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Kinetic and thermodynamic behavior of partially purified cellobiase from *Humicola fuscoatra* MTCC 1409

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The study was undertaken with the objective to purify and characterize cellulases from thermophilic fungus *Humicola fuscoatra* in order to find out its potential to increase hydrolysis of lignocellulosic biomass especially paddy straw. Cellobiase, an important component of cellulase enzyme complex, was produced by solid-state fermentation on Mandel media under optimized conditions using rice straw as substrate and was partially purified by ammonium sulphate saturation followed by DEAE-cellulose chromatography. Two isoforms of cellobiase, C-I and C-II, which were most kinetically efficient at their optimum pH of 6.0 and temperature of 50°C were identified after purification,. These isoforms were thermostable at a temperature range of 30–70°C. Co^{2+}, Zn^{2+}, and Mn^{2+} activated whereas, EDTA and Hg^{2+} inhibited the activity of cellobiase. Na^{+}, Mg^{2+}, K^{+}, and Fe^{2+} did not influence the activity of isoforms. The molecular weight of isoforms C-I & C-II was 48 and 44 kDa, respectively. Activation energy (E_a) values for C-I and C-II isoforms were 7.65 and 9.57 KJ/mol and corresponding enthalpy change (ΔH) values were 16.27 and 11.49 KJ/mol, respectively. Change in entropy (ΔS) values at 50°C for C-I and C-II were 0.098 KJ/K/mol and 0.085 KJ/K/mol, respectively. The pK values of ionizing groups in free enzyme and enzyme-substrate complex were between 4.3 and 6.8, indicating the possible precipitation of carboxyl groups of aspartate and glutamate and imidazolium group of histidine in the cellobiase catalyzed the hydrolysis of cellobiose by both isoforms.

Keywords: Cellobiase, Culture conditions, *Humicola fuscoatra*, Isoforms, Purification

Agricultural biomass considered as the cheapest and renewable source for the production of industrially important products worldwide. Bioconversion of lignocellulosic biomass leads to the production of organic chemicals, which act as intermediates for the manufacture of various polymers, resins, and other chemicals. These residues, which are consisting of lignin, cellulose, and hemicelluloses, can be used as feedstock for the biorefinery, aromatic compounds (from lignin), and low molecular weight aliphatic compounds from ethanol produced from cellulose and hemicellulose. Three types of cellulases; namely, exoglucanase, endoglucanase, and cellobiase are required for complete enzymatic degradation of lignocellulosic waste. Endoglucanase (endo-1,4-glucano-hydrolase) creates free side chains by randomly cleaving the β-(1,4) glycosidic linkages of cellulose fiber at regions of low crystallinity, whereas, exoglucanase or cellobiohydrolase (1,4-glucan cellobiohydrolase) further removes cellobiose units from the free chain ends created by endoglucanase. Cellobiase hydrolyses cellobiose to produce glucose units. This enzymatic hydrolysis of cellulosic waste is considered as a most promising approach since very high temperature and pressure are not required in this process and it prevents the accumulation of unwanted by-products. To make the process economically viable, it is essential to produce cellulases from cheap and easily available sources. Microbial cellulases have shown their potential application in various industries including pulp and paper, textile, laundry, biofuel production, food and feed industry, brewing, and agriculture[1]

Bacteria and fungi are the most common microbial sources for production of cellulolytic enzymes[2]. However, fungi are better agents of decomposition of organic mass of agricultural residues[3] because they secrete free cellulase complexes having all the three components of cellulases such as endoglucanases, exoglucanases, and cellobiases. Cellobiase is an important component of cellulase enzyme complex

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Abbreviations: DEAE, diethylaninoethyl; DNS, 3,5 dinitrosalicylic acid

because it is essential for complete hydrolysis of cellulose to glucose units. Formation of sophorose and gentiobiose by this enzyme induces cellulase enzyme system[4]. It also regulates of the whole cellulolytic process, being a rate-limiting factor during enzymatic hydrolysis of cellulose, as cellobiase often inhibits both endoglucanase and exoglucanase activities[5]. Hence, high cellobiase activity is required for efficient enzymatic hydrolysis of cellulose. This enzyme has a key attention to the scientists because the most widely used fungus for hydrolysis of lignocellulosic biomass, *Trichoderma reesei*, lacks this particular enzyme. Mostly, the fungal sources are preferred over bacterial sources for the production of the β-glucosidases due to ease in the downstream process with high activity and their stability over a wide range of pH and temperature[6]. Since most industrial processes are carried out at high temperatures, there is a great interest in thermophilic enzymes. For understanding the mechanism of cellulose degradation by these enzymes, it is necessary to produce, purify and characterize these enzymes. However, so far the data on the production and purification of cellulases by thermophilic fungi are very scarce. Production of cellulases by thermophilic fungus *Humicola fuscoatra* MTCC 1409 has been optimized[7,8] and potential of the produced enzyme had been evaluated for degradation of paddy straw[7]. Since the activity of cellobiase was found to be highest among all three type of cellulases[8], the focus of the current study was to purify and characterize the kinetic and thermodynamic behaviour of the cellobiase [EC 3.2.1.21] from *Humicola fuscoatra* MTCC 1409.

