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Glucose production from cellulose through biological simultaneous enzyme production and saccharification using recombinant bacteria expressing the β-glucosidase gene

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Efficient cellulosic biomass saccharification technologies are required to meet biorefinery standards. Biological simultaneous enzyme production and saccharification (BSES), which is glucose production from cellulosic biomass by *Clostridium thermocellum*, can be a reliable cellulose saccharification technology for biorefineries. However, the current BSES processes require purified β-glucosidase supplementation. In this study, recombinant bacteria expressing the β-glucosidase gene were developed and directly applied to BSES. The engineered *Escherichia coli* expressing the thermostable β-glucosidase gene from *Thermoanaerobacter brockii* exhibited 0.5 U/ml of β-glucosidase activities. The signal peptide sequence of *lytF* gene from *Bacillus subtilis* was the most appropriate for the β-glucosidase secretion from *Brevibacillus choshinensis*, and the broth exhibited 0.74 U/ml of β-glucosidase activities. The engineered *E. coli* and *B. choshinensis* expressing the thermostable β-glucosidase gene produced 47.4 g/L glucose and 49.4 g/L glucose, respectively. Glucose was produced by the hydrolysis of 100 g/L Avicel cellulose for 10 days through BSES, and the product yield was similar to that obtained through BSES with purified β-glucosidase supplementation. Our findings indicate that the direct supplementation of β-glucosidase using bacterial cells expressing β-glucosidase gene or their broth was applicable to BSES, suggesting the potential of this process as a cost-effective approach to cellulose saccharification.

[**Key words:** Biorefinery; Cellulosic biomass; Biological simultaneous enzyme production and saccharification; *Clostridium thermocellum*; β-Glucosidase]

Cellulosic biomass is an abundant, renewable, and underutilized resource (1). Biorefinery, which refers to chemical production from cellulosic biomass, is an alternative strategy to resolve problems of resource depletion and global warming caused by carbon dioxide emission from oil refineries (2). Synthetic biological approaches to biorefinery processes have used microorganisms, such as *Escherichia coli* or *Saccharomyces cerevisiae*, to produce chemicals (e.g., fuel compounds) through bioprocessing (3). During the bioprocessing of cellulosic biomass, diverse carbohydrate-active enzymes are used to convert the cellulosic biomass into fermentable sugars. However, the efficiency of the enzymatic degradation of cellulosic biomass remains low (4).

Clostridium thermocellum is a candidate bacterium for cellulosic biomass saccharification (5). Reportedly, *C. thermocellum* completely degrades 4.4 g/L of purified cellulose within 1 day (6), 65% of 5 g/L of switchgrass within 5 days, and 70% of 10 g/L corn hulls within 7 days (7–9). Multicellulolytic enzyme complexes, called cellulosomes, constitute diverse carbohydrate-active enzymes. Cellulosomes largely contribute to the cellulose degradation ability of *C. thermocellum* (10–12). The main product of cellulose degradation is cellobiose, which leads to the feedback inhibition of cellulosomes. The supplementation of β-glucosidase (BGL) leads to the hydrolysis of cellobiose into two glucose molecules, thereby resolving the feedback inhibition, which in turn results in increased glucose production from purified cellulose, pretreated switchgrass, and pretreated rice straw (13,14). *C. thermocellum* preferentially uses cellooligosaccharide, and glucose tends to accumulate in the culture broth (15). Glucose production by *C. thermocellum* from 100 g/L cellulose or 120 g/L alkali pretreated rice straw remarkably increases to approximately 76.7 g/L over 10 days with the supplementation of purified BGL, suggesting that BGL supplementation can be a reliable cellulose saccharification approach for biorefineries (16). This technology is referred to as biological simultaneous enzyme production and saccharification (BSES), and it does not require the addition of diverse carbohydrate-active enzymes for the saccharification of cellulosic biomass.

