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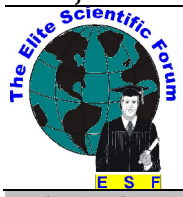


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RESEARCH ARTICLE

# Cloning of a β-glucosidase gene from thermophilic fungus *Cheatomium thermophilum*

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**ABSTRACT**

The investigation was focused on cellulolytic enzymes from *Cheatomium thermophilum*, a thermophilic fungus that produces cellulose degrading enzymes and has been exploited by the industry. It was found that the fungus showed maximum growth on Eggins and Pugh medium at pH 5 and by using carboxymethyl cellulose (CMC) as carbon source. RNA was isolated from mycelia of *Cheatomium thermophilum* and cDNA was synthesized. The subsequent PCR products were purified through agarose gel electrophoresis and ligated into pUC19. The recombinant vectors containing the *bgl* gene were transformed into *E coli* DH10B for further characterization.

## INTRODUCTION

Cellulases and hemicellulases are the two important classes of enzymes produced by filamentous fungi and secreted into the cultivation medium (Ahmed et al., 2003; Ahmed et al., 2005). Cellulase enzymes, 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), exoglucanse (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 a, b). β-glucosidase is considered to be an important component of the cellulase system hydrolyzing a broad variety of aryl- and alkyl-β-glucosides as well as cellobiose and cello-oligosaccharides to glucose with only a carbohydrate moiety (Bhatia et al., 2002). There is a strong interest in cellulases because of their applications in industries of starch processing, grain alcohol fermentation, malting and brewing and extraction of fruit and vegetable juices (Jamil et al., 2005). Xylanases and cellulases together with pectinases account for 20% of the world enzyme market (Ahmed et al., 2007; Sheikh et al., 2003).

Filamentous fungi have been used for more than 50 years in the production of industrial enzymes (Saadia et al. 2008). *Chaetomium thermophilum* is among the main cellulase and xylanase producing microorganisms (Saleem et al., 2008) with thermophilic properties.

In Pakistan, cellulosic residues are produced to as much as 50 million tons every year (Ghori et al., 2011; Ahmed et al., 2010) that could be utilized for bulk production of various enzymes including cellulases. For industrial use, thermophilic enzymes are preferred in most of the processes due to high temperatures. Therefore, we are investigating a thermophilic fungus *Chaetomium thermophilum* for the production of cellulases and xylanases. Isolation and cloning of the genes for the enzymes not only enhanced our efforts to produce such enzymes at pilot and industrial scale but also helped in characterization of the enzymes in more detail. In an effort to produce and characterize the cellulases and xylanases from the thermophilic fungus *Chaetomium thermophilum* we report in this paper the cloning and screening of β-glucosidase encoding gene from the fungus.

## MATERIALS AND METHODS

**Chemicals:** All chemicals were purchased from Sigma Chemical Co., Missouri USA except where stated otherwise. The restriction and modifying enzymes and genomic DNA extraction kit were from Fermentas.

**Induction of cellulase genes**: *Chaetomium thermophilum* ATCC 28076 was obtained from ATCC

USA. For the isolation of total RNA, the fungus was grown in 500 mL Erlenmeyer flasks containing 100 mL of Eggins & Pugh medium (Eggins & Pugh, 1962); g/L 1.0 $KH_2PO_4$, 0.5 KCl, 0.5 $(NH_4)_2SO_4$, 0.2 $MgSO_4.7H_2O$, 0.1 $CaCl_2.2H_2O$, 0.5 L-asparginine, 0.5 yeast extract; 1% carboxymethyl cellulose was added as a carbon source to induce the cellulolytic genes. pH of the medium was adjusted to 5 (Saleem et al., 2008). The culture was grown for 4 days at 45 °C with shaking at 150 rpm and harvested by centrifugation at 10000 rpm for 20 min, at 4°C (Ahmed et al., 2007). The resulting pellet was used for RNA extraction.

**RNA extraction and cDNA synthesis**

For extraction of RNA the *C. thermophilum* pellet was frozen in liquid nitrogen before ground in an ice-cold mortar until powdery consistency was achieved. TRI reagent (Molecular research center, USA) was used for total RNA extraction following the manufacturer's instructions. The RNA was treated with DNase1 and again purified. RNA quality and purity was checked by gel electrophoresis and spectrophotometer.

