



## ISOLATION AND PURIFICATION OF CELLULASE

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**ABSTRACT**

The production of cellulase enzymes on carboxymethyl cellulase (CMCase, endoglucanase activity) by an isolated fungus *Aspergillus terreus* evaluated. The intracellular cellulase from *A. terreus* was isolated purified and some properties were characterized. Employing a purification scheme consisting to the following steps isolated it: Crude extract after centrifugation, Ammonium sulphate precipitation & DEAE – cellulase chromatography and ultra gel column chromatography. By adopting these step a fold purification of 270 with 22.11 % overall yield of were obtained. PAGE, SDS- PAGE, immunodiffusion and immunoelectrophoresis and isoelectric focusing confirmed the homogeneity of the enzyme. The extracted fractions analyzed based on soluble protein amounts and enzymes activities. The cellulase enzymes from isolated *A. terreus* showed 80% (according to CMCase). The best enzyme activities were in pH 4-7 and the temperatures between 40°C to 50°C. By the purification process, 40% of protein, 77% of CMCase activities extracted from concentrated enzyme solution from *A. terreus*.

**KEY WORDS:** Cellulase, *Aspergillus terreus*, DEAE cellulase, Ultrogel, Immunodiffusion.

**INTRODUCTION**

Cellulases (1,4)-(1,3:1,4)-/ J-D-glucan 4-glucanohydrolase (EC 3.2.1.4) or the enzyme essential for the Carbon cycle in nature. These cellulases are commercially used in the food industry for clarification of fruits and juices and pharmaceuticals industry. It is almost necessary to purify with 100% purity and yield. In the last decayed much attention is given on cellulase degradation, because of its purpose as a renewable energy alternative for fossil fuel. It is the abundant biomass on earth. Cellulase is composed of Beta-1-4 linked D-glucose units which could be hydrolysed by cellulases resulting in fermentable sugar for bioethanol bioconversion. The widely accepted mechanism of enzymatic cellulase hydrolysis involved the synergistic action of 3 types of cellulases including endo-beta 1,4-glucanase (EC 3.2.1.4), Exoglucanase (EC 3.2.1.91) and beta Glucosidase (EC 3.2.1.21), Which step by step nicked the intermolecular beta 1,4 glycosidic bonds cleaved cellulase chain in to release of cellobiose units and cut cellobiose and oligosaccharides to produce glucose. Many fungal and microbe sources where known for decomposing cellulase, the available application of them was far from satisfactory because the properties and production of their known cellulases could not conform to the specified requirements such as high catalytic efficiency of insoluble cellulosic substrate, superior stability at elementary temperature and at a certain pH, and strong tolerance to end product inhibition (Zhang *et al.*, 2006). Since, Cellulases are commercially and medically used enzyme high purity with maximum yield is required. Various laboratories attempted to purify cellulases. No single method was satisfactory for any purification biochemist should concentrate and maximum possible yield, the maximum catalytic activity and the maximum

possible purity is required and the strategy for enzyme purification good source for the enzyme production good method of homogenization and a perfect method of separation is required. Hence in this chapter cellulases is purified with LKB column (Sweden) with Carboxy CM-trisacryl, ultrogel column with maximum yield and the chart is presented. The purified enzyme is tested for its purity with the Ouchterloney immunodiffusion technique, Rocket electrophoresis, Immunoelectrophoretic technique, PAGE, SDS-PAGE and isoelectric focusing.

**MATERIALS & METHODS****Purification of Cellulase****Crude enzyme preparation**

In the present study, the culture medium which was standardized earlier (chapter I) was used for culturing the mold. To obtain maximum quantity of enzyme one 1000ml of medium in 5 litre Haffkin's Flask. This 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 *A. terreus* 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 cheese cloth. 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 ammonium sulphate precipitation.

**Ammonium sulphate precipitaion**

To 1 liter of dialysed supernatant solution ammonium sulphate (AR) was added slowly with a constant stirring in

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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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 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 4°C 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 micro perpex 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 (A<sub>280</sub>) were carried out in a Shimadzu spectrophotometer 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 carboxy methyl cellulase using reducing sugar method of Miller by Di Nitro Salicylic acid. One unit of Cellulase activity is defined as micro gram of reducing sugar released from carboxy methyl cellulase per mg of enzyme in one hour at 32±1°C under assay conditions.