The supplementation of purified BGL is not a cost-effective method of cellulose saccharification. To circumvent the need for supplemental purified BGL, *C. thermocellum* has been engineered to exhibit increased BGL activity. Maki et al. (17) have reported that

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the BGL activity of *C. thermocellum* transformed with a plasmid harboring the *C. thermocellum bglA* gene was 2.3-fold greater than that of the wild-type during the late log phase of growth. Moreover, *C. thermocellum* expressing the *cglT* gene from *Thermoanaerobacter brockii* showed a 4.8-fold increase in BGL activity (0.04 U/g cellulose) compared with the control strain although the recombinant strain did not show increased glucose production from Avicel cellulose (Ichihara et al., unpublished data). The *bglA* gene from *Caldicellulosiruptor* sp. F32 was fused with the *celS* gene of *C. thermocellum* genomic DNA, which conferred significant BGL activity (7.23 U/g cellulose), resulting in 68.6 g/L glucose production from 100 g/L Avicel cellulose. However, this process lasted 20 days (18).

Here, we studied the applicability of model bacteria, *E. coli*, *Bacillus subtilis*, and *Brevibacillus choshinensis* engineered with the *bgl* gene for glucose production from cellulosic substrates. Our findings suggest that the direct supplementation of bacterial cells expressing the *bgl* gene or their broth is applicable for BSES processes and can be a cost-effective approach to cellulose saccharification.

MATERIALS AND METHODS

Plasmid construction and transformations of *E. coli*, *B. subtilis*, and *B. choshinensis* The *cglT* gene (WP_012269734.1) (19) encoding a thermostable BGL in *T. brockii* DSM1457 (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany) was amplified and inserted into the plasmid pQE30 (Qiagen, Hilden, Germany) at *Bam*HI and *Pst*I sites (Fig. S1). *E. coli* JM109 (Toyobo, Osaka, Japan) was transformed with the resulting plasmid DNA. *E. coli* transformants were cultured in Luria broth (LB) supplemented with 50 μg/mL ampicillin at 37°C.

The *cglT* gene was also inserted into the plasmid pBE-S (Takara Bio, Shiga, Japan) at *Xho*I and *Pst*I sites. A plasmid library harboring diverse signal peptide sequences was constructed by replacing the *aprE* signal peptide sequence in the resultant plasmid DNA with 173 signal peptide sequences from *B. subtilis* through In-Fusion cloning (Takara Bio) (Fig. S1). *B. subtilis* RIK1285 (Takara Bio) was transformed with this plasmid library. *B. subtilis* transformants were cultured in LB supplemented with 10 μg/mL kanamycin at 37°C. *B. subtilis* strains exhibiting BGL activity were screened and isolated (20).

DNA encoding the signal peptide sequence and *cglT* gene from the isolated *B. subtilis* strain exhibiting the highest BGL activity were amplified and inserted into the pBIC1 plasmid (Takara Bio) (Fig. S1). *B. choshinensis* SP3 (Takara Bio) was transformed with the resultant plasmid and cultured in TM medium (10 g/L glucose, 10 g/L Phytone peptone, 5 g/L bonito extract, 2 g/L yeast extract, 10 mg/L $FeSO_4 \cdot 7H_2O$, 10 mg/L $MnSO_4 \cdot 4H_2O$, and 1 mg/L $ZnSO_4 \cdot 7H_2O$; pH 7.0) supplemented with 50 μg/mL neomycin at 30°C (21,22).

BGL purification Isolated *E. coli* transformants were cultured in LB supplemented with 50 μg/mL ampicillin at 37°C overnight. Once the cells reached an optical density of approximately 0.5 at 600 nm, 1 mM isopropyl-1-thio-β-D-galactopyranoside was added to the cultures. Cells were subsequently collected and sonicated, and the expressed proteins in cell-free extracts were purified using Profinia IMAC protein purification system (Bio-Rad, Hercules, CA, USA) (Fig. S2). The purified protein was dissolved in 20 mM potassium phosphate buffer (pH 7.0) and stored at −80°C until further use.

BGL assay BGL assays were performed with *p*-nitrophenyl β-D-glucoside in 0.1 M succinate buffer (pH 5.7) at 60°C for 10 min. BGL activity was determined on the basis of the measurement of *p*-nitrophenol release: one unit of enzyme releases 1 μmol equivalent of *p*-nitrophenol per minute (16).

