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Characteristics of bifunctional acidic endoglucanase (Cel5B) from *Gloeophyllum trabeum*

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Abstract The endoglucanase (Cel5B) from the filamentous fungus *Gloeophyllum trabeum* was cloned and expressed without a signal peptide, and alanine residue 22 converted to glutamine in *Pichia pastoris* GS115. The DNA sequence of *Cel5B* had an open reading frame of 1,077 bp, encoding a protein of 359 amino acid residues with a molecular weight of 47 kDa. On the basis of sequence similarity, Cel5B displayed active site residues at Glu-175 and Glu-287. Both residues lost full hydrolytic activity when replaced with alanine through point mutation. The purified recombinant Cel5B showed very high specific activity, about 80- to 1,000-fold and 13- to 70-fold in comparison with other endoglucanases and cellobiohydrolase, on carboxymethylcellulose and filter paper, respectively, at pH 3.5 and 55°C. Cel5B displayed bifunctional characteristics under acidic conditions. The kinetic properties of the enzyme determined using a Lineweaver–Burk plot indicated that Cel5B is a catalytically efficient cellulolytic enzyme. These results suggest that Cel5B has high bifunctional endo- and exoglucanase activity under acidic conditions and is a good candidate for bioconversion of lignocellulose.

Keywords *Gloeophyllum trabeum* · Endoglucanase · Bifunctional · Point mutation · *Pichia pastoris*

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Introduction

Microorganisms employ the modes of energy generation and metabolism that are best suited for their adaptation to the diverse habitats and conditions they are exposed to in nature. Microbes produce a range of hydrolytic enzymes that allow them to utilize a wide variety of substrates for their nutrition. For example, some microorganisms have evolved mechanisms, such as expression of cellulolytic enzymes, to efficiently utilize cellulosic biomass that is abundantly available in nature. Research on microbial cellulase, in addition to enhancing our understanding of behavioral microbiology, can lead to technological developments for industrial applications, such as production of bioethanol from cellulosic biomass.

Generating alternative energy has become a central issue because of limited fossil fuels and global warming. Many alternatives to fossil fuels are available that are less toxic, renewable, and have eco-friendly properties [8, 9, 28]. Of these, cellulosic bioethanol is a major alternative energy source. Enzymatic hydrolysis of cellulosic and lignocellulosic biomass constitutes the largest portion of the bioethanol

production price because of the high enzyme prices, and lowering the costs can accelerate advances in the industrial scale production of bioethanol. Progress is being made to clone cellulases using a broad range of organisms, and exploiting bacteria, yeast, and plant protein expression systems to hydrolyze cellulose [13, 17, 21]. However, some critical factors for cellulase selection include strong cellulase activity, high expression, and stability [3, 14].

Degradation of cellulose requires a hydrolytic enzyme complex involving the synergistic action of endoglucanase (EC 3.2.14) and cellobiohydrolase (EC 3.2.1.91). Cellobiohydrolase releases cellobiose from reducing and non-reducing ends, whereas endoglucanase randomly cleaves internal glucosidic bonds. On the basis of structural characteristics and the mechanism of action, endoglucanases have been classified into 14 glycosyl hydrolase families (GHF), i.e., GHF5, 6, 7, 8, 9, 10, 12, 26, 44, 45, 48, 51, 61, and 74 (see http://www.cazy.org/Glycoside-Hydrolases.html) [11, 12]. GHF5 is one of the largest GHFs and is widespread in fungi, bacteria, insects, and plant-parasitic nematodes [4, 19]. The three-dimensional structure of GH5 cellulases exhibits the $(\beta/\alpha)_8$ barrel fold, and Glu residues have been identified as catalytic nucleophile/bases with catalytic protons (see http://www.cazy.org/GH5.html).

Numerous fungal cellulase proteins have been studied at the molecular level. Efficient extracellular cellulolytic enzymes from *Tricoderma* and *Aspergillus* enable these organisms to survive on cellulosic biomass. The endoglucanases of *Tricoderma* and *Aspergillus* show high activity on carboxymethylcellulose (CMC) and β-glucan under moderate acidic conditions [10, 25, 27]. In this study, Cel5B, which belongs to GHF5 of *Gloeophyllum trabeum*, was expressed in *Pichia pastoris*; the recombinant Cel5B showed efficient bifunctional endo- and exo-type cellulase activity under strongly acidic conditions.

