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Characterization of a Multimodular Endo-β-1,4-Glucanase (Cel9K) from *Paenibacillus* sp. X4 with a Potential Additive for Saccharification

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An endo-β-1,4-glucanase gene, *cel*9K, was cloned using the shot-gun method from *Paenibacillus* sp. X4, which was isolated from alpine soil. The gene was 2,994 bp in length, encoding a protein of 997 amino acid residues with a predicted signal peptide composed of 32 amino acid residues. Cel9K was a multimodular enzyme, and the molecular mass and theoretical pI of the mature Cel9K were 103.5 kDa and 4.81, respectively. Cel9K contains the GGxxDAGD, PHHR, GAxxGG, YxDDI, and EVxxDYN motifs found in most glycoside hydrolase family 9 (GH9) members. The protein sequence showed the highest similarity (88%) with the cellulase of *Bacillus* sp. BP23 in comparison with the enzymes with reported properties. The enzyme was purified by chromatography using HiTrap Q, CHT-II, and HiTrap Butyl HP. Using SDS-PAGE/activity staining, the molecular mass of Cel9K was estimated to be 93 kDa, which is a truncated form produced by the proteolytic cleavage of its C-terminus. Cel9K was optimally active at pH 5.5 and 50°C and showed a half-life of 59.2 min at 50°C. The CMCase activity was increased to more than 150% in the presence of 2 mM Na^+, K^+, and Ba^{2+}, but decreased significantly to less than 50% by Mn^{2+} and Co^{2+}. The addition of Cel9K to a commercial enzyme set (Celluclast 1.5L + Novozym 188) increased the saccharification of the pretreated reed and rice straw powders by 30.4% and 15.9%, respectively. The results suggest that Cel9K can be used to enhance the enzymatic conversion of lignocellulosic biomass to reducing sugars as an additive.

Keywords: *Paenibacillus* sp. X4, multimodular endo-β-1,4-glucanase, GH9, saccharification

Introduction

Cellulose is one of the most abundant polysaccharide and consists of β-1,4-linkages of glucose, and it is found in various cellulosic materials as well as hemicellulosic polymers. The polymeric complexes have been of particular interest as valuable sources for the production of biofuels, and therefore cellulases have been investigated and used in the fermentation of biomass into biofuels because they yield fermentable sugars [1–3]. Cellulases are also widely used in the textile, pulp, and paper industry and for pharmaceutical and food applications, as well as in laundry detergents [4, 5] and in the cell-surface display technique [6]. Generally, cellulases are classified into three groups; endo-β-1,4-glucanase (E.C. 3.2.1.4), exo-β-1,4-glucanase (E.C. 3.2.1.91), and β-glucosidase (E.C. 3.2.1.21). Among these enzymes, endo-β-1,4-glucanase is responsible for the cleavage of internal bonds in glucose chains [7]. Among many bacterial endo-β-1,4-glucanases, several have been cloned from *Paenibacillus* sp. and produced heterologously in *Escherichia coli* [8–16].

In cellulosic biofuel production, the main obstacle is the cost of the conversion of the cellulosic biomass, and the thermochemical pretreatment and enzymatic hydrolysis are the key factors in terms of the cost barrier [3]. The pretreatment disrupts the recalcitrant structures of the cellulosic biomass and makes it more accessible to the enzymes, using various physicochemical methods [17]. In the bacterial cellulase system, glycosyl hydrolase family 9 (GH9) cellulases are generally regarded as the major

cellulose-degrading enzymes [18]. It was reported that synergistic proteins, including carbohydrate-binding modules (CBMs), increase the enzymatic hydrolysis of cellulose [19, 20].

Previously, we isolated *Paenibacillus* sp. X4 from soil of an alpine region, near Takakkaw Falls [21]. In this study, we report on the cloning of a multimodular endo-β-1,4-glucanase, a member of GH9, containing two CBMs, from the strain. The enzyme might contribute to the hydrolysis of cellulosic materials in the environment. We have also investigated its biochemical properties, including its potential use in the conversion of pretreated biomass to fermentable sugars.

