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Molecular cloning and expression of fungal cellobiose transporters and β-glucosidases conferring efficient cellobiose fermentation in *Saccharomyces cerevisiae*

Yi-Hyun Bae[a,b,1], Kyeong-Hyeon Kang[a,1], Yong-Su Jin[c], Jin-Ho Seo[a,b,*]

[a] Department of Agricultural Biotechnology and Center for Food and Bioconvergence, Seoul National University, Seoul 151-742, Republic of Korea
[b] Interdisciplinary Program for Bioengineering, Seoul National University, Seoul 151-742, Republic of Korea
[c] Department of Food Science and Human Nutrition, University of Illinois at Urbana-Champaign, 1206 West Gregory Dr., Urbana, IL 61801, USA

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ABSTRACT

Cellobiose was once regarded as a byproduct that should be removed from biomass hydrolysates because of its inhibitory activity to cellulases. It was revealed, however, that cellobiose could serve as a co-substrate for xylose fermentation by engineered *Saccharomyces cerevisiae*. Despite its advantages, to date, little is known about cellodextrin transporters that endow *S. cerevisiae* with cellobiose transporting ability. In this study, engineered *S. cerevisiae* strains capable of fermenting cellobiose were constructed by expressing various fungal cellobiose transporters and intracellular β-glucosidases. Among them, the strain expressing a putative sugar transporter from *Penicillium chrysogenum* (Pc_ST) and β-glucosidase from *Thielavia terrestris* (Tt_BG) showed an improved cellobiose fermentation performance compared to the strain expressing a cellodextrin transporter from *Neurospora crassa* (Nc_CDT-1) and β-glucosidase from *N. crassa* (Nc_GH1-1). Cellobiose fermentation by *S. cerevisiae* Pc_ST/Tt_BG under microaerobic conditions resulted in 14.5 ± 0.5 g/L of final ethanol concentration with a yield of 0.37 ± 0.01 g ethanol/g cellobiose, which are 22% and 26% higher than the corresponding values of *S. cerevisiae* Nc_CDT-1/Nc_GH1-1. These results suggest that the yield and rate of cellobiose fermentation can be improved by adopting optimal pairs of cellobiose transporters and β-glucosidase.

1. Introduction

Cellobiose is a disaccharide of two glucose molecules linked by a β-(1 → 4) bond. The amount of cellobiose present in cellulosic hydrolysates depends on the activity of β-glucosidase added in the enzymatic saccharification process. Previously, cellobiose was considered as an undesirable byproduct because of its inhibitory effect on cellulase activity (Holtzapple et al., 1990). But several research groups have shown that direct fermentation of cellobiose instead of glucose might have several advantages, especially when cellobiose is used as a co-substrate for lignocellulosic ethanol production by engineered *Saccharomyces cerevisiae* (Ha et al., 2011; Li et al., 2010; Saitoh et al., 2010).

In general, cellobiose is metabolized through three modes in microorganisms: First, some fungi and bacteria secrete β-glucosidase and hydrolyze cellobiose into glucoses in the extracellular medium, and then the glucose molecules are transported into the cells (Singhania et al., 2013). Second, some *Clostridium* species produce cellodextrin transporters and cellobiose phosphorylases that phosphorylyze cellobiose into one glucose molecule and one glucose-1-phosphate molecule that are both further metabolized through glycolysis (Demain et al., 2005). Third, some cellulolytic fungi express both cellodextrin transporters and intracellular β-glucosidases to transport cellobiose into the cells and then break the cellobiose into glucose molecules in the cytoplasm (Galazka et al., 2010).

S. cerevisiae is a well-known host that has been used for commercial production of ethanol from starch- and sugar-based feedstocks but the yeast needs genetic engineering for cellobiose metabolism. Intriguingly, although the second above-mentioned mode is the most energetically favorable pathway in theory (Sadie et al., 2011), it was shown that the cellobiose transporter/cellobiose phosphorylase system is less efficient compared to the cellobiose

* Corresponding author at: Department of Agricultural Biotechnology and Center for Food and Bioconvergence, Seoul National University, Seoul 151-742, Republic of Korea. Tel.: +82 2 880 4855; fax: +82 2 873 5095.
E-mail address: jhseo94@snu.ac.kr (J.-H. Seo).
[1] These authors contributed equally to this work.

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Table 1
Sources of genes coding for putative cellodextrin transporters and β-glucosidases.

Encoded protein	Notation	GI number	CDS size (bp)	Relevant work
Neurospora crassa cellodextrin transporter	Nc_CDT-1	85111152	1740	Ha et al. (2011)
Trichoderma reesei sugar transporter	Tr_ST	340519623	1530	This study
Trichoderma reesei hexose transporter	Tr_HT	340515449	1617	This study
Penicillium chrysogenum sugar transporter	Pc_ST	255936523	1644	This study
Thielavia terrestris sugar transporter	Tt_ST	367053195	1671	This study
Neurospora crassa β-glucosidase	Nc_GH1-1	85078541	1431	Ha et al. (2011)
Penicillium chrysogenum hypothetical protein	Pc_BG	255941825	1452	This study
Thielavia terrestris glycoside hydrolase family 1 protein	Tt_BG	367050955	1431	This study

transporter/intracellular β-glucosidase system when both systems were introduced to *S. cerevisiae* (Ha et al., 2012).

