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Production and purification of Pectinase by fungal strain in solid-state fermentation using agro-industrial bioproduct

[1*]Dange V.U., [2]Harke S.

Institute of Bioscience and Technology, M.G.M.College, Aurangabad

Abstract: Pectin lyase and polygalacturonase production by fungal strain *Aspergillus oryzae* was carried out in solid-state fermentation. *Aspergillus oryzae* produced polygalcturonase (PG) and pectin lyase (PL) on mixture of agro-industrial bioproduct in solid state fermentation. To select the optimum growth condition for maximum enzyme production, Mosambi baggage, wheat bran, Mosambi peel and lemon peel were used as substrate for the solid state fermentation process. These substrates were used as sole constituent of media. The Enzyme (wheat bran) was purified by ammonium sulphate precipitation, dialysis and ion exchange chromatography. In the present studies the main objective was to explore new natural source as a substrate for the production of pectinase. Wheat bran act as a best natural source for the production of enzyme from *Aspergillus oryzae.* The enzyme was purified up to 67.2% fold successfully. It could be employed for extraction of juices as well as clarification with improved quality.

Keywords: *Aspergillus oryzae*, polygalacturonase, pectin lyase, agro-industrial bioproduct, solid-state fermentation.

1. 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 is fungi (Zheng and Shetty, 2000; Pandey et al., 2001). Several agro-industrial waste 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.

Pectinase are enzymes which degrade pectic substances and are of great importance to the food industry. These enzymes are used to facilitate extraction, filtration and clarification to increase yield in the production of fruit juices and beverages. Pectinase is extensively used in food processing industry, souring of cotton, degumming of plant fibers, waste water treatment, vegetables oil extractions, tea and coffee fermentation, bleaching of paper, in the alcoholic beverage. These enzymes classified according to the criteria whether Pectinase act by transelimination or hydrolysis or whether cleavage is random.

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 b -elimination. Both endo types of PGs and PALs (EC 3.2.1.15 and EC 4.2.2.2, respectively) are known by splitting 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 a combination of PE with PG or PAL (Sarkanen et al, 1991, Pilnik and Voragen, 1993).

The aim of this work was to study of pectolytic enzymes production by newly isolated strains of fungi by solid-state fermentation using agro-industrial by-products. Some physico-chemical characteristics of the enzymes produced have been discussed.

2. MATERIAL AND METHODS

Screening of pectinase producing fungi:

Screening of Pectinase production was done by plate assay. The isolates were screened for pectinase activity. This was done by inoculating the organisms on the Pectinase Screening Agar Medium (PSAM) plates containing 1% of pectin (in case of agro-industrial substrate i.e. Mosambi baggage, wheat bran, Mosambi peel and lemon peel); 0.14% of $(NH_4)_2SO_4$; 0.20% of K_2HPO_4; 0.02% of $MgSO_4.7H_2O$; 0.10% of nutrient solution (5 mg/l $FeSO_4.7H_2O$; 1.6 mg/l $MnSO_4.H_2O$; 1.4 mg/l $ZnSO_4.7H_2O$; 2.0 mg/l $CoCl_2$), pH 5.0. The mixture was incubated at 30°C for 24. The plates were flooded with 50mM iodine solution and incubated for 15 min at 37^0C. A clear zone around the growth of the fungi was indicated to Pectinase activity.

Production of Pectinase using different substrate:

For the production of pectinase, solid state fermentation was performed. To select the optimum growth condition for maximum enzyme production, Mosambi baggage, wheat bran, Mosambi peel and lemon peel were used as substrate for the solid state fermentation process. These substrates were used as sole constituent of media.

Substrate sources:

A microorganism requires a lot amount of carbohydrate source for enzyme productions. Five different substrates sources were used for the productions of Pectinase.

1. Pectin,

2. Wheat bran

3. Mosambi peel extract,

4. Mosambi Baggage extract

5. Lemon peel extract, etc.

Pectin as substrate source was actually the chemical source available at various laboratories. Whereas Wheat bran, Mosambi peel extract, Mosambi Baggage extract, Lemon peel extract, etc. as substrate were agriculture waste which was used as major substrate source for the Pectinase productions.

