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Evaluation of Ethanol Production Activity by Engineered *Saccharomyces cerevisiae* Fermenting Cellobiose through the Phosphorolytic Pathway in Simultaneous Saccharification and Fermentation of Cellulose

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In simultaneous saccharification and fermentation (SSF) for production of cellulosic biofuels, engineered *Saccharomyces cerevisiae* capable of fermenting cellobiose has provided several benefits, such as lower enzyme costs and faster fermentation rate compared with wild-type *S. cerevisiae* fermenting glucose. In this study, the effects of an alternative intracellular cellobiose utilization pathway—a phosphorolytic pathway based on a mutant cellodextrin transporter (CDT-1 (F213L)) and cellobiose phosphorylase (SdCBP)—was investigated by comparing with a hydrolytic pathway based on the same transporter and an intracellular β-glucosidase (GH1-1) for their SSF performances under various conditions. Whereas the phosphorolytic and hydrolytic cellobiose-fermenting *S. cerevisiae* strains performed similarly under the anoxic SSF conditions, the hydrolytic *S. cerevisiae* performed slightly better than the phosphorolytic *S. cerevisiae* under the microaerobic SSF conditions. Nonetheless, the phosphorolytic *S. cerevisiae* expressing the mutant CDT-1 showed better ethanol production than the glucose-fermenting *S. cerevisiae* with an extracellular β-glucosidase, regardless of SSF conditions. These results clearly prove that introduction of the intracellular cellobiose metabolic pathway into yeast can be effective on cellulosic ethanol production in SSF. They also demonstrate that enhancement of cellobiose transport activity in engineered yeast is the most important factor affecting the efficiency of SSF of cellulose.

Keywords: Cellulosic ethanol, simultaneous saccharification and fermentation, cellobiose transporter, cellobiose phosphorylase, engineered *Saccharomyces cerevisiae*

Introduction

To achieve economic production of ethanol from cellulosic biomass, fermentation of various intermediate sugars (*e.g.*, cellobiose and cellodextrins) released during cellulose hydrolysis is important, which suggests that *Saccharomyces cerevisiae*—the most suitable host for ethanol fermentation—should be engineered because it cannot metabolize intermediate sugars without modification of sugar metabolism [1–3]. For instance, engineering *S. cerevisiae* to utilize intracellular cellobiose has been revealed as an efficient strategy for cellulosic ethanol production because it can lead to simultaneous fermentation of mixed sugars (*e.g.*, xylose and cellobiose) without glucose repression [1–5]. Moreover, intracellular utilization of cellobiose by engineered *S. cerevisiae* can reduce enzyme costs for the simultaneous saccharification and fermentation (SSF) of cellulosic biomass, because supplementation of extracellular β-glucosidase is not necessary when compared with the traditional SSF process employing non-engineered *S. cerevisiae* supplied with extracellular β-glucosidase [4, 6, 7].

Until now, two different intracellular cellobiose metabolic pathways—a hydrolytic pathway using intracellular β-glucosidase (GH1-1 from *Neurospora crassa*) with various

cellobiose transporters (CDT-1 from *N. crassa*, CltA from *Aspergillus nidulans*, Stp1 from *Trichoderma reesei*, and HXT2.4 from *Pichia stipitis*, *etc.*), and a phosphorolytic pathway using intracellular cellobiose phosphorylases (SdCBP from *Saccharophagus degradans* and CepA from *Clostridium stercorarium*, *etc.*) with several sugar transporters (CDT-1 from *N. crassa* and Lac12 from *Kluyveromyces lactis*, *etc.*)—have been introduced into *S. cerevisiae* for successful ethanol production from cellobiose [1, 2, 8–14]. Whereas the hydrolytic pathway is known to spend 2 ATP molecules to initiate glycolysis with cellobiose (cellobiose + H_2O + 2 ATP → 2 glucose-6-phosphate + 2 ADP), the phosphorolytic pathway is known to have energetic advantages with the expense of only 1 ATP molecule (cellobiose + phosphate + ATP → 2 glucose-6-phosphate + ADP), suggesting that the phosphorolytic pathway would provide several benefits (*e.g.*, higher cell growth yield and higher ethanol yield by saving energy) compared with the hydrolytic pathway under anaerobic and stressful conditions [9, 13, 15, 16]. However, phosphorolysis of cellobiose is a thermodynamically unfavorable reaction because it has a positive Gibbs free energy change value (ΔG^o = 3.6 kJ/mol), whereas ΔG^o for the hydrolysis reaction is negative (ΔG^o = −12.5 kJ/mol) [9, 15, 16], indicating that an excess of cellobiose must be supplied to the yeast expressing cellobiose phosphorylase in order to drive the non-spontaneous phosphorolysis of cellobiose [9, 15].