In co-fermentation of cellobiose and xylose by engineered *S. cerevisiae* expressing both cellobiose transporter and β-glucosidase, individual sugars are transported through independent transporters which enables cellobiose and xylose to be simultaneously transported in contrast to the case of glucose and xylose co-fermentation. Subsequently, cellobiose is hydrolyzed to two glucose molecules in the cytosol, while not disturbing xylose transport (Ha et al., 2011; Li et al., 2010). Despite the advantages of cellobiose as a co-substrate in ethanol fermentation from lignocellulosic biomass, only a limited number of sugar transporters have been verified to confer a cellobiose transporting ability to *S. cerevisiae*. One of them is the lactose permease from *Kluyveromyces lactis* (Sadie et al., 2011) and the others are the cellodextrin transporters from *N. crassa* (Galazka et al., 2010) and *Scheffersomyces stipitis* (Ha et al., 2013). So far, engineered strains expressing a cellodextrin transporter encoded by *cdt-1* and a β-glucosidase encoded by *gh1-1* from *N. crassa* are known to be the most efficient in terms of cellobiose consumption and ethanol production (Ha et al., 2012, 2013; Sadie et al., 2011). During cellobiose fermentation, a substantial amount of cellodextrin accumulated presumably due to a transglycosylation activity of *N. crassa* β-glucosidase, which might lead to low ethanol yield and productivity from cellobiose (Ha et al., 2011). Therefore, it is necessary to discover novel cellobiose transporters and β-glucosidases for improved utilization of cellobiose derived from lignocellulosic biomass.

The present study explored novel cellodextrin transporters and intracellular β-glucosidases from diverse cellulolytic fungi in order to achieve efficient and rapid fermentation of cellobiose by engineered yeast. To this end, we excluded some fungi including *Aspergillus* species that are known to secrete an enormous amount of β-glucosidases, considering that cellodextrin transporters may not exist in these microorganisms or lose their functionality due to the lack of evolutionary advantages of harboring cellobiose transporters (Arber, 2000). Candidates were selected among putative cellodextrin transporters from *Trichoderma reesei*, *Penicillium chrysogenum* and *Thielavia terrestris* that are known to show remarkable lignocellulosic biomass decomposition ability. *T. reesei* is an industrially important cellulolytic fungus that has a capability to secrete a large quantity of cellulases and hemicellulases but displays limited β-glucosidase activity. *P. chrysogenum* is a well-characterized penicillin producer, which also possesses a broad ability to degrade lignocellulosic biomass (Jami et al., 2010). Although *Penicillium* species are closely related to *Aspergillus* species, they secrete a relatively small amount of β-glucosidases. *T. terrestris* is a novel thermophilic fungus that is phylogenetically related to *N. crassa*. Cellulases and hemicellulases from *T. terrestris* have been shown to exhibit superior catalytic characteristics and stability (Berka et al., 2011). The candidate genes coding for cellodextrin transporters and β-glucosidases were selected based on the amino acid sequence homology with *N. crassa* CDT-1 and *N. crassa* GH1-1 (Table 1), and the genome sequences of the fungi were obtained from the database of the JGI genome project (http://genome.jgi-psf.org/). Next, the effect of different combinations of the cellodextrin transporters and the β-glucosidases on cellobiose utilization ability was investigated based on the fact that cellodextrin transporters could be inhibited by cellotriose and cellotetraose (Galazka et al., 2010), which implies that β-glucosidase activities could affect cellobiose uptake.

Finally, among various combinations evaluated for ethanol production from cellobiose, it was confirmed that *S. cerevisiae* Pc_ST/Tt_BG utilized cellobiose more efficiently than *S. cerevisiae* Nc_CDT-1/Nc_GH1-1.

2. Materials and methods

2.1. Stains and plasmids

Escherichia coli TOP10 (Invitrogen, Carlsbad, CA, USA) and *S. cerevisiae* D452-2 (Nikawa et al., 1991) were used for gene cloning and cellobiose fermentation, respectively. Plasmids p423GPD (2 μm plasmid, *HIS3* marker) and p425GPD (2 μm plasmid, *LEU2* marker) (Mumberg et al., 1995) were used to express the genes encoding putative cellodextrin transporters and β-glucosidases under the regulation of the constitutive GPD promoter. The strains and plasmids used in this study are listed in Table 2.

2.2. Genetic manipulation

To acquire coding DNA sequences (CDS) encoding fungal cellodextrin transporters and β-glucosidases, total RNAs were extracted from *N. crassa*, *T. reesei*, *P. chrysogenum*, *T. terrestris* with an RNeasy mini kit (QIAGEN Inc., Valencia, CA, USA). cDNAs were synthesized with a RevertAid Premium First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada). Each gene was amplified with the primers listed in Supplementary data 1 with the corresponding cDNA as the template. After amplified with PCR, each gene was treated with the restriction enzymes as indicated in its primer names. For expression of cellodextrin transporter genes, p423GPD was used for cloning genes coding for cellodextrin transporters of *N. crassa* (Nc_CDT-1), *T. reesei* (Tr_ST, Tr_HT), *P. chrysogenum* (Pc_ST) and *T. terrestris* (Tt_ST). For cloning and expression of genes for β-glucosidase, p425GPD was used. The genes of Nc_GH1-1 (*N. crassa*), Pc_BG (*P. chrysogenum*), and Tt_BG (*T. terrestris*) were cloned by the same way for cellodextrin transporters. Plasmids were transformed into *S. cerevisiae* with an EZ-yeast transformation kit (MP Biomedicals, Solon, OH, USA). A minimal medium (6.7 g/L Yeast Nitrogen Base without amino acids and 20 g/L glucose) without histidine and leucine was used to select the transformants containing both the cellodextrin transporter gene and the β-glucosidase gene cloned in p423GPD and p425GPD, respectively.

2.3. Culture conditions

LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl) containing 50 μg/mL ampicillin was used for engineered *E. coli* cultivation. YEPC medium (10 g/L yeast extract, 20 g/L bacto-peptone,

Table 2
Strains and plasmids used in this study.