Media, cultivation of microorganism and enzyme production:

The solid substrates were prepared as follows:

a) Mosambi bagasse extract: The pellet of orange bagasse (pressed mixture of pulp and peel) was obtained by local market. Chemical analysis revealed that the dry material was composed of 11.8% fiber, 6.3% nitrogen, 6.7% ash, 19% total sugar (9% reducing sugar) and 0.1% pectin. The material was ground and particles sieved by a Bender USS 230 strainer and dried at 80°C.

b) Mosambi peel and Lemon peel extract: Mosambi peel and Lemon peel extract were purchased by market. Chemical analysis revealed that the dry material was composed of 75% fiber, 5% nitrogen, 3.4% ash, 10.36% total sugar (3.53% reducing sugar).The material was washed in tap water and the same procedure described above was followed.
c) Wheat bran: Wheat bran was obtained commercially, dried and used untreated. Chemical analysis revealed that the dry material was composed of 8.12% fiber, 6.5% nitrogen, 4.57% ash, 16.7% total sugar (5.22% reducing sugar).

The substrates for fermentations consisted of sugar cane bagasse and wheat bran mixtures in proportions of 1:9 and 9:1, respectively, and of sugar cane bagasse, orange bagasse and wheat bran in proportions of 1:1:1.

Solid-state fermentation (SSF) was carried out using a 250 mL Erlenmeyer flask containing 5g of sterilized substrate (120°C/40min) inoculated with 10 mL aliquots of conidia suspension (approx.10^7 spore/g dry substrate) which was obtained from a 7-day agar slant culture suspended in sterile Tween 80 solution. After inoculation, 10 mL of nutrient solution, composed of 0.1% NH_4NO_3; 0.1% $NH_4H_2PO_4$; 0.1% $MgSO_4$ x $7H_2O$, were added to each of the flasks. The final moisture content of the medium was approximately 67%.

The cultivation was carried out at 30°C for 15 days. At 24h intervals the solid fermented material, corresponding to one Erlenmeyer flask, was mixed with 30 mL of distilled water (6g of fermented material/mL), stirred for 40 min, filtered under vacuum and centrifuged. The supernatant was used as crude enzyme solution.

Purification of Pectinase:

Ammonium Sulfate Precipitation:

Pectinase in the crude extract was purified between 40-70% of ammonium sulfate precipitation. Various percentage of ammonium sulfate was being used for the precipitation of the enzyme sample. 10ml of crude enzyme solution was taken in centrifuge tube then added (40%,50%,65%and70%)ammonium sulfate to enzyme solution keep at 4^0C for overnight then centrifuged at 12000 rpm for 15min. pellet was dissolved using sodium acetate buffer. precipitated enzyme was purified by using the procedure of Yogesh Khairnar and 2009.

Dialysis:

Precipitated enzyme solution was filled into the dialysis bag and both the ends of bag were sealed with the help of cotton thread. Then the bags were placed on sugar beds for 2 hours to remove impurities and excess of buffer solution from enzyme solution. Then the dialysis a bag containing concentrated enzyme solution was incubated in phosphate buffer solution Sucrose is used for the sugar bed preparation. Dialyzed enzyme solution is used for further purification process in Ion Exchange Chromatography.

Ion Exchange chromatography:

The basic principle behind the use of the column preparation was to treat the enzyme sample with various concentration of salt solution this process actually help with removal of various bound and unbound protein. DEAE cellulose was used for column preparation. 2.5gm of DEAE cellulose was prepared in 12ml of 10mM tris HCL having pH 7.5 and the beds were allowed to settle for 30 min. The bottom of burette was packed with glass wool. The matrix was poured into column to the level of 7.5 cm and allowed to settle. The pH was maintained at 7.2 by addition of phosphate buffer. Column was always filled with phosphate buffer to avoid from drying the matrix. The column was used again for enzyme purification. The fraction of enzyme solution was collected and the enzyme assay was done. (Yogesh Khairnar and 2009)

Glucose estimation and Pectinase assay:

The reducing sugar of the extra cellular enzyme was determined according to Stiles 1926. Pectinase was measured as follows: 2ml pectin solution, 1 ml distilled water, 1 ml acetate buffer (0.05 M, PH 4.0) was incubated at 40◦C in water bath for 10 minutes then 1 ml enzyme solution was added and kept it for 60 minutes and the increase of reducing sugar was estimated by the usual method. One unite of Pectinase is defined as1µ mol reducing sugar liberated per minute under assay condition. The concentration of enzyme in solution was detected by using spectrophotometer. Absorbance of enzyme solution against phosphate buffer was taken at 280nm. Pectinase activity was determined by Qualitative and Quantitative methods of sugar estimation. Quantitative estimation of sugar was done by DNSA method and absorbance was taken at 575nm.

