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FOOD ENZYME CELLULASE FROM PINE APPLE BLIGHT CAUSED BY CERATOCYSTIS AND ITS ISOLATION, PRODUCTION AND PURIFICATION

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ABSTRACT
In this study cellulase enzyme was isolated from *Ceratocystis paradoxa* a pathogen infecting pine apple crop causing a huge crop lose all over the world. The cellulase enzyme was produced from pine apple sources in the medium cultured with *Ceratocystis paradoxa*. The enzyme was isolated from crude extract by ammonium sulphate precipitation, DEAE column chromatography, ultragel column, SDS PAGE. After purification a purification fold of 8.75 and a yield of 53.74% were obtained. SDA PAGE of purified sample showed a molecular weight of 25 k Da. The homogeneity of the purified sample checked by immunological techniques.

KEYWORDS : *Ceratocystis paradoxa*, Cellulase**,** Dialysis, DEAE column chromatography, SDS PAGE.

INTRODUCTION

Cellulose is the principal constituent of the cell wall of most terrestrial plants. The source of cellulose is in plants and it is found as micro-fibrils (2-20 nm in diameter and 100 – 40,000 nm long). These form the structurally strong frame work in the cell walls. Cellulose molecules are arranged parallel to each other and are joined together with hydrogen bond. This long cable like structure of cellulose produce such a strong support structure for plants. Cellulose is a complex polysaccharide consisting of 4000 or more glucose units. Cellulose is non digestible to human beings but they are food for herbivorous animals which have microorganisms in there digestive tract for the digestion. Cellulose are also food for termites which have protozoan's for the digestion. Cellulose from various sources is all the same at the molecular level. However, they differ in the crystalline structures and bindings by other biochemical molecules. Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulose containing raw materials and waste products that are not exploited or which could be used more efficiently.

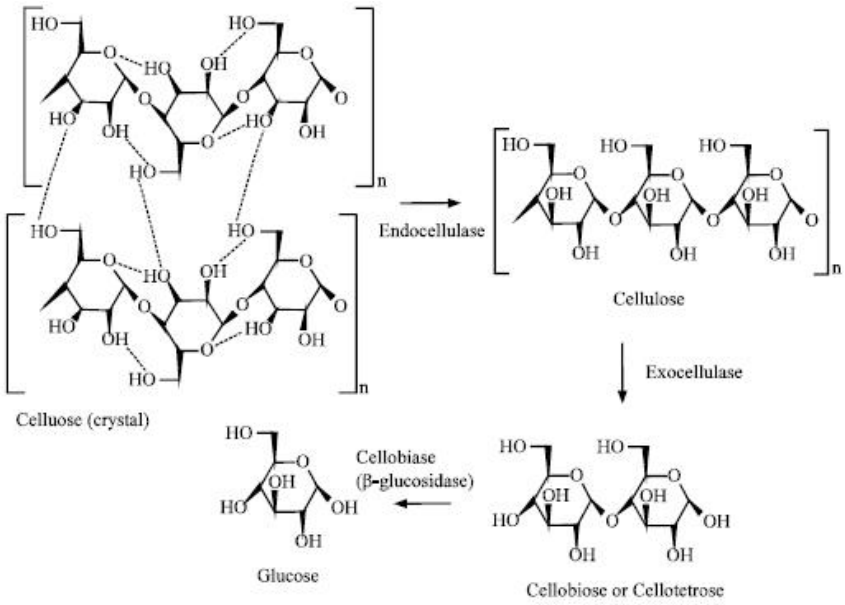


FIGURE 1: Hydrolysis of cellobiose

The problem in this respect is however to develop processes that are economically profitable. Cellulose containing wastes may be agricultural, urban, or industrial in origin, sewage sludge might also be considered a source of cellulose. Agricultural wastes include crop residue, animal excreta and crop processing wastes slashing generated in logging, saw dust formed in timber production and wood products in forestry originated

