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# Characterization of acidic endoglucanase Cel12A from *Gloeophyllum trabeum* and its synergistic effects on hydrogen peroxide–acetic acid (HPAC)-pretreated lignocellulose

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**Abstract**

*Gloeophyllum trabeum* is a potent filamentous fungus that rapidly decomposes lignocellulose. In the present study, we cloned the *G. trabeum cel12a* gene and expressed it in *Pichia pastoris* strain GS115. The purified recombinant *Gt*Cel12A exhibited high pH stability and very high specific enzymic activity against β-glucan (6546 U $mg^{-1}$) and carboxymethyl cellulose (1129 U $mg^{-1}$) compared to *Gt*Cel5B, endoglucanases from *Trichoderma reesei*, and other glycoside hydrolase family 12 (GH12) enzymes. *Gt*Cel12A exhibited high enzymic activity with regard to hydrogen peroxide–acetic acid (HPAC)-pretreated lignocellulose biomass, and produced cellobiose as a major product with a small quantity of glucose. In combination with commercial cellulase, this enzyme also showed synergistic effects of 14.5, 16.1, 29.0, and 13.4% on filter paper, HPAC-pretreated pine, corn stover, and rice straw, respectively. The acidic endoglucanase *Gt*Cel12A from *G. trabeum* is a promising tool that can be used in combination with cellulase against HPAC-pretreated lignocellulose.

**Keywords:** HPAC-pretreatment, Endoglucanase, Synergistic effects, *Pichia pastoris*

## Introduction

Approximately 200 billion tons of lignocellulose are produced every year. These renewable polymers can be used to produce biofuel or biochemicals without competing with the first-generation feedstocks [1]. Enzymatic hydrolysis, which converts lignocellulose into its monomeric sugars, requires a hydrolytic enzyme complex [2]. A synergistic cooperation of cellulases, comprising exo-1,4-β-glucanases (EC 3.2.1.91 and EC 3.2.1.176) and endoglucanase (EC3.2.1.4), is important for the complete hydrolysis of lignocellulose. Endoglucanases, which hydrolyze the internal glucosidic bonds, provide exo-1,4-β-glucanases with new reducing and nonreducing ends. Enzymes have been widely used in the pulp and paper, textile, bioethanol, wine and brewery, food processing, animal feed, agricultural, biomolecular chemical products, and pharmaceutical industries [1, 3, 4]. Cellulases comprise approximately 20% of the global enzyme market [5]. Currently, the main source for commercial and industrial cellulases is *Trichoderma reesei*, a well-studied and -established fungus used to produce cellulase at a scale of more than 100 g $L^{-1}$ [6, 7]. However, the production cost of cellulase accounts for approximately 40% of the total cost of bioethanol production from lignocellulose biomass [1]. Therefore, the development of effective enzymes or an enzyme cocktail is essential to enhance enzyme efficiency.

Among bacteria and fungi, brown rot basidiomycetes have been studied extensively for their ability to rapidly decompose lignocellulosic biomass, despite lacking the processive cellobiohydrolases [8]. Cellulase-encoding gene sequences have been reported in *Gloeophyllum trabeum* [9]. This fungus is also one of the most potent and a well-studied microorganism that degrades lignocelluloses [8, 10–12]. *G. trabeum* is able to depolymerize lignocellulose biomass using a unique system combining

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nonenzymatic lignocellulose degradation, which is Fenton-type reactions mediated by reducing $Fe^{3+}$ and $O_2$ with two hydroquinones [11, 12], with enzymatic lignocellulose degradation [8, 10].

Endoglucanases are grouped into 14 glycoside hydrolase (GH) families within the Carbohydrate-Active enZYmes database (CAZy; http://www.cazy.org/Glycoside-Hydrolases.html). Among these, the GH 12 family has a β-jelly roll structure with relatively low molecular mass and several catalytic activities, including endoglucanase (EC 3.2.1.4), xyloglucan hydrolase (EC 3.2.1.151), β-1,3-1,4-glucanase (EC 3.2.1.73), and xyloglucan endotransglucosylase (EC 2.4.1.207). Most of GH12 have two conserved cystines region that form a disulfide bridge, which offers enzyme structures local stabilization. This family of endoglucanases plays a critical role during the hydrolysis of β-1,3-1,4 and β-1,4-glucan with high activities [13].

A variety of recombinant endoglucanases and xyloglucan-specific endoglucanases in the GH12 family have been cloned and studied using various hetero-expression hosts [14–18]. A *G. trabeum* endoglucanase expressed in a host, *Aspergillus niger*, has been characterized in terms of molecular weight, thermal stability, and optimal activity conditions [16, 19]. Tambor et al. [19] have also reported a synergistic relationship between endoglucanases and *T. reesei* Cel7A with regard to the digestion of filter paper. However, there is insufficient information about enzyme activity with regard to the digestion of lignocellulose substrates and synergistic relationship with cellulase. *Pichia pastoris* provides a well-studied eukaryotic heterologous expression system with numerous advantages, including protein folding, protein processing, posttranslational modification, and high expression [20–22]. In the present study, we describe the expression, purification, and biochemical characterization of acidic *Gt*Cel12A from *P. pastoris*. We also studied the synergistic effects of *Gt*Cel12A with commercial cellulase on hydrogen peroxide–acetic acid (HPAC)-pretreated lignocellulose from pine, corn stover, and rice straw.