Strains	Features	Relevant work
S. cerevisiae D452-2	*Matα, leu2 his3 ura3 can1*	Nikawa et al. (1991)
S. cerevisiae Nc_CDT-1/Nc_GH1-1	D452-2/p423GPD-Nc_CDT-1/p425GPD-Nc_GH1-1	Ha et al. (2011)
S. cerevisiae Tr_ST/Nc_GH1-1	D452-2/p423GPD-Tr_ST/p425GPD-Nc_GH1-1	This study
S. cerevisiae Tr_HT/Nc_GH1-1	D452-2/p423GPD-Tr_HT/p425GPD-Nc_GH1-1	This study
S. cerevisiae Pc_ST/Nc_GH1-1	D452-2/p423GPD-Pc_ST/p425GPD-Nc_GH1-1	This study
S. cerevisiae Tr_ST/Nc_GH1-1	D452-2/p423GPD-Tr_ST/p425GPD-Nc_GH1-1	This study
S. cerevisiae Nc_CDT-1/Pc_BG	D452-2/p423GPD-Nc_CDT-1/p425GPD-Pc_BG	This study
S. cerevisiae Nc_CDT-1/Tt_BG	D452-2/p423GPD-Nc_CDT-1/p425GPD-Tt_BG	This study
S. cerevisiae Pc_ST/Pc_BG	D452-2/p423GPD-Pc_ST/p425GPD-Pc_BG	This study
S. cerevisiae Pc_ST/Tt_BG	D452-2/p423GPD-Pc_ST/p425GPD-Tt_BG	This study

20 g/L cellobiose) was used to cultivate and induce genes related to cellobiose utilization in *N. crassa*, *T. reesei*, *P. chrysogenum* and *T. terrestris*. YNBD medium (6.7 g/L Yeast Nitrogen Base without amino acids, 1.4 g/L of yeast synthetic drop-out medium supplements with appropriate amino acids (85 mg/L, whereas leucine was added at 170 mg/L), 20 g/L glucose) was used for selection of yeast strains. Yeast extract, tryptone and bacto-peptone were purchased from Becton Dickinson (Sparks, MD, USA) and the other ingredients were obtained from Sigma (St. Louis, MO, USA).

2.3.1. Flask culture

Flask culture was performed in a 250 mL flask with a working volume of 50 mL in YEPC medium (10 g/L yeast extract, 20 g/L bacto-peptone, 40 g/L cellobiose) at 30 °C, 80 rpm in a shaking incubator (Vision, Korea).

2.3.2. Batch fermentation

The glycerol stock of the engineered *S. cerevisiae* was transferred to a test tube containing YNBD selection medium and incubated overnight at 30 °C, 250 rpm in a shaking incubator. Preculture was performed in a 500 mL baffled flask with a working volume of 100 mL at 30 °C, 250 rpm in YNBD selection medium. The inocula for batch fermentations were prepared by growing the cells around OD_{600} = 10. The cells were harvested by centrifugation at 3000 rpm for 5 min and washed in sterilized water. The washed cells were transferred to a fermentor to have an initial OD_{600} of approximately 1.0 after inoculation. Batch fermentation was performed in YEPC medium (10 g/L yeast extract, 20 g/L bacto-peptone, 40 g/L cellobiose) in a bench-top 1 L multi-fermentor (KoBioTech, Korea) with a working volume of 500 mL. The pH of the medium was automatically controlled at pH 5.5 by addition of 1 N HCl and 1 N NaOH and the temperature was maintained at 30 °C throughout the batch fermentation. The fermentation was performed under microaerobic condition at 0.3 vvm aeration and 200 rpm.

2.4. Activity assay

The β-glucosidase activity was determined by a modification of the procedure described previously (Wood and Bhat, 1988). All reagents were purchased from Sigma (St. Louis, MO, USA). In order to prepare the sample solutions, the cells were harvested and adjusted to OD_{600} of 10 in 1 mL and washed twice with distilled water, and then treated with Y-PER solution (Thermo scientific, Rockford, IL, USA) for 20 min. To prevent degradation of the proteins during sample preparation, the protease inhibitor cocktail (Roche Diagnostics Ltd, Mannheim, German) was added. Cell debris was removed by centrifugation at 13,000 rpm for 10 min at 4 °C. The supernatant was used for assay of intracellular β-glucosidase activity. To determine β-glucosidase activity in the medium during the fermentation, the samples were obtained from the broth after removing the cell pellet. The β-glucosidase activity was measured using a 96-well microplate. Each well was supplied with reaction mixtures of 0.5 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) dissolved in 50 mM potassium phosphate buffer (pH 6.0) by mixing 100 μL of 100 mM potassium phosphate buffer (pH 6.0), 20 μL of 5 mM *p*-NPG, and 80 μL of the sample solution collected by the above procedure. Then, the β-glucosidase activities were determined by measuring the changes of optical density at 405 nm with the microplate reader (VERSAmax, Molecular Device Co., CA, USA) to quantify the release of *p*-nitrophenol (*p*-NP) from *p*-NPG for 1 h at 30 °C. The experiments were carried out in triplicate.

One unit of β-glucosidase activity was defined as the amount of the enzyme required to release 1 μmol of *p*-NP per minute from *p*-NPG in 50 mM potassium phosphate buffer (pH 6.0) at 30 °C. The specific activity of the sample solution was determined by dividing the enzyme activity of the sample solution added to the reaction mixture (unit/mL) by the protein concentration (mg/mL).

