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Full Length Research Paper

Production of recombinant cellulase enzyme from *Pleurotus ostreatus* (Jacq.) P. Kumm. (type NRRL-0366)

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Successful utilization of *Pleurotus ostreatus* (Jacq.) P. Kumm. (type NRRL-0366) mushroom as a type of edible locally isolated mushroom in Egypt at the Mushroom Research Center (Mubarak City for Scientific Research and Technology Applications), to produce extensive hydrolyzing cellulase complex enzymes. This hydrolysis was approached in submerged culture supplemented with avicel PH101 as a substrate for endo-,exoglucanase production. The avicel concentration 6% yielded the maximum enzyme activities (2.46, 1.80 U/ml) for both endo-and exoglucanase activities on basal medium at 27°C, initial pH value of 5.5 for 12 days on rotary shaker (180 rpm) incubation period. Cellulase enzyme was amplified using specific PCR and the amplicone was cloned using TOPO TA cloning vector. The cellulolytic activity of the recombinant protein was examined and high activity was obtained compared with the standard ones. The avicel was used as a sole carbon source in the fermentation medium and the results revealed that, avicel induced the cellulolytic activity of the examined organism compared with those grown on medium deficient of avicel.

Key words: *Pleurotus ostreatus,* mushroom, avicel, recombinant cellulolytic enzymes.

INTRODUCTION

Mushrooms were known more than three thousand years ago by the ancient Egyptians (Hassan et al., 2010). They were considered a luxury food, were eaten only by the nobility, and known as the food of the gods. In the 1940's some European foreigners live in Egypt, they cultivated mushrooms on a very small scale and collected wild mushrooms during the winters. In the 1980's the mushroom cultivation farms were established in Tanta and Faquos could not satisfy the demands from the hotels, tourists, and local residents. Mushroom became a new and alternative demand for poultry and animal protein fresh mushrooms. The demand is increasing rapidly as consumers discover the delicious meaty flavor of mushrooms (Daba et al., 2008).The commercial cultivation began in 1988, when several universities and Food technology institutes established research that are responsible for training growers in mushroom cultivation and marketing.

Mushrooms have been used as medicinal materials from 100 years ago. In the fields of Chinese the oriental medicine, dried mushrooms are used as diuretics and some other species have recently been getting attention as carcinostatic substances (Mizuno et al., 1995; Wasser and Wise, 1999). The control and improvement of edible fungus cultures have provoked considerable interest in the past few years because mushroom production is economically important. *Pleurotus spp*. is third place in worldwide production of edible mushrooms after *Agaricus bisporus* and *lentinula edodes* (Chang, 1999), these mushrooms yield the possibility of successful cultivation on a variety cheap substrates such as rice straw (Kaul and Janardhanon, 1970; Ghada et al., 2008), banana pseudostems (Jandaik and Kapoor, 1974).

Mycelial growth of *Pleunotus spp*. is fast, and various lignocellulosic waste products can be used as a culture substrate (Yildiz et al., 2002). Cellulose is the only renewable carbon source that is available in large quantities and can be a solution to the problems of energy, chemicals, and food. Cellulose can be hydrolyzed by acid or enzymatic treatment, yielding soluble products of low molecular weight such hexoses

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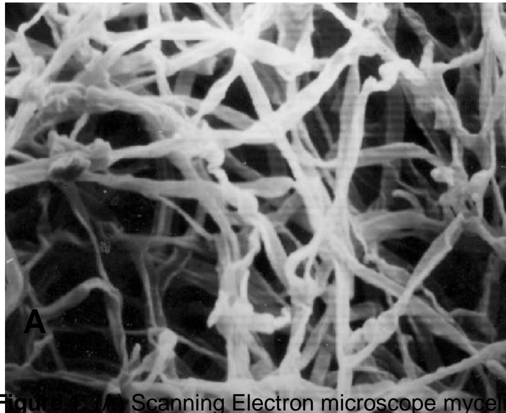

Figure 1. (A) Scanning Electron microscope mycelial net work, (B) Mushroom fruit bodies *Pleurotus ostreatus* cultivated on rice straw.

and pentoses, the high cost of the production of these enzymes has hindered the industrial application of cellulose bioconversion (Lange, 2007). One of the different approaches to overcome this hindrance is to make continuous search for organisms with secretion of cellulase enzymes in copious amounts and to optimize enzyme production with them.

