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Transporter engineering for cellobiose fermentation under lower pH conditions by engineered *Saccharomyces cerevisiae*

Eun Joong Oh[1,2], Suryang Kwak[1,2], Heejin Kim[1,2], and Yong-Su Jin[1,2]

[1]Department of Food Science and Human Nutrition, [2]Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

Correspondence should be addressed to Yong-Su Jin (ysjin@illinois.edu).

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Abstract

The aim of this study was to engineer cellodextrin transporter 2 (CDT-2) from *Neurospora crassa* for improved cellobiose fermentation under lower pH conditions by *Saccharomyces cerevisiae*. Through directed evolution, a mutant CDT-2 capable of facilitating cellobiose fermentation under lower pH conditions was obtained. Specifically, a library of CDT-2 mutants with GFP fusion was screened by flow cytometry and then serial subcultured to isolate a CDT-2 mutant capable of transporting cellobiose under acidic conditions. The engineered *S. cerevisiae* expressing the isolated mutant CDT-2 (I96N/T487A) produced ethanol with a specific cellobiose consumption rate of 0.069 g/g cell/h, which was 51% and 55% higher than those of the strains harboring wild-type CDT-1 and CDT-2 in a minimal medium with 2 g/L of acetic acid.

Keywords *Saccharomyces cerevisiae*, Cellobiose, Cellodextrin transporter, Low pH conditions, Transporter engineering

Highlights

- Directed evolution of cellobiose transporter CDT-2 was attempted
- A mutant CDT-2 facilitating cellobiose fermentation at lower pH levels was isolated
- The mutant CDT-2 showed better fermentation under both neutral and acidic conditions

1. Introduction

Microbial fermentation of lignocellulose-derived sugars has been intensively studied to enable the economic and sustainable production of value-added products (FitzPatrick et al., 2010; Kumar et al., 2008). *Saccharomyces cerevisiae* is a traditional glucose-fermenting strain and robust microorganism which has been widely used to produce ethanol and other chemicals in many industrial processes (Dhamankar & Prather, 2011; Du et al., 2011; Nevoigt, 2008). While *S. cerevisiae* can perform efficient fermentation of glucose into ethanol, this yeast cannot utilize xylose—the second most abundant sugar component in lignocellulosic hydrolysates—because of the lack of a xylose-utilizing pathway (Jeffries, 2006). However, engineered *S. cerevisiae* harboring heterologous xylose-assimilating pathways can utilize xylose. Intensive metabolic engineering efforts have further improved xylose fermentation by engineered *S. cerevisiae* (Kim et al., 2013; Lee et al., 2014). Nonetheless, one of the problems of xylose fermentation by engineered *S. cerevisiae* is the inhibition of xylose uptake under the presence of glucose. Because of the glucose repression on xylose fermentation, engineered *S. cerevisiae* ferments xylose only after depletion of glucose (Subtil & Boles, 2012). This sequential utilization of glucose and xylose resulted in low yields and productivity of ethanol (Ha et al., 2011). In order to convert hexose and pentose in plant cell wall hydrolysates into biofuels and chemicals, engineered *S. cerevisiae* capable of fermenting xylose and cellobiose simultaneously has been proposed (Galazka et al., 2010; Ha et al., 2011). Engineered yeast expressing heterologous genes coding cellodextrin transporter (*cdt-1*) and intracellular β-glucosidase (*gh1-1*) from *Neurospora crassa* and xylose metabolic enzymes assimilated cellobiose and xylose simultaneously.

Simultaneous fermentation of cellobiose and xylose resulted in higher ethanol yields and productivities than the sequential utilization of glucose and xylose (Galazka et al., 2010; Ha et al., 2011).