Indeed, the phosphorolytic cellobiose-fermenting *S. cerevisiae* with CDT-1 and SdCBP showed significantly slower cellobiose fermentation than the hydrolytic cellobiose-fermenting *S. cerevisiae* with CDT-1 and GH1-1 [9]; however, the cellobiose fermentation rate and ethanol yield of the phosphorolytic *S. cerevisiae* were dramatically enhanced by a mutant cellobiose transporter, CDT-1 (F213L), resulting in the phosphorolytic *S. cerevisiae* expressing CDT-1 (F213L) showing similar or even better cellobiose fermentation compared with the hydrolytic *S. cerevisiae* strains expressing either CDT-1 (F213L) or CDT-1 [9]. These results clearly demonstrated that efficient ethanol production from cellobiose could be achieved by pushing the phosphorolytic reaction in the presence of an enhanced cellobiose transporter.

However, considering that the cellobiose concentration in SSF of cellulose is maintained at a much lower level than cellobiose fermentation, it is questionable whether engineered *S. cerevisiae* employing the phosphorolytic pathway can push the phosphorolysis of cellobiose and perform efficient ethanol production in SSF. This concern might be the reason why many studies have mainly focused on introducing the hydrolytic pathway to develop yeast systems producing ethanol from cellulosic biomass [1, 2, 6, 10–12, 14, 18].

In this study, we intended to examine whether similar or better ethanol production could be achieved in SSF of cellulose by the phosphorolytic *S. cerevisiae* expressing mutant CDT-1 compared with the hydrolytic *S. cerevisiae* expressing mutant CDT-1. We also intended to check whether SSF with the phosphorolytic *S. cerevisiae* expressing mutant CDT-1 could accomplish better ethanol production than the traditional SSF with non-engineered yeast and extracellular β-glucosidase.

Materials and Methods

Strains, Plasmids, and Cultivation Conditions

S. cerevisiae D452-2 (*MATα*, *leu2*, *his3*, *ura3* and *can1*) [19] was used as the host strain expressing cellobiose phosphorylase (SdCBP) from *S. degradans* along with either the wild-type cellobiose transporter (CDT-1) or the mutant cellobiose transporter (CDT-1 (F213L)) from *N. crassa* FGSC 2489 [1, 9]. The plasmids for overexpressing SdCBP, CDT-1, and CDT-1 (F213L) were constructed previously (pRS425-SdCBP, pRS426-cdt1, and pRS426-cdt1 (F213L), respectively) [1, 9]. All the strains and plasmids used in this study are listed in Table 1.

Synthetic complete medium (6.7 g/l of yeast nitrogen base without amino acids, 0.625 g/l of complete supplement mixture without leucine, tryptophan, and uracil, pH 6.0) containing 20 g/l of glucose was used for seed cultivation. Yeast extract-peptone (YP) medium (10 g/l of yeast extract, 20 g/l of Bacto peptone, pH 6.7) with 50 g/l of cellobiose was used for pre-cultivation. Yeast cells at exponential growth in the pre-cultivation were harvested and used in SSF. Seed cultivation and pre-cultivation were carried out at 30°C and 250 rpm.

