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Production and Characterization of Cellobiohydrolase from a Novel Strain of *Penicillium purpurogenum* KJS506

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Abstract A high cellobiohydrolase (CBH)-producing strain was isolated and identified as *Penicillium purpurogenum* KJS506 according to the morphology and comparison of internal transcribed spacer rDNA gene sequence. When rice straw and corn steep powder were used as carbon and nitrogen sources, respectively, a maximum CBH activity of 2.6 U mg-protein^{-1}, one of the highest among CBH-producing microorganisms, was obtained. The optimum temperature and pH for CBH production were 30 °C and 4.0, respectively. The increased production of CBH in *P. purpurogenum* culture at 30 °C was confirmed by two-dimensional electrophoresis followed by MS/MS sequencing of the partial peptide. The internal amino acid sequences of *P. purpurogenum* CBH showed a significant homology with hydrolases from glycoside hydrolase family 7. The extracellular CBH was purified to homogeneity by sequential chromatography of *P. purpurogenum* culture supernatants on a DEAE-sepharose column, a gel filtration column, and then on a Mono Q column with fast-protein liquid chromatography. The purified CBH was a monomeric protein with a molecular weight of 60 kDa and showed broad substrate specificity with maximum activity towards *p*-nitrophenyl β-D-cellobiopyranoside. *P. purpurogenum* CBH showed $t_{1/2}$ value of 4 h at 60 °C and V_{max} value of 11.9 μmol min^{-1} mg-protein^{-1} for *p*-nitrophenyl-D-cellobiopyranoside. Although CBHs have been reported, the high specific activity distinguishes *P. purpurogenum* CBH.

Keywords Cellobiohydrolase · Enzyme production · Glycoside hydrolase · *Penicillium purpurogenum* · Purification

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Introduction

Cellulose, a main component of plant cell wall, represents the most abundant renewable biomass available on earth [15, 37]. It is a linear bio-polymer composed of 100–10,000 D-glucopyranosyl units linked by β-1,4-D-glucosidic bonds. Microbial cellulases catalyzing the hydrolysis of plant polysaccharides are industrially important enzymes used to saccharify industrial and agricultural cellulose-containing residues, treat cellulose pulp wastes in the paper industry, enhance the extraction of fermentable substances in the beer brewing and alcohol fermentation industries, etc. [3]. The cellulose degradation requires a multienzymatic system composed of three activities: endo-1,4-β-glucanase (EG, EC 3.2.1.4), cellobiohydrolase (CBH, EC 3.2.1.91), and β-glucosidase (BGL, EC 3.2.1.21) [5]. EG and CBH act cooperatively and synergistically in depolymerizing cellulose to cellobiose and short oligosaccharides, which are converted by BGL to glucose [31, 38]. CBHs are most efficient on highly ordered crystalline cellulose and cleave mainly cellobiose from the opposite ends of the glucose chains, whereas EGs act more randomly in the middle of the chains, probably in the more amorphous regions of cellulose [21, 29].

In the process of cellulose hydrolysis, enzyme production is still the most crucial and costly step. There is still a large gap between market price of enzyme and what would be economically feasible for a bio-refinery or bio-ethanol production facility. Filamentous fungi have demonstrated a great capability for secreting a wide range of cellulases, with the genera *Trichoderma* being the most extensively studied and reviewed among the cellulase producing fungi. Although the cellulolytic enzymes of *Trichoderma reesei* have been investigated thoroughly [24, 30], the amount of CBH secreted by this fungus is insufficient for effective conversion of cellulose to glucose [32]. In addition, there is an increasing demand for the production of CBH in the conversion of cellulose to cellobiose for the subsequent production of fuel ethanol [31].

Researchers are especially interested in fungal cellulases because they are secreted extracellularly and their activity is much higher than the cellulases from yeasts and bacteria [16]. *Penicillium* species have been known as a strong producer of cellulase [27]. However, only a few CBHs have been produced and characterized from the genus *Penicillium* [8, 35]. In the present study, a potent CBH-producing fungus was isolated and identified as *Penicillium purpurogenum*. Although CBH production by *P. purpurogenum* has been reported [28], the environmental conditions to enhance CBH production and characterization of CBH have never been thoroughly reported. Here, we optimized the environmental conditions to enhance CBH production by *P. purpurogenum*, purified and characterized an extracellular CBH from *P. purpurogenum* cultures. Under optimized conditions, *P. purpurogenum* produced CBH with a 2.6 U mg-protein^{-1} specific activity, which is one of the highest specific activities among CBH-producing microorganisms. The properties of the enzyme, including its substrate specificity, molecular form, and partial amino acid sequence revealed that this enzyme is a CBH, a member of GH7.

Materials and Methods

Isolation of Microorganism

The soil samples collected from Sorak Mountain (South Korea) by the capillary tube method were diluted in sterile dilution solution (0.9% saline), aliquots were spread on potato dextrose agar plate, and the plates were incubated for 3 days. The morphologically

different colonies were inoculated into a 3 mL of the growth medium containing (g L^{-1}) peptone 8, yeast extract 2, KH_2PO_4 5, K_2HPO_4 5, $MgSO_4 \cdot 7H_2O$ 3, thiamine·HCl 0.005, and microcrystalline cellulose 20 (Sigma), and cultivated at 28 °C with agitation at 200 rpm for 5 days. CBH activity of the culture broth was analyzed using *p*-nitrophenyl-D-cellobiopyranoside (pNPC, Sigma) as described previously [12]. One unit of pNPC-hydrolyzing activity was defined as the amount of enzyme equivalent to release 1 μmol of *p*-nitrophenol per minute. After analyses, the strain with the highest CBH activity was selected.

Identification of Microorganism

Fatty acid composition was analyzed by a gas chromatography (Agilent 6890 N, CA, USA), and the identification of the isolated strain was determined using the MIDI database. For the sequence analysis, the ITS1-5.8S-ITS2 rDNA region of the fungus was amplified by PCR using primer set pITS1 (5′-TCCGTAGGTGAACCTGCCG-3′) and pITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [36]. The 700-bp amplicon thus obtained was cloned and sequenced. The sequences were proofread, edited, and merged into composite sequences using the PHYDIT program version 3.1 (http://plaza.snu.ac.kr/~jchun/phydit). The identified strain *P. purpurogenum* KJS506 was deposited at the Korean Agricultural Culture Collection (KACC) and was given the KACC accession number 93053P.