2.5. Analysis

Dry cell weight was calculated by measuring the optical density of the culture broth after appropriate dilution with a spectrophotometer (Ultrospec 2000, Amersham Phamacia Biotech, Uppsala, Sweden) at 600 nm and by applying a conversion factor of 0.3 g dry cell weight per liter with OD_{600} = 1 (Oh et al., 2012). Concentrations of sugars, alcohols and acids in culture broth were determined by HPLC (1100LC, Agilent, Santa Clara, CA, USA) equipped with an RI detector. The components in the sample were separated by a carbohydrate analysis column (Rezex ROA-organic acid, Phenomenex, Rancho Palos Verdes, CA, USA) heated at 60 °C. 5 mM H_2SO_4 was used as mobile phase at the rate of 0.6 mL/min. Protein concentrations were determined by a protein assay kit (Biorad Laboratories, Hercules, CA, USA).

3. Results

3.1. Exploration of novel cellodextrin transporter from cellulolytic fungi

In order to discover more fungal cellodextrin transporters other than Nc_CDT-1 encoded by *N. crassa cdt-1*, several candidate genes which might code for a cellodextrin transporter were identified from genome sequences of cellulolytic fungi. The candidate genes were selected based on amino acid sequence similarity to Nc_CDT-1 using the protein database at the National Center for Biotechnology Information (NCBI) linked to the protein blast program (pblast). Heterologous proteins such as Tr_ST (EGR49861) from *T. reesei*, Tr_HT (EGR45703) from *T. reesei*, Pc_ST (XP_002559288) from *P. chrysogenum*, and Tt_ST (XP_003656976) from *T. terrestris* were expressed in *S. cerevisiae* (Table 1). Interestingly, most candidate genes were annotated as hypothetical lactose permease in the results of pblast analysis, which is plausible in that many transporters exhibit substrate ambiguity. For example, *E. coli* uses the same transporter to take up cellobiose and lactose. Cellobiose and

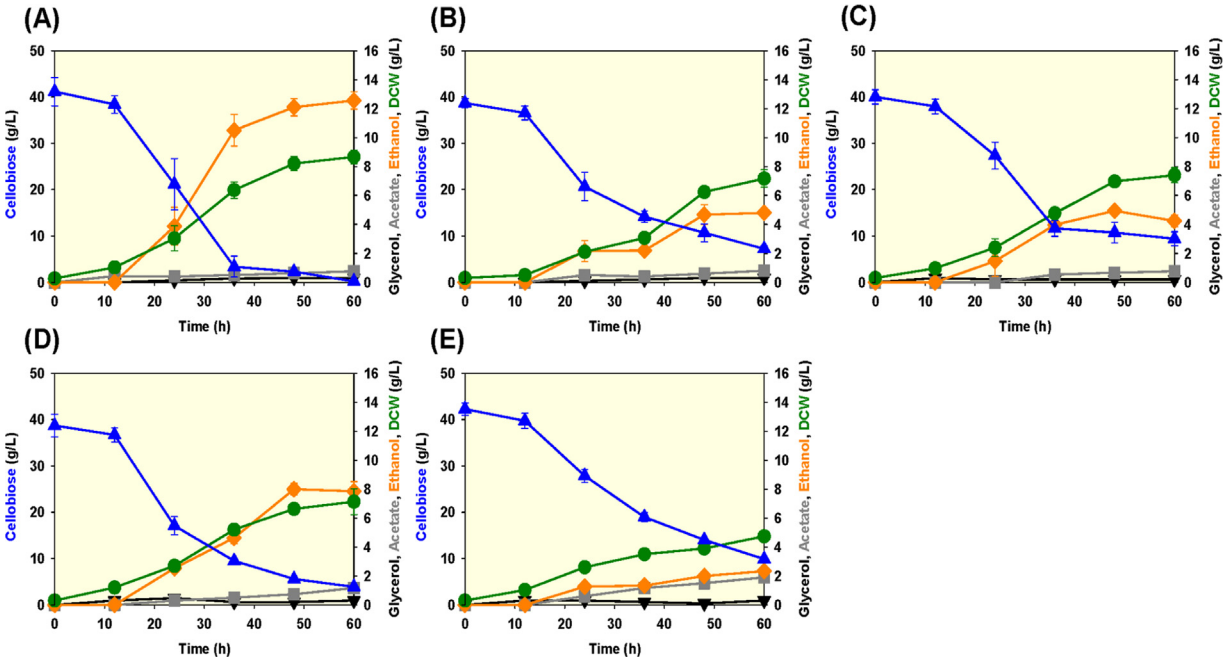

Fig. 1. Profiles of engineered *S. cerevisiae* D452-2 strains expressing *N. crassa* β-glucosidases and various cellodextrin transporters; Nc_CDT-1 and Nc_GH1-1 (A), Tr_ST and Nc_GH1-1 (B), Tr_HT and Nc_GH1-1 (C), Pc_ST and Nc_GH1-1 (D), Tt_ST and Nc_GH1-1 in YEPC (YP and 40 g/L cellobiose) medium at 30 °C, 80 rpm. Symbols denote as follows; blue triangle, cellobiose concentration (g/L); orange diamond, ethanol concentration (g/L); green circle, dry cell weight (g/L); gray square, acetate concentration (g/L); black inverted triangle, glycerol concentration (g/L). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

lactose are disaccharides whose monomeric units are connected by a β-(1 → 4) bond and differ only in the position of the hydroxyl group on C4 of galactose or glucose of which the hydroxyl group is up in galactose and down in glucose.

To utilize cellobiose after uptake into the cells, intracellular β-glucosidase should be introduced. Therefore, plasmids containing each candidate gene of cellodextrin transporter and plasmids carrying *N. crassa* β-glucosidase, namely p423GPD-Nc_GH1-1, were co-transformed. As a result, engineered strains of *S. cerevisiae* Tr_ST/Nc_GH1-1, Tr_HT/Nc_GH1-1, Pc_ST/Nc_GH1-1, Tt_ST/Nc_GH1-1 were constructed along with the control strain *S. cerevisiae* Nc_CDT-1/Nc_GH1-1 (Table 2). For comparison of cellobiose fermentation capabilities of engineered *S. cerevisiae* strains expressing different cellobiose transporters, flask cultures were performed in YEPC medium (Fig. 1 and Table 3).