Evolution of Enzyme effect:

The partially purified enzymes of wheat bran and pectin rate of 0.5, 1, 1.5, 2, 2.5 percentages were added to the respective pulp of apple. The control without enzyme was kept along with other sample at 40^0C for 4 hour of the incubation for extraction of juice. Treated apple pulp was passed through a muslin cloth and juices were analyzed for juice yield (V.K. joshi, Mukesh parma 2011)

Juice clarification:

Partially purified enzyme at the rate of 0.5 & 1% each was added to the apple. Treated juice was kept at overnight at room temperature (27^0C) and was analyzed for clarification.

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Protocol for apple juice clarification:

Chopped medium sized apple into small pieces, roughly 5mm x 5mm x 5mm.Weighted half of the apple into beaker and half into another about 50g of apple in each). Diluted 1ml of pectinase by adding 1ml distilled water and add to one of the beakers of apple.Add 2ml of distilled water to the other beaker of apple. Stirred the beaker contents with a plastic spoon. Cover the beakers with cling film, then incubate them at 40°C for 15 - 20 minutes. Place funnels lined with coffee filter papers into measuring cylinders. Add the contents of the beakers to the filter funnels and record the volume of juice obtained from both lots of apple at 5 minute intervals.

3. RESULTS AND DISCUSSION

Screening of best isolate:

Fungi were used for the production of pectinase. Extensive screening was carried out by measuring glucose and pectinase activity. *Aspergillus oryzae* demonstrated a large zone of hydrolysis around the large colony on pectin agar medium. Similarly wheat bran, mosambi peel, mosambi baggage, lemon peel containing media was used for screening of positive pectinase producers (Plate 1, 2). On the basis of screening program, *A.oryzae* was selected for further experiments. *Aspergillus oryzae* demonstrated a large zone of hydrolysis around the large colony on pectin agar medium. The zone of clearance was seen, degradation was evidenced by a clear zone around fungal growth (Diameter of zone of clearance was 4.3mm).

Plate-1 screening of best isolate for production of pectinase

Control ***Aspergillus oryzae***

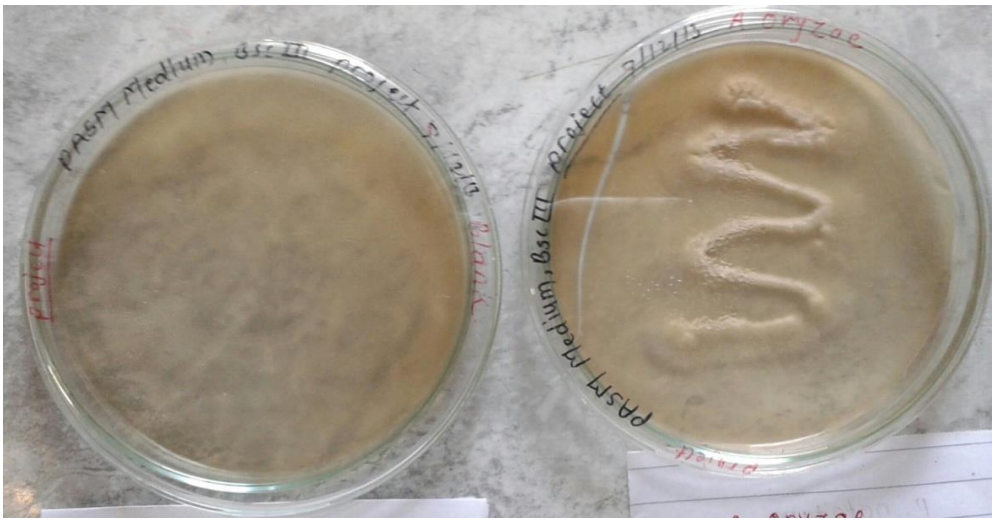
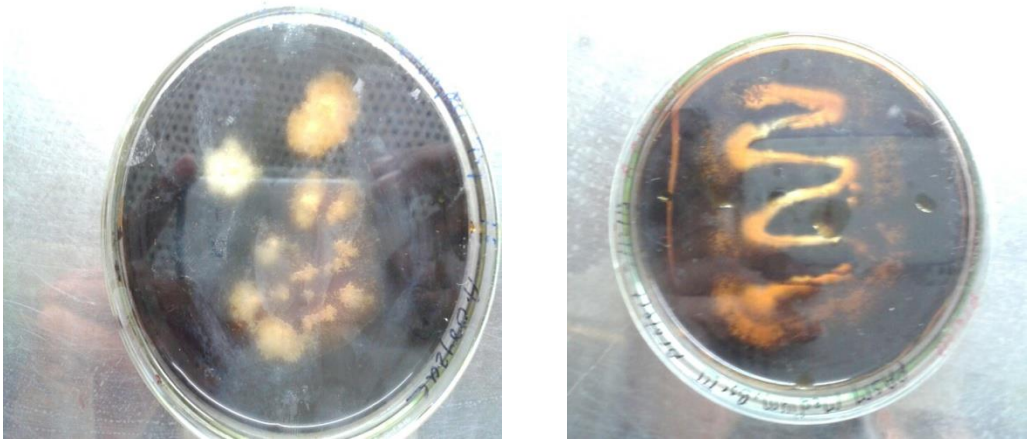