activities. Currently, there are two major ways of converting cellulose to glucose: chemical versus enzymatic. Cellulose rich plant biomass is one of the foreseeable and sustainable sources of fuel, animal feed and feed stock for chemical synthesis (Bhat, 2000). Therefore the conversion of cellulosic biomass to fermentable sugars and alcohols through biocatalyst cellulase produced by various cellulolytic organisms has attracted a worldwide attention (Ladisch *et al*., 1983). The mode of action of the enzyme and the actual role of each component of the enzyme in performing the specific reaction. A cellulose enzyme system consists of three major components: endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). of which endoglucanase (EC 3.2.1.4; 1,4-β-D-glucanglucanohydrolase) acts on carboxy methyl cellulose, causing random scission of cellulose chains yielding glucose and cello-oligosaccharides; exoglucanase (EC 3.2.1.91; 1,4-β-D-glucancellobiohydrolase) acts on microcrystalline cellulose (avicel),imparting an exo-attack on the non-reducing end of cellulose, liberating cellobiose as the primary product and beta-glucosidases (EC 3.2.1.21) that facilitates the hydrolysis of cellobiose to glucose (Fig. 1). Cellulase complexes located on the cell surface mediate adherence of anaerobic cellulolytic bacteria to the substrate. In bacteria cellulose is a supramolecular complex called Cellulosome. It contains five enzymatic subunits called endocellulase, exocellulase, cellobiase, oxidative cellulases and cellulose phosphorylases. Of these enzymes, only endocellulases and cellobiases participate in β (1, 4) linkage hydrolysis. Cellulosomes derived from different bacteria show a divergent type of architecture, owing to the number of interacting (Lameed, 1988). Cellulases have a wide range of enormous potential applications in biotechnology and many thermo stable endoglucanase appeared to have a great potentiality for industrial use (Karmakar and Ray, 2011). In spite of their commercial importance, the high cost of production of these enzymes has hindered the industrial application of cellulose bioconversion therefore; there is an ever increasing demand for more stable, highly active, specific enzymes of nominal cost. Biotechnology of cellulases began in early 1980s, first in animal feed (Ali *et al*., 1995) followed by food applications (Grassin and Fauquembergue, 1996) later in the textile, laundry (Singh *et al*., 2007)as well as in the pulp and paper industries (Godfrey and West, 1996, Mai *et al*., 2004). During the last two decades, the use of cellulases has increased considerably, especially in textile, in the bioprocessing of natural fibers, such as for the hydrolysis of cellulose to fermentable sugars and ethanol production deinking of recycled paper, biopolishing of cotton fabrics to enhance softness and appearance and treatment of recycled fibers to restore fiber texture and flexibility lost during operations (Bhat *et al*., 1997), in pulp and paper industries (Godfrey and West, 1996; Harman and Kubicek, 1998; Uhlig, 1998). It has also been shown that cellulase treatment in combination with physical refining can provide a means for altering the morphology of coarse wood fibers (*e.g.*, Douglas fir) to produce fine paper products (Mansfield *et al*., 1996). Mixture of different cellulase along with hemi-cellulase and pectinase have immense potential and application in research and development area for controlling plant diseases and enhancing plant growth. The discarded biomass and agro wastes are successfully utilized for the production of enzymes, sugar and alcohols (Karmakar and Ray, 2011; Acharya *et al*., 2008; Milala *et al*., 2009). Pineapple fruits are compound, oval fruits, six to eight inches long with spiky robust leaves at the top of the fruit. The tough waxy rind is green, brown and yellowish in colour with a scale-like appearance. The flesh of the pineapple is juicy and yellow to white in colour. Pineapple is a tropical plant and fruit. Its scientific name is *Ananascomosus* and it belongs to the class: Liliopsida and family Bromeliaceae. Pineapple is the source of the proteolytic enzyme bromelain which is used in commercial meat tenderizers and which constitutes practice as a soft tissue anti-inflammatory and for topical debridement.

Pineapple is a good source of minerals and vitamins. Fruit rot of pineapple caused by *Ceratocystis paradoxa*is a plant pathogen which leads to huge crop losses in different parts of world. The fungus enters through wounds and the crevices between individual fruits. The affected initially developed a reddish colour, which later turned to brownish black due to the fungus *Ceratocystic paradoxa* infection had reported in other crops like maize, coconut and sugarcane (Daniel *et al*., 2013). In *Ceratocystic paradoxa*, cellulase is the key virulent factor for the infection (Swaroop 2007).The aim of this study was to isolate, produce and purify cellulose enzyme from pine apple waste using *Ceratocystis paradoxa.*