Unexpectedly, a long lag phase was observed in all strains before starting to consume cellobiose, although a constitutive promoter (GPD promoter) was used to express both cellodextrin transporters and β-glucosidase. An additional experiment showed that the lag time could be shortened by culturing the engineered strains in YEPC medium before the flask culture (data not shown), although the reason has to be further studied.

Among the strains containing the cellodextrin transporter other than Nc_CDT-1, *S. cerevisiae* Pc_ST/Nc_GH1-1 showed an ethanol fermentation performance comparable with *S. cerevisiae* Nc_CDT-1/Nc_GH1-1. However, substantial amounts of cellobiose remained unused even after 60 h of fermentation (Fig. 1). Although the superior strain to *S. cerevisiae* Nc_CDT-1/Nc_GH1-1 could not be found in combination with Nc_GH1-1, it would be worth investigating other intracellular β-glucosidases, considering the possibility that Pc_ST is more vulnerable to the accumulated cellodextrin supposedly resulted from insufficient β-glucosidase activity of Nc_GH1-1.

3.2. Investigation on novel intracellular β-glucosidases from cellulolytic fungi

To discover novel β-glucosidases to further improve the cellobiose utilization ability of the engineered *S. cerevisiae* expressing the cellodextrin transporter gene (*Pc_ST*) from *P. chrysogenum*, putative β-glucosidase genes were obtained from cDNA of *P. chrysogenum* from which Pc_ST was also originated, and *T. terrestris* which is renowned for its extraordinary capability to degrade plant biomass. By analyzing with the pblast program, Pc_BG (XM_002561636) and Tt_BG (XM_003655809) encoding genes were selected based on the protein homology with Nc_GH1-1 from the corresponding hosts. The genes were amplified from the cDNAs after cellobiose induction in YEPC medium, because both of the genes coding for putative β-glucosidases and cellodextrin

Table 3
Summary of cellobiose fermentation performances of *S. cerevisiae* Nc_CDT-1/Nc_GH1-1, Tr_ST/Nc_GH1-1, Tr_HT/Nc_GH1-1, Pc_ST/Nc_GH1-1, and Tt_ST/Nc_GH1-1 strains.

Strains	Dry cell weight (g/L)[b]	Cellobiose consumption rate (g/L h)[a]	Final ethanol concentration (g/L)[b]	Ethanol yield (g/g cellobiose)[b]	Productivity (g/L h)[b]
Nc_CDT-1/Nc_GH1-1	8.2 ± 0.5	1.00 ± 0.05	12.1 ± 0.6	0.31 ± 0.01	0.25 ± 0.01
Tr_ST/Nc_GH1-1	6.2 ± 0.4	0.72 ± 0.08	4.7 ± 0.7	0.17 ± 0.01	0.10 ± 0.01
Tr_HT/Nc_GH1-1	7.0 ± 0.4	0.76 ± 0.03	4.9 ± 0.3	0.17 ± 0.02	0.10 ± 0.01
Pc_ST/Nc_GH1-1	6.6 ± 0.4	0.86 ± 0.04	8.0 ± 0.4	0.24 ± 0.01	0.17 ± 0.01
Tt_ST/Nc_GH1-1	3.9 ± 0.2	0.71 ± 0.04	2.0 ± 0.3	0.07 ± 0.01	0.04 ± 0.01

Each value is the average of three replicates ± standard deviation.
[a] The cellobiose consumption rates were calculated according to the data obtained from 12 h to 48 h.
[b] The values for dry cell weight, final ethanol concentration, ethanol yield, and productivity were calculated based on 48 h fermentation time.

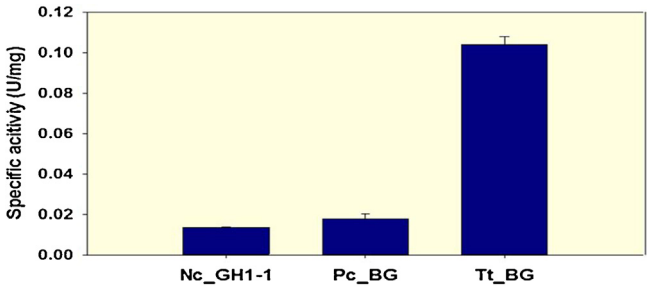

Fig. 2. Specific enzyme activities of β-glucosidases from *N. crassa*, *P. chrysogenum* and *T. terrestris*. The experiments were repeated in triplicate. Values are the mean of three replicates. Error bars show the standard deviation from the mean value.

transporters were reported to contain introns and translated after splicing only in presence of cellobiose. Each β-glucosidase gene was cloned in p425GPD and co-transformed with p423GPD-Nc_CDT-1 into *S. cerevisiae* D452-2.

After introducing Pc_BG and Tt_BG into *S. cerevisiae*, intracellular β-glucosidases activities were measured with the samples from Y-PER treated cell lysates. The extracellular activities were also checked in the medium to eliminate the possibility of cellobiose hydrolysis by secreted β-glucosidases. The *S. cerevisiae* strains showed negligible extracellular β-glucosidase activities. In addition, the strains expressing only β-glucosidases, but not cellodextrin transporters, could not grow on cellobiose as a sole carbon source (data not shown).