Materials and Methods

Procurement and maintenance of culture

The culture of *Humicola fuscoatra* MTCC 1409 was procured from the Institute of Microbial Technology, Chandigarh, India. The culture was maintained on potato dextrose agar (PDA) slants at 45±2°C by monthly transfers.

Production of enzyme cellobiase

Mandel media was used for the production of the enzyme from *Humicola fuscoatra*[9]. Paddy straw (3 g) was added as a substrate in 12 mL of Mandel media for enzyme production and the medium was autoclaved at pressure 15 psi for 20 min after adjusting the pH to 6.0. Flasks in triplicates were cooled to room temperature and were inoculated with 1 mL inoculums containing 10^7 spores/mL and were incubated at 45±2°C for 6 days. After incubation, the enzyme was extracted with 30 mL of acetate buffer and was centrifuged at 10 000 rpm for 20 min at temperature 4°C. The supernatant was used as a source of crude enzyme.

Assay of enzyme cellobiase

For measuring cellobiase activity, 0.5 mL of enzyme extract was mixed with 0.5 mL of 10 mM cellobiose (substrate) solution and the mixture was incubated at 50°C for 10 min in a water bath. After incubation, 3 mL of DNS reagent was added. Then the mixture was placed in a boiling water bath for 15 min followed by addition of 1 mL of 40% sodium potassium tartrate. The contents were cooled at room temperature and 2 mL distilled water was added. The absorbance was recorded at 575 nm in a UV-VIS spectrophotometer[10]. The corresponding enzyme activity was calculated from the standard curve prepared simultaneously using glucose as a standard (20–100 µg/mL). One unit (U) of cellobiase activity is defined as the amount of enzyme, which release 1 µmole of reducing sugar in one min per gram paddy straw. The specific activity of the enzyme was measure by dividing activity/mg protein.

Protein assay

The method of Lowry *et al.*[11] was used for estimation of protein concentration of enzyme extracts. The standard curve was prepared simultaneously using bovine serum albumin as a standard (20–100 µg/mL) and was used to calculate protein concentration in enzyme extracts.

Enzyme Purification

The enzyme was partially purified using $(NH_4)_2SO_4$ precipitation, dialysis and Ion exchange chromatography. Crude enzyme preparation produced under optimized conditions was saturated with 0-30% and 30-90 % ammonium sulphate and was centrifuged at 10 000 × g for 15 min. The precipitates were dissolved in 0.1 M acetate buffer (pH 5.4), dialysed against the excess of diluted (5X) acetate buffer for 48 h at 4°C. The activity of cellobiase was tested in the protein dialysate and was further purified by DEAE-Cellulose column chromatography. DEAE-cellulose column was equilibrated with 0.1 M acetate buffer (pH 6.0) and the protein dialysate was loaded in the column. The enzyme was eluted with the same buffer using a stepwise gradient (0.1 M each) of increasing molarity of NaCl from 0.1 M to 1.0 M. The flow rate

was maintained at 1 mL per min. The 30 mL buffer of each molarity was used for elution. Fractions (5 mL) of each molar solution were collected and were analysed for cellobiase activity and protein content. The fractions containing the sufficient high activity of cellobiase were separately pooled for their further characterization. After each step of purification, yield, specific activity, and purification fold were calculated. Yield % was calculated by dividing units at each purification step with total activity (units) in the homogenate at each purification step multiplied by 100. Purification fold was obtained by dividing the specific activity of the enzyme at every purification step by the specific activity of crude enzyme extract.

Electrophoretic analyses

For determining the purity of the enzyme and its molecular mass native gel electrophoresis was done by to the method of Laemmli[12]. Samples were loaded in sample buffer containing 0.5 M Tris–HCl (pH 6.8), sucrose, 2-mercaptoethanol, and bromophenol blue. After loading the samples, the electrodes were connected to DC power pack and the current was adjusted to 1.5 mA per cm. Electrophoresis was continued until the bromophenol blue (dye) reached 1 cm from the lower end of the gel. The standard protein ladder with molecular range 14–95 kD was run along with the sample. Urease (95.0 kDa) bovine serum albumin (66 kDa), ovalbumin (43 kDa), L-lactate dehydrogenase (35 kDa), c chymotrypsinogen (25 kD), trypsin inhibitor (20kD and lysozyme (14 kDa),) were used as markers. After the termination of electrophoresis, the gel was stained with a solution of Coomassie brilliant blue R-250 for 4–5 h and de-stained with many changes of a de-staining solution containing methanol, glacial acetic acid, and water.

Characterization of enzyme cellobiase

Effect of pH and temperature on enzyme activity

Effect of pH on cellobiase activity was measured by varying the pH from 3.0 to 7.0. For the pH range, 4.0–5.0, sodium acetate buffer was used; whereas, sodium phosphate buffer was used for pH range 6.0–7.0. For ascertaining the effect of temperature on enzyme activity, incubation of reaction mixture was done at a temperature range from 20 to 70°C and enzyme activity was measured as described. The optimum pH and optimum temperature were determined by plotting percent of maximum activity versus pH and temperature, respectively.

