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Cloning of Endoglucanase Gene from *Trichoderma harzianum* in *E. coli*

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Abstract

Recycling of agro-industrial wastes to produce high quality protein is an efficient and economical way to solve the problem of deficiency in good quality protein. Cellulases are attractive source for utilization of the agro-industrial waste materials. Endoglucanase (EC 3.2.1.4) was isolated from *Trichoderma harzianum* (E-58 Strain). It was grown on Vogel's medium with different carbon sources. Maximal production of endoglucanase gene was achieved at 28°C, pH 5.5 under continuous shaking at 120 rpm for 5 days. Glucose repressed the synthesis of enzyme whereas carboxymethylcellulose produced the enzyme in substantial amounts. RNA was isolated from mycelia of *T. harzianum*. First strand of cDNA was synthesized using oligo dT (18) primer and subjected to PCR with sequence specific primers. The amplified products were purified through agarose gel electrophoresis and ligated into pUC18. The plasmids containing endoglucanase genes were transformed into *E. coli* for further characterization.

Key words: Cloning, Endoglucanase, *E.coli*, *T.harzianum*

Introduction

Despite the fact that the world community is no longer pre-occupied with fossil fuel shortages, there is still a considerable research and development directed towards understanding and commercializing enzymatic hydrolysis of cellulose (Walker and Wilson, 1991). Cellulose is the most abundant organic polymer (Goyal *et al.,* 1991) in this planet and is an important renewable energy source along with sugars and starches. Energy production from cellulosic raw material involves its hydrolysis into glucose. As compared to acid or alkali hydrolysis, enzymatic hydrolysis of cellulose is more favorable because enzymes are more stable, recoverable, specific, low in energy requirements and non-polluting.

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Cellulase system contains three enzymes that can degrade crystalline cellulose. Exocellobiohydrolase or Exoglucanase (EC 3. 2. 1. 91), 1-4 ß, glucan cellobiohydrolase or endo-1-4, ß-D-glucanase (CMCase) (E.C. 3.2.1.4) and ß-glucosidase or cellobiosidase or glucohydrolase (E.C. 3.2.1.21). Highly ordered cellulose substrates are converted into soluble sugars only when endoglucanases, exoglucanases and ß-glucosidases are present in solution simultaneously in right proportion. The function of endoglucanase is to increase the effective concentration of accessible end groups for the end wise action of cellobiohydrolase. Hence endoglucanases are assigned the role of initiating the attack on native cellulose (Madhavi *et al.,* 1984). Endoglucanases hydrolyze cellulose chains at random to produce a rapid change in degree of polymerization. Substrates include CMC, H_3PO_4 or alkali swollen amorphous cellulose, H_3PO_4 but crystalline cellulose such as cotton fibre or avicel is not attached to significant extent. Hydrolysis of amorphous cellulose yields a mixture of glucose, cellobiose and other soluble cello-oligosaccharides. The rate of hydrolysis of the longer chain cello-oligosaccharides is high and the rate increases with degree of polymerization. Glucose and cellobioside are principal products of the reaction (Fan *et al.,* 1987). A number of fungal species are known for the production of cellulases such as *Aspergillus niger,Sporotrichum* spp,*chaetomium thermophile,Trichoderma* species etc. (Szozodark, 1988). Gene cloning is recently being employed for studying the structure and function of a number of enzymes and proteins and their over expression. This strategy has been found very efficient as compared to other traditional methods. Present study was designed to layout strategy for enhanced production of endoglucanase. In this paper we have reported the isolation and cloning of endoglucanase gene from *Trichoderma harzianum*.