Cellulose saccharification by BSES *C. thermocellum* DSM 1313 (DSMZ) was inoculated in BM7CO medium (1.5 g/L KH_2PO_4, 2.9 g/L K_2HPO_4, 2.1 g/L urea, 6.0 g/L yeast extract, 0.5 g/L L-cysteine-HCl·H_2O, 4.0 g/L Na_2CO_3, 0.5 mg/L $MgCl_2 \cdot 6H_2O$, 7.5 μg/L $CaCl_2 \cdot 2H_2O$, and 250 μg/L resazurin; pH 7.0) containing 1% Avicel cellulose (Sigma–Aldrich, St. Louis, MO, USA) and cultured at 60°C for 2 days under anaerobic conditions with nitrogen gas (23). For the estimation of glucose production, 100 μL of the culture was transferred into 5 mL of BM7CO medium containing either ball-milled cellulose or Avicel cellulose and cultured at 60°C under anaerobic conditions. BGL was subsequently added to the broth, and the cultures were incubated for 20 days. Glucose concentrations in the broth were measured by the mutarotase-glucose oxidase method with Glucose C2 kit (Wako, Osaka, Japan).

Cellulase assay The broth during BSES was mixed with 1% carboxymethyl cellulose (Sigma–Aldrich). The reactions were carried out in 300 μL of 50 mM potassium phosphate buffer (pH 7.0) at 60°C. The amounts of released reducing sugars were determined by the dinitrosalicylic acid method. One unit of activity was defined as micromoles of glucose equivalents per minute per mL of broth.

RESULTS AND DISCUSSION

cglT* gene expression in *E. coli*, *B. subtilis*, and *B. choshinensis First, the *cglT* gene from *T. brockii* was expressed in *E. coli*. Cells expressing the *cglT* gene were collected, resuspended in BM7CO medium (6×10^8 cells/mL), and incubated at 60°C. The suspension showed more than 0.5 U/mL of BGL activity at the incubation time longer than 0.5 h compared with the activity of 0.011 U/mL before incubation (Fig. 1). This result suggests that BGL molecules were released from the *E. coli* cells after the heat treatment at 60°C.

B. subtilis was transformed with the plasmid library harboring the *cglT* gene and the 173 *B. subtilis* signal peptide sequences, and the transformed strains were screened for BGL activity in the culture supernatants. Eight signal sequences allowed CglT secretion in *B. subtilis* (Fig. 2A, Table S1). The culture supernatant of the transformants carrying the *B. subtilis lytF* signal sequence exhibited the highest BGL activity (0.042 U/mL) among the library strains. *B. choshinensis*, which is an effective host for heterologous gene expression and protein secretion (21,22), was transformed with the DNA encoding the *cglT* gene and *lytF* signal sequence. The culture of the *B. choshinensis* transformants showed 0.74 U/mL of the BGL activity, which was 6.2-fold higher than that of the *B. subtilis* transformants (Fig. 2B).

Saccharification of ball-milled cellulose through BSES by *E. coli* cells expressing the *cglT* gene *C. thermocellum* was cultured in BM7CO medium containing 50 g/L ball-milled cellulose, and the culture was supplemented with *E. coli* cells expressing the *cglT* gene (6×10^8 cells/mL; 10 U/g cellulose). After a 3-day incubation, ball-milled cellulose was completely degraded (Fig. 3A), and the glucose concentration in the broth was 37.3 g/L, which was equal to that obtained with purified BGL supplementation (Fig. 3B). This result indicates that the direct supplementation of bacterial cells is applicable for cellulose degradation through BSES. The *E. coli* cells supplementation was able to be decreased to 1.2×10^8 cells/mL (2 U/g cellulose) for the saccharification of 50 g/L ball-milled cellulose through the BSES (Fig. S3).

The cultivation period of *C. thermocellum* prior to BGL addition for longer than 12 h resulted in a significant increase in glucose concentrations compared with cultivation periods of 0 and 6 h (Fig. 4). The significant increase in glucose concentrations was not observed between the 12- and 24-h cultivation periods. These results demonstrate that cultivation of *C. thermocellum* prior to BGL supplementation is essential, with 12-h incubation period being optimal for the saccharification of 50 g/L of ball-milled cellulose.

