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Cloning and Expression of Cellulase Gene from *Trichoderma reesei* in to *Escherichia coli*

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A gene encoding cellulase enzyme involved in carboxymethylcellulose (CMC) degradation was isolated and sequenced from the ûlamentous fungus, *Trichoderma reesei.* A DNA fragment of 764bp conferring cellulase activity was cloned in *E. coli* BL21 and it contained a 726 bp ORF which encoded a protein belonging to glycosyl hydrolase family 45 and was classified as endoglucanase. It showed a maximum cellulase activity of 11.75 IU/g, at pH 5.5 and at 37°C. The SDS–PAGE and zymogram analyses showed a protein band with a molecular weight of about 24,420 Da confirming endoglucanase expression.

Key words: Cellulase, Endoglucanase gene, cDNA, *E.coli* BL21 and *Trichoderma reesei.*

Traditionally hydrolysis of cellulose by fungal cellulase systems is thought to require the cooperative action of endoglucanases (EC 3.2.1.4, endo-b-D-1,4-glucanases) (EG), cellobiohydrolases (EC 3.2.191) (CBH) and β-glucosidases (EC 3.2.1.21) (BGL). Endoglucanases belong to the cellulose degrading enzymes and hydrolyse the β -D-1,4-linkages in cellulose chain. In addition to cellulose, at least the glucose to mannose and glucose to glucose linkages in glucomannan are hydrolysable by endoglucanases. Substrate specificity of enzymes vary depending on their structural features and endoglucanases from certain glycoside hydrolase (GH) families are able to hydrolyse hemicelluloses (Atte *et al.,* 2013). *T. reesei* (teleomorph *Hypocrea jecorina*) is a mesophilic soft-rot ascomycete fungus that is widely used in industry as a source of cellulases and hemicellulases for the hydrolysis of plant cell wall polysaccharides. However, it was subsequently shown to be the anamorph of the pantropical ascomycete Hypocrea jecorina (Kuhls *et al.,* 1996). Endoglucanases are the main components of the *T.reesei* cellulase system and they comprise <10% of the secreted proteins of the organism (Heikinheimo and Buchert, 2001). *T. reesei* expresses five endoglucanases, Cel5A (EG II) (Saloheimo *et al.*, 1988), Cel7B (EG I) (Penttila *et al.,* 1986), Cel12A (EG III) (Ward *et al.,* 1993; Okada *et al.*, 1998), Cel45A (EG V) (Saloheimo *et al.*, 1994) and Cel61A (EG IV) (Saloheimo *et al.*, 1997) (EC 3.2.1.4). A low molecular weight endoglucanase has previously been described (Karlsson *et al.*, 2002). The high capability of digesting cellulosic materials by this microorganism has attracted a great deal of attention to investigations on cellulase system. Now, it has enjoyed a long history of safe use for industrial enzyme production (Nevalainen *et al.*, 1994) and as an important model system for studying lignocellulosic deconstruction (Diego Martinez *et al.*, 2008). Here in this study, we isolated

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and cloned a cellulase gene from *T. reesei* SJVTR which might provide further understanding on the cellulase system in this strain.

METHODS

Fungal Strains, plasmids

Environmental new isolate *T. reesei* SJVTR was used throughout this study. The plasmid pUC18 and *Escherichia coli* BL 21 strains were used for the construction of the expression vector. Luria–Bertani (LB) medium supplemented with ampicillin (100 µg/mL) was used for propagation of *E. coli*. *T. reesei* SJVTR conidia were produced from glycerol stocks of *T. reesei* grown on potato dextrose agar medium (PDA: 4.0 g/L potato extract, 15.0 g/L agar, 20.0 g/L) at 45°C. Conidia were harvested with 2 ml 0.01% (w/v) Tween 80. Liquid batch cultures were inoculated at a concentration of 10^5spores/mL. Strain was grown on 100 ml *Trichoderma* Minimal Media [TMM: 15 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 10 g/L of the respective carbon source, 0.005 g/L $FeSO_4$.7H20, 0.0016 g/L $MnSO_4.H_20$, 0.0014 g/L $Zn.SO_4.H_20$, 0.0037 g/L $CoCl_{12}.6H_20$, 0.6 g/L $MgSO_4$, 0.6 g $CaCl_2$] in 250 mL Erlenmeyer flasks at 45°C, shaking at 200 rpm (Laure *et al.,* 2013). Mycelia were grown for 48 h in 1% (w/v) glucose, after which they were removed by filtration through Miracloth (Genei, Bangalore), washed with double distilled water (ddH_2O), and transferred to fresh media (Delmas *et al.,* 2012).