Materials and Methods

Chemicals and Enzymes

Lichenan (Icelandic moss) was purchased from Megazyme (Ireland). Isopropylthio-β-D-galactoside (IPTG) and 5-bromo4-chloro-3-indolyl-β-D-galactoside (X-gal) were from Bioneer (Korea). The Celluclast 1.5L and Novozym 188 were obtained from Novozyme (Denmark). Glucose, cellobiose, barley β-glucan, carboxymethyl-cellulose (CMC), laminarin, birchwood xylan, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), *p*-nitrophenyl-β-D-cellobioside (*p*NPC), *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX), and other chemicals were from Sigma-Aldrich (USA).

Cloning of a Gene for CMC-Degrading Enzyme

The gene source in this study was the isolate *Paenibacillus* sp. X4 described previously [21]. The chromosomal DNA was isolated, partially digested with Sau3AI, and separated by agarose gel electrophoresis. Fragments of 3−5 kb in size eluted from the gel were ligated to BamHI-digested pUC19, and introduced into *E. coli* DH5α competent cells (Yeastern Biotech. Co., Taiwan). *E. coli* transformants were grown on LB agar plates supplemented with ampicillin (50 μg/ml), IPTG, and X-Gal (4 μl of 200 mg/ml and 40 μl of 20 mg/ml, respectively, for a premade plate). The transformants were tooth-picked, inoculated onto LB containing 0.5% CMC (LBC) agar plates, and grown for about 24 h at 37ºC, and the colonies with halos were selected after staining with Congo red. Recombinant plasmids of the clones were isolated and analyzed.

Sequence Analysis of the Gene

The nucleotide sequence of the insert DNA was determined by SolGent Co. Ltd. (Korea). The conserved region of the gene was identified with BlastN or BlastP of BLAST, and a phylogenetic tree of the gene was constructed at the NCBI (http://www.ncbi.nlm.nih.gov). The signal peptide was predicted with SignalP 4.1 in CBS (http://www.cbs. dtu.dk/services/SignalP/) [22]. The molecular mass and pI of the encoded protein were predicted, and multiple alignment was constructed using DNA/MAN (ver. 4.11; Lynnon Biosoft, Canada).

Purification of the Enzyme

The selected clone was grown in LB broth supplemented with 50 μg/ml of ampicillin for 12 h at 37ºC with rotary agitation at 200 rpm, and cells were harvested by centrifugation. After dispersion in 50 mM sodium citrate buffer (pH 5.5), the crude enzyme solution was prepared by sonication and dialyzed against 50 mM Tris-HCl buffer (pH 8.0), as previously described [23]. The crude enzyme was loaded onto a HiTrap Q column (5 ml; GE Healthcare, Sweden) and eluted with a linear gradient of NaCl (0–1.0 M) in 50 mM Tris-HCl buffer (pH 8.0) at a flow rate of 1.0 ml/min. The active fractions collected were purified by CHT-II column (5 ml; Bio-Rad, USA) chromatography with a gradient of sodium phosphate buffer (10–500 mM, pH 6.8) at a flow rate of 0.5 ml/min [24]. Then, the active fractions were further purified by HiTrap Butyl HP (5 ml; GE Healthcare, USA) and eluted with a reverse linear gradient of 1.5–0 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min. The protein concentration was determined using the method of Bradford [25], and SDS-PAGE was carried out on 11.5% polyacrylamide gels [26].

