

## Protein and Sugar Assay, Characterization and Expression Endoglucanase Gene in *Trichoderma longibrachiatum* 36MS and *Aspergillus terreus* 31MS

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**Abstract:** In this study CMCase activity of different fungal species was investigated. Several fungal isolate from cultural soils were screened to CMCase activity in CMCase medium. The released sugar and protein in culture medium were assayed which started four days after inoculation for one month. The proteins in culture medium were precipitated by addition solid ammonium sulphate. The highest CMCase activity was observed 13 day after inoculation for *Trichoderma longibrachiatum* 36MS and *Aspergillus terreus* 31MS. A full-length cDNA of the  $\beta$ -1,4- Endoglucanase gene were isolated and sequenced. Endoglucanase gene of *T. longibrachiatum* 36SM and *A. terreus* MS31 have 1386 bp and 1416 open reading frame fragment that which encode to 462 and 472-amino-acid proteins respectively. Further bioinformatics information showed that *T. longibrachiatum* species with high cellulose activity has 7 protein prosites and two domain and *A. terreus* has 4 protein prosites and two domains. The results indicate that *T. longibrachiatum* 36MS and *A. terreus* MS31 have a high potential for industrial application and biotechnological manipulations.

**Key words:** *T. longibrachiatum* • *A. terreus* • Endoglucanase gene • CBD • CMCase activity

### INTRODUCTION

Cellulose is the most important polysaccharide in cell wall composition and is one of the most abundant hydrocarbon in nature. Biological digestion of cellulose happens gradually in soil [1]. A complex of cellulase acts synergistically and breaks  $\beta$ -1, 4 linkages of cellulose chain. At least three cellulase enzymes which named  $\beta$ -glucosidase (EC 3.2.1.21), endoglucanase (EC 3.2.1.4) and exoglucanase (EC 3.2.1.91) are taken part in cellulose biodegradation [2, 3]. Some microorganisms with their enzymatic activity can convert lignocellulosic materials that could be used for energy source [4]. There are more than hundred fungal species which are capable to grow on cellulose as the sole source of carbon but some of them could secrete cellulose enzymes to cellulose degradation [5]. We have reported that *Fusarium solani*,

*Trichoderma viride*, *T. reesei*, *T. konningii* and *Aspergillus terreus* can produce cellulose with high activity too [6]. Screening of soilborn fungi for cellulose activity result to *Trichoderma harzianum* B17 and *Aspergillus sp.* A23 isolates showed high activity in enzymatic degradation of cellulose [7]. Because of various industrial and commercial applications of cellulose enzymes with microbial sources, they have an important role in current economic value. So, to improve the applications of secreted cellulose in industrial applications, gene technology has been employed. In this study we screened and evaluated three hundred fungal isolates collected from different cultural soils to protein and sugar assay. Also we attempted to identify cellulose isoenzyme based on molecular weight, determining their homology, active domain and protein prosites by different bioinformatics approaches.

## MATERIALS AND METHODS

**Culture and Growth Medium:** Two fungi isolates (*Trichoderma longibrachiatum* 36MS and *Aspergillus terreus* 31MS) with a cellulose high activity selected between several soil born fungal isolate were used in this research. A basic liquid mineral medium with the following composition 0.05 g FeSO<sub>4</sub> 7H<sub>2</sub>O, 0.25 g MnSO<sub>4</sub> H<sub>2</sub>O, 0.25 g CoCl<sub>2</sub>, 0.25 g ZnSO<sub>4</sub>, 0.25 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 g KH<sub>2</sub>PO<sub>4</sub>, 0.25 g MgSO<sub>4</sub> 7 H<sub>2</sub>O, 0.4 g CaCl<sub>2</sub>, 0.3 g urea and 0.2 ml Tween 80 was used for Carboxy Methyl Cellulose (CMC) medium.