Conditions for SSF of Cellulose

SSF experiments were performed with pretreated corn stover (PCS) obtained from National Renewable Resource Laboratory (NREL) and Avicel PH-101 (Sigma, USA) as the substrates. PCS was washed to reduce the inhibitory effects of fermentation inhibitors during SSF [20]. In addition, the SSF experiments were performed under two different conditions: anoxic and microaerobic. The following are the specific conditions for each SSF experiment: (i) Anoxic SSF of pretreated cellulosic biomass was carried out in 100-ml bottles containing 20 ml of YP, 10% (w/v) washed PCS, and Celluclast 1.5L (10 filter paper unit (FPU)/g glucan). Celluclast 1.5L was used as the cellulase mixture for cellulose saccharification. The initial concentration of yeast cells was adjusted to 10.5 g/l for a high cell density SSF. After inoculation of yeast cells into the bottles containing media, the bottles were tightly closed using caps equipped with a wireless gas production measurement system (Ankom Technology, USA), which only allowed CO_2 release without air intake. This prevention of air intake changes the environmental condition in the bottle from initially aerobic to

Table 1. List of plasmids and *S. cerevisiae* strains used in this study.

Plasmids and strains	Relevant features	References
Plasmids		
pRS425PGK	*LEU2*, P_{PGK}-MCS-T_{CYC}, 2 μ origin, Amp^r	[1]
pRS425-gh1-1	*LEU2*, P_{PGK}-*gh1-1*-T_{CYC}, 2 μ origin, Amp^r	[1]
pRS425-SdCPB	*LEU2*, P_{PGK}-*SdCBP*-T_{CYC}, 2 μ origin, Amp^r	[9]
pRS426PGK	*URA3*, P_{PGK}-MCS-T_{CYC}, 2 μ origin, Amp^r	[1]
pRS426-cdt1	*URA3*, P_{PGK}-*cdt1*-T_{CYC}, 2 μ origin, Amp^r	[1]
pRS426-cdt1 (F213L)	*URA3*, P_{PGK}-*cdt1* (F213L)-T_{CYC}, 2 μ origin, Amp^r	[9]
Strains		
D452-2	*MATa*, *leu2*, *his3*, *ura3* and *can1*	[19]
D-56	D452-2/pRS425PGK /pRS426PGK	[17]
D-56+188	D-56 with extracellular β-glucosidase	[17]
D-BTm	D452-2/pRS425-gh1-1/pRS426-cdt1 (F213L)	[9]
D-CTw	D452-2/pRS425-SdCBP/pRS426-cdt1	[9]
D-CTm	D452-2/pRS425-SdCBP/pRS426-cdt1 (F213L)	[9]

finally anaerobic (called anoxic condition). Since CO_2 formation by yeast cells can be directly related to ethanol production under anoxic condition, CO_2 production was continuously monitored by measuring the accumulation of gas pressure [17, 21]. The temperature and agitation speed were maintained at 30°C and 100 rpm, respectively. At the end of SSF, samples were collected to analyze the residual sugars and ethanol; (ii) Anoxic SSF of pure cellulose was performed in 100-ml bottles containing 20 ml of YP, 13% (w/v) Avicel PH-101, and Celluclast 1.5L (10 FPU/g cellulose). After inoculating yeast cells at the final concentration of 10.5 g/l, the bottles were tightly closed with caps containing the gas-measuring module for SSF to be carried out under anoxic conditions. The temperature and agitation speed were maintained at 30°C and 100 rpm, respectively. At the end of SSF, samples were collected to analyze the residual sugars and ethanol; and (iii) Micro-aerobic SSF of pure cellulose was performed in 250-ml flasks containing 50 ml of YP, 13% (w/v) Avicel PH-101, and Celluclast 1.5L (10 FPU/g cellulose) with (or without) Novozyme 188 (5.4 cellobiase unit (CBU)/g cellulose). Novozyme 188 was supplemented as the extracellular β-glucosidase for the conversion of cellobiose to glucose in the traditional type of SSF with parental *S. cerevisiae* containing empty plasmids. The flasks were equipped with an air-lock device (3-piece airlock with silicone stopper (Amazon, USA)) to minimize air inflow while releasing CO_2 during SSF. The temperature and agitation speed were maintained at 30°C and 100 rpm, respectively. All SSF experiments were performed in duplicates.