### DEAE column chromatography

The DEAE-cellulase column chromatography was carried out according to the following procedure. A DEAE cellulase (Sigma chemicals fine grade) column of 3 X 25 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<sub>4</sub>)<sub>2</sub> SO<sub>4</sub> 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. Both fractions of peak I and peak II were found to possess the cellulase activity. Fractions of Endoglucanase and Endoglucanase II were individually pooled lyophilized dissolved in buffer pH 7.0 containing 0.05M NaCl.

### Ultrogel Column

Further purification of Cellulase I fractions was carried out by gel filtration on ultrogel ACA 44 (LKB) column. The column (1.6 X 88cm) was equilibrated with 0.01M 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 ultrogel column. 3 ml fractions per tube at the flow rate of 9ml/ hour were collected. The individual fractions were analysed for Endoglucanase activity. The Endoglucanase active fractions were pooled and lyophilized. The lyophilized and dialysed Endoglucanase from DEAE column was loaded on to the ultrogel column (1.6 X 88.0 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 A<sub>80</sub> 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.

### Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was carried out according to the method of Davis (1964). The detailed procedure has been given in general material and methods.

### SDS- Polyacrylamide gel electrophoresis (SDS- PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out to determine the homogeneity of purified cellulase according to procedure of Laemmli (1970). The detailed procedure is explained in general materials and methods.

### Isoelectric focusing

The isoelectric focusing was carried out in a semi preparative manner 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 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).

### Determination of Molecular weight of cellulase

The molecular weight of the purified cellulase I and cellulase II was determined by gel filtration on Sephadex G-100 by the procedure which was adopted by Andrews (1964). The column was standardized by using low molecular weight kit containing ribonuclease (MW 43,000), Albumin (BSA) (Mw 67,000) and Blue dextran 2000. The Kav values of standard protein were plotted against corresponding molecular weight in semi logarithmic graph. The straight line obtained was used for the determination of molecular weight of purified cellulase I and cellulase II.

### Ultraviolet absorption spectrum of cellulase

The purified lyophilized sample of cellulase I and cellulase II was dissolved in distilled water (2 mg/ 1 ml) in the quartz cell of Shimadzu spectrophotometer model UV 260 and the absorbance was scanned from 190 nm to 300

nm. A print out of the scanned diagram on the screen of shimadzu spectrophotometer was obtained.

**RESULTS & DISCUSSION**

Table 1, Shows the precipitation of cellulase activity at different percentages of ammonium sulphate concentration. The pellet obtained shows maximum cellulase activity.

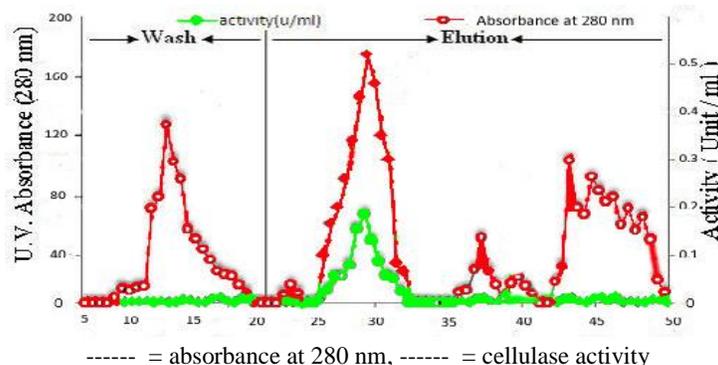
**TABLE 1.** Ammonium sulphate precipitation of crude enzyme preparation of Cellulase from natural onion medium

Sl No	Percentage of (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (%)	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-1, Significance ++ = p < 0.001  
 + = p < 0.05, NS = Not significant

Fig 2 shows the elution profile of DEAE Cellulase column chromatography of cellulase. The precipitate obtained between 80- 95% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction was dialyzed and applied on DEAE cellulase 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.

