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PURIFICATION AND CHARACTERIZATION OF CELLULASE FROM *ASPERGILLUS FUMIGATUS* (FRESENIUS, 1863)

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ABSTRACT

In the present work purification and characterization of cellulase was studied which was produced from *Aspergillus fumigatus* (Fresenius, 1863) when it was grown in 50 mL of culture medium containing sunflower waste powder as substrate after 120 h, temperature 30° C, at initial pH 5.0, agitation rate 50 rpm and inoculum size $6x10^6$ conidia. Celluase 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 58.6 % having specific activity 268.2 U/mg. Kinetic constants (K_m 3.32 mM and V_{max} 188.7 U/mL/min) were determined by Lineweaver-Burk Plot and molecular mass (33 kDa) by 12 % SDS-PAGE. Cellulase showed maximum activity at pH 6.0 (121.7 U/mL) and at the temperature of 55° C (106.8 U/mL). The purified cellulase was activated by $MnCl_2$ (139.6 U/mL) and $CoCl_2$ (148.4 U/mL) but inhibited by $HgCl_2$ (21.6 U/mL), $CdCl_2$ (32.7 U/mL), $FeSO_4$ (29.5 U/mL) and $Fe_2(SO_4)_3$ (49.5 U/mL The value of K_m of the purified cellulase shows that its natural substrate is carboxymethyl cellulose and therefore it is considered to be endocellulase which is highly benefits to the industrial application.

Key words: Purification of cellulose, endocellulase, *Aspergillus fumigatus*.

INTRODUCTION

Cellulose is a linear polymer of glucose which is the most produced organic matter by plants on the planet earth using sunrays. In cellulose, glucose units are connected together by beta-1, 4-glycosidic linkage (Beguin and Aubert, 1994). A large number of bacteria and fungi can utilize cellulose by converting this 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), Cellulose phosphorylase (EC 2.4.1.20), Cellobiase (EC 3.2.1.21) and Oxidative cellulose (EC 1.1.99.18). They all at the same moment convert cellulose into glucose and therefore are used in industries (Bhat, 2000; Wood and Bhat, 1998).

Enzyme recovery is essential 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 (Mawadza *et al*., 2000). In the present work cellulase (endocellulase) was obtained from locally isolated strain of *Aspergillus fumigatus* (Fresenius, 1863) at optimized conditions by utilizing cellulosic wastes in submerged fermentation and then it was purified and characterized.

MATERIALS AND METHODS

Strain and fermentation conditions

Strain of *Aspergillus fumigatus* (was isolated from the soil of NED University of Engineering & Technology Karachi and it was grown for cellulase production after 120 h in 50 mL of culture medium containing sunflower cellulosic waste at 30° C, at initial pH 5.0 and inoculum size $6x10^6$.

Enzyme Activity

Cellulase activity was determined by following Wood and Bhat (1998) using carboxymethyl cellulose (Sigma-Aldrich, USA) as a substrate. The reaction mixture contains 0.5 mL (1 % w/v) of substrate in sodium citrate buffer solution (0.1 M .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 stopped by adding 1.0 mL of 3, 5-dinitrosalicyclic acid (DNSA) and boiled the contents of test tube for 15 minutes. The absorbance was noted at 540 nm spectrophotometer (Tomos). One unit (U) of cellulase

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activity was defined as the quantity of enzyme (Cellulase) which is required to release 1 µmol reducing sugars (glucose) per mL per minute under the condition of assay.

Determination of total protein

Protein in the enzyme sample was determined by using the standard method (Lowry *et al*., 1951). Specific activity was determined by dividing total enzyme activity (u) by total quantity of protein in milligram.

Purification and characterization of cellulase

All steps which were involved in the purification were completed at 4° C. Purification was included ammonium sulphate precipitation and column chromatography.

Ammonium sulphate precipitation

In the first step varying amounts (60-80 %) of ammonilum 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 kD molecular mass cut off dialyzing bag against a large volume of H_2O. The purified enzyme was stored at -5° C.

Column chromatography

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

Determination of kinetic parameters ($K_{m\ \&}\ V_{max}$)

With the help of Lineweaver-Burk Plot (Lineweaver and Burk, 1934) the kinetic constant, K_m of purified cellulase was determined by following conditions; The reaction mixture contains 0.5 mL (1 % w/v) of substrate in sodium citrate buffer solution (0.1 M .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 carboxymethyl cellulose hydrolysis by purified enzyme was also determined by the same conditions.