Plate-2 Following Plates showing zone of clearance with addition of iodine containing different substrates



Wheat Bran as a substrate **Pectin as a substrate**

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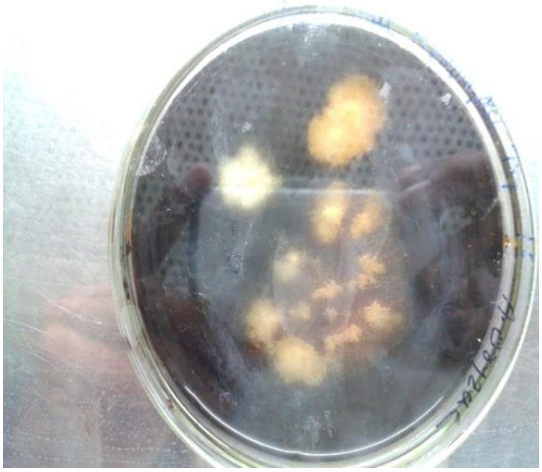
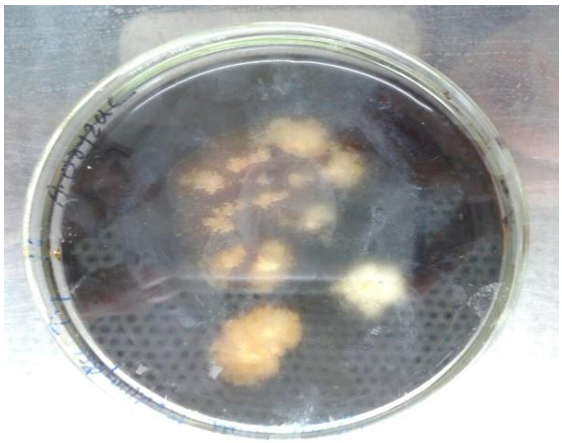


Mosombi Peel as substrate



Mosambi Baggage as a substrate

Production of enzyme:

To find out the maximum production of enzyme in different media, enzyme assay was done. (Plate 3, 4). Following table showing the maximum production of enzyme was achieved by wheat bran.

Sr. No.	Different substrate	Enzyme activity (IU) ml^{-1}
1.	Wheat Bran	1.12
2.	Pectin	0.65
3.	Mosambi Peel	0.30
4.	Mosambi Baggage	0.22
5.	Lemon Peel	0.28

Extensive literature survey reveals that, production and purification was done by using pectin as a substrate. Therefore our study was continuing for purification and application of pectinase by using wheat bran as a natural source.