Thermostability of enzyme

For determining the thermostability of enzyme, the enzyme was incubated for 1 h at different temperatures ranging from 0–70°C prior to the reaction of the enzyme with cellobiose and enzyme activity was measured at optimum temperature and pH.

Effect of metals and non-metals on enzyme activity

For ascertaining the effects of metals and non-metal ions on the activity of enzyme in each pooled fraction, 0.1 mL of enzyme, 0.1 mL of metal salt solutions (10 mM), 0.1 mL substrate, and 0.7 mL acetate buffer (pH 6.0) was added to make the reaction mixture (1 mM). Similarly, the second set of 5 mM concentration was made by adding 0.5 mL of metal salt solutions and 0.3 mL of acetate buffer to the 0.1 mL enzyme and 0.1 mL substrate. The activity of enzyme cellobiase was assayed as to ascertain the percent inhibition and stimulation of enzyme activity with different chemicals like EDTA, NaCl, $MgCl_2$, $CaCl_2$, KCl, $CoCl_2$, $FeCl_3$, $HgCl_2$, $MnSO_4$, and $ZnSO_4$.

Kinetic properties of enzyme cellobiase

Determination of K_m and V_{max} at different temperatures and pH

Enzyme activity was measured in reaction mixture using varying concentrations of cellobiose (1–20 mM) at (i) optimum temperature and varying pH from 4.0 to 7.0 (ii) at optimum pH and varying temperature from 40 to 60 °C. Michaelis constant Km (substrate concentration at a velocity of the reaction was 1/2 V_{max}) and V_{max} were determined at different pH and temperatures by the double reciprocal plot of Lineweaver and Burk[13].

Determination of Activation energy (E_a)

The activation energy was determined for temperature range 30–50°C and was calculated from the slope of linear plot of the log of the enzyme activity V_{max} *vs* 1/T according to the Arrhenius law

$E_a = -2.303\ R \times$ slope (from log V_{max} *vs* 1/T graph)

where, T is an absolute temperature in Kelvin (K), and R is the gas constant (8.314 $JK^{-1}mol^{-1}$). The enzyme activity (V) was expressed in units mg $protein^{-1}$, and the activation energy in KJ mol^{-1}.

Change in enthalpy (ΔH), change in entropy (ΔS), and change in free energy (ΔG)

The enthalpy, entropy, and free energy changes were calculated as $\Delta H = -2.303\ R \times$ slope (from pK_m *vs* 1/T graph), $\Delta G = R\ T\ 2.303 \times \log Km$ ($K_m = 1/K_{eq}$), and $\Delta S = (\Delta H - \Delta G)/T$, respectively. All the values

reported in this paper were the mean of three replicates.

Results and Discussion

Purification of cellobiase

The crude enzyme was extracted using optimized conditions[8] and was initially analyzed for cellobiase activity. The activity of cellobiase in crude enzyme extract was 314.39 U with the specific activity of 0.676 U/mg proteins (Table 1). The enzyme was further purified using ammonium sulphate precipitation and DEAE cellulose column chromatography. The two steps purification process resulted in improvement in specific activity of cellobiase. After dialysis step, purification achieved was 1.54 and 4.33-fold for 0–30% and 30–90% ammonium sulphate precipitate dialysate, respectively as the specific activity of enzyme increased from 0.676 U/mg in the crude enzyme to 1.04 and 2.93 U/mg protein for 0–30% and 30–90% precipitate dialysate, respectively (Table 1). Precipitate dialysate of 30–90% saturation was then loaded to DEAE-cellulose column for further purification, After anion exchange chromatography on DEAE-cellulose column, two different cellobiase isoforms named C-I and C-II were identified with the purification folds of 18.08 and 12.11, respectively (Table 1). These isoforms C-I and C-II were eluted at 0.4–0.5 M and 0.7–0.8 M of NaCl concentrations, respectively. Elution pattern of the precipitate of ammonium sulphate (30–90%) in DEAE-cellulose chromatography had been shown in Fig. 1. Dariot *et al.*[14] purified cellobiase with 13-fold purification from *Monascus purpureus* using gel filtration chromatography technique, whereas, Ahmed *et al.* [15] purified cellulases from *Trichoderma harzianum* by ammonium sulphate precipitation, gel filtration chromatography on Sephadex G-200 and Sephadex G-50 column with the purification fold of 1.74. Cellobiase from *Paecilomyces thermophila* had also been purified by gel filtration chromatography with 105-fold purification with the final recovery of 21.7%[16]. Chauve *et al.*[17] purified β-glucosidase enzyme from two fungal species with purity yield of 95% and purification fold of 53 using anion exchange chromatography. The molecular mass of crude and purified isoforms of cellobiase was determined using native-PAGE. The purified cellobiase isoforms resulted in single protein band with a mobility corresponding to molecular mass about 48 kDa and 44 kDa for C-I and C-II, respectively (Fig. 2). The

