GLOBAL JOURNAL OF BIO-SCIENCE AND BIOTECHNOLOGY

www.scienceandnature.org

FOOD ENZYMES PECTINASE FROM LEAF BLIGHT DISEASE OF ONION CAUSED BY *ALTERNARIA CEPULAE*

Megha, S.V., Maragathavalli, S., Brindha, S. and Annadurai, B.
Research and Development Centre, Bharathiar University, Coimbatore

ABSTRACT
In this study pectinase enzyme was isolated from *Alternaria cepulae* in onion medium. The organism causes leaf blight disease in onion. The organism was cultured in natural onion medium. Medium optimization, optimum pH, temperature optimization were conducted and the purification of pectinase enzyme was done by ammonium sulphate precipitation, DEAE cellulose chromatography, ultragel column method. T hen a purification fold of 245.18 and 270.93 were obtained for two fractions. A yield of 26.16% and 22.11% were also obtained. The homogeneity was checked by SDS PAGE and a molecular weight of 66 kDa was obtained. The homogeneity of the purified enzyme sample was also checked by immunological techniques

KEYWORDS : Pectinase, *Alternaria cepulae***,** DEAE cellulose chromatography, ultragel column method, SDS PAGE.

INTRODUCTION

Pectin is a structural heteropolysaccharide in the primary cell wall of plants. Pectin is the major component of the middle lamella which helps the plant cells to bind together. The structure and chemical composition of pectin differ among plants and within plant. It helps in the cell wall extension and plant growth. These pectins are degraded by pectinase enzymes during infection on plants by different micro organisms. Among pectinase enzymes endopolygalacturonase plays a significant role in pathogenecity of many plants. Endopolygalacturonase is one of the prime macerating enzyme produced by *Alternaria cepulae* during leaf blight disease of onion (Annadurai, 1998, 1999).The leaf blight disease in onion caused by *Alternaria cepulae*. *Alternaria* are major plant pathogen, they are ubiquitous genus containing saprophytes in soil air and plant pathogens..The colonies are fast growing, black to olivaceous black or greyish. The optimum temperature for the growth is 25-28^{0}C and maximum 31-32^{0}C.The pectic enzyme secreted by the pathogen degrades the pectic constituents of the cell wall and middle lamella when the pathogen invades the host in the leaf blight disease (Srinivasan *et al*., 1960, Bilgrami, 1963). Leaf Blight disease occurs on the radial leaves of transplanted seedlings at 3-4 leaf stage. It causes small yellowish to orange flecks in the middle of the leaves, which soon developed in to elongated spindle shaped spots surrounded by pinkish margin.The disease on the inflorescence stalk causes severe damage to the seed crops. The fungus survives in the plant debris or soil. Fungi possess a diverse array of secreted enzymes to depolymerise the main structural polysaccharide components on the cell wall like cellulose, hemicelluloses and pectin (Kubicek *et al*., 2014).

FIGURE 1: Leaf Blight disease in Onion

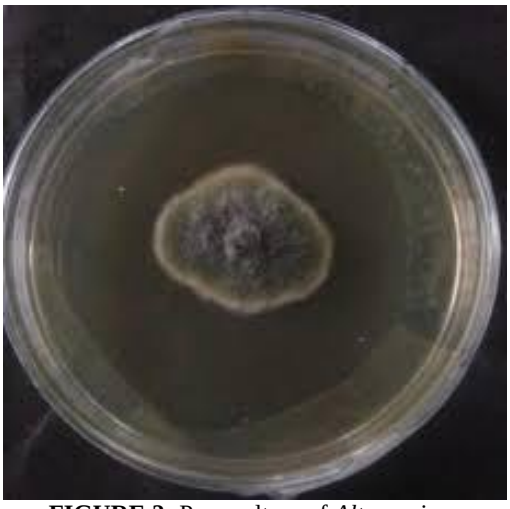


FIGURE 2: Pure culture of *Alternaria sp.*

The involvement of pectic enzymes in degradation of pectic constituents of cell wall and middle lamella of plant tissue has been reported for diverse types of diseases such as soft rot, dry rot, wilts, blights and leaf spots which are caused by plant pathogens like fungi, bacteria and nematodes. The cementing substance in vegetables induce pectinase production which hydrolyses the pectin to produce mushy consistency (Jay, 2005; Hoodal *et al*., 2002).The breakdown of the polygalacturonic chains in pectic substances is caused by two groups of enzymes. The production of pectic enzymes *invivo* is usually proven by removal of infected tissue from sick plants, purification of crude enzyme and determination of its ability to reproduce disease symptoms in healthy plants. The hydrolases or glycosidases are depolymerases, which are able to hydrolyse the α-1, 4 glycosidic bonds. They are produced by yeasts, molds, bacteria and higher plants. In order to form an active enzyme-substrate complex different enzymes call for different structure around the glycodsidic bonds, e.g. the presence of free or esterifies adjacent carboxyl groups, of a specific position in the polygalacturonic chain. Accordingly the glycosidases can be divided into polygalacturonases (PGs) which preferentially attack pectic acid and pectinic acids with a low degree of esterification, whereas the polymethyl galacturonases (PMGs) preferentially attack pectic acids with a high degree of esterification (Deuel and Stutz, 1943a). Endopolygalacturonases which split the available glycodsidic linkages more or less at random, have been found in the sub-groups of PGs and PMGs; up till now exopoloygalacturonases, which hydrolyse pectinic acids or pectic acids from one end of the chains, were detected only in the sub-group of PGs. The existence of a second main group of depolymerizing enzymes, the lyases or transeliminases, was discovered in 1960 (Albersheim *et al.*, 1960b); the first name is preferred by the International Union of Biochemistry. These enzymes cause a transelimination reaction similar to that as described for pectic acids during heating in neutral or alkaline environment.