Isolation of RNA and synthesis of cDNA

Mycelia from each condition were snap-frozen and ground to a fine powder under liquid N_2 using a mortar and pestle. 100 mg of mycelial powder was used for RNA extraction, the procedure of which was described elsewhere (Delmas *et al.*, 2012). Briefly, total RNA was extracted from mycelial powder using TriZol reagent (Invitrogen) according to manufacturer's instructions. Extracted RNA was purified using the Qiagen RNeasy Mini Kit following the manufacturer's instructions. One step RT-PCRs were performed using the SuperScript ® III One step RT-PCR kit from Invitrogen.

Amplification and sequencing of Cellulase gene

The coding sequence of cellulase was amplified from the RNA template of *T. reesei* by one step RT PCR method. The forward primer containing a *Hin*dIII restrictionsite (TGCAAAGCTTGGTGAGGTTATAGCT) (underlined) and reverse primer containing a *Pae* I restrictionsite (CTGAGCATGCTCAAGGAAG ACACTGGGAGT) (underlined) were employed. A 50 µl reaction mixture of RT PCR was performed with 25 µ1 2X Reaction Mix (a buffer containing 0.4 mM of each dNTP, 3.2 mM MgSO4), 2 µ1 of SuperScript® III RT / Platinum® Taq mix, each 1 µ1 of forward and reverse primers at 0.5 µM, 5 ¼l of 100 ng RNA template of *T. reesei* and enough autoclaved distilled water to bring the total volume up to 50 µl. The amplification reaction was performed in a C1000 thermal cycler (Bio-Rad). The temperature profile was as follows: Efficient cDNA synthesis was achieved by an initial incubation 55°C at 30 sec, first cycle followed by initial denaturation at 94°C for 2 min. 40 cycles, PCR amplification consisting of 94°C for 15 sec denaturation, 56°C for 30 sec annealing and 72°C for 1 min extension, finally the reaction was held at final extension at 72°C for 5 min. Following amplification, 15 µl of the product was electrophoresed in a 1% agarose gel, using TAE buffer, stained with 2.5 mg L^{-1} of ethidium bromide and documented using AlphaImager gel documentation system.

Transformation of cellulase gene into *E. coli* pUC18 vector

The PCR products amplified from the total RNA of *T. reesei* were cut with restriction site, and the pUC18 vector was cut with the same enzymes A standard ligation reaction system was made up of 1µl 10 x ligation buffer, 1µl 50% PEG,1 µl Vector, 4 µl doubly digested PCR product, 1 µl T4 DNA ligase. A final volume of 10 µl was formed via addition of sterile double distilled water (ddH_2O). The recombinant Plasmid pUC18 SJVE was obtained by ligation reactions which were conducted by a BioRad thermal cycler for 3h at

Table 1. Endoglucanase and Total cellulase activities of the strains.

Strain Name	Endoglucanase activity (CMC/ml)	Total cellulase activity (FPU/ml)
T. reesei 992	8.95	0.458
T. reesei SIV TR	10.32	0.512
E. coli	3.5	0.012
pUC18 SJVE	11.75	0.567

18°C, then the ligated mix was transformed into competent *E. coli* BL 21 cells using the method of Tu *et al* (Tu *et al* ., 2005). Transformants were plated onto Lysogenic agar containing 1% of CMC and ampicillin (50µg/ml) and grown at 37°C. The plates were flooded with 5 ml of aqueous solution of Congo red (1 mg/ml), incubated for 30 min and washed a few times with 5 ml of 1 M NaCl (Wood, 1980). Colonies surrounded by a clear zone were considered cellulase positive. The transformants designated pUC18 SJVE was found to be the highest producer of cellulase among all recombinant strains generated.

Endoglucanase assay

The endoglucanase activity was determined using CMC as substrate .The reaction mixture consisted 800 µl of 1% (w/v) CMC in 50 mM Na-acetate buffer at pH 5.0 and 200 µl of cultured supernatant after centrifugation. The substrate was hydrolysed for 30 min incubation at 50°C and the production of reducing ends was determined with the DNS method using glucose as a standard. 1 unit of endoglucanase (EC 3.2.1.4) cellulase, CMCase is the amount of protein in milligrams enough to produce 1 mmol of reducing sugars from the substrate by minute.

