

## PRODUCTION OF ENDOGLUCANASE FROM A THERMOPHILIC FUNGUS

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Many species of fungi are good source of several important enzymes. In the present studies, an endoglucanase (endo-1,4-D-glucanase, EC 3.2.1.4) was produced from a thermostable fungus *Chaetomium thermophile*. It was grown on Vogel's medium with different carbon sources like xylan, carboxymethylcellulose, corncobs and glucose for 5-days at 180 rpm at 28 °C in orbital shaker. Production of endoglucanase was very low with glucose as carbon source, whereas xylan and carboxymethylcellulose produced the enzymes in appreciable amount. Growth conditions of *Chaetomium thermophile* were optimized for maximal production of endoglucanase (EG): pH 5.0, temperature 50°C, incubation period 120 h, substrate 1% CMC. The enzyme was produced and isolated from the culture filtrate through centrifugation. The crude enzyme extract had 0.064 IU/mL endoglucanase activity. Total protein in the crude extract was 0.103 mg/mL and the specific activity was 0.615 IU/mg.

**Key words:** Endoglucanase, Thermophilic fungi, *C. thermophilum*

### INTRODUCTION

Cellulose is the most abundant and renewable source of energy on earth (Ryu and Mandels, 1980; Suto and Tomita, 2001; Guedon *et al.*, 2002). Its conversion to soluble sugars is preferred by enzymic hydrolysis (Ryu and Mandels, 1980; Wood and McCrae, 1982; Rao and Mishra, 1989) and involves cellulases that provide a key opportunity for achieving the tremendous benefits of biomass utilization (Himmel *et al.*, 1999). Cellulases interactively promote the cellulose degradation (Wood and McCrae, 1982; Rao and Mishra, 1989), to cope with the problems of food and energy shortages expected in near future with explosive increase in human population (Sakka *et al.*, 2000). A number of fungi and bacteria capable of utilizing cellulose as a carbon source have been identified (Wood and McCrae, 1982; Rao and Mishra, 1989). In general, bacterial cellulases are produced constitutively by bacteria, whereas fungal cellulases are produced only in the presence of cellulose as a substrate (Mandel and Reese, 1957). The cellulolytic organisms constitutively produce three main types of enzymes, namely endoglucanase (1,4- $\beta$ -D-glucan glucanohydrolase; EC 3.2.1.4), exoglucanase (1,4-B-D-glucan cellobiohydrolase; EC 3.2.1.91) and  $\beta$ -glucosidase (cellobiase or  $\beta$ -D-glucoside glucosyl hydrolase; EC 3.2.1.21) (Ryu and Mandels, 1980; Wood, 1985; Wood, 1992; Tomme *et al.*, 1995; Ohmiya *et al.*, 1997; Sakka *et al.*, 2000). Cellulose, by the synergistic action of endo- and exo-glucanases, is converted to oligosaccharides and cellobiose (Nisizawa *et al.*, 1972; Wood *et al.*, 1989; Bhat and Bhat, 1997), while  $\beta$ -glucosidase completes the

hydrolysis by converting cellobiose and oligosugars into glucose (Sternberg, 1976).

Although cellulases are produced by a number of microorganisms, the yields are still low due to catabolite repression and end-product inhibition (Yoshihiko and Takahisa, 2002). Cellulase synthesis is regulated by induction and repression (Lin and Wilson, 1987). At least five different transcription factors and two signaling components for cellulase gene regulation have been discussed by Schmoll and Kubicek (2003). Cellulase regulation involves i) basal level expression, ii) mass secretion of cellulases induced by inducers, and iii) glucose or catabolite repression (Suto and Tomita, 2001). Regardless of origin, a cellulase usually consists of two or more structural and functional domains, i.e., a combination of a catalytic domain and a cellulose-binding domain connected by a linker sequence is a common arrangement (Tomme *et al.*, 1995; Ohmiya *et al.*, 1997).