Plate-3 Production Of pectinase by using different substrates

A)Pectin as a substrate **B) Wheat bran as a substrate**

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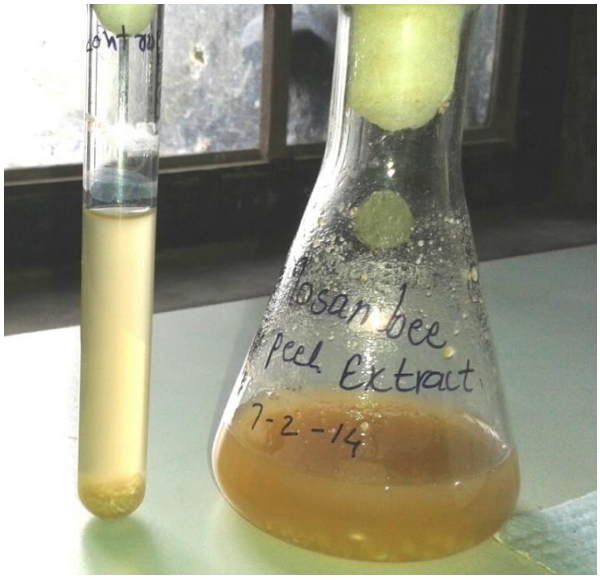
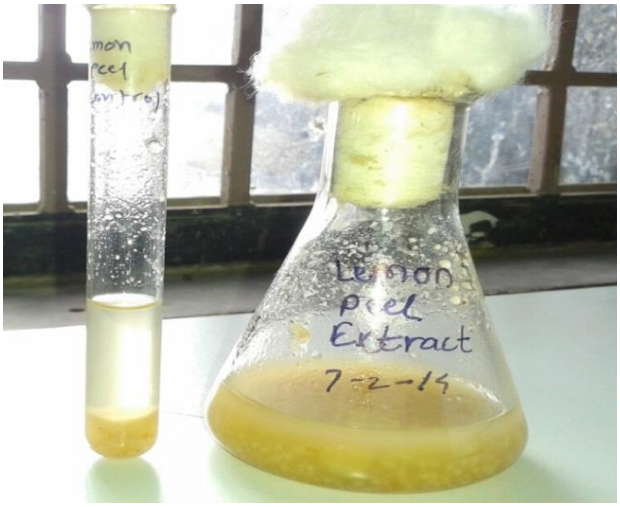
C) Mosombi Baggage as substrate



D) Mosambi Peel as a substrate

Plate-4



E) Lemon Peel as a substrate

F) Comparative analysis of enzyme production

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Purification of enzyme:

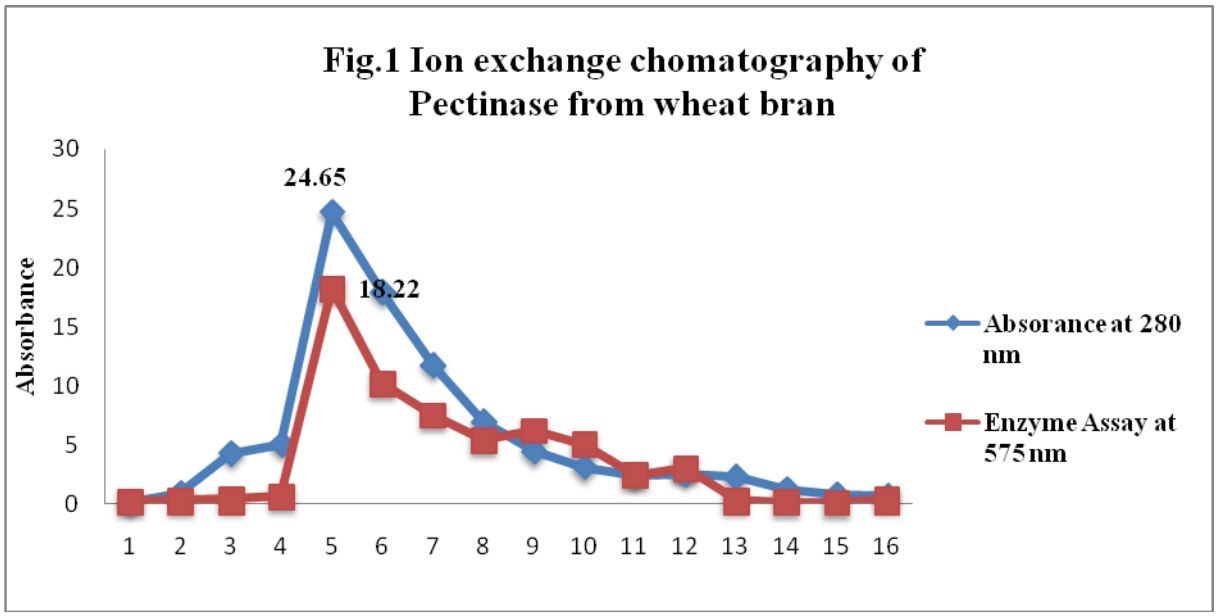
The Enzyme (wheat bran) was purified by ammonium sulphate precipitation, dialysis and ion exchange chromatography.

