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ISOLATION OF A XYLAN DEGRADING GENE FROM GENOMIC DNA LIBRARY OF A THERMOPHILIC FUNGUS *CHAETOMIUM THERMOPHILE* ATCC 28076

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Abstract

The most abundant hemicellulosic polymers xylans, constitutes about 20-40% of total plant biomass. Xylanases are involved in fruit softening, seed germination and plant defense systems. In this study, genomic DNA of *Chaetomium thermophile* ATCC 28076 was isolated and a genomic library was created in *E.coli* DH10B-pUC 19 host vector system. Positive clones were screened by Congo red staining. The *E.coli* harboring xylanase gene showed clear zone with Congo red clearance assay on xylan plates.

Introduction

Plant cell walls are the most abundant renewable source of fermentable sugars on earth (Himmel *et al*., 1999) and are the major reservoir of fixed carbon in nature (Yang *et al*., 2007). Plant biomass comprises on average 23% lignin, 40% cellulose, and 33% hemicellulose by dry weight (Sa-Perriera *et al*., 2003). Biomass is an alternative natural source for chemical and feedstocks with a replacement cycle short enough to meet the demand in the world fuel market (Kulkarni *et al*., 1999). Complete degradation of the complex heteropolysaccharides requires the action of several main-chain and side-chain-cleaving enzymes such as endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan 1, 4-β-xylosidase, E.C. 3.2.1.37), α-glucuronidase (α-glucosiduronase, E.C. 3.2.1.139), α-arabinofuranosidase (α-L-arabinofuranosidase, E.C. 3.2.1.55) and acetylxylan esterase (E.C. 3.1.1.72) (Choi *et al*., 2000).

The most abundant hemicellulosic polymers are xylans, made up of β-1,4-linked xylose units. Xylan constitutes about 20-40% of total plant biomass (Ninawe *et al*., 2008). Xylan represents an immense resource of biopolymers for practical applications accounting for 25-30% of the dry biomass of woody tissues of dicots and lignified tissues of monocots and occurs up to 50% in some tissues of cereal grains (Moure *et al*., 2006). Among the annual plants, hard woods and softwoods contain 20-25% and 7-12% xylan, respectively (Whistler & Richards, 1970). Xylan has a high potential for degradation to useful end products.

Endo-β-1, 4 xylanases catalyze the hydrolysis of the backbone of xylan to produce xylooligosaccahrides, which in turn can be converted to xylose by β-xylosidase (Zhang *et al*., 2007). Currently, xylanases and cellulases together with pectinases account for 20% of the world enzyme market (Polezeli *et al*., 2005). One of the most important large-scale biotechnological applications of recent years is the use of xylanases as bleaching agents in pulp and paper industry (Szendefy *et al*., 2006).

The fungi are eukaryotic, achlorophyllous and heterotrophic thallopytes comprising about 1.5 million species, of which only 74,000 species are described (Hawksworth, 2001; Jamil *et al*., 2007). Filamentous fungi have been used for more than 50 years in the production of industrial enzymes (Dalboge, 1997). A number of fungal species are known for the production of xylanases such as *Aspergillus niger*, *Sporotrichum* spp., *Chaetmoium thermophile* and *Trichoderma harzianum* (Ahmed *et al*., 2005; Jamil *et al*., 2005). *Chaetomium thermophile*, a thermophilic fungus is well known for the production of xylanases (Hakulinen *et al*., 2003; Latif *et al*., 2006; Katapodis *et al*., 2007). *Escherichia coli* is commonly used for cloning (Saadia *et al*., 2008) or for the construction of genomic DNA libraries for the isolation of fungal xylanases. We report here construction of *C. thermophile* ATCC 28076 genomic library in *E. coli* for the isolation of a xylan degrading gene.

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.

