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Degradation of Crystalline Cellulose by the Brown-rot Basidiomycete *Fomitopsis palustris*

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This study demonstrated that the brown rot basidiomycete *Fomitopsis palustris* was able to degrade crystalline cellulose (Avicel). This fungus could also produce the three major cellulases (exoglucanases, endoglucanases, and β-glucosidase) when the cells were grown on 2.0% Avicel. Avicel degraded by *F. palustris* showed a decrease in relative crystallinity from 83% to 78.5% after 14 days of incubation. The characterization study indicated that optimum pH was 4.5 and optimum temperature was 70°C for exoglucanase (cellobiohydrolase) activity. Hydrolysis of Avicel by the crude enzyme from *F. palustris* yielded 1.6 mg/ml of glucose after 43 h, which corresponded to a cellulose conversion degree of 3.2%. Therefore, this study revealed for the first time that the brown rot basidiomycete *F. palustris* produces cellulases capable of yielding soluble sugars from crystalline cellulose.

Key words: β-Glucosidase, brown rot basidiomycete, cellobiohydrolase, crystalline cellulose, endoglucanase, *Fomitopsis palustris*

Agricultural wastes and forest materials containing high levels of lignocellulose are particularly abundant in nature and have a potential for bioconversion. They constitute a renewable resource from which many useful biological and chemical products can be derived. Accumulation of this biomass in large quantities every year results not only in deterioration of the environment but also in loss of potentially valuable materials that can be processed to yield energy, food, and chemicals.

Cellulose biodegradation by fungi has generally been considered to involve only three types of hydrolytic enzymes: endoglucanases (EGs), cellobiohydrolases (CBHs), and β-glucosidase (BGL). EGs randomly cleave the internal β-1,4-glucosidic links, CBHs act on the free ends of cellulose polymer chains, and BGL hydrolyzes cellobiose and other water-soluble cellodextrins to glucose (Eriksson *et al*., 1990). Cellulases belong to a large group of glycosyl hydrolases (GHs), which have been classified into several families based on amino acid sequence similarities (Henrissat, 1993; Henrissat and Bairoch, 1993; Henrissat and Davies, 1997).

Cellulolytic enzymes from soft-rot and white-rot fungi have been extensively studied in some model organisms such as *Trichoderma reesei* and *Phanerochaete chrysosporium* (Eriksson and Petttersson, 1975; Claeyssens *et al*., 1989; Eriksson *et al*., 1990; Uzcategui *et al*., 1991). White-rot fungi in particular are known to be capable of degrading not only lignin, but also a variety of relatively recalcitrant environmental pollutants (Han *et al*., 2004; Shin, 2004). Recently, the genome sequence of the white-rot fungus *P. chrysosporium* strain RP8 has been revealed and its genomic information is available at http://genome.jgi-psf.org/whiterot1/ (Martinez *et al*., 2004). The availability of the *P. chrysosporium* genome data may greatly facilitate the identification of the extracellular proteins produced during cellulose degradation by the wood-rot fungi.

Brown rot basidiomycetes cause the most destructive type of wood decay and are important contributors to biomass recycling (GreenIII and Highley, 1997; Gilbertson and Ryvarden, 1986). These basidiomycetes are unusual in that they rapidly depolymerize the cellulose in wood without removing the surrounding lignin that normally prevents microbial attack (Kerem *et al*., 1999). However, there is little information available on the mechanism of hydrolysis of cellulose by brown-rot fungi. To date, only a few EGs have been isolated, including those from *Gloeophyllim* (*Lenzites*) *sepiarium* (Bhattacharjee and Majumder, 1993), *G. trabeum* (Herr *et al*., 1978), *Polyporis schweinzii* (Keilich *et al*., 1969), and *Tyromyces* (*Fomitopsis*) *palustris* (Ishihara and Shimizu, 1984). These fungi are generally thought to lack the exoglucanases that can hydrolyze crystalline cellulose. They are known to produce classical EGs (Eriksson *et al*., 1990)

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that can disrupt crystalline cellulose extensively (Rättö *et al*., 1997) when they act together with extracellular reactive oxygen species generated by these fungi (Cohen *et al*., 2002). A recent work has reported that crude extracts from wood colonized by two brown rot fungi, *Wolfiporia cocos* and *Laetiporus sulphreus*, were able to release soluble reducing sugars from crystalline cellulose (Machuca and Ferraz, 2001). Moreover, Cohen *et al.* (2005) has shown that the brown rot basidiomycete *G. trabeum* produces EGs capable of yielding assimilable glucose from crystalline cellulose.

