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Full Length Research Paper

Kinetics of exoglucanase and endoglucanase produced by *Aspergillus niger* NRRL 567

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In this study we reported for the first time kinetics of exoglucanase (EXG) and endoglucanase (EG) from *Aspergillus niger* NRRL 567. The optimum pH and temperature for crude EXG and EG was found to be 3.5 and 30°C respectively. Zn^{2+}, Ca^{2+}, Mn^{2+} and Co^{2+} enhanced the crude activity of EXG and EG whereas Mg^{2+}, Fe^{2+} and Hg^{2+} showed various degree of inhibitory effects. Cu^{2+} enhanced crude EXG activity and inhibited crude EG activity. The energy of activation (Ea) for the EXG and EG were 21.20 and 22.52 kJ mol^{-1}, respectively. The Q_{10} values obtained for the EXG and EG were 1.38 and 1.4, respectively. These enzymes had lower K_m value that shows their high affinity for the substrates. Overall, the studies demonstrate that these enzymes may be suitable for industrial use.

Key words: Exoglucanase, endoglucanase, kinetics, characterization, *Aspergillus niger* NRRL 567.

INTRODUCTION

Cellulosic biomass is the largest amount of waste produced by human activities and the most attractive substrate for 'biorefinery strategies' to produce high-value products (example fuels, bioplastics or enzymes) through fermentation processes (Mazzoli et al., 2012). These cellulosic waste products can be used by fermentation for the production of useful products. Various alternatives are exercised to diminish this waste by elimination, purification and recycling (Ahmed et al., 2010; Athar et al., 2009). Cellulose is the most abundant organic polymer on this planet and is an important renewable energy source along with sugars and starches (Ahmed et al., 2009b). Cellulose degradation and its subsequent utilizations are important for global carbon sources. The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest (Ahmed et al., 2009a).

Cellulases and hemicellulases are two important classes of enzymes produced by microorganisms including filamentous fungi and secreted into the cultivation medium. Cellulase which can hydrolyze cellulose forming glucose and other commodity chemicals can be divided into three types: Endoglucanase (endo-1, 4-β-D-glucanase, EG, EC 3.2.1.4); exoglucanase (also called as cellobiohydrolase) (exo-1, 4-β-D-glucanase, CBH, EC 3.2.1.91) and β-glucosidase (1,4- β -D-glucosidase, BG, EC 3.2.1.21) (Ahmed et al., 2009 b).

Cellulases are important enzymes in many proposed processes for producing fuels and chemicals from plant biomass (Wilson, 2012). They also play a key role in increasing the yield of the fruit juices, oil extraction and in improving the nutritive quality of bakery products and animal feed (Bhat, 2000). The application of cellulases to the hydrolysis of lignocellulosic materials (biomass) in order to further convert the released fermentable sugars into ethanol has increased because of their worldwide demand for renewable fuels (de Castro et al., 2010). Xylanases and cellulases together with pectinases account for 20% of the world enzyme market (Ahmed et al., 2007).

Filamentous fungi have been used for more than 50 years for the production of industrial enzymes. *Trichoderma*, *Humicola* and *Aspergillus* species were

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Abbreviations: EXG, Exoglucanase; **EG,** endoglucanase; **Ea,** energy of activation.

shown to be interesting cellulase and xylanase producers (Ghori et al., 2011; Saadia et al., 2008; Irshad et al., 2008; Saleem et al., 2008). *Aspergillus* sp. is an important commercial source of cellulases for food, textile, and the pharmaceutical industries (Naika and Tiku, 2010). *A. niger* has been widely used for the production of cellulases, but limited information is available on the kinetics of EXG and EG from *A. niger* (Hanif et al., 2004).

For fermentation processes, use of intensive raw material and process yield (product produced/substrate consumed), in addition to productivity are critical measures of performance and economic viability (Sattar et al., 2008; Ali et al. 2009). In a search for new and efficient cellulases to be used in industry, we optimized various fermentation conditions for the production of cellulases from *A. niger* NRRL 567 in our previous study (Ghori et al., 2011). As a continuation of our previous study, here we reported some properties and kinetics of the crude exoglucanase (EXG) and endoglucanase (EG) from *A. niger* NRRL 567.

MATERIALS AND METHODS

Microorganism

A. niger NRRL 567 was taken out from our frozen glycerol stock stored at -80°C. *A. niger* was maintained on agar slants medium which consisted of (g/L); corn stover 20; $CaCl_2.2H_2O$ 0.05; $MgSO_4$, 0.05; KH_2PO_4, 1.5; Urea, 3; agar, 20 at 30°C (Ghori et al., 2011).

