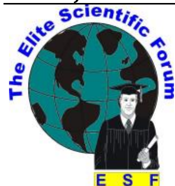

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RESEARCH ARTICLE

Kinetics of Cellobiohydrolase from a Phytopathogenic Fungus *Trichoderma harzianum*

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ABSTRACT
Cellulases from fungal sources have industrial importance. Kinetics of cellobiohydrolase from a fungus, *Trichoderma harzianum* was studied. The enzyme showed its maximum activity at pH 5 and temperature of 60°C, indicating its potential for use in different industrial applications. Lower value of Michealis-Menten constant (K_M = 2.8 mM) obtained from Line weaver Burk Plot was indicative of the affinity of enzymes for the substrate. The value of energy of activation (E_a) obtained from the Arrhenius plot was very small (5.9 $kJK^{-1}mol^{-1}$). This may be interpreted in terms of good relationship between enzyme and substrate. Enthalpy for the hydrolysis of the enzyme at optimum temperature was (2.2 $kJK^{-1}mol^{-1}$). Lower Q_{10} value (0.0044) shows very high catalytic activity on substrate concentration gave a good agreement between the theoretical and experimental values. The enzyme activity was inhibited at varying levels of the metal ions viz Cu^{2+}, Hg^{2+} & Fe^{2+} and activated by Ca^{2+} & Co^{2+}. The kinetics and thermodynamic properties of the enzyme demonstrate that it may have industrial applications.

INTRODUCTION

Trichoderma (*T.*) *harzianum* is a fungus which produces bioactive compounds against phytopathogenic fungi (de Souza et al., 2018; Peltola et al., 2004). The fungus also produces cellulases that are of industrial importance. The cellulase complex consists of three enzymes viz, Endo-1,4-β-D-glucanase or carboxymethylcellulase (CMCase) (E.C.3.2.1.4), Exo-1,4-β- D-glucanase or cellobiohydrolase (E.C.3.2.1.91) and β-glucosidase or cellobiosidase (E.C.3.2.2.1). These enzymes work synergistically to hydrolyze cellulose into glucose (Limon et al., 2004).
The cellulases degrade the cellulose, which is the much abundant and renewable entity in the biosphere obtained as a result of photosynthesis (Borisova et al., 2018; Gupta and Verma, 2015). Economical production of cellulases is a key for successful utilization of cellulosic material as renewable carbon source (Lopez-Ramirez et al., 2018; Mushtaq and Jamil, 2012). Massive amount of lignocelluloses stuff relinquished each year as scrap which is a worldwide distress for nature (Ghori et al., 2000). Cellulosic material such as cereal, grain residue, stalk, husk, bagasse, sawdust etc. are disposed as agricultural wastes or burnt which cause massive pollution in the environment (Karigar and Rao, 2011).
This study focused on the utilization of *T. harzianum* cellulases to evaluate different kinetics and thermodynamic parameters of a cellobiohydrolase from the fungus that could have industrial importance.

MATERIALS AND METHODS

Enzyme production

The *T. harzianum* was grown in enzyme production medium g/100 mL (avicel 4.0, dextrose 2.0, $CaCl_2.2H_2O$ 0.005, $MgSO_4.7H_2O$ 0.005, KH_2PO_4 0.15, urea 0.3) pH- 5.5 for 96 h at 120 rpm. The culture was filtered through Whatman filter paper No: 1 followed by centrifugation at 4000 rpm for 10 minutes. The spore free supernatant was assayed for cellobiohydrolase using avicel as substrate at pH 5.5, incubated at 60 °C. Free glucose produced by the enzyme was complexed with dinitrosalicylic acid and determined spectrophotometrically at 550 nm (Gadgil et al., 1995).

One unit of enzyme activity was defined as µmol of glucose equivalent released/mL of the enzyme extract. The culture was grown in growth medium at varying pH from (2-9) and temperature (10-70°C) and assayed for the cellobiohydrolase.

Kinetic studies

Determination of Maximal Velocity (V_{max}) and Michaelis-Menten Constant (K_M): Cellobiohydrolase produced by the fungus was assayed in 0.5 M acetate buffer (pH 5.0) with variable amount (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 ml) of 1% avicel as substrate. The data obtained were plotted according to Line weaver-Burk Plot.