Purification and characterization of pectinolytic enzymes produced by *Aspergillus sp.* on raw materials by solid state fermentation has been reported. The pectic enzymes like polygalacturonase, pectin methyl esterase and pectatelyase were produced and purified.(Dinci *et al*., 2007). Alkaline pectinase are important industrial enzyme with great significance in current biotechnological application like textile, waste water treatment, mineralize pectic substances in the environment. Using onion waste as the waste substrate in solid state fermentation pectinase production and Pleurotussajor-caju (Giordana *et al*., 2017). Pectic enzyme production was induced by culturing of *Pseudomonas flourescens* in minimal salt medium containing citrus pectin as soul carbon source and *invivo* inoculation of the culture in to the healthy onion bulb (Simbo, 2009). The application of alkaline pectinases in the textile industry for the retting and degumming of fiber crops, production of good quality paper, fermentation of coffee and tea, oil extractions and treatment of pectic waste water fruit juice industries in clarification purpose, textile processing, paper and pulp industry, animal feed *etc*. Enzymatic treatment for juice extraction is most common now a day. Enzymatic treatments prior to mechanical and thermal treatments significantly improve juice recovery (Sharma *et al*., 2017). Hence in this chapter pectinases produced was purified from the crude extract by different techniques like centrifugation, ammonium sulphate precipitation, dialysis, gel column chromatography and ultrogel column. The homogeneity of the enzyme is tested using SDS-PAGE and purity with the immunological methods like Ouchterlony immune-diffusion technique, Immuno electrophoretic technique etc.

MATERIALS & METHODS

Wet Lab

Cleaning of glassware's, maintenance of the fungus, preparation of PDA, subculturing, preparation of glassware for sterilization, sterilization Potato Dextrose Agar ,Tryptophan Broth, Tyrosine-Casein Nitrate Agar, Water Agar, Mycelial Dry Weight Determination, methodologies were adopted according to the methods of Annadurai *et al*. (1998, 99, 2000).

Culture Media

Medium Optimization

Different compositions of mediums were used for the optimization of the culture. The mediums like Potato Dextrose Agar Medium, Czapek Dox medium, Pectin medium, Natural Onion medium are used to identify the ideal medium for the production of organism and also for the production of pectinase enzyme.

Potato dextrose agar media

The microorganisms were isolated and sub cultured in Petri plates and test tube slants in the PDA medium in the following combination.

Peeled potato = 250.0 g
Glucose = 20.0 g
Agar = 15.0 g
Water = 1000 ml.
pH 6.0 – 6.5

Peeled potatoes were made into thin chips. They were boiled in 500 ml of water and the extract was taken. The agar is melted in the other half of the water and mixed with the potato glucose solution and the volume made up to 1 liter.

CzapekDox Medium

Sucrose	30g
Sodium nitrate	2g
Dipotassium phosphate	1g
Magnesium sulphate	0.5g
Potassium chloride	0.5g
Ferrous sulphate	0.01g

pH (at 25°C)7.3 ±0.2

The weighed ingredients were suspended in 1000 ml distilled water. It was heated for boiling to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes (Eaton et al 1998).

Pectin medium

Three different pectin medium differing in pH were designed by the author in this laboratory for pectic enzyme production.

Pectin - 5. 0 g
Yeast extract - 5. 0 g

KH_2PO_4 - 2. 0 g
$(NH_4)_2SO_4$ - 1. 0 g
$MgSO_4$ - 0. 5 g
KCl - 0. 2 g
H_2O -1000 ml.

Onion Medium

Three different onion medium differing in pH were designed by the author in this laboratory for pectic enzyme production

K_2HPO_4 2g
KH_2PO_4 2g
$(NH_4)_2SO_4$ 1g
$MgSO_4.7H_2O$ 1.1g
Yeast extract 0.6g
Chopped onion

Enzyme Activity on Culture period.

The médium with máximum enzyme activity was selected for the study of enzyme production on different periods of the culture. The enzyme activity was calculated on equal intervals of the culture fíltrate to identify the period in which máximum enzyme production has occured.

Optimization of pH

The médiums showing high enzyme activity was selected for the optimization of pH. The desired médium with different pH ranges were prepared and inoculated with culture organism. The enzyme activity was checked on the optimum day for calculating the optimum pH for the production of maximum enzyme.

Optimization of Temperature

The médiums showing high enzyme activity was selected for the optimization of temperature. The desired médium was selected and inoculated with the culture and stored in different temperatures. The enzyme activity was checked on the optimum day for calculating the optimum temperatura for the production of máximum enzyme.

Culture Methods

20 ml of each of the onion media supplemented with pectin were taken in 250 ml Erlenmeyer flasks. After autoclaving in Barnslead sterilizer (Boston) at 15 lb pressure for 20 minutes, it was cooled to room temperature (32±1°C) and all the microorganisms obtained from various sources were inoculated and was grown in still culture.

After 16 days of growth, the contents of the flask was filtered through a glass funnel fitted with a coarse grade sintered glass filter and was thoroughly washed with water. The mat was pressed in filter paper to remove the excess of moisture. This was transferred to a previously weighed filter paper. It was dried in an oven at 70°C over night. It was cooled to room temperature (32±1°C) in desiccators and weighed.

Inoculation of the medium

To the Haffkins flasks of 5 liter capacity, 1liter liquid culture medium was employed. Inoculums prepared in PDA slants containing mostly conidia and mycelia were transferred to a sterile flask. Culture was incubated at 32±1°C. in glass incubation cupboard for 16 days.

Preparation of crude extract

The microorganisms along with the culture medium were triturated in a mortar with acid washed sand. This was centrifuged and the supernatant was collected. The residue was triturated again with distilled water, centrifuged and the supernatant was mixed with previous extract. The total liquid collected was made up to a known volume and analyzed for protein activity and pectinase activity.