Materials and methods

Strains, plasmid, medium, and growth conditions

E. coli TOP10F' was used as the host cell for DNA cloning, and *P. pastoris* GS115 was used as the host strain for protein expression. The pGEM-T Easy vector (Promega, Madison, WI, USA) and pPICZαA vector (Invitrogen, Carlsbad, CA, USA) were used for TA cloning and protein expression, respectively. *G. trabeum* was obtained from Korea University (KUC 8013). It was grown on PDA agar plate at 25°C for 5 days and then inoculated into 100 mL of Avicel medium containing 1% Avicel, 0.42% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.02% urea, 0.03% $CaCl_2$, 0.03% $MgSO_4{\cdot}7H_2O$, 0.1% protease peptone, 0.2% Tween 80, and 0.2% trace element solution (0.5% $FeSO_4{\cdot}7H_2O$, 0.16% $MnSO_4{\cdot}H_2O$, 0.14% $ZnSO_4{\cdot}7H_2O$, 0.2% $CoCl_2$) in a 500-mL baffle flask for 5 days at 25°C with shaking at 200 rpm.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from the filamentous fungus *G. trabeum* using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and subjected to long-distance PCR for cDNA synthesis using the SMART™ cDNA Library Construction kit (Clontech, Mountain View, CA, USA).

Cloning of the full-length *Cel5B* by rapid amplification of cDNA ends (RACE) PCR

After aligning sequences of the GH5 fungal endoglucanases, one degenerate primer was designed. 3′ RACE PCR was conducted to clone the full-length *Cel5B* using the degenerate primer (5′-GGNWSNGGNTAYTGYGAYGC NCARTG-3′) (N = A, G, C, or T; W = A or T; S = G or C; Y = C or T; R = A or G) and the CDS III/3′ PCR primer (5′-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30 N– 1 N-3′) (N = A, G, C, or T; N–1 = A, G, or C) in a 20-μL volume containing 3 μL first-strand cDNA, 2 μL 10× Ex Taq buffer, 1 μL gene-specific primer, 1 μL CDS III/3′ PCR primer, 1.6 μL 2.5 mM dNTP, and 0.1 μL Ex Taq DNA polymerase (5 U/μL). The PCR amplification was conducted as follows: 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 1 min, with a final extension for 5 min at 72°C. The PCR product was cloned using the pGEM-T Easy vector and sequenced. The 5′ RACE-PCR primers were used in combination with the 3′ target primer (5′-TAGGCACCCGTGTTCAAGCAAGC TTGGACG-3′), which was designed to align against the 3′ RACE-PCR products and 5′ PCR primer (5′-AAGCAG TGGTATCAACGCAGAGT-3′). PCR was then performed using the same conditions, and the amplicon was cloned into the pGEM-T Easy vector. The full-length *Cel5B* gene was amplified using primers Cel-F (5′-GAATTCATGTTCAA GGCACTCCTCAG-3′) and Cel-R (5′-TCTAGATCATG CGTTGGCAATCGGAG-3′) in a 20-μL volume containing 3 μL first-strand cDNA, 2 μL 10× Ex Taq buffer, 1 μL Cel-F primer, 1 μL Cel-R primer, 1.6 μL 2.5 mM dNTP, and 0.1 μL Ex Taq DNA polymerase (5 U/μL). The PCR amplification was conducted as follows: 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final extension for 5 min at 72°C. The PCR product was cloned using the pGEM-T Easy vector and sequenced.

Expression and purification of Cel5B in *P. pastoris*

The target fragment of Cel5B without the signal peptide, and alanine residue 22 converted to glutamine was inserted

into the pPICZαA vector, and transformed in *P. pastoris*. Several transformant colonies were generated, and Cel5B expression was confirmed by Western blot analysis using anti-His antibody (Abfrontier, Seoul, South Korea). Selected colonies were inoculated into 3 mL YPD (1% yeast extract, 2% protease peptone, 1% dextrose) medium at 30°C for 24 h, and then inoculated into 100 mL YPG (1% yeast extract, 2% protease peptone, 1% glycerol) medium incubated under the above conditions. After 24 h, cell pellets were harvested and resuspended in 100 mL YPM (1% yeast extract, 2% protease peptone, 0.5% methanol). After a 24-h incubation at 30°C, the supernatant was separated from the cells by centrifugation for 20 min at 26,000×*g* (Avanti J-E, Beckman, Fullerton, CA, USA), and Cel5B was purified using a Ni–NTA matrix (Qiagen, Hilden, Germany).

Enzyme assay and kinetic analysis

Optimal pH and temperature were determined by checking activity on CMC at various ranges of pH (2.0–7.0) and temperature (30–70°C). To determine thermal stability, Cel5B was initially preincubated at various temperatures (30–60°C) for 0, 10, 30, 60, 90, and 120 min at pH 3.5 without the substrate after the residual activity was checked for 0.5 h under optimal conditions. The specific activity was determined on 1% CMC, 1% β-glucan, filter paper, and 1% xylan as a substrate by measuring the reducing sugars liberated after hydrolytic action of Cel5B under optimal conditions using the dinitrosalicylic acid reagent and a glucose standard curve [23]. Kinetic properties of Cel5B were determined using various concentrations of CMC. K_m and V_{max} were calculated using the Lineweaver–Burk plot [22].

Solubilizing ability of filter paper

A total of 2 mL of reaction mixture containing a 20-mg filter-paper disk was supplemented with 20 and 50 μg/mL of purified Cel5B. The solubilizing process was monitored after 1 h incubation at 37°C.

