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Molecular Cloning of β-glucosidase Gene from *Trichoderma harzianum*

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Abstract: *Trichoderma harzianum* (E-58) was grown on Vogel's medium containing glucose and xylan as carbon source. Glucose repressed the *β*-glucosidase production while xylan produced the enzyme in substantial amounts. Total RNA was isolated from mycelia of *Trichoderma harzianum* grown in medium containing xylan as carbon source. RT-PCR was performed on the isolated RNA. cDNA strand was synthesized by using oligo $(dT)_{18}$ primer. Specific primers were used to amplify *bgl* gene from cDNA. The *bgl* gene was ligated at SmaI site of pUC18 and transformed into *E. coli*.

Key words: β-glucosidase gene isolation, β-glucosidase gene cloning, *Trichoderma harzianum*, β-glucosidase production

INTRODUCTION

Cellulose is the world's most abundant organic polymer. Cellulases are the enzymes that hydrolyze cellulose polymer into glucose monomers[1]. Cellulose may be hydrolyzed enzymatically by the combined action of at least three enzymes i.e. exoglucanase (EC 3.2.1.91), endoglucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21). Synergism between different cellulase components exists when they are acting simultaneously on insoluble cellulosic substrates[2]. Endocellulases attack randomly at internal glucosidic bonds of cellulase chains, thereby producing polymer chain ends and soluble oligosaccharides. Exoglucanases cleave cellobiosyl residues from the ends of cellulose chains. β-glucosidases catalyze the hydrolysis and the assimilation of cellobiose, biodegradation product of cellulose and other plant derived glucosides such as arbutin and salicin[3]. β-glucosidases are also responsible for hydrolysis of isoflavonoid conjugates to release fungal toxic compounds when legumes are attacked by pathogens and serve as bait in pest control system[4-7]. More than 14,000 fungi have been reported in degradation of cellulose and other insoluble fibre materials[9]. Out of these, *Trichoderma* has received the highest attention for basic research and also for the industrial production of cellulolytic enzymes[8].

It can grow on an inorganic medium supplemented with only an organic carbon source. The biosynthesis of cellulases is induced by cellulose and other inducers and regulated via catabolic repression and their activity is influenced by end-product inhibition[10]. Understanding of fungal β-glucosidase has advanced considerably over the last few years through combination of biochemical and molecular approaches[1]. The present work describes the isolation of β-glucosidases gene from *Trichoderma harzianum* and its cloning in *E. coli*.

MATERIALS AND METHODS

Strain and culture conditions: *Trichoderma harzianum* (E-58) was maintained on agar slants (Vogel's medium) containing (g L^{-1}) Trisodium citrate, 0.5; KH_2PO_4, 0.5; NH_4NO_3, 0.2 $(NH_4)_2SO_4$, 0.4; $MgSO_4$, 0.02; Peptone, 0.1; yeast extract, 0.2; Xylan, 0.2; Agar 2.5. Freshly inoculated slants were incubated at 28°C for 5 days and then stored at 4°C. Spores from slants were transferred to inoculum (Vogel's medium) containing glass beads for uniform suspension. The flasks were incubated for 24 h at 28°C on a rotatory shaker at 180 rpm. The pH was adjusted to 5.5. The inoculum (20 mL) was transferred to 200 mL of Vogel's medium in 1000 mL Erlenmeyer flasks. The flasks were incubated for 5 days in rotatory shaker at 180 rpm at 28°C. The mycelia were harvested by filteration and stored at –40°C. The filtrate (stored at 4°C) was used for enzyme assay.

Enzyme assay: β-glucosidase was assayed by monitoring the rate of formation of p-nitrophenol from p-nitrophenyl-β-D-glucopyranoside (pNPG). One unit of activity was defined as the amount of the enzyme that catalyzes the formation of 1 μ mol p-nitrophenol per min at 40°C and pH 5.

To 1 ml of 10 mM p-nitrophenyl β-D glucopyranoside solution (substrate) in buffer, 1 ml of appropriate dilute enzyme was added and incubated at 40°C for 10 min.

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Three mL of 1 M Na_2CO_3 solution (reaction stopper) was added to reaction mixture and volume was made upto 10 mL with distilled water. Absorbance was noted at 400 nm.