Analytical Methods

The concentrations of sugars and ethanol were determined by high-performance liquid chromatography (HPLC; Agilent Technologies 1200 Series) equipped with a refractive index detector using a Rezex ROA-Organic Acid H^+ (8%) column (Phenomenex Inc., USA). The column was eluted with 0.005 N of H_2SO_4 at a flow rate of 0.6 ml/min at 50°C.

Results and Discussion

SSF of Pretreated Cellulosic Biomass by Engineered *S. cerevisiae* Fermenting Cellobiose Via the Phosphorolytic Pathway

In the previous studies, we found that ethanol production by the hydrolytic *S. cerevisiae* with wild-type CDT-1 (the D-BTw strain) became faster as the amount of inoculum was increased in SSF of cellulose [6]. We also found that ethanol production by the hydrolytic *S. cerevisiae* could be improved by expressing the mutant CDT-1 (the D-BTm strain) instead of the wild-type CDT-1 in SSF of cellulose [17]. Furthermore, the effects of intracellular cellobiose utilization of the hydrolytic *S. cerevisiae* strains in SSF could be maximized by decreased loading of cellulolytic enzymes, which led to better ethanol production by the D-BTm strain than the parental *S. cerevisiae* with extracellular β-glucosidase (the D-56+188 strain) [17]. Consequently, based on the previous SSF conditions (high inoculum size, expression of mutant cellobiose transporter, and reduced loading of the cellulase mixture) [6, 17], the feasibility of ethanol production in SSF of cellulose by the phosphorolytic *S. cerevisiae* employing mutant CDT-1 (the D-CTm strain) was investigated in this study.

Fig. 1 shows the CO_2 accumulation profiles (≈ the profiles of ethanol production) in SSF of washed PCS by two phosphorolytic yeast strains—D-CTw (*S. cerevisiae* expressing SdCBP with wild-type CDT-1) and D-CTm (*S. cerevisiae*

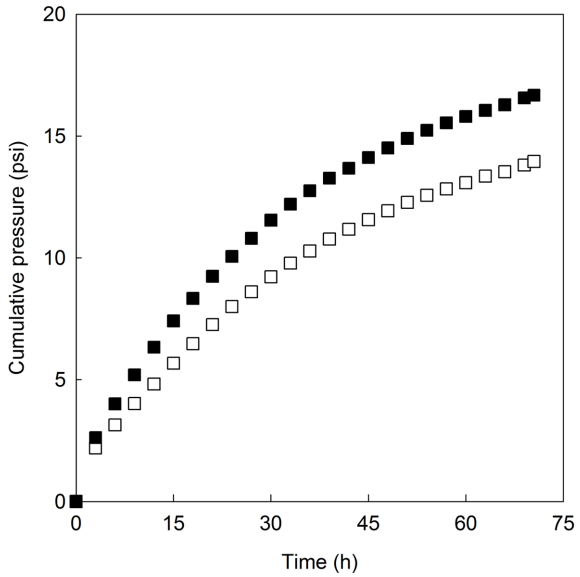

Fig. 1. Gas production profiles in anoxic SSF of 10% pretreated corn stover from NREL.

SSF was carried out with 10.5 g/l of initial cell concentration at 30°C and 100 rpm. Celluclast 1.5L (10 filter paper unit (FPU)/g glucan) was used for saccharification of cellulose. The yeast strains used in SSF are as follows: D-CTw (phosphorolytic *S. cerevisiae* expressing wild-type CDT-1; open square) and D-CTm (phosphorolytic *S. cerevisiae* expressing mutant CDT-1; closed square). Gas production was measured in two independent experiments, and the symbols in the figure show mean values.

