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Utilization of recombinant *Trichoderma reesei* expressing *Aspergillus aculeatus* β-glucosidase I (JN11) for a more economical production of ethanol from lignocellulosic biomass

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The capacity of *Trichoderma reesei* cellulase to degrade lignocellulosic biomass has been enhanced by the construction of a recombinant *T. reesei* strain expressing *Aspergillus aculeatus* β-glucosidase I. We have confirmed highly efficient ethanol production from converge-milled Japanese cedar by recombinant *T. reesei* expressing *A. aculeatus* β-glucosidase I (JN11). We investigated the ethanol productivity of JN11 and compared it with the cocktail enzyme *T. reesei* PC-3-7 with reinforced cellobiase activity by the commercial Novozyme 188. Results showed that the ethanol production efficiency under enzymatic hydrolysis of JN11 was comparable to the cocktail enzyme both on simultaneous saccharification and fermentation (SSF) or separate hydrolysis and fermentation (SHF) processes. Moreover, the cocktail enzyme required more protein loading for attaining similar levels of ethanol conversion as JN11. We propose that JN11 is an intrinsically economical enzyme that can eliminate the supplementation of BGL for PC-3-7, thereby reducing the cost of industrial ethanol production from lignocellulosic biomass.

[**Key words:** *Trichoderma reesei*; *Aspergillus aculeatus* BGL I; Simultaneous saccharification and fermentation; Separate hydrolysis and fermentation; Ethanol]

The second-generation biofuel ethanol from lignocellulosic biomass offers a promising alternative renewable and environmentally friendly energy due to its cost benefits, abundance and renewability (1–3). Japanese cedar is a promising lignocellulosic substrate belonging to the softwood type because it contains a high proportion of glucose and mannose units that can be utilized by hexose-utilizing *Saccharomyces cerevisiae*. Compared to hardwood or agricultural waste, it scarcely contains xylose units (4,5). Hemicellulose contained in Japanese cedar is glucomannan and galactoglucomannan. A backbone of randomly arranged mannose and glucose units linked by β-1,4-glycosidic bonds, and galactose units attached to mannose units linked by α-1,6-glycosidic bonds, comprise the structural units of this softwood substrate (6).

Conversion of cellulosic materials to glucose is the key step of lignocellulosic ethanol production, which mainly depends on the degradation capacity of cellulases secreted by cellulolytic microorganisms and the accessibility of these enzymes to cellulose (7–9). An important cellulolytic organism, capable of secreting a large amount of cellulolytic enzymes is the filamentous fungus *Trichoderma reesei* (10). Three types of cellulases act in conjunction with each other to effectively convert cellulose to glucose in *T. reesei*. These include endo-β-1,4-glucanases (EG, EC 3.2.1.4), which randomly cleave internal bonds of the amorphous region of the cellulose chain and expose the chain end to create new targets for the second set of enzymes, the cellobiohydrolases (CBH, EC 3.2.1.91). These enzymes, in turn, hydrolyze both the crystalline and amorphous celluloses from the reducing and non-reducing end to liberate cellobiose, which then is cleaved to glucose by the third set of enzymes, the β-glucosidases (BGL, EC 3.2.1.21) (11–13). *T. reesei* cellulases act synergistically to hydrolyze cellulose to glucose effectively. Among them, the β-glucosidase is considered an important enzyme because it could relieve the cellobiose inhibition of EG and CBH (14). However, end-product inhibition by glucose limits the activity and cellulose hydrolysis by endogenous *T. reesei* β-glucosidases, and this, combined with their extremely low endogenous activity, subsequently results in inefficient ethanol production (15,16). In addition, the adsorption of cellulases to the accessible surface of cellulose can enhance the efficiency of enzyme hydrolysis (17,18).

Another approach to overcoming insufficient BGL activity and improving glucose yield is the addition of extracellular β-glucosidase, such as Novozyme 188 with Celluclast 1.5L to *T. reesei* cultures (19). However, such approaches result in increased cost of ethanol production. Utilization of recombinant technology for increased BGL activity has been very successful in recent years. A recombinant strain X3AB1, constructed from a *T. reesei* strain expressing *Aspergillus aculeatus* β-glucosidase I (AaBGL1) under the control of *xyn3* promoter, produced an enzyme preparation called JN11, which

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yielded over 60 times more BGL activity than the PC-3-7, the parental strain when grown on Avicel cellulose (20). Subsequently, Kawai et al. (21) showed that JN11 exhibited the best balance of cellulase and hemicellulase activities and therefore is very effective for saccharification and is preferred for enzymatic hydrolysis of various cellulosic biomasses. These characteristics make JN11 worthy of further investigation of its potential in the conversion of lignocellulose into ethanol.