Optimum temperature: Enzyme solution (1mg/mL) in 0.5 M acetate buffer was assayed at various temperatures (10- 70° C) with 10° C internal for the enzyme activity as described previously (Rajoka et al., 1992; Rajoka and Malik, 1986).

Activation energy: Activation energy (Ea) of the enzyme was determined by using the data for optimum temperature as under: $E_a = -Slope \times R$ where R= Gas constant, Slope=-Ea/R

Q_{10}: The value of activation energy was used to calculate the rise in reaction rate for every 10°C increase in temperature as follows:

$$Q_{10} = \frac{E_a}{K}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)$$

RESULTS AND DISCUSSION

Enzyme production

Phytopathogenic fungus *T. harzianum* was grown using avicel as a carbon source at different time intervals and fermentation modes (data not shown) to optimize the conditions for maximal production of the cellobiohydrolase. Maximum enzyme activity was achieved after 96 hours of fermentation with continuous shaking method. Optimal pH and temperature of the growth medium for maximal enzyme production were found to be 5 and 30 °C, respectively (Fig. 1 and 2).

Kinetics of the cellobiohydrolase

The cellobiohydrolase produced from the fungus was subjected to kinetics study.

Enzyme-substrate interaction

The dependence of the reaction on concentration of the enzyme substrate was studied using Line weaver-Burk transformation of Michaelis-Menten equation in the form of Line weaver Burk Plot,$\left(\frac{1}{v}\ vs\ \frac{1}{(s)}\right)$. The V_{max} and K_M values obtained were 1.32 IU/mL/min and 2.8 mM, respectively (Fig. 3). The results indicated small K_M values for cellobiohydrolase, which demonstrated high affinity of the enzyme with the substrate.

Similar to our studies, Ghori et al. (2000) observed the effect of substrate level on the cellulase activity of another fungus, *Aspergillus niger*, with optimum substrate level at 4% CMC. However, the activity may further be increased by addition of a cellulose binding domain to the protein in *T. harzianum*.

Optimal temperature of the enzyme

Optimal temperature of the cellobiohydrolase was determined by studying the enzyme activity after incubation of the enzyme extract at varying temperatures. The optimal temperature of the enzyme was found 60°C (Fig. 4) which indicated that the enzyme might have industrial potential for the catalytic processes.

Energy of activation and enthalpy of activation

Energy of activation of cellobiohydrolase from *T. harzianum* was found to be 5.907 kJ K^{-1} mol^{-1} as calculated from Arrhenius plot (Atkins, 1995). Slope for the enzyme is shown in Fig. 5. It was observed that at 60°C, cellobiohydrolase had maximum catalytic activity in the conversion of avicel into glucose. At a temperature higher than 60°C, the enzyme becomes prone to denaturation, therefore showed lower activity. The small amount of activation energy indicates a good relationship between enzyme and the substrate.

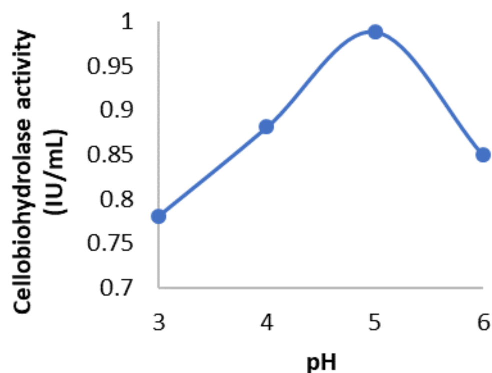

Fig. 1: Effect of *Trichoderma harzianum* culture pH on cellobiohydrolase activity

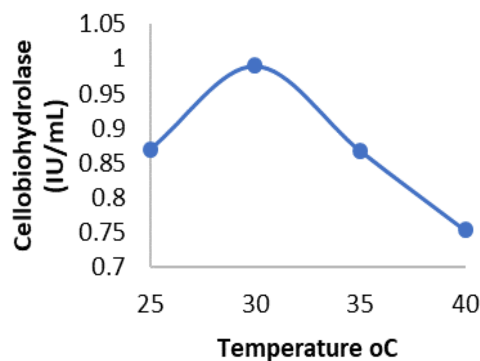

Fig. 2: Effect of *Trichoderma harzianum* culture temperature on cellobiohydrolase activity