As endoglucanases initiate the cellulose degradation so their enhanced production is of utmost importance. The enzymes isolated from thermophiles have been especially focused because of their inherent stability and industrial applications. Endoglucanases have mostly been isolated and expressed from mesophilic fungi. No report on endoglucanase production from thermophilic microorganisms has so far been found in literature.

*Chaetomium thermophilum* is a fungus that can resist high temperature. Therefore, cellulases produced from this fungus are thermostable and can be of great industrial importance. We report here production and isolation of endoglucanase from this thermophilic fungus.

## MATERIALS AND METHODS

### Organisms and Growth Conditions

*Chaetomium thermophilum* ATCC 28076, a filamentous fungus, was obtained from the American type culture collection, USA. The fungus was maintained on potato carrot agar at 45 °C. For production of endoglucanase (EG), the fungus was grown in Eggins and Pugh medium (Eggins and Pugh, 1962) with 1% (w/v) carboxymethyl cellulose (CMC) (pH 5.0). The cultures were incubated at 45 °C in an orbital incubator shaker operated at 150 rpm for 120 h. Induction of endoglucanase (EG) was studied by growing the fungus in the growth medium supplemented with 1% (w/v) CMC, glucose, xylan or corn cobs. Cultures were harvested daily by filtering through cheese cloth and the mycelia washed with distilled water. After removing excess water by gentle hand-squeezing, the mycelia were stored at -70 °C and the filtrate was centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was assayed for endoglucanase activity and protein, as discussed below.

### Enzyme Assay

EG activity was determined by measuring the amount of reducing sugars released from CM-cellulose by the DNS-method using glucose as standard (Han *et al.*, 1995; Deshpande, 1984; Ding *et al.*, 2001). Reaction mixtures contained 1.0 mL enzyme solution and 1.0 mL 1% (w/v) CM-cellulose in 0.05 M citrate phosphate buffer (pH 6.0). After incubation at 50 °C for 30 min, the reaction was terminated by the addition of 3 mL DNS reagent. The mixture was boiled for 10 min., cooled under tap water, and the absorbance was noted at 550 nm against blank. One unit of enzyme activity was defined as the amount of enzyme that produced one  $\mu$ mole reducing sugar equivalents per min under the assay conditions. Protein was determined by the method of Bradford method (Bradford, 1976) with BSA as standard. Protein in column effluents during gel filtration was monitored by measuring  $A_{280}$ .

## RESULTS AND DISCUSSION

### Growth of *Chaetomium thermophilum*

The revived culture of *Chaetomium thermophilum* was maintained on potato-carrot agar and cultured on Eggins and Pugh medium, pH 5.0, temperature 45 °C. After 5 days the shake flask culture was harvested and enzyme activity was determined that was found to be 29.06 IU/mL with 6.18 IU/mg specific activity. *Chaetomium thermophilum* growth conditions viz. pH, temperature and incubation period were optimized for

the maximal production of the enzyme. Fig. 1 shows that the endoglucanase activity (28.4 IU/mL) was increased with increase in pH from 4 (17.03 IU/mL) to 5 (23.84 IU/mL), whereas above this pH a marked decrease in enzymatic activity was observed. Similarly, growth for varying temperature conditions (40 – 70 °C) was also harvested and optimal temperature was found to be 50 °C for maximal production of the enzyme. The enzymatic activity was increased from 21.73 to 29.13 IU/mL with an increase in temperature from 40 to 50 °C. Above 50 °C a decreasing trend was observed, when the temperature was raised to 70 °C the enzyme decreased to 20.79 IU/mL (Fig. 2). To check the effect of incubation period, the growth was harvested after fixed time intervals of 24, 48, 72, 96, 120 and 144 h (Fig. 3). The maximum enzyme activity

