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Isolation and Characterisation of Cellobiohydrolase Gene from *Aspergillus terreus* SUK-1

Nik Marzuki Sidik, Siti Nurmi Nasir, Siti Nurbayan Rusiman and Shaiful Adzni Sharifuddin

School of Biosciences and Biotechnology, Faculty of Science and Technology 43600 Universiti Kebangsaan Malaysia (UKM), Bangi, Malaysia.

Abstract: *Aspergillus terreus* SUK-1 isolated from oil palm waste was reported to produce cellulolytic enzymes for the degradation of cellulosic materials. RNA Ligase Mediated Rapid Amplification of cDNA Ends methodology was used to facilitate the isolation and cloning of a full length cDNA. The cDNA sequence consists of 1623 nucleotides complete coding sequence encoding a cellobiohydrolase of 540 amino acids. Analysis showed that the gene had high identity with various cellobiohydrolase genes and proteins. Catalytic and cellulose binding domains were detected by Pfam and the NCBI Conserved Domain Search. Study of the gene expression showed that the highest gene expression was seen 24 h after fungal inoculation, and then the expression was subsequently reduced until the fifth day when the fungus reached the stationary growth phase. Addition of glucose into growth media completely suppressed the gene expression. The gene was designated as *CBHat1* and the GenBank accession number is AY864863.1.

Key words: *A. terreus* SUK-1, cellobiohydrolase, RLM-RACE, RT-PCR, expression.

INTRODUCTION

Cellulose is the world's most abundant renewable biomass and the major component of plant cell walls (Lynd, 2002). Biodegradation of cellulose is catalysed by the cellulase enzyme complex composed of endoglucanase, cellobiohydrolase (exoglucanase) and β-glucosidase. Numerous fungal cellulases have been studied, and genes encoding cellobiohydrolase were isolated from *Fusicoccum* sp. (Kanokratana *et al*., 2008), *Talaromyces emersonii* (Murray, *et al*., 2003) and *Chrysosporium lucknowence* (Gusakov, *et al*., 2005). Some cellobiohydrolase genes have been cloned, and recombinant cellobiohydrolases have been produced (Kanokratana *et al*., 2008; Dienes *et al*., 2006; Voutilainen, *et al*., 2007). A global economical demand for cellulase and cellobiohydrolase in particular is justified. Cellulases are used in a wide range of industries such as textiles (Ibrahim, *et al*., 2005; Miettinen-Oinonen, *et al*., 2005), pulp and paper (Xu, *et al*., 2009), animal feed (Ng, *et al*., 2002), detergent (Anish, *et al*., 2007) and biofuel (Sukumaran, *et al*., 2009).

Aspergillus terreus SUK-1 is a local fungal strain isolated from palm oil waste sludge. The presence of extracellular cellulolytic activities from *A. terreus* SUK-1 was reported by Omar *et al*., (1984) and Kader *et al*., (1988). In fact, it was suggested that *A. terreus* SUK-1 had better cellulolytic activities for complete degradation of cellulose than other isolated cellulases due to high activity of β-glucosidase in the system (Omar, *et al*., 1984). Since this result was first reported (Omar, *et al*., 1984), study of this fungus has been limited. *A. terreus* SUK-1 has only recently been studied for application in facilitating composting and partial degradation for animal feed production from oil palm empty fruit bunches. These two projects only recently commenced, and have yet been published.

The cellobiohydrolase of *A. terreus* SUK-1 has never been isolated and studied in detail. Here, we report the isolation of a cDNA fragment containing a complete coding sequence for cellobiohydrolase via the RNA Ligase-Mediated Rapid Amplification of cDNA Ends (RLM-RACE) approach (Gubler and Hoffman, 1983). The expression pattern of the cellobiohydrolase gene during a growth period of 7 days was also reported.

MATERIALS AND METHODS

Preparation of Fungal Strain:

The *A. terreus* SUK-1 culture used for this study was obtained from School of Bioscience and Biotechnology, Universiti Kebangsaan Malaysia, Malaysia. It was maintained on Potato Dextrose Agar (PDA) and subcultured every 3 months. The fungus was first inoculated into a broth medium containing glucose and minerals and incubated overnight at 30 °C while shaking at 250 rpm. A sample of 12.5 ml of the overnight culture was transferred into a 1-L conical flask containing 500 ml of growth medium (Mandel and Reese, 1957) and then incubated at 30 °C with shaking at 250 rpm.

Corresponding Author: Nik Marzuki Sidik, School of Biosciences and Biotechnology Faculty of Science and Technology 43600 Universiti Kebangsaan Malaysia, Malaysia. Ph: +60389215998/5955 Fax: +603 80252698 E-mail: nms@ukm.my

RNA Isolation and cDNA Synthesis:

A three-day culture of cells was harvested by filtering through nylon gauze. The harvested biomass was then frozen and ground. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and used to generate cDNA with SuperScript™ cDNA Synthesis Systems (Invitrogen).