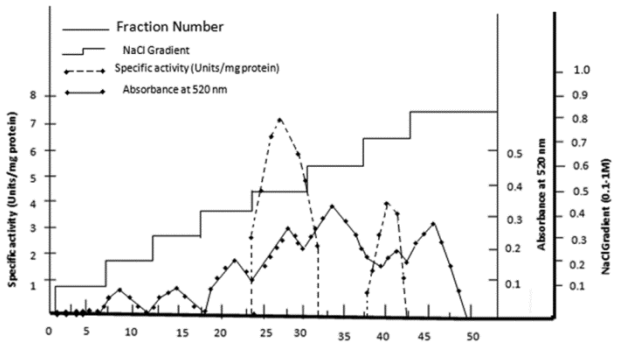

Fig 1 — Elution pattern of the precipitate of ammonium sulphate (30–90%) in DEAE-cellulose chromatography

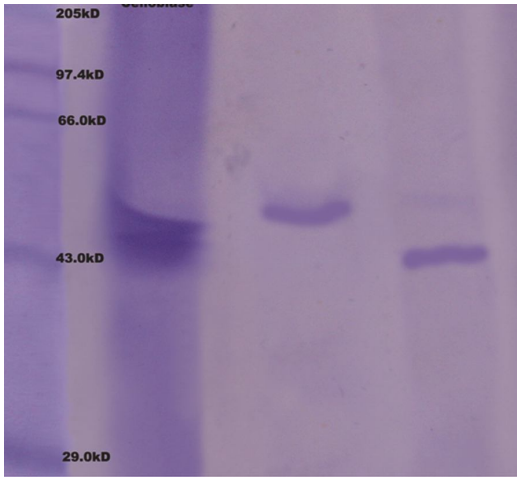

Fig. 2 — Native–PAGE of crude and purified cellobiase

Table 1 — Purification profile of cellobiase

Purification Step	Total Enzyme Activity (Units)	Protein (mg)	Specific Activity (Units/mg protein)	Purification fold	Yield (%)
Crude enzyme	314.39	465.27	0.676	1	100
(0-30%) $(NH_4)_2SO_4$ precipitated enzyme	71.36	68.35	1.04	1.54	22.7
(30-90%) $(NH_4)_2SO_4$ precipitated enzyme	206.56	70.53	2.93	4.33	65.7
Anion Exchange Chromatography					
C-I	57.54	4.71	12.22	18.08	18.3
C-II	14.0	1.71	8.19	12.11	3.33

results regarding the molecular mass of the enzyme were close to the findings of Saha and Bothast[18] who purified β-glucosidase from *Candida peltata* with a molecular mass of 43 kDa. Takashima *et al.*[19] purified six β-glucosidases, two endoglucanases, and an exoglucanase from a thermophilic fungus *Humicola grisea* and reported that molecular mass of the purified enzymes estimated by SDS-PAGE was from 38500 to 115000 Da. However, the molecular mass of 92 kDa of β-glucosidase from *Melanocarpus* sp.[20] and about 126.0 kDa of purified β-glucosidase from a fungus *Penicillium simplicissimum*[21] had been reported.

Effect of pH and temperature on the activity of cellobiase isoforms

pH activity profile of two isoforms of cellobiase was obtained in the pH 3.0–7.0 (Fig. 3A). The specific activity of both isoforms increased with increase in pH up to 6.0 and then decrease with increase in pH. The specific activity of isoforms C-I and C-II was 73.1% and 60.5% at pH 3.0, 78.4% and 71.9% at pH 4.0, 89.6% and 85.2% at pH 5.0, respectively with respect to maximum specific activity (100%) at pH 6.0. With further increase in pH to 7.0, isoforms C-I and C-II showed the activity of 83.6% and 80.7% as compared to maximum activity. Hence, optimum pH of both isoforms was found to be 6.0. Similar results were obtained by Kaur *et al.*[20] who also observed maximum cellobiase activity at pH 6.0 at 50°C produced from *Melanocarpus sp.* Yin *et al.*[22] reported that cellulase produced from *Bacillus subtilis* YJ1 exhibited the highest activity at pH 6.0–6.5 and was stable between pH 6.5 and 7.5. Optimum pH of β-glucosidase/cellobiase isolated from *Penicillium simplicissimum* H-11 was found to be about from 4.4–5.2[21]. Acidic pH was found to be favorable for the activity of cellobiase isolated from *Monasus sanguineus*[23].