Estimation of Protein

Total protein was determined by the Lowry method (Lowry *et al.*, 1951) using BSA as the standard.The procedure was explained in general materials and method.

Determination of Pectinase Enzyme

Estimation of Endopolygalacturonase (EPG EC 3.2. 1. 15)

Reducing sugar method

Endo PG activity was estimated according to the method of Nelson (1944) and Somogyi (1952)

Alkaline Copper reagent

Reagent I

Solution A: 15gms of Rochelle salt and 30 gms of anhydrous Na_2CO_3 was dissolved in about 300ml of water.
Solution B: 20gms of anhydrous Na_2SO_4 were dissolved in 500 ml of water and heated to expel air and cooled.
The solution A was combined with solution B and the final volume was made up to 1 liter with distilled water.

Reagent II

5 gms of $CuSO_4$. $5H_2O$ and 45 gms of anhydrous Na_2SO_4 were dissolved in water and made up to 250 ml with water.

Alkaline copper reagent was prepared by mixing 4 volumes of reagent I with one volume of reagents II (V/V) just prior to use.

Reagent III (Arsenomolybdate reagent)

25 gms of ammonium molybdate was dissolved in 450 ml of water, 21 ml of concentrated H_2SO_4 and 3 gms of Na_2SO_4.6H2O were dissolved in 25 ml of water. This was added to the molybdate solution. This solution was incubated for 24 – 48 hours at 32 ±1°C and stored in a glass stoppered brown bottle.

1 volume of this solution was diluted with 2 volumes of 1.5 N H_2SO_4 (V/V) just prior to use.

Procedure

The incubation mixture containing 1.0 ml of sodium polypecate of different pH from 3.0 – 9.0, 0.5 ml of $CaCl_2$ or water and 0.5 ml of enzyme were incubated at 32 ±1°C for 1hour. The reaction was stopped by adding 2.0 ml of Alkaline copper reagent. The tubes were kept in boiling water bath for 30 minutes. The control treatments were carried out in the same manner except that the enzyme was added after adding the Alkaline copper reagent. The tubes were then cooled to room temperature and 1.0 ml of Arsenomolybdate reagent was added and then it was read at 520 nm in uv – 260 Shimadzu spectrophotometer.

The activity was expressed as µ moles of galacturonic acid released per ml per 30 minutes as assessed from galacturonic acid standard curve.

Purification Procedures

Crude enzyme preparation

In the present study, the natural onion medium which was standardized was used for culturing the mold. To obtain maximum quantity of enzyme one kilogram of onion was cut into small pieces with onion cutter. The onion chips were taken along with 500ml of water in 5litre. Haffkin's flask. This was autoclaved in barslead (Boston) autoclave at 15lb pressure for 20 minutes. After sterilization the flasks were cooled to 32 ±1ºC. Then a slant of *A. cepulae* was inoculated into medium in a sterile chamber. After 16

days of incubation at 32plus or minus 1°C the contents were taken out by adding 500 ml of 0.25M NaCl. This was blended in warning blender for 5 second at 4 ºC. the blended juice was filtered through 2 layers of cheese cloth. The filtrate was centrifuged at 20,000 rpm for minutes at 4ºC. The clear supernatant was dialysed against 2liter. 0.01 M phosphate buffer at pH 7.0 for 48 hours with two changes at 4 ±1ºC.

Ammonium sulphate precipitation

To 1 liter of dialysed supernatant solution ammonium sulphate (AR)was added slowly with a constant stirring in a magnetic stirrer for 4 to 6 hours at 4^0C to give 0- 40 %, 40- 80%, 80-95% saturation. After the desired percentage of saturation the contents of each step was centrifuged at 20,000 rpm for 20 minutes at 4 degree C. The supernatant solution was taken for the next $[NH_4]_2\ SO_4$ precipitation. The precipitate obtained from each step after centrifugation was dissolved in 0.01 M phosphate buffer at pH 7.0 containing 0.1 M NaCl. The dark brown color solution thus obtained was dialysed at 4ºC for 24 hours against the same buffer with at least three changes of 3 liter buffer. The activity of EPG and protein content were estimated.

Dialysis

The protein suspension was transferred to visking dialysis tubing and dialyzed against two changes of buffer against 200ml of buffer A and allowing 8-10 h for equilibration after adjustment the pH (pH.8 as the buffer A). Dialysis was repeated against the distilled water 200ml for 2h. The dialyzed sample was centrifuged at 5000g for 15min.

Chromatographic procedures

The sample containing maximum endoPG [EPG] activity was selected for further purification by chromatography. The entire chromatography operations were carried out at 4^0 C. Addition of sample to the column, elution of proteins and maintenance of column flow rate were performed using peristaltic pump (LKB model 2132 microperpex pump). The column effluents were continuously monitored at 280 nm using a UV monitor (LKB UV cord S II model 2338). The recording of absorbance was simultaneously done by LKB model 2210 recorder. Constant volume fractions were collected by fraction collector (LKB Redirac model 2112). Measurements of absorbance of collected fractions (A280) were carried out in a shimadzu spectrophometer UV- 260 Japan with 1 cm cuvette at 280 nm. Polygalacturonase activity was quantified by measuring the micro gram of galacturonic acid released ml of enzyme in 30 minutes from sodium polypectate using reducing sugar method of Nelson Somogyi. One unit of polygalacturonase activity is defined as micro gram of galacturonic acid released from sodium polypectate per mg of enzyme in 30 minutes at 32 ±1ºC under assay conditions.

DEAE column chromatography

The DEAE–cellulose column chromatography was carried out according to the following procedure.