Thin-layer chromatography (TLC) analysis

The enzymatic hydrolysis products of the cellodextrins were analyzed by TLC. The samples, including cellobiose (G2), cellotriose (G3), cellotetraose (G4), cellopentaose (G5), cellohexaose (G6), CMC, and filter paper were separated on a TLC aluminum sheet silica gel 60 plate with a solvent solution of *n*-butanol/acetic acid/H_2O (2:1:1, v/v). The sugars were detected by a staining solution (95 mL methanol, 5 mL H_2SO_4, 30 mg *N*-(1-naphthyl)ethylenediamine dihydrochloride).

Scanning electron microscopy (SEM) for filter paper degradation

The control and enzyme-treated samples were prepared and then fixed using a mixture of 2% paraformaldehyde (v/v) and 2% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2) for morphological analysis. After washing in phosphate buffer, samples were dehydrated in a graded ethanol series and then lyophilized. Samples were sputter coated with a thin layer (20 nm) of gold. All samples were observed and photographed with SEM (S2400, Hitachi, Japan).

Protein structure and active site residues analysis

The three-dimensional structure of Cel5B was predicted using the EsyPred3D Web Server 1.0 [20] listed on Expasy and based on sequence similarity. Active site residues were confirmed by creating mutations at E175A and E287A using mutagenic primers; (E175A-F 5′-GCATCATGAATGCG CCGCATGACC-3′ and E175A-F 5′-GGTCATGCGGCGC ATTCATGATGC-3′), and (E287A-F 5′-GATCAACACT GCGACTGGCGGAGG-3′ and E287A-R 5′-CCTCCGCC AGTCGCAGTGTTGATC-3′). Both mutants were transformed in *P. pastoris* and were tested for activity and expression.

Results

Gene cloning of *Cel5B* and sequence analysis

The *Cel5B* gene was amplified using 3′ RACE and 5′ RACE PCR. The full-length *Cel5B* gene was cloned with restriction enzyme sites into the pGEM T-Easy vector, and the *Cel5B* sequence was confirmed by Genotech, Inc. (Daejeon, Korea). The full-length *Cel5B* gene of *G. trabeum* had an open reading frame of 1,077 bp encoding a protein of 359 amino acid residues. *Cel5B* shares 99% amino acid sequence identity with Cel5A (GenBank accession number ADH 51728), which indicates that *Cel5B* is most likely an isoform of Cel5A. *Cel5B* exhibited 73, 67, and 70% amino acid sequence identity with other endoglucanases from *Polyporus arcularius* (GenBank accession number BAF 75943), *Trichoderma* sp. *C-4* (AAR 29981), and *T. viride* (ADJ 10627), respectively. The amino acid sequence of *Cel5B* had no N- or C-terminal cellulose binding domains (Fig. 1a).

Expression, purification, and characterization of Cel5B

The target fragment of Cel5B without the signal peptide was inserted into the pPICZαA vector, and the recombinant