Plasmid: The plasmid pUC19 predigested with *Bam*H1 was obtained form Fermentas. This plasmid carries *Lac Z* promoter and ampicillin resistance gene**.**

Microorganism and Culture conditions: *Chaetomium thermophile* ATCC 28076 was used in this study. For the isolation of genomic DNA the fungus was grown in 500 mL Erlenmeyer flask containing 100 mL of Eggins & Pugh medium in which the concentrations of the nutrients were g/L; 1.0 KH_2 PO_4, 0.5 KCl, 0.5 $(NH_4)_2$ SO_4, 0.2 $MgSO_4$. $7H_2$ O, 0.1 $CaCl_2$. $2H_2$ O, 0.5 L-asparginine, 0.5 yeast extract and 1% glucose was added as a carbon source (Eggins & Pugh, 1962). The pH of the medium was adjusted to 5. The culture was grown for 4 days at 45^{o}C with shaking at 150 rpm. Culture was harvested by centrifugation at 10,000 rpm for 20 min., at 4^{o}C (Ahmed *et al*., 2007). The resulting pellet was used for DNA extraction.

Genomic library construction and screening: *Chaetomium thermophile* ATCC 28076 genomic DNA was isolated by Fermentas DNA extraction kit following the manual instructions. Genomic DNA of *C. thermophile* was partially digested by *Bam*HI and the resulting fragments were cloned in pUC19/*E. coli* DH10B host-vector system to construct genomic DNA library. The tranformants were screened on LB-Amp-xylan-plates (containing 100 µg/mL ampicillin and 0.1% (w/v) oat spelt xylan). After 20 h of incubation at 37^{o}C, the colonies were lifted off with Whatman No 1 filter paper disc and stained with Congo red followed by destaining with 1 *M* NaCl (Teather & Wood, 1982). The positive clone was isolated by alkaline lysis method and tested for xylanase activity.

Xylanase assay: Xylanase activity was assayed using 1% (w/v) of birchwood xylan as a substrate. Reaction mixture contained 1 mL of appropriately diluted enzyme and 1% xylan in citrate phosphate buffer. The mixture was incubated at 50^{o}C for 30 min. After predetermined periods the releasing sugars were estimated with 3, 5-dinitrosalysilic acid using xylose as standard (Miller, 1959). One unit of xylanase activity was defined as the amount of enzyme that released 1 µmol reducing sugars equivalent xylose per min^{-1}.

Results and Discussions

Cloning of genomic DNA in *E. coli*: DNA was isolated from *C. thermophile* ATCC 28076 grown in Eggins & Pugh medium for 4 days (45°C, shaking at 150 rpm) with 1% glucose as carbon source. For the construction of genomic DNA library both quantity and quality of DNA are extremely important. Using the current method of genomic DNA extraction by Fermentas DNA extraction kit high quality DNA was obtained. It has been found in this study that it is crucial to start with fresh mycelium for DNA isolation. Cells were kept frozen after mycelium was disrupted in liquid nitrogen. The isolated DNA was of high quality and good yield with no apparent degradation (Fig. 1). Genomic DNA was also treated with RNAses to remove RNA contamination.

Genomic DNA was digested with *Bam*HI and the resulting fragments of size 0.5-2 kb were separated by agarose gel electrophoresis. A library of these fragments was constructed by ligation to *Bam*HI digested pUC19 with T_4 DNA ligase.

Screening of xylanase activity: In order to isolate a xylan degrading gene, genomic library of *C. therm*ophile ATCC 28076 in pUC19 was intensively screened for xylanase positive clone. Positive tranformants were screened by ampicillin and oat spelt xylan. Congo red staining showed presence of clear zones of hydrolysis around colonies suggesting that xylan hydrolysis was obtained (Fig. 2).