## Materials and methods

### Substrate materials

Pine wood and chips were obtained from the arboretum of Chonnam National University, South Korea. Rice straw and corn stover were purchased online (http://www.gmarket.co.kr/). Lignocellulose biomass were pretreated with HPAC, according to a procedure reported previously [23]. Following pretreatment, residues after the pretreatment were washed with hot water until attaining neutral pH and then freeze-dried.

### Microbial strains, media, and cultivation conditions

The filamentous fungus *G. trabeum* strain ATCC11539 was used as a gene source for *cel12a*. The fungus was grown on potato dextrose agar (PDA) medium at 30 °C for 5 days and then transferred into 100 mL Avicel medium (10 g $L^{-1}$ Avicel, 5 g $L^{-1}$ $(NH_4)_2SO_4$, 2 g $L^{-1}$ $KH_2PO_4$, 0.3 g $L^{-1}$ $CaCl_2$, 0.3 g $L^{-1}$ $MgSO_4{\cdot}7H_2O$, 1 g $L^{-1}$ protease peptone, 0.2% (*v*/*v*) Tween 80, and 0.2% (*v*/*v*) trace elements solution) trace elements solution; 5 g $L^{-1}$ $FeSO_4{\cdot}7H_2O$, 1.6 g $L^{-1}$ $MnSO_4{\cdot}H_2O$, 1.4 g $L^{-1}$ $ZnSO_4{\cdot}7H_2O$, and 2 g $L^{-1}$ $CoCl_2$) contained in a 500-mL baffle flask at 30 °C for 5 days in a 180 rpm shaking incubator. The TOP10 *E. coli* strain was transformed and grown on Luria–Bertani (LB) medium at 37 °C for 18 h. Yeast strain *Pichia pastoris* GS115 was grown step-by-step on YPD media (10 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ protease peptone, and 10 g $L^{-1}$ dextrose), YPG (10 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ protease peptone, and 1% (*v*/*v*) glycerol) at 30 °C for 24 h, respectively. After cultivation on the YPG medium, the yeast cell was harvested and transferred onto YP medium (10 g $L^{-1}$ yeast extract, 20 g $L^{-1}$ protease peptone) with 0.8% (*v*/*v*) methyl alcohol added every 24 h, for heterologous protein expression at 30 °C for 4 days.

### *Gtcel12a* gene cloning

The *G. trabeum* was harvested for total RNA extraction by centrifuging at 13,000 rpm (Avanti J-E, Beckman, Fullerton, CA, USA) for 30 min after cultivation in PDB medium with 1% (*w*/*v*) Avicel. To extract total RNA from *G. trabeum*, the TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA) was used. The SMART cDNA library construction kit (TaKaRa, Mountain View, CA, USA) was used for cDNA synthesis with isolated RNA. Gene sequences of *GtCel12a* were amplified by Polymerase chain reaction (PCR) using designed primers (*Gt*Cel12A-F 5′-GAA TTC GCG ACC GTG CTC ACT GGT CAA TAC-3′ and *Gt*Cel12A-R 5′-CTG CAG CCC GCT CAA GCT GAC GCT GAA-3′) without the signal peptide. PCR was performed in a volume of 20 µL, containing 3 µL first strand cDNA, 2 µL 10× Ex Taq buffer, 2 µL dNTP, 1 µL each of forward and reverse primers, and 0.1 µL Ex Taq DNA polymerase (5 U $\mu L^{-1}$) under the following conditions: 1 cycle of denaturation at 95 °C for 30 s, and 35 cycles of annealing at 60 °C for 30 s, extension at 72 °C for 60 s. The resulting PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) with *Eco*RI and *Pst*I restriction enzymes and then sequenced and transformed into TOP10F *E. coli* strain. The transformants were selected on LB medium with ampicillin.

### Heterologous enzyme expression, purification, western blot, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The *Gtcel12a* gene was subcloned into the pPICZαA vector (Invitrogen) with restriction enzymes, *Eco*RI and *Pst*I, and then it was transformed into host strain, *P. pastoris* GS115, for heterologous expression. The transformants were selected on YPD medium with the antibiotics zeocin. Cells were harvested by centrifuging at 13,000 rpm for 30 min. The supernatant was then filtered through a GF/C glass microfiber filter (1.2 μm) to remove any remaining yeast cells, and then purified using a column, containing 1 mL Ni–NTA matrix (Qiagen, Hilden, Germany). *Gt*Cel12A expression was confirmed through a western blot assay with mouse anti-His-tagged antibody (Abfrontier, Seoul, South Korea). The purified enzymes were dialyzed in 20 mM tris-hydrochloride buffer (pH 8.0). The protein concentration was determined by a Bradford assay at 595 nm with bovine serum albumin (BSA) as a standard. SDS-PAGE analysis was conducted to determine the molecular mass of a heterologous expressed enzyme using a 12% (*w*/*v*) polyacrylamide gel stained with Coomassie brilliant blue R-250.

### Biochemical characterization

The optimal pH was determined by measuring the relative activity of enzymes in 50 mM buffers ranging from pH 2.0 to 9.0, glycine-hydrochloric acid (pH 2.0–3.0), sodium acetate (pH 4.0–5.0), sodium phosphate (pH 6.0–7.0), and tris-hydrochloride (pH 8.0–9.0) in 1% (*w*/*v*) solution of carboxymethyl cellulose (CMC) at 50 °C, following a dinitrosalicylic acid (DNS) assay by using a spectrophotometer (Multiskan EX, Thermo Fisher Scientific, Vantaa, Finland) at 550 nm. The optimal temperature was determined by monitoring the relative activity at temperature ranging from 20 to 70 °C in 50 mM glycine-hydrochloric acid buffer (pH 2.5) with CMC. pH stability was assessed according to the residual enzyme activity after preincubation in various buffers (ranging from pH 2.0 to 9.0) at 4 °C for 18 h. Thermal stability was likewise determined by monitoring residual enzyme activity after preincubation at 30–60 °C for 0–120 min.

