Chem. Soc.*, pp. 247-269.

11)Silva, D.; Martins, E. S.; Silva, R. and Gomes, E (2002). Pectinase production from *Penicillium viridicatum* RFC3 by solid state fermentation using agricultural residues and agro-industrial by-product. *Braz. J. Microbiol.*, 33, 318-324.

12)Soares, M. M. C. N.; Silva, R.; Carmona, E. C. and Gomes, E. (2001), Pectinolytic enzymes production by *Bacillus* species and their potential application on juice extraction. *World J. Microbiol. Biotechnol.*, **17**, 79-82.

13)Venugopal C,Jayachandra T,Appaiah KA. 2007.Effect of aeration on the production of endopectinase from coffee pulp by a novel thermophilic fungi mycotypha sp. Strain No.AKM1801 6(2),245-250

14)Zhen, Z. and Shetty, K (2000), Solid state production of polygalacturonase by Lentinus edodes using fruit processin wastes. *Process Biochem.*, **35**, 825-830

Figure 1:Effect of various solid substrates on pectinase production

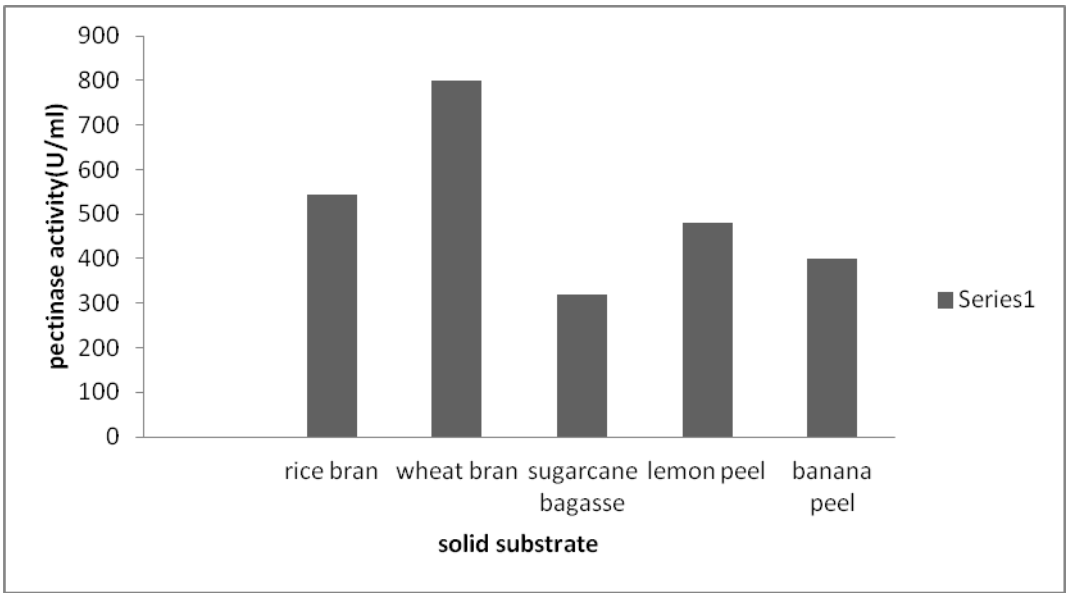


Figure2 : Effect of wheat bran concentration on pectinase production

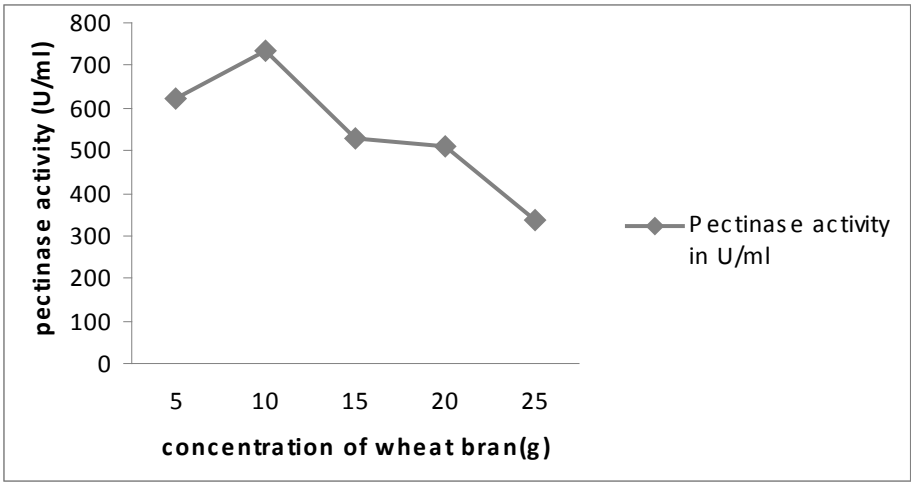


Figure3: Effect of incubation time on pectinase production

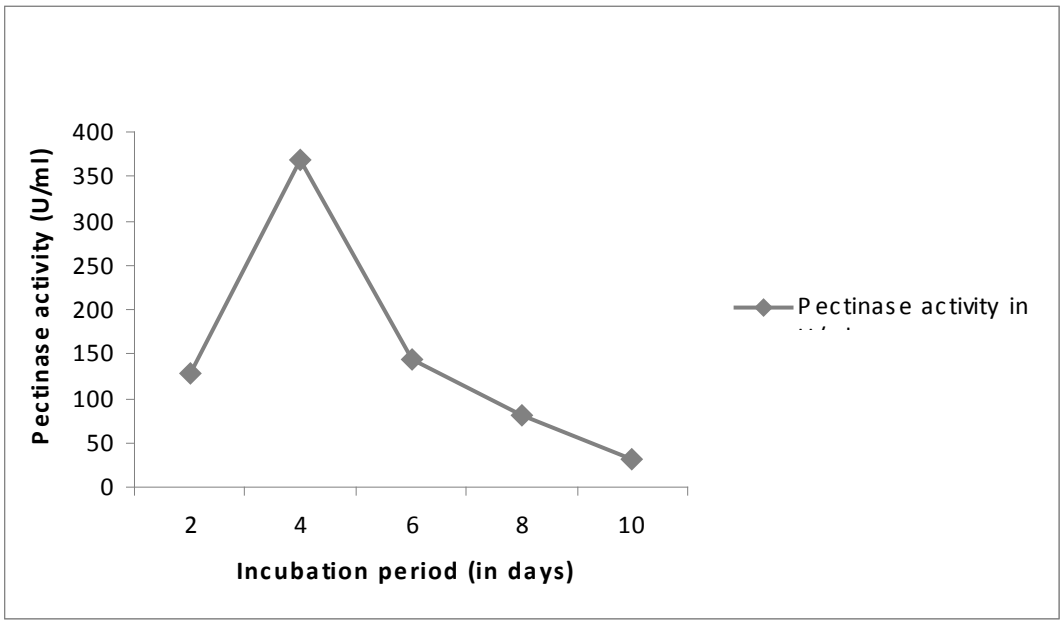


Figure 4: Effect of temperature on pectinase production

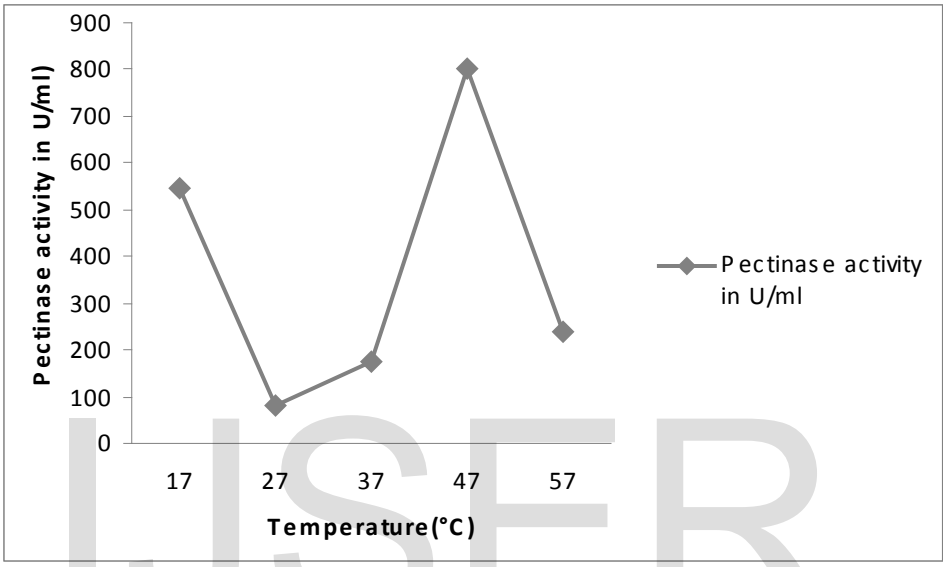


Figure5: Effect of pH on pectinase production

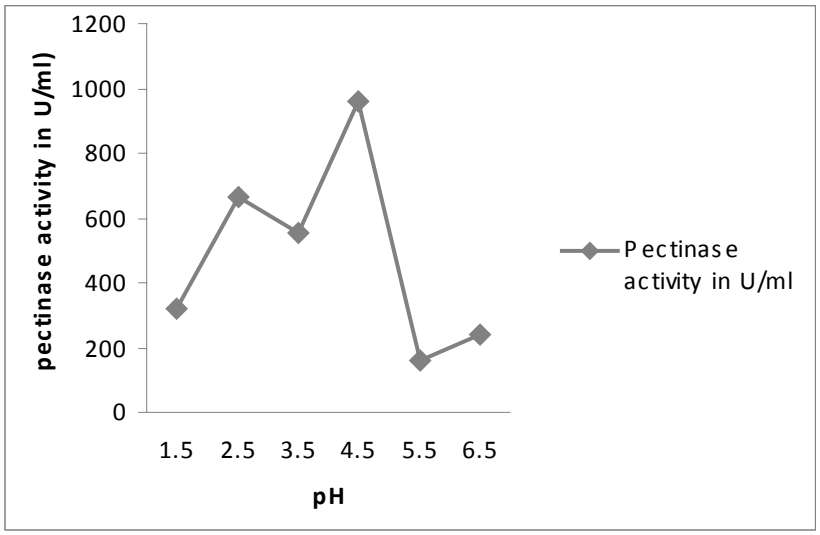
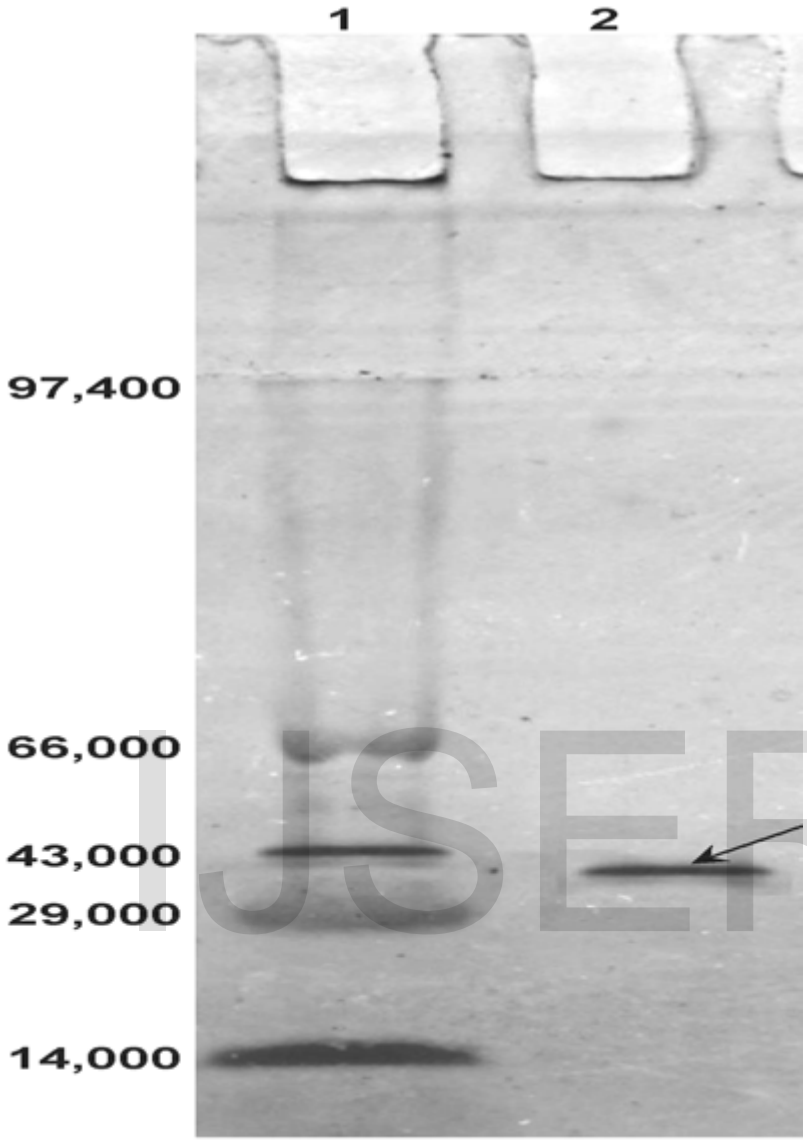


