

PURIFICATION AND CHARACTERIZATION OF CELLULASE FROM *ASPERGILLUS NIGER* (VAN TIEGHEM, 1867).

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ABSTRACT: In the present work purification and characterization of cellulase was studied which was produced from *Aspergillus niger* (van Tieghem 1867) when it was grown on 50 mL of culture medium containing sugar cane bagasse as cellulosic substrate after 96 h, at temperature of 30° C, at initial pH 5.0, agitation rate 100 rpm and inoculum size 6×10^6 conidia. Cellulase was purified first by ammonium sulfate precipitation and then Bio-Gel P-100 chromatography to about 9.8 folds than crude enzyme with the recovery of 59.8 % having specific activity 176.2 U/mg. Kinetic constants (K_m 2.7 mM and V_{max} 169.5 U/mL/min) were determined by Lineweaver-Burk Plot and molecular mass (34 kDa) by 12 % SDS-PAGE. Cellulase showed maximum activity at pH 6.0 (136.8 U/mL) and at the temperature of 55° C (80.6 U/mL). The purified cellulase was activated by $MnCl_2$ (142.7 U/mL) and $CoCl_2$ (150.8 U/mL) but inhibited by $HgCl_2$ (23.7 U/mL), $CdCl_2$ (31.5 U/mL), $FeSO_4$ (30.9 U/mL) and $Fe_2(SO_4)_3$ (52.4 U/mL). K_m value shows the purified cellulase has high specificity to carboxymethyl cellulose and was considered to be endocellulase which is highly benefits to the industrial application.

Key words: purification of cellulase, endocellulase *Aspergillus niger* (van Tieghem 1867)

1. INTRODUCTION

Cellulose is a lined polymer of simple sugar units (glucose) which are connected together by beta-1, 4-glycosidic linkage [1]. A large variety of fungi and bacteria can consume cellulose by converting it insoluble substrate into soluble compounds with the help of group of enzymes, cellulases, which are released by them. The term cellulases means a group of enzymes which includes Endocellulase (EC 3.2.1.4), Exocellulase (EC 3.2.1.91), Cellobiase (EC 3.2.1.21), Oxidative cellulase (EC 1.1.99.18) and Cellulose phosphorylase (EC 2.4.1.20). They all synergistically convert cellulose into glucose and therefore are used in industries [2, 3].

Enzyme recovery is very important step after fermentation. It includes separation of biomass and suspended solid particles, enzyme extraction, concentration and purification of enzyme. Purification of enzymes is usually performed by chromatography. Ion-exchange chromatography is the most common technique which is widely used for the purification of cellulase due to economical, high resolving power, simple to operate and high capacity [4]. In the present work cellulase (endocellulase) was obtained from locally isolated strain of *Aspergillus niger* (van Tieghem 1867) at optimized conditions by utilizing cellulosic wastes in submerged fermentation and then it was purified and characterized

2. MATERIALS AND METHODS

2.1 Strain

Strain of *Aspergillus niger* (van Tieghem 1867) was isolated from the soil of NED University of Engineering & Technology Karachi and it was grown for cellulase production on 50 mL of culture medium containing sugar cane bagasse as cellulosic substrate after 96 h, at temperature of 30° C, at initial pH 5.0, agitation rate 100 rpm and inoculum size 6×10^6 conidia.

2.2. Enzyme Activity

Cellulase activity was determined by following Wood and Bhat [3] using carboxymethyl cellulose (Sigma-Aldrich, USA) as a substrate. The reaction mixture contains 0.5 mL of 1 % (w/v) substrate in 0.1 M sodium citrate buffer (pH 4.8) and 0.5 mL of

culture broth in a test tube which was then incubated at 40° C for 30 min. The reaction was terminated by adding 1.0 mL of 3, 5-dinitrosalicylic acid (DNSA) and boiled the contents of test tube in water bath for 15 minutes. The absorbance was noted at 540 nm at UV-Visible spectrophotometer. One unit of cellulase activity was expressed as the amount of enzyme required to release 1 μ mol reducing sugars per mL per minute under the assay condition by using glucose as a standard curve.

2.3. Determination of total protein

Protein in the enzyme sample was determined by the method of Lowry *et al.* [5].