Media and culture conditions

The inoculum for *A. niger* NRRL 567 consisted of (g/L); corn stover 20; $CaCl_2.2H_2O$ 0.05; $MgSO_4$, 0.05; KH_2PO_4, 1.5; $(NH4)_2SO_4$, 2 at pH 3.5 and grown at 30°C on an orbital shaker working at 120 rpm. A 10 mL of liquid culture from the inoculum was transferred to 1000 mL Erlenmeyer flasks containing 250 mL fermentation medium under the same conditions as discussed above.

The strain was grown with corn stover (4% w/v), 0.1 % molasses and 1% yeast at pH 3.5 at 30°C. Biomass was harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. Resulting supernatant was tested for crude EXG and EG activity.

Enzyme assay

EXG activity was assayed in reaction mixture (1 mL) containing 1% (w/v) avicel, 0.05 M acetate buffer, pH 5.0 and appropriately diluted enzyme solution. After incubation at 60°C for 30 min, the reaction was stopped by adding 3 mL dinitrosalycilic acid reagent (DNS) (Shamala and Sereekanth, 1985). EG activity was assayed in reaction mixture (1 mL) containing 1% (w/v) carboxymethylcellulose (CMC), 1 mL of sodium acetate buffer, pH 5.0 and at 60°C. The reaction was stopped by adding 3 mL DNS reagent (Shamala and Sereekanth, 1985). One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 µmole of glucose or p-nitrophenol from the appropriate substrates under the standard conditions.

Effect of pH and temperature on crude exoglucanase and endoglucanase

Optimum pH of the crude EXG and EG activity was determined by measuring the enzyme activities in buffers at different pH (3, 3.5, 4, 4.5, 5). Optimum temperature of the crude EXG and EG activity was determined by measuring the enzyme activities at different temperatures (10 to 70°C, with 10°C interval).

Effect of metal ions on enzyme activities

The effect of several metal ions (Zn^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+} and Hg^{2+}) on the crude activity of EXG and EG was studied by adding each ion at a concentration of 1 m*M*. The enzyme solution was pre-incubated with cations in sodium acetate buffer at 4°C for 30 min and the activity was measured under the standard conditions.

Determination of kinetic parameters of crude exoglucanase and endoglucanase

EXG was assayed in 0.5*M* acetate buffer of pH 5.0 with variable concentrations of avicel as substrate. EG was assayed in 0.5*M* sodium acetate buffer, pH 5.0 with variable concentrations of carboxymethylcellulose (CMC). The data was plotted according to Lineweaver-Burk plot, Woolf-Augustinsson-Hofstee plot and Hanes-Woolf plot.

Activation energy (Ea), increase in reaction rate per 10°C rise in temperature (Q10) and heat of ionization (ΔH)

Activation energy of the EXG and EG was determined by using the data for optimum temperature as:

Ea = Slope x R

Where, R is the gas constant;

Slope = ΔH/R

The value of activation energy was also used to calculate the increase in reaction rate for every 10°C rise in temperature with the help of following formula (Atkins, 1994):

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

Where, K is the velocity constant (proportional to the rate of reaction).

Heat of ionization of cellulases was determined by checking enzyme activities at optimal pH at different temperatures (10, 15, 22, 28, 30, 35 and 40°C). ΔH was calculated as: ΔH = Slope x R x T.

RESULTS AND DISCUSSION

Cellulase production by *A. niger*

In our previous study, we found that maximum cellulase production from *A. niger* NRRL 567 was achieved with 4% corn stover, 0.1% molasses and 1% yeast sludge at optimal pH, temperature and incubation time of 3.5 and 30°C and 96 h, respectively (Ghori et al., 2011). We