Enzyme Assay

For the standard assay, the enzyme activity was analyzed using 0.5% CMC as the substrate in 50 mM sodium citrate buffer (pH 5.5), as described previously [27], except at 50ºC. The amount of reducing sugar released was determined after incubation for 15 min at 50ºC using the dinitrosalicylic acid (DNS) method [28]. For the substrate specificity of Cel9K, the activities were determined using 0.5% barley β-glucan, lichenan, laminarin, and birchwood xylan. The hydrolyzing activities for Avicel (0.5%) and filter paper (a strip, 1 cm × 1 cm) were observed after 2 h reaction at 50ºC. One unit of the enzyme activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per minute under the designated conditions. Glucose was used as the standard. Hydrolyzing activities for *p*NPG, *p*NPC, and *p*NPX were assayed using 2.0 mM of each substrate, as described previously [27], except at 50ºC. One unit of the hydrolyzing activity was defined as the amount of enzyme liberating 1 μmol of *p*NP in 1 min.

Characterization of the Enzyme

The optimum temperature and thermostability for the enzyme were determined by measuring the enzyme activities at designated temperatures and preincubated conditions, respectively [24]. Optimum pH levels for the enzyme activities were determined using 50 mM sodium citrate (pH 3.5−6.0) and universal (pH 3.0−12.0) buffers. The influences of various cations and effectors on the enzyme activity were analyzed as described previously [24].

Enzymatic Saccharification

The enzymatic hydrolysis of reed (*Phragmites communis*) and rice (*Oryza sativa*) straw powders was carried out as described previously [29] with slight modifications. In short, the pretreated materials (*i.e.*, reed or rice straw powders) were suspended to 6%

A

B

Fig. 1. Multiple alignment of the amino acid sequences of Cel9K and other endo-β-1,4-glucanases (**A**); putative conserved domains of Cel9K (**B**); and phylogenetic tree of the enzyme with other endo-β-1,4-glucanases (**C**).

In (**A**), five catalytic residues are shown as red circles. The inverted triangle indicates the expected cleavage site for a signal peptide in Cel9K. The conserved motifs are represented as orange boxes. CBM_3, carbohydrate binding module_3; FN3, fibronectin type-3 domain.
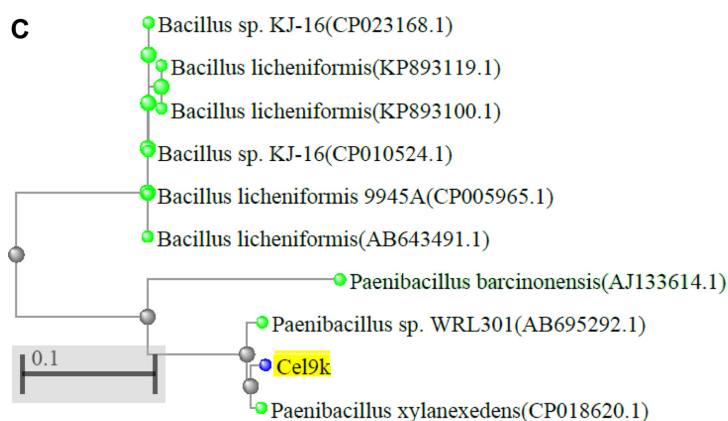
Fig. 1. Continued.

in 5 ml of 50 mM sodium citrate buffer (pH 5.5) in a 50-ml Falcon tube. The concentrations of enzymes used were 0.2 U/ml of Celluclast 1.5L; 0.2 U/ml of Novozyme 188; and 0.33 U/ml of Cel9K. The reactions were carried out for up to 72 h at 50°C with shaking at 150 rpm. Aliquots of the reaction mixtures were withdrawn at designated times (0, 6, 12, 24, 48, and 72 h) and centrifuged for 10 min at 12,000 ×g; the amount of released soluble sugar was measured by the DNS method using D-glucose as a standard [28]. All experiments were performed in duplicate.

Nucleotide Sequence Accession Number

The nucleotide sequence of cel9K has been deposited in the GenBank under the accession number MG570051.