Two processes, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF), are widely used in ethanol production, each with its own merits and problems. In SHF, conditions for saccharification and fermentation can be optimized independently of each other, but the process is limited by product inhibition of enzymes during the hydrolysis of sugar, while in SSF, end product inhibition is greatly reduced due to immediate glucose consumption by yeast, resulting in enhanced cellulosic ethanol conversion (22). However, this process requires scouting for the best thermal conditions that are suitable for both the enzymatic reaction and fermentation of yeast, which could be a drawback for this process (23). The fermentative yeast that is favored in ethanol production is *S. cerevisiae* due its high tolerance to ethanol and inhibitors produced during the hydrolysis of cellulose and high ethanol productivity (5,24–26).

In this study, we evaluated the efficient conversion of the lignocellulosic material, Japanese cedar, into ethanol by using JN11 for the first time. We determined the optimal protein concentration required to produce a comparable yield of glucose and ethanol for both JN11 and the cocktail enzyme, *T. reesei* PC-3-7 culture supernatant (PC-3-7) reinforced by commercial BGL from *Aspergillus niger* (Novozyme 188), for both SHF and SSF processes. In addition, we have also demonstrated in this study, the surprising result of JN11 adsorption to biomass during the saccharification step.

MATERIALS AND METHODS

Lignocellulosic raw material The lignocellulosic substrate, Japanese cedar was pretreated by converge-milling pretreatment in Ichinoseki National College of Technology. Converge mill (Type ET2 manufactured by Earthtechnica Co. Ltd.: pot (SUS), 1 L; media balls (SUJ-2), φ8 mm; ball-filling ratio, 10%; sample input, 30 g; rotation speed, 800 rpm) was used in this study. The components of converge-milled Japanese cedar were 40% (wt.) of cellulose, 13% (wt.) of hemicellulose, which constitutes to 44% (wt.) of glucose, 7.6% (wt.) of mannose, 1.2% (wt.) of galactose and 4.8% (wt.) of xylose, and 36% (wt.) of lignin.

***T. reesei* cultivation and cellulase preparation** *T. reesei* PC-3-7 cellulase hyper-producing strain (ATCC 66589) obtained from Kyowa Hakko Kogyo Co. Ltd. was used in this research. The cultivation of *T. reesei* PC-3-7 was carried out as described previously (27) with 1% (w/v) Avicel (Funacel; Funakoshi Co., Ltd., Tokyo, Japan) as a carbon source. 1×10^7 conidia were inoculated in 50 mL culture medium and incubated at 28°C for 6 days on a rotary shaker at 220 rpm. The culture supernatant as enzyme preparation was obtained as filtrate through Miracloth (Calbiochem, Darmstadt, Germany). Novozyme 188 purchased from Sigma (St. Louis, MO, USA) was used to reinforce the BGL activity in *T. reesei* PC-3-7 culture supernatant (PC-3-7). The culture supernatant from recombinant *T. reesei* expressing *A. aculeatus* β-glucosidase I, X3A1 strain was prepared as using methods described previously (20).

Yeast strain and culturing conditions *S. cerevisiae* INVSc1 (*MATa, his3-D1, leu2, trp1-289, ura3-52,* Invitrogen, Carlsbad, CA, USA) was used as the fermentative organism. A single colony of the subcultured *S. cerevisiae* INVSc1 on YPD agar plate was aerobically cultivated in a sterilized 50 mL of YPD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) medium, its pH was adjusted to 5.2 by HCl (Nacalai Tesque, Inc., Kyoto, Japan), and incubated at 30°C, 130 spm (stroke per minute) for 24 h (exponential growth phase). The optical density of yeast in culture cell was determined by UV-VIS spectrophotometer with OD 600 nm and used for the estimation of dry cell weight (dcw) of cultures added in the fermentation experiments.

Enzyme assay and ethanol analysis The protein concentration of PC-3-7, JN11, and Novozyme 188 preparation were measured by Lowry method using bovine serum albumin as a standard. Total cellulase activity reported in filter paper unit (FPU), was determined according to the Laboratory Analytical Procedure published by the National Renewable Energy Laboratory (28). The cellobiase activity was determined in 200 mM of acetate buffer (pH 5.0) at 50°C with final concentration of 20 mM of cellobiose (Sigma) as a substrate in total volume of 500 μL. The amount of glucose released by enzyme reaction was determined by Glucose C2 test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). One unit of cellobiase activity (CBU) was defined as the amount of enzyme that produced 2 μmol of glucose per minute. The β-glucosidase, β-mannosidase and β-xylosidase activities were determined using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc), *p*-nitrophenyl-β-D-mannopyranoside (*p*NPMan), and *p*-nitrophenyl-β-D-xylopyranoside (*p*NPXyl), (all from Sigma), respectively, as substrates at 50°C for 10 min (pH 5.0). The released *p*-nitrophenol was detected at 410 nm after adding 1 M sodium carbonate to stop the reaction. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of *p*NP per minute. The Avicelase, carboxymethylcellulase (CMCase), xylanase and mannanase activities were measured as reducing sugar produced using the Somogyi-Nelson method (29). The final concentration of Avicel, carboxymethylcellulose (low viscosity), and birch wood xylan (Sigma) was 1% and that of glucomannan (low viscosity) was 0.5% in 50 mM acetate buffer (pH 5.0) at 50°C for 10 min of CMCase activity, 15 min of mannanase activity, and 30 min of Avicelase and xylanase activities. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of glucose or xylose equivalent.