Figure 6:SDS-PAGE of purified Pectinase of *Aspergillus niger*



Lane No. 1: Standard protein molecular weight marker;

Lane No 2: Purified pectinase of *Aspergillus niger*

Table 1:Effect of various solid substrates on pectinase activity

Various solid substrates	Pectinase activity **(units)** *
Rice bran	544
Wheat bran	800
Sugarcane bagasse	320
Lemon peel	480
Banana peel	400

*One unit- The amount of enzyme required to liberate 1µ mole of reducing groups per minute with galacturonic acid as standard.

Table2 : Effect of wheat bran concentration on pectinase production

Wheat bran concentration (g)	Pectinase activity **(units)** *
5	624
10	736
15	528
20	512
25	336

*One unit- The amount of enzyme required to liberate 1µ mole of reducing groups per minute with galacturonic acid as standard.

Table 3: Effect of incubation time on pectinase production

Incubation period (no of days)	Pectinase activity **(units)** *
2	128
4	368
6	144
8	80
10	32

*One unit- The amount of enzyme required to liberate 1µ mole of reducing groups per minute with galacturonic acid as standard.

Table 4: Effect of Temperature on pectinase activity

Temperature**(**ºC)	Pectinase activity **(units)** *
17	544
27	80
37	176
47	800
57	240

*One unit- The amount of enzyme required to liberate 1µ mole of reducing groups per minute with galacturonic acid as standard

Table 5: Effect of pH on pectinase production

pH	Pectinase activity (units) *
1.5	320
2.5	665
3.5	1552
4.5	960
5.5	160
6.5	240

*One unit- The amount of enzyme required to liberate 1µ mole of reducing groups per minute with galacturonic acid as standard

Table 6:Purification scheme of extracellular Pectinase from *Aspergillus niger* grown on wheat bran medium.

Purification steps	Total units	Total protein (mg)	Specific activity (U/mg protein)	Recovery %	Fold purification
Crude filtrate	887.2	170.0	5.22	100	6.85
Partial purification	609.0	17.05	35.72	68.64	