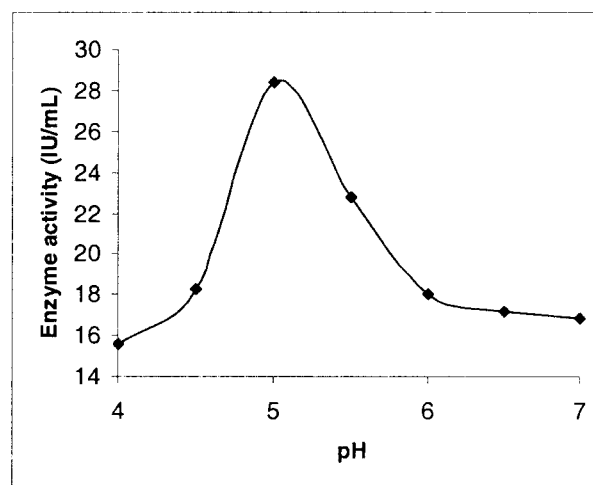


Fig. 1. Effect of pH on growth of *Chaetomium thermophilum* with reference to endoglucanase activity. Each value represents mean from at least three independent experiments.

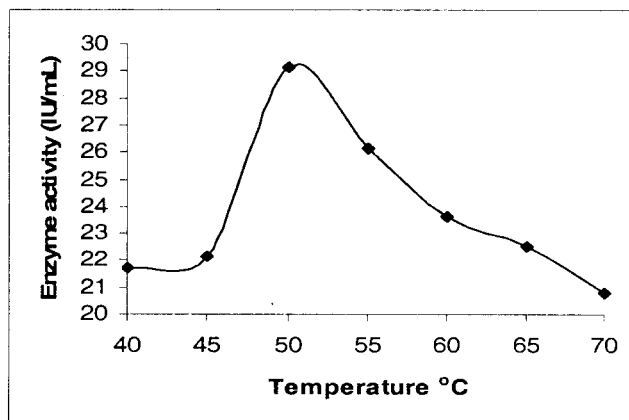


Fig. 2. Effect of temperature on growth of *Chaetomium thermophilum* with reference to endoglucanase activity. Each value represents mean from at least three independent experiments.

(30.87 IU/mL) was yielded by *C. thermophilum* after 96 h. Fig. 3 shows that the enzyme activity was increased after each 24 h increase in incubation period. However, after 96 h further increase in incubation time decreased the activity of endoglucanase (21.38 IU/mL enzyme activity was found after 144 h of incubation). No reports on the production of endoglucanase from *C. thermophilum* could be found in literature. However, other cellulases have been purified from this fungus. Ganju *et al.* (1989) found two cellobiohydrolases in culture filtrate of *C. thermophilum*. Similarly, Liu *et al.* (2005) found high amounts of cellobiohydrolase II from *C. thermophilum*. Large amounts of extracellular and intracellular  $\beta$ -glucosidase activity from *C. thermophilum* were produced and separated by Venturi *et al.* (2002).

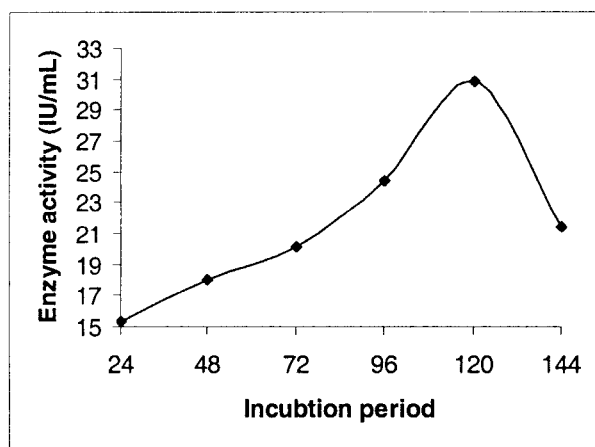


Fig. 3. Effect of incubation period on growth of *Chaetomium thermophilum* with reference to endoglucanase activity. Each value represents mean from at least three independent experiments.