Reagents

DEAE –cellulose in 3 X25 cm column
0.5 N HCl
0.5 N Na OH
0.01 M phosphate buffer, pH 7.0
0.05 M to 0.2 M NaCl

Procedure

A DEAE –Cellulose (Sigma chemicals fine grade) column of 3X25 cm was prepared after pretreatment with 0.5 N Na OH and 0.5 N HCl. The column was equilibrated with 0.01 M phosphate buffer pH 7.0.50 ml of the dialysed 80-95% $(NH_4)_2\ SO_4$ precipitation solution which shows high EPG activity was loaded in the column and washed with the same buffer till the washing showed the absorbance almost zero at 280 nm. The elution of the enzyme was carried with 0.01 M phosphate buffer containing stepwise gradient of 0.05 M, 0.1 M and 0.2 M NaCl. The flow rate was 30 ml per hour. 6 ml of fractions were collected at the interval of 12 minutes.

All the individual fractions obtained from DEAE column were analysed for endoPG activity. Both fractions of peak I and peak II were found of possess the PG activity. Fractions of EPG I and EPG II were individually pooled lyophilized dissolved in buffer pH 7.0 containing 0-05 M NaCl.

Ultra gel Column

Further purification of EPG I fractions was carried out by gel filtration on ultrogel ACA 44 (LKB) column. The column (1.6 X 88 Cm) was equilibrated with 0.01 M phosphate buffer pH 7.0 containing 0.05 M NaCl.

5 ml of the lyophilized and dialysed fraction of EPG I was loaded on the ultrogel column. 3 ml fractions per tube at the flow rate of 9 ml/ hour were collected. The individual fractions were analysed for EPG activity. The EPG active fractions were pooled and lyophilized.

The lyophilized and dialysed EPG II from DEAE column was loaded on to the ultrogel column (1.6 X 88.0 xm).3 ml fractions per tube were collected at the flow rate of 9 ml per hour using the same buffer. The individual fraction was analysed for EPG activity. The EPG active fractions were pooled and lyophilized.

Test for purity of the enzyme

The homogeneity of the purified Cellulase was determined by SDS-polyacrylamide gel electrophoresis, Ouchterlony immunodiffusion method and Graber and Burtin immunoelectrophoresis.

SDS- Polyacrylamide gel electrophoresis (SDS- PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out to determine the homogeneity of purified cellulase. The detailed procedure is explained in general materials and methods.

Isoelecteric focussing

The isoelectric focusing was carried out in a semi preparative manner using polyacrylmide gel rods (11 X165 mm) by adopting the method of Wrigley (1971) at 4°C. The procedure is explained under general materials and methods.

Immunological studies

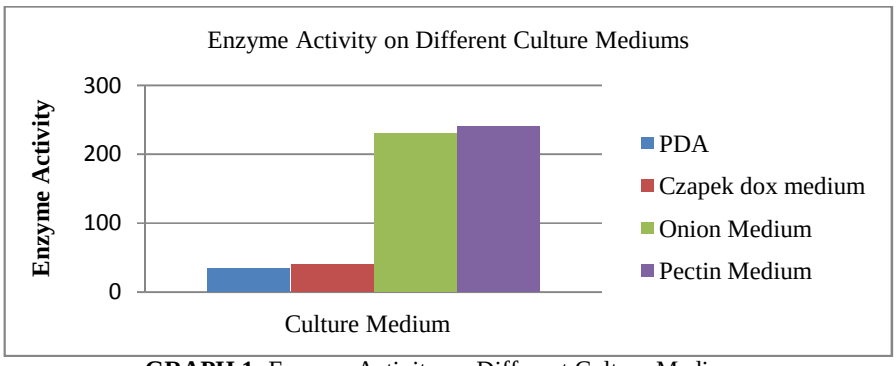
To study the homogeneity of the enzyme, double immuno diffusion technique was carried out according to the method of Ouchterlony and Nilsson (1973) and immuno electrophoresis was performed according to the technique of Graber and Burtin (1964).

RESULTS

Media Optimization

The enzyme activity on different types medium like Potato Dextrose Agar medium, CzapekDox medium, Natural

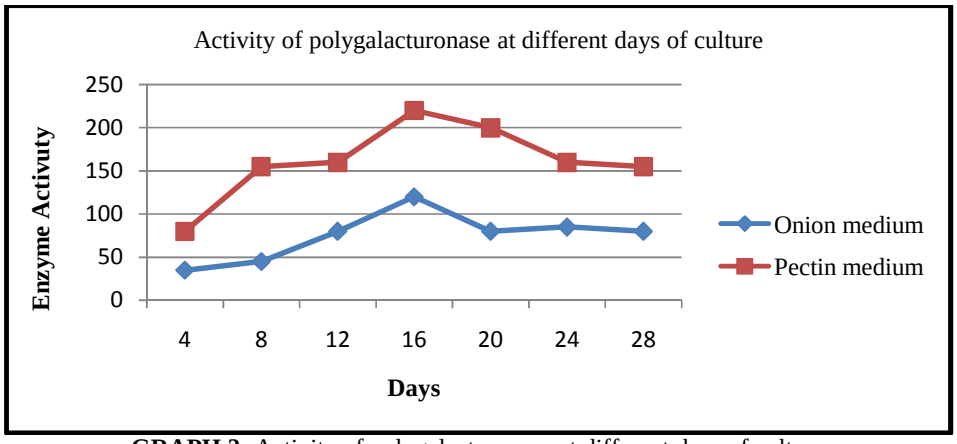
onion medium and Pectin medium were studied. Maximum pectinase activity was obtained on Onoin medium and Pectin medium. The results obtained were shown in Graph 1.