Culture Conditions

Pre-cultures, inoculated with 1×10^8 *P. purpurogenum* KJS506 conidiospores, were grown in 100 mL of potato dextrose broth. Pre-cultures (5 mL) were inoculated into 200 mL of cellulolytic medium in a fermenter. The effect of carbon or nitrogen source on CBH production was investigated after 7 days of cultivation in flasks containing medium composed of 50 g L^{-1} of carbon sources and various nitrogen sources. The concentration of nitrogen source was adjusted to the same content of nitrogen using Kjeldahl method. The effect of temperature and pH on CBH production was analyzed in a 7-L fermenter with varying the growth temperatures (24 to 36 °C) and pH values (3.0 to 7.0). For fermenter culture, the mycelia of *P. purpurogenum* were inoculated into 100 mL of potato dextrose broth. Pre-cultures (5 mL) were inoculated into 3 L of medium in a 7-L fermenter. This culture media contained (g L^{-1}) yeast extract 10, KH_2PO_4 5, $(NH_4)_2SO_4$ 5, $MgSO_4 \cdot 7H_2O$ 3, and microcrystalline cellulose 40. The effect of dissolved oxygen tension (DOT) on CBH production was investigated at pH 7.0 and 30 °C for 7 days. DOT was controlled at 0~5%, 0~10%, 10~20%, 20~30%, and 30~40% by adjusting agitation speed. The other conditions were the same as described in fermenter culture. The enzyme activity was assayed after 6 days of cultivation in fermenters, and all the fermentation trials were carried out in triplicate.

Two-Dimensional Electrophoresis and Protein Identification

P. purpurogenum culture supernatant was concentrated using ultrafiltration (MWCO 10 kDa), washed with 20 mM Tris–HCl buffer (pH 7.5), and the supernatant was used as the protein solution. The protein sample was subjected to Isoelectricfocusing (Amersham Biotech, Uppsala, Sweden) following the manufacturer's instructions. The proteins were further separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-

PAGE) and stained with Coomassie brilliant blue R-250. The sliced protein spots were submitted and analyzed by nano-LC-MS/MS for internal amino acid sequencing as described [23]. To identify protein sequence, homology search method was employed with a MS data analysis program, SEQUEST (ThermoFinnigan, San Jose, CA, USA) against fungi protein database obtained from National Center for Biotechnology Information protein sequence database.

Enzyme Assay

CBH activity was assayed using pNPC (Sigma), cellotetraose, cellopentose, carboxymethylcellulose (CMC), and avicel (Fluka) as substrate. The enzymatic reaction mixtures (1 mL) containing 100 μL of enzyme solution and 10 mM pNPC (final concentration) in 100 mM sodium acetate buffer (pH 5.0) were incubated for 15 min at 50 °C. The amount of *p*-nitrophenol released was measured at A_{415} (ε_{415}=17.0 mM^{-1} cm^{-1}) after addition of 2 M Na_2CO_3 to the reaction mixtures. One unit of pNPC-hydrolyzing activity was defined as the amount of enzyme equivalent to release 1 μmol of *p*-nitrophenol per minute. Specific activities of the purified CBH for hydrolysis of 1% avicel or CMC were determined at 50 °C, pH 5.0 for 1 h. Avicel or CMC was diluted to appropriate concentrations with 0.1 M sodium acetate buffer [10], and CBH activity was measured by dinitrosalicylic acid method or GOD-POD method [18].

Purification of CBH

All procedures were performed at 4 °C, and 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol was used in the purification procedures unless otherwise stated. Protein was measured by the the Bradford method [4], using bovine serum albumin as a standard. Protein in the column effluents was monitored by measuring the absorbance at 280 nm. All chromatographic separations were performed using a BioLogic FPLC system (Bio-Rad, CA, USA).

Step 1: Preparation of Crude Enzyme Cells from the culture broth were harvested by centrifugation at 10,000×*g* for 30 min. After washing with 20 mM sodium acetate buffer (pH 5.0), the washes and supernatants were combined, concentrated, and desalted by ultrafiltration through a polyether sulfone membrane (50 kDa cut off) in a stirred cell (Amicon Inc. Beverly, MA, USA).

Step 2: DEAE-Sepharose Chromatography The dialyzed enzyme solution was loaded on a DEAE SepharoseTM Fast Flow column (1.6×10 cm, Amersham Biosciences) equilibrated with 20 mM sodium acetate buffer at pH 5.0, and protein was eluted with a 180-min linear gradient of 0–0.5 M NaCl in the same buffer at a flow rate of 1.0 mL/min. Fractions of 1 mL each were collected and assayed for CBH activity. Active fractions were pooled, dialyzed, and concentrated with ultrafiltration.

Step 3: Sephacryl Gel Filtration Chromatography The concentrated enzyme solution was loaded on a HiPrep 16/60 Sephacryl S-300 HR column (1.0 cm×120 cm, Amersham Biosciences) equilibrated with 20 mM sodium acetate buffer containing 100 mM NaCl at pH 5.0, and proteins were eluted with the same buffer at a flow rate of 0.5 mL/min. Active fractions were pooled, dialyzed against the same buffer, and concentrated with ultrafiltration.

Step 4: MonoQ Ion Exchange Chromatography The enzyme was further purified with a MonoQ ion exchange column 5/50 GL (1.0×10 cm, Amersham Biosciences) previously equilibrated with 20 mM sodium acetate buffer (pH 5.0). The enzyme was eluted with a 180-min linear gradient of 0–0.5 M NaCl in the same buffer at a flow rate of 0.5 mL/min. The combined active fractions were pooled, concentrated, and dialyzed against the same buffer and concentrated with a Centricon (Millipore Corp., Bedford, MA, USA) ultrafiltration device with a molecular mass cutoff of 50 kDa, and then used as a purified enzyme in the following experiments.

Determination of pH and Temperature Optima

The optimal pH of CBH activity was determined by incubating the purified enzyme at 50 °C for 15 min in different buffers: citrate (100 mM, pH 3–4), sodium acetate (100 mM, pH 4–6), and phosphate (100 mM, pH 6–8). To determine the optimal temperature, the enzyme was incubated in sodium acetate buffer (100 mM, pH 5) for 15 min at different temperatures: from 40 to 80 °C. To determine the thermo-stability of CBH activity, the purified enzyme was incubated at different temperatures (40, 50, 60, 70, and 80 °C) in the absence of substrate. After incubating them for certain periods of time (0–72 h), the residual CBH activity was determined as described above.