### Enzyme assays

Nine materials were used as enzyme assay substrates: Avicel PH-101 (microcrystalline cellulose, ca. 50 μm), xyloglucan from tamarind, β-glucan from barley (Megazyme), filter paper (Whatman number 1), locust bean gum from *Ceratonia* siliqua seeds, xylan from beech wood, carrageenan, CMC, and soluble starch (all purchased from Sigma). These substrates were prepared as 1% stock solutions. Each enzymatic assay was performed in triplicate in a final volume of 100 μL, containing 50 μL substrate stocks, 50 mM glycine-hydrochloric acid buffer (pH 2.5), and diluted enzyme. The kinetic parameters, $K_m$ and $V_{max}$, were evaluated with 0.25–2.0% CMC as a substrate for 5 min under optimal conditions (pH 2.5, 50 °C) and estimated using Lineweaver–Burk plot. To determine the effects of chemical and ions, 50 mM of EDTA, NaCl, LiCl, KCl, $CoCl_2$, $ZnCl_2$, $CaCl_2$, $MnCl_2$, $HgCl_2$ $AlCl_3$, and $FeCl_3$ were tested. Reducing sugars were measured using dinitrosalicylic acid reagent with a glucose standard curve [24]. One unit (U) was defined as the amount of enzyme required to produce 1 μmol of reducing sugars per min. Products degraded from oligosaccharides were prepared by purified enzymes in a 100 μL final volume with 50 mM glycine-hydrochloric acid buffer (pH 2.5), diluted enzyme, and each substrate, containing 1% (*w*/*v*) cellobiose, cellotriose, cellotetraose, or cellopentaose. These mixtures were incubated at 50 °C for 1 to 20 min, followed by loading 1 μL of released products on thin-layer chromatography (TLC) silica gel 60 plates and separated with *n*-BuOH/$CH_3O_2H$/$H_2O$ (2:1:1 *v*/*v*). Sugars were detected using a 5% (*v*/*v*) $H_2SO_4$ in methanol staining solution [25].

### Enzymatic hydrolysis and synergistic effect with commercial cellulase

Hydrolysis was performed under their optimal conditions of *Gt*Cel12A and *Gt*Cel5B [24]. Synergistic effects were examined using a 3% (*w*/*v*) solution of HPAC-pretreated lignocellulose and filter paper, 50 mM citrate buffer (pH 4.8), 10 FPU $g^{-1}$ commercial cellulase (Celluclast 1.5L, Novozymes, Denmark), and 30 U $g^{-1}$ in pine, 50 U $g^{-1}$ *Gt*Cel12A in filter paper, corn stover, and rice straw at 48 °C for 24 h. The reducing sugars were determined by a DNS assay and high-performance liquid chromatography (HPLC) (Waters, Milford, MA, USA) with a Rezex RPM column (300 × 4.6 mm; Phenomenes, Torrance, CA, USA). The analysis conditions were 85 and 40 °C in the column and refractive index detector, respectively, with deionized water at a flow rate of 0.6 mL $min^{-1}$.

## Results and discussion

### Gene cloning and sequence analysis

We obtained the *cel12a* gene (National Center for Biotechnology Information (NCBI) accession number HQ163778) from *G. trabeum* ATCC 11539 and confirmed it by sequence analysis. This gene includes 741 nucleotides, encoding 246 amino acids with a calculated molecular weight of 26.17 kDa and a theoretical isoelectric point of 4.10. The SignalP 4.1 server (http://www.cbs.dtu.dk/server/SignalP) predicted that *Gt*Cel12A has signal peptides from amino acids 1 to 21. The amino acid sequence of the *Gt*Cel12A was aligned by BLAST

(http://www.uniprot.org/blast/) with 12 other fungal family endoglucanases and visualized using the Vector NTI software. Multiple alignment analysis showed that the *Gt*Cel12A had high identities with other brown-rot fugal GH12 endoglucanases, *Neolentinus lepideus* (87.3% identity), *Laetiporus sulphureus* (65.2% identity), *Postia placenta* (67.4% identity), and *Daedalea quercina* (63.8% identity) (Additional file 1: Fig. S1). Therefore, these results indicate that *Gt*Cel12A belongs to the typical glycolyl hydrolase family 12, which is a specific endoglucanase of brown-rot fungi.

### Expression and purification of recombinant *Gt*Cel12A in the *P. pastoris*

*Gt*Cel12A has been reported in its native form, and in heterologous protein expression from *Aspergillus niger* [10, 16, 19]. The *Gtcel12a* gene was expressed in the host *P. pastoris* GS115 using a pPICZαA vector without signal peptides, owing to an unfavorable N-terminal secretion signal in the yeast [25]. The expressed enzyme was purified using a Ni–NTA agarose affinity column and dialyzed with 20 mM tris-hydrochloride buffer (pH 8.0). The expressed *Gt*Cel12A protein was confirmed by western blot assay (Additional file 1: Fig. S2a). SDS-PAGE showed that the molecular weight of the purified protein from *P. pastoris* was approximately 31 kDa, which was larger than the calculated molecular mass (Additional file 1: Fig. S2b). Recombinant *Gt*Cel12A expressed from *A. niger* strain CBS513.88 and py11 had different protein molecular masses, of 29 and 26.1 kDa, respectively [16, 19]. Recombinant EGIIIs (Cel12A) of *Trichoderma reesei* was expressed in heterologous hosts *E. coli*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*, which likewise showed different molecular masses (25, 28, and 29 kDa, respectively) due to N-glycosylation by the different hosts. The molecular mass was decreased to 25 kDa by endoglycosidase H and α-mannosidase treatment [26]. These increases in molecular weight may be at least partly related to glycosylation by a heterologous host in the recombinant *Gt*Cel12A protein.