Materials and Methods

Strains and Cultivation Conditions

A fungal strain *Trichoderma harzianum* (E58) was employed for the isolation of endoglucanse gene. It was grown at 28°C with shaking (120 rpm) in Vogel's medium (0.5% Trisodium citrate, 0.5% KH_2PO_4, 0.2% NH_4NO_3, 0.4% $(NH_4)_2SO_4$, 0.02% $MgSO_4$, 0.1%

peptone, 0.2% yeast extract pH 5.50), containing 1% glucose or carboxymethycellulose or xylan as a carbon source (Ikram-ul-Haq *et al.,* 2001)

Enzyme Assay

Assay of endoglucanase (CMC ase) was performed by the method described by Gadgil et al. (1995). In Assay, 1 mL of diluted enzyme was incubated for 30 min with 1 mL of CMC and 1 mL of 0.1Mcitrate buffer of pH 4.8 at 50°C. Reaction was stopped by adding 3 mL of Dinitro Salicylate (DNS) reagent and boiled for 15 min and cooled in ice. The absorbance was measured at 540 nm. The international units of endoglucanase produced per mL were calculated by factor obtained from standard curve of glucose (Fig.1).

Hot Phenol Method for the Isolation of Total RNA

Mycelia (1g) were measured in the presence of dry ice and homogenized with resuspension buffer (sodium acetate, 0.01M, NaCl 0.05M). SDS was added to the suspension to make a final concentration of 0.1% and centrifuged at 10,000 rpm for 10 minutes. Supernatant was collected and one volume of prewarm phenol solution (at 65°C) was added. Incubated at 65°C for 3-5 minutes under shaking and then incubated at -15°C for 1-3 minutes under shaking. It was then centrifuged at 10,000 rpm for 3 minutes and aqueous phase was collected. Phenol extraction was repeated and RNA was precipitated with ethanol. The pellet was rinsed with 70% ethanol and resuspended in Diethyl pyrocarbonate (DEPC) treated water.

RT-PCR (Reverse Transcription Polymerase Chain Reaction)

First strand synthesis of cDNA was made with the help of cDNA synthesis kit from MB1 Fermentas, Lithuania. Approximately 10 µg of isolated RNA sample and 0.5 µg of oligo dT primer were mixed and centrifuged for 4 seconds. It was incubated at 70°C for 5 minutes in water bath and chilled in ice. Reaction buffer (5X), 20 units of RNase inhibitor, 2µL of dNTPs (10 mM) were added and mixed by tapping. It was incubated at 37°C for 5 minutes. Reverse transcriptase (200 units) was added and incubated at 42°C for 60 minutes. The reaction was stopped by heating at 70°C for 10 minutes. 180uL of 10mM Tris HCl/10mM EDTA was added and stored at -40°C. The RT reaction (20 µL) was amplified by PCR. Primers were designed according to sequence of endoglucanase gene from *T. reesei* as information for *Trichoderma harzianum* was not available. The information for primer designing was obtained from Medline library available on world wide web. Following primers were designed

Primers Designed for Endoglucanase gene (eglI)

Primer 1 5' atggcgccctcagttacactg 3'
Primer 2 5 aaggcattgcgagta 3'

The primers were obtained from MBI Fermentas. PCR was done with following conditions. 10x buffer (MBI fermentas), 200 µM dNTPs/each, 1U Taq polymerase were added to DNA template and 500 nM each primer, various concentrations of $MgCl_2$ (1.0-9.0 mM) were used. Thermocycler (Perkin Elemer) conditions were set as: Melting 95°C for 90 sec; Annealing 50°C for 60 sec; Extension 72°C for 60 sec. PCR product, were separated by electrophoresis on agarose gel and visualized by ethidium bromide staining.

Ligation of the genes into plasmid

The RT-PCR amplified endoglucanase gene was ligated into pUC18 plasmid digested with Smal at 30°C for 2 hours. The vector was dephosphorylated using 10 units of calf intestinal alkaline phosphatase. The reaction was incubated at 37°C for 1 hour and the enzyme was denaturated at 65°C for 1 hour. Dephosphorylated DNA was purified with the help of DNA purification kit (MBI Fermentas).

Insert vector ratio of 3:1 was used for ligation. The reaction was incubated in DNA ligase buffer (MBI Fermentas) with final concentration of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP and 1mM DTT containing 1U of DNA ligase for 24 hours at 18°C.

Transformation into *E. coli*

The ligated vector was transformed into *E. coli* (10 b) competent cell by heat shock method (Ausubel *et al.,* 1988) and plated on agar-ampicillin plates.