2.4 Purification and characterization of cellulase

All steps of purification were performed at 4° C. Purification was included ammonium sulphate precipitation and column chromatography.

2.5. Ammonium sulphate precipitation

In the first step varying amounts (60-80 %) of ammonium sulphate were added to cell free supernatant and then precipitates were obtained by spinning at 10,000 x g for 20 minutes. Precipitates were then dissolved in 0.5 M Tris Hydrochloric acid buffer at pH 8.0 and dialyzed. The salts from precipitates were removed by using 5 kDa molecular mass cut off dialyzing bag against a large volume of H₂O. The purified enzyme was stored at -5° C.

2.6. Column chromatography

The ammonium sulphate precipitated enzymes were then load onto Bio-Gel P-100 column (1.6 × 100 cm) with 20 mM Tris-HCl buffer (pH 8.0) at a flow rate of 4 mL/h. Fractions with cellulase activity were collected and analyzed for enzyme activity. Only those fractions having high enzyme activities were pooled, dialyzed and examined on SDS-PAGE.

2.7 Determination of kinetic parameters (K_m & V_{max})

With the help of Lineweaver-Burk Plot [6] the kinetic constant, K_m of purified cellulase was determined by following conditions; The reaction mixture contains 0.5 mL of 1 % (w/v) carboxymethyl cellulose in 0.1 M sodium citrate buffer (pH 4.8) and 0.5 mL of culture broth in a test tube which was then incubated at 40° C for 30 min. The maximum velocity, V_{max} of

Table 1: Purification summary of extracellular cellulase from *Aspergillus fumigatus* (Fresenius 1863)

Treatment	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude enzyme	500	3561	139.28	25.6	100	1
(NH ₄) ₂ SO ₄ (80 %) treated	37	2720	54.2	50.2	76.4	1.96
Bio-Gel P-100	7.5	2031	11.53	176.2	59.8	6.9

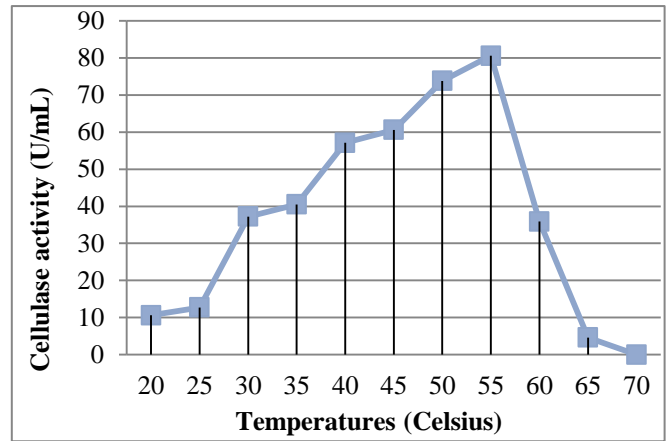


Fig. 1. Effects of Temperatures on the activity purified cellulase from *Aspergillus niger* (van Tieghem 1867).

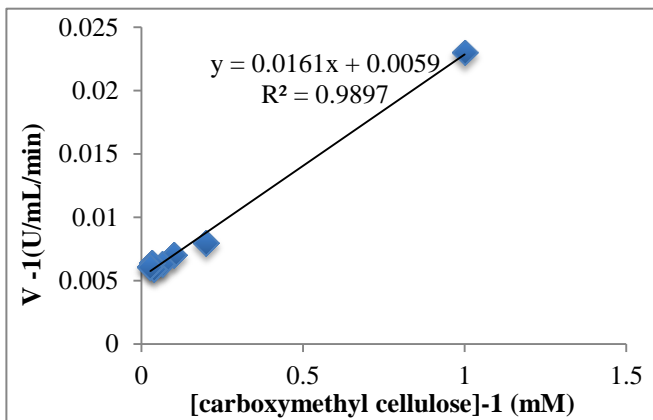


Fig. 1: Line weaver-Burk Plot for the determination of K_m and V_{max} values of cellulase from *Aspergillus niger* (van Tieghem 1867).