Reverse Transcription-PCR and Cloning:

A reverse transcriptase-polymerase chain reaction (RT-PCR) approach was used to clone the gene. Two primers were synthesised based on information obtained from Genbank (http://www.ncbi.nlm.nih.gov/) about the conserved regions of CBH genes. The sense primer was CBHf1, 5'GTCATTGACGCCAACTGGCGCTGG3', and the antisense primer was CBHr1, 5'ACGCTCCCAGCCCTCAACGTTGG3'. The PCR cycles for amplification were: pre-denaturing at 94 ºC for 3 min. for one cycle; then 94 ºC for 30 sec, 65 ºC for 45 sec and 72 ºC for 1 min. for 30 cycles; and finally, 72 ºC for 10 min for one cycle. The PCR product was then separated on a 1% agarose gel, stained with ethidium bromide and visualised under UV light. The amplified DNA was then purified using QIAquick Gel Extraction Kit (Qiagen). The purified PCR product was cloned into pCR®II-TOPO (Invitrogen) and transformed into *E. coli* DH5*α* made competent as described by Nishimura *et al*. (1990).

RLM-RACE and Cloning of 5' and 3' end of CBHat1:

The GeneRacer™ Kit (Invitrogen, USA) was used in the amplification of 5' and 3' ends of CBH. Experiments were conducted according to the recommendation protocols of the supplier. The purified PCR products were then cloned into pCR®II-TOPO (Invitrogen) and transformed into *E. coli* DH5*α* as mentioned.

DNA Sequencing and Analyses:

Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen) and sent for sequencing. The sequences were edited with BIOEDIT (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The sequences were then analysed with the Blast program (http://www.ncbi.nlm.nih.gov/BLAST/) and the NCBI Conserved Domain Search. The Pfam protein families database (http://www.sanger.ac.uk/software/pfam) was used to search the sequences for conserved domains. Sequence analysis for gene prediction was done using GENSCAN (http://genes.mit.edu/GENSCAN.html).

Expression of CBHat1:

Total RNA of growing *A. terreus* SUK-1 was isolated 7 hours after inoculation and subsequently every 24 hours after inoculation for 7 days. Total RNA was also isolated from *A. terreus* SUK-1 grown in Mandel's medium in the presence of 0.04 M glucose. A reverse transcriptase-polymerase chain reaction (RT-PCR) approach using gene-specific primers was used to study the expression of CBH gene. A total RNA sample of 1 µg was then used to generate cDNA with the SuperScript™ cDNA Synthesis System (Invitrogen). The sense primer was CBHf1, 5'GTCATTGACGCCAACTGGCGCTGG 3', and the antisense primer was CBHr1, 5'GGGCTCCCAGCCCTCGACATTGG 3'. The PCR cycles used for amplification were as described above. A housekeeping gene, E*f1α* for transcriptional elongation factor 1-α was also amplified as a control.

RESULTS AND DISCUSSION

The designed primers successfully amplified a DNA band of 486 bp. Amplification from the 5' end yielded a 635 bp sequence, while amplification from the 3' end yielded a 1609 bp sequence. The sequences were aligned with BIOEDIT to obtain a full sequence alignment, of 1843 bp, which includes the 5' and 3'UTRs. The complete cDNA sequence was analysed with GENSCAN, which predicted a single-exon gene beginning at nucleotide 70 and ending at nucleotide 1692, giving a complete coding sequence of 1623 bp for the CBH gene (Figure 1). The putative signal sequence is located between amino acid Met1-Ala23. This gene was designated as *CBHat1* (GenBank Accession number AY864863).

Analyses of the CBHat1 gene and protein sequences matched closely with the sequences of cellobiohydrolase genes and proteins from various sources. The CBHat1 gene of *A. terreus* SUK-1 had 97% sequence identity to cellobiohydrolase gene from *A. terreus* NIH2624 and 82% sequence identity to cellobiohydrolase genes from both *Neosartorya fischeri* NRRL 181 and *A. fumigatus* Af293. A 78% identity match was also observed between nucleotide sequences for *CBHat1* and cellobiohydrolase genes from both *P. decumbens* and *P. oxalicum* (Table 1). In addition, the CBHat1 protein of *A. terreus* SUK-1 was 94% matched to the cellobiohydrolase protein from *A. terreus* NIH2624. High identity match or 74-79% was also observed between CBHat1 protein and cellobiohydrolase protein from various sources such as *A. fumigatus* Af293, *M. fischeri* NRRL 181, *P. aurantiacus*, *P. axalicum* and *P. chrysogenum* (Table 1).