Effect of pH and temperature on purified cellulase activity

Effects were observed 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 (Wood and Bhat, 1998).

Effects of various additives on cellulase activity

Various compounds such as $Fe_2(SO_4)_3$, $MnCl_2$, $HgCl_2$, $FeSO_4$, $CdCl_2$, $BaCl_2$, $CoCl_2$, $AgNO_3$, and $NiSO_4$, (all 1 mM) were incubated with purified enzyme at 55° C for thirty minutes and then cellulase activities were determined.

Molecular mass determination .

Molecular mass of purified enzyme (Cellulase) was determined by 12 % SDS-PAGE and reported in kilo Dalton. Molecular markers were 14.4 kDa (Alpha-lactalbumin), 20.1 kDa (trypsin inhibitor), 30 kDa (carbonic anhydrase), 45 kDa (ovalbumin), 66 kDa (albumin) and 97 kDa (Phosphorylase B).

RESULTS AND DICUSSIONS

Purification

The extracellular cellulase from *Aspergillus fumigatus* 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 58.6 %. It has specific activity 268.2 U/mg.
- The summary of purification can be seen (Table 1).

Table 1. Summary of Purification steps of extracellular cellulase by *Aspergillus fumigatus.*

Treatment	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude enzyme	500	4212	154.49	27.3	100	1
$(NH_4)_2SO_4$ (80 %) treated	42	3021	69.20	43.7	71.7	1.60
Bio-Gel P-100	9.8	2470	9.21	268.2	58.6	9.8

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 = 3.32 mM and V_{max} = 188.7 U/mL/min) of purified cellulase from *Aspergillus fumigatus* were determined by Lineweaver-Burk Plot (Fig. 1) for carboxymethyl cellulose. Varying K_m and V_{max} values were reported in literature. Ekperigin (2007) reported 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.* (2005) reported value of 3.6 mg/mL and 1.1 mM for *Pseudomonas fluorescens*. The difference in K_m and V_{max} values of the presently purified cellulase from *Aspergillus fumigatus* and other fungal species may be due to the difference of genetic constitution among different species even different samples of same specie (Iqbal *et al*., 2011).

Molecular mass of Purified Cellulase

The molecular mass of purified cellulase from *A. fumigatus* was found 33 kDa as determined by SDS-PAGE (Fig. 2). Akiba *et al.* (1995) reported molecular mass of cellulase from *Aspergillus niger* 40 kDa while Mawadza *et al*. (2000) reported 25-45 kDa from *Bacillus sp.*

Effect of temperature and pH on cellulase activity

The temperature (Fig. 3) and pH (Fig. 4) optima for the purified cellulase from *Aspergillus fumigatus* (Fresenius 1863) were found 55° C and 6.0 respectively. Temperature and pH optima of 55C and 6.0 respectively have also been reported by Saha (2004) for *Mucor circinelloides.*

Effect of Additives

Effects of various additives on the activity of purified cellulase from *A. fumigatus* are plotted (Fig. 5). Most of additives such as KCl, NaCl and NH_4Cl negligibly affect the cellulase activity, while $CoCl_2$ (148.4 U/mL) and $MnCl_2$ (139.6 U/mL) increased and $HgCl_2$ (21.6 U/mL), $Fe_2(SO_4)_3$ (49.5 U/mL), $FeSO_4$ (29.5 U/mL) and $CdCl_2$ (32.7 U/mL) decreased the purified cellulase activity. The similar results were also reported by Smriti and Sanwal (1999) from *Catharanthus roseus.*

CONCLUSION

From the present work it can be concluded that *Aspergillus fumigatus* could utilize natural cellulosic wastes as substrate (such as sunflower waste) for its growth and production of high levels of cellulase. The crude enzyme was purified first by ammonium sulfate precipitation and then by Bio-Gel P-100 chromatography. The temperature and pH optima of purified cellulase were 55° C and 6.0 respectively. $CoCl_2$ (148.4 U/mL) and $MnCl_2$ (139.6 U/mL) activated and $HgCl_2$ (21.6 U/mL) , $Fe_2(SO_4)_3$ (49.5 U/mL) , $FeSO_4$ (29.5 U/mL) and $CdCl_2$ (32.7 U/mL) deactivated the cellulase activity. K_m and V_{max} values for carboxymethyl cellulose were 3.32 mM 188.7 U/mL/min respectively. The molecular mass was about 33 kDa as determined by 12 % SDS-PAGE. The produced cellulase was endocellulase useful in industrial application.