Results and Discussion

Growth of *T. harzianum*

Spores of *T. harzianum* were maintained on agar slants containing 0.2% CMC or 0.2% xylan. *T. harzianum* was grown in Vogel's medium for 5 days at 28°C at 120 rpm. The pH of the medium was adjusted to 5.5 to obtain maximal production of endoglucanase. It was found earlier that maximum induction of endoglucanase gene was achieved at pH 5.5 (Ikram-ul-Haq *et al.,* 2001). Temperature of the growth medium was kept at 28°C which is in accordance with previous work conducted on *T. harzianum* (Esterbauer *et al.,* 1991) who found that optimum temperature for cellulase production was 25-28°C and optimal temperature for growth was 30°C for *T. reesei*.

After getting growth, the medium was filtered, residues containing growth was stored for the isolation of RNA and from the filtrate endoglucanase assay was performed. The enzyme activity was calculated by factor obtained from standard curve of glucose (Fig 1). Present results (Table1) suggest that when glucose was used as a carbon source, the production of endoglucanase from *T. harzianum* was inhibited, on the other hand when CMC was used as a carbon source the production increased but the maximum production of endoglucanase was marked when xylan was used as a carbon source. The results are in agreement with earlier reports where it was found by Ximenes et al. (1996) that glucose repressed the enzyme production. They

used xylan and CMC for the growth of *T. harzianum*. Similarly Malik et al. (1986) noted that with glucose as a carbon source maximum growth was obtained but negligible cellulases were produced. They found that substantial amounts of cellulases were produced with other carbon sources. Production of highly active cellulases has also been found by Esterbauer et al. (1991). Present work is in accordance with Li and Ljungdahal et al. (1994) who used xylan as a carbon source for the isolation of RNA from *Aureobasidum pullulans*.

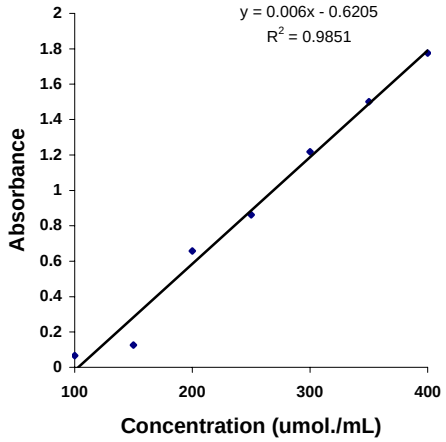

Fig 1: Standard curve of glucose

Gene Amplification by RT-PCR

RNA isolated from *T. harzianum* was used for endoglucanase gene amplification through RT-PCR.

Table 1 Endoglucanase produced from *T. harzianum* grown on different Carbon sources

Carbon source	Enzyme activity (IU/mL/min)
1% glucose	9.04
1% CMC	11.2
1% xylan	14.4

Isolation of RNA

RNA was isolated by Hot phenol method from mycelia of *T. hazrianum* grown in Vogel's medium.

The results of RNA quantification are given in Table 2.

Table 2 Quantification of RNA isolated from *T. harzianum*

Sr. No.	Carbon source for growth of *T. harzianum*	RNA Concentration (mg/mL)
1	1% glucose	0.716
2	1% CMC	0.918
3	1% xylan	1.995

RNA samples were treated with DNase to remove DNA. RNA isolated form *T.harzianum* grown on 1% xylan was used for gene amplification through RT-PCR. Earlier workers isolated mRNA from *T. reesei* by hot phenol method (La-Grange *et al.,* 1996) whereas total cellular RNA from mycelia of *T. reesei* grown on liquid culture was isolated by Taina et al. (1993). Li and Ljungdahl (1994) purified RNA for making cDNA from *A. pullulans*. Endoglucanase production was also found repressed or induced when glucose or xylan was used a carbon source respectively (Ximenes *et al.,* 1996; Malik *et al.,* 1996).