FIGURE 2: *Ceratocystis paradoxa* infected Pine apple

FIGURE 3: Morphology and Culture of *Ceratocystis paradoxa*

Two methods were compared for the isolation from soil fungus *Ceratocystis paradoxa*, causal agent of pineapple disease of sugarcane. The semi-selective V8-A medium permitted both a qualitative and a quantitative assessment and colonies characteristic of the pathogen were observed within two days (Moutia and Saumtally, 1999). Cellulase production from pine apple using *Trichoderma ressei* was done under submerged fermentation and the enzyme activity was checked using DNSA method according to Miller where reducing sugar was calculated (Saravanan *et al*., 2012). Hydrolytic enzymes produced by *Ceratocystis paradoxa* extracellularly were partially purified and its cellulolytic and proteolytic enzyme assay was done using carboxy methyl cellulose and casein (Adisa, 1987). Hence in this chapter cellulases produced was purified from the crude extract by different techniques like centrifugation, ammonium sulphate precipitation, dialysis, gel column chromatography and ultragel column .The homogeneity of the enzyme is tested using SDS-PAGE and purity with the immunological methods like Ouchterlony immunodiffusion technique, Immuno electrophoretic technique etc.

MATERIALS & METHODS

Glasswares

All glasswares were washed first with water and detergent and further cleaned by soaking in 5% nitric acid for 24 hours and finally rinsed with deionised water (Milli Q water system and stored in sterile place for the experiments.

Crude enzyme preparation

In the present study, the culture medium supplemented with pine apple waste as the source was used for culturing the fungus. To obtain maximum quantity of enzyme one 1000ml of medium in 5litre Haffkin's Flask was autoclaved in bars lead (Boston) autoclave at 15 lb pressure for 20 minutes. After sterilization the flasks were cooled to 32 ±1°C. Then a slant of *Ceratocystis paradoxa* cultured in PDA was inoculated into medium in a sterile chamber. After 16 days of incubation at 32 ±1°C the contents were taken out by adding 500 ml of 0.25M NaCl. This was blended in waring blender for 5 second at 4°C the blended juice was filtered through 2 layers of cheesecloth. The filtrate was centrifuged at 20,000 rpm for minutes at 4°C. The clear supernatant was dialyzed against 2 liter. 0.01 M phosphate buffer at pH 7.0 for 48 hours with two changes at 4±1°C. The dialysed crude enzyme extract was used for initial enzyme assay and for purification using ammonium sulphate precipitation.

Estimation of Cellulase

Flasks containing growing culture of fungal isolates were withdrawn and the filtrate obtained after removal of mycelial mat by filtration through Whatman No.1 filter paper was used as an enzyme source.

Activity of endoglucanase in the culture filtrates was quantified by carboxymethyl cellulose method. The reaction mixture with 1.0 ml of 1% carboxymethyl cellulose in 0.2 M acetate buffer (pH 5.0) was pre-incubated at 50°C in a water bath for 20 minutes. An aliquot of 0.5 ml of culture filtrate with appropriate dilution was added to the reaction mixture and incubated at 50°C in water bath for one hr. An appropriate control without enzyme was simultaneously run. The reducing sugar produced in the reaction mixture was determined by Dinitrosalicylic acid (DNS) method (Miller 1959). 3, 5-dinitro-salicylic acid reagent was added to aliquots of the reaction mixture and the color developed was read at wavelength 540 nm by using the spectrophotometer (ELICO, SL 171). One unit of endoglucanase activity was defined as the amount of enzyme releasing one µ mole of reducing sugar /ml /h.

Reagents

Carboxymethyl cellulose

(1%) Carboxymethyl cellulose was used as substrate for enzyme assay. The substrate was prepared by dissolving 1g of CMC in 50 ml acetate buffer pH 5.0 by heating in a water bath for 10 min under constant stirring. After cooling to room temperature, the solution was made up to 100ml with pH 5.0 acetate buffer. The substrate could be stored at room temperature for maximum life of 4 weeks.

Acetate buffer

For the preparation of acetate buffer, we need 0.1 M of acetic acid which can be made by addition of 3 ml of glacial acetic acid to make 100 ml of solution with the help of distilled water. Take 1.36 gm of sodium acetate and dissolves it in 100 ml distilled water. Now pipette out 64.3 ml of sodium acetate solution in a standard flask and add 35.7 ml of acetic acid solution. Now make the volume 100 ml with the addition of distilled water. The pH of the

buffer will be 5. The buffer is stable and can be stored at room temperature for two weeks.

Sodium Potassium hydroxide

16g of sodium hydroxide and 22.49g of potassium hydroxide was dissolved in deionised water and made upto 100ml with the same. This could be stored in a plastic bottle for indefinite time.