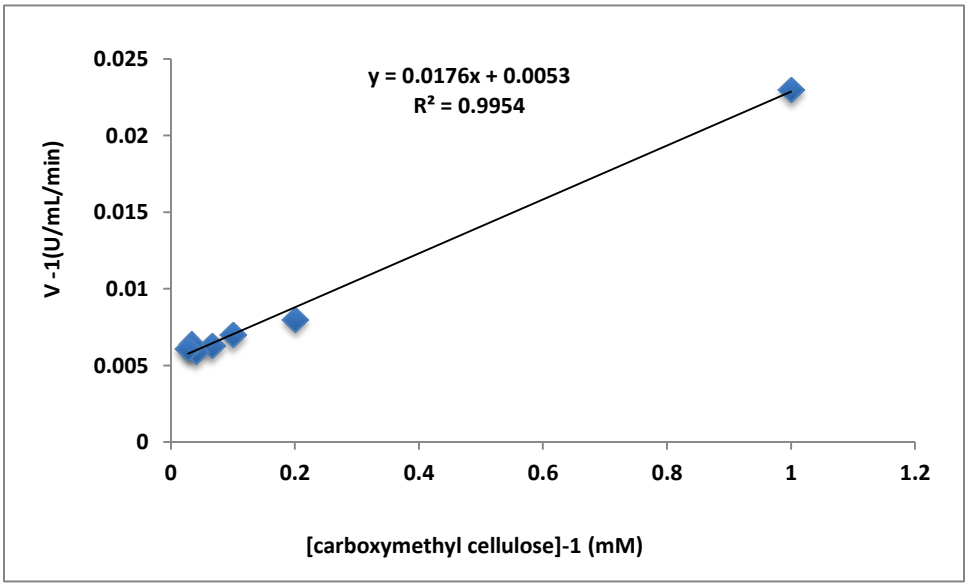

Fig.1. Line weaver-Burk Plot for the determination of K_m and V_{max}values of cellulase from *Aspergillus fumigatus.*

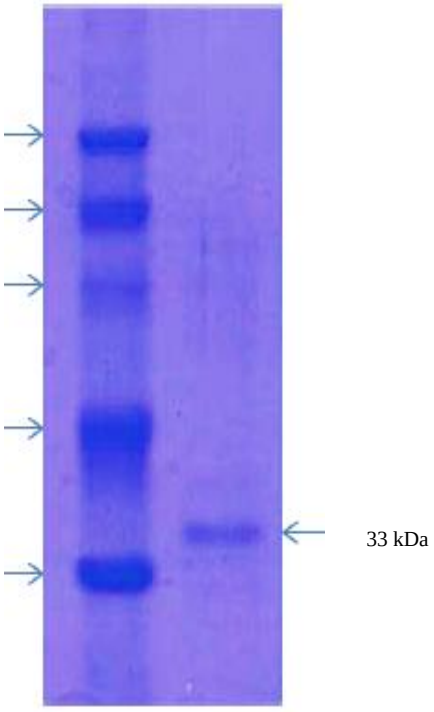

Fig.2. Molecular mass determination of cellulase from *A. fumigatus*, 12 % SDS-PAGE. First lane is the molecular weight marker [from bottom to top: Carbonic anhydrase (30 kDa), ovalbumin (45 kDa), albumin (66 kDa) and Phosphorylase B (97 kDa)] Second lane is purified enzyme.

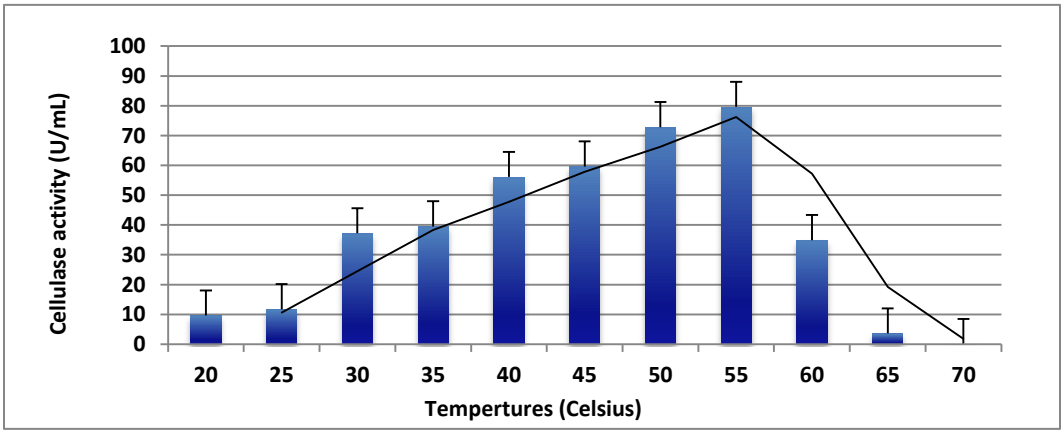

Fig.3. Effects of Temperature on the activity of purified cellulase from *A. fumigatus*.