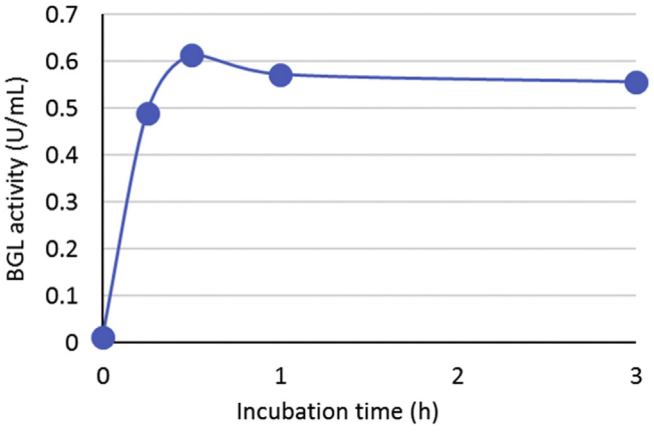

FIG. 1. β-Glucosidase activity of the *E. coli* cell suspension expressing the *cglT* gene (6×10^8 cells/mL) during incubation at 60°C.

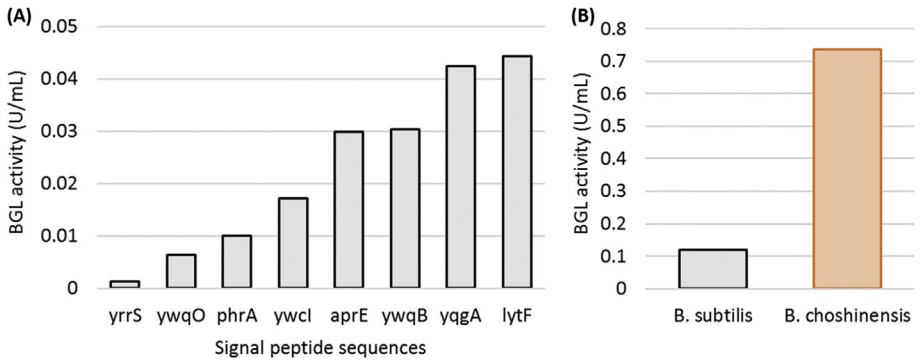

FIG. 2. β-Glucosidase activities of *B. subtilis* and *B. choshinensis* expressing the *cglT* gene. (A) *B. subtilis* was transformed with the plasmid library harboring the *cglT* gene with diverse signal peptide sequences (Fig. S1). The isolated *B. subtilis* strains were screened for β-glucosidase (BGL) activities in the culture supernatants. (B) *B. choshinensis* was transformed with the plasmid DNA harboring the *cglT* gene with *lytF* signal peptide sequences. BGL activities of the broth of *B. subtilis* and *B. choshinensis* strains are represented.

C. thermocellum cells enter the stationary phase for at least 24 h during the cultivation with 100 g/L cellulose (16), and it is expected that purified BGL supplementation inhibits the growth of *C. thermocellum* within the exponential phase.

Saccharification of Avicel cellulose through BSES by *E. coli* and *B. choshinensis* expressing the *cglT* gene Subsequently, we attempted the degradation of Avicel cellulose through BSES. Under experimental conditions in this study, 40.8 g/L and 47.4 g/L of glucose were produced from 100 g/L Avicel cellulose through BSES supplemented with purified CglT (10 U/g cellulose) within 9 days and 14 days, respectively (Fig. S4). Without BGL supplementation, glucose yields from the degradation of 100 g/L Avicel cellulose by *C. thermocellum* were 13.2 g/L and 17.5 g/L within 10 and 18 days, respectively (Fig. 5).

Experiments with the supplementation of *E. coli* cells (1.4×10^9 cells/mL; 10 U/g cellulose) yielded glucose concentrations of 47.4 g/L and 50.1 g/L within 10 and 18 days, respectively. In the case of the use of *B. choshinensis*, CglT is secreted and is not concentrated by the cell collection, and 2.1 U/g cellulose of BGL activity was applied to the BSES by the supplementation of the *B. choshinensis* broth. A total of 2 U/g cellulose of BGL activity is enough for the improvement of the glucose production from 100 g/L Avicel cellulose compared to that without the BGL supplementation (Fig. S4). Glucose concentrations of 49.4 g/L and 51.9 g/L within 10 and 17 days were measured, respectively (Fig. 5). These glucose productivities were similar to those obtained through the supplementation of purified BGL (Fig. S4), indicating that the direct supplementation of bacterial cells expressing the *bgl* gene or their broth is applicable for glucose production from Avicel cellulose through BSES.