### Characterization of the *Gt*Cel12A

To determine the biochemical characterization of *Gt*Cel12A, the effect of pH and temperatures was conducted on the CMC substrate. The *Gt*Cel12A expressed from *P. pastoris* exhibited enzyme activity over a range of extremely acidic conditions (pH 2.0–4.0) with an optimal pH 2.5, showed residual relative enzyme activity exceeding 80% across the range of pH conditions tested, and was highly stable at base conditions of pH 8.0–9.0 (Fig. 1a). In contrast, the *Gt*Cel12A expressed by *A. niger* has an optimal pH of 4.5, indicating N-glycosylation of the protein molecule [16, 19]. Similarly,

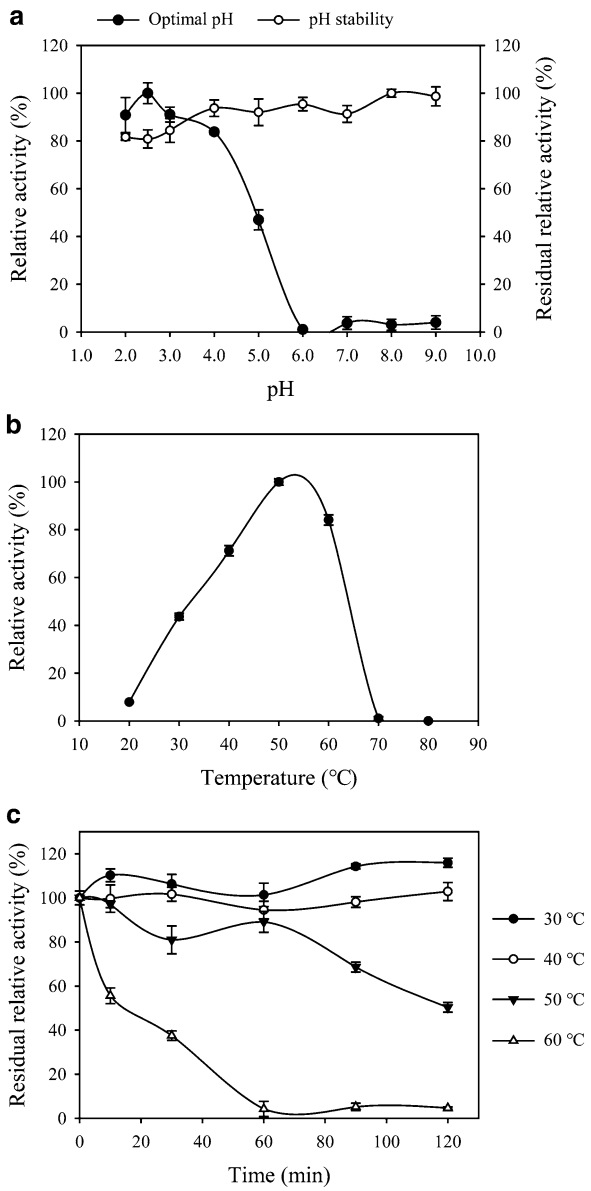


**Fig. 1** Characterizations of optimal pH, temperature, and stability of recombinant *Gt*Cel12A. **a** The optimal pH and pH stability were measured at 50 °C with 50 mM buffers at a pH range of pH 2.0–9.0 on CMC; pH stability was determined by monitoring residual activity after preincubation without substrate for 18 h at 4 °C. **b** Optimal temperature was determined at various temperatures (20–80 °C) in 50 mM glycin–HCl (pH 2.5). **c** Thermal stability was determined at 50 °C in 50 mM glycin–HCl (pH 2.5) by the preincubated enzyme in 30–60 °C for various time (0, 10, 30, 60, 90, and 120 min). All of error bars indicate ± SD

other GH12 endoglucanases from fungi have been reported to have an optimal (pH values pH 5.0–6.0) [17, 27]. Optimal enzyme activity was found to occur at 50 °C within a tested range of 20–80 °C (Fig. 1b). *Gt*Cel12A was stable below 40 °C but lost approximately half of its activity at 50 °C after 120 min (Fig. 1c).