Dinitro salicylic acid reagent (DNSA)

About 2.5 g of DNSAwas dissolved in 150ml of deionised water. To this 25ml Sodium potassium hydroxide was added drop-wise. Then 75g of potassium sodium tartaratetetrahydrate (RocheIle's salt) was added and the solution cooled to room temperature and filled upto 500ml with deionised water. The reagent was stored at room temperature in dark. Maximum storage life is one month.

100mM Glucose stock standard solution

1.8016 g of D glucose was dissolved and made upto 100ml with acetate buffer. Molecular weight of glucose: 180.16.

Working standard solution

Glucose standard stock solution was diluted to give concentrations of 5, 10, 15, 20 and 30 micromole per ml of the solution. 10% Tween 20: 10 g of Tween 20 was made upto 100ml with deionised water. Stored at room temperature. Maximum storage life is one week.

Procedure

50 µl of the enzyme solution was added to 0.5ml of 1% CMC dissolved in 0.1 M sodium acetate buffer, pH 5.5. After incubation at 40°C for 30 min, 1ml of DNSA reagent was added, kept for 5 minutes in a boiling water bath and cooled on ice for 5 minutes. The contents were centrifuged at 3000 rpm for 10 minutes to remove unreacted carboxymethyl cellulose and diluted with 1.5ml of deionised water. The degree of enzymatic hydrolysis of the CMC was determined spectrophotometrically by measuring the absorbance at 530nm. A reaction blank was prepared similarly but the reaction mixture without incubation was heated in a boiling water bath for 5 min to denature the endoglucanase. For preparing standard curve, 50 µl of glucose standard solution (5, 10, 15, 20 and 30 µmol/ml) was mixed with 0.5ml of 1% CMC and incubated similarly followed by addition of 1ml DNSA reagent, boiling, centrifuging and estimation of the color at 530nm. A blank was prepared with 50ul acetate buffer instead of glucose standards. The amount of sugar produced by enzyme reaction was calculated from its optical density using standard curve obtained from glucose standards.

Ammonium sulphate precipitaion

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 ° C 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°C. The supernatant solution was taken for the next $(NH_4)_2SO_4$ 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 endoglucanase and protein content were estimated.

Chromatographic procedures

The sample containing maximum Cellulase activity was selected for further purification by chromatography. The entire chromatography operations were carried out at 4° 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 Rediracmoderl, 2112). Measurements of absorbonce of collected fractions (A_{280}) were carried out in a shimadzu spectrophometer uv-260 Japan with 1 cm cuvette at 280 nm. Cellulase activity was quantified by measuring the microgram of reducing sugar released per ml of enzyme in one hour from carboxymethyl cellulose using reducing sugar method of Miller by dinitro salicylic acid. One unit of Cellulase activity is defined as micro gram of reducing sugar released from carboxymethyl cellulose per mg of enzyme in one hour at 32 ±1°C under assay conditions.

DEAE column chromatography

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

Reagents

1. DEAE – Cellulose in 3 X25 cm column
2. 0.5 N HCl
3. 0.5 N NaOH
4. 0.01 M phosphate buffer, pH 7.0
5. 0.05 M to 0.2 M NaCl

Procedure

A DEAE cellulose (Sigma chemicals fine grade) column of 3 X25 cm was prepared after pretreatment with 0.5 N NaOH and 0.5 N HCl. The column was equilibrated with 0.01M phosphate buffer pH 7.0 50 ml of the dialysed 80-95% $(NH_4)_2$ SO_4 precipitation solution which shows high cellulase activity was loaded in the column and washed with the same buffer till the washing showed the absorbance almost zero at 280nm. 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 Cellulase activity.

Ultragel Column

Further purification of Cellulase I fractions was carried out by gel filtration on ultragel 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 Endoglucanase I was loaded on the ultragel column. 3 ml fractions per tube at the flow rate of 9 ml/ hour were collected. The individual fractions were analysed for Endoglucanase activity. The Endoglucanase active fractions were pooled and lyophilized.

The lyophilized and dialyzed Endoglucanase from DEAE column was loaded on to the ultrogel column (1.6X88.0 cm). 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 cellulase activity. The Cellulase active fractions were pooled and lyophilized.