Results and Discussion

Cloning and Characterization of the Gene for a CMC-Degrading Enzyme

One CMCase-positive clone, YA 1-139, was selected from about 140 transformants based on the size of the halo on an LBC agar plate. The insert DNA in the recombinant plasmid was 6.9 kb in length and had three open reading frames (ORFs) (data not shown). One ORF of 2,994 bp was identified as an endo-β-1,4-glucanase gene with high similarities to the GH9 enzymes and named *cel*9K. The gene was predicted to

encode a protein of 997 amino acid residues with a putative signal peptide of 32 amino acid residues. The molecular mass and the theoretical pI of the mature Cel9K were calculated to be 103.5 kDa and 4.81, respectively. In comparison with the enzymes with reported properties, the protein sequence showed the highest similarity (88%) to the cellulase CelB of *Paenibacillus* sp. BP23 (later *P. barsinonensis*, AJ133614) [8], followed by 61% similarity to the Cel9A of *Bacillus licheniformis* GXN151 (AAR29083) [30], and 60% to the EglA of *Bacillus pumilus* CL16 (AY339624) [7]. Cel9K was found to be a multimodular endoglucanase; Cel9K contained GGxxDAGD, PHHR, GAxxGG, YxDDI, and EVxxDYN motifs in its catalytic domain (43–474th amino acid residues), and the motifs were found in most GH9 proteins (Fig. 1A). Similarly to CelB, Cel9K had two CBM_3 domains (505–585th and 851–931st) at the C-terminal end of the catalytic domain and an fibronectin type-3 domain (663–748th) between the CBMs (Fig. 1B), whereas Cel9A and EglA possessed only a CBM_3.

In the phylogenetic tree of Cel9K and other related proteins using DNA sequences, Cel9K was closest (98%) to the endo-β-1,4-glucanase of *Paenibacillus xylanexedens* (CP018620), of which the enzymatic properties have not been reported (Fig. 1C).

Table 1. Purification of an endo-β-1,4-glucanase (Cel9K) of *Paenibacillus* sp. X4 from an *E. coli* transformant YA 1-139 clone.

Procedure	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Supernatant	15	41.7	27.8	0.67	1.0	100
(NH$_4$)$_2$SO$_4$ precipitation	2	7.04	24.7	3.51	5.2	88.8
HiTrap Q chromatography	4	0.41	4.92	12.0	17.9	17.7
CHT-II chromatography	8	0.17	3.63	21.4	31.9	13.1
HiTrap Butyl HP chromatography	3	0.012	1.93	160.8	240.0	6.9

Purification of Cel9K

Cel9K produced by recombinant *E. coli* cells was purified using HiTrap Q, CHT-II, and HiTrap Butyl HP chromatographies. Higher enzyme purity was obtained with HiTrap Q column chromatography than with High-Q column chromatography (data not shown). The recovery yield, purification fold, and specific activity of the enzyme purified by the methods were 6.9%, 240.0-fold, and 160.8 U/mg protein, respectively (Table 1). The purified

Fig. 2. SDS-PAGE of purified Cel9K and of samples obtained during purification (**A**); and activity staining of the enzyme by Congo-red after SDS-PAGE and renaturation (**B**).
In (**A**), M, size markers; 1, crude extract; 2, ammonium sulfate 40% fractionate; 3, HiTrap Q chromatography; 4, CHT-II chromatography; 5, HiTrap Butyl HP chromatography. The numbers left of the figures represent the molecular masses of the proteins in kilodaltons (kDa). In (**B**), M, size markers; 1, ammonium sulfate 40% fractionate; 2, HiTrap Butyl HP chromatography.

Cel9K appeared as a nearly single band on the SDS-PAGE gel, and it had a molecular mass of 93 kDa (Fig. 2A). The value corresponded to that of a cleaved enzyme, comprising about 868 amino acid residues. That might mean that the truncated protein was a polypeptide comprising the 33rd–900th amino acid residues, and a fragment of 65 amino acid residues containing about half of the second CBM_3 was cleaved by proteolytic cleavage, as likely the cases in foreign protein expression in *E. coli* [27, 31]. In fact, in activity staining after SDS-PAGE, three extra minor bands (corresponding to one larger and two smaller molecular masses) were observed in the ammonium sulfate precipitate, and the major band was isolated to near homogeneity after the purification (Fig. 2B), similarly to the results of CelB where one intense and three less intense bands were detected [8]. The truncation was observed in other multimodular enzymes [32, 33].