RNA isolation: Hot phenol method was used to isolate total RNA. Mycelia (1g) were macerated in the presence of dry ice and homogenized with re-suspension buffer (Sodium acetate 0.01 M, pH 4.0; Nacl 0.05 M). SDS was added to the solution to make final concentration of 0.1% and centrifuged at 10,000 rpm for 10 min. Supernatant was collected and one volume of prewarm (65°C) phenol solution (90%, phenol; 10% resuspension buffer) was added. The reaction was incubated at 65°C for 3-5 min under shaking and then incubated at -15°C for 1-3 min with intermittent shaking. It was then centrifuged at 10,000 rpm for 3 min and aqueous phase was collected. Phenol extraction was repeated and RNA was precipitated with 0.3 M NaCl and cold ethanol. After centrifugation at 10,000 rpm for 20 min at 4°C the pellet was rinsed with 70% ethanol and centrifuged again at 10,000 rpm for 10 min. The pellet was dried and resuspended in 500 μL DEPC treated H_2O. For Dnase treatment 80 μL $MgCl_2$/DTT (100 mM each), 1.6 μL DNase, 0.8 μL, RNase inhibitor and 317.6 μL autoclaved H_2O were added in an eppendorf tube having 400 μL of isolated RNA sample. The eppendorf was inverted twice and incubated at 37°C for 20 min. 200 μL of DNase stop solution (50 mM EDTA,1.5M sodium acetate, 1% SDS) was added and Phenol/chloroform extraction was performed twice RNA was ethanol precipated and washed with 70% ethanol. It was resuspended in 500 μL DEPC treated H_2O.

RT-PCR: First strand synthesis of cDNA was made with the help of cDNA synthesis kit from MBI Ferments. Approximately 10 μg of RNA sample was added to 0.05 μg of oligo dT primer. After incubation for 5 min at 70°C, the tube was chilled in ice and centrifuged briefly to collect the mixture. The tube was then placed in ice and 5 x reaction buffer (4 μL), RNAase inhibitor (1 μL) and 10 mM dNTPs (2 μL) were added. It was incubated at 37°C for 5 min and 200 units of reverse transcriptase were added. After incubation at 42°C for 60 min, the reaction was stopped by heating at 70°C for 10 min. To this 180 μL of 10 mM Tris-chloride/10 mM EDTA (pH 7.5) was added and kept at -40°C. Twenty μL of the RT was amplified by PCR by using specific primers[11], 5' atgttgcccaaggatttcag and 5'cgccgccgcaatcagctcgtc. PCR was done under following conditions: 10x buffer (MBI Ferments) 200 μM dNTPS/each, 1 U Taq polymerase were added to DNA template and 500 nM each primer. Thermocylcer (Perkin Elemer) conditions were set as melting 95°C for 90 sec, annealing 50°C for 60 sec and extention 72°C for 60 Sec. PCR products were separated by electrophoresis on agarose gel and visualized by ethiduim bromide staining.

Ligation and transformation of gene: Approximately 10 μg plasmid(pUC18) was digested with SmaI using appropriate buffer and dephosphorylated using 10 units of CIAP. The reaction was incubated at 37°C for 1 h and the enzyme was denatured at 65°C for 1 h. Dephosphorylated samples were phenol/chloroform extracted and ethanol precipitated before ligation. 3:1 (molar) insert: vector was used for ligation.

The reaction was incubated in DNA ligase buffer (MBI Ferments) with final concentration of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$ 1 mM ATP and 1 mM DTT containing IU of DNA ligase for 24 h at 18°C. The ligated vector was transformed into *E. coli* competent cells (10B) by heat shock method[12] and plated on agar-ampicillin plates. The colonies from successful transformations were picked up and grown.

RESULTS AND DISCUSSION

Trichoderma harzianum (E-58) produces highly active cellulases[19]. It was tested for its ability to produce β-glucosidase on different carbon sources like glucose and xylan (Table 1). The enzyme was produced from *T. harzianum* grown on 1% glucose and 1% xylan in Vogel's medium[13] at pH 5.5 for 5 days at 28°C in shake flasks. This work is in consistent with previous work conducted on *T. harzianum*[14,15]. Xylan induced high cellulases activities and glucose suppressed these enzymes. This is consistent with one reported by Ximenes *et al.*[7]. RNA was isolated from mycelia which were grown in Vogel's medium containing xylan as carbon source. The enzyme activity (IU) was calculated by factor obtained from standard curve of p-nitrophenol (Fig. 1). Present work suggests that when glucose was used as a carbon source, the production of β-glucosidase from *T. harzianum* was inhibited and when insoluble carbon source was used, the production increased significantly.