#### Effect of different carbon sources

Carbon catabolite repression is an ubiquitously occurring important regulatory mechanism that leads to the expression of genes encoding enzymes that are involved in the utilization of complex carbon sources when simple sugars like glucose are available. Such a situation was also observed with *C. thermophile* cellulase system that, in the presence of high levels of glucose, failed to induce expression of the cellulase genes. Enzymatic analysis of *C. thermophile* ATCC 28076 grown on medium with carboxymethyl cellulose, glucose, corncobs and xylan as carbon and energy source demonstrated the presence of 32.49, 4.21, 28.15 and 25.23 IU/mL endoglucanase activities, respectively (Table 1). The lowest endoglucanase activity was observed with glucose.

Table 1. Growth of *C. thermophile* for maximal production of EG using different carbon sources

Substrate (1%)	EG (IU/mL)
Glucose	4.21 $\pm$ 0.08
CMC	32.49 $\pm$ 0.22
Corncoobs	28.15 $\pm$ 0.19
Xylan	25.23 $\pm$ 0.23

Effect of these carbon sources on EG expression was also studied at various time intervals. Glucose repressed the expression drastically (Fig. 4). The

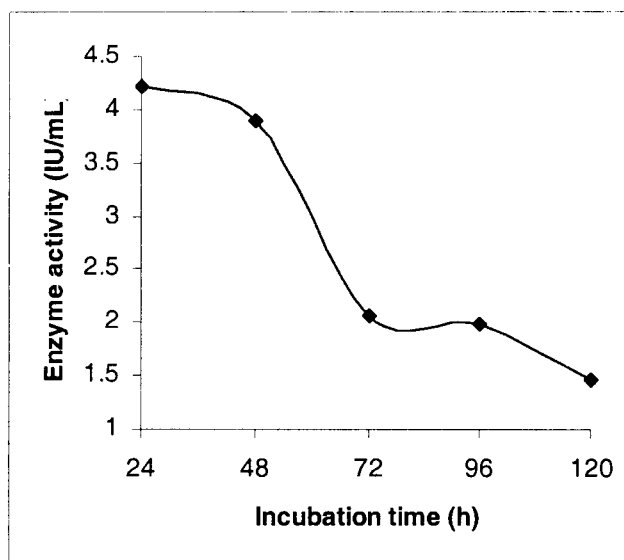
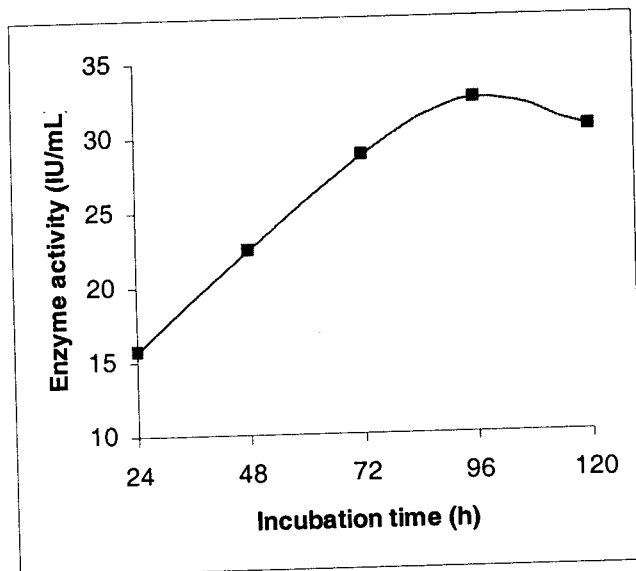