Estimation of protein

Protein content was determined by adopting the procedure of Lowry *et al*. (1951) described under general materials and methods with crystalline bovine serum albumin (BSA) as standard. The fractions collected after chromatographic column were directly read at A80 in a shimadzu spectrometer for monitoring the protein

Test for purity of the enzyme

The homogeneity of the purified Cellulase was determined by polyacrylamide gel electrophoresis, SDS-polyacrylamide gel electrophoresis, isoelectric focusing, immuno diffusion and immuno electrophoretic techniques.

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.

Isoelectericfocussing

The isoelectric focusing was carried out using polyacrylamide gel rods (11 X 165 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 immunodiffusion technique was carried out according to the method of Ouchterlony and Nilsson (1967) and immunoelectrophoresis was performed according to the technique of Graber and Burtin (1964).

RESULTS & DISCUSSION

Table 2 shows the precipitation of cellulase activity at different percentages of ammonium sulphate concentration and the pellet obtained shows maximum cellulase activity.

DEAE cellulose chromatography

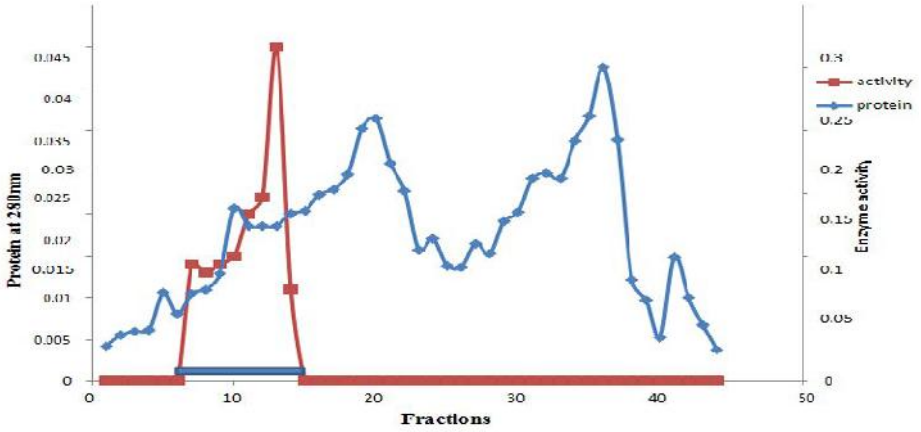
The precipitate obtained between 60-80% $(NH_4)_2SO_4$ fraction was dialyzed and applied on DEAE cellulose column. The stepwise gradient elution shows that first fraction of EG activity (designated as cellulase) eluted at the concentration of 0.1M NaCl eluted at the concentration of 0.2 M NaCl.

Fractions 6 ml per tube were collected at the flow rate of 30 ml per hour in DEAE cellulose column (3 X 25 cm). Eluted with 0.2 M sodium acetate buffer at pH 7.0 containing 0.05 M to 0.2 Na Cl gradient. The elution profile (Graph 3) shows only the one peak, which contains both protein and cellulase activity. Elution profile of EG from DEAE cellulose column after pooling and dialysis against 0.01 M phosphate buffer (pH 7.0) was lyophilized. It was later checked for homogeneity.

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

Sl No	Percentage of $(NH_4)_2 SO_4$ (%)	Volume (ml)	Cellulase activity units up of reducing sugar rel/ml/1 hr	Protein content µg/ml	Specific activity in units/µg
1	Crude enzyme	900	56.4	71.2	0.79
2	0-20	130	1.32	26.4	0.05
3	20-40	100	1.85	25.8	0.07
4	40-60	80	2.04	29.1	0.07
5	60-80	60	976.4	76.9	12.69
6	80-100	50	345.6	73.5	4.7

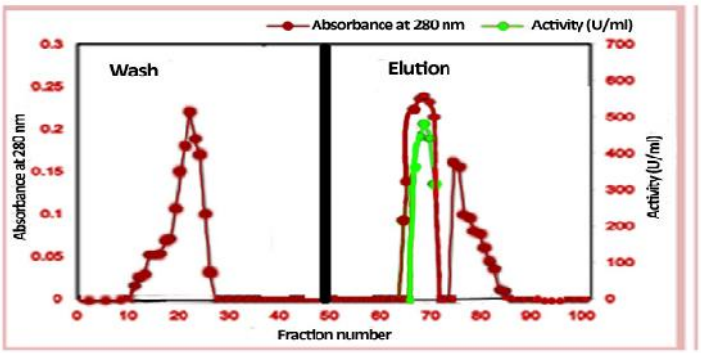
Values given are the mean value ($\bar{X}$) of 4 datas
d.f. = degrees of freedom = n-1Significance ++ = $p < 0.001$,+ = $p < 0.05$
NS = Not significant