**Table 1 Comparison of optimal conditions and specific activity of *Gt*Cel12A to those of other endoglucanases**

| Source | Protein | GH family | Host | Specific activity (U $mg^{-1}$) | | | | Optimal condition | | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | β-Glucan | CMC | Xyloglucan | Filter paper | Temperature (°C) | pH | |
| *Gloeophyllum trabeum* | Cel12A | 12 | *P. pastoris* | 6546 ± 33.5 | 1129 ± 16.3 | 10.1 ± 0.03 | 0.21 ± 0.001 | 50 | 2.5 | This study |
| | Cel5B | 5 | *P. pastoris* | 3675 ± 198.2 | 483 ± 12.0 | – | 1.004 ± 0.04 | 55 | 3.5 | [25] |
| *Trichoderma reesei* | EGIII | 12 | * | – | 20 | 17 | – | – | 5.7 | [31] |
| | | | *E. coli* | 60 | 15 | 64 | – | – | 6.0 | [15] |
| | EGII | 5 | *P. pastoris* | – | 220.2 | – | ND | 55 | 5.0 | [32] |
| *Penicillium oxalicum* | XEG12A | 12 | *P. pastoris* | ND | ND | 172 | – | 55–60 | 4.5–5.5 | [18] |
| *Aspergillus terreus* | GH12 | 12 | *A. nidulans* | 256 ± 0.11 | 3.33 ± 0.04 | 115 ± 0.04 | – | 55 | 5.0 | [17] |

Specific activities were determined by measuring the reducing sugars using the dinitrosalicylic acid (DNS) reagent in triplicate

–, indicates that enzyme activity or data are not shown; *, the native form of an enzyme; ND, no detection

This result consistent with the observed stability of *Gt*Cel12A expressed from *A. niger* [19].

The specific activity of the purified recombinant enzyme was assayed using various substrates (Additional file 1: Table S1). The enzyme exhibited significantly higher specific activity toward β-glucan (6546 U $mg^{-1}$) and CMC (1129 U $mg^{-1}$), which represent the β-1,3-1,4- and β-1,4-linkages, respectively, than toward xyloglucan (10.1 U $mg^{-1}$) and filter paper (0.21 U $mg^{-1}$). It did not degrade Avicel, xylan, soluble starch, locust bean gum, or carrageenan. The results of substrate preference were similar to that of *Gt*Cel12A expressed by *A. niger* [16]. The *Gt*Cel12A had detectable enzyme activities against both β-glucan and xyloglucan. Thus, *Gt*Cel12A can be classified into SF 12-1 of the GH12 family [28]. The kinetic parameters of *Gt*Cel12A were as follows: $V_{max}=3452$ μmol $min^{-1}$ $mg^{-1}$, $K_m=13.83$ mg $mL^{-1}$, and $k_{cat}=1726$ $s^{-1}$ as determined by a Lineweaver–Burk plot. Table 1 shows the characteristics of *Gt*Cel12A in comparison with other endoglucanases from various fungal genera including *Trichoderma*, *Aspergillus*, and *Penicillium*. Such fungi have been widely studied because they produce large quantities of cellulases and accepted commercial enzymes [29, 30]. The endoglucanases *Tr*EGIII, *Tr*EGII, *Po*XEG12A, and *At*GH12 had low or zero enzyme activities with respect to β-glucan and CMC, while *Gt*Cel5B in previous study showed high specific activities. However, *Gt*Cel12A exhibited notable specific activities on both substrates compared to them. [15, 17, 18, 25, 31, 32].

The influences of metal ions and EDTA were tested. The relative activity of *Gt*Cel12A was 126.9% when EDTA was added, and relative activities in the presence of these metal ions ($Ca^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $K^{+}$, $Li^{+}$, or $Na^{+}$) were 90–100% of the relative activities observed in the absence of these cofactors. However, relative activity was entirely lost following exposure to $Al^{3+}$, $Fe^{3+}$, and $Hg^{2+}$. *Gt*Cel12A did not employ metal ions as a cofactor. Naika and Tiku [33] have shown that enzyme activity of GH12 endoglucanase was enhanced in the presence of EDTA, which can affect molecule to form an intermediate state that is well disposed for protein–substrate interaction.

The hydrolysis pattern of oligosaccharides, including cellobiose, cellotriose, cellotetraose, and cellopentaose, was assessed by TLC analysis (Fig. 2). *Gt*Cel12A