Table 1: β_Glucosidase activity in culture extracts of *T. harzianum*

Carbon source	Enzyme activity (U mL^{-1})
1% Glucose	0.0635
1% Xylan	0.5370
1% Xylan	0.6929

Table 2: Quantification of RNA isolated from *Trichoderma harzianum*

Carbon source	Concentration (mg mL^{-1})
1% Glucose	0.716
1% Glucose	0.918
1% Glucose	0.502
1% Xylan	1.262
1% Xylan	1.995
1% Xylan	1.022

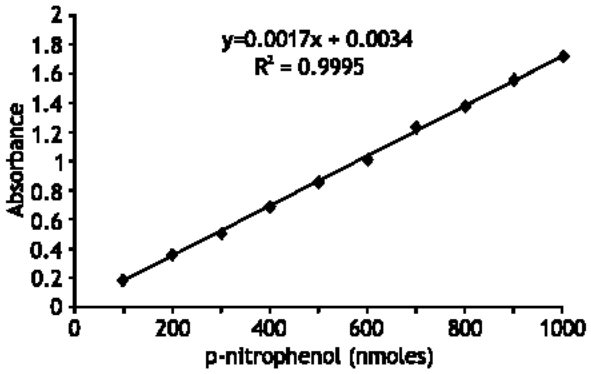

Fig. 1: Standard curve for p-nitrophenol

Fig. 2: RT-PCR for amplification of *bgl* gene from *Trichoderma harzianum*

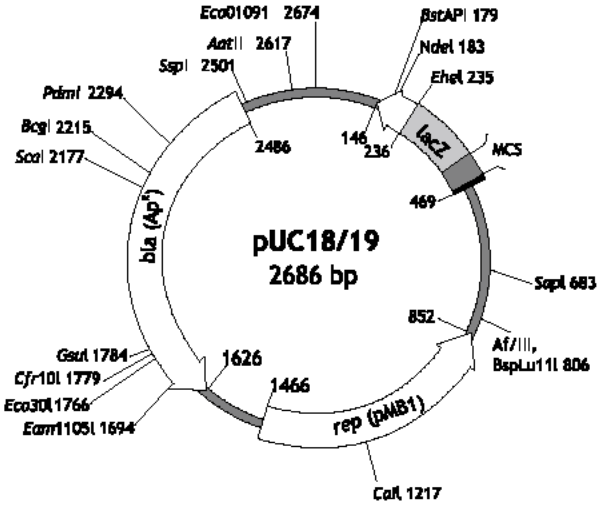

Fig. 3: Restriction map of pUC18

Fig. 4: pUC18 digested with SmaI

The results are in agreement with earlier reports in which growth conditions are reported[8,17,18]. They found that the optimum temperature for cellulase production was 25-28°C and optimum temperature for growth was 30°C. Total RNA extraction was performed according to hot phenol method. RNA was isolated both from *T. harzianum* grown on both xylan and glucose as carbon source. The quantification of different RNA samples is shown in Table 2. Better results were observed with the samples that were grown on xylan[7,18]. RNA isolated from *T. harzianum* grown on xylan was subjected to DNase treatment to digest the DNA.

First cDNA strand was synthesized using Oligo $(dT)_{18}$ primer and was directly subjected to PCR along with the specific primers. The amplified product was run on gel (Fig. 2) The isolated β-glucosidase gene as a result of RT-PCR was cloned into pUC18 plasmid (Fig. 3). SmaI restriction enzyme was used to linearize the pUC18 to obtain blunt ends for ligation of the *bgl* gene into plasmid. The product of the restriction digestion was run on 1% agarose gel (Fig. 4). The band was excised from gel and purified using DNA extraction kit. The techniques used in the present study are discussed by Bashir *et al.*[19]. Rajoka *et al.*[20] and Winters *et al.*[21] also used pUC18 for cloning of β-glucosidase gene.

The amplified gene was ligated into pUC18 plasmids with the help of DNA ligase. The ligated products were transformed into *E. coli* competent cells. Colonies from successful transforments were picked up and grown. The *bgl* gene from *T. harzianum* was, therefore, successfully amplified with the help of RT-PCR, ligated into pUC18 and transformed into *E. coli*. It can now be used for expression and sequencing of the gene.

Several workers have isolated β-glucosidase gene from various organisms and cloned into different hosts. β-glucosidase gene from *T. reesei* was cloned and sequenced by Barnett *et al.*[22]. Similarly *bgl* gene from *Xanthomonas compestris* XA5-5 was cloned in *E. coli* by

Zou *et al.*[23]. Tajima *et al.*[16] also cloned *bgl* gene from genomic DNA of *Acetobacter xylinum* ATCC 23769 and determined its neucloestide sequence. *bgl*-I and *bgl*-II genes from *Humicola grisea* and *T. reesei* were cloned and expressed by Takashima *et al.*[11].

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