Substrate Specificity of Cel9K

The relative activities of Cel9K toward CMC (β-1,4-glucan), barley β-glucan (β-1,3-1,4-glucan), and lichenan (β-1,3-1,4-glucan) were 100%, 311.4%, and 157.3%, respectively (Table 2). The enzyme showed low hydrolytic activities of 15.2% and 2.3% toward birchwood xylan (β-1,4-xylose) and laminarin (β-1,3-1,6-glucan), respectively. The substrate specificity of the enzyme indicates that Cel9K is able to hydrolyze β-1,4 linkages of mixed-linked β-1,3-1,4-glucans, as well as pure β-1,4-glucan, CMC. Cel9K showed lower activity for lichenan (157.3%) than for barley β-glucan (311.4%). The difference in enzyme activity might come from the higher ratio of β-1,4 to β-1,3 linkages in β-glucans than in lichenans, as mentioned by Akita *et al.* [34].

Table 2. Substrate specificity of endo-β-1,4-glucanase (Cel9K).

Substrate	Relative activity (%)
CMC	100[a]
Barley β-glucan	311.4 ± 8.52
Lichenan	157.3 ± 7.10
Laminarin	2.3 ± 0.57
Xylan	15.20 ± 3.31
Avicel	0.98 ± 0.15
Filter paper	0.88 ± 0.15
*p*NPC	3.6 ± 0.86
*p*NPG	ND
*p*NPX	2.8 ± 0.57

[a]The value corresponding to 159.7 U/mg protein in specific activity.
ND, not detectable.

The hydrolyzing activities for the filter paper or Avicel of Cel9K were relatively low, likely multimodular endoglucanases from *B. pumilus* and *Paenibacillus barcinonensis* [7, 35], but contrary to those from *Caldicelllulosiruptor bescii* and *Clostridium thermocellum*, which were highly active for the crystalline cellulose [36, 37].

Properties of Cel9K

Cel9K exerted its maximal CMCase activity at 50ºC and showed much less activity at 40ºC and 60ºC (*i.e.*, 56.0% and 67.0% of its maximal activity, respectively) (Fig. 3B). The optimum temperature of Cel9K was slightly lower than those (55ºC) for the cellulase Pgl8A of *Paenibacillus cookii* [13] and the endo-β-1,4-glucanase (Egl-257) of *Bacillus circulans* KSM-N257 (BAB69035) [38], and that (60ºC) for EglA [7]. Cel9K showed the characteristics of a weakly acidic enzyme, showing maximal CMCase activity at pH 5.5 (Fig. 3A). The optimum pH was similar to that of CelB [8] and Pgl8A [13] but quite different from that of Egl-257 (pH 8.5) [38]. Cel9K retained 36.0% of its CMCase activity after 10 min of heat treatment at 60ºC (Fig. 3C). The half-life for the activity was 59.2 min at 50ºC and was lower than that of Egl-257 (15 min at 60ºC) [38].

Mono- and divalent cations tested had significant influence on the CMCase activity of Cel9K (Fig. 4); at 2 mM concentration, Na^+, K^+, and Ba^{2+} increased the activities to 152.9%, 158.1%, and 151.1%, respectively, and Mg^{2+}, Ca^{2+}, Fe^{2+}, and Zn^{2+} increased them by more than 20%. However, at 5 mM concentration, the stimulation effects were not observed, maintaining control levels. Interestingly, Mn^{2+} and Co^{2+} showed inhibitory activities to less than 50% at 2 mM (25.0% and 48.9%, respectively) and 5 mM concentrations (32.5% and 42.5%, respectively). The effects of Ca^{2+} and Mn^{2+} on Cel9K activity were similar to those on CelB; however, the effect of Ba^{2+} on Cel9K was different from that on CelB [8]. No inhibition of Cel9K was observed by Zn^{2+}, contrary to the results of CelB and EglA [7, 8].