SDS–PAGE analysis and Zymogram

Cells were grown in liquid medium harvested by centrifugation, washed, suspended with sodium acetate buffer (pH 5.2), and homogenized by ultrasion treatment. Then the mixture was centrifuged at 10,000 rpm (4°C) for 10 min, supernatant was further purified by the method of Kaur *et al*., (Kaur *et al*., 2007). Recombinant protein was dissolved in protein loading buffer, for 5 min and then run on a 12.5% SDS–PAGE following modified Laemmli method (Laemmli,1970). Zymogram analysis of cellulase protein from the transformants was resolved in a native PAGE (without SDS in any of the solution). For the detection of CMCcase activity, 1% CMC was included in the gels before polymerization. Samples were prepared with 2X sample buffer and heated at 95°C for 3 min before being applied to the gel. Gels were then stained with 0.2% Congo red for 30 min and washed with 1M NaCl until CMCase bands became visible. Then the gels were dried, visualized and photographed.

RESULTS AND DISCUSSION

Characterization of *Trichoderma reesei* SJVTR cellulolytic fungi

Among the 12 cellulase producing fungal isolates, SJVTR forming large yellow zone on 1% CMC agar Plate. It showed the highest cellulase activity than others and it has determined by the DNS method. The optimum pH and temperature were 5.5 and 45°C, respectively (Figs.1 and 2).The results suggested that pH range at 5 to 6.5 range and temperature from 40°C to 50°C was more favourable conditions for cellulase activity of *T. reesei*. The 18S rRNA gene sequence of this isolate was 100% identical to that of *T. reesei* strain IPBCC93_260 and IPBCC06_325 (KC847188.1 and KC847186.1) (Fig.3). The hydrolysis pattern of low molecular weight endoglucanase has previously been described on CMC as substrate (Karlsson *et al.*, 2002).

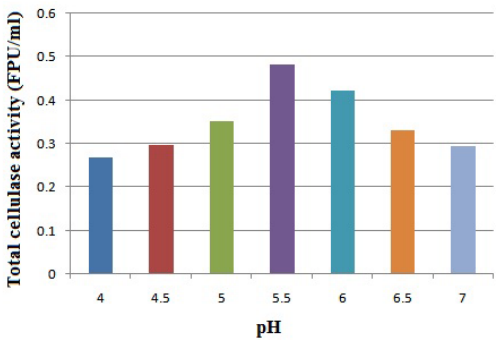

Fig. 1. Filter paper (Total cellulase) activity of SJVTR from pH 4 to 7

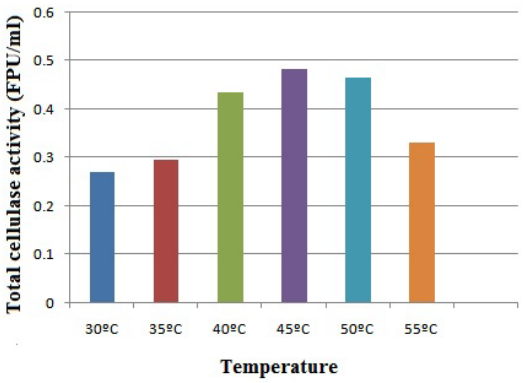

Fig. 2. Filter paper (Total cellulase) activity of SJVTR from temperatures 30°C to 55°C

Cloning and expression of cellulase gene in to pUC 18 vector

The recombinant cellulase-producing *E. coli* harbored a *T. reesei* derived 764bp *Hin*dIII and *Pae* I fragment. Sequencing of this insert demonstrated the presence of a 726 bp open reading frame potentially encoding a protein with a molecular mass of 24,420 Da. The 18 N-terminal amino acids likely encode a signal peptide. The predicted amino acid sequence was 100% identical to that of the endo-1,4-beta-glucanase (P43317) *T. reesei* and endoglucanase V (AAQ21385) *Trichoderma viride*. The *E. coli* cells harboring pUC18 SJVE exhibited cellulase activity compared with wild type *T. reesei* (NCIM 992)and *E. coli* BL21 (Table 1). The clearing zones around the colonies containing pUC18 SJVE were larger than the clearing zones found in wild type colonies of *T. reesei* (NCIM 992) and *E. coli* but smaller than *T. reesei* SJVTR *T. reesei* produces a complete set of cellulases which can hydrolyse cellulose to soluble sugars (Ilme´n, 1997). It might be the reason for the higher expression of the cellulase in *T. reesei* SJV TR and *T. reesei* .The endoglucanase activity and total cellulase activity were recorded. The endoglucanase activity of pUC18 SJVE with CMC as a substrate was 11.75 IU/g at pH 5.5, Temperature 37 °C (Table 1, Figs.4 and 5).