Among three β-glucosidases tested in this study, the β-glucosidase from *T. terrestris* (Tt_BG) showed the highest activity that was 10 times higher than that of *N. crassa* β-glucosidase (Nc_GH1-1). The activity of the β-glucosidase from *P. chrysogenum* (Pc_BG) was similar to that of Nc_GH1-1 (Fig. 2).

3.3. Different combinations of cellodextrin transporters and β-glucosidases

As mentioned above, cellobiose transport could be inhibited by cellodextrin. Moreover, it was proved that the intracellular activity to hydrolyze cellobiose is closely related to cellobiose consumption ability (Ha et al., 2012). Therefore, introduction of different β-glucosidases into engineered *S. cerevisiae* may exert an influence on cellobiose-fermenting ability. To investigate the effect of different combinations of the cellodextrin transporters and β-glucosidases, six engineered *S. cerevisiae* strains expressing cellobiose transporters and β-glucosidases with combinations of two cellobiose transporters (Nc_CDT-1 and Pc_ST) and three β-glucosidases (NC_GH1-1, Pc_BG and Tt_BG) were evaluated. Mostly, the engineered strains expressing Nc_CDT-1 exhibited higher final ethanol concentrations as well as cellobiose consumption rates than the strains harboring Pc_ST (Fig. 3). However, the *S. cerevisiae* Pc_ST/Tt_BG strain showed the best performance among six strains. Moreover, the *S. cerevisiae* Pc_ST/Tt_BG could consume all cellobiose in the medium.

The *S. cerevisiae* Pc_ST/Tt_BG strain showed a similar cellobiose consumption rate of 1.02 ± 0.06 g/L·h to the strains harboring Nc_CDT-1 (0.98–1.03 g/L h) but resulted in higher final ethanol concentration of 14.6 ± 0.5 g/L and ethanol yield of 0.38 ± 0.01 g ethanol/g cellobiose, whereas *S. cerevisiae* Nc_CDT-1/Nc_GH1-1 gave 12.4 ± 0.4 g/L final ethanol concentration with yield of 0.33 ± 0.01 g ethanol/g cellobiose. The results are summarized in Table 4.

3.4. Comparison of S. cerevisiae Nc_CDT-1/Nc_GH1-1 and S. cerevisiae Pc_ST/Tt_BG strains

To confirm the improved cellobiose fermentation performance of *S. cerevisiae* Pc_ST/Tt_BG compared to *S. cerevisiae* Nc_CDT-1/Nc_GH1-1, microaerobic batch fermentations using a bioreactor were conducted in YEPC media containing 40 g/L of cellobiose. During 60 h culture, both strains consumed all cellobiose in the medium. As in the flask cultures, the strain expressing both Pc_ST and Tt_BG showed higher productivity and final ethanol concentration as well as ethanol yield compared to the *S. cerevisiae* Nc_CDT-1/Nc_GH1-1 strain (Fig. 4 and Table 5). Cellodextrins including cellotetraose and cellotriose were formed from cellobiose by the transglycosylation activity of the β-glucosidases (Supplementary data 2), and the higher final ethanol concentration could be explained by the merged HPLC profiles of the samples taken at the end of the fermentation (Fig. 5). In the culture broth of *S. cerevisiae* Pc_ST/Tt_BG, reduced amounts of cellodextrin were detected, which could elucidate the reason for the increase in a final ethanol concentration and ethanol yield in *S. cerevisiae* Pc_ST/Tt_BG. The batch fermentation of *S. cerevisiae* Pc_ST/Tt_BG resulted in 14.5 ± 0.5 g/L of final ethanol concentration with a yield of 0.37 ± 0.01 g ethanol/g cellobiose and productivity of 0.30 ± 0.01 g/L·h, while the *S. cerevisiae* Nc_CDT-1/Nc_GH1-1 strain resulted in 12.5 ± 0.4 g/L of final ethanol concentration with a yield of 0.31 ± 0.03 g ethanol/g cellobiose and productivity of 0.26 ± 0.01 g/L h.

4. Discussion

Economic viability is an important issue for commercial production of lignocellulosic bioethanol. Hence, efficient utilization of pentoses (primarily xylose) is one of the significant milestones to achieve economical processes. Because *S. cerevisiae* lacks specific xylose transporters, engineered *S. cerevisiae* harboring xylose metabolizing enzymes could take up xylose only after glucose concentration was below 4 g/L (Krahulec et al., 2010). As a result, several metabolic problems with regard to cofactor-imbalance (Lee et al., 2012) and energy deficiency (Bergdahl et al., 2012; Kim et al., 2013) would occur during co-fermentation of glucose and xylose. In contrast, cellobiose could serve as a beneficial co-substrate for xylose utilization, although heterologous genes coding for a cellodextrin transporter and β-glucosidase should be introduced to endow *S. cerevisiae* with the ability to utilize cellobiose. Extracellular cellobiose did not interfere with xylose transport and the intracellular glucose produced from cellobiose hydrolysis by intracellular β-glucosidase could supply NADPH and ATP, while maintaining glucose signaling involved in rapid cell growth and metabolism (Zaman et al., 2008). But as shown in previous reports on cellobiose utilization by the engineered *S. cerevisiae*, direct cellobiose fermentation by engineered yeast has not yet been optimized. Ethanol yields and productivities from cellobiose are still lower than those from glucose partially because of cellodextrin accumulation (Ha et al., 2011).