Saccharification of the Biomass

In the previous report, the pretreated materials were

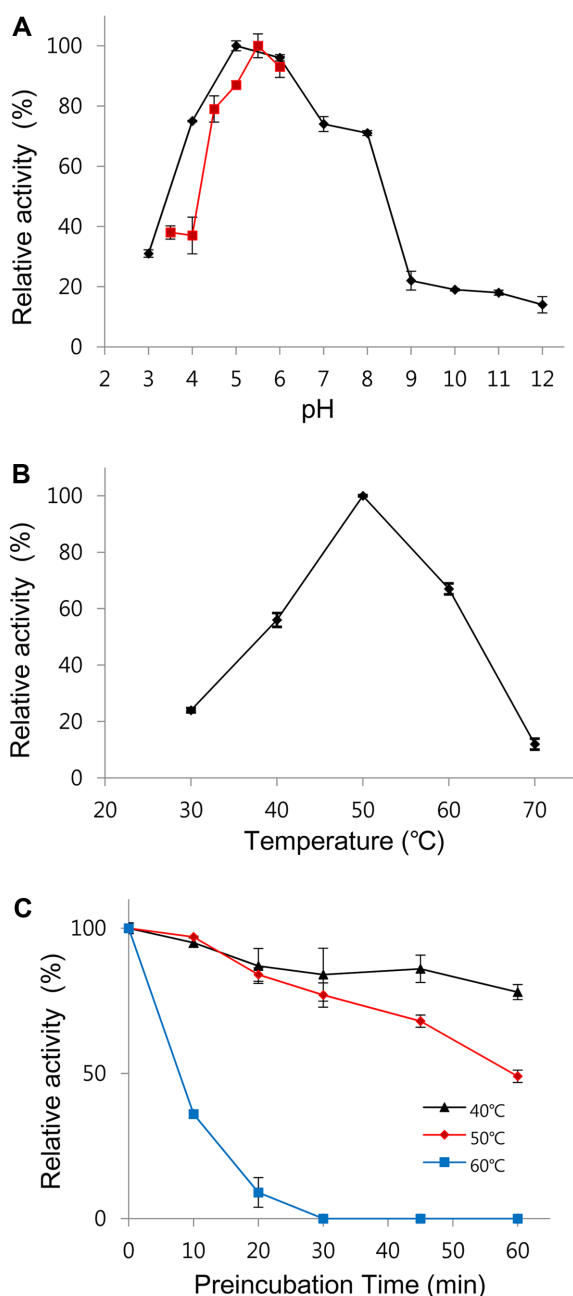**Fig. 3.** Optimum pH (**A**), optimum temperature (**B**), and thermostability of Cel9K (**C**).
In thermostability experiments, Cel9K was preincubated at different temperatures for the indicated times without substrate, and then the residual activity was measured under the standard assay condition. ◆, Universal buffer; ■, sodium citrate buffer.
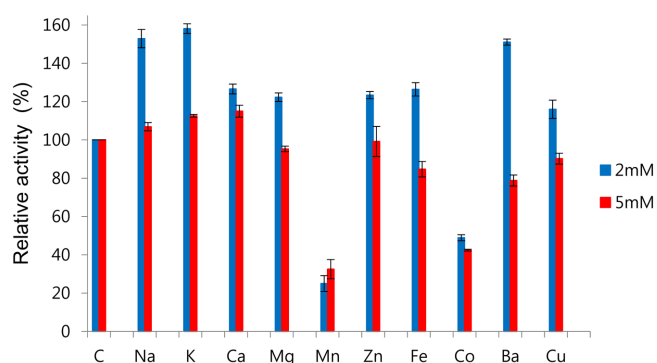

Fig. 4. Effects of metal ions on Cel9K activity.