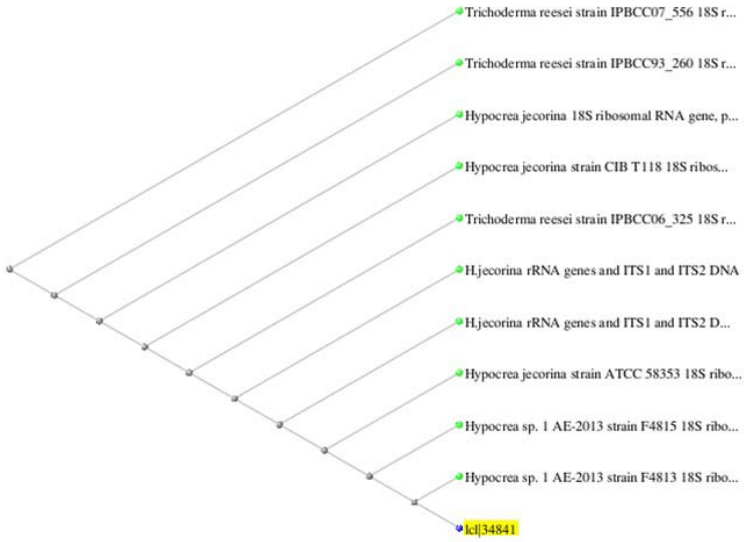

Fig.3. Phylogenetic tree based on 18SRNA partial gene sequence showing the positions of the strain vs related strains. The scale indicates Neighbor joining, slanted method and maximum sequence difference 0.5.

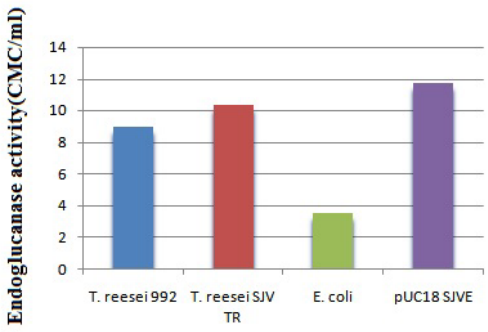

Fig.4. Endoglucanase activity of wild type and recombinant strains CMC as substrate

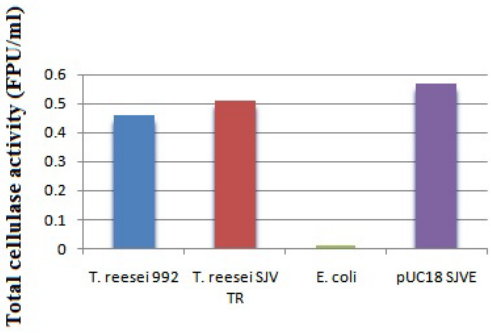

Fig.5. Total cellulase activity of wild type and recombinant strains using filter paper as substrate

SDS-PAGE and Zymogram analysis

Cellulase enzyme expressed by the recombinant clone was analysed by both SDS-PAGE and Zymograms. The relative molecular mass of the endoglucanase enzyme expressed by the recombinant pUC18 SJVE strains (harboring cellulase) was found as 24.42 kDa. The cellulase enzyme secreted by the recombinant pUC18 SJVE strains degraded CMC efficiently. Molecular weight of the cellulase enzyme was in accordance with the deduced amino acid sequence of the cloned cellulase genes. The cellulase gene expression in the recombinant strain was confirmed by a band 24.42kDa size in the zymogram.

DISCUSSION

T. reesei CAZy-encoding genes, representing 17 different Glycoside hydrolases (GH families) involved in lignocellulose deconstruction. *T. reesei* belonging to GH families such as 39, 115 and 45 assist in the degradation of cellulose (Hakkinen *et al.,* 2012). *T. reesei*, low levels of mRNA from genes encoding hydrolytic enzymes (GH) involved in the degradation of complex carbohydrates, including hemicellulose and cellulose, are present when the fungus is cultivated using glucose as carbon source. In medium containing glucose, many of these enzymes in *T. reesei* are likely to be involved in cell wall remodeling during hyphal extension as high growth rates are achieved in the presence of glucose (Fujii *et al.,* 2010). To develop a recombinant strain encoding cellulase gene with high efficiency, high activity and high stability is in a great demand and also a very challenging mission in the field of biology. *T. reesei* SJVTR was isolated from agricultural field in an around Tiruchirappalli. The isolated *T. reesei* strain was found to have high levels of cellulase activity. An efficient cellulose hydrolysis needs different enzymes working systematically, several endoglucanase families including EGI, EGII, EGIII, EG IV and EGV had been reported previously. In order to find more efficient gene encoding cellulase, a novel cellulase gene from *T. reesei* (SJV TR) was cloned and successfully expressed in *E. coli* BL 21. A gene encoding this cellulase belonging to glycosyl hydrolase family 45 and was classified as endoglucanase. This gene could be used for the expression of endoglucanase enzyme in industrial scale.

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