Cellulase is used for commercial food processing in coffee. It performs hydrolysis of cellulose during drying of beans. Furthermore, cellulases are widely used in textile industry and in laundry detergents (Elisashvili et al., 2009). They have also been used in the pulp and paper industry for various purposes, and they are even used for pharmaceutical applications. Cellulase is used as a treatment for phytobenzoars, a form of cellulose benzoar found in human stomach. In this study, we investigated the influence of avicel PH101 concentration (as a carbon source) on the production of different enzymatic activities of cellulase complex, complete enzymatic hydrolysis of enzymes require 3 types of enzymes, namely cellobiohydrolase, endoglucanase or carboxymethyl cellulase (CMCase) and 6- glucosidases (Bhat, 2000), in this paper we are dealing with the endo, and exoglucanase (avicelase) activities by *Pleurotus ostreatus* under submerged condition. The effect of fermentation time on the growth and enzymatic cellulase activities of the fungus in a laboratory study was also represented. The total protein was extracted and determined from 8 and 4 samples with avicel and the other without avicel.

MATERIALS AND METHODS

Mushroom cultivation

Strain *P. ostreatus* (Jacq.) P. Kumm. (type NRRL-0366) was provided by the Agricultural Research Service (Peoria, U.S.A.), and reactivated monthly in Petri dishes containing sterile solid potato-dextrose agar medium. The mycelia growing in dishes were incubated at 25°C for 7 days, and then stored in a refrigerator at 4°C. Potato dextrose agar slant was inoculated with spores from wild *P. ostreatus* strain, slants were incubated at 25°C for 10 days. The mycelium culture (Figure 1A) obtained was used for production of mushroom fruit bodies (Figure 1b) using rice straw as a substrate according to the method described by Chang and Milles (1982).

Enzyme production

Cultivation was preformed on culture basal submerged medium, (mushroom medium) (gm/100 ml) Containing of 0.2% yeast extract, 0.2% peptone, 0.1% K_2HPO_4, 0.05% KH2PO4, 0.05% $MgSO_4$, and supplemented with 2% microcrystalline cellulose (Avicel PH101) as a carbon source, pH adjusted to 5.5, the culture medium was sterilized by autoclaving at 121°C for 15 min, inoculated by aseptically adding 2 ml of seed culture to 100 ml of sterilized media and incubated at 27°C on rotary shaker (180 rpm) for 12 days, cultures were centrifuged at 4000 Xg for 15 min at 4°C, the supernatant was used as the crude enzyme to measure the activity of endoglucanase (CMCase), exoglucanase (avicelase) activities and extracellular protein.

Enzyme assays

Endoglucanase activity (Carboxymethyl – cellulase, CMCase)

Activity of endoglucanase was estimated according to technique proposed by (Ghose, 1987), using a reaction mixture containing 1 ml of 2% carboxymethyl cellulose (CMC) in 0.05 M acetate buffer (pH 4.8) and 1 ml of culture supernatant. The reaction mixture was incubated at 50°C for 60 min and the reducing sugar produced was determined by dinitrosalicylic acid–DNS method of (Miller, 1959) using glucose as a sugar standard, Blanks were prepared with inactivated enzymes. One unit (1U) of endoglucanase activity was defined as the amount of enzyme releasing 1 mg of reducing sugar per min.

Exoglucanase (Avicelase) activity

The activity of exoglucanase was determined as described previously, for endoglucanase one, but the incubation was carried

out with 1 ml of 1% avicel suspension instead of carboxymethyl cellulose. Extracellular protein was measured in culture supernatant by the method of (Lowry et al., 1951) with bovine serum albumin as standard.