Temperature activity profile of two isoforms of cellobiase was obtained at a temperature range of 20°C to 70°C (Fig. 3B). Catalytic activities, in temperature range, are evaluated and expressed as the percentage of maximum activity. The rate of enzyme catalysed reaction increased with increase in temperature up to 50°C and then declined thereafter, might be to denaturation of the enzyme. The optimum temperature for both isoforms was found to be 50°C. At 40°C, C-I retained 84.0% and C-II retained 82.1% of their maximum specific activity. Above 50°C specific activity started decreasing. At 60°C, C-I retained 76.6% and C-II retained 86.9% of their maximum specific activity. Ma *et al.*[24] reported that β-glucosidase/cellobiase activity gradually increased 40−60°C, but a sharp decrease was found at 65°C, with an activity of only 60% of the maximal value and 15% at 70°C when produced from *Aspergillus glaucus*. However, in our results, cellobiase produced from *Humicola fuscoatra* showed no sharp decline in its activity beyond optimum temperature. At 70°C, the isoforms C-I and C-II, showed 70.3 and 70.5%, respectively of the specific activity with respect to the maximum activity. The activity of enzyme at higher temperature range is an advantageous factor for saccharification of biomass and can also prevent contamination[23]. Maximum activity of β-glucosidase/cellobiase at 60°C had been reported from *Penicillium simplicissimum* H-11[21] and from *Melanocarpus sp.*[20].

Thermostability of enzyme

Both isoforms were pre-incubated prior to reaction for 1 h at temperature 0–70°C to study the thermostability of both isoforms. Maximum enzyme activity was found when the enzyme was pre-incubated at 50°C. Increase or decrease in pre-incubation temperature from 50°C resulted in a small loss of activity of enzyme. At 0–20°C pre-incubation temperature, 25–55% reduction in activity of cellobiase was observed in both the

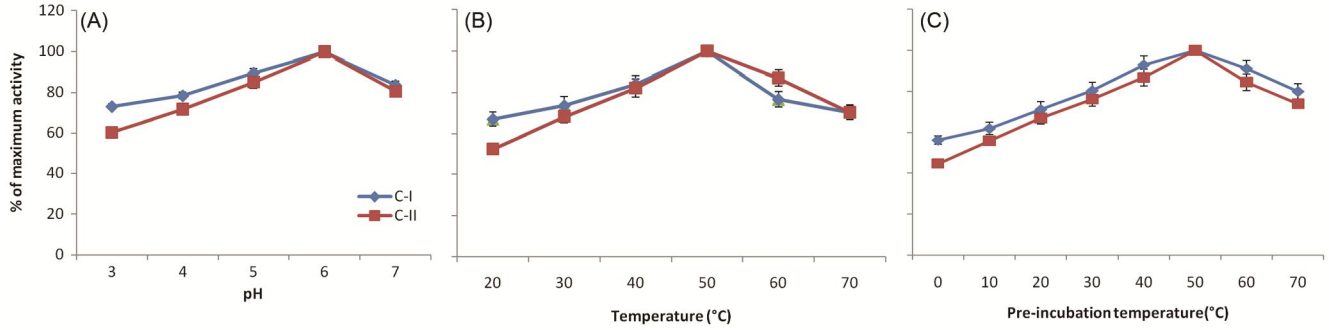

Fig. 3 — Effect of pH (A), temperature (B), and pre-incubation temperature (C) on the activity of cellobiase isoforms

isoforms. At 30°C, enzyme lost 19.6% and 23.7 % of its activity for C-I and C-II, respectively. At 40°C and 60°C, loss in enzyme activity was almost negligible *i.e.* 7–9% for C-I, whereas, C-II isoform showed 13–16% reduction in its activity at this temperature. At 70°C cellobiase retained 80% and 74.1% of its activity for both isoforms C-I and C-II, respectively (Fig. 3C). Hence, cellobiase produced from *Humicola fuscoatra* was found to be stable within a wide range of temperatures. Cellobiase produced from *Melanocarpus sp*. was found to be stable at 50°C[20]. Mawadza *et al.* [25] reported that cellulases produced by *Bacillus* strains were stable up to 50°C above which a rapid decrease in stability was seen particularly after incubation for 1 h. However, Saha and Bothast[18] reported that cellobiase produced from *Candida peltata* was fairly stable at temperatures up to 45°C. β-glucosidase produced from *Penicillium simplicissimum* H-11 was also stable at temperature up to 50°C for 4 h of pre-incubation period[21].