First strand cDNA was synthesized using RevertAid H Minus cDNA synthesis kit (Fermentas) according to manufacturer's instructions. First strand cDNA was then used as a template for PCR.

**Gene amplification and cloning of β-glucosidase gene**

Molecular cloning techniques and restriction digestions were performed as described by Sambrook and Russel (2001). Using cDNA as a template β-glucosidase gene was amplified by PCR using *Taq* DNA polymerase (Fermentas). PCR primers were designed from the sequences of β-glucosidase gene for different fungi available on NCBI. Several sets of primers were tried out of which following primers successfully amplified *bgl* gene from the fungus:

Forward:

5′C C A G A A T T C A C G C C G C C G C A A T C A G

Reverse:

5′G C G A A T T C G C A T G T T G C C C A A G G A C

The amplification conditions were optimized as: Initial denaturation at 94 °C for 3 min, 35 cycles of 1 min at 94 °C, 1 min at 49 °C, and 1 min at 72 °C, with a final extension of 10 min at 72 °C. PCR products electrophoresed on 1% agarose gel and visualized on gel documentation system after ethidium bormide staining. The amplicon of expected size was gel purified by DNA extraction kit.

The PCR products were ligated into plasmid pUC19 digested by *Sma*1 and dephosphorylated by calf intestinal alkaline phosphatase. Recombinant plasmids were transformed into *E. coli* DH10B by heat shock method and screened by Congo red method (Teather and wood., 1982).

**β-glucosidase Assay**

BGL activity was assayed in 1 mL reaction mixture containing 1% salicin in 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 dinitorsalycilic acid solution (Shamala & Sereekanth 1985) and absorbance was noted at 560 nm. One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate 1 μmole of sugars under the standard conditions.

## RESULTS AND DISCUSSION

**Growth and enzyme activity in culture extract of *Chaetomium thermophilum***

*Chaetomium thermophilum* ATCC 28076 a thermostable fungus was used in this study. Eggins and Pugh medium (1962) was proved to be a good source for growth of the fungus. Carboxymethyl cellulose was used as carbon source because it is an inducer of cellulase genes in fungi. A basal level expression of cellulases exists in the fungi due to which the enzymes are secreted out into the medium to some extent. As soon as the fungi come in contact with some cellulosic material the enzymes degrade it into smaller units that enter in the fungal cells and elicit expression of the genes from the relevant promoters (Schmoll and Kubicek, 2003). The culture was grown and monitored for the enzyme activity for five days. Maximum activity was found to be 0.462 U/mL on fourth day (Figure 1).

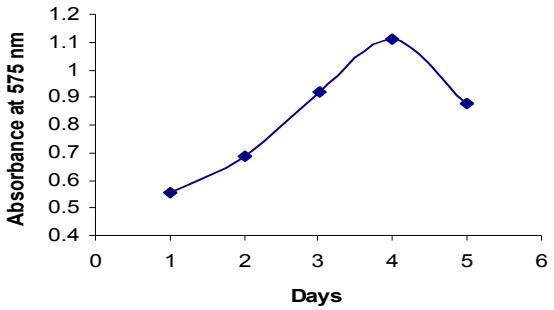


**Fig. 1: Growth curve for *Chaetomium thermophilum* at different time intervals for the production of β-glucosidase**

**RNA isolation and cDNA synthesis**

TRI reagent (Molecular research center, USA) was used for total RNA extraction from *C. thermophile* that yielded good quality RNA. However, some DNA contamination was observed that was removed by treating the RNA with DNase1. Quantity of RNA was found to be in the range of 4.23 ug/mL. The $A_{260/280}$ ratio was also in a reasonable range so that the RNA could be used in further analysis.