Fomitopsis palustris causes a typical brown rot and is a model fungus for wood-preservative efficacy tests both in Korea and Japan. It has been reported that extracellular enzyme preparations from this fungus degraded carboxymethylcellulose (CMC), hemicelluloses, and several β-glycosides, but did not exhibit cellulolytic activity on crystalline cellulose (Ishihara and Shimizu, 1984). However, we found that this fungus was able to degrade both the crystalline and amorphous forms of cellulose from wood (unpublished data). Here we report that the major cellulases are produced by *F. palustris* grown on microcrystalline cellulose (Avicel) cultures, resulting in changes in crystallinity of these substrates.

Materials and Methods

Microorganism and culture conditions

The brown rot basidiomycete *F. palustris* (formerly *Tyromyces palustris*) (Berkeley et Curtis) Murill, a standard fungus for wood preservative efficacy tests in Japan and Korea, was used in this study. *F. palustris* was maintained on agar plates as described previously (Yoon *et al*., 2002). To prepare the precultures, 5 agar-mycelium plugs of 5 mm in diameter were punched out and inoculated into 100 ml of liquid medium containing 2% (w/v) dextrose, 2% (w/v) maltose, and 0.5% (w/v) peptone in a 500-ml Erlenmeyer flask. The plugs were incubated in this medium at 28°C for 7 days on a rotary shaker at 105 rpm. For growth on liquid culture, media contained microcrystalline cellulose (Avicel; Fluka, Switzerland) as a carbon source. The control culture medium contained 2.0% (w/v) glucose as carbon source. About 5 ml of *F. palustris* precultures were aseptically inoculated into 100 ml of liquid media containing 2% (w/v) Avicel in 500-ml shaking flasks as described previously (Yoon *et al.*, 2002) The cultures were then incubated at 28°C for 30 days. After incubation, the fungal culture was squeezed through cheesecloth and centrifuged at 10,000 × g for 30 min at 4°C. The resulting supernatant was concentrated 20-fold by ultrafiltration with Amicon (10 kDa cut-off). The concentrate was used for enzyme assays.

Enzyme assays

Endo-1,4-β-glucanases (EGs) were assayed in 100 mM sodium acetate (pH 5.0) by using Ostazin Brilliant Red H-3B hydroxyethyl cellulose (OBR-HEC) (Sigma, USA) as described by Biely *et al*. (1985). The enzyme solution (30 μl) was incubated with 120 μl of 2.5 mg/ml OBR-HEC in a final volume of 300 μl for 15 min at 40°C. The enzymatic reaction was stopped by adding 900 μl of ethanol and the solution was further incubated for 5 min at room temperature. After the solution was centrifuged for 5 min at 15,000 × g, the absorption of the supernatant was measured at 550 nm. The enzyme activity was expressed as the increase in ΔA_{550} per min.

β-Glucosidase (BGL) activity was assayed in 100 mM sodium acetate buffer (pH 5.0) by using *p*-nitrophenyl-β-D-glucoside (*p*NPG) (Sigma, USA) as a substrate (Herr *et al*., 1978). Reaction mixtures (1 ml) containing 100 μl of extracellular solution and 1 mM *p*NPG (final concentration) in 100 mM sodium acetate buffer (pH 5.0) were incubated for 15 min at 40°C. The amount of *p*-nitrophenol was measured by monitoring of changes in A_{405} (ε_{405}=17.0/mM/cm) after addition of Na_2CO_3 to the reaction mixtures. One unit (U) of *p*NPG- hydrolyzing activity was defined as the amount of enzyme necessary to release 1 μmol of *p*-nitrophenol per minute.

Exocellulase (CBH) was determined with *p*-nitrophenyl-β-D-lactoside (*p*NPL) (Sigma, USA) as a substrate (Schubot *et al*., 2004). Reaction mixtures (1 ml) containing 100 μl of extracellular solution and 1 mM *p*NPL (final concentration) in 100 mM sodium acetate buffer (pH 5.0) were incubated for 15 min at 40°C. The amount of *p*-nitrophenol was measured as mentioned above. One unit (U) of *p*NPL- hydrolyzing activity was defined as the amount of enzyme necessary to release 1 μmol of *p*-nitrophenol per minute.

Protein concentration

The protein concentration in the culture solution was measured with a Protein Assay kit II (Bio-Rad, USA) based on Bradford's method (Bradford, 1976).