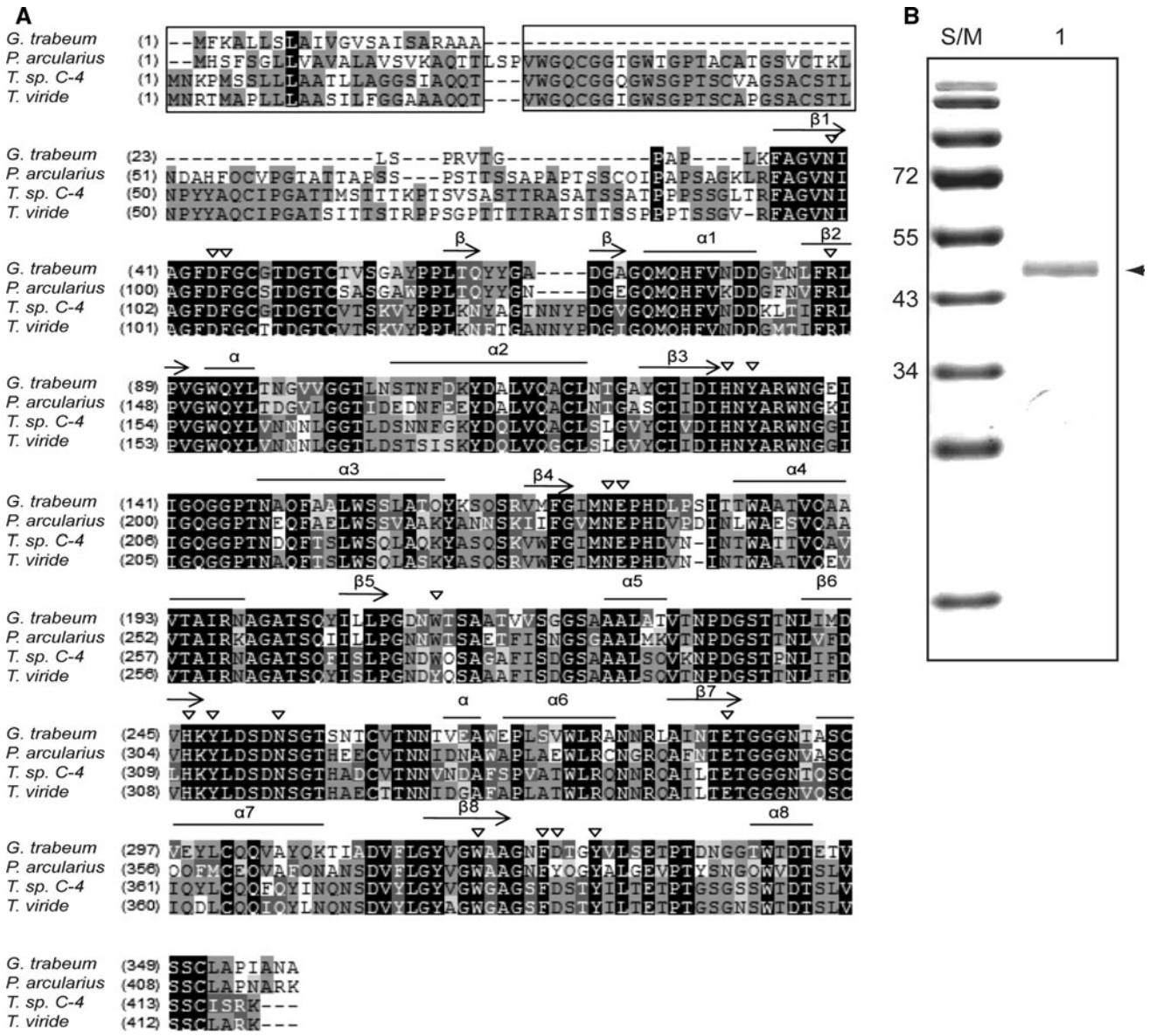

Fig. 1 **a** Alignment of the *Gloeophyllum trabeum Cel5B* deduced amino acid sequence with other endoglucanases. *Box* regions are indicated in front of the signal peptide regions and cellulose binding modules. *β* and *α* indicate the *β*-sheet and α-helix. **b** SDS-PAGE analysis of Cel5B after Ni–NTA purification shows an approximately 47-kDa protein. *S/M* protein ladder, *lane 1* purified Cel5B

Cel5B protein showed a molecular mass of approximately 47 kDa on SDS-PAGE (Fig. 1b). Physical characteristics of Cel5B showed that the protein was acidic and mesophilic in nature. Cel5B was most efficient under strongly acidic conditions of pH 3.5 at 55°C (Fig. 2a, b). However, Cel5B failed to display good thermal stability. Cel5B was stable below 40°C but showed complete loss of activity at 60°C after 90 min (Fig. 2c). The specific activities of the purified enzyme on various substrates are shown in Table 1. Cel5B showed characteristics of both endo- and exo-type cellulases by acting on CMC and *β*-glucan as well as filter paper. It had no activity on xylan. Cel5B showed very high specific activity when compared with other in-house-produced endo- and exo-type cellulases (Table 2). The kinetic properties of Cel5B showed high V_{max} (2,828.85 μmol min^{-1} mg^{-1}), K_m (31.10 mg), and K_{cat} (686.39 min^{-1}), which indicated high catalytic efficiency against CMC.

Hydrolytic properties of Cel5B for different substrates

The hydrolysis patterns of cello-oligosaccharides were analyzed by TLC. Cel5B did not degrade cellobiose, whereas