PAGE and Molecular Mass Determination

For the determination of subunit molecular mass, SDS-PAGE was performed as described by Laemmli [11] with 10% gels. Protein bands were visualized with Coomassie brilliant blue R-250 (Sigma). Isoelectric focusing was performed with 7.5% polyacrylamide gels (0.5×10 cm) supplemented with ampholytes (0.4% pH 3–10) as described by O'Farrel et al. [19]. The molecular mass of the purified enzyme was determined by size exclusion chromatography using a SuperoseTM 12 (Amersham Pharmacia Biotech, Uppsala, Sweden) column attached to a BioLogic FPLC system (Bio-Rad). The enzyme was eluted with 20 mM sodium acetate (pH 5.0) at a flow rate of 0.5 mL/min.

Determination of Kinetic Parameters and Inhibition Constants

The values of the Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined for CBH by incubating in 100 mM sodium acetate buffer pH 4.5 at 50 °C with pNPC and CMC at concentrations ranging from 0.5 to 50 mM. Values for K_m and V_{max} were determined from Lineweaver–Burk plots using standard linear regression techniques.

Internal Amino Acid Sequence of CBH

The purified protein was resolved by SDS-PAGE and then electroblotted onto a polyvinylidene trifluoride membrane (Bio-Rad). Protein cleavage for peptide mapping was carried out at 37 °C for 4 h with 100 ng of endoproteinase Asp-N or endoproteinase Lys-C or trypsin (Promega, Madison, WI, USA) to digest 20 μg of purified enzyme in 50 μL of 100 mM $(NH_4)_2CO_3$ (pH 8.5). The resulting peptide fragments were separated by SDS-PAGE (15% polyacrylamide), and the separated peptides were transferred to a polyvinylidene trifluoride membrane by electroblotting. Peptide bands were visualized by 0.1% Coomassie brilliant blue R-250 staining in 40% methanol. The partial amino acid

sequence was determined by Edman degradation with an automatic protein sequencer (model 491A; Applied Biosystems, Division of Perkin-Elmer) at The National Instrumentation Center for Environmental Management (Seoul, South Korea). The partial amino acid sequence was used to identify analogous proteins through a BLAST search of the nonredundant protein database. Genomic DNA was isolated using Wizard nucleic acid purification kit (Promega). Degenerate primers (PpuCBHDF 5′-ACRCYGGHR TTGGMWCCTGC and PpuCBHDR 5′-GKRCCRAACTTRATGTTSG) were designed based on the partial peptide sequences EGWVPST and SNIKFGT, respectively. PCR was performed as follows: All PCRs contained 1× buffer, 4 μL of 10 mM dNTPs, 10 pmol/μL of each primer, and 5 units of *Taq* DNA polymerase in 100 μL. Amplification was performed with one cycle of 94 °C for 5 min followed by 35 cycles of denaturation (60 s at 94 °C), annealing (60 s at 45–55 °C), and extension (180 s at 72 °C), with a final extension of 72 °C for 10 min. For analysis, 10 μL of reaction mixture was electrophoresed on a 1% agarose gel and stained with ethidium bromide solution (5 μg mL^{-1}). The purified PCR products were ligated to TA cloning vector (RBC Bioscience, Taipei, Taiwan) and sequenced with M13 primers from both strands at Macrogen (Seoul, South Korea).

Results and Discussion

Identification of the Isolated Strain

Among 340 strains screened for CBH activity, 20 strains were selected based on the fluorescence observed when the agar plates containing 10 mM of MUC were exposed under UV. Out of 20 isolates, an efficient CBH-producing microorganism was selected for further study. Sequencing of the ITS rDNA region of the isolated fungus was performed, and the ITS sequence was submitted to GenBank with the accession no. GQ292537. The strain showed the highest identity (99%) with *P. purpurogenum*. Phylogenetic relationships were inferred using alignment and cladistic analyses of homologous nucleotide sequences of known microorganisms, and the approximate phylogenetic position of the strain is shown in Fig. 1. The probability that the isolated fungus and *P. purpurogenum* belong to the same branch is 99%. In addition, the composition of the cellular fatty acids of the isolated strain was similar to those of the *Penicillium* species (Table 1). The colonies are flat, filamentous, and velvety, or cottony in texture. The conidiophores (3–3.5 μm in diameter) are simple or branched and are terminated by clusters of flask-shaped phialides. Based on its morphology, the composition of its cellular fatty acids, and a comparison of the ITS rDNA gene sequences, the isolated strain was identified as a strain of *P. purpurogenum* and was named as *P. purpurogenum* KJS506.

Optimization of Carbon and Nitrogen Sources for CBH Production in Flasks

To select suitable carbon source for CBH production, *P. purpurogenum* KJS506 was cultivated in the medium containing various carbon sources (cellulose, rice straw, wheat bran, xylan, avicel, carboxymethylcellulose, cellobiose, glucose, maltose, lactose, and sucrose). Among various carbon sources, rice straw was the best carbon source for CBH production. And the optimum concentration was determined to be 50 g L^{-1} leading to CBH specific activity of 1.1 U mg-protein^{-1} in a flask culture.