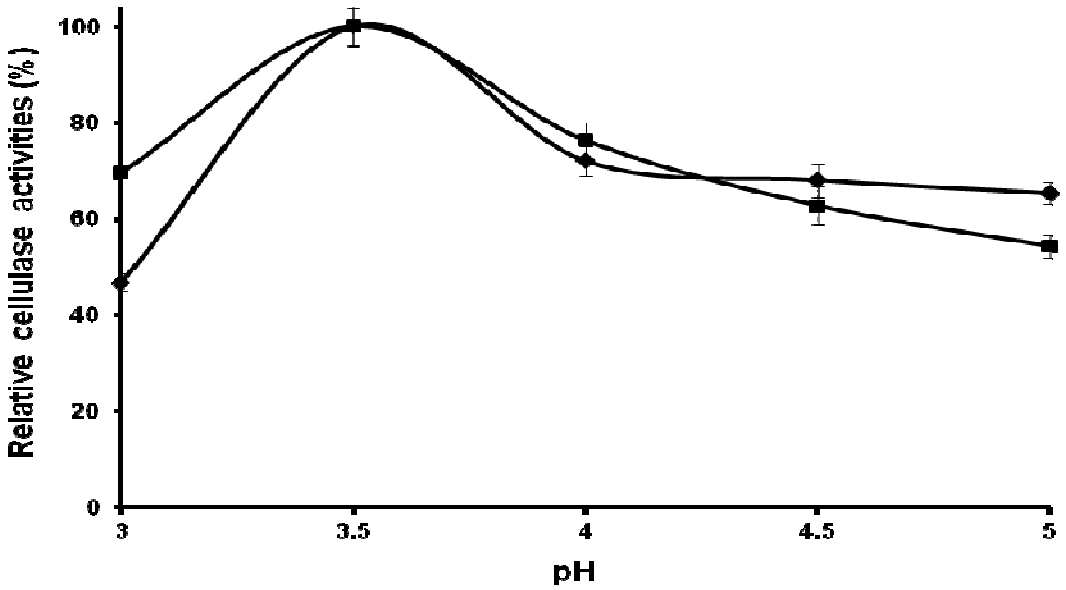

Figure 1. Optimum pH for crude cellulases from *A. niger* NRRL 567. ♦, EXG; ■, EG.

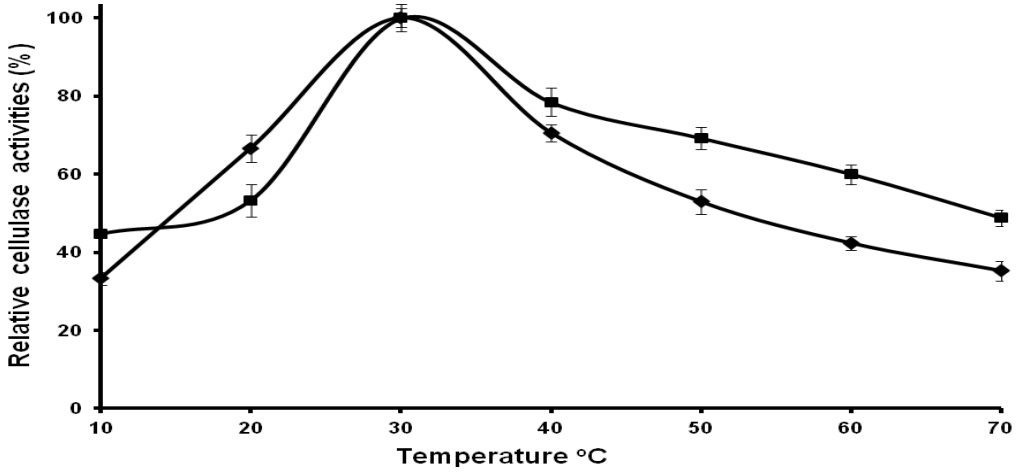

Figure 2. Optimum temperature for crude cellulases from *A. niger* NRRL 567. ♦, EXG; ■, EG.

used the same conditions in our current study for the kinetics studies of the crude EXG and EG from *A. niger* NRRL 567.

Effect of pH and temperature on crude exoglucanase and endoglucanase

Optimum pH for the crude EXG and EG from *A. niger* NRRL 567 was found to be 3.5 (Figure 1). Temperature optimum for crude EXG and EG was found to be 30°C (Figure 2).

The optimal pH for fungal cellulases varies from species to species though in most cases the optimum pH ranges from 3.0 to 6.0 (Niranjane et al., 2007; Ahmed et al., 2003, 2005). Our results are in agreement with the observation that stability of the fungal cellulases is commonly between pH 3.0 and pH 8.0 (Xu et al., 2006; Peng et al., 2009). Cellulases in general show optimum temperature between 30 to 55°C (Xu et al., 2006; Peng et al., 2009).

Effect of metal ions on crude EXG and EG activities

Several metal ions were assayed for their effects on crude EXG and EG activities. The results are shown in Table 1. Zn^{2+}, Ca^{2+}, Mn^{2+} and Co^{2+} enhanced EXG activity whereas Mg^{2+}, Fe^{2+} and Hg^{2+} showed various

Table 1. Effect of metal ions on activity of exoglucanase (EXG) and endoglucanase (EG).

Effector[a]	Relative activity (%) of EXG	Relative activity (%) of EG
Control	100 ± 2.1	100 ± 2.3
Zn^{2+}	121.2 ± 3.1	131.3 ± 3.1
Ca^{2+}	148.1 ± 2.3	154.6 ± 2.3
Mn^{2+}	155.6 ± 0.1	145.9 ± 0.1
Co^{2+}	107.7 ± 1.7	123.7 ± 1.7
Cu^{2+}	121.2 ± 3.1	58.3 ± 3.1
Mg^{2+}	74.3 ± 2.0	70.9 ± 2.0
Fe^{2+}	67.5 ± 3.4	61.3 ± 3.4
Hg^{2+}	60.6 ± 2.3	49.6 ± 2.3

[a]The final concentration of the various cations was 1m*M*. Data are given as mean ± SD.