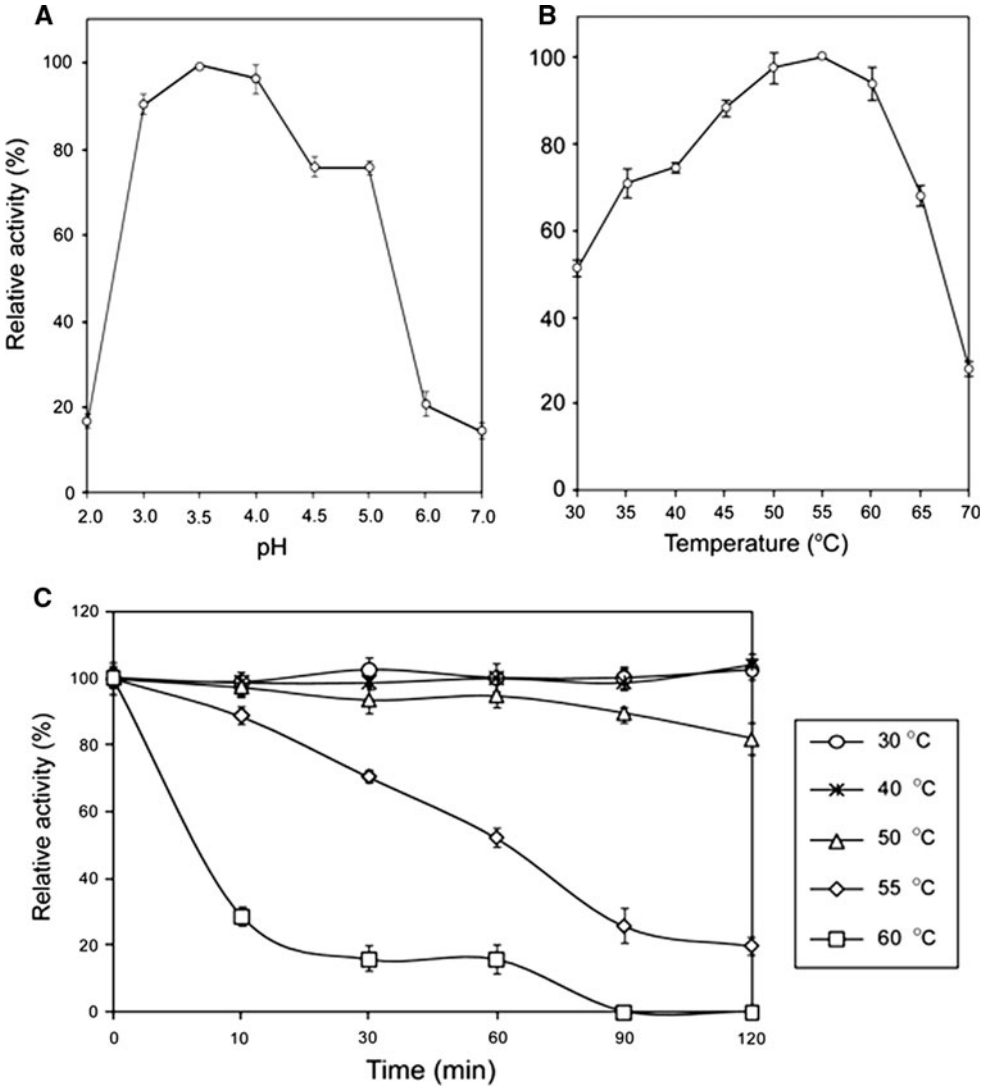

Fig. 2 Optimal pH, temperature, and thermal stability of recombinant Cel5B. **a** The optimal pH was determined in the range 2.0–7.0 using 50 mM hydrochloric acid/potassium chloride (pH 2), 50 mM glycine/hydrochloric acid (pH 3–3.5), 50 mM sodium acetate (pH 4–5), and 50 mM sodium phosphate (pH 6–7) at 50°C against CMC for 0.5 h. **b** Optimal temperature for CMC was determined at 30–70°C, pH 3.5 for 0.5 h. **c** Thermal stability of the Cel5B enzyme was determined by preincubating the apoenzyme at various temperatures (30–60°C) for 0, 10, 30, 60, 90, and 120 min at pH 3.5, and residual activity was checked on CMC under optimal conditions

Table 1 Specific activities of Cel4 towards CMC, *β*-glucan, filter paper, and xylan

Substrate	Backbone linkage (backbone sugar)	Specific activity (U/mg)
CMC	*β*-1,4 (glucose)	482.94 ± 12.03
β-Glucan	*β*-1,3 (glucose)	3,674.58 ± 198.16
Filter paper	*β*-1,4 (glucose)	1.004 ± 0.042
Xylan	*β*-1,4 (xylose)	NA

Specific activities were determined by measuring the reducing sugar using the dinitrosalicylic acid reagent in triplicate

NA no activity

cellotriose weakly degraded to G1 and G2. Cellotetraose hydrolyzed to release mainly G2 and G3 and some G1. Cellopentaose was primarily hydrolyzed into G2 and G3 and a trace amount of G4 and G1. Cellohexaose mainly degraded into G2 and G3 and faintly into G1, G4, and G5 (Fig. 3a and Supplementary Fig. S1). The CMC (Fig. 3b) and filter paper (Fig. 3c) hydrolyzed into G2 and slightly into G1 and G3. It was clear that Cel5B was mainly randomly hydrolyzed at the glucosidic bond into G2 or G3.

Solubilizing ability and degradation pattern of filter paper by Cel5B

The solubilizing ability of Cel5B is shown in Fig. 5a. Cellobiohydrolase degraded the filter paper, but the bifunctional characteristics of Cel5B allowed the solubilization of filter paper very efficiently after 1 h incubation at 37°C. Morphological changes were observed when the filter paper was treated with Cel5B. After enzyme treatment, the microfilaments of filter paper appeared rougher than the surface of the untreated filter paper (Fig. 4).