Determination of cellulase enzymes molecular weight using (Sodium dodecyl sulfate polyacrylamide Gel electrophoresis) SDS- PAGE

Gel electrophoresis (BioRad, USA) was carried out according to Laemmli (1970) method on 12% SDS-PAGE. Suitable volume of 12% SDS polyacrylamide separating gel was prepared by mixing 37.5 ml of 30% stock solution of acrylamide (acrylamide BDH, 146 g; Bis-acrylamide, 4 g in 500 ml distilled water), 22.5 ml of 1.5 M Tris-HCl, pH 8.8, 29.1 ml of distilled water and 0.9 ml of 10% SDS solution.0.5% final concentration of TEMED (v/v) and 1.5 ammonium sulphate (w/v) were added just before pouring the gel. The mixture was poured using a pipette into Biorad Buchler electrophoresis unit (BioRad, USA), and then overlaid carefully with isopropanol to the level of the gel surface. The gel was left for polymerization at room temperature for about 30 min. Staking gel (5%) was prepared by mixing 5 ml of 30%, 1 acrylamide, 0.3 ml of 10% SDS, 37.5 ml 1 M Tris-HCl buffer (pH 6.8), and 20.5 ml of distilled water. TEMED was added at concentration of 0.5% (v/v) and ammonium sulphate was added also at concentration of 1.5 (w/v). The polymerization was carried out as previously described, where a comb was inserted to prepare the wells. The comb was removed from the staking gel after polymerization, then the gel was installed to the reservoir containing buffer solution of 0.025 M Tris-HCl, 0.192 M glycine (pH 8.3) and 0.1% SDS (w/v). Samples were prepared by mixing small volume of *P. ostreatus* mushroom sample containing about 1 mg/ml protein with (X2) application buffer, 0.125 M Tris- HCl (pH 6.8), 4% SDS, 10% 2-mercapto ethanol, 10% glycerol and 0.02% bromophenol blue, and then exposed to 100°C in water bath for 1 min. Each sample was applied to a separate well in the slab gel along with a prestained SDS molecular weight marker (14-205 K Daltons). Electrophoresis was carried out at constant current 25 mA for about 1.5 h. The gel was stained with comassie blue, 0.06% comassie brilliant blue R-250 in 50% methanol and 10% acetic acid. The gel was destained overnight in a mixture of 60 ml methanol, 40 ml acetic acid, and 800 ml distilled water. The gel will viualized on gel documantation system.

DNA extraction from fungal mycelium

DNA extraction was performed using 10 to 15 mg (wet weight) of freshly subcultured fungi. The fungal mycelium were grinded in liquid nitrogen using mortar and pestle and then the ground mycelium was subjected to DNA extraction using QIAGene, DNA extraction kit (QiaGen, Germany).

Cellulase amplification using specific PCR

Polymerase chain reaction PCR amplification was carried out using two cellulase specific primers, primer F; 5-ATA GAA TTC TTR TCN GCR RTT YTG RTG RAA CAA and the reverse primer R; ATA GAA TTC ATY TGG GAY TGY TGY AAR CC-3. PCR reaction was performed in a total volume of 50 l and contain 5 l (5 x Green Go Taq flexi buffer (promega, USA), 5 l (5 x colorless Go Taq flexi buffer (Promega, USA), (100 mM Tris-HCl (pH 8.8 at 25°C) 500 mM KCl),5 l $MgCl_2$ (25 Mm) (promega, USA), 2 l 4 dNTPs mixture (10 mM of each) (BIORON, Germany), 4 l C. DNA of chymosin,4 l of each primer (20 pmol / l), 2 U Taq polymerase (5 U / l) (promega, USA), the reaction volume was completed to 50 l with sterile distilled H_2O. The reaction mixtures were subjected to amplification as follows: initial denaturation step at 95°C for 3 min, followed by 35 cycles of amplification with denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min ending with extension at 72°C for 10 min, the thermocycler (PTC-200 Peltier, USA) was used for performing PCR amplification, as described by Shih et al. (2002).

Cloning and subcloning cellulase gene

Cellulase of amplified PCR products was done by T/A based cloning protocol by using TOPO TA Cloning® (with pCR® 2.1-TOPO® Cloning vector) and (a TOP 10 E-*coli* strain) (Invitrogen™, USA). The chymosin gene was released from the pCR® 2.1-TOPO® vector using *Eco*RI restriction enzyme, meanwhile the released fragment was purified by EzWay™Gel Extraction kit (KOMBIOTECH. Korea) and ligated to the lineraized prokaryotic expression pPROEX HT (life technologies, USA) and the cloning was done according to the protocols outlined by Life Technologies, Invitrogen. For more acceptable protein for the human, we tried to subclone the functional gene into PichiaPink™ Yeast Expression system (Invitrogen company) according to the manufacture procedure.