X-ray diffraction analysis

The measurement of crystallinity in Avicel during various cultivation periods was carried out by powder X-Ray Diffractometry (XRD, Bruker D5005, Germany) as described by Segal *et al*. (1959). Crystallinity (%) was defined as $[1-I_{am}/I_{002}]\times100$, where I_{002} is the crystalline peak of the maximum intensity at 2θ between 22° and 23° for cellulose I, and I_{am} is the minimum intensity at 2θ between 18° and 19° for cellulose I.

Characterization of exoglucanase

To characterize the enzyme involved in biodegradation of microcrystalline cellulose, a 14-day old culture of *F. palustris* grown in liquid medium containing 2.0% (w/v) Avicel as a carbon source was used. The effect of pH on enzymatic activities was tested at pH values from 3.5 to

8.0 using 100 mM sodium acetate buffer at 40°C under standard assay conditions as described above. To determine the optimal incubation temperature, the reaction mixtures were incubated at temperatures ranging from 30 to 80°C at pH 5.0.

Avicel hydrolysis

Hydrolysis of Avicel (50 mg/ml) in a crude solution containing the exocellulase activity from *F. palustris* was measured for 2 days at pH 5.0 and 50°C. An aliquot of the suspension (0.1 ml) was taken and centrifuged for 10 min at 12,000 × g. The concentration of glucose in supernatant was determined by a Wako Glucose CII-Test (Wako Pure Chemical, Japan).

Results and Discussion

Production of β-1,4-endoglucanase, cellobiohydrolase, and β-glucosidase

Previous reports indicated that fungal cellulases were produced only in the presence of cellulose as a growth substrate (Mandels and Rees, 1957). The ability of the brown rot basidiomycte *F. palustris* to produce extracellular cellulolytic enzymes (EG, CBH, and BGL) was tested using a liquid culture containing 2.0% Avicel. The data presented in Fig. 1 shows that the levels of all three enzymes increased sharply after 8 days of cultivation. The results also showed that *F. palustris* produces CBH along with EG to degrade lignocellulosic substrates (Table 1), although this fungus has long been thought to lack the cellulases that degrade crystalline cellulose (Ishihara and Shimizu, 1984).

When this fungus was incubated in culture containing 2.0% glucose instead of Avicel as a carbon source, these enzyme activities were not detected (Table 1). Since glucose is an end product of cellulolysis and inhibits extracellular cellulolytic enzymes, our study also suggests that the enzymes from *F. palustris* are inducible and regulated by catabolite repression.

X-ray diffraction analysis of crystalline cellulose

Various methods and approaches for determining the crystallinity index of a sample have been proposed (Segal *et al*., 1959; Roberts, 1991; Buschle-Diller and Zeronian, 1992, 1994). Fig. 2 shows the crystallinity values of Avicel degraded by *F. palustris*. It was observed that the crystallinity of Avicel decreased from 83% to 78.5%, indicating biodegradation of crystalline cellulose by a cellulase complex from *F. palustris*. When *Cryptomeria japonica* trees were degraded by this fungus, crystallinity of woody cellulose deceased from 48.9% to 9.1% after 12 weeks of incubation (unpublished data). The biodegradation of crystalline cellulose generally involves the action of both endo- and exo-acting cellulases. Exoglucanases act processively on cellulose free ends while remaining attached to the cellulose. They release soluble cellobiose molecules, which are subsequently hydrolyzed to assimilable glucose by BGLs (Beguin and Aubert, 1994). The results also indicate that exoglucanases are also involved in the degradation of crystalline cellulose by *F. palustris*.