Table 2 Comparison of specific activity of Cel5B with endo- and exo-type cellulases on CMC and filter paper

Protein	Specific activity (U/mg)		Source	Reference
	CMC	Filter paper		
Cel5B	482.94 ± 12.03	1.004 ± 0.042	*Gloeophyllum trabeum*	This study
Cel5A	6.70 ± 0.14	NA	*Thermotoga maritima*	[24]
EGIII	4.76 ± 0.18	NA	*Trichoderma reesei*	[25]
EGIV	0.462 ± 0.010	NA	*Ruminococcus albus*	[15]
Cel5-CBM6	0.480 ± 0.006	NA	*Ruminococcus albus*	[1]
E4	0.937 ± 0.032	0.074 ± 0.0025	*Thermobifida fusca YX*	[26]
CBH2	NA	0.014 ± 0.0005	*Trichoderma reesei*	[29]

Each enzyme assay was carried out under its optimal conditions. Specific activities were determined by measuring the reducing sugars using the dinitrosalicylic acid reagent in triplicate

NA no activity

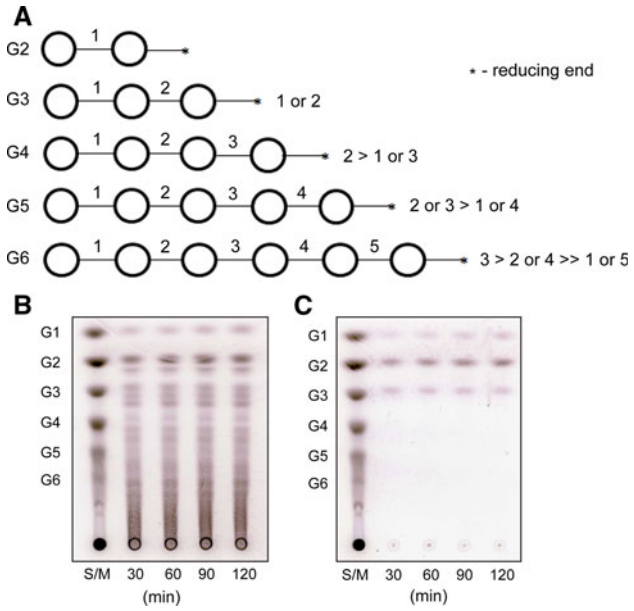

Fig. 3 TLC analysis of products and hydrolysis patterns of cellodextrins, CMC, and filter paper (Whatman no. 1) by Cel5B. **a** Preferential cleavage of Cel5B for cellodextrins. **b** CMC (0.5%) was incubated with 6.7 μg/mL Cel5B at 37°C for 30, 60, 90, and 120 min. **c** Filter paper (2.2 mg) was incubated with 55 μg/mL Cel5B at 37°C for 30, 60, 90, and 120 min. *G1* glucose, *G2* cellobiose, *G3* cellotriose, *G4* cellotetraose, *G5* cellopentaose, *G6* cellohexaose, *CMC* carboxymethylcellulose

Protein structure and active site residues

A computed three-dimensional model of Cel5B was based on the template structure of *Thermoascus aurantiacus* (PDB: 1H1N) [30]. It showed a $(\beta/\alpha)_8$ barrel fold, which is a general characteristic of GH5. On the basis of sequence similarity and mutational analysis, Glu-175 and Glu-287 represented active site residues (Fig. 5b). Both residues lost full hydrolytic activity when replaced with alanine (Fig. 5c).

Discussion

Renewable energy production, such as bioethanol obtained from lignocellulosic materials, is being widely studied, but an enzyme complex is essential for the production of monosaccharides by hydrolysis of lignocellulosic materials. To make renewable energy production feasible, enzymes with broad substrate specificity and high cellulolytic activity are being screened. Fungi are widely known to produce cellulase [2]. Brown rot fungi are considered important microbes that maintain soil fertility, and *G. trabeum* is widely reported brown rot wood-decaying fungus. *G. trabeum* has an excellent ability to generate the Fenton system (Fe^{2+} and H_2O_2), which degrades lignocelluloses, but limited knowledge on cellulose-degrading enzymes is available [16]. It was believed that *G. trabeum* relies on the Fenton degrading system, but the present study, as well as the findings by Cohen et al. [5], demonstrated that *G. trabeum* also has the ability to express cellulolytic enzymes. *G. trabeum* can express various isoforms of cellulases, which are responsible for crystalline cellulose degradation.

Pichia pastoris is widely known as an important host organism for recombinant protein expression and production [6, 31, 34]. Therefore, we used *P. pastoris* for efficient protein purification and expression of Cel5B. When we tried to express full-length Cel5B, the target was not detected. So, after removing signal peptide, Cel5B was expressed and purified in a soluble form. Many endoglucanses can be separated from their signal peptides for protein expression [32]. Here, we expressed and characterized Cel5B without the signal peptide because of an unfavorable N-terminal coding sequence which disturbs recombinant expression in yeast, and the hydrophobic alanine residue 22 was also converted to a hydrophilic glutamine. Cel5B displayed bifunctional characteristics by showing affinity for CMC and filter paper. Multifunctional hydrolysis enzymes have been reported from several microorganisms