**Sugar, Protein Assay and Enzyme Assay:** Five hundred µl of CMC medium in each clean test tube were subjected to released proteins and sugars assays. Concentrations of released fungal extracellular proteins and sugars were determined using Bradford method and Arsenate-Molybdate reagent, respectively [9, 10]. All detected protein bands were incubated in 1.5 ml CMC medium at 37°C for 12 h. Therefore, total released protein and cellulose enzyme activity was determined using Kossem and Nannipieri method.

**Ammonium Sulphate Precipitation and Electrophoresis:** The proteins in medium were precipitated by the addition of solid ammonium sulphate to 80% saturation. The precipitate was allowed to 4°C for 10 h and was collected by centrifugation of 7000 rpm in a cold centrifuge at 4°C for 40 min. The precipitate was dissolved in a minimal amount of 50 mM acetate buffer (pH 5) containing 1 mM EDTA and then dialyzed for 24 h by three times changes Dialysis dissolved Samples in buffer, in 10 KDa diameter pores dialysis packs which stay overnight in the same buffer at pH 4°C on stirrer. To determine molecular weight of cellulase enzymes, a polyacrylamide gel electrophoresis was performed in the absence of SDS in Laemmli system [11].

**RNA Isolation and RT-PCR:** Fungal mycelium induced by CMC after 114 h was subjected to RNA extraction. Total RNAs were isolated using standard phenol-chloroform procedure and were purified using Guanidinethiosulfat RNA purification Kit (TriPure Isolation Reagent, Roch, Germany). Total RNA was quantified using a Nanodrop (ND-100) spectrophotometer and RNA quality was assessed by 1% agarose gel electrophoresis stained by ethidium bromide. First-strand cDNA was synthesized from total RNA using Omniscript® Reverse Transcription System (Qiagen, Germany). Amount of 50-100 ng of the total RNA from the induced hyphae was

used to prepare double-strand cDNA using Qiagenec DNA Kit (Qiagene, Germany) according to manufacturer's protocol.

**Primer Design and PCR Amplification:** Primers used in this study were designed with NTI software. For cDNA amplification, we designed the specific primer of *Trichoderma longibrachiatum* strains: TL1, TL11 (GenBank accession number GU144298.1fp 5'-ATGGCGCCCTCAGTTACACT-3' and rp 5'-GCTACTCGCAATGCCTTTTA-3') and for *Aspergillus terreus*, AT1, AT11 (GenBank accession number XM001217290.1fp 5'-ATGATGTCTCTTCTCAGCAGC3-3' and rp 5'-GTGGTACTCTTAGTGCTGGTAA-3'). PCR was performed on cDNA templates using a lightcycler (Eppendorf, Germany). PCR reactions (5 min 94°C - 35 cycles 1min 57°C 1 min 72°C 1 min 94°C - 10 min 72°C for TL1 & TL11 and 5 min 94°C - 35 cycles 1min 59°C 1 min 72°C 1 min 94°C - 10 min 72°C for AT1 & AT11) using relative primers were followed by electrophoresis of amplicons on agarose 1.5%.

**Sequence Analysis:** DNA extraction and purification was performed using Silica Bead DNA Gel Extraction Kit (Fermenthas, USA) according to manufacturer's protocol. The cDNA of interest were further analysis by DNA sequencing (Macrogen, Korea). To translate the obtained sequences into a deduced amino acid sequence the Bioedit sequence Alignment edit program and Mega4 software was used. DNA sequences were queried against the BLASTN (nucleic acid), TBLASTX (translated protein query search), Swissprot using the NCBI standalone Blast All program [12]. Sequence similarities above 50% with an E value less than 1E<sup>-10</sup> were considered as statistically significant positive matches.

## RESULTS AND DISCUSSION

The growth process was started 12 hours after inoculation. Increasing mycelia mats showed substrate breakdown takes place and fungal isolates can produce extracellular enzymes to prepare their growth requirements. Released extracellular enzymes of both species caused various increase in sugar levels produced from CMC degradation. The investigated isolates shown high growth rate, resulting grand biomass in culture medium which these observation are confirmed by protein and sugar assay. The maximum rates of sugar released in medium correspond to 13<sup>th</sup> day after fungal inoculation for each isolate (Table 1).