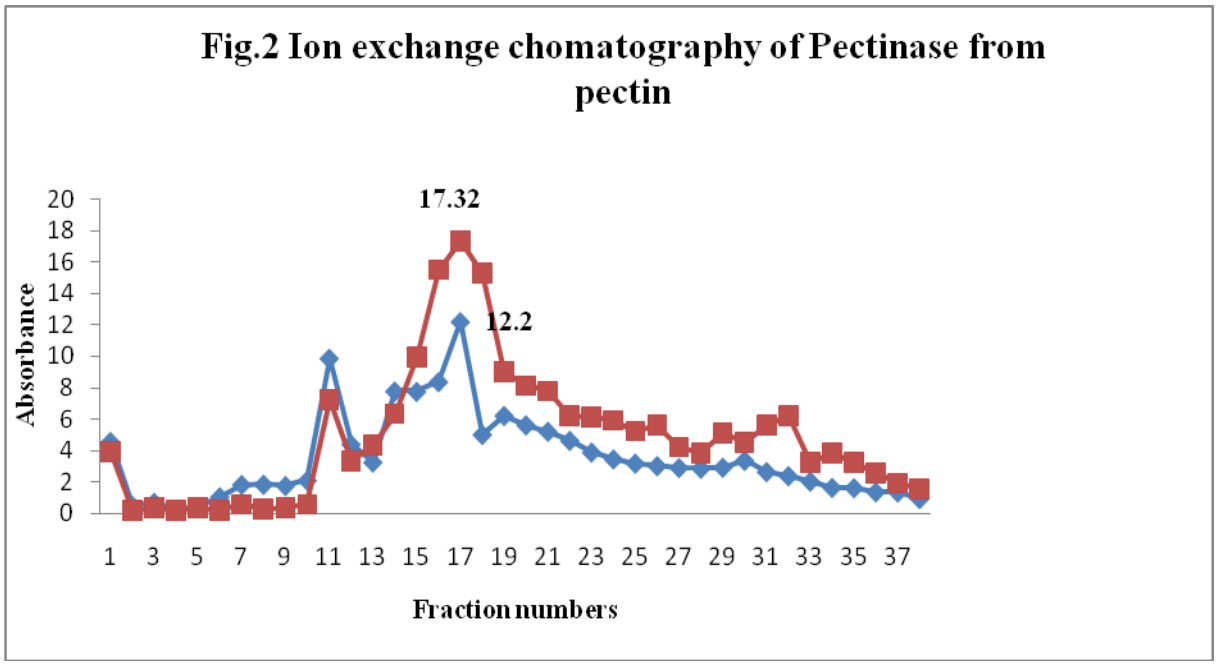
Ammonium Sulfate Fractionation

It is often used to remove proteins other than the one being purified. The technique involves adding increasing concentrations of ammonium sulfate to the protein solution and centrifuging out the precipitated material in certain concentration ranges. Highest degree of precipitations was achieved by 65% concentration of ammonium sulphate. Ammonium sulfate fractionation; proteins can be further purified by the sequential application of chromatography.

Ion Exchange Chromatography

This fraction was loaded onto the anion exchanger, DEAE- Cellulose equilibrated with 20 mM Tris HCl (pH 7.5) buffer. The proteins were eluted with a NaCl gradient (0±2 M). Most of the protein was present in two major peaks (Figure 1), but pectinase activity was detected only in the one fraction (Figure 2). Using DEAE-Cellulose chromatography, 67.2-fold purification of the enzyme was achieved.





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Evolution of Efficiency of Pectinase and its application:

Juice extraction from Apple fruit pulp:

The effect of enzyme treatment on extraction of pulp of apple juice as shown in (Plate-7(B)). Addition of enzyme at different concentration increases the juice recovery in apple fruit. The increase in juice yield was minimum for pectin but in case for wheat bran, it was increased more than 50% with respect to control at maximum enzyme concentration. The maximum juice recovery was obtained at 7.5% of concentration in wheat bran. Further apricot showed the highest juice recovery of 75% followed by Wheat Bran and 55% by Pectin compared to that untreated apple.

V. K. Joshi 2011 have studied on purification and characterization of pectinase produced from apple pomace and evaluation of its efficacy in fruit juice extraction and purification.

Plate-5 (A)Enzyne treated and untreated apple juice filtration by muslin cloth

(B) Enzyme treated clarified juice with untreated sample

4. CONCLUSION

In the present studies the main objective was to explore new natural source as a substrate for the production of pectinase. It was concluded from the study that Wheat bran act as a best natural source for the production of enzyme from *Aspergillus oryzae.* The enzyme was purified up to 67.2% fold successfully. It could be employed for extraction of juices as well as clarification with improved quality.

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