In this study, efficient and rapid cellobiose utilization was achieved through discovering novel cellodextrin transporters and β-glucosidases and finding the optimal combination. New cellodextrin transporters were selected from among sugar transporters of *T. reesei* (Tr_ST, Tr_HT), *P. chrysogenum* (Pc_ST), *T. terrestris* (Tt_ST) based on high protein similarity to *N. crassa* CDT coded by *cdt-1*. Among the candidates, the strain expressing Pc_ST resulted in a comparable cellobiose consumption rate compared with the strain containing Nc_CDT-1. However, the combination of Pc_ST and Nc_GH1-1 showed still lower cellobiose utilization rates and final ethanol concentration as compared with the combination of Nc_CDT-1 and Nc_GH1-1. But it was hypothesized that cellobiose transporting ability might be underestimated because of

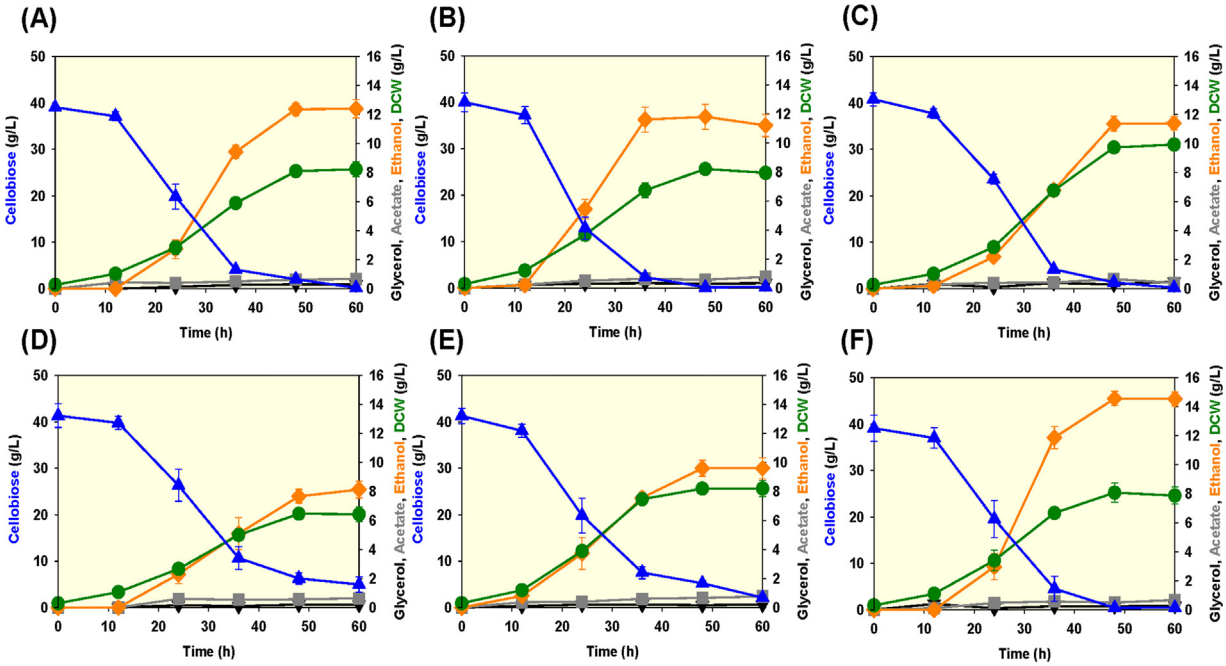

Fig. 3. Profiles of engineered *S. cerevisiae* D452-2 strains containing different combinations of cellodextrin transporters and β-glucosidases; Nc_CDT-1 and Nc_GH1-1 (A), NC_CDT-1 and Pc_BG (B), Nc_CDT-1 and Tt_BG (C), Pc_ST and Nc_GH1-1 (D), Pc_ST and Pc_BG (E), Pc_ST and Tt_BG (F) in YEPC (YP and 40 g/L cellobiose) medium at 30 °C, 80 rpm. Symbols denote as follows; blue triangle, cellobiose concentration (g/L); orange diamond, ethanol concentration (g/L); green circle, dry cell weight (g/L); gray square, acetate concentration (g/L); black inverted triangle, glycerol concentration (g/L). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 4
Cellobiose fermentation performances of *S. cerevisiae* Nc_CDT-1/Nc_GH1-1, Nc_CDT-1/Pc_BG, Nc_CDT-1/Tt_BG, Pc_ST/Nc_GH1-1, Pc_ST/Pc_BG, and Pc_ST/Tt_BG strains.

Strains	Dry cell weight (g/L)[b]	Cellobiose consumption rate (g/L h)[a]	Final ethanol concentration (g/L)b	Ethanol yield (g/g cellobiose)[b]	Productivity (g/L h)[b]
Nc_CDT-1/Nc_GH1-1	8.1 ± 0.3	0.98 ± 0.02	12.4 ± 0.4	0.32 ± 0.01	0.26 ± 0.01
Nc_CDT-1/Pc_BG	8.2 ± 0.3	1.03 ± 0.05	11.8 ± 0.9	0.30 ± 0.03	0.25 ± 0.02
Nc_CDT-1/Tt_BG	9.7 ± 0.4	1.01 ± 0.03	11.4 ± 0.5	0.29 ± 0.01	0.24 ± 0.01
Pc_ST/Nc_GH1-1	6.5 ± 0.3	0.93 ± 0.07	7.7 ± 0.5	0.22 ± 0.01	0.16 ± 0.01
Pc_ST/Pc_BG	8.2 ± 0.4	0.91 ± 0.03	9.6 ± 0.6	0.26 ± 0.01	0.20 ± 0.01
Pc_ST/Tt_BG	8.1 ± 0.7	1.02 ± 0.06	14.6 ± 0.5	0.38 ± 0.01	0.30 ± 0.01

Each value is the average of three replicates ± standard deviation.
[a] The cellobiose consumption rates were calculated according to the data obtained from 12 h to 48 h.
[b] The values for dry cell weight, final ethanol concentration, ethanol yield, and productivity were calculated based on 48 h fermentation time.