Table 1: Rat of sugar and protein release at 13<sup>th</sup> day after induction in CMC medium

Isolates	Sugar assay (g/L)	Protein assay (mg/L)
<i>Trichoderma longibrachiatum</i> 36MS	0.08934	0.08345
<i>Aspergillus terreus</i> 31MS	0.08123	0.07986

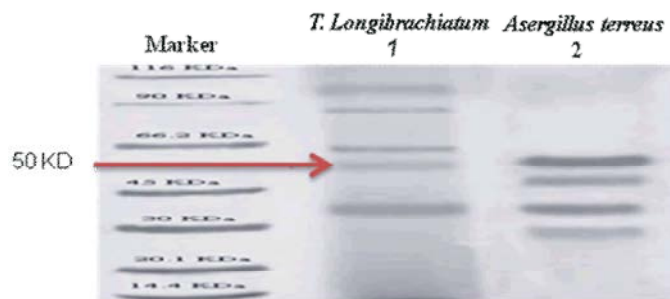


Fig. 1: SDS-PAGE of crude protein concentrated by ammonium sulfate. 12% Polyacrylamide gel was used, stained with Coomassie R-250 brilliant blue. A protein profile produced by *T. longibrachiatum* (lane 1). and *Aspergillus terreus* at CMC (lane 2). M: protein size marker

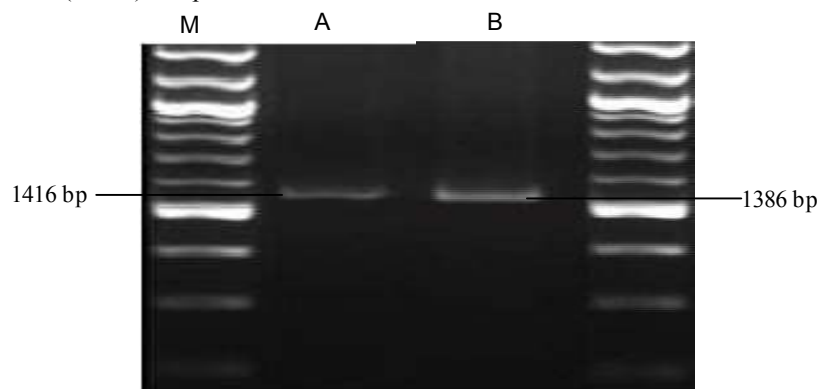


Fig. 2: PCR product using specific primers for *A. terreus* (A) and *T. longibrachiatum* (B) Marker SMO311 (250-20000bp)

As the screening tests results, *A. terreus* MS31 and *T. longibrachiatum* SM 36 highest to cellulose degradation and both could possess immense potential in modifying cellulosic substratum in artificial and natural condition and could use in biotechnology because of their potential value in biotechnology including use as enzyme supplements for livestock and in food and beverage. Protein assays were carried out for two isolates with high cellulose activity. The results revealed that the expression of endoglucanase isoforms was increased on the carbon sources used (Table 1). Protein profiles produced by both strains were visualized using Coomassie R-250 brilliant blue. This profile showed that mass protein corresponded to  $\beta$ -1,3-Endoglucanase enzyme (50kD) are present in both fungi but in *T. longibrachiatum* 36SM the quantity of protein was less than *A. terreus* MS31 isolates (Fig. 1). These results revealed related protein size expected, approximately 50 kD weight in two fungi. In this limit,

*T. longibrachiatum* 36SM showed one feeble protein band while *A. terreus* 36SM has two strong bands indicating more cellulose activity. Protein assays during experiments showed some gradual changes in released protein concentration.