Effect of metal ions and other reagents on the activity of cellobiase

Enhancement in the activity of both isoforms was observed with Ca^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+} while EDTA and Hg^{2+} caused the inhibition of enzyme activity at 1 and 5 mM concentration. Na^{+}, Mg^{2+}, K^{+}, and Fe^{2+} did not influence the activity of both isoforms. Addition of Co^{2+} caused the increase in activity of both isoforms C-I and C-II by 79.8 and 82.4% at 1 mM and 81.6 and 85.8% at 5 mM concentration, respectively. Mn^{2+} increased the activity by 80% and 79% at 1 mM concentration and 88 and 83.3% at 5mM concentration, respectively in both the isoforms. Ca^{2+} increased the activity by 5-15% ,whereas, Zn^{2+} increased the activity by 15-25% in both the isoforms. Some enzymes require metal ions apart from the requirement of co-enzymes to complete its activity. Metals are common inorganic modifiers. They can enhance or can decline the rate of reaction. EDTA caused 50% inhibition of the enzyme activity of both the isoforms, whereas, Hg^{2+} was observed as a stronger deactivator of the enzyme as it caused a loss in activity by 98–99% in both the isoforms. K^{+}, Mg^{2+}, and Fe^{2+} showed enhancement in activity by 1–8% in both the isoforms (Table 2). Our findings were mostly in accordance with the findings other studies. β-glucosidases of many other fungal species were also strongly activated by Zn^{2+} ion[4]. According to Saha[26] and Lucas *et al.* [27], Co^{2+} and Mn^{2+} also showed enhancement in the cellulase activity from *Mucor circinelloides* and *Chalara paradoxa*, respectively. An inhibitory effect of EDTA to the activities of cellulase from wild-type of *Pseudomonas fluorescens* had been reported[28]. Iqbal *et al.* [29] reported that EDTA and Hg^{2+} showed inhibitory effect on purified cellulase whereas addition of Co^{2+} and Mn^{2+} resulted in an increase in the activity. However, K^{+} and Na^{2+} did not affect the cellulase from *Rhizopus oryzae*[26,27,30]. Ma *et al.*[24] observed that Na^{2+}, K^{+}, and Mg^{2+} have no effect on β-glucosidase activity, however, Fe^{2+} caused the activation of the enzyme and EDTA abolished nearly half of the activity of β-glucosidase.

Kinetic and thermodynamic properties of cellobiase

Michaelis constant K_m and V_{max} were determined at different temperatures by the double reciprocal plot of Lineweaver and Burk[13]. For this, effect of different

Table 2 — Effect of metals and non-metals on the activity of cellobiase

Chemical	% of maximum activity C-I		% of maximum activity C-II	
	1mM	5mM	1mM	5mM
Control	100.0	100.0	100.0	100.0
EDTA	50.4± 0.23	49.7±0.38	50±0.58	48.8±0.69
Na^{+}	99.7±0.75	98.5±1.26	99.4±0.69	97.9±0.73
Mg^{2+}	102.1±1.32	102.7±1.64	101.5±1.50	102.0±0.87
Ca^{2+}	111.6±1.46	111.9±1.96	106.3±0.87	108.4±1.39
K^{+}	104.6±0.92	105.9±2.04	105±0.98	106.0±1.15
Co^{2+}	179.8±3.50	181.6±4.12	182.4±2.54	185.8±1.27
Fe^{2+}	105.2±1.94	107.3±1.78	104.9±1.21	107.0±1.04
Hg^{2+}	2.6±0.13	2.0±0.10	1.5±0.12	1.8±0.14
Zn^{2+}	117.7±2.06	120.9±0.98	119.0±1.73	122.5±1.44
Mn^{2+}	180.0±3.72	188.0±3.67	179.0±2.02	183.3±2.14

[Values are mean ± SE of three replications]

substrate concentrations (5–20 mM) on the activity of two isoforms of cellobiase was studied at different temperatures ranging from 30–50°C. The curve of the double reciprocal plot was used to calculate the values of kinetic constants K_m and V_{max}. The K_m value decreased from 5.0 mM at 30°C to 3.33mM at 50°C whereas V_{max} increased from 49.0 at 30°C to 60.6 at 50°C. A similar trend was observed for C-II (Table 3). Smaller K_m value is an indication of higher affinity towards the substrate. Both the isoforms were found to be kinetically most perfect at temperature 50°C amongst the all other temperatures as V_{max}/K_m values for both C-I and C-II isoforms at this temperature were highest *i.e.* 18.2 for isoform C-I and 14.6 for isoform C-II. C-I was found to be more kinetically perfect than C-II as V_{max}/K_m values at all the temperatures were higher in comparison to those of C-II.

The values of log V_{max} and pK_m (−log K_m) for both C-I and C-II isoforms at different temperature (30–50°C) were plotted against 1/T to calculate the Ea and ΔH. From the graphs (Fig. 4A–D), the linear relationship between log V_{max} and pK_m with 1/T were obtained for both isoforms. These linear plots suggested that V_{max} and K_m are simple constants rather than complex functions of many velocity constants. It may also be concluded from these results, that single-intermediate enzyme–substrate complex was the rate-limiting step.

The energy of activation E_a, calculated for C-I and C-II was 7.65 and 9.57 KJ/mol, respectively. Lower E_a of C-I than C-II, also explained the higher specific activity and more kinetic perfection of C-I as in comparison to C-II. The corresponding enthalpy change (ΔH) values for C-I and C-II calculated were found to be 16.27 and 11.49 KJ/mol, respectively. The entropy change (ΔS) values for C-I and C-II at 50°C were calculated to be 0.098 and 0.085 KJ/K/mol. The small entropy change suggested that both C-I and C-II had undergone the negligible conformational change during cellobiose–cellobiase complex formation. Free energy change (ΔG) values calculated for C-I and C-II at 50°C were −15.34 and −16.08 KJ/mol, respectively (Table 4). The almost negligible difference was found between changes in free energy ΔG values for C-I and C-II indicated that ΔG did not provide any information regarding the rate of reaction.