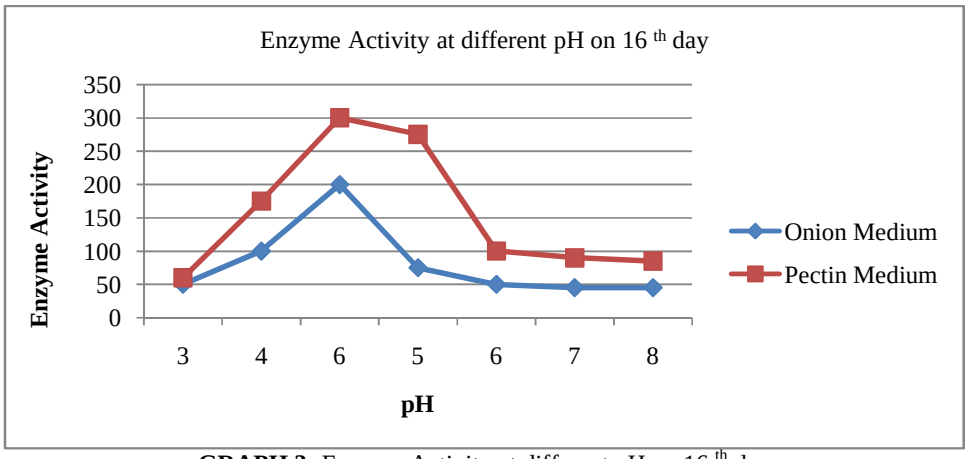
GRAPH 1: Enzyme Activity on Different Culture Mediums

Enzyme activity on different days of culture

The poly galacturonase activity on the culture mediums over the period of growth of the organism was studied. The two mediums Natural onion medium and Pectin medium was inoculated with the organism and the poly galacturonase enzyme activity was checked on different intervals of time. Maximum activity was obtained on 16th day of the incubation perion. The result obtained was shown in Graph 2.



GRAPH 2: Activity of polygalacturonase at different days of culture



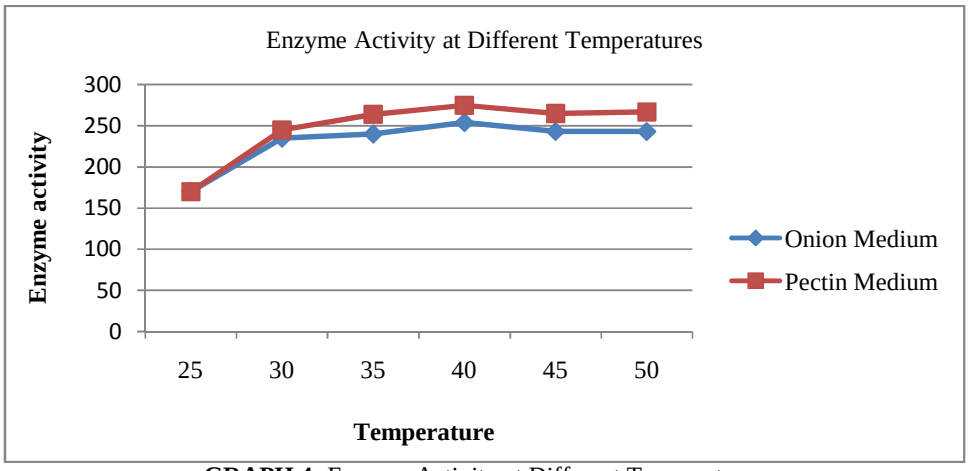
GRAPH 3: Enzyme Activity at different pH on 16 th day

Determination of optimum pH

Natural onion medium and Pectin mediums of different pH values were prepared and inoculated with the organism. The enzyme activity on different periods of growth on different pH mediums were determined. Maximum enzyme activity was shown on 16th day of pH 6 on both mediums. Graph 3 shows that the enzyme production is maximum on medium with pH 6.

Determination of Optimum Temperature

Natural onion medium and Pectin mediums were inoculated with the culture organism and stored in different temperatures for the growth and enzyme production. Maximum enzyme activity was shown on temperature between 35-45^{0}C. The Graph 4 shows results obtained for the enzyme activity on different temperatures.



GRAPH 4: Enzyme Activity at Different Temperatures

Purification of Pectinase

Table 1: shows the precipitation of pectinase activity at different percentages of ammonium sulphate concentration. The pellet obtained with 80-95% ammonium sulphate shows maximum pectinase activity.

TABLE 1: Ammonium sulphate precipitation of crude enzyme preparation of EPG from natural onion medium

Sl No	Percentage of $(NH_4)_2 SO_4$ (%)	Volume (ml)	EPG activity units up of gal. acidrel/ ml/ 30 min	Protein content µg/mi	Specific activity in units/ug
1	Crude enzyme	900	56.4	71.2	0.79
2	0-40	30	1.32	26.4	0.05
3	40-80	40	2.04	29.1	0.07
4	80-95	50	976.4	76.9	12.0

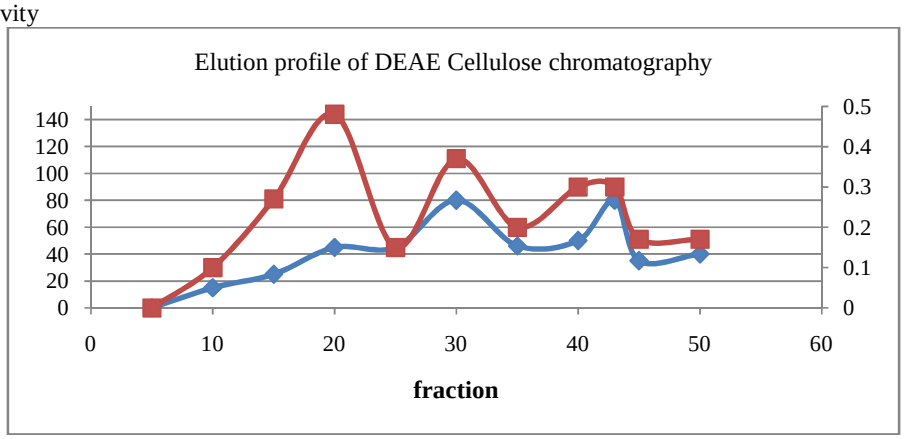
Elution profile of the purification of EPG from crude enzyme fraction of 80-95% ammonium sulphate fraction by ion exchange chromatography on DEAE cellulose column was conducted.