**Isolation of a β-glucosidase encoding gene**

RNA isolated from *C. thermophilum* was used for *bgl* gene amplification through Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). First strand of

cDNA was made with the help of cDNA synthesis kit. The amplified products were analyzed by agarose gel electrophoresis. The RT-PCR product was about 1.5 kb and no other non-specific bands were found. Figure 2 shows RT-PCR from RNA of *C. thermophilum* grown on CMC as a substrate. It is clearly demonstrated from the figure that significant amplification was achieved from the cDNA. The corresponding bands were excised from the gel by DNA extraction kit and purified for ligation in pUC19 plasmid.

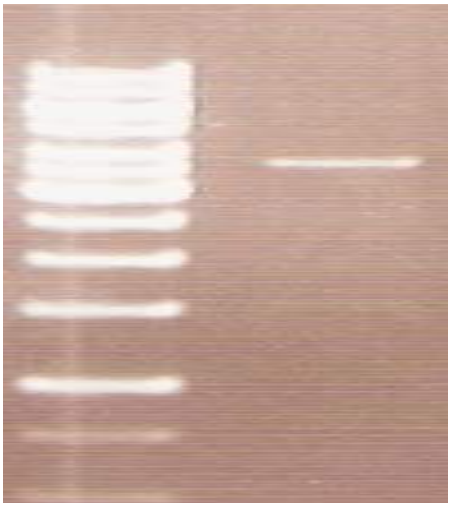

**Fig. 2: RT-PCR amplification of *bgl* gene from *C. thermophilum.* Lane 1: DNA Molecular weight marker, Lane 2: *bgl* gene amplified by RT-PCR**

**Cloning and screening of *bgl* gene**

The *bgl* gene was ligated into pUC19 and transformed in *E. coli* DH10B and plated on LB-ampicillin-IPTG-X-Gal-salicin plates. Congo red staining showed the presence of clear zones of hydrolysis around colonies suggesting that β-glucosidase hydrolysis was obtained (Fig. 3).

Earlier, a thermostable β-glucosidase gene (*bgl*) from *Chaetomium thermophilum* CT2 was cloned and expressed in *Pichia pastoris* by Xu et al. (2011). However, it was a different strain as compared to the work reported in this paper. Moreover, the expression was carried out in yeast whereas we got the expression in *E. coli*. Although the expression level was achieved at Perti plate level, but the same may be scaled up to fermenter level. Main advantage of the expression in *E. coli* is simplicity of the host that is helpful in further processing of the expressed protein. Molecular cloning and characterization of two intracellular β-glucosidases belonging to glycoside hydrolase family 1 from the basidiomycete *Phanerochaete chrysosporium* have been reported by Tsuda et al. (2006). Cloning, expression, and characterization of two β-glucosidases from isoflavone glycoside-hydrolyzing bacterium *Bacillus subtilis* natto has also been reported (Kuo and Lee, 2008).

Conclusively, the *bgl* gene was isolated from thermophilic fugus that yielded the enzyme at 45 $^{o}$C. Although some *bgl* genes have been reported from different fungi, but none has been reported so far from the strain used in this study. This will help in expression studies of *bgl* gene that may yield thermophilic enzymes for industrial use in future.

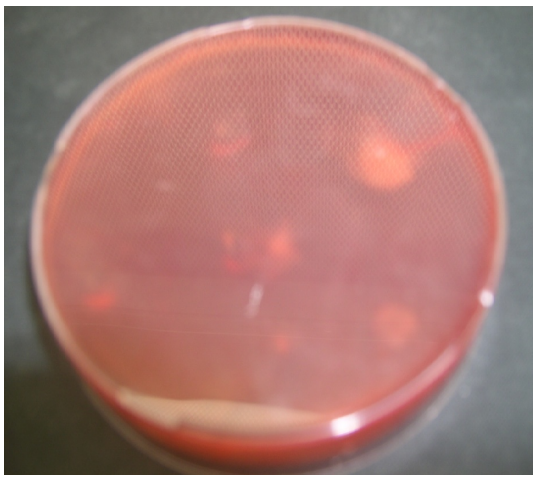

**Fig. 3: Salicin hydrolysis by recombinant vectors on LB-Salicin plates. β-glucosidase plate assay of transformed *E. coli* cells containing *C. thermophilum bgl*. Congo red staining of agar plate containing 1% salicin and destaining with 1 M NaCl revealed haloes representing β- glucosidase activity.**

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