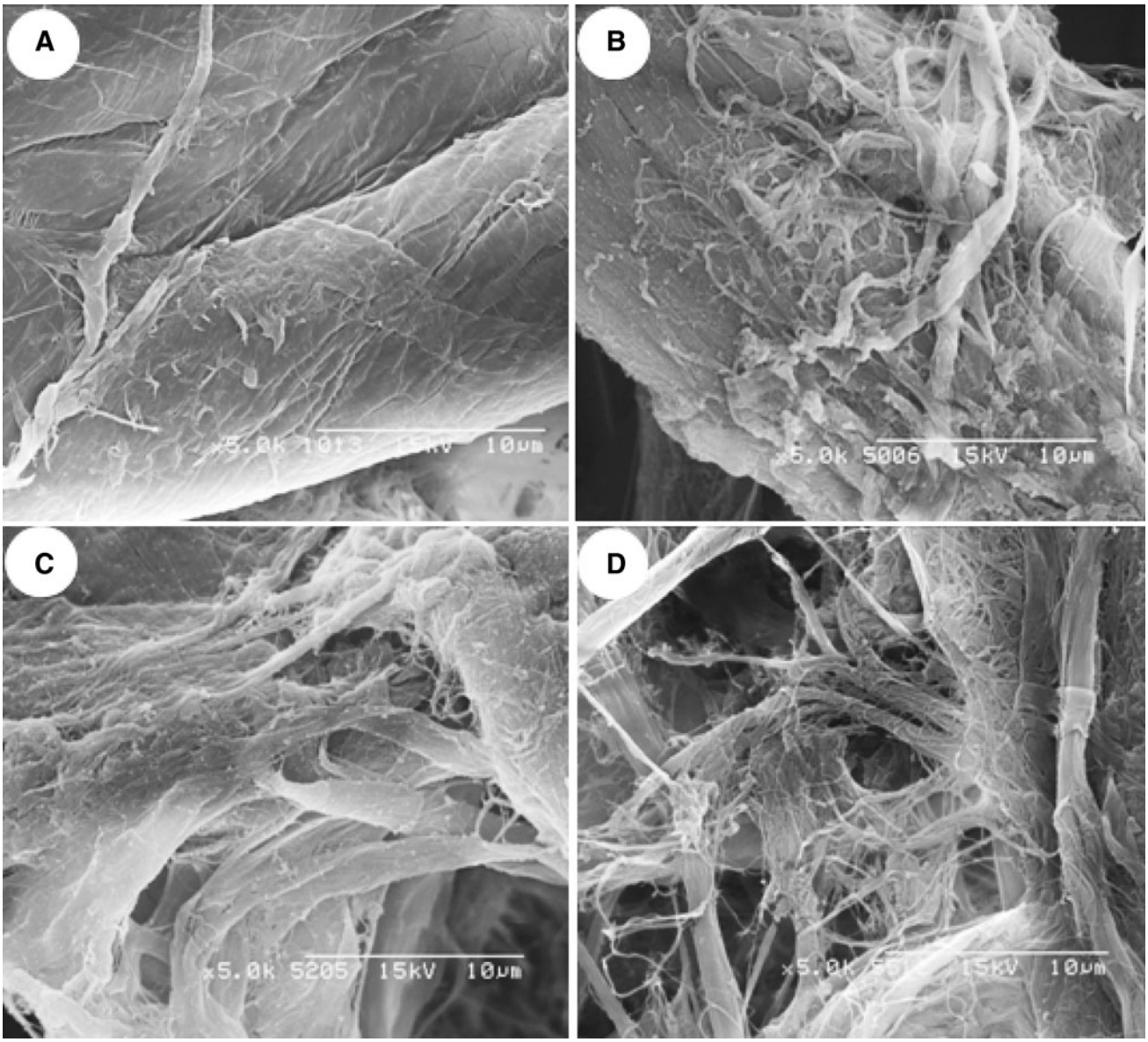

Fig. 4 Scanning electron photomicrographs of filter paper treated with Cel5B: **a** control; **b** 1 h; **c** 2 h; **d** 5 h. *Scale bar* 10 µm

and characterized with high endocellulase and exocellulase activity [18, 33]. However, their degrading activity is at a lower rate than that of Cel5B. Cel5B showed very high specific activity compared with other endoglucanases and cellobiohydrolase (about 80- to 1,000-fold and 13- to 70-fold on CMC and filter paper, respectively). Cel5B worked most efficiently under strongly acidic conditions and retained activity for a longer period of time below 40°C, which revealed that Cel5B could be a candidate for cellulose degradation under acidic treatment conditions.

To confirm the important active site residues we tried point mutation analysis. According to Davies and Henrissat [7], enzymatic hydrolysis of the glycosidic bond requires two critical residues (catalytic nucleophile/base and catalytic proton donor). On the basis of the predicted three-dimensional structure, Cel5B has two active site residues. One is a catalytic nucleophile/base and the other is a catalytic proton donor. We confirmed by mutational analysis that active site residues Glu-175 and Glu-287 of Cel5B are a major factor in the enzyme's hydrolytic activity.