Fractions 6 ml / tube were collected at the flow rate of 30 ml/ hour in DEAE cellulose column (3 X25 cm). Eluted with 0.2 M sodium acetate buffer at ph 7.0 containing 0.05 M to 0.2 NaCl gradient.

------ = absorbance at 280 nm

------ = EPG activity

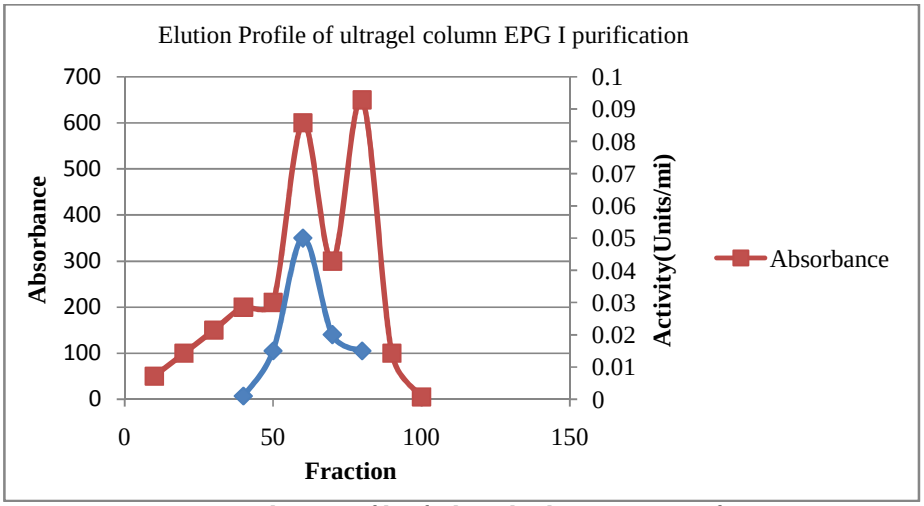
Graph 5 shows the elution profile of DEAE-Cellulose column chromatography of EPG. The precipitant obtained between 80-95 % ammonium sulphate fractions was dialyzed and applied on DEAE-Cellulose column. The stepwise gradient elution shows that first fraction 25th to 35th with EPG activity (designated as EPG I) was eluted at the concentration of 0.1 M NaCl and the second fraction 38th to 45th with EPG activity (designated as EPG II) eluted at the concentration of 0.2M NaCl.



GRAPH 5 Elution profile of DEAE Cellulose chromatography (Red line shows the Absorgance and Blue line shows the Activity (Units/ml))

Elution profile of the purification of EPG I from DEAE – Cellulose on Ultrogel column. Fractions corresponding to peak I of DEAE – cellulose column were pooled together, concentrated and rechromatographed on ultrogel column (1.6 X 88 cm). Fraction of 3 ml / tube was collected at the flow rate of 9 ml / hour eluted with sodium acetate buffer.
--- = Protein absorbance at 280 nm-------- = EPG activity

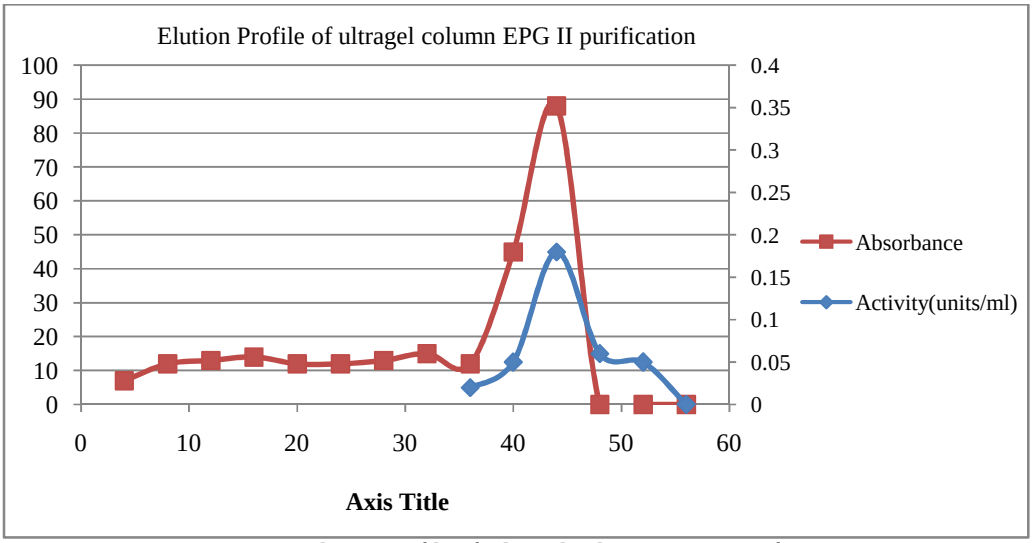
Elution profile of EPG I from DEAE- Cellulose column after pooling and dialysis against 0.01M phosphate buffer (pH 7) is shown Graph 6. The sample was then loaded on ultragel column and the fraction fron 41st to 70th showed EPG activity.This fraction was eluted out and stored for identification of homogeneity.



GRAPH 6: Elution profile of Ultragel column EPG I purification

Elution profile of the purification of EPG II from DEAE – Cellulose on Ultrogel column. Fractions corresponding to peak II of DEAE – cellulose column were pooled together, concentrated and rechromatographed on Ultrogel column (1.6 X 88 cm). Fraction of 3 ml / tube was collected at the flow rate of 9 ml / hour eluted with sodium acetate buffer.
-- = Protein absorbance at 280 nm----- = EPG activity

Elution profile of EPG II from DEAE- Cellulose column after pooling and dialysis against 0.01M phosphate buffer (pH 7) is shown in Graph 7.The sample was then loaded on ultragel column and the fraction fron 37thto 47th showed EPG activity. This fraction was eluted out and stored for identification of homogeneity.