Prawitwong et al. (16) have reported that 76.7 g/L glucose was produced from 100 g/L Avicel cellulose or 120 g/L alkali pretreated rice straw by *C. thermocellum* with the supplementation of purified BGL for 10 days. Zhang et al. (18) have reported the glucose production of 68.6 g/L from 100 g/L Avicel cellulose over 20 days by *C. thermocellum* expressing the *bglA* gene from *Caldicellulosiruptor* sp. F32. The glucose productivities measured in this study (49.4 g/L glucose from 100 g/L Avicel cellulose for 10 days) (Fig. 5) were lower than those presented by Prawitwong et al. (16) and Zhang

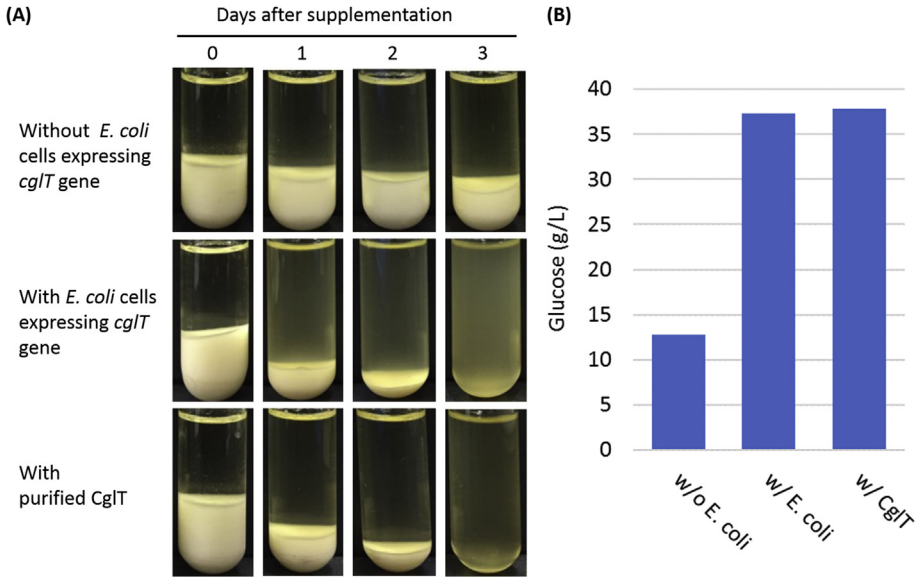

FIG. 3. Saccharification of 50 g/L ball-milled cellulose though biological simultaneous enzyme production and saccharification (BSES) by *E. coli* cells expressing the *cglT* gene. (A) *C. thermocellum* was cultured in the medium containing 50 g/L ball-milled cellulose at 60°C under anaerobic conditions. *E. coli* cells expressing the *cglT* gene (10 U/g cellulose) were subsequently added to the broth, and the cultures were incubated for 3 days. (B) Glucose concentrations produced from 50 g/L ball-milled cellulose through BSES at 3 days are represented.

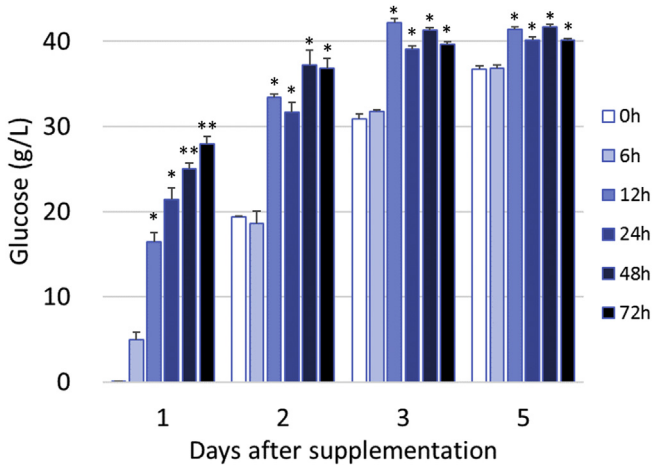

FIG. 4. Effects of the timing of supplementation with *E. coli* cells expressing the *cglT* gene on glucose production from 50 g/L ball-milled cellulose through biological simultaneous enzyme production and saccharification (BSES). *C. thermocellum* was cultured for 0–72 h prior to the supplementation of *E. coli* cells expressing *cglT* gene (10 U/g cellulose). Glucose concentrations in the cultures were measured. Asterisks and double asterisks represent values significantly larger than values at 6- and 12-h cultivation periods of *C. thermocelum* prior to *E. coli* supplementation, respectively (Student's *t*-test, $p < 0.01$). Bars indicate standard errors. The experiment was performed in triplicate.

et al. (18). However, the BGL activity was maintained during the BSES, Avicel cellulose was incompletely degraded in this study. Increasing the supplementation of collected *E. coli* cells (20 U/g cellulose of BGL activity) did not increase glucose productivity (data not shown). Thus, the cellulose-degrading ability of *C. thermocellum* which depend on the culture condition may affect glucose productivity.