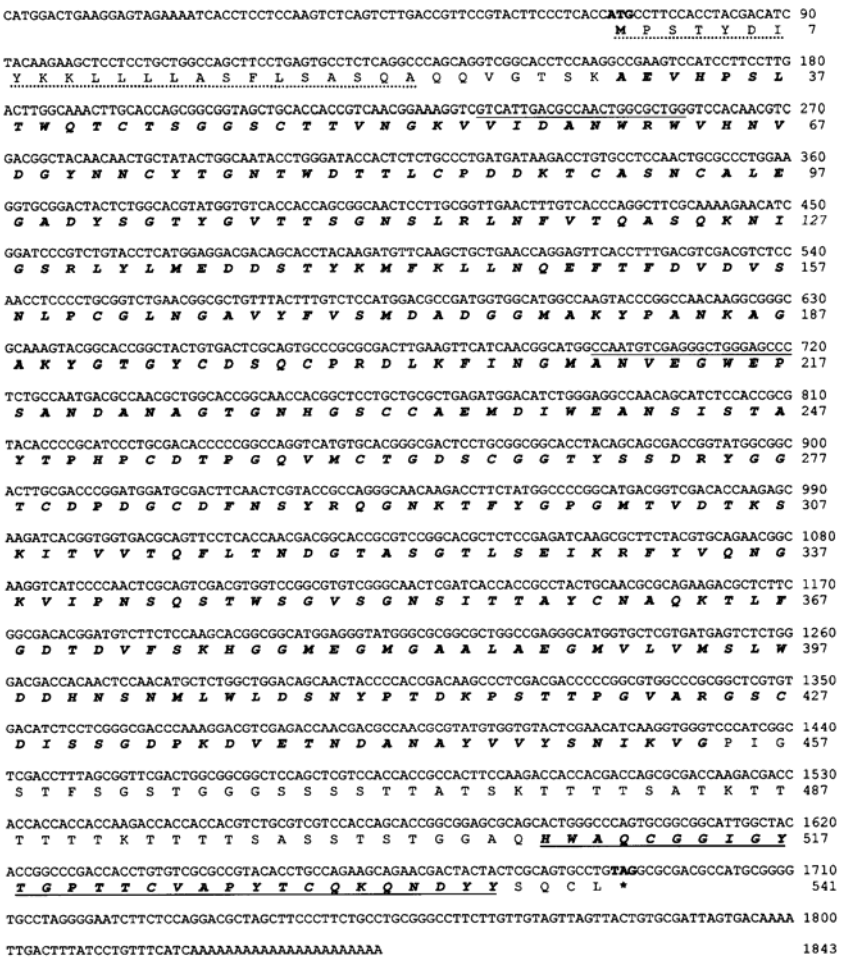

Fig. 1: Nucleotide and deduced amino acid sequences of *CBHat1*. Nucleotide and amino acid positions in *CBHat1* are numbered on the right. The putative signal sequence (residues Met1-Ala23) are indicated by dotted line. The catalytic domain is indicated by italic and bold letters. The CBD region is shown by italic, bold letters and underlined. Annealing sites for the CBHf1 and CBHr1 primers are underlined in the nucleotide sequence. The start (ATG) and stop (TAG) codons are shown in bold letters. * indicates the stop codon.

Table 1: Sequence similarity matches between *CBHat1* gene and protein to other registered cellobiohydrolase genes and proteins.

Gene/Protein	Identity (%)	Organism	GenBank Accession Number
Gene			
Exoglucanase 1 precursor mRNA	97	*A. terreus* NIH2624	XM_001214180
Glycosyl hydrolase family 7 protein mRNA	82	*Neosartorya fischeri* NRRL 181	XM_001258277
1,4-beta-D-glucan-cellobiohydrolyase mRNA	82	*A. fumigatus* Af293	XM_745951
Glycosyl hydrolase family 7 protein mRNA	79	*A. clavatus* NRRL 1	XM_001268256
Cellobiohydrolase gene	78	*P. decumbens*	GQ844299
Exo-cellobiohydrolase (*cbhI*) mRNA	78	*P. oxalicum*	EU727171
Protein			
Exoglucanase 1 precursor	94	*A. terreus* NIH2624	XP_001214180
1,4-beta-D-glucan-cellobiohydrolyase	79	*A. fumigatus* Af293	XP_751044
Cellobiohydrolase	78	*N. fischeri* NRRL 181	XP_001258278
Cellobiohydrolase	77	*A. clavatus* NRRL 1	XP_001268257
Exo-cellobiohydrolase	75	*P. chrysogenum*	AAV65115
Exo-cellobiohydrolase	74	*P. oxalicum*	ACE60553
Cellobiohydrolase precursor	79	*T. aurantiacus* var. levisporus	AAW27920
Cellobiohydrolase I	78	*T. aurantiacus*	AF478686_1