expressing SdCBP with mutant CDT-1)—under anoxic conditions. The D-CTm strain showed faster gas production until the end of SSF compared with the D-CTw strain, obviously due to the enhanced cellobiose transport activity of the mutant cellobiose transporter, CDT-1 (F213L) [9]. Interestingly, CO_2 formation by D-CTw was not significantly poorer than D-CTm, which was quite different from the observation in the previous study where D-CTw showed much slower cellobiose consumption and ethanol production (about 3.5-times slower) than D-CTm during cellobiose fermentation [9]. This result might be due to the inoculation of considerably higher amounts of yeast cells in SSF compared with cellobiose fermentation in the previous study (10.5 g/l in SSF *vs.* 0.35 g/l in cellobiose fermentation). In addition, since small amounts of glucose could be released in SSF of cellulose, whereas only cellobiose was present in cellobiose fermentation, D-CTw might also utilize glucose for ethanol production. Determination of the Monod constant (K_S) and maximum specific growth rate (μ_{max}) for the phosphorolytic *S. cerevisiae* strains showed that D-CTw and D-CTm have similar affinities to cellobiose (0.04 g cellobiose/l of K_S for D-CTw *vs.* 0.06 g cellobiose/l of K_S for D-CTm), but D-CTw grows 30% slower than D-CTm (0.19 h^{-1} of μ_{max} for D-CTw *vs.* 0.27 h^{-1} of μ_{max} for D-CTm) (Fig. S1). Consequently, D-CTw might be able to take up cellobiose well even at a low level of cellobiose, explaining why D-CTw did not exhibit significantly worse CO_2 production than D-CTm in SSF compared with cellobiose fermentation. However, a slower growth rate might be the reason why D-CTm produced CO_2 faster than D-CTw despite similar affinity to cellobiose.

In order to verify whether the phosphorolytic cellobiose-fermenting *S. cerevisiae* performed better than the hydrolytic cellobiose-fermenting *S. cerevisiae* and the glucose-fermenting *S. cerevisiae* under the same SSF conditions, the gas profiles from the previous study—CO_2 production by the hydrolytic yeast expressing mutant CDT-1 (D-BTm) and the parental yeast with extracellular β-glucosidase (D-56+188) in anoxic SSF of washed PCS [17]—were compared (Fig. S2). Interestingly, both D-CTw and D-CTm showed faster gas production than D-56+188 until the end of SSF, which is a similar pattern to the gas production in SSF of washed PCS with the hydrolytic yeast strains (D-BTw and D-BTm) in the previous study [17]. Based on these results, it can be strongly proposed that the benefits of intracellular cellobiose utilization by the engineered *S. cerevisiae* strains in SSF can be maximized when cellobiose formation is limited by reducing the amount of cellulase mixture. Moreover, D-CTm showed a similar profile of CO_2 production compared with D-BTm (became slightly faster from the late period of SSF), demonstrating that the phosphorolytic yeast expressing mutant CDT-1 can produce ethanol as efficiently as the hydrolytic yeast expressing mutant CDT-1 in SSF of cellulose. It also demonstrates that the most important factor influencing ethanol production in SSF is the activity of the cellobiose transporter in the engineered yeast. The final ethanol concentrations and yields are summarized in Table 2. Comparable to the gas production profiles, D-CTm produced 14.6 g/l of ethanol with 0.247 g/g yield, which was slightly higher than D-BTm (14.2 g g/l of ethanol with 0.242 g/g yield) but 26% higher than D-56+188 (11.6 g/l ethanol with 0.197 g/g yield) [17].

SSF of Pure Cellulose by Engineered *S. cerevisiae* Fermenting Cellobiose Via Phosphorolytic Pathway

It was expected that fermentation inhibitors would not be released from pure cellulose compared with PCS that contains considerable amounts of lignin [20]. Consequently, SSF of pure cellulose (Avicel PH-101) was performed under similar conditions to check whether the same patterns of CO_2 production by the phosphorolytic *S. cerevisiae* strains

Table 2. Summarized results from SSF of cellulose with engineered *S. cerevisiae* strains.