Table 2. Kinetics parameters for crude EXG and EG from *A. niger* NRRL 567

Enzyme	Lineweaver-Burk plot		Hanes-Woolf plot		Woolf-Augustisson-Hofstee plot	
	K_m (mg/mL)	V_{max} (µmol/mL/min)	K_m (mg/mL)	V_{max} (µmol/mL/min)	K_m (mg/mL)	V_{max} (µmol/mL/min)
EXG	7.35	1.9	7.48	1.91	4.53	1.46
EG	4.75	1.7	4.13	1.58	4.09	1.57

degrees of inhibitory effects. Cu^{2+} enhanced EXG activity and inhibited EG activity.

The presence of Mg^{2+}, Fe^{2+} and Hg^{2+} is reported to inhibit the activity of cellulases as cited in other literature (Mawadza et al., 2000; Elshafei et al., 2009; Maheshwari et al., 2000; Murashima et al., 2002). It seems that heavy metals attack some certain groups at the active site of enzyme, for example the thiol groups, leading to the inactivation, whereas Mn^{2+} could enhance the substrate binding affinity of the enzyme and stabilize the confirmation of the catalytic site (Chauvaux et al., 1995; Tao et al., 2010). According to these results , Mg^{2+}, Fe^{2+} and Hg^{2+} must be avoided in future cultivations for higher exoglucanase and endoglucanase production from *A. niger* NRRL 567.

Effect of substrate concentration on crude EXG and EG

Avicel was used as substrate for EXG whereas CMC was used as a substrate for EG. Kinetics parameters of EXG and EG from *A. niger* NRRL 567 are presented in Table 2.

For crude EXG, by using the Lineweaver-Burk plot (1/V vs 1/S), the K_m was 7.35 mg/mL and V_{max} was 1.9 µmol/mL/min. By using Hanes-Woolf plot (S) vs. [S]/V), the K_m was found to be 7.48 mg/mL and V_{max} 1.91 µmol/mL/min. By using Woolf-Augustinsson-Hofstee plot (V/[S] vs. V), the K_m was 4.53 mg/mL and V_{max} was 1.46 µmol/mL/min (Table 2).

For crude EG, by using the Lineweaver-Burk plot (1/V vs 1/S) the K_m was 4.75 mg/mL and V_{max} was 1.7 µmol/mL/min. By using Hanes-Woolf plot (S) V [S]/V), the K_m was found to be 4.13 mg/mL and V_{max} 1.58 µmol/mL/min. By using Woolf-Augustinsson-Hofstee plot (V/S vs. V), the K_m was 4.09 mg/mL and V_{max} was 1.57 µmol/mL/min (Table 2).

Generally, fungal exoglucanases and endoglucanases show specificity toward various substrates. Literature suggests that the kinetic behavior of cellulases might be affected in the presence of other proteins (or substances) in the medium (Rastogi et al., 2010). Our results indicate small K_m values for EXG and EG which demonstrates high affinity of EXG and EG with their respective substrate. It is, therefore, concluded that the EXG and EG produced during the present study were good catalytic agents for bioconversion of waste materials into useful products.

Energy of activation (Ea) and enthalpy of activation energy (ΔH*)

The energy of activation (Ea) for EXG and EG was 21.20 and 22.52 kJ mol^{-1}; the slope was –2.551 and–2.7092, respectively. From these results, it was found that at 30°C, EXG and EG had maximum catalysis in the conversion of avicel into glucose by using activation energy (Ea) mentioned above. After this temperature, the enzyme starts becoming denatured and show less activity towards the conversion of substrate into product. The small amount of activation energies indicates a good relationship between the enzymes and their substrates.

The enthalpy (ΔH*) of activation of EXG and EG was 18.68 and 20.00 kJ mol^{-1}. It was concluded that kinetically

EXG and EG from *A. niger* 567 were favorably good for the conversion of cellulose into glucose.

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

The Q_{10} values obtained for both EXG and EG were 1.38 and 1.4, respectively. These values show that there was an average of 1.37 times increase in the reaction rate of these enzymes when the temperature was increased from 20 to 30°C. Lower Q_{10} values demonstrate high catalysis as a distinctive feature of the enzymes catalysis as the Q_{10} of a catalyzed reaction is lower as compared to the same reaction uncatalyzed.

Conclusion

This study led us to conclude that this organism may serve as good source of exoglucanase and endo-glucanase production, often deficient in many organisms. The optimum pH and temperature for the crude EXG and EG were found to be 3.5 and 30°C, respectively; hence these enzymes can be used in food industry where high temperature is not required. This study will help in the future production of cellulases at industrial scale from *A. niger* NRRL 567.

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