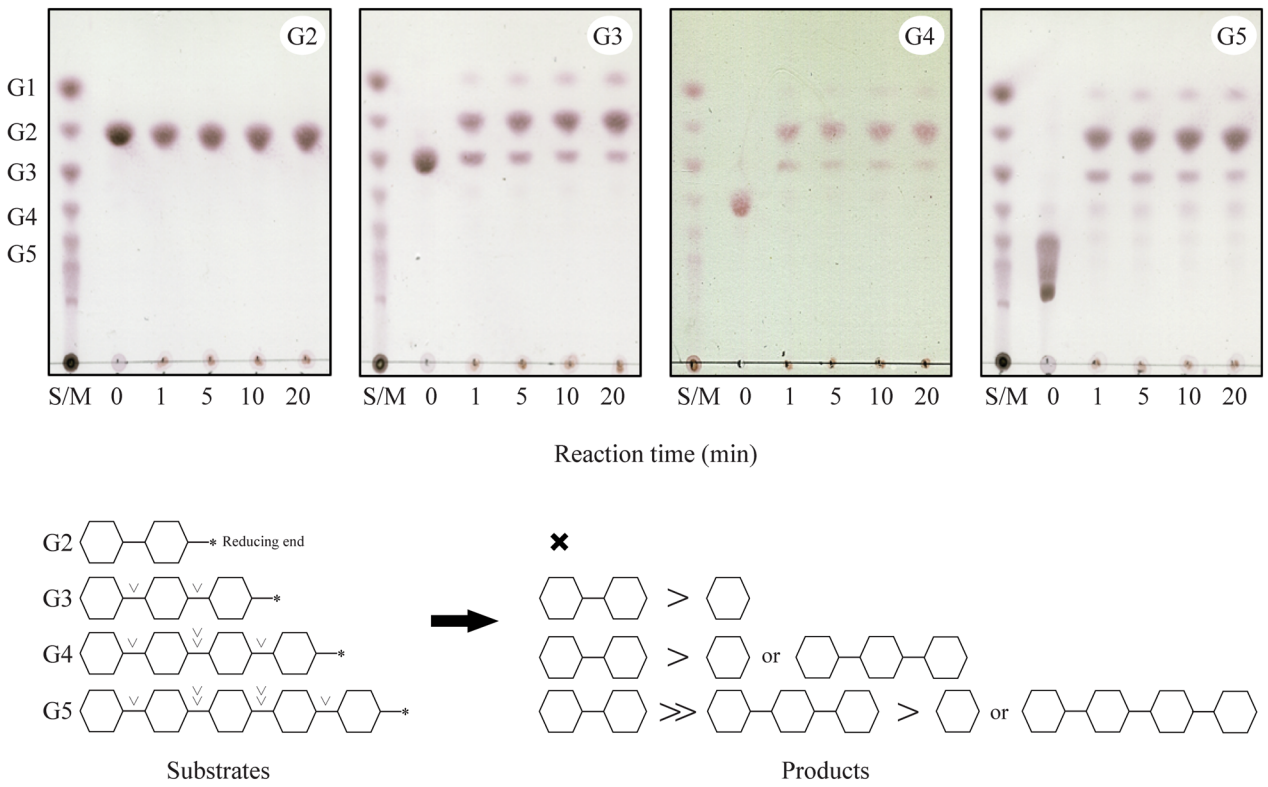


**Fig. 2** Thin-layer chromatography (TLC) analysis of hydrolyzed products of cello-oligosaccharides. TLC was conducted after digestion with *Gt*Cel12A for different incubation times ranging from 1 to 20 min. The diagram shows substrate hydrolysis and the resulting hydrolyzed products. *S/M* standard marker, *G* glucose, *G2* cellobiose, *G3* cellotriose, *G4* cellotetraose, *G5* cellopentaose

did not hydrolyze cellobiose into glucose; however, it did exhibit hydrolytic activity toward cellotriose, converting it into glucose and cellobiose. Cellotetraose was mainly degraded into cellobiose and minimally into glucose and cellotriose. Cellopentaose was likewise hydrolyzed by attacking its internal bonds, releasing cellobiose and cellotriose. In a previous study, *Tr*Cel12A did not degrade cellotriose into cellobiose and glucose, unlike *Gt*Cel12A [34]. However, the *Gt*Cel5B exhibited similar cello-oligomers hydrolysis patterns to those of *Gt*Cel12A, but with weaker hydrolysis activity for cellotriose [25]. For these reasons, recombinant acidic *Gt*Cel12A could be a candidate for use in numerous commercial industrial applications, as a superior enzyme with high catalytic efficiency and stability across a wide range of pH conditions.

### Enzymatic hydrolysis and synergistic effects of *Gt*Cel12A on the HPAC-pretreated lignocellulose

Enzymatic hydrolysis of lignocellulose is impaired by several factors such as lignin, cellulose crystallinity, enzyme accessibility, and degree of polymerization. Lignin is the most important limiting factor for enzymatic hydrolysis because it limits the accessibility of cellulases to their substrates and directly inhibits cellulases [35, 36]. As a result, the enzymatic hydrolysis efficiency of lignocellulose is reduced by lignin. Therefore, an effective pretreatment method for lignocellulose is crucial to ensure a high conversion rate during enzymatic hydrolysis. A pretreatment method for lignocellulose biomass has been reported to effectively eliminate (97.2%) of lignin from biomass. [23]. In the present study, we used the HPAC-pretreated lignocellulose as a substrate to assess enzyme activity and synergistic effects. Purified *Gt*Cel12A exhibited enzyme activities on HPAC-pretreated pine, rice straw, and corn stover and its characteristics were compared to those of *Gt*Cel5B endoglucanases because the *Gt*Cel5B showed more higher enzyme activities than other family 12 of glycoside hydrolase (GH12s) against β-glucan and CMC [25] (Fig. 3a). The pine substrate released 0.219 and 0.185 mg $mL^{-1}$ reducing sugars via *Gt*Cel5B and *Gt*Cel12A, respectively. As a result, similar reducing sugar yield were achieved by both endoglucanases with respect to the pine substrates. However, *Gt*Cel12A achieved reducing sugar yields of 0.270 and 0.292 mg $mL^{-1}$ from rice straw and corn stover, respectively, which were higher than the 0.144 mg $mL^{-1}$ of reducing sugars that *Gt*Cel5B produced from each substrate. HPLC showed that *Gt*Cel12A produced cellobiose as a major product, and glucose as a minor product, from HPAC-pretreated lignocellulose (Fig. 3b). This result may correspond with the hydrolysis patterns of cello-oligomers determined by TLC analysis. Cellulose