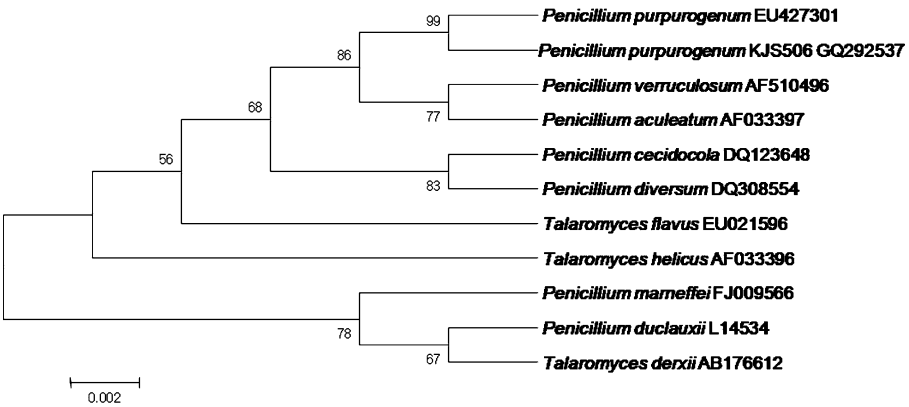

Fig. 1 The phylogenetic dendrogram for *P. purpurogenum* KJS506 and related strains based on the ITS rDNA sequence. *Numbers following the names of the strains* are accession numbers of published sequences

Since the mechanisms that govern the formation of extracellular enzymes are influenced by the availability of precursors for protein synthesis, the effect of inorganic and organic nitrogen sources on CBH synthesis was also studied. Among the various nitrogen sources (peptone, tryptone, corn steep powder, yeast extract, urea, ammonium sulfate, sodium glutamate, and sodium nitrate), corn steep powder (10 g L^{-1}) favored maximum CBH (1.3 U mg-protein^{-1}) production, followed by yeast extract, while inorganic nitrogen sources were poor sources of nitrogen in the absence of pH control. When nitrogen sources were combined, it showed inhibitory effect on CBH activity. CBH production was maximal (1.3 U mg-protein^{-1}) at 50 g L^{-1} of rice straw and 10 g L^{-1} of CSP in the production medium.

Effect of pH and Temperature on CBH Production in a Fermenter

The influence of culture medium pH on CBH production was studied in the range of pH 3.0–6.5. As the pH of culture media increased from 3.0 to 4.0, CBH activity increased with the maximal specific activity of 1.6 U mg-protein^{-1} at pH 4.0; after pH 4.5, CBH production significantly declined until it became almost zero at pH 6.5 (Fig. 2a). In addition, there were distinct differences in cell growth between the cultures grown at pH 4.0 and those grown at 5.0 or 6.0. The cultures at pH 4.0 showed the higher concentrations of cells, suggesting that lower pH stimulated the growth of *P. purpurogenum.*

Table 1 Fatty acid compositions of *Penicillium* strains.

	% of fatty acid contents									Reference
	14:0	15:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	
Penicillium paraherquei	0.29	0.80	13.73	0.46	2.42	2.67	3.30	25.41	40.46	[25]
Penicillium restrictum	0.34	0.22	19.52	0.34	0.43	0.21	7.20	34.49	36.42	[25]
Penicillium simplicissimum	0.49	0.61	16.93	0.39	0.77	0.48	5.90	25.98	46.81	[25]
Penicillium verruculosum	0.25	–	28.07	0.47	–	–	5.86	20.56	44.19	[25]
Penicillium purpurogenum KJS506	0.27	0.66	16.92	0.41	–	–	5.76	26.30	42.72	This study

Each value represents the mean of triplicate measurements and varied from the mean by not more than 15%

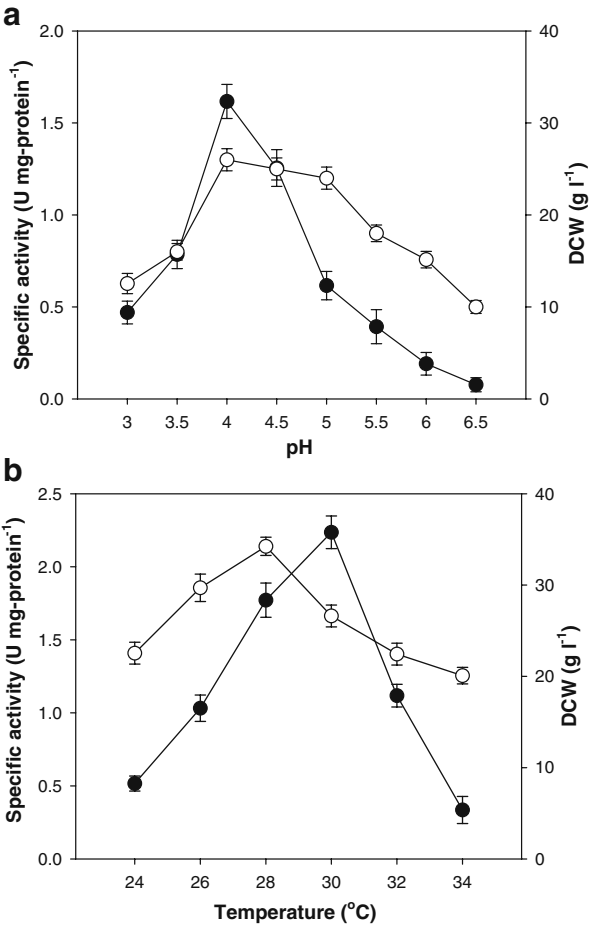

Fig. 2 **a** Effect of pH on the specific activity of CBH in *P. purpurogenum* culture. Cultivation of *P. purpurogenum* KJS506 was performed for 6 days in a 7-L fermenter. Cultivation temperature and rpm were controlled as 28 °C and 200 rpm, respectively. **b** Effect of temperature on the specific activity of BGL in *P. purpurogenum* culture. Cultivation pH and rpm were controlled as pH 4.0 and 200 rpm, respectively. Specific activity (*closed circles*), DCW (*open circles*)

To investigate the effect of temperature on CBH production, *P. purpurogenum* KJS506 was grown at various temperatures in a fermenter containing 50 g rice straw L^{-1} medium (Fig. 2b). At 30 °C, *P. purpurogenum* showed a significantly higher CBH activity (2.2 U mg-protein^{-1}) than that at 24 °C (0.5 U mg-protein^{-1}), suggesting that temperature is a critical factor for CBH production in *P. purpurogenum.*

Proteome Analysis of the *P. purpurogenum* Culture at 24 and 30 °C

Figure 3 shows the proteome expression profiles of the culture at 24 and 30 °C. Expression levels of several protein spots around p*I* 3.5–6.0 [24, 30, 32], which is the p*I* range of various CBHs, were significantly changed. Especially, the spots mentioned as 1, 2, and 3 in the culture at 30 °C were significantly increased in the expression level compared with those at 24 °C. The sliced protein spots were submitted and analyzed by nano-LC-MS/MS for internal amino acid sequencing as described [23]. Among them, spot 2 with two fragment sequences of CEADNCGGT and MVLVLSPWDD was identified as CBH by nano-LC-MS/MS sequencing, suggesting the increased production of CBH due to the

change of the growth temperature. In the same way, spots 1 and 3 were identified as peptidase and acyl CoA esterase, respectively.