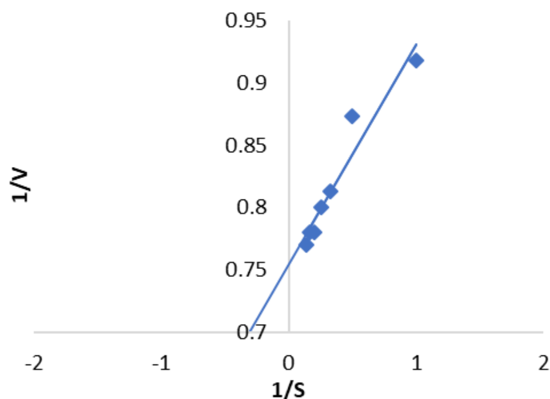

Fig. 3: Line weaver-Burk plot for cellobiohydrolase activity from *Trichoderma harzianum.*

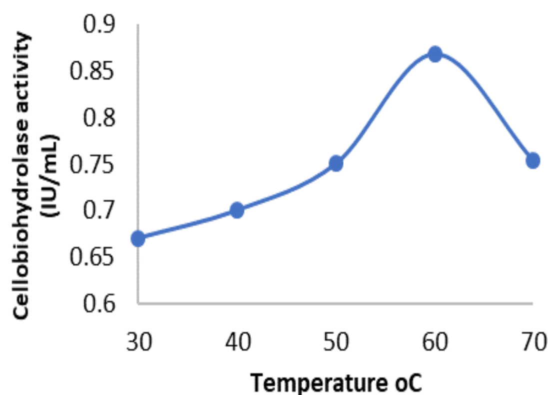

Fig. 4: Optimal temperature of cellobiohydrolase from *Trichoderma harzianum.*

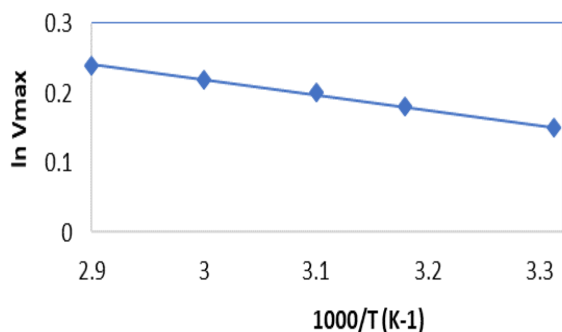

Fig. 5: Arrhenius plot for activation energy of cellobiohydrolase catalyzed reaction.

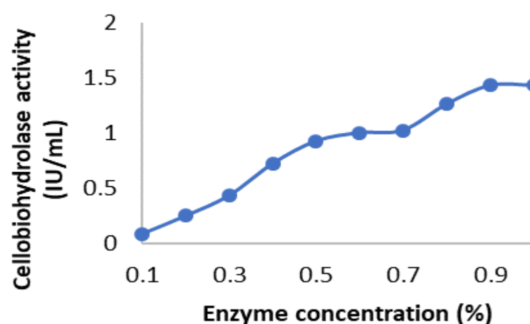

Fig. 6: Effect of enzyme concentration on cellobiohydrolase activity.

Enthalpy of activation was found to be 2.2 $kJK^{-1}mol^{-1}$ for cellobiohydrolase which demonstrates that kinetically the fungus *T.harzianum* was favorably good for conversion of cellulose into glucose.

Increase in reaction rates per 10°C rise in temperature (Q_{10})

Increase in reaction rate for every 10°C rise in temperature was calculated for cellobiohydrolase with the help of activation energy. The Q_{10} value obtained for cellobiohydrolase was 0.0044. Lower Q_{10} values demonstrated high catalysis in that the Q_{10} of a catalyzed reaction was lower as compared to the same uncatalyzed reaction (Segel, 1975). The Q_{10} value of another cellulase from *T. harzianum* and carboxymethylcellulase, was 1.7 that was higher as compared to our enzyme.