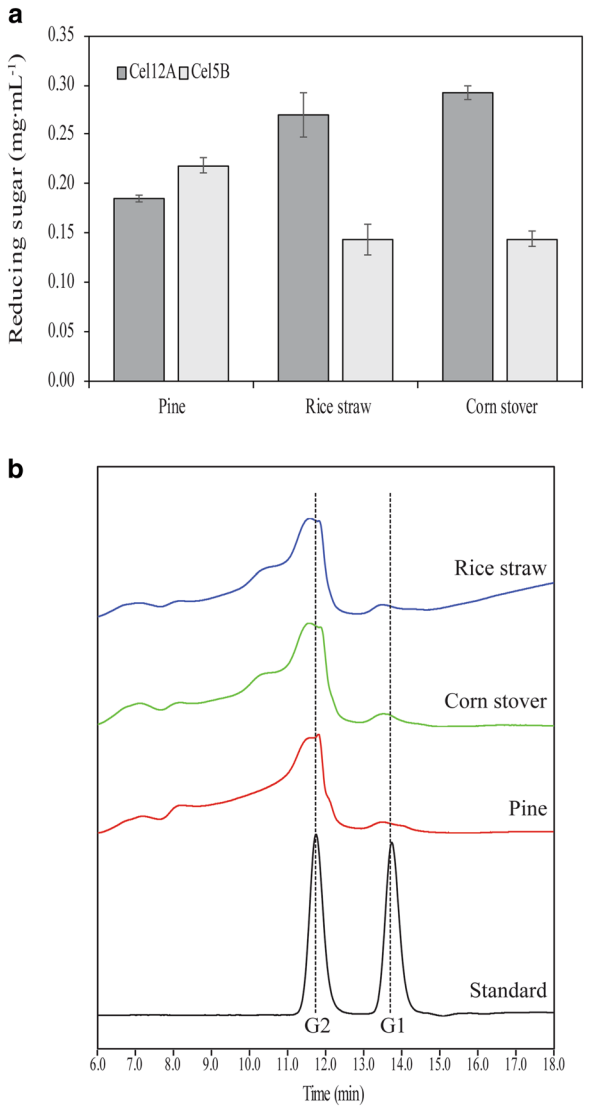


**Fig. 3** Comparison of enzymatic hydrolysis on the HPAC-pretreated pine, corn stover, and rice straw. **a** Enzyme activities of *Gt*Cel12A and *Gt*Cel5B was compared under their optimal conditions for 24 h on HPAC-pretreated lignocellulose. Error bars indicate ± SD. **b** Soluble sugars were analyzed by HPLC following enzymatic hydrolysis with purified *Gt*Cel12A. *G1* glucose, *G2* cellobiose

comprises a combination of crystalline and amorphous forms that can be easier to degrade than the crystalline regions [37]. The relative ratio of crystalline and amorphous cellulose regions varies according to the biomass source, which results in different degrees of degradability by enzymes. Certain grass-based biomasses contain significant quantities of β-glucan component in their cell walls. These conditions enabled *Gt*Cel12A to exhibit a high specific activity on β-glucan and CMC (Table 1). Miotto et al. [16] also reported that *Gt*Cel12A expressed

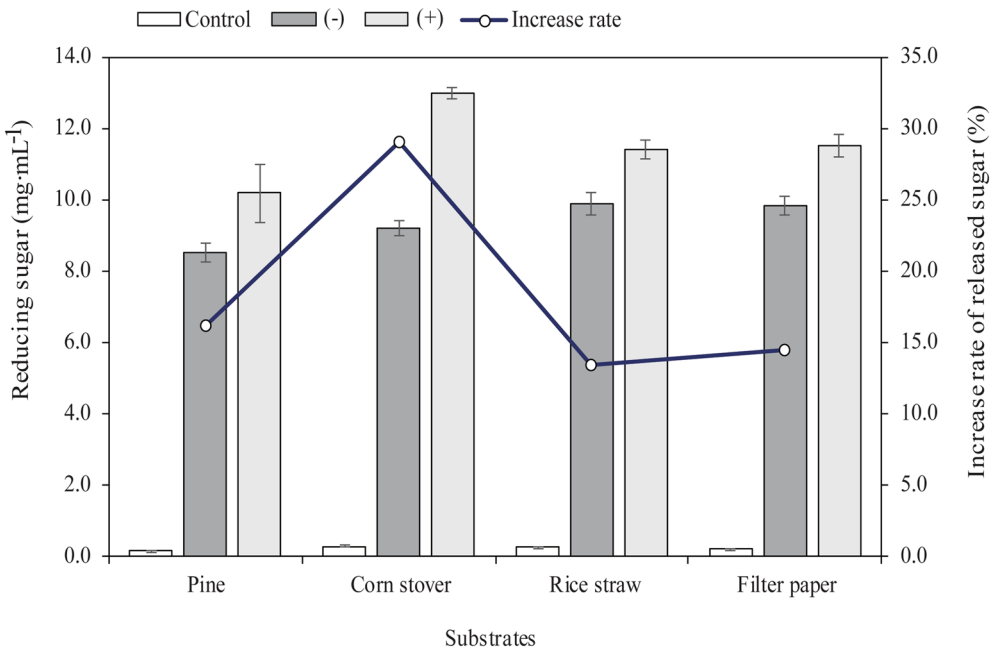


**Fig. 4** Synergistic effects of *Gt*Cel12A with commercial cellulase on filter paper and HPAC-pretreated lignocellulose. Purified *Gt*Cel12A with 10 FPU $g^{-1}$ cellulase was used on the 3% (w/v) of the HPAC-pretreated substrates in 50 mM citrate buffer (pH 4.8) at 48 °C for 24 h. Control: *Gt*Cel12A alone, (−): cellulase alone, (+): cellulase with *Gt*Cel12A, increase rate: increase in the amount of released sugar (%). Error bars indicate ± SD

by *A. niger* had high enzyme activity with respect to β-glucan, and exhibited hydrolytic effects with respect to oat spelt. These results suggest that *Gt*Cel12A hydrolyzed grass-based biomass more efficiently than pine wood compared to *Gt*Cel5B. Furthermore, *Gt*Cel12A exhibited a higher specific activity on CMC, which is amorphous cellulose, compared to *Gt*Cel5B. However, *Gt*Cel5B had higher specific activity on filter paper (Table 1). These results suggest that *Gt*Cel12A hydrolyzes amorphous cellulose more efficiently than *Gt*Cel5B, perhaps due to the cellulose crystallinity of the cellulose in HPAC-pretreated lignocellulose.