Domain search was done with NCBI Conserved Domain Search and Pfam. Both platforms identified two types of domains consisting of a set of similar separate catalytic domains of the glycosyl hydrolase family 7 (core protein) and a set of cellulose-binding domains (CBD) separated by a linked peptide. The catalytic domain has been identified as extending from amino acids Ala31 to Gly454, while the cellulose-binding domain was located between amino acids His508 and Tyr536 (Figure 1). Glycosyl hydrolases are a widespread group of enzymes that hydrolyse the glycosidic bonds between two or more carbohydrates, and the glycosyl hydrolase family 7 comprises enzymes with several known activities, such as endoglucanase and cellobiohydrolase. Therefore, *CBHat1,* like other isolated cellobiohydrolase proteins (18-20), consists of a catalytic domain responsible for the hydrolysis reaction and a cellulose-binding domain mediating the binding of the enzyme to the substrate.

The expression study of *CBHat1* showed that the CBHat1 mRNA transcript was not detected up to 7 h after inoculation. The transcript was detected and reached its highest level of expression 24 h after inoculation. The level of expression decreased beyond 24 h and continually to decrease up through 96 h after incubation. A sharp decrease in the level of transcript was observed at 120 h, and the level of transcript fluctuated after 120 h (Figure 2).

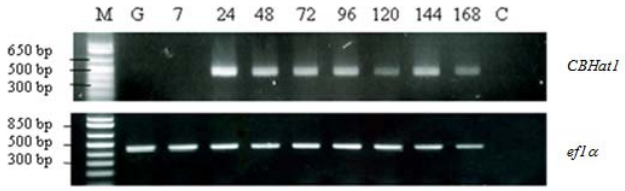

Fig. 2: Expression of *CBHat1* (top) and transcriptional elongation factor 1-α gene (*ef1α*) (bottom) of *A. terreus* SUK-1 in Mandel's medium. M, 1 kb DNA ladder (Invitrogen); G, *A. terreus* SUK-1 in Mandel's medium in the presence of glucose. Numbers on the top of the figure indicate incubation times in hours. Size of DNA markers (bp) is shown on the left.

Early expression of the cellobiohydrolase gene at a high level is required for *A. terreus* SUK-1 to utilise carbon sources for living. Cellobiohydrolase catalyses the production of cellobiose residues from cellulose chains, which are subsequently catalysed by β-1,4 glucosidase to produce glucose monomers (Teeri, 1997; Zhang *et al*., 2006). The level of gene expression is gradually reduced as more and more cellobiose subunits accumulate. Few studies have shown that cellobiohydrolase activity is reduced or repressed by its product, i.e., cellobiose. Niranjane *et al.* (2007) showed that addition of cellobiose to the growth medium reduced expression of the cellobiohydrolase gene in *Phlebia gigantea*. In *A. niger*, cellobiohydrolase gene expression was induced in the presence of cellobiose. However, gene expression was suppressed when cellobiose was present in a greater excess (>5%) than the amount expected to be released from complex carbon sources during growth (Hanif *et al*., 2004). In contrast, induction of cellobiohydrolase gene expression by cellobiose was also reported by Suto and Tomita (2001) and Lynd *et al.* (2002) at a lower concentration.

In addition, there have been reports showing that cellobiohydrolase activity was also repressed by the final product of cellulase degradation of cellulose, i.e. glucose. Figure 2 shows that CBHat1 gene expression was not detected when *A. terreus* SUK-1 was grown in glucose-containing media. Similarly, Hanif *et al,* (2004) showed that exogenous supply of glucose inhibited the synthesis of the cellobiohydrolase enzyme in cultures of *A. niger* grown on wheat bran. Lockington *et al.* (2002) reported that the cellobiohydrolase gene transcript was not detected when *A. nidulans* was cultured in the presence of glucose. In *Penicillium decumbens*, the transcriptional level of the cellobiohydrolase gene was lower under glucose-repression conditions (Sun *et al*., 2008). A similar effect of glucose on cellobiohydrolase gene expression was also reported (Niranjane, *et al*., 2007; Beguin, 1990; Bhiri, *et al*., 2010). These modes of inhibition and/or repressions may explain the sharp reduction of gene expression at 120 h. However, in this study, the accumulation of neither cellobiose nor glucose was measured. We proposed that further studies be conducted in order to observe the inhibition effect of cellobiose and glucose on cellobiohydrolase activity in *A. terreus* SUK-1.

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Conclusion:

We have isolated a complere coding sequence of cellobiohydrolase gene, *CBHat1* of 1623 bp encoding 540 amino acids. Both nucleotide and amino acid sequences showed high similarity toward published cellobiohydrolase gene and protein sequences. *CBHat1* expression was induced by the substrate and suppressed by end product.

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