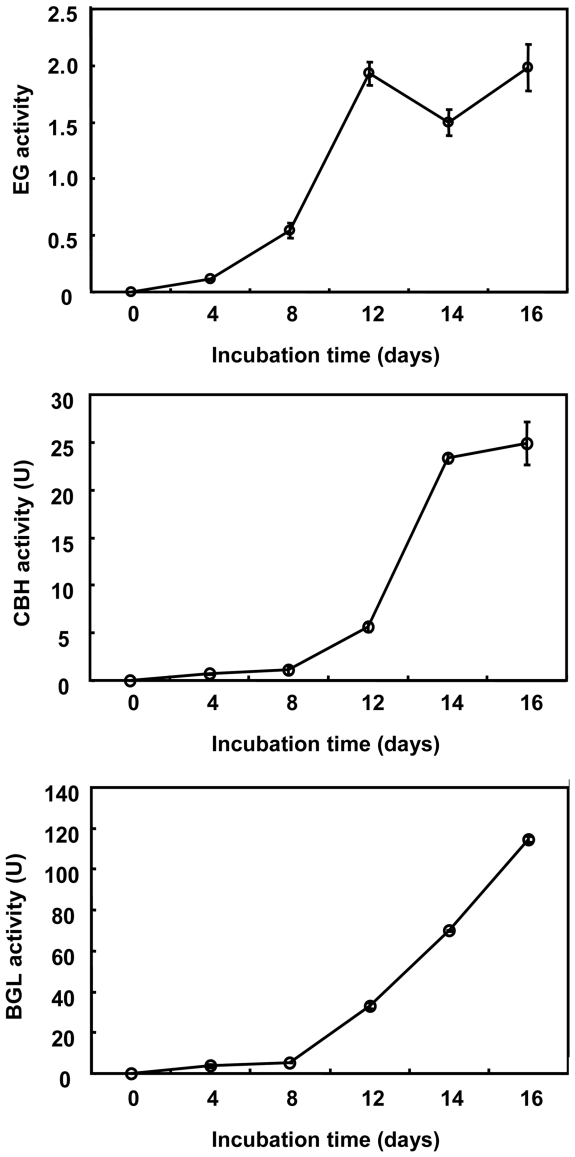

Fig. 1. Changes in the activity of extracellular endoglucanase, cellobiohydrolase, and β-glucosidase of *Fomitopsis palustris* grown on 2.0% Avicel. The EG activity was expressed as ΔA_{550} per minute. Values represent the mean of three replicate determinations; error bars indicate standard deviation.

Effects of pH and temperature on enzyme activity

As shown in Fig. 3A, the optimum pH value for the CBH was 4.5. The optimum pH value for this enzyme was very close to the initial pH of the medium (5.0). The optimum temperature for this enzyme was 70°C at pH 5.0 (Fig. 3B). These results are similar to those obtained from *Talaromyces emersoni*i (Tuohy *et al*., 2002) and *Chrysosporium*

Table 1. Production of β-1,4-endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (BGL) from fungal growth on different carbon sources[b]

Carbon source (2%, w/v)	Enzyme activity			Protein concentration (mg/ml)
	EG[a] (ΔA_{550}/min)	CBH (U)	BGL (U)	
Avicel	1.50 ± 0.12	23.3 ± 0.5	69.8 ± 0.1	0.31
Corrugated cardboard	0.077 ± 0.004	12.9 ± 0.9	117 ± 5.9	0.49
Cotton	2.46 ± 0.22[c]	10.8 ± 2.6	20.2 ± 0.2	0.35
Glucose	-[d]	-	-	-
Waste paper	0.044 ± 0.006	26 ± 3.2	356 ± 12.6	0.16

[a]The enzyme activity was expressed as ΔA_{550} per minute.
[b]Cultures were grown at 28°C for 14 days. Values shown are averages of triplicate experiments for each substrate.
[c]The enzyme solution was diluted by 100 fold and then EG activity was measured, because the value of A_{550} was too much high (over 2.0).
[d]not detected.

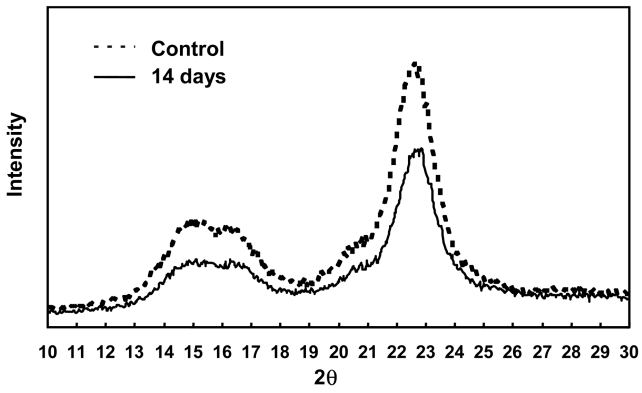

Fig. 2. Change in the crystallinity value of Avicel after 14- day incubation. Crystallinity was determined by X-Ray Diffractometry. Control samples were incubated without *F. palustris*.