GRAPH 7: Elution profile of Ultragel column EPG II purification

A summary of purification presented in Table 3.2 reveals that pectinase was purified to 245.18 fold with 26.16 % yield for EPG I and 270.93 fold of purification with 22.11% yield for EPG II.

TABLE 2: purification of endopolygalacturonase

Steps	Volume (ml)	Total Protein (µg)	Total endoPG Activity units (µgofgal.acid.rel)	Specific activity Units/µg protein	Purification fold	Yield (%)
Culture filtrate (supernatant)	900	64080	50760	0.79	1	100
80-95% $(NH_4)_2SO_4$ Precipitation	50	3845	48820	12.7	16.0	96.18
DEAE-column EPG I-fraction	138	201	21618	107.55	135.5	42.58
EPG II-fraction	40	162	20825	128.18	161.34	41.01
Ultragel column EPG I-fraction	34	68	13285	195.36	245.18	26.16
EPG II-fraction	16	52	11226	215.88	270.93	22.11

From each step of purification the enzyme samples were tested for homogeneity using SDS-PAGE. The Fig 3 shows that the crude sample, partially purified sample and the ultra gel purified sample showed only one band in SDS-PAGE with a molecular weight of 66kDa. Thus its homogeneity was confirmed by comparing with a protein marker.

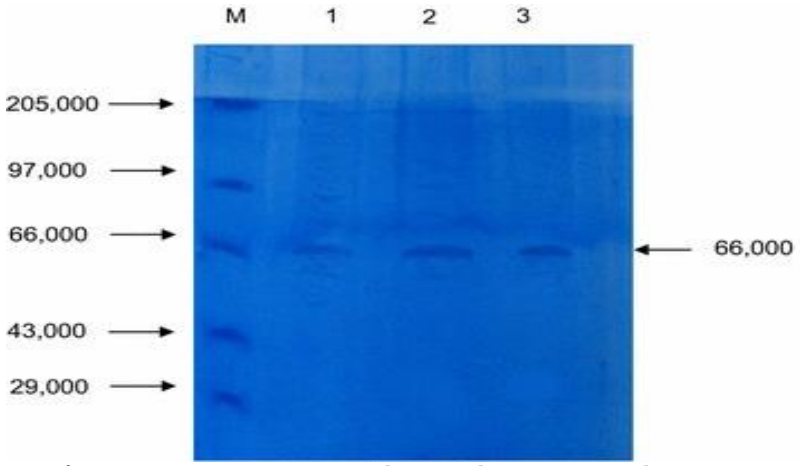
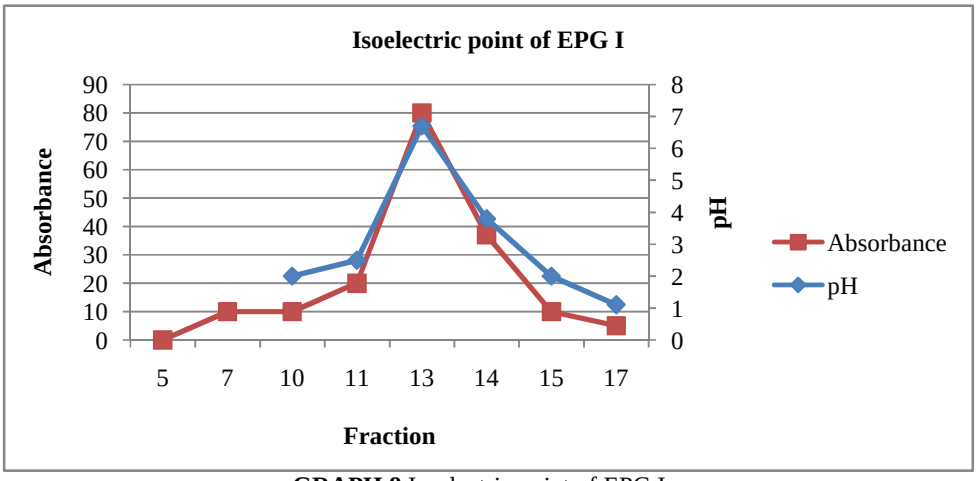


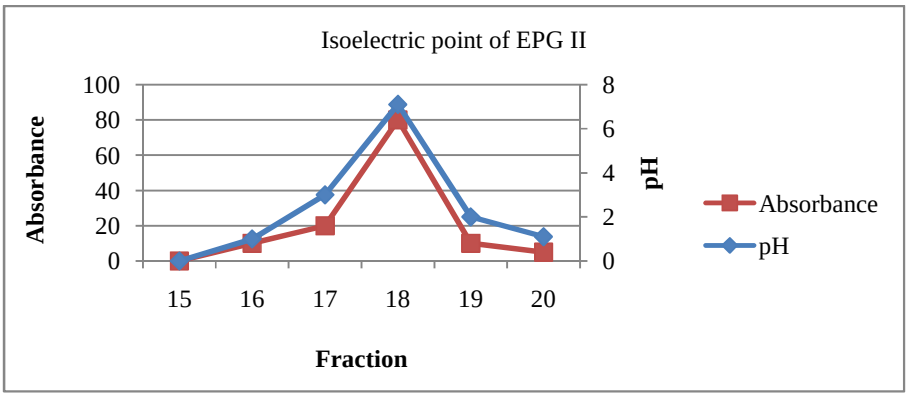
FIGURE 3: SDS PAGE of Pectinase, Lane M protein marker in Dalton, Lane 1 crude pectinase, Lane 2 partially purified pectinase and Lane 3 Ultra gel purified pectinase

Determination of isoelectric point

The isoelectric point of pectinase in a semi preparative manner of EPG I is shown in Graph 10. It indicates a single peak of pectinase activity between 11th and 12th fractions at isoelectric pH 6.7. The semi preparative isoelectric focusing of EPG II is shown in Graph 11. It shows a single of EPG activity between fractions 16 and 17 and its isoelectric pH is 7.1.