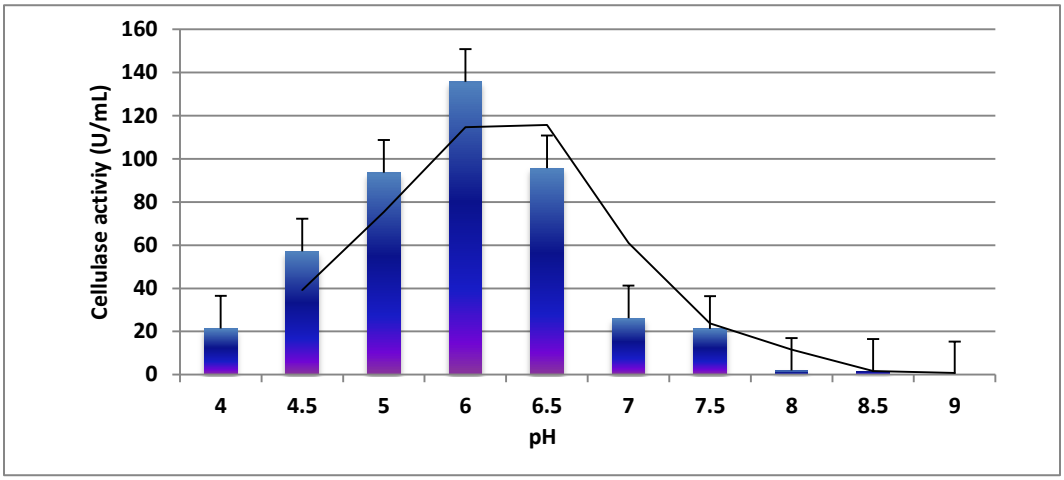

Fig.4. Effects of pH on the activity of purified cellulase from *A. fumigatus*.

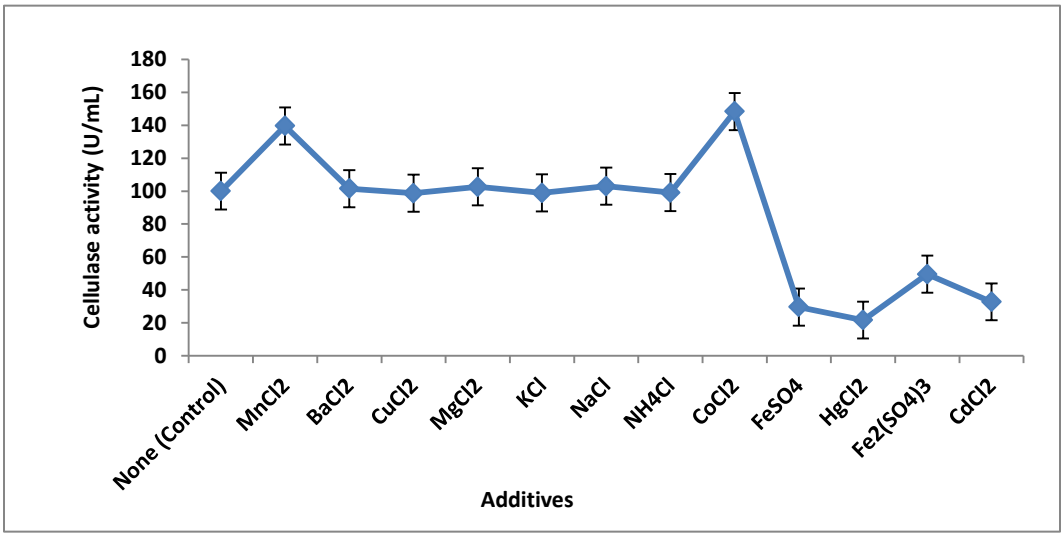

Fig. 5. Effects of additives on the activity of purified cellulase from *A. fumigatus*.

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