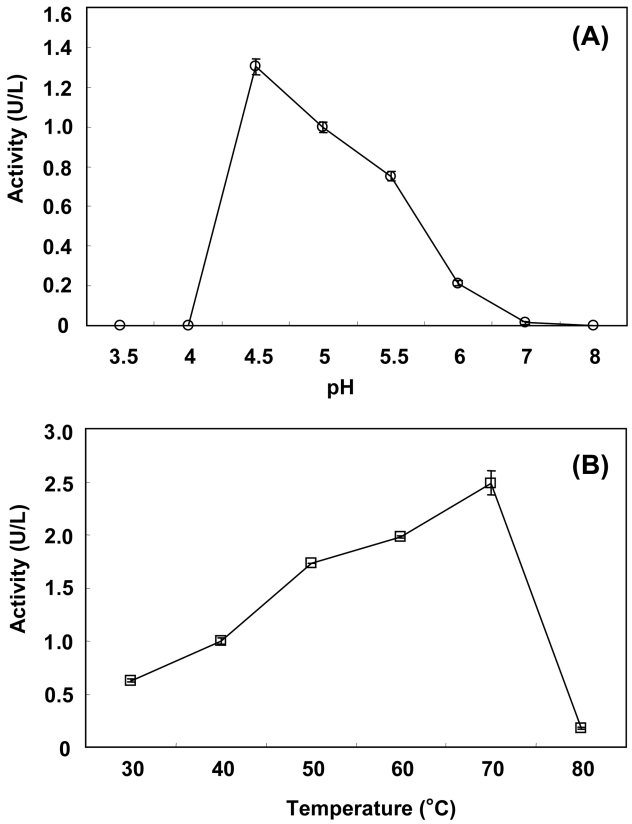

Fig. 3. Effects of pH (A) and temperature (B) on the activities of extracellular cellobiohydrolase (CBH) of *F. palustris* grown on 2.0% Avicel. Values represent the mean of three replicate determinations; error bars indicate standard deviation.

lucknowense (Gusakov *et al.*, 2005). The crude CBH activity from *F. palustris* also has a high temperature optimum.

Glucose production by Avicel hydrolysis

Avicel hydrolysis by the crude enzyme from *F. palustris* was studied for 2 days at 50°C and pH 5.0. The time course of glucose concentration by hydrolysis of Avicel is shown in Fig. 4. The initial hydrolysis rate was fast for the first 11 h and then the yield of glucose after 43 h was 1.6 mg/ml, which corresponded to a 3.2% degree of conversion of cellulose .This data also shows that the brown rot fungus *F. palustris* has an exocellulase activity to degrade crystalline cellulose.

In general, efficient hydrolysis of crystalline cellulose requires the cooperative action of three kinds of enzymes: CBHs, EGs, and BGL (Woodward, 1991; Tomme *et al.*, 1995). Data from this study indicates that *F. palustris* has these three major cellulases (Fig. 1) and is able to degrade crystalline cellulose (Fig. 2). Our results differed from those obtained by Ishihara and Shimizu (1984) who could not find enzymes active on crystalline cellulose in *F. palustris*. Recent reports have shown that the brown rot fungi *C. puteana* (Schmidhalter and Canevascini, 1993) and *G. trabeum* (Cohen *et al.*, 2005) secreted cellulases hydrolyzing crystalline cellulose to produce cellobiose. From these reports, therefore, it is now clear that *F. palustris* and other brown rot basidiomycetes produce cellulases capable of yielding soluble sugars from crystalline cellulose. The findings reported in this paper clearly suggest that *F. palustris* produces all of the known cellulase components necessary for wood decay. Further studies on

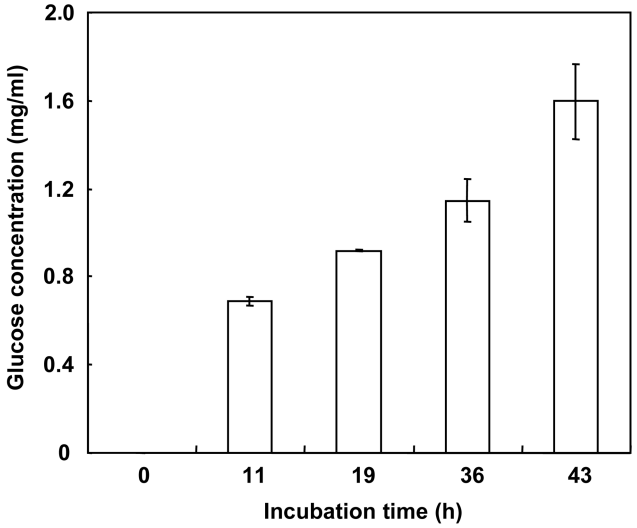

Fig. 4. The time course of glucose production from hydrolysis of Avicel by extracellular enzymes of *F. palustris*. Values represent the mean of three replicate determinations; error bars indicate standard deviation.

purification and characterization of the enzyme will be required to elucidate whether the major extracellular enzyme produced by *F. palustris* grown on crystalline cellulose is exocellulase.

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