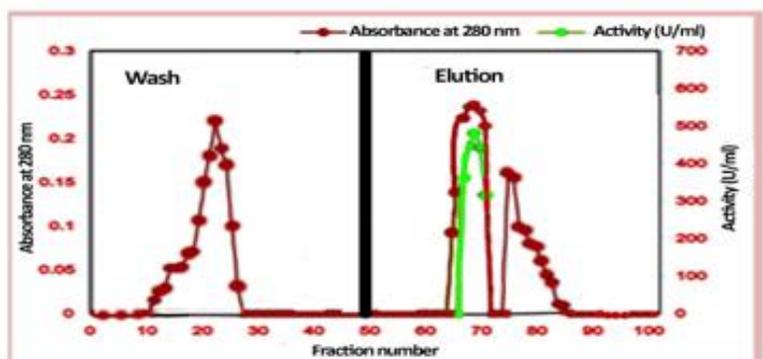
Elution profile of the purification of Endoglucanase from crude enzyme fraction of 80 to 95% ammonium sulphate fraction by ion exchange chromatography on DEAE cellulase column. Fractions 6 ml per tube were collected at the flow rate of 30 ml per hour in DEAE cellulase column (3 X 25 cm). Eluted with 0.2 M sodium acetate buffer at pH 7.0 containing 0.05 M to 0.2 M NaCl gradient.



**FIGURE 2:** Elution profile of DEAE-cellulase column chromatography for the purification of Cellulase

Elution profile of EG I from DEAE cellulase 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 ultrogel column (Fig 3) shows two protein fraction but cellulase activity is seen only in 1<sup>st</sup> fraction, ie from 36<sup>th</sup> to 52<sup>nd</sup> fraction.



**FIGURE 3:** Elution profile of ultrogel column for cellulase purification

Elution profile of the purification of cellulase from DEAE cellulase on ultrogel column. Fractions corresponding to peak I of DEAE cellulase column were pooled together,

concentrated and rechromatographed on ultrogel column (1.6 X 88 cm). Fractions of 3 ml / tube were collected at

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the flow rate of 9 ml / hour eluted with sodium acetate buffer.

----- = protein absorbance at 280 nm

----- = cellulase activity

The second fraction of DEAE column after conducting the same procedure as described above was loaded to the

ultragel column. The elution profile (Fig. 4) shows only the one peak, which contains both protein and cellulase activity. The cellulase activity is seen. A summary of purification presented in Table 5 reveals that cellulase is purified to 245 fold with 26% yield. cellulase is purified to 270 fold and with 22% yield.

**TABLE 5.** Purification of Cellulase

Steps	Volume (ml)	Total Protein (µg)	Total cellulase Activity units (µg of gal. acid rel)	Specific activity Units/µg protein	Purification fold	Yield (%)
Culture filtrate (Supernatant)	900	64080	50760	0.79	1	100
80-95% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> Precipitation	50	3845	48820	12.7	16.0	96.18
DEAE-column CXI-fraction	138	201	21618	107.55	135.5	42.58
CX II - fraction	40	162	2085	128.54	161.34	41.01
Ultragel column CX I -fraction	34	68	13285	195.36	245.18	26.16
CX II - fraction	16	52	11226	215.88	270.93	22.11

From each step of purification the enzyme samples were tested for homogeneity using PAGE and SDS-PAGE. After final purification CX I show only one band in PAGE (Fig 6) and SDS – PAGE (Fig 7). Similarly CX II also shows single band in PAGE (Fig 8) and SDS – PAGE (Fig 9).

**FIGURE 6:** Polyacrylamide gel Electrophoresis of cellulase



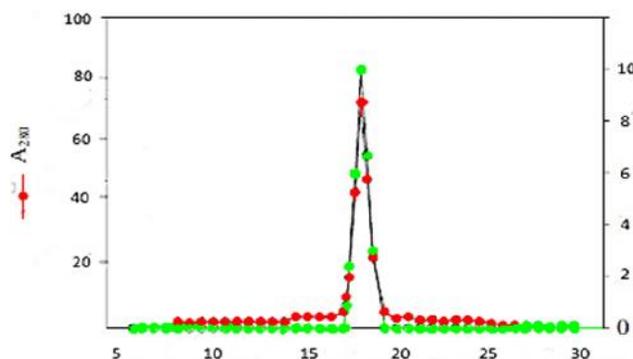
**FIGURE 7:** Sodium Dodecyl Sulphate Polyacrylamide gel Electrophoresis of Cellulase