Cellulase purification using 6x Histidine affinity-tagged method

Cellulase purification was carried out by Ni-NTA resin matrix (QIAGEN Inc., USA). The induced bacterial cells was pelleted and resuspended in 4 volumes of lysis buffer (50 mM Tris-HCl (PH 8.5 at 4°C), 5 mM 2-mercaptoethanol, 1 mM PMSF). The suspension was sonicated until 80% of the cell was lisyed. The cell debris was removed by centrifugation, the supernatant was removed to a new tube (crude supernatant) . Affinity purification was done according to the protocols outlined by Life Technologies, Invitrogen.

Solubilization and renaturation of chymosin protein

The procedures developed by Marston et al. (1984), the inclusion pellets were solubilized in 8 M urea buffer (pH 8). The urea mixture was incubated at 25°C for 1 h before the insoluble molecules were removed by centrifugation. The urea solution was then diluted in a high pH buffer (pH 10.7) for renaturation of chymosine. After the insolubilization in 8 M urea, the inclusion body solution was diluted with phosphate buffer pH 10.7, the solution was incubated at 25°C for 1 h and then adjusted to pH 8 and incubation was continued at 25°C for 1 h. The solution was transferred to dialyze against buffer (20 mM Tris/HCl pH 8.0, 50 mM NaCl, 1 mM EDTA) at 4°C overnight. The folded streptokinase were then assay as thrombolytic agent.

Plasmid miniprep

Plasmid miniprep (QIAGEN, Germany) was performed on the clones to harvest the vectors potentially with insert. The spectrophotometry was applied to estimate the quantity and quality of the plasmid samples. The plasmids were restriction digested at 37°C for 4 h by NdeI (4 unit/ug DNA) (NEB, USA) and SacII (4 unit/ug DNA) (NEB, USA) then heat inactivated at 65°C for 20 min to further confirm the insert. 6 ul of the digested products were loaded and resolved on agarose-TAE gel after electrophoresis. Each clone was also submitted for DNA sequencing done by a local vendor.

The recombinat cells were cultivated on LB (Lysogeny broth) medium, the positive transformants carrying the pGEM-T Easy Vector were Ampicillin resistant.

Table 1. Extracellular protein content and cellulolytic activities of *P.ostreatus* at different avicel concentration. Under shaked condition.

Avicel concn (%) (g/100 ml)	Endoglucanase activity (U/ml)	Exoglucanase activity (U/ ml)	Extracellular protein content (mg/ml)
0	0.66	0.53.	0.78
2	1.45	0.89	1.82
4	1.93	1.29	2.16
6	2.46	1.80	2.50
8	1.72	1.35	2.05
10	0.97	0.76	1.36

Table 2. Enzymatic activities and extracellular protein of *P. ostreatus* versus the time of fermentation.

Days	Time of fermentation (h)	Endoglucanase activity (U/ ml)	Exoglucanase activity (U/ ml)	Extracellular protein content (mg/ ml)
2	48	1.98	0.95	1.05
4	96	2.43	1.56	2.70
6	144	3.13	2.0	3.22
8	192	4.0	2.50	3.50
10	240	3.06	2.87	2.42
12	288	2.46	1.80	2.50

RESULTS AND DISCUSSION

Influence of avicel concentration

An evaluation of carbon source utilization by *P. ostreatus* suggested that the production of high - titer cellulase necessitated an increase in the concentration of avicel as a carbon source, by adding different levels of avicel ranging from 0 to 10%. The results of this investigation are represented in Table 1 these results collectively indicate that the substrate concentration has a variable effect on the metabolic activities of the tested organism. The highest activities (2.46 and 1.80 U/ml) for endo- ,exoglucanase respectively, were detected at substrate concentration 6%. Some cellulases that were lower than the expected maximum cellulases titer were however obtained at avicel concentration greater than 6%, the activities decreased gradually and 10% of avicel concentration showed a low end, exoglucanase activities representing 39.43 and 42.22% of the activity obtained at 6% substrate level this results according to Duboism et al. (1956) This relatively lower titer may be attributed to the adsorption of cellulase produced on to the avicel, the repression was concentration – dependant (Bindu et al., 2006; Suzuki et al., 2008; Waeonukul et al., 2009).