For two highest active species, cDNA molecules synthesized from total RNA were amplified using specific primers. For *A. terreus* MS31 and *T. longibrachiatum* 36MS isolates. Product size of this molecule in *A. terreus* and *T. longibrachiatum* were 1416 and 1386 bp respectively (Fig. 2). Corresponding amplicons were cut from agarose gel then purified to separate DNA molecules of cellulase gene. These products were sequenced then screened for quality. The alignment of resulting sequence obtained from cDNA with that of other  $\beta$ , 1-4 endoglucanase nucleotide sequence in different fungi species showed high similarity. These data confirm the presence of related enzyme in cultural medium in experimental condition. The nucleotide sequence data

Table 2: Prosite name for *A. terreus* and *T. longibrachiatum*

Fungi species	Rate	Number	Prosite function
<i>A. terreus</i>	High	4	N-glycosylation site* Protein kinase C phosphorylation site* Casein kinase II phosphorylation site* N-myristoylation site*
<i>T. longibrachiatum</i>	High	7	N-glycosylation site* cAMP- and cGMP-dependent protein kinase phosphorylation site* Protein kinase C phosphorylation site* Casein kinase II phosphorylation site* Tyrosine kinase phosphorylation site* N-myristoylation site* CBM1 (carbohydrate binding type-1) domain signature*

reported for *T. longiderarum* and *A. terreus* are appeared in the EMBL and NCBI GenBank nucleotide sequence databases with the accession number HQ628311 and JF309108 respectively. According to analyzed whit Pfam site ([www.pfam.sanger.ac.uk](http://www.pfam.sanger.ac.uk)) and Clcbio software, these genes encodes protein which has two domains. Catalytic domain and cellulose-binding domain (CBD).

Bioinformatic analysis of protein sequence obtained showed that two fungal species have different prosite in  $\beta$ , 1-4 endoglucanase protein domains. *T. longideratum* 36MS isolates having high cellulose function showed 7 prosite and *A. terreus* with same cellulose function has 4 prosite. Comparing of these prosite show that in both fungi N-glycosylation site, protein kinase C phosphorylation site, N-myristoylation site and Casein kinase II phosphorylation site are common ( Table 2 ). The two blasts on the protein sequence database of ncbi these two proteins have 99% homology.

In current research we investigated CMCase activity of some soil fungi [6, 13]. We demonstrated that CMC is a good inducer for extracellular CMCase production by the fungi, clearly. Based on these experiences we attempted to develop our project in all isolated which were unknown from cellulose function in our laboratory. So three hundred soil fungi were used to evaluate their potential in cellulose activity. The regulation of cellulase biosynthesis in several fungal has been investigated. These observation demonstrated that formation of EG, AAEG and  $\beta$ - glucosidase are inducible in fungi [14,15] the results also show that two high active isolate is belonged to different genera and the variability for cellulose activity is not noticeable in the same genus. In biotechnology, there is an enormous potential for use of the cellulose-binding domains (CBDs) [16]. This domain of cellulose enzyme, CBD, can increase the effective concentration of enzyme on insoluble cellulose substrates [5]. In this study to further recognition of released protein structure, sequence analysis of corresponding cDNA was done. Based on molecular mass of secreted protein, the expression of endoglucanase (EG) in both fungi was inducible. These finding suggest that the possibility that

despite sugar and protein assay could use to screen fungi to enzyme activity though gene expression analysis of one of the three important genes may be exploited to select the suitable fungi industrial application. The prosite analysis of two protein sequences showed different part in studied domain. So this result indicate that numerous of prosite can determine the cellulose activity. For more investigation of cellulose activity of fungi in different medium conation various easily metabolizable carbon source such as glucose. Though, the fructose, a furanose form of sugar is recommended [17]. Also, two obtained cDNA were similar to that are submitted in Genbank. In this research, protein analysis by SDS-PAGE on all native and active isolates in north of Iran could lead to access to a greatgenetically resources of gene responsible to cellulose degradation and recognize the isolates with punctual mutation which are compatible to natural growth condition having high cellulose activity.

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