GRAPH 8 Isoelectric point of EPG I



GRAPH 9: Isoelectric point of EPGII

Immunological tests of Homogenity

Ouchterony double diffusion patterns of purified pectinase

The ouchterlony double immunodiffusion pattern indicates the homogeneity of the pectinase (Fig 4). The highly purified enzyme gave a single immune-precipitation line in reflected light when reacted with its rabbit antiserum showing the presence of only one component.

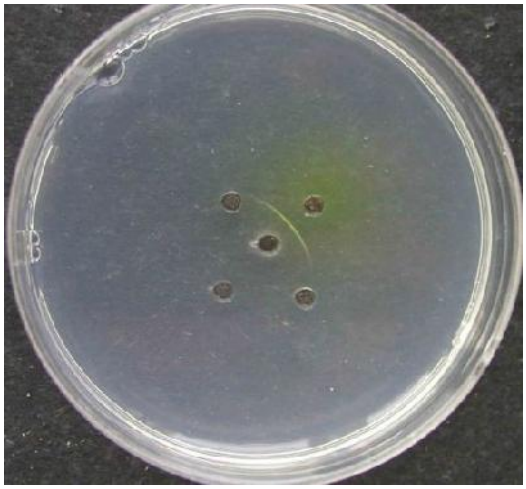


FIGURE 4: Ouchterony double diffusion patterns

Graber and burtin immunoelectrophoresis pattern of purified pectinase

Graber and Burtin qualitative analysis by immunoelectrophoresis also confirmed the homogeneity of the enzyme as depicted in Fig. 5

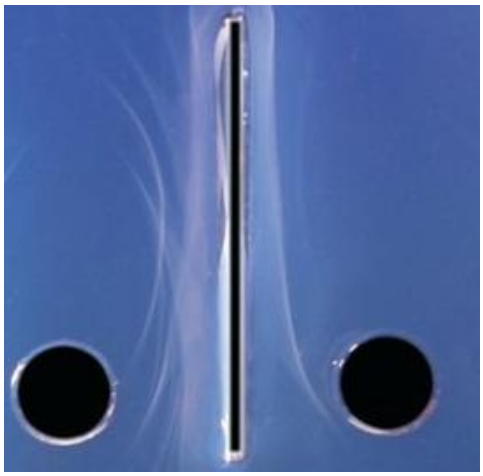


FIGURE 5: Graber and burtin immunoelectophoresis

DISCUSSION

In the present study pectinase enzyme was isolated from *Alternaria cepulae* causing blight disease in onion, using natural onion medium supplemented with pectin. The optimization of medium, maximum enzyme production day of the culture, optimum pH and Temperature for the culture for maximum enzyme production was also studied. The enzyme was cultured in submerged fermentation method. There are researches done on the production of pectinase in submerged and solid state fermentation (Sharma *et al*., 2006, Carolina, 2007).The supernatant was purified by precipitation using ammonium sulphate, dialysis and DEAE cellulose column chromatography. After purification its homogenity was checked using SDS PAGE and isoelectric focusing was also done. The purity of the sample obtained was confirmed by immune diffusion techniques which obtained a single precipitation line on the plates. In the present study we assayed the enzyme activity using Lowry and Nelson and Somogyi method according to the amount of protein and galacturonic acid produced in the media respectively. This method is introduced as the most satisfactory procedure in the previous studies (Sumi *et al*., 2015) .In the purification of pectinase, procedure like ammonium sulphate precipitation, gel filtration and ion exchange were used in various studies before (Kashyap *et al*., 2000).In this study the adsorbed fraction of enzyme was eluted out and its enzyme activity was checked. Two fractions were shown maximum enzyme activity and they were again purified in Ultra gel column. When crude enzyme from *Alternaria cepulae* was subjected sequential purification steps, the purified sample showed an 245 fold purification for EPG I and 270 fold purification for EPG II (Table 2).

The results of SDS-PAGE (Fig. 3) suggest that the purified enzyme is homogeneous. A molecular weight of 66kDa was obtained. In previous studies the molecular weight of polygalacturonase was observed as 68kDa which is similar to this study (Christina 1992), therefore the band obtained was confirmed as PG. Isoelectric focussing pattern (Graph 10 and 11) also confirmed the previous findings of the homogeneity of the enzyme) The immunodiffusion and immnoelectrophoresis results showing single precipitation line confirm the homogeneity of the purified polygalacturonase. The broad application of pectinase in different industries makes this study relevant now a day. Various fruit and vegetable processing wastes have been used for the production of pectinase (Bari, 2010, Kumar, 2012).

CONCLUSION

Onion fields were often affected by leaf blight disease caused by *Alternaria cepulae* in and around Coimbatore district. Much of the damage caused economical loss. During the host pathogen relationship of onion and *Alternaria cepulae* a commercial enzyme pectinase is produced. This pectinase is used in food industries. Hence, *Alternaria cepulae* was grown in natural onion medium in Haffkin flask. The culture filtrate was precipitated with 80-95% ammonium sulphate. After dialysis this was subjected to DEAE cellulose chromatography and ultra gel chromatography and purified to the extent of 26.16% yield and 245.18 fold purity for EPG I and 22.11% yield and 270.93 purification fold for EPG II fraction. The enzymes were tested for homogeneity with usual tests.

ACKNOWLEDGEMENT

The author S V Megha is grateful to R & D Bharatiyar university and Biotechnology department.Megha extend her gratitude to Dr B Annadurai, Dr R Puvanakrishnan, Scientist at department of Biotechnology CLRI, Chennai, Dr R Easwaramoorthy and all the well wishers for useful suggestions.

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