Earlier different researchers have used pUC18/19 vector for cloning of xylanase genes in *E. coli* successfully. Basaran *et al*., (2001) used pUC19 as expression vector for cloning and characterization of xylanase gene from *Pichia stipitis*. They screened xylanase activity from the recombinant pUC19 containing xylanase gene on RBB-xylan plate and found that recombinant pUC19 having xylanase gene produced 10 times more xylanase than the culture fluid of *Pichia stipitis*. Quereshy *et al*., (2000) isolated a xylanase gene from genomic library constructed in pUC19 from *Bacillus circulanas*. The supernatant produced by *E. coli* harboring recombinant plasmid had 79 U of xylanase activity whereas intracellular fraction showed only 10 U indicating that 88% of the enzyme was secreted extracellularly. Huang *et al*., (2006) cloned a 642 bp xylanase gene from *Bacillus subtilis* in pUC18. They screened xylanase gene by Congo red method. Crude recombinant xylanase obtained from *E. coli* showed high xylanase activity. Majority of xylanase activity (49.6%) was found in the periplasm, 22.4% of the activity was extracellular while 28% was associated with cytoplasmic fractions. Similarly, a 981 bp *xln*C gene from *Aspergillus nidulans* was ligated in plasmid pUC18 and transformed into *E. coli* for the expression of xylanase gene (MacCabe *et al*., 1996).

Recombinant xylanase activity: A positive recombinant clone containing xylan degrading gene was obtained. This positive transformant was designated as pUC19-*xyn* and tested for xylanase activity. Extracellular and intracellular xylanase activity from pUC19-*xyn* was 2.13 ± 0.10 IU/mL and 1.40 ± 0.80 IU/mL, respectively.

E. coli has been used for the construction of genomic libraries for the isolation of fungal xylanase genes. Kimura *et al*. (2000) isolated a *xynA* gene from *Penicillum* sp. 40 via construction of genomic DNA library in *E. coli* DH5α. The structural part of *xynA* gene was found to be 721 bp. Xylanase genes have been isolated from genomic library of *Helminthosporium turcicum* and *Helminthosprium turcicum* (Defugu *et al*., 2001; Defugu *et al.,* 2004). Likewise *xynF*3 gene was isolated from genomic library of *Aspergillus oryzae* KBN616 (Kimura *et al*., 2002).

Fig. 1. DNA extraction by Genomic DNA Extraction kit (Fermentas). Lane 1, 2: *C. thermophile* DNA, Lane 3: Marker.

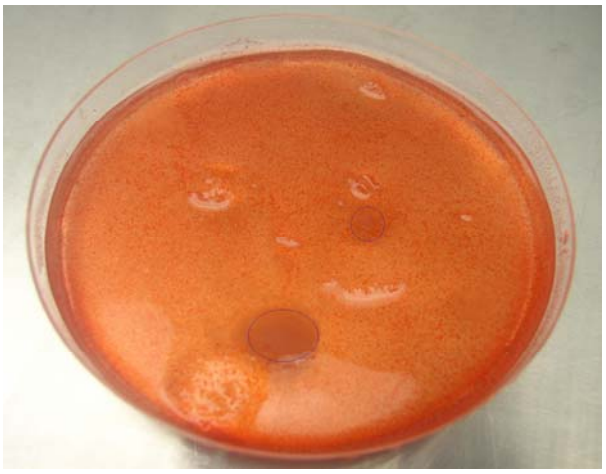

Fig. 2. Xylan hydrolysis by recombinant vectors on LB-Xylan plates. Xylanase plate assay of transformed *E. coli* cells containing *C. thermophile* xylanase gene. Congo red staining of agar plate containing 1% oat spelt xylan and distaining with 1 *M* NaCl revealed halos representing xylanase activity.

Although several yeasts and fungi are now used as hosts for fungal xylanase gene expression, *E. coli* will continue to be used for the construction of genomic DNA libraries for the isolation of xylanase genes. Improvement in vectors and *E. coli* strains will help increase cloning of fungal xylanase genes. We have successfully isolated a xylan degrading gene from genomic DNA library of *C. thermophile* ATCC28076. Further studies will be focused on characterization and expression of this gene.

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