Effect of different substrate concentrations (5–20 mM) on the activity of two isoforms of

Table 4 — Variation in kinetic and thermodynamic properties for two isoforms of cellobiase

Thermodynamic parameters	C-I	C-II
Activation energy (E_a)(KJ/mol)	7.65	9.57
Enthalpy change (ΔH)(KJ/mol)	16.27	11.49
Entropy change (ΔS) at 50°C(KJ/K/mol)	0.098	0.085
Free energy change (ΔG)at 50°C(KJ/mol)	−15.34	−16.08

Table 3 — K_m, V_{max}, and V_{max}/K_m values of cellobiase at different temperature

Temperature (°C)	C-I			C-II		
	K_m (mM)	V_{max} (Units/mg protein)	V_{max}/K_m	K_m (mM)	V_{max} (Units/mg protein)	V_{max}/K_m
30	5.0±0.12	49.0±0.58	9.8	3.33±0.11	29.24±0.27	8.78
40	4.0±0.09	55.2±0.92	13.8	2.82±0.10	32.48±0.34	11.5
50	3.33±0.14	60.6±0.69	18.2	2.5±0.10	36.50±0.26	14.6

[Values are mean ± SE of three replications]

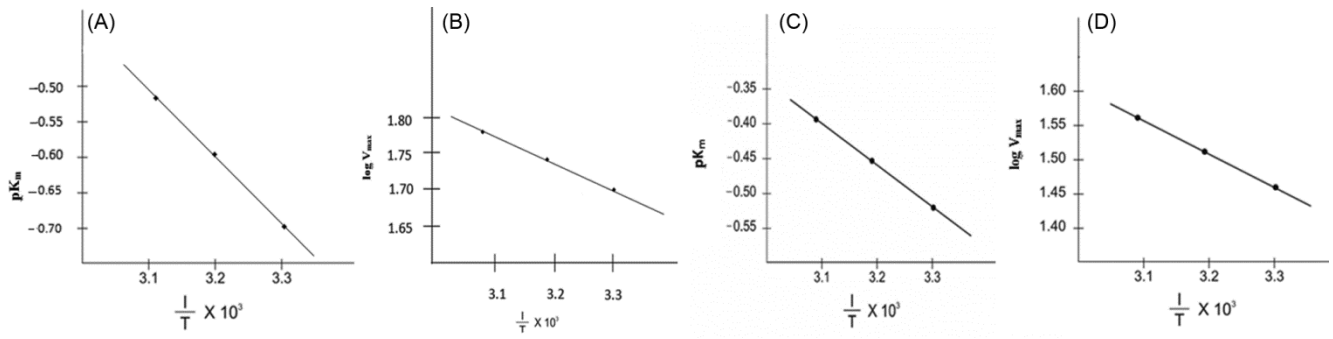

Fig. 4 — Relationship between pK_m and 1/T of C-I (A), Relationship between log V_{max} and 1/T of C-I (B), Relationship between pK_m and 1/T of C-II (C),and Relationship between log V_{max} and 1/T of C-II (D)

cellobiase was studied at different pH. From the reaction velocity (V) and substrate concentration (S), apparent Michaelis constant K_m and V_{max} were calculated by the double reciprocal plot of Lineweaver and Burk[13]. From Km and V_{max}, values of V_{max}/K_m were also calculated. The results are given in Table 5. K_m values for both the isoforms decreased with increase in pH up to 6.0 then increased with further increase in pH, whereas, the reverse trend was found in V_{max}. K_m values (mM) for C-I isoforms were 4.00, 3.78, 3.33, 2.61, 2.22, 2.33, and 2.80 and for C-II isoforms were 2.86, 2.61, 2.22, 1.82, 1.46, 2.00, and 2.31, respectively at pH 4.0–7.0 (with 0.5 unit interval). Lowest K_m values for isoforms C-I and C-II were obtained at pH 6.0, thus indicating that at this pH the enzyme was in its more appropriate ionic form and had a greater affinity for the substrate. V_{max} values (U/mg protein) at different pH ranging from (4-7) at 0.5 unit interval were 20.5, 26.9, 36.4, 42.5, 48.2, 41.9 & 35.4 for isoform C-I and 13.8, 17.3, 21.5, 24.9, 27.3, 20.4 & 11.8 for isoform C-II. Highest values of V_{max} and V_{max}/K_m were obtained at pH 6.0 for both isoforms indicating that at pH 6.0 both isoforms were having maximum kinetic efficiency. However C-I isoform was found to have higher V_{max}/K_m value at all the pH, indicating that C-I isoform had more kinetic perfection than C-II. According to Ekperigin[31], K_m values for *Acinetobacter antiratus* and *Branhamella* sp. were 0.32 and 2.54 mM for cellobiose as substrate. Similarly, K_m value of 3.6 mg/mL for *Pseudomonas fluorescens* and 1.1 mM for *Trichoderma reesei* were reported by Bakare *et al* [28] and Cascalheira and Queiroz[32], respectively. In literature, different ranges of K_m and V_{max} for different fungal species have been reported. Purified β-glucosidase from the culture supernatant of a fungus *Penicillium simplicissimum* had K_m and V_{max} of 14.881 mg/mL and 0.364 mg/mL/min against salicin as a substrate[21].