Optimization of Dissolved Oxygen Tension for CBH Production

The effect of DOT on growth and CBH production was investigated in 7-L fermenters. Fermentation was carried out without DOT control until 36 h since DOT was maintained above 40%. After 36 h of culture, DOT was controlled to be maintained within the established value (0–5%, 5–10%, 10–20%, 20–30%, and 30–40%) by varying the agitation speed. Growth of all culture showed very similar pattern, regardless of their DOT limit. However, specific CBH activity increased as DOT limit rose up to 10–20%. When DOT was controlled above 10%, the highest CBH specific activity (2.6 U mg DCW^{-1}) was attained.

Purification of a CBH from *P. purpurogenum* Culture

The enzyme from the culture supernatant of *P. purpurogenum* grown on rice straw was purified 7.5-fold to homogeneity with an overall enzyme yield of 9.9% and a specific activity of 10.8 U mg-$protein^{-1}$ (Table 2). Analysis of the enzyme by gel electrophoresis in the presence of SDS revealed one band with a M_r of 60,000 (Fig. 4a). Size exclusion chromatography on a Sephacryl S-300 high-resolution column resulted in the elution of the enzyme activity as a symmetrical peak corresponding to a M_r of approximately 61,000 (Fig. 4b). These results indicate that the enzyme migrates as a monomer in gel filtration under the mild conditions used, and thus may also be present and active as a monomer in solution.

Optimum pH and Temperature for CBH Activity

The purified CBH showed a significant activity from pH 4 to 7. The optimum pH for *P. purpurogenum* CBH was 5.0, with 82% and 79% of the maximum activity at pH 4.0 and

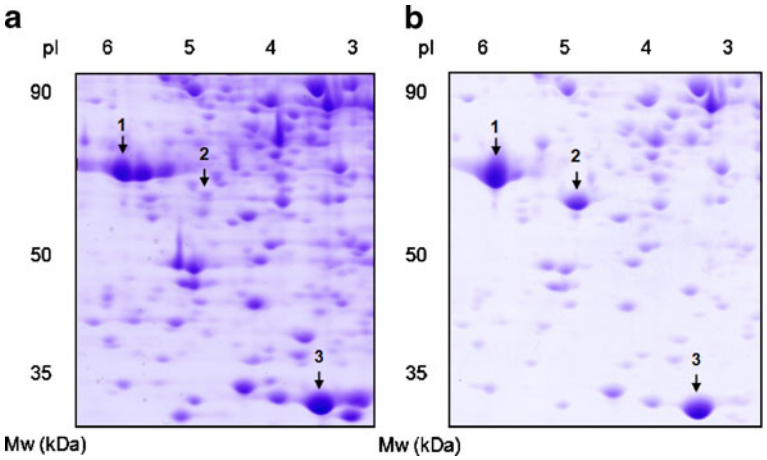

Fig. 3 Proteome expression profiles of *P. purpurogenum* KMJ601 grown at 24 °C (**a**) and 30 °C (**b**). Each composite image is based on 2-D gels from replicate cultures. The *horizontal axis* represents the approximate pH range of isoelectric focusing first dimension. The *vertical axis* represents the molecular mass in kilodaltons. *1* peptidase, *2* CBH, *3* acyl CoA esterase

Table 2 Purification of CBH from culture broth of *P. purpurogenum* KJS506.

Procedure	Total protein (mg)	Total activity (U)	Specific activity (U mg-protein^{-1})	Yield (%)	Purification fold
Crude	89	232	2.6	100	1
Ultrafiltration (PES50)	64	186	2.9	80.2	1.1
DEAE ion exchange chromatography	22.7	109	4.8	47.0	1.8
Sephacryl gel filtration chromatography	6.4	52	8.2	22.4	3.2
MonoQ ion exchange chromatography	2.1	23	10.8	9.9	4.2

pH 6.0, respectively. Most of the reported CBHs showed maximum activity in acidic pH range 4.0–6.0. The isoelectric point, p*I*, was determined to be 4.8, which is also typical (3.5–6.0) for extracellular CBHs (http://www.brenda.uni-koeln.de) [2]. These properties are similar to those found for the majority of fungal cellulases. Highest CBH activity was detected at 60 °C which decreased rapidly at temperatures over 60 °C. The stability of purified CBH was studied at various temperatures from 30 to 70 °C, and the enzyme showed $t_{1/2}$ values of 72, 46, 22, 4, and 0.5 h at 30, 40, 50, 60, and 70 °C, respectively.

Effects of Metal Ions and Various Compounds on CBH Activity

The effects of various metal ions and reagents at 0.1 mM on CBH activity were determined by preincubating the enzyme with the individual reagents in 20 mM sodium acetate buffer pH 5.0 for 30 min. $ZnCl_2$ and $CoCl_2$ (each at 0.1 mM) inhibited CBH activity by 21.2%

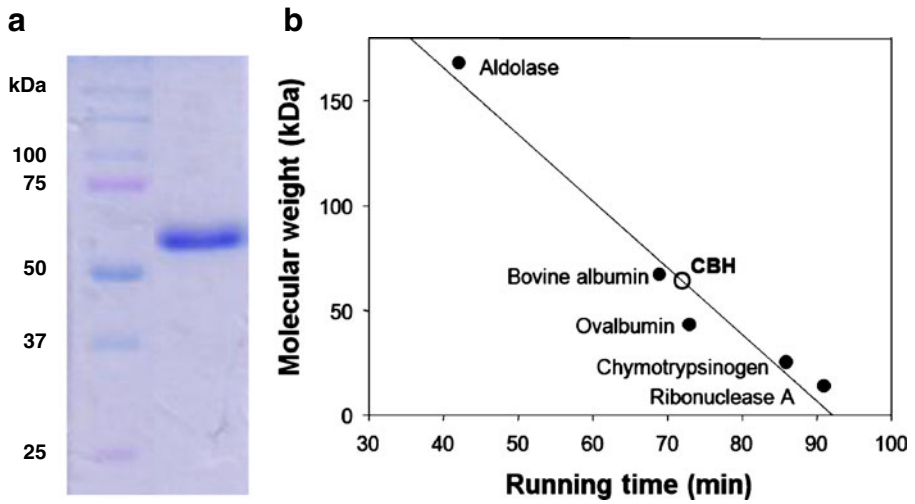

Fig. 4 SDS-PAGE and molecular mass determination of CBH purified from the newly isolated *P. purpurogenum*. **a** SDS-PAGE of purified CBH. **b** Determination of native molecular mass of *P. purpurogenum* CBH by gel filtration chromatography on a Sephacryl S-300 high-resolution column. The column was calibrated with standard molecular weight proteins such as aldolase (168 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa)

Table 3 Properties of CBHs from various sources.