The amount of cellobiose, mannose, xylose and ethanol derived from the SSF or SHF experiment was determined using a high performance liquid chromatography (HPLC) LC-20AD (Shimadzu Corporation, Kyoto, Japan) system equipped with HPX-87P Aminex column (Bio-Rad Laboratories, Inc., CA, USA) and refractive index detector of RID-10A (Shimadzu). The temperature was set at 80°C, and water (Millipore) was used for the mobile phase at a flow rate of 0.6 mL/min. All samples were filtered with 0.45 μm disposable hydrophobic filter before being subjected to HPLC (Dismic-13 HP, Advantec, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The data presented in the tables are the mean values of three independent experiments.

Saccharification Saccharification was conducted in a 50 mL polyethylene bottle. 10% (w/v) of converge-milled Japanese cedar was used as substrate. JN11 was loaded at 5 and 15 mg protein corresponding to 17.25 and 51.75 CBU, respectively/g-converge-milled Japanese cedar. The protein loading amount of cocktail enzyme was determined under 5 and 15 mg protein of PC-3-7, corresponding to 0.28 and 0.84 CBU, respectively, with various supplementing protein amounts of Novozyme 188 to reinforce cellobiase activity in PC-3-7. The amounts of protein supplements with Novozyme 188 increased the CBU up to 0.5, 1, 2, 3, 5 and 10 for 5 mg protein loading and 1, 2, 3, 5, 10 and 20 for 15 mg protein loading of PC-3-7/g-converge milled Japanese cedar. 50 mM final concentration of acetate buffer (pH 5.0) and distilled water were added to set the total volume to 10 mL. The saccharification bottles were incubated in a shaking incubator (Bioshaker BR-43FL, Taitec Corporation, Saitama, Japan) at 50°C, 200 spm for 24 h.

For the SHF process, the saccharification step was performed under similar cellobiase activity of JN11 and cocktail enzyme. JN11 was loaded at 5, 10 and 15 mg protein corresponding to 17.25, 34.50 and 51.75 CBU of JN11/g-converge-milled Japanese cedar. The cocktail enzyme was loaded at 5, 10 and 15 mg protein of PC-3-7 and reinforced by 5.7, 11.4 and 17.1 mg protein of Novozyme 188, which made the total protein loading amount of cocktail enzyme to 10.7, 21.4 and 32.1 mg for corresponding to 17.25, 34.50 and 51.75 CBU, respectively. After incubation at 50°C, 200 spm for 72 h, the saccharification samples were boiled for 10 min to denature the enzyme and centrifuged at 3000 rpm for 10 min. The amount of glucose in the saccharified supernatant was measured by Glucose C2 test solution. The conversion of glucose was calculated as the concentration of glucose released in the total reaction volume compared to the initial glucose contained in converge-milled Japanese cedar.

Fermentation Fermentation was performed by adding *S. cerevisiae* INVSc1 culture at 1 OD_{600} unit (exponential growth phase), which was equivalent to 9 mg dcw, into the boiled, saccharified liquid supernatant. The fermentation bottles were sealed to prevent air transfer into the bottle (limiting oxygen) and incubated in a shaking incubator at 30°C, 100 spm for 12–24 h. The amount of glucose in the supernatant was measured by Glucose C2 test solution.

Enzyme characterization For examining cellulase adsorption of JN11 and cocktail enzyme to the biomass precipitate, saccharification was tested under similar cellobiase activity of JN11 and cocktail enzyme. JN11 was loaded at 5.2 CBU, corresponding to 1.5 mg protein/0.1 g-converge-milled Japanese cedar and the cocktail enzyme was loaded at 0.1 CBU, corresponding to 1.5 mg protein of PC-3-7 with 5.1 CBU of Novozyme 188. 50 mM final concentration of acetate buffer (pH 5.0) and distilled water were added for a total volume of 500 μL, dispensed into a 96 deep-well plate, and incubated in a shaking incubator (Bio-shake M BR-024, Taitec) at 50°C, 1200 spm for 2–72 h. Saccharified supernatant and biomass precipitate were separated by centrifugation and the biomass precipitate was washed three times by Millipore water. The free proteins in the saccharified supernatant and proteins adsorbed to the saccharified biomass precipitate were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel slabs as described by Laemmli (30). Gels were stained with Coomassie blue R250. The molecular mass marker used was the Precision Plus