Culture conditions	Strains	Final ethanol (g/l)	Ethanol yield from cellulose[b] (g/g)	Reference
Anoxic, 10% pretreated corn stover (PCS), Celluclast 1.5L (10 filter paper unit (FPU)/g glucan), [a]Novozyme 188 (5.4 cellobiase unit (CBU)/g glucan)	D-56+188	11.6 ± 0.35	0.197	[17]
	D-BTm	14.2 ± 0.41	0.242	[17]
	D-CTw	11.9 ± 0.44	0.203	This study
	D-CTm	14.6 ± 0.39	0.247	This study
Anoxic, 13% Avicel, Celluclast 1.5L (10 FPU/g cellulose), [a]Novozyme 188 (5.4 CBU/g cellulose)	D-56+188	33.2 ± 0.62	0.280	[17]
	D-BTm	37.3 ± 0.92	0.315	[17]
	D-CTw	28.2 ± 0.59	0.239	This study
	D-CTm	37.1 ± 0.71	0.313	This study
Microaerobic, 13% Avicel, Celluclast 1.5L (10 FPU/g cellulose), [a]Novozyme 188 (5.4 CBU/g cellulose)	D-56+188	33.5 ± 0.19	0.283	This study
	D-BTm	36.1 ± 0.16	0.305	This study
	D-CTw	27.7 ± 0.21	0.235	This study
	D-CTm	35.0 ± 0.17	0.296	This study

[a]Novozyme 188 was only used in SSF with the glucose-fermenting strain (D-56+188, the parental *S. cerevisiae* with extracellular β-glucosidase) for degradation of cellobiose to glucose.

[b]Based on the previous report where the glucan (cellulose) content of PCS was determined to be 58.9% [20], and the ethanol yield from 10% PCS in SSF was calculated from the final ethanol concentration divided by 58.9 g/l of the glucan concentration. Because Avicel was determined to have 9% of water content [17], the ethanol yield from 13% Avicel was calculated from the final ethanol concentration divided by 118.3 g/l of the actual cellulose concentration.

could be observed in comparison with the SSF of pretreated cellulosic hydrolysate (washed PCS). Since SSF of non-pretreated cellulose was reported to require a longer time than SSF with pretreated cellulose [6, 17], SSF of non-pretreated Avicel PH-101 was continued until 192 h (about 3 times longer than SSF of washed PCS).

The CO_2 accumulation profiles in SSF of Avicel PH-101 by the two phosphorolytic yeast strains, D-CTw and D-CTm, are illustrated in Fig. 2. Similarly to the above SSF of washed PCS, the D-CTm strain showed faster gas production than the D-CTw strain. However, the difference of final CO_2 accumulation between D-CTw and D-CTm was 3.5-fold (9.6 psi in SSF of Avicel PH-101 *vs.* 2.7 psi in SSF of washed PCS). Compared with the hydrolysis of pretreated cellulose (washed PCS), cellobiose formation might be limited but the formation of several cellodextrins (*e.g.*, cellotriose and cellotetraose) might be stimulated in the hydrolysis of non-pretreated cellulose (Avicel PH-101). Because wild-type CDT-1 is reported to have significantly lower transport activity for cellodextrins than cellobiose [1, 9], D-CTw might not be able to take up cellodextrins as efficiently as cellobiose, which might cause considerably slower CO_2 production by D-CTw.

To verify whether the phosphorolytic *S. cerevisiae* performed better than the hydrolytic *S. cerevisiae* (D-BTm) and the parental *S. cerevisiae* with β-glucosidase (D-56+188)

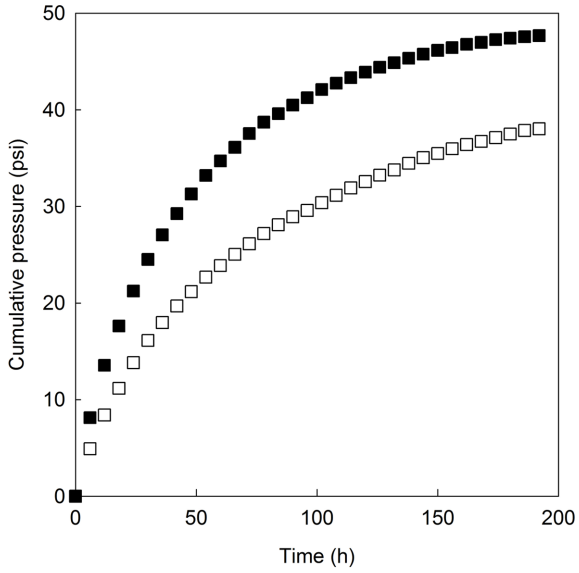

Fig. 2. Gas production profiles in anoxic SSF of 13% Avicel PH-101.