	M_r (kDa)	Quaternary structure	Opt. pH	Opt. temp (°C)	Specific activity (U mg^{-1})	p*I*	Reference
Dichomitus Squalens Ex-1	39	Monomer	5	60	13.64	4.6	[22]
Dichomitus Squalens Ex-2	36	Monomer	5	60	12.06	4.5	[22]
Talaromyces emersonii CBH IA	66	Monomer	3.6	78	7.7	4.54	[33]
Talaromyces emersonii CBH IB	56	Monomer	4.1	66	2.4	3.7	[33]
Talaromyces emersonii CBH II	56	Monomer	3.8	68	NR	6.09	[33]
Trametes versicolor	55	Monomer	5	40	0.8	NR	[12]
Penicillium occitanis CBH I	60	NR	4~5	60	1.09	5.2	[14]
Penicillium occitanis CBH II	55	NR	4~5	65	0.03	5.9	[14]
Thermoascus aurantiacus	48	NR	6	65	0.11	NR	[7]
Penicillium purpurogenum KJS506	60	Monomer	5	60	10.8	4.8	This study

All specific activities were obtained with pNPC as a substrate.

NR not reported

and 33.7%, respectively, and EDTA inhibited the enzymatic activity by 24.6%. In contrast, Fe^{2+} and Hg^{2+} ions caused complete inhibition of *P. purpurogenum* CBH. The effects of sulfhydryl compounds on CBH activity were also examined. The addition of 0.5 mM β-mercaptoethanol to the reaction mixture increased the enzymatic activity by 36.5%.

Substrate Specificity and Kinetic Parameters of *P. purpurogenum* CBH

P. purpurogenum CBH exhibited activity with pNPC (10.8 U mg-protein^{-1}), avicel (6.5 U mg-protein^{-1}), and filter paper (8.1 U mg-protein^{-1}). These substrates are known to be preferred substrates for CBHs and, to a lesser extent, for EGs. In contrast, CMC, a model substrate for the detection of EGs [30], was not a substrate for *P. purpurogenum* CBH. These results suggest that the enzyme is a CBH. In addition, the enzyme readily hydrolyzed cellooligosaccharides including cellotetraose and cellopentaose, and cellobiose was the major product. Therefore, the purified *P. purpurogenum* enzyme is a CBH, rather than an endo-type cellulase or β-glucosidase, which hydrolyzes cellooligosaccharides to glucose. This hypothesis is further supported by the analysis of the partial peptide sequence, which indicates that *P. purpurogenum* CBH belongs to GH7.

Initial velocities were determined in the standard assay mixture at pH 5.0. The substrates tested had hyperbolic saturation curves, and the corresponding double-reciprocal plots were linear. The concentration of pNPC varied from 0 to 50 mM. CBH activity with increasing pNPC concentrations followed typical Michaelis–Menten-type kinetics. The Lineweaver–Burk plot obtained for the conversion of pNPC under standard assay conditions showed a