Graph 1: Elution profile of DEAE-cellulose column chromatography for the purification of Cellulase

Ultragel column Chromatography

Elution profile of EG I from DEAE cellulose column after pooling and dialysis against 0.01 M phosphate buffer (pH 7.0) was lyophilized. A small amount was dissolved in the same buffer and loaded on ultragel column (Graph 4) shows two protein fraction but cellulase activity is seen only in 1st fraction, *i.e*. from 65rd to 70th fraction.



GRAPH 2: Elution profile of ultrogel column for cellulase purification

Purification of Cellulase

A summary of purification presented in Table 2.1 reveals that cellulase was purified to 8.75 fold with 53.74 % yield.

TABLE 2: Purification of Cellulase

Steps	Volume (ml)	Total Protein (µg)	Total Cellulase Activity units (µg of glu rel)	Specific Activity units per µg of protein	Purification fold	Yield (%)
Culture filtrate (Supernatant)	200	223.4	14202	63.57	1	100
60-80% $(NH_4)_2 SO_4$ Precipitation	140	81.8	9483	115.92	1.82	66.77
DEAE- cellulose column fraction	65	26.2	8326	317.78	4.92	58.62
Ultra gel fractionation	40	13.5	7634	565.48	8.75	53.74

Elution profile of the purification of Endoglucanase from crude enzyme fraction of 60 to 80% ammonium sulphate fraction by DEAE cellulose column. From each step of purification the enzyme samples were tested for homogeneity using SDS-PAGE. After final purification CX I show only one band in SDS-PAGE and the molecular weight was found to be 25k Da (Fig 2.4)

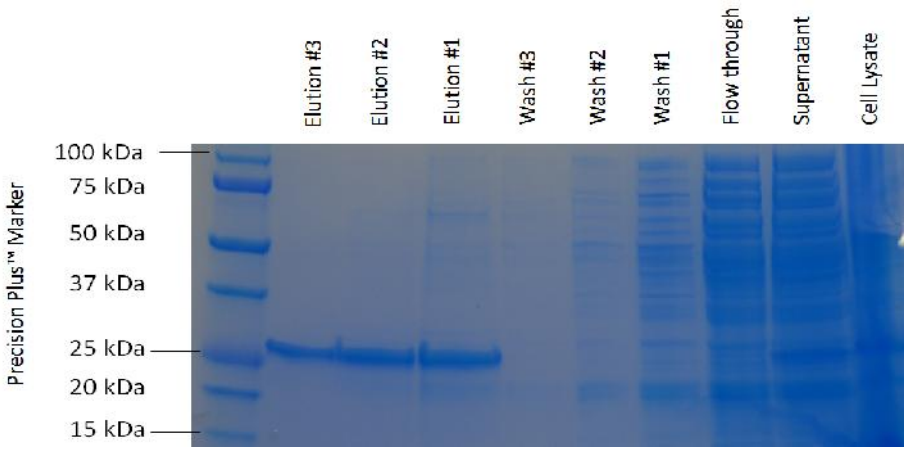


FIGURE 4: Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis of Cellulase

Cellulase activity is expressed in units as µg of glucose released /ml/30 miinutes. Isoelectric focusing was carried out in semipreparative manner using polyacrylamide gel rods (11 X 165 mm) based on the method of Wrigley (1971) at 4° C.

Determination of isoelectric point of cellulase

Isoelectric forcussing was carried out in a semi preparative manner using polyacrylamide gel rods (11 X 165 mm) based on the method of Wrigley (1971)at 4^0C