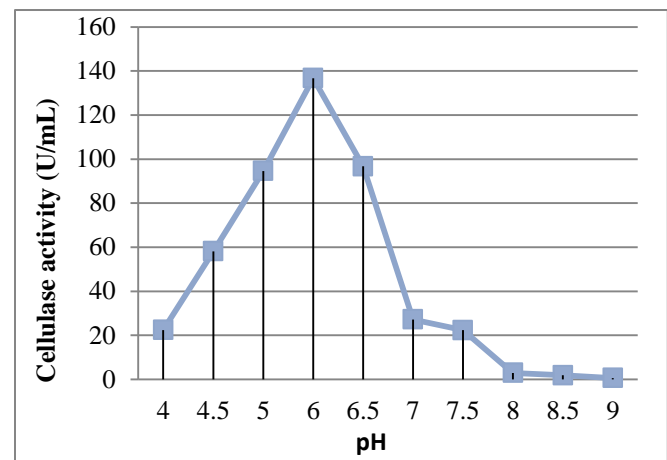


Fig. 2. Effects of pH on the activity purified cellulase from *Aspergillus niger* (van Tieghem 1867).

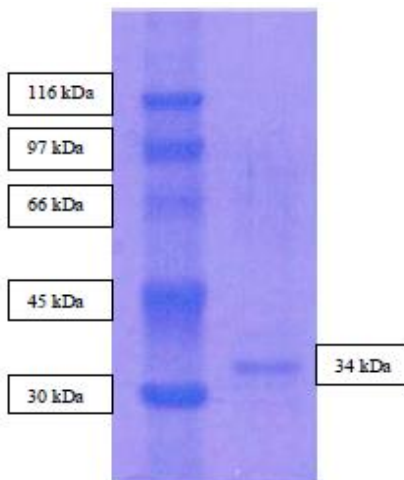


Fig. 2: Molecular mass determination of cellulase from *Aspergillus niger* (van Tieghem 1867) 12 % SDS-PAGE. First lane is the molecular weight marker and Second lane is purified enzyme.

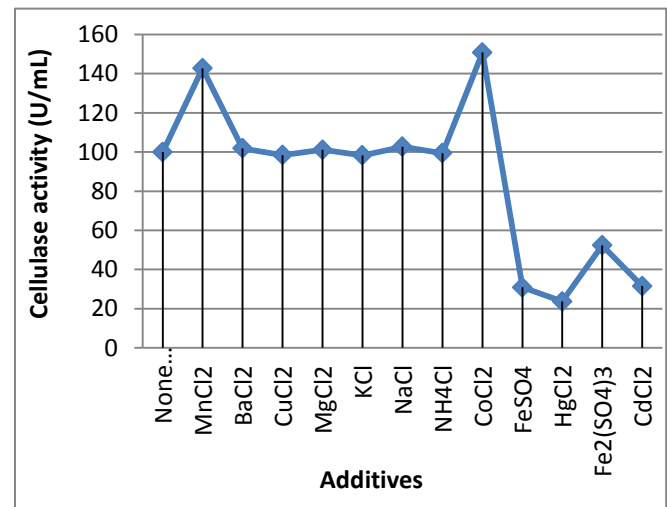


Fig. 5. Effects of additives on the activity purified cellulase from *Aspergillus niger* (van Tieghem 1867).

carboxymethyl cellulose hydrolysis by purified enzyme was also determined by the same conditions.

2.8 Effect of pH and temperature on cellulase activity

The effects were examined by incubating enzyme in 0.05 M acetate buffer at different pH from 3.0-9.0 at 40° C for fifteen minutes. After the determination of optimum pH cellulase activity was checked at different temperatures from 20-70° C [3].

2.9 Effects of various additives on cellulase activity

Various compounds such as Fe₂(SO₄)₃, MnCl₂, HgCl₂, FeSO₄, CdCl₂, BaCl₂, CoCl₂, AgNO₃, and NiSO₄, (all 1 mM) were incubated with purified enzyme at 55° C for thirty minutes and then cellulase activities were determined.

2.10 Determination of molecular mass

Molecular mass of purified enzyme was determined by 12 % SDS-PAGE and reported in kilo Dalton. Carbonic anhydrase (30 kDa), ovalbumin (45 kDa), albumin (66 kDa), Phosphorylase B (97 kDa) and β-Galactosidase (116 kDa), were used as protein markers.