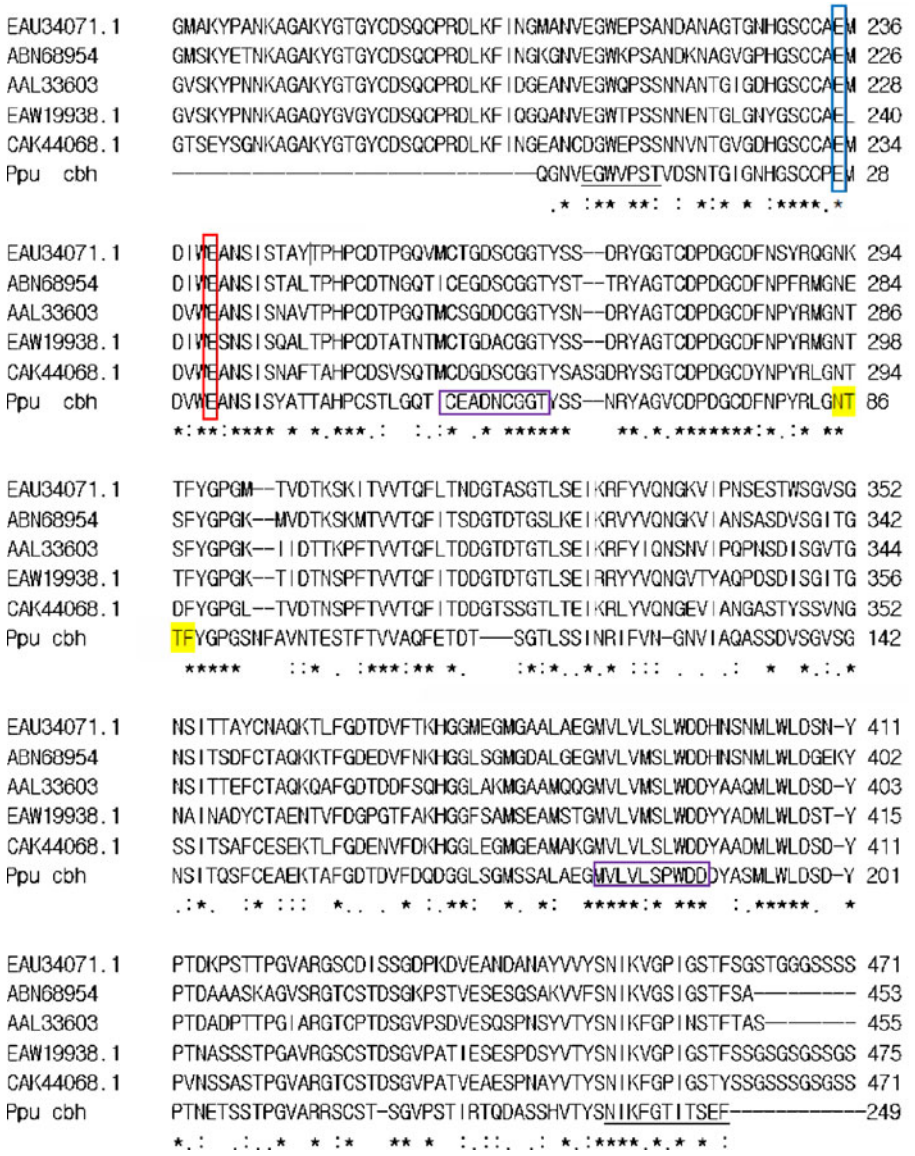

Fig. 5 Sequence alignment of *P. purpurogenum* CBH-like protein with other cellulases. The amino acid sequences of CBH like protein and five other glycoside hydrolases in family 7 were aligned. Residue positions that are identical in all six sequences are indicated with *asterisks*. The accession numbers are CAK44068.1 (*A. niger*), AAL33603 (*T. emersonii*), EAU34071.1 (*A. terreus*), EAW19938.1 (*N. fischeri*), and ABN68954 (*P. decumbens*). The Glu-27 equivalent residue was identified as a potential nucleophile in the displacement reaction (*blue-framed residue*), and the Glu-32 equivalent residue was identified as a potential proton donor (*red-framed residue*). Potential N-glycosylation sites are shown in *yellow color*. The *purple-framed residues* indicate the amino acids deduced from spot 2 in Fig. 3. The *line* indicates the amino acids obtained from LC-MS/MS sequencing

K_m of 1.1 mM and V_{max} of 11.9 U mg-protein^{-1}. The K_m value of *P. purpurogenum* CBH for pNPC is similar to those obtained with the *T. reesei* CBH1 (0.25 mM), the *Talaromyces emersonii* CBH1A and CBH1B (2.1 mM and 0.8 mM), the *Trametes versicolor* TvCel7a (0.58 mM), and the *Phanerochaete chrysosporium* CBH1 (0.38 mM) [9, 12, 26, 33]. Specific activity or V_{max} value of *P. purpurogenum* CBH is also within the range of those reported for CBHs (Table 3).

Identification of the Partial Gene Product

A partial 623-bp amplicon was obtained by PCR using a degenerate primer pair, PpuCBHDF and PpuCBHDR, which were designed based on the peptide sequences identified by nano-LC-MS/MS sequencing. Based on amino acid sequence similarities, glycosidases have been classified into several families, with most CBHs belonging to either family 6 or family 7 [6]. Partial gene product amplified by PCR from the fragment sequences contained the pfam00840 CBH-like domain present in GH family. It also contained E_{27}MDVWE_{32}AN residues (Fig. 5), which are highly conserved in GH7 [20]. In the fragment E_{27}MDVWE_{32}AN, Glu-27 residue has been identified as a potential nucleophile in the displacement reaction and Glu-32 identified as a potential proton donor [1, 7]. The consensus sequence (N-Xaa-S/T) corresponding to the potential N-glycosylation sites was also found in the partial gene product (N_{85}TTF_{88}) of *P. purpurogenum* CBH (26). A homology search revealed that the deduced gene product had 63%, 63%, 61%, 60%, and 58% amino acid identity with GH7 CBHs of *Aspergillus niger* (CAK44068.1), *T. emersonii* (AAL33603), *Aspergillus terreus* (EAU34071.1), *Neosartorya fischeri* (EAW19938.1), and *Penicillium decumbens* (ABN68954), respectively (Fig. 5). The evidence from enzymology and bioinformatics studies strongly suggests that *P. purpurogenum* CBH should be classified as a member of GH7.

In conclusion, a potent CBH-producing strain was isolated and identified as *P. purpurogenum* KJS506. Under the optimal culture condition, *P. purpurogenum* KJS506 is one of the highest among CBH-producing microorganisms [12, 13, 17, 34]. Although CBH-producing microorganisms have been reported from other sources, *P. purpurogenum* KJS506 is distinguished by its high CBH specific activity of the culture broth, which makes *P. purpurogenum* KJS506 a good candidate for the industrial applications including textile finishing, paper/pulp, and saccharification for bio-ethanol production. Further studies on the cloning of the full-length gene encoding CBH and structural characterization of CBH from *P. purpurogenum* KJS506 are in progress.

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References

1. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research, 25*, 3389–3402.
2. Baldrian, P., & Valaskova, V. (2008). Degradation of cellulose by Basidiomycetous fungi. *FEMS-Microbiology Reviews, 32*, 501–521.