Mawadza *et al* [25] observed K_m of 1.5 and 1.7 mg/mL, respectively, and V_{max} of 0.93 and 1.70 mM glucose min^{-1} mg $protein^{-1}$, respectively for purified cellulases produced by two *Bacillus* strains, CH43 and HR68. In order to obtain a clearer picture of the effect of pH on K_m and V_{max}, the results were plotted according to the method of Dixon[33]. The values of log V_{max}/K_m and log V_{max} were plotted against pH (4–7) for both C-I and C-II. In these plots (Fig. 5A–D), the straight-line portions (with slope +1, 0, −1) of the curves intersect on extrapolation. The application of Dixon's rule[34] showed that pK values of ionizing group existing in free enzyme were 4.7 and 6.8 and that in the enzyme-substrate complex there were two ionizing groups with pK values 4.4 and 6.8 for C-I. Similarly, for C-II, the pK values for

Table 5 — K_m, V_{max}, and V_{max}/K_m values of cellobiase isoforms at different pH

pH	C-I			C-II		
	K_m (mM)	V_{max} (Units/mg protein)	V_{max}/K_m	K_m (mM)	V_{max}(Units/mg protein)	V_{max}/K_m
4	4.00±0.10	20.5±0.32	5.12	2.86±0.12	13.8±0.15	4.82
4.5	3.78±0.12	26.9±0.36	7.13	2.61±0.10	17.3±0.21	6.65
5	3.33±0.10	36.4±0.51	10.9	2.22±0.07	21.5±0.28	9.68
5.5	2.61±0.07	42.5±0.44	16.2	1.82±0.09	24.9±0.22	13.7
6	2.22±0.08	48.2±0.38	21.7	1.46±0.05	27.3±0.26	18.7
6.5	2.33±0.08	41.9±0.26	17.9	2.00±0.06	20.4±0.18	10.2
7	2.80±0.13	35.4±0.18	12.6	2.31±0.08	11.8±0.12	5.11

[Values are mean ± SE of three replications]

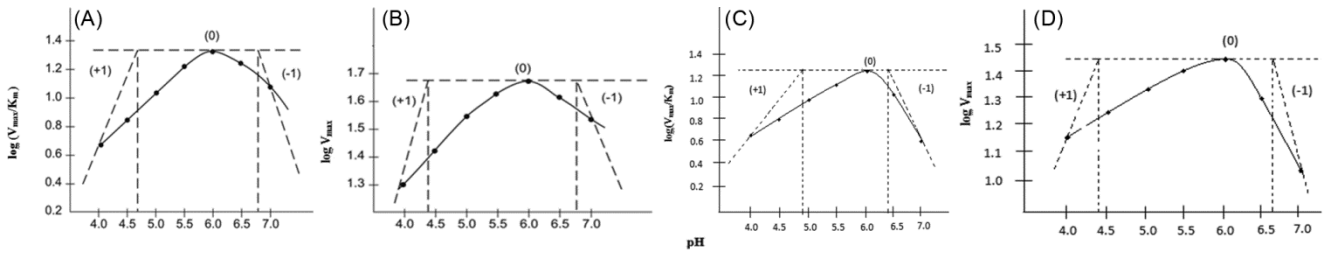

Fig 5 — Relationship between log (V_{max}/K_m) and pH of C-I (A), Relationship between log V_{max} and pH of C-I (B), Relationship between log (V_{max}/K_m) and pH of C-II (C), and Relationship between log V_{max} and pH of C-II (D)

ionizing groups existing in the free enzyme were 4.8 and 6.4 and that in the enzyme–substrate complex there were two ionizing groups with pK values 4.3 and 6.7. Comparison of the above pK values of ionizing groups with those listed by Dixon and Webb[34] indicated the possible participation of a carboxyl group of aspartate and glutamate (pK 3.0–5.0) and a histidine imidazolium group (pK 5.6–7.0) in the cellobiase catalyzed the hydrolysis of cellobiose by both C-I and C-II.

Conclusion

In the present study, cellobiase was produced and partially purified from *Humicola fuscoatra.* Kinetic and thermodynamic properties of cellobiase were also studied. Two different cellobiase isoforms named C-I and C-II were identified after purification of enzyme cellobiase. Both isoforms were most efficient kinetically at pH 6.0 and at temperature of 50°C and were also found to be thermostable within a temperature range of 30–70°C. Negligible difference in change in free energy (ΔG) values and very small entropy change for both isoforms suggested that ΔG did not provide any information regarding the rate of reaction and enzyme had undergone a negligible conformational change during cellobiose–cellobiase complex formation. The pKa values indicated the possible participation of carboxylate groups of aspartate/ glutamate and imidazolium group of histidine in *Humicola fuscoatra* catalysed the hydrolysis of cellobiose by both the isoforms of cellobiase. Cellobiase produced from *Humicola fuscoatra* can be used for efficient degradation of lignocellulosic biomass.

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