Effect of enzyme concentration

Ten levels of enzyme concentration were studied to find the effect of enzyme concentration on the cellobiohydrolase activity. The activity was enhanced linearly up to 0.9% of the enzyme concentration after which it became constant (Fig. 6). It indicated that all the available substrate had been converted into product with 9% of the enzyme extract (Murtaza et al., 2003).

Effect of metal ions

Effect of varying concentrations of metal ions viz Ca^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+} and Co^{2+} was investigated on cellobiohydrolase activity at pH 5 and temperature 60°C (Table 1).

Ca^{2+} worked as activator of the enzyme and enhanced the activity up to 1.0 mM concentration whereas the increase in activity was reduced to some extent at 1.5 mM. Similar trend was found earlier where Ca^{2+}activated a cellulase at lower concentration and had an inhibitory activity at higher concentration (Murtaza et al., 2003). Cu^{2+}at 0.5 mM lowered the enzyme activity to 27.7%, whereas the activity was further decreased to 7.6% at 1.0 mM concentration. Further increase in the Cu^{2+}concentration to 1.5 mM decreased the activity in a similar fashion. Therefore, Cu^{2+}served as inhibitor of the cellobiohydrolase activity. Similar trend was observed by Okoshi et al. (1990) in cellulase enzymes from a fungus. Co^{2+} enhanced the cellobiohydrolase activity to a significant level up to 1 mM concentration. However, adverse effect on the enzyme activity was observed with further increase in the metal ion concentration to 1.5 mM, and activity was decreased to 13.6%. Nawaz et al. (2006) also reported

Table 1: Effect of varying concentrations of metal ions on cellobiohydrolase activity

Substrate (mL)	Metal ion (mM)	Enzyme activity (IU/mL)				
		Ca^{2+}	Co^{2+}	Cu^{2+}	Fe^{2+}	Hg^{2+}
0.5		1.2	1.2	0.9	0.6	0.4
1.0	0.00	1.5	1.02	1.3	1.1	0.9
1.5		1.6	1.01	1.4	1.2	1.4
0.5		1.4	1.4	0.65	0.55	0.26
1.0	0.5	1.8	2.0	0.8	0.94	0.87
1.5		2.0	2.0	1.1	1.1	0.82
0.5		1.9	1.8	0.6	0.48	0.2
1.0	1.0	3.0	2.7	0.78	0.9	0.78
1.5		2.3	2.2	0.92	0.91	0.71
0.5		1.6	1.3	0.59	0.42	0.19
1.0	1.5	2.5	1.9	0.67	0.87	0.49
1.5		1.9	1.9	0.8	0.86	0.6

Co^{2+} as activator of cellobiohydrolase activity from a different fungus. A decrease in the enzyme activity was found with Fe^{2+}, the activity was decreased to 8.33, 12.7 and 12.5 percent at 0.5 mL volume of the substrate with 0.5 mM, 1.0 mM and 1.5 mM Fe^{2+} concentration respectively. Lin and Stutzenberger (1995) also demonstrated that Fe^{2+} acted as inhibitor of the cellulase enzymes with increase in the metal ion concentration. Hg^{2+} was also found to inhibit the cellobiohydrolase activity at varying concentration of the metal ion. Highest decrease in the enzyme activity was observed at 1.5 mM concentration. Cellobiohydrolase activity has been reported to be inhibited by Hg^{2+} that might be due to interaction of the metal ions with proteins within a cell (Sharma et al., 1990).

In conclusion, *T. harzianum* produced cellobiohydrolase to a significant level. The enzyme showed its maximal activity at pH 5 and temperature 60 °C. The enzyme activity was inhibited at varying levels of the metal ions viz Cu^{2+}, Hg^{2+} & Fe^{2+} and activated by Ca^{2+} & Co^{2+}. Kinetic analysis revealed that the enzyme followed first order kinetics. Lower K_M value of the enzyme demonstrated high affinity to the respective substrate, hence the enzyme may be of industrial application.

Authors' contributions

KYB performed the research and data analysis. KBS conducted a part of research. SRG planned the research. MIG performed the data analysis. All authors read and approved the final draft of manuscript.

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