SSF was carried out with 10.5 g/l of initial cell concentration at 30°C and 100 rpm. Celluclast 1.5L (10 FPU/g cellulose) was used for saccharification of cellulose. The yeast strains used in SSF are as follows: D-CTw (phosphorolytic *S. cerevisiae* expressing wild-type CDT-1; open square) and D-CTm (phosphorolytic *S. cerevisiae* expressing mutant CDT-1; closed square). Gas production was measured in two independent experiments and the symbols in the figure show mean values.

under the same conditions, the gas profiles from the previous study—CO_2 productions by D-BTm and D-56+188 in anoxic SSF of non-pretreated Avicel PH-101 [17]—were compared (Fig. S3). Interestingly, gas production by D-56+188 became faster than D-CTw in the middle of SSF. These gas production profiles were in good accordance with the previous report where CO_2 production by the hydrolytic *S. cerevisiae* with the wild-type CDT-1 (D-BTw) became slower than D-56+188 after the middle period of SSF of Avicel PH-101 [17]. Whereas the D-CTw strains might not be able to take up cellodextrins well, supplementation of extracellular β-glucosidase to D-56+188 might enable degradation of cellodextrins to glucose, because most β-glucosidases are reported to exhibit a broad range of specificities to cellodextrins with good activities [22, 23], which might lead CO_2 production by D-56+188 to become faster than D-CTw. However, CO_2 production by D-CTm did not become slower than D-56+188 during the SSF regardless of substrate type. Mutant CDT-1 (F213L) was demonstrated to transport cellodextrins with considerably higher transport activity than wild-type CDT-1 [9], suggesting that the D-CTm strain could uptake several intermediate sugars as efficiently as cellobiose. Consequently, it can be emphasized that the enhanced transport activity of mutant CDT-1 on several intermediate sugars might be the most important factor for cellobiose-fermenting yeast to show better ethanol production than the parental yeast with extracellular β-glucosidase. Notably, D-CTm showed similar CO_2 production profiles compared with D-BTm (became slightly slower from the late period of SSF), suggesting that there might be no difference between the hydrolytic and phosphorolytic pathways in terms of ethanol production in the SSF of cellulose when the mutant CDT-1 was expressed in the engineered yeast. The final concentrations and yields of ethanol are summarized in Table 2. Comparable to the profiles of gas production, D-CTm produced 37.1 g/l of ethanol with 0.313 g/g yield, which was almost the same as D-BTm (37.3 g g/l of ethanol with 0.315 g/g yield) but 12% higher than D-56+188 (33.2 g/l ethanol with 0.280 g/g yield).

Another SSF of pure cellulose was performed under microaerobic conditions in order to evaluate whether the monitoring of CO_2 accumulation could directly correlate with monitoring ethanol production. Fig. 3 shows the ethanol production profiles in SSF of Avicel PH-101 by the two phosphorolytic yeast strains (D-CTw and D-CTm), the hydrolytic yeast strain (D-BTm), and the parental yeast strain with extracellular β-glucosidase (D-56+188). Similarly to the previous anoxic SSF of Avicel PH-101, ethanol production by D-56+188 became faster than D-CTw after 48 h of SSF. However, both D-BTm and D-CTm showed better ethanol production than D-56+188 until the end of SSF, again demonstrating that SSF employing cellobiose-fermenting *S. cerevisiae* with the mutant cellobiose transporter showed better ethanol production than the traditional SSF. One different observation from the above anoxic SSF is that D-BTm produced more ethanol (about 1.1 g/l) than D-CTm during the whole SFF time. In contrast to the anoxic condition, D-BTm might be able to synthesize more ATP because oxygen supply was not tightly restricted in the microaerobic conditions, supporting that hydrolytic *S. cerevisiae* easily produced ethanol even though it spent one more ATP to start glycolysis using cellobiose compared with the phosphorolytic *S. cerevisiae* [9, 15, 16]. Possibly, the energetic advantages of cellobiose phosphorolysis in D-CTm might not be featured under microaerobic conditions. In addition, most cellobiose phosphorylases are reported to cleave cellobiose exclusively with lower activity to