more efficient in the enzymatic hydrolysis of the biomass, and doubling the concentrations of the commercial enzymes showed less than 10% increases in the production of reducing sugar [29]. Based on the results, in the present study, pretreated samples and a set of concentrations of the commercial enzymes (Celluclast 1.5L + Novozyme 188) were used for the experiments. The production of reducing sugar from the pretreated reed or rice straw powders by hydrolysis with commercial enzymes reached its plateau after 24–48 or 48 h reaction, respectively (Fig. 5). The influence of the addition of Cel9K on the saccharification was determined by the addition of Cel9K to the control (Celluclast 1.5L + Novozyme 188). The addition of Cel9K increased the saccharification of the biomasses, reed and rice straw powders, by 30.4% (2.3–3.0 g/l) and 15.9% (4.4–5.1 g/l), respectively (Fig. 5). The additive effects of Cel9K were greater than the increases with the doubling of the commercial enzymes [29]. It has been reported that saccharification efficiency was increased using an optimized crude enzyme cocktail in the enzymatic hydrolysis of pretreated rice straw [39], and using an ionic liquid-tolerant cocktail in the hydrolysis of the ionic liquid-pretreated biomass at higher temperatures [40]. In this study, the use of an additive enzyme, Cel9K, was found to increase the saccharification of the pretreated biomass by the commercial enzymes. Cel9K was able to hydrolyze pure β-1,4-glucans, such as CMC, as well as β-1,4 linkages of mixed-linked β-1,3-1,4-glucans. From these results, it can be suggested that using additive enzymes, such as Cel9K, is the way to improve the saccharification of lignocellulosic substrates.

In conclusion, an endo-β-1,4-glucanase gene of *Paenibacillus* sp. X4 was cloned and characterized. The endo-β-1,4-glucanase (Cel9K) belonged to the GH9 family and comprised a multimodular structure. The enzyme showed a 30.4% enhancement in the pretreated reed powder following its addition to the commercial preparations. These results suggest its potential use as an additive in the saccharification of cellulosic biomass.

Acknowledgments

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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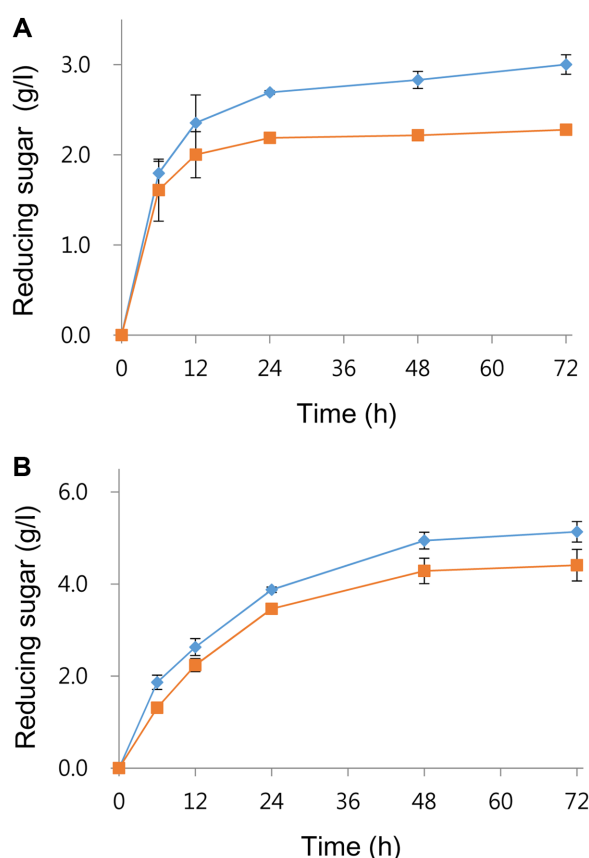
Fig. 5. Saccharification of reed (**A**) and rice straw (**B**) powders. ■, A commercial enzyme set (Celluclast 1.5L + Novozyme 188); ◆, the commercial enzyme set + Cel9K.

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