The synergistic cooperation of different types of cellulases is important for a high lignocellulose conversion rate. Endoglucanase, which acts on the internal glycosidic bonds in cellulose, creates new reducing and nonreducing ends for the *exo*-1,4-β-glucanases. When acting alone, *Gt*Cel12A achieved a reducing sugar yield of only 0.185 mg $mL^{-1}$ from HPAC-pretreated pine. However, we detected a synergistic effect when we compared the amount of reducing sugars released from HPAC-pretreated pine lignocellulose by commercial cellulase alone with that released by commercial cellulase together with purified *Gt*Cel12A with enzymic activity of 10 filter paper units per gram (FPU $g^{-1}$). An external supplement of purified *Gt*Cel12A increased reducing sugar production by 16.1, 29.0, 13.4, and 14.5% on the HPAC-pretreated pine, corn stover, rice straw, and filter paper, respectively, compared to commercial cellulase alone (Fig. 4). In a previous study, synergistic effects of endoglucanases were reported to enhance hydrolysis efficiency: the GH12 family endoglucanase from *A. terreus* increased hydrolysis efficiency by 38.7% compared to crude cellulase alone on delignified rice straw [38]. Endoglucanase from *Armillaria gemina* KJS114 additionally showed enhanced reducing sugar production when combined with commercial cellulase mixtures on the pretreated poplar [39]. Increased enzymatic hydrolysis may be related to a synergistic action between the purified endoglucanase and commercial cellulase, and could also be enhanced by added endoglucanase, which causes an increase in the total amount of enzyme in the mixture [38]. Therefore, acidic *Gt*Cel12A could be a superior candidate for enzymatic hydrolysis of HPAC-pretreated lignocellulose.

## Conclusion

The recombinant *G. trabeum* acidic endoglucanase Cel12A expressed by *P. pastoris* GS115 had an approximate molecular weight of 31 kDa, which differs from that of the enzyme expressed by *A. niger*. The purified *Gt*Cel12A expressed by *P. pastoris* had a more acidic

optimal pH than that expressed by *A. niger* and exhibited superior pH stability across a range of pH values (2.0–9.0). Furthermore, *Gt*Cel12A exhibited a notable specific activity with regard to CMC and β-glucan compared to *Gt*Cel5B, *Tr*Cel12A, and other GH12 endoglucanases. *Gt*Cel12A exhibited better enzymatic hydrolysis on HPAC-pretreated rice straw and corn stover than *Gt*Cel5B. Moreover, it showed synergistic effects of 16.1, 29.0, 13.4, and 14.5% on HPAC-pretreated pine, corn stover, rice straw, and filter paper, respectively, when *Gt*Cel12A was added as a supplement to cellulase. The *Gt*Cel12A with superior pH stability and enzyme activity could be a potential candidate as an additive enzyme for biotechnological application.

## Additional file

**Additional file 1: Figure S1.** Multiple alignment of *Gloeophyllum trabeum* Cel12A amino acid sequences. Alignment of *Gt*Cel12A with *Gloeophyllum trabeum* Cel5B, *Daedalea quercina* GH12, *Laetiporus sulphureus* GH12, *Neolentinus lepideus* GH12, *Postia placenta* GH12, *Trichoderma reesei* Cel12A, Cel5A, and Cel7B. Identical regions are indicated by black background. Conserved and similar regions are indicated in dark and light gray, respectively. A arrows, helices, and stars indicate β-sheet regions, α-helix regions, and deduced catalytic residues (E122 and E207), respectively. **Figure S2.** Western blot analysis and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of *Gt*Cel12A. (a) The purified enzyme was detected by western blot assay with a mouse anti-His tag. (b) Purified recombinant *Gt*Cel12A from *Pichia pastoris* was loaded on a 12% (*w/v*) SDS-PAGE gel and stained with Coomassie blue. Lane S/M: molecular weight size marker, lane 1: purified recombinant *Gt*Cel12A, lane 2: purified recombinant *Gt*Cel5B.

### Abbreviations

BSA: bovine serum albumin; CAZy: Carbohydrate-Active enZYmes database; CMC: carboxymethyl cellulose; DNS: dinitrosalicylic acid; GH: glycoside hydrolase; HPAC: hydrogen peroxide–acetic acid; HPLC: high-performance liquid chromatography; LB: Luria–Bertani; PCR: Polymerase chain reaction; PDA: potato dextrose agar; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC: thin-layer chromatography; YPD: yeast extract peptone dextrose; YPG: yeast extract peptone glycerol; 3D: three-dimensional.

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### Authors' contributions

CHO, YS, and H-JB designed and planned the research. CHO, CSP, and YGL performed the experiments. CHO wrote the manuscript with contributions of CSP. H-JB supervised the experiments and manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

All data generated or analyzed during this study are included in this published article and Additional file 1.

### Consent for publication

Informed consent was obtained from all individual participants in the study.

### Competing interests

The authors declare that they have no competing interests.

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