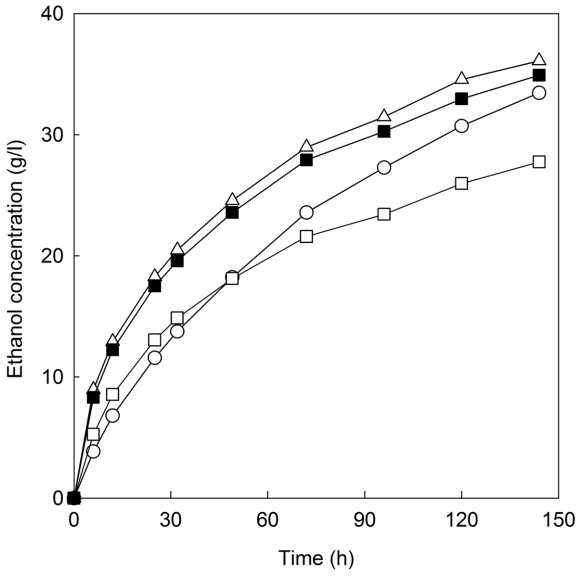

Fig. 3. Ethanol production profiles in microaerobic SSF of 13% Avicel PH-101.

SSF was carried out with 10.5 g/l of initial cell concentration at 30°C and 100 rpm. Celluclast 1.5L (10 FPU/g cellulose) was used for saccharification of cellulose, and Novozyme 188 (5.4 cellobiase unit (CBU)/g cellulose) was used for degradation of cellobiose to glucose. The yeast strains used in SSF are as follows: D-56+188 (parental *S. cerevisiae* with extracellular β-glucosidase; open circle), D-BTm (hydrolytic *S. cerevisiae* expressing mutant CDT-1; open triangle), D-CTw (phosphorolytic *S. cerevisiae* expressing wild-type CDT-1; open square), and D-CTm (phosphorolytic *S. cerevisiae* expressing mutant CDT-1; closed square). Ethanol concentration was measured in two independent experiments, and the symbols in the figure show mean values.

cellodextrins [24, 25], whereas GH1-1 is known to cleave not only cellobiose but also cellodextrins with good activity [1], which might also be a probable reason why D-CTm produced slightly less amount of ethanol than D-BTm in microaerobic SSF. The final ethanol concentrations and yields are summarized in Table 2. The D-CTm strain produced 35.0 g/l of ethanol with 0.296 g/g yield, which was lower than D-BTm (36.1 g/l of ethanol with 0.305 g/g yield) but still higher than D-56+188 (33.5 g/l ethanol with 0.283 g/g yield).

In this study, we observed that the phosphorolytic *S. cerevisiae* expressing mutant CDT-1 showed almost the same ethanol production performance in anoxic SSF, but slightly lower ethanol production in microaerobic SSF, compared with the hydrolytic *S. cerevisiae* expressing mutant CDT-1. We also observed that the cellobiose-fermenting yeast expressing mutant CDT-1 showed better ethanol production than the parental yeast with extracellular β-glucosidase, regardless of the cellobiose metabolic pathway. Because CDT-1 is the energy-dependent active transporter, ethanol production in SSF of cellulose with cellobiose-fermenting *S. cerevisiae* may be further improved by the introduction of another cellobiose transporter (energy-independent facilitator, CDT-2 from *N. crassa*) if CDT-2 is developed to exhibit enhanced cellobiose transport activity.

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