Influence of fermentation time

The enzymes were produced both during the growth and stationary phases, so the process of these enzymes production and secretion is a growth associated one (Domingues et al., 2000). As it is previously observed, the values of cellulases activities and extracellular protein were higher at 6% avicel concentration, in case of endoglucanase, a low activity was obtained when the fungus was growing and when the stationary phase was reached. Results shown in Table 2 represent a sudden increase in activity until it reached a maximum at 192 h (8 days). A decrease was observed after wards probably due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes (Nochure et al., 1993), as in the case of exoglucanase, reaching a minimum at 280 h. The highest value of activity was achieved at 240 h.

Determination of the molecular weight of protein

The molecular weight of cellulase enzyme was determined by SDS polyacrylamide gel electrophoresis applied to 12% SDS polyacrylamide gel. The molecular weight of one band was calculated from the relation between molecular weight of the marker and the relative mobility of the calculated molecular weight was found to be 45,000 daltons, (Figure 2). These results agree with (Cailler 1986). However, gel electrophoresis in the presence of sodium dodecyl sulphate, as described by Weber and Osborn (1969) and Nadla et al. (1979)

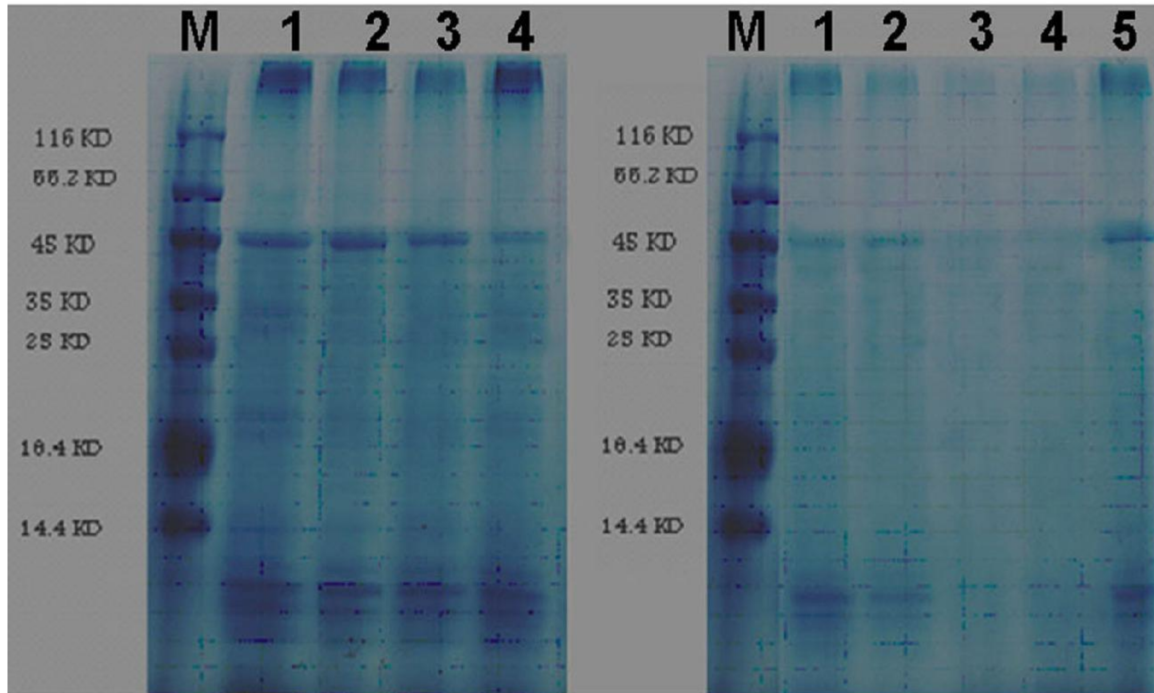

Figure 2. SDS-PAGE protein pattern with avicel (2, 4, 6 and 8% from left to right) and the other without avicel .