3. Bhat, M. K., & Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. *Biotechnology Advances, 15*, 583–620.
4. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.
5. Eriksson, K., Blanchette, R. A., & Ander, P. (1990). *Microbial and enzymatic degradation of wood and wood components*. Berlin: Springer.
6. Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal, 280*, 309–316.
7. Hong, J., Tamaki, H., Yamamoto, K., & Kumagai, H. (2003). Cloning of a gene encoding thermostable cellobiohydrolase from *Thermoascus aurantiacus* and its expression in yeast. *Applied Microbiology and Biotechnology, 63*, 42–50.
8. Hou, Y., Wang, T., Long, H., & Zhu, H. (2007). Cloning, sequencing and expression analysis of the first cellulase gene encoding cellobiohydrolase 1 from a cold-adaptive *Penicillium chrysogenum* FS010. *Acta Biochim Biophys Sin (Shanghai), 39*, 101–107.
9. Igarashi, K., Samejima, M., & Eriksson, K. E. (1998). Cellobiose dehydrogenase enhances *Phanerochaete chrysosporium* cellobiohydrolase I activity by relieving product inhibition. *European Journal of Biochemistry, 253*, 101–106.
10. Kanokratana, P., Chantasingh, D., Champreda, V., Tanapongpipat, S., Pootanakit, K., & Eurwilaichitr, L. (2008). Identification and expression of cellobiohydrolase (CBHI) gene from an endophytic fungus, *Fusicoccum sp.* (BCC4124) in *Pichia pastoris*. *Protein Expression and Purification, 58*, 148–153.
11. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680–685.
12. Lahjouji, K., Storms, R., Xiao, Z., Joung, K. B., Zheng, Y., Powlowski, J., et al. (2007). Biochemical and molecular characterization of a cellobiohydrolase from *Trametes versicolor*. *Applied Microbiology and Biotechnology, 75*, 337–346.
13. Li, Y. L., Li, D. C., & Teng, F. C. (2006). Purification and characterization of a cellobiohydrolase from the thermophilic fungus *Chaetomium thermophilus* CT2. *Wei Sheng Wu Xue Bao, 46*, 143–6.
14. Limam, F., Chaabouni, S., Ghrir, R., & Marzouki, N. (1995). Two cellobiohydrolases of *Penicillium occitanis* mutant Pol 6: Purification and properties. *Enz Microbial Technol, 17*, 340–346.
15. Lynd, L. R., Laser, M. S., Bransby, D., Dale, B. E., Davison, B., Hamilton, R., et al. (2008). How biotech can transform biofuels. *Nature Biotechnology, 26*, 169–172.
16. Mathew, G. M., Sukumaran, R. K., Singhania, R. R., & Pandey, A. (2008). Progress in research on fungal cellulases for lignocellulose degradation. *Journal of Scientific and Industrial Research, 67*, 898–907.
17. Medve, J., Lee, D., & Tjerneld, F. (1998). Ion-exchange chromatographic purification and quantitative analysis of *Trichoderma reesei* cellulases cellobiohydrolase I, II and endoglucanase II by fast protein liquid chromatography. *J Chromatography A, 808*, 153–165.
18. Miller, G. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analy Chem, 31*, 426–428.
19. O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry, 250*, 4007–4021.
20. Ohnishi, Y., Nagase, M., Ichiyanagi, T., Kitamoto, Y., & Aimi, T. (2007). Transcriptional regulation of two cellobiohydrolase encoding genes (cel1 and cel2) from the wood-degrading Basidiomycete *Polyporus arcularius*. *Applied Microbiology and Biotechnology, 76*, 1069–1078.
21. Parkkinen, T., Koivula, A., Vehmaanpera, J., & Rouvinen, J. (2008). Crystal structures of *Melanocarpus albomyces* cellobiohydrolase Cel7B in complex with cello-oligomers show high flexibility in the substrate binding. *Protein Science, 17*, 1383–1394.
22. Rouau, X., & Odier, E. (1986). Purification and properties of two enzymes from *Dichomitus squalens* which exhibit both cellobiohydrolase and xylanase activity. *Carbohydrate Research, 145*, 279–292.
23. Shevchenko, A., Wilm, M., Vorm, O., & Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Analytical Chemistry, 68*, 850–858.
24. Shoemaker, S., Schweickart, V., Ladner, M., Gelfand, D., Kwok, S., Myambo, K., et al. (1983). Molecular cloning of exo–cellobiohydrolase I derived from *Trichoderma reesei* strain l27. *Bio/Technology, 1*, 691–696.
25. Stahl, P., & Klug, M. (1996). Characterization and differentiation of filamentous fungi based on fatty acid composition. *Applied and Environmental Microbiology, 62*, 4136–4146.
26. Stahlberg, J., Divne, C., Koivula, A., Piens, K., Claeyssens, M., Teeri, T. T., et al. (1996). Activity studies and crystal structures of catalytically deficient mutants of cellobiohydrolase I from *Trichoderma reesei*. *Journal of Molecular Biology, 264*, 337–349.

27. Sun, X., Liu, Z., Qu, Y., & Li, X. (2008). The effects of wheat bran composition on the production of biomass-hydrolyzing enzymes by *Penicillium decumbens*. *Applied Biochemistry and Biotechnology, 146*, 119–128.
28. Takao, S., Kamagata, Y., & Sasaki, H. (1985). Cellulase production by *Penicillium purpurogenum*. *Journal of Fermentation Technology, 63*, 127–134.
29. Teeri, T. T., Koivula, A., Linder, M., Wohlfahrt, G., Divne, C., & Jones, T. A. (1998). *Trichoderma reesei* cellobiohydrolase: Why so efficient on crystalline cellulose? *Biochemical Society Transactions, 26*, 173–178.
30. Teeri, T. T., Lehtovaara, P., Kauppinen, S., Salovuori, I., & Knowles, J. (1987). Homologous domains in *Trichoderma reesei* cellulolytic enzymes: Gene sequence and expression of cellobiohydrolase II. *Gene, 51*, 43–52.
31. Tomme, P., Kwan, E., Gilkes, N. R., Kilburn, D. G., & Warren, R. A. (1996). Characterization of CenC, an enzyme from *Cellulomonas fimi* with both endo- and exoglucanase activities. *Journal of Bacteriology, 178*, 4216–4223.
32. Tomme, P., Van Tilbeurgh, H., Pettersson, G., Van Damme, J., Vandekerckhove, J., Knowles, J., et al. (1988). Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. *European Journal of Biochemistry, 170*, 575–581.
33. Tuohy, M. G., Walsh, D. J., Murray, P. G., Claeyssens, M., Cuffe, M. M., Savage, A. V., et al. (2002). Kinetic parameters and mode of action of the cellobiohydrolases produced by *Talaromyces emersonii*. *Biochimica Et Biophysica Acta, 1596*, 366–380.
34. Uzcategui, E., Ruiz, A., Montesino, R., Johansson, G., & Pettersson, G. (1991). The 1, 4-beta-D-glucan cellobiohydrolases from Phanerochaete chrysosporium. I. A system of synergistically acting enzymes homologous to Trichoderma reesei. *Journal of Biotechnology, 19*, 271–285.
35. Wang, T., Wang, C., Gao, P., Zhong, L., & Zou, Y. (1998). Subcloning and expression of coding region for cellulase binding domain of CBH I from *P. janthinellum* in *E. coli*. *Wei Sheng Wu Xue Bao, 38*, 269–75.
36. White, T., Bruns, T., & Lee, S. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. Innis & T. White (Eds.), *PCR protocols: A guide to methods and applications* (pp. 315–322). New York: Academic.
37. Zhang, Y. H. P. (2009). A sweet out-of-the-box solution to the hydrogen economy: Is the sugar-powered car science fiction? *Energy Environ Sci, 2*, 272–282.
38. Zhang, Y. H. P., Himmel, M. E., & Mielenz, J. R. (2006). Outlook for cellulase improvement: Screening and selection strategies. *Biotechnology Advances, 24*, 452–481.