RT-PCR

Reverse transcription was performed on RNA obtained from *T. harzianum*. First strand cDNA synthesis was done by using oligo (dT)18 primer from MBI Fermentas. It was directly applied to PCR for amplification in which sequence specific primers for endoglucanase genes were used. It is clearly demonstrated from Figure (2) that significant amplification was achieved from the cDNA. Expected fragment size of endoglucanase gene was 1503bp. The corresponding bands were excised from gel and purified for ligation in pUC18. Same strategy was used by Li ans Ljungdahl, 1994. They purified RNA to make cDNA from *A. pullalans*. Similarly LA Grange et al. (1996) isolated xylanase gene from *T. reesei*. They synthesized first strand of cDNA with the help of reverse transcription and amplified it through RT-PCR. Isolation of cellulase genes from *Trichoderma* and *Agaricus* species by PCR based strategy using degenerate primers has also been reported (Jia et al. 1999). Negative amplification of the genes with glucose and positive amplification with CMC and xylanase as carbon source growth medium was also supported by work done by Bin et al. (1996). They found that the expression of endoglucanase gene was regulated at transcription level by either cellulose or one of its degradative products.

Fig 2 RT-PCR of RNA isolated from *T. harzianum* grown on xylan.

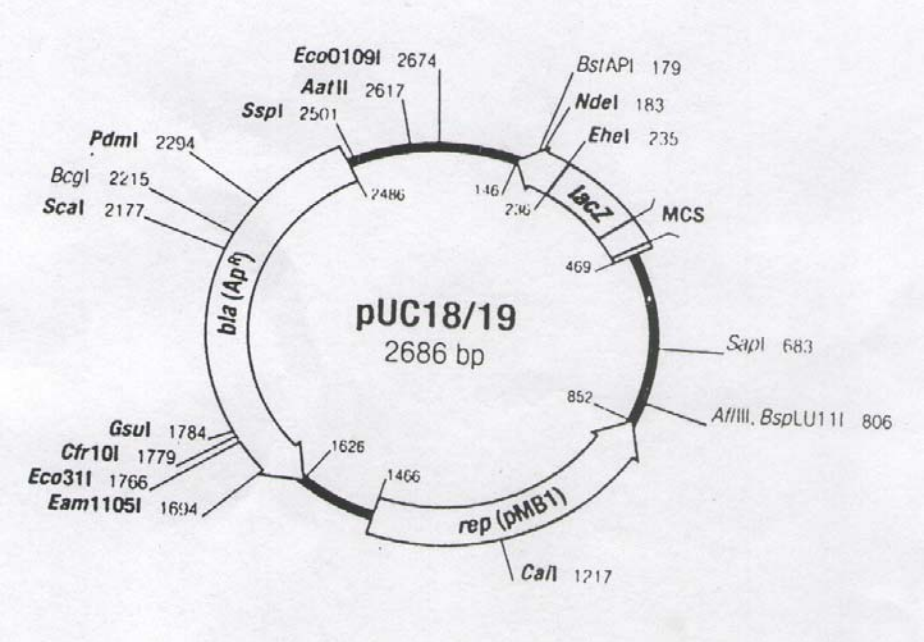

Fig 3 Restriction map of pUC18.

Ligation and Transformation

The amplified endoglucanase gene was ligated into MCS (SmaI) of pUC18 plasmid (Fig. 3) with the help of DNA ligase. The ligated products were transformed into *E. coli* (10 b) competent cells (Fig. 4). Earlier, cellulase genes have been cloned into *E. coli* to get better production of large quantities of pure cellulases by Benguin et al. (1987). Migheli et al. (1998) studied nine transformants of *Trichoderma longibrachiatum* with extra copies of egl1 gene for endoglucanse production. Similarly Blanco et al. (1998) cloned the gene cellA, encoding an endoglucanase from strain *Bacillus* sp. BP-23 and expressed it in *E. coli*.

Endoglucanase gene from *Trichoderma harzianum* was therefore successfully amplified with the help of RT-PCR and cloned into *E.coli*.

Fig 4 Colonies of *E. coli.* having cloned endoglucanase gene.

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