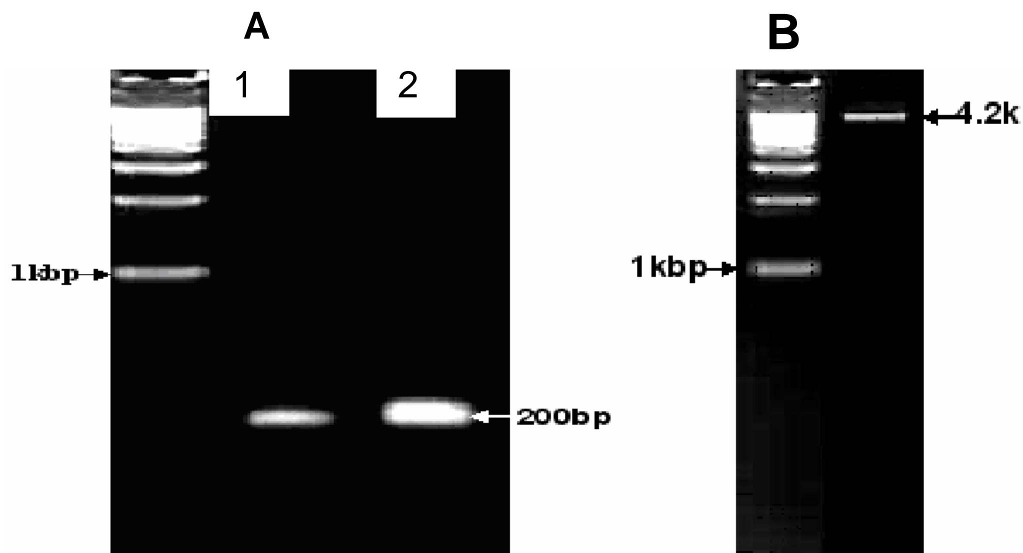

Figure 3. (A) PCR amplification for the cellulase gene from the *P. ostreatus* mycelium. Lanes; lane1: DNA marker 10 kbp ladder. Lane (1); amplifed cellulase gene from grown organism on medium deficent on avicel. Lane (2); the amplicone from organism grown on medium contains avicel as a carbon source. (B) Plasmid miniprep of the recombinant cellulase gene.

indicated that at least five proteins were present in cellulase. Activity assays on the SDS-polyacrylamide gel showed that the protein with the highest molecular weight had cellulolytic activity. The presence of several components, revealed by SDS-polyacrylamide electrophoresis, and of both carboxymethyl cellulose and avicelase activities could indicate that the protein obtained by preparative electrophoresis is a multi-enzyme complex.Protein extraction was carried out for the examined 8 samples, 4 samples with avicel and the other without. The data presented in Figure 3 revealed that, the protein pattern is the same in the examined 8

samples but it differ in band intensity. The band intensity which considered as indicator for protein expression was broad and strong in case of the medium contains avicel (2, 4, 6 and 8%), but it was so narrow and weak in the medium free of avicel. Moreover, the avicel induced the cellulolytic activity of the examined organism compared with these grown on the other medium.

Cloning and *in vitro*-transcription of the cellulase enzyme

About 200 bp of the cellulase enzyme was amplified using specific PCR, and the obtained amplicone was cloned using the TOPO TA cloning kit. The recombinant clones were selected on medium contains the avicel as a sole carbon source. The recombinant purified protein was assayed for it cellulolytic activity and the results revealed that a high activity was obtained compared with the standard ones (Stahl, 1997). We used PCR-based methods to clone and sequence four previously unidentified cellulase cDNAs: *cbhI-I*, *cbhI-II*, *cbhII-I* and *egII*. *CbhI-I*, *cbhI-II* and *cbhII-I* consist of 1710, 1610 and 1453 bp, respectively, and encode for 512, 458 and 442 amino acids, respectively. *EgII* consists of 1180 bp encoding for 310 amino acids, and belongs to family 61 of the glycosyl hydrolases. *CbhI- I, cbhII-I* and *egII* all have a modular structure, with the catalytic domain (CD) and cellulose-binding domain (CBD) located at the C-terminus in *cbhI-I* and *egII*, and at the N-terminus in *cbhII- I*. *CbhI-II* shows high homology to *cbhI-I* but lacks a CBD. Northern blotting revealed that *cbhI-I, cbhI-II and cbhII-I* were coordinately expressed at various stages of the mushroom developmental cycle (substrate colonization to mature fruit body), although the number of *cbhI-I* transcripts was much smaller. No *egII* expression was detectable during the substrate colonization phase but transcription levels increased as fruit body morphogenesis progress (Shaojun et al., 2006).

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