the cellodextrin accumulated in the medium. Therefore, in order to find other intracellular β-glucosidases of high enzymatic activity, two candidate genes encoding putative β-glucosidases were newly cloned from *P. chrysogenum* (*Pc_BG*) and *T. terristeris* (*Tt_BG*). Each Nc_CDT-1 and Pc_ST was co-expressed with a combination of Nc_GH1-1, Pc_BG or Tt_BG, respectively. Among the six engineered strains, the Pc_ST/Tt_BG combination resulted in the best performance in terms of the final ethanol concentration and ethanol yield. *S. cerevisiae* Pc_ST/Pc_BG carrying proteins from the homologous source of *P. chrysogenum* showed a similar performance to

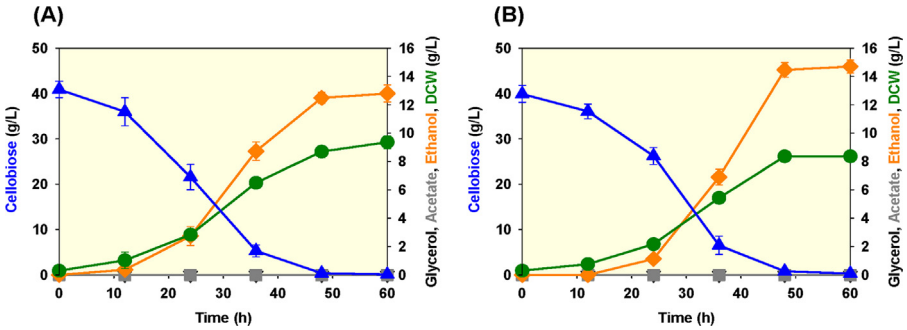

Fig. 4. Batch fermentations profiles of engineered *S. cerevisiae* Nc_CDT-1/Nc_GH1-1 (A), *S. cerevisiae* Pc_ST/Tt_BG (B) in YEPC (YP and 40 g/L cellobiose) medium at 30 °C, 0.3 vvm aeration and 200 rpm. Symbols denote as follows; blue triangle, cellobiose concentration (g/L); orange diamond, ethanol concentration (g/L); green circle, dry cell weight (g/L); gray square, acetate concentration (g/L); black inverted triangle, glycerol concentration (g/L). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 5
Batch fermentation performances of *S. cerevisiae* Nc_CDT-1/Nc_GH1-1 and Pc_ST/Tt_BG strains.

Strains	Dry cell weight (g/L)[b]	Cellobiose consumption rate (g/L h)[a]	Final ethanol concentration (g/L)[b]	Ethanol yield (g/g cellobiose)[b]	Productivity (g/L h)[b]
Nc_CDT-1/Nc_GH1-1	8.7 ± 0.4	0.99 ± 0.08	12.5 ± 0.4	0.31 ± 0.03	0.26 ± 0.01
Pc_ST/Tt_BG	8.4 ± 0.3	0.98 ± 0.04	14.5 ± 0.5	0.37 ± 0.01	0.30 ± 0.01

Each value is the average of three replicates ± standard deviation.
[a] The cellobiose consumption rates were calculated according to the data obtained from 12 h to 48 h.
[b] The values for dry cell weight, final ethanol concentration, ethanol yield, and productivity were calculated based on 48 h fermentation time.

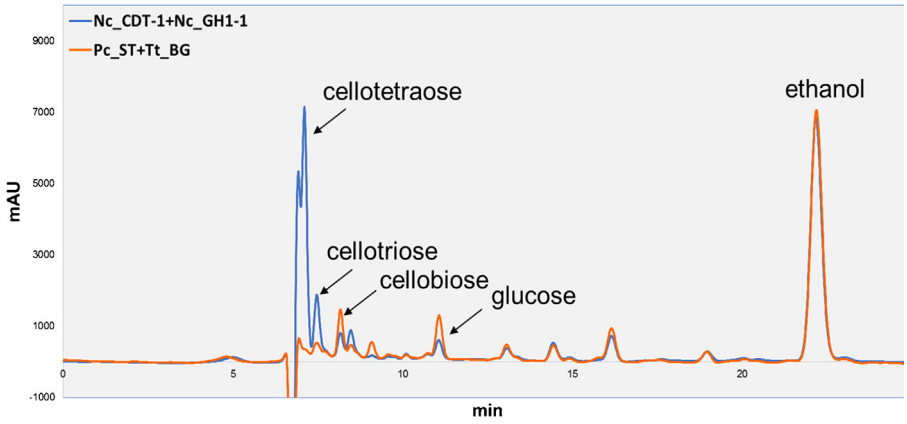

Fig. 5. HPLC chromatogram of the samples taken after the batch fermentation presenting cellotriose and cellotetraose during cellobiose fermentation.

Pc_ST/Nc_GH1-1 combination. In case of the strains expressing a cellodextrin transporter from *P. chrysogenum*, it seems that the β-glucosidase activities are closely related to cellobiose utilization. The increased final ethanol concentration and ethanol yield could partly be explained by the high activity of Tt_BG, but the reason of the different results of Nc_CDT-1/Tt_BG and Pc_ST/Tt_BG systems remains unsettled. It would be worthwhile to verify the intrinsic cellobiose uptake capacity of each cellodextrin transporter as well as the susceptibility to accumulated cellodextrin.

The expression of Pc_ST and Tt_BG in engineered *S. cerevisiae* led to the remarkable reduction of cellodextrin accumulation and ultimately resulted in an increase in ethanol yield by 27% and final ethanol concentration by 22%. It is expected that the improvement of the cellobiose fermenting ability of a *S. cerevisiae* system with a novel combination of a cellodextrin transporter and β-glucosidase could facilitate economic production of lignocellulosic bioethanol.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2013.10.030.

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