Holwerda et al. (24) have reported that *C. thermocellum* achieved 80% degradation of 100 g/L Avicel cellulose in the absence of BGL supplementation during incubation, whereas the supplementation of vitamins increased cellulose degradation to 93%. The influence of oxygen and redox potential on the activity of cellulosomes may also play a role in these processes because aeration during cellulose saccharification by *C. thermocellum* cultures has been reported to

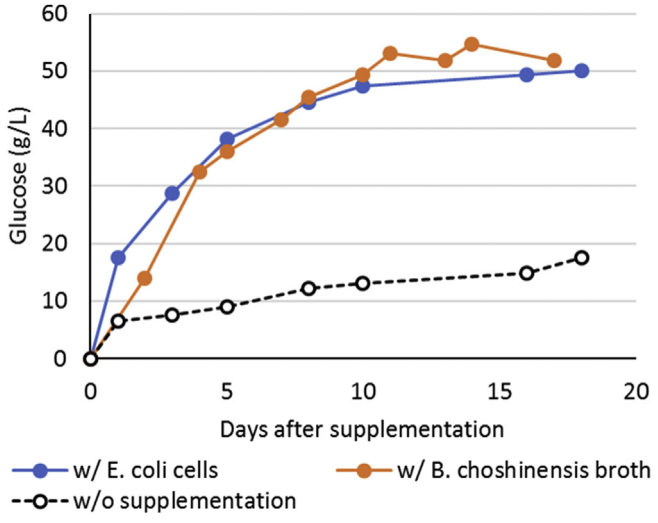

FIG. 5. Saccharification of 100 g/L Avicel cellulose through biological simultaneous enzyme production and saccharification (BSES) by recombinant bacteria expressing the *cglT* gene. *C. thermocellum* was cultured in medium containing 100 g/L Avicel cellulose, and the collected *E. coli* cells (10 U/g cellulose) or the cell cultures of *B. choshinensis* (2.1 U/g cellulose) were subsequently supplemented.

significantly inhibit degradation (18,25,26). Additionally, low pH conditions during incubation as a result of organic acids production by *C. thermocellum* could inhibit cellulose degradation through BSES (16,18).

The pH of *C. thermocellum* broth during BSES was decreased to 6.0. BSES in the medium at pH 8.0 and with stirring at 170 rpm improved the productivity: glucose concentrations of 62.1 g/L and 70.8 g/L within 10 and 21 days, respectively (Fig. S5). Carboxymethyl cellulase activity of the broth at 3 days after the BGL supplementation significantly increased when the BSES was conducted at pH 8.0 with the stirring (Fig. S5). These suggest that pH and stirring conditions are important for the glucose productivity through BSES, which may be the result of improvement of cellulase activity of *C. thermocellum* culture.

The sterilization of waste containing recombinant bacterial cell would be an important and potentially energy-consuming step in biorefineries. However, cellulose saccharification in this study used recombinant *E. coli* or *B. choshinensis* cells that can be effectively killed with heat treatment (i.e., 1 h at 60°C). Following heat treatment, recombinant cells could not form colonies on LB agar plates or TM agar plates at 37°C (Fig. S6), indicating that the heat treatment at 60°C following BSES effectively killed both the *E. coli* and *B. choshinensis* cells.

BSES can be used as a reliable cellulose saccharification process in biorefineries. Although cellulose saccharification by BSES has required purified BGL supplementation so far, we demonstrated in this study that the supplementation of the engineered *E. coli* or *B. choshinensis* produced significant glucose yields from 100 g/L Avicel cellulose over 10 days through BSES. The observed productivities were similar to those recorded with the supplementation of purified BGL. Overall, our findings suggest that the direct supplementation of bacterial cells expressing the *bgl* gene or their broth was applicable to BSES, indicating the potential of this process as a cost-effective cellulose saccharification approach to bioprocessing.

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The authors declare that they have no conflict of interest.

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