In summary, the present investigation provided information on the hydrolytic capacity of *G. trabeum*. The prominent advantages of Cel5B are its bifunctional character, high specific activity, K_{cat}, and V_{max} on CMC. Many endoglucanases have been isolated from various sources, but only some have been expressed and purified. In this study,

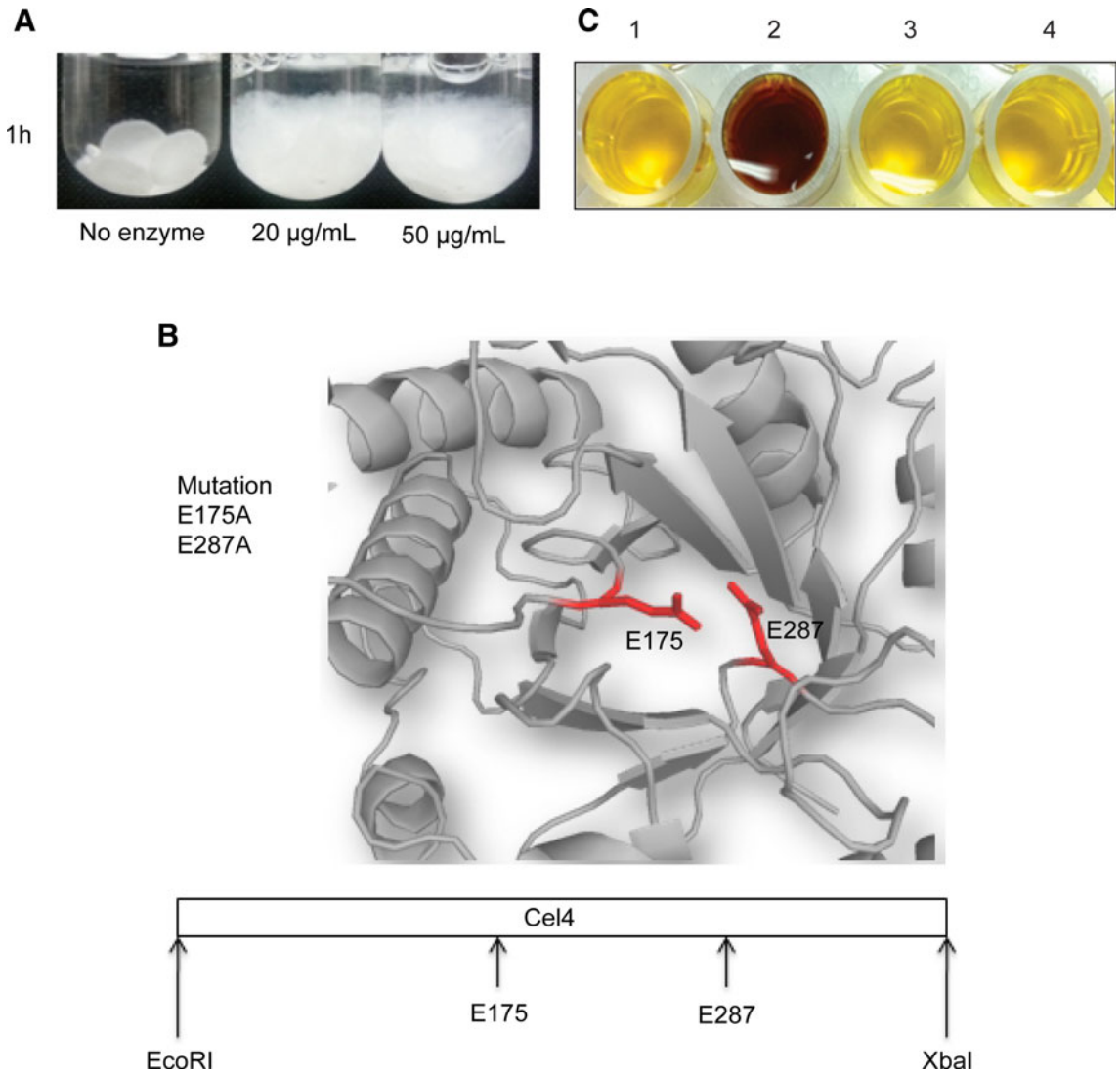

Fig. 5 Solubilization ability of Cel5B on filter paper (Whatman no.1) and the mutation of active site residues. **a** Solubilization of filter paper by Cel5B after 1 h at 37°C incubation. **b** Active site residues (Glu-175 and Glu-287) of Cel5B through the predicted three-dimensional structure. **c** Activities were determined by measuring the reducing sugar using the dinitrosalicylic acid reagent: *lane 1* control, *lane 2* purified Cel5B, *lane 3* E175A, *lane 4* E287A

we reported the gene expression analysis and characteristics of this particular endoglucanase.

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