3. RESULTS AND DISCUSSIONS

3.1 Purification

The extracellular cellulase from *Aspergillus niger* (*van Tieghem 1867*) was purified as per following results:

- At 20-70 % (w/v) saturation of ammonium sulphate, no enzyme activity was found while at 80 % (w/v) saturation the maximum specific activity (43.7 U/mg of protein) with 1.6 fold purification was obtained. Above 80 % (w/v) no pellets were obtained.
- Pellets of cellulase were dialyzed.
- They run on Bio-Gel P-100 column and were eluted by 0.2 M NaCl.
- The enzyme was purified about 9.8 folds than crude enzyme with the recovery of 59.8 %. It has specific activity 176.2 U/mg.
- The summary of purification can be seen (Table 1).

3.2 Determination of kinetic parameters (K_m & V_{max})

The Michaelis-Menten Constant (K_m) is defined as the substrate concentration at half of the maximum velocity V_{max}. Both kinetic parameters (K_m = 2.7 mM and V_{max} = 169.5 U/mL/min) of purified cellulase from *Aspergillus niger* (*van Tieghem 1867*) were determined by Lineweaver-Burk Plot (Fig. 1) for carboxymethyl cellulose [6]. Varying K_m and V_{max} values were reported in literature. Ekperigin reported [7] K_m and V_{max} values of cellulase from *A. anitratus* and *Branhamella sp.* 4.97 mM and 7.90 mg/mL respectively for carboxymethyl cellulose while Bakare *et al.* [8] reported values of 3.6 mg/mL and 1.1 mM for *Pseudomonas fluorescens*. The difference in K_m and V_{max} value of the presently purified cellulase from *Aspergillus niger* (*van Tieghem 1867*) and other reported fungal species may be due to the genetic variability among different species [9].

3.3 Molecular mass of Purified Cellulase

The molecular mass of purified cellulase from *Aspergillus niger* (*van Tieghem 1867*) was found 34 kDa as determined by SDS-PAGE (Fig. 2). Akiba *et al.* [10] reported molecular mass of cellulase from *Aspergillus niger* 40 kDa while Mawadza *et al.* [4] reported 25-45 kDa from *Bacillus sp.*

3.4 Effect of temperature and pH on cellulase activity

The temperature (Fig. 3) and pH (Fig. 4) optima for the purified cellulase from *Aspergillus niger* (*van Tieghem 1867*) were found 55° C (80.6 U/mL) and 6.0 (136.8 U/mL) respectively. Similar temperature and pH optima (55° C and 6.0) were also reported by Saha [11] for cellulase from *Mucor circinelloides*.

3.5 Effect of Additives

Effects of various additives on the activity of purified cellulase from *Aspergillus niger* (*van Tieghem 1867*) are plotted (Fig. 5). Most of additives such as KCl, NaCl and NH₄Cl negligibly affect the cellulase activity, while CoCl₂ (150.8 U/mL) and MnCl₂ (142.7 U/mL) increased and HgCl₂ (23.7 U/mL), Fe₂(SO₄)₃ (52.4 U/mL), FeSO₄ (30.9 U/mL) and CdCl₂ (31.5 U/mL) decreased the purified cellulase activity. The similar types of results were also reported by Smriti and Sanwal [12] for cellulase from *Catharanthus roseus*.

3. CONCLUSION

From the results obtained in this study, *Aspergillus niger* (*van Tieghem 1867*) could utilize natural wastes such as sunflower waste as substrate for growth and produce high levels of cellulase. The cellulase was purified by ammonium sulfate precipitation and then Bio-Gel P-100 chromatography. The temperature and pH optima of purified cellulase were 55° C and 6.0 respectively. CoCl₂ (150.8 U/mL) and MnCl₂ (142.7 U/mL) activated while HgCl₂ (23.7 U/mL), Fe₂(SO₄)₃ (52.4 U/mL), FeSO₄ (30.9 U/mL) and CdCl₂ (31.5 U/mL) deactivated the cellulase activity. K_m and V_{max} values for carboxymethyl cellulose were 2.7 mM 169.5 U/mL/min respectively. The molecular mass was about 34 kDa as determined by 12 % SDS-PAGE. The produced cellulase was endocellulase and highly benefits to the industrial application.

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