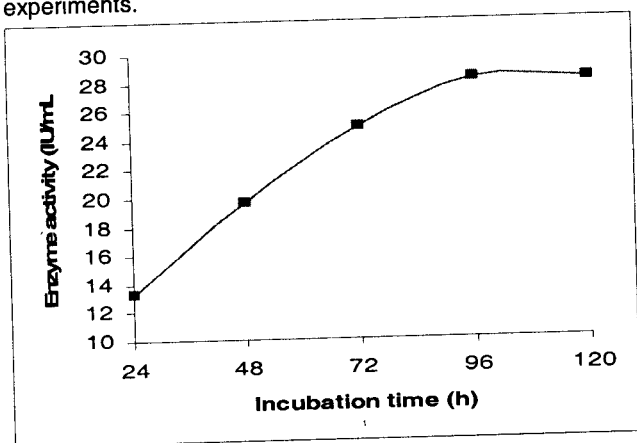
Fig. 4. Effect of glucose on endoglucanase expression at different time intervals. Each value represents mean from at least three independent experiments.

enzyme activity after 24 hours might have obtained due to constitutive expression of the enzyme. Carboxymethyl cellulose (Fig. 5), corn cobs (Fig. 6) and xylan (Fig. 7) enhanced the endoglucanase expression with time up to 120 h, after which decrease in expression was observed.

The study on regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei* also supports the present findings (Ilmen *et al.*, 1997). The production of endoglucanase in *Pseudomonas fluorescens* subsp. cellulosa was also induced by CMC, avicel or filter paper, and repressed by glucose (Shimada *et al.*, 1994).  $\beta$ -glucosidase production from *Chaetomium thermophilum* var. *coprophilum* was also repressed by glucose and induced by cellobiose (Venturi *et al.*, 2002). Cellulase synthesis appears to be regulated at the level of mRNA transcription where small soluble molecules, released from cellulose, act as inducing compounds (Mach and Zeilinger, 2003).

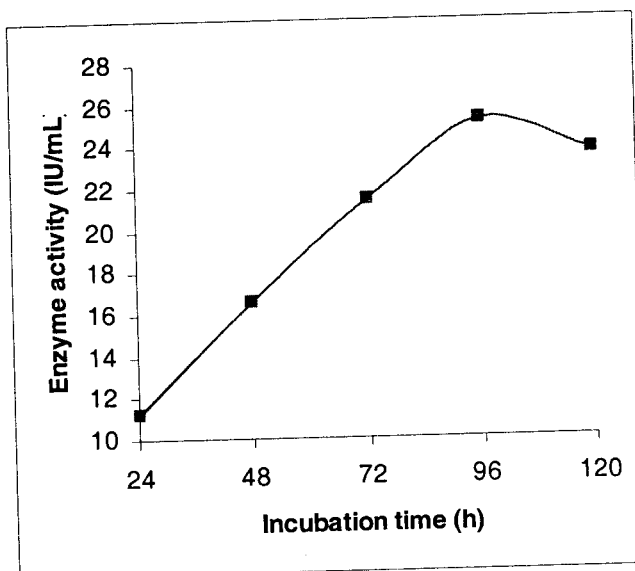


**Fig. 5.** Effect of carboxymethyl cellulose on endoglucanase expression at different time intervals. Each value represents mean from at least three independent experiments.



**Fig. 6.** Effect of corn cobs on endoglucanase expression at different time intervals. Each value represents mean from at least three independent experiments.

Cellulose is generally believed to induce the synthesis of cellulases (Jamil *et al.*, 2006). However, cellulose being insoluble polymer, cannot traverse the cell membrane. It must be converted into soluble inducers for induction of the genes (Beguin, 1990). Similar induction/repression mechanism may exist for the endoglucanase from *C. thermophilum*. The complex polysaccharides might be converted into smaller molecules because of basal level expression of genes (Henrique-Silva *et al.*, 1996). Repression in enzyme expression in the presence of glucose might be due to the involvement of Cre1 (carbon catabolite repressing) type protein that has been found to cause repression of cellulase-encoding genes in *T. reesei* (Ilmen *et al.*,



**Fig. 7.** Effect of xylan on endoglucanase expression at different time intervals. Each value represents mean from at least three independent experiments.

1996; 1997). However, further investigation is needed to explore the exact mechanism of repression of the enzyme in the presence of glucose in this fungus.

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