The gel was cast by mixing the following solution

1.30% 9W/V) acrylamide + 1% (W/V) N, N'methylene bisacrylamide in water = 4.0ml

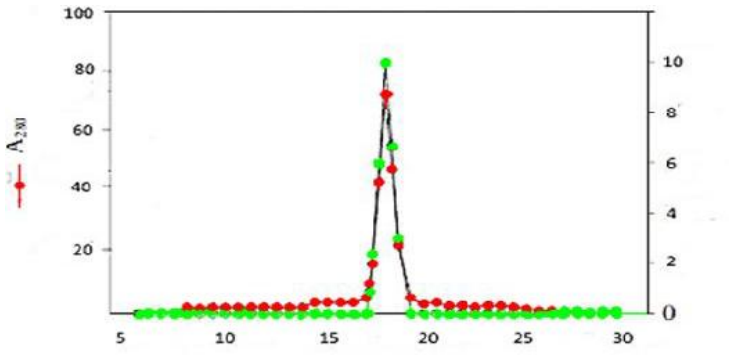
2. Ampholine (ph 3.5 – 10.0) = 0.4 ml

3. Enzyme protein 5 mg = 1.0 ml

4. Water = 9.6 ml

5. Ammonium per sulphate (1% w/v) = 1.0 ml

It indicates a single peak of cellulase activity between 16^{th} and 17^{th} fractions at isoelectric pH 6.7. (Graph 5)



GRAPH 3*:* Determination of isoelectric point of cellulase

Ouchterony double diffusion patterns of purified cellulase

The Ouchterlony (1973) double immunodiffusion pattern indicates the homogeneity of the cellulase (Fig 2.5). The highly purified enzyme gave a single immunoprecipition line in reflected light when reacted with its rabbit antiserum showing the presence of only one component.

FIGURE 5: Ouchterony double diffusion patterns

Graber and burtin immunoelectrophoresis pattern of purified cellulase

Graber and Burtin (1964) qualitative analysis by immunoelectrophoresis also confirmed the homogeneity of the enzyme as depicted in Fig 2.6.

FIGURE 6: Graber and Burtin immunoelectophoresis

DISCUSSION

In the present study cellulase enzyme was isolated from *Ceratocystis paradoxa* leaf blight causing organism in pine apple using pine apple waste material as the source. The enzyme was cultured in submerged fermentation method. Researchers have suggested the production of cellulase using submerged fermentation conditions (Akinycle and Olaniyi, 2013; Sajitha *et al*., 2016). 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 immunodiffusion techniques which obtained a single precipitation line on the plates. In the present study we assayed the enzyme activity using Lowry and DNSA method according to the amount of protein and reducing sugar concentration produced in the media respectively. This method is introduced as the most satisfactory procedure in the previous studies (Srilakshmi and Narasimha, 2012; and Anuradhasingh 2014).The DEAE cellulose column treatment at pH 5.0 selectively removed the brown pigments from the crude enzyme extract giving appreciable increase in specific activity. Similar affinity column was used for the purification of cellulose (Pressey, 1973 and Takahashi, 1985). The DEAE cellulose column chromatography at pH 7.0 selectively adsorbed the entire EG.

The adsorbed enzyme was eluted form the stepwise column gradient as a major fraction **(**Graph 4). Similar

elution pattern was reported for the purification of Cellulase (Gaur *et al*., 2015, Sajitha *et al*., 2016, Ajay *et al*., 2012and Hafiz *et al*., 2011). When crude enzyme from *Ceratocystis paradoxa* was subjected sequential purification steps, the purified sample showed a 565.48 fold increase in specific activity and a yield of 53.74% were obtained (Table 2).

The results of SDS-PAGE (fig .4) suggest that the purified enzyme is homogeneous. A molecular weight of 25kDa was obtained. Isoelectric focussing pattern (Graph 5) also confirmed the previous findings of the homogeneity of the enzyme. The presence of single protein band suggests that cellulase consists of a single polypeptide chain. Researchers have suggested that molecular weight of cellulase ranges from 12 kDa to 126kDa previously (Sajitha *et al.*, 2016, Gaur *et al.*, 2015, Hatif *et al.*, 2011) the immunodiffusion and immunoelectrophoresis results showing single precipitation line confirm the homogeneity of the purified endoglucanase

CONCLUSION

Pine apple is affected by a blight disease caused by *Ceratocystis paradoxa*. This fruit rot in pineapple leads huge crop loss in different parts of the world. The same infection is reported in maize, coconut and sugarcane (Daniel et al 2013). Hence this *Ceratocystis* was isolated and cultured in Haffkins flask for large scale production and purification of cellulase. Then it is filtered, precipitated with ammonium sulphate and were subjected to DEAE cellulose column chromatography and ultra gel column to purified to an extent of 565.48 enzyme activity, a purification fold of 8.75 and a yield of 53.74%. Homogeneity test were conducted by SDS PAGE, Ouchterlony immunodiffusion and immunoelectrophoresis..

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