Determination of isoelectric point in a semi preparative manner of CX I shown in Fig 10. It indicates a single peak of cellulase activity between 11<sup>th</sup> and 12<sup>th</sup> fractions at isoelectric pH 06.7. The semi preparative isoelectric focusing of cellulase II is shown in Fig 11. It shows a single of CX activity between fractions 16 and 17 and its isoelectric pH is 7.1. The outchterlony's double immunodiffusion pattern indicates the homogeneity of the

cellulase I and cellulase II (Fig 12 and 14). The highly purified enzyme gave a single immunoprecipitin line in reflected light when reacted with its rabbit antiserum showing the presence of only one component. Graber and Burtin qualitative analysis by immuno electrophoresis also confirmed the homogeneity of the enzyme as depicted in Fig 13 and 15.

### Determination of isoelectric point of cellulase



Cellulase activity is expressed in units as µg of galacturonic acid released /ml/30 minutes. Iso electric focusing was carried out in semipreparative manner using

polyacrylamidegel rods (11 X 165 mm) based on the method of Wrigley (1971) at 4°C.

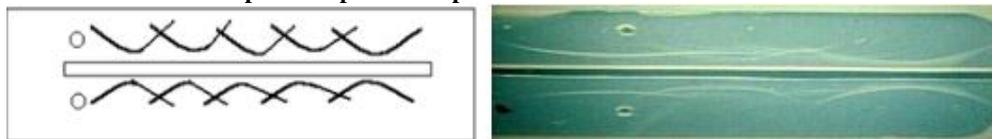
### Determination of isoelectric point of cellulase

Isoelectric focussing was carried out in a semi preparative manner using polyacrylamide gel rods (11 X 165 mm) based on the method of Wrigley (1971)



The central well contained antiserum of highly purified enzymes. The peripheral wells 1,2,3,4 and 5 contained 6 micro liter antigens.

### Graber and burtin immunoelectrophoresis pattern of purified cellulase



The through contained antiserum of highly purified cellulase I and the wells contained 3 micro liter of the antigen. The central well contained antiserum of highly purified enzymes. The peripheral wells 1, 2, 3,4 and 5 contained 6 micro liter of antigen.

### DISCUSSION

In the previous study, we reported the isolation of *A. terreus* and the effects of the cellulase enzymes of this fungus on the biodegradation of lignocellulosic wastes (Emitiazi, G. 2001). In the present study an optimization of cellulase activity of this fungus due to the best pH and temperature, showed an elevated cellulase production (80% for CMCase) by *A. terreus*. The fungi showed the best enzyme production in pH 4-7 and temperatures 40-50°C. Coral *et al.*, 2002 extracted the carboxy methyl cellulase from *Aspergillus niger* Z10 with the activity temperature of 40°C and optimum pH of 5 (Coral *et al.*, 2002). Other researches also determined similar optimum conditions for cellulase activity by aerobic fungi (Chinedu *et al.*, 2008. Acharya *et al.*, 2008). In the present study we assayed the enzyme activity according to the amount of reducing sugar concentration produced in the media. This method is introduced as the most satisfactory procedure in the previous studies (Emitiazi and west, 1996, Mandel *et al.*, 2001). The DEAE cellulase 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 by Pressey, 1973, Strand *et al.*, 1976, and Takahashi, 1985). The DEAE cellulase column chromatography at pH 7.0 selectively adsorbed the entire EG. The adsorbed enzyme was eluted from the stepwise column gradient as a major fraction (fig. 2). Similar elution pattern was reported for the purification of Cellulase (Magro *et al.*, 1980, Hoffman *et al.*, 1982). When crude enzyme from *A. niger* was subjected sequentially to six different treatments, the purified CX I having 245 fold increases in specific activity with 26.16% yield and cellulase II having 270 fold increases in activity with 22.11% yield were obtained (table 5). The results of polyacrylamide gel electrophoresis and SDS-PAGE (fig. 6, 7, 8 and 9) suggest that the purified enzyme is

### Ouchterony's double diffusion patterns of purified cellulase

homogeneous. Isoelectric focussing pattern (10 and 11) also confirmed the previous findings of the homogeneity of the enzyme. The presence of single protein band suggests that cellulase I and cellulase II consists of a single polypeptide chain (Cervone *et al.*, 1977). The immuno diffusion and immno electrophoresis results showing single precipitation line confirm the homogeneity of the purified endoglucanase.

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