In prior studies, two cellodextrin transporters (CDT-1 and CDT-2) from *N. crassa* have been expressed in *S. cerevisiae* for direct fermentation of cellobiose. CDT-1 is a proton symporter, requiring ATP to pump out the protons by the plasma membrane ATPase during cellobiose fermentation. In contrast, CDT-2 is a facilitator—transporting cellobiose by a concentration gradient—has a much lower uptake activity (V_{max}) than CDT-1 (Galazka et al., 2010; Kim et al., 2014) . Although CDT-2 has a lower transporter activity, CDT-2 has a potential to surpass the performance of CDT-1 due to energetic benefits under ATP-limited conditions, such as anaerobic and acidic conditions. For example, a mutated CDT-2 obtained by directed evolution showed improved cellobiose fermentation performances under anaerobic conditions as compared to CDT-1 (Lian et al., 2014).

During the depolymerization of lignocellulosic biomass, fermentation inhibitors are generated in addition to fermentable sugars. Among the inhibitors, acetic acid ranging from 1 to 15 g/L inhibits the growth and fermentation of fermenting microorganisms (Almeida et al., 2007; Klinke et al., 2004; Palmqvist & Hahn-Hägerdal, 2000). Also, acidic conditions are advantageous for preventing contamination as well as cellulase reactions. As such, it would be desirable if cellobiose fermentation can be done under acidic conditions. However, engineered yeast expressing a cellobiose transporter does not ferment cellobiose well under lower pH conditions because cellobiose transporters cannot transport cellobiose (Klingmuller & Huh, 1972). In this

study, directed evolution was attempted to obtain CDT-2 mutants which can transport cellobiose under acidic (pH 3–4) conditions. A sequential screening and selection method was used to isolate CDT-2 mutants showing enhanced expression and facilitating cellobiose fermentation under acidic conditions. Through the combined screening and selection strategy, a mutant CDT-2 capable of enabling cellobiose fermentation at pH 3 was isolated. The CDT-2 mutant contained two mutations (I96N/T487A) and facilitated improved cellobiose fermentation regardless of pH (3 and 6).

2. Methods

2.1. Strains, media, and culture conditions

The yeast plasmids and strains used in this study are summarized in Table 1. *Escherichia coli* DH5 (*F– recA1 endA1 hsdR17 [rK– mK+] supE44 thi-1 gyrA relA1*) (Invitrogen, Gaithersburg, MD) was used to amplify the plasmids. *S. cerevisiae* D452-2 (*MAT α leu2 his3 ura3 can1*) (Hosaka et al., 1992) was used as a host strain for library construction and cellobiose fermentation. The *E. coli* was grown in the Luria-Bertani medium; 50 μg/mL of ampicillin was added if necessary. The yeast strains were routinely cultivated at 30℃ in YP medium (10 g/L of yeast extract and 20 g/L of peptone) with 20 g/L of glucose or 20 g/L of cellobiose. To select the transformants using an auxotrophic marker, yeast synthetic complete (SC) medium, which contained 6.7 g/L of yeast nitrogen base plus 20 g/L of cellobiose, 20 g/L of agar, and CSM-Leu-Trp-Ura-His (MP Biomedicals, Santa Ana, CA) with a supply of appropriate nucleotides and amino acids, was used.

2.2. Fermentation experiments

Yeast cells were grown in YP medium containing 20 g/L of cellobiose to prepare inoculums for fermentation experiments. The yeast cells were harvested in a mid-exponential phase and inoculated into experimental flasks after washing them twice with sterilized water. All of the flask fermentation experiments were performed using 25 mL of medium with the appropriate sugars at 30℃ with initial optical densities (OD) at 600nm of 1.0 or 0.1 under oxygen-limited conditions. SC medium containing 20 or 40 g/L of cellobiose was used for flask fermentations after pH adjustments at 3 or 6.

2.3. Construction of CDT-2 mutant library

All of the primers used for constructing a CDT-2 mutant library are listed in Table S1. *In vivo* homologous recombination in yeast was used for constructing a CDT-2 mutant library (Swers et al., 2004). The pRS426 plasmid was used as a backbone, and mutated CDT-2 was translationally fused with green fluorescent protein (GFP) to monitor the expression level of mutant CDT-2 in yeast (Galazka et al., 2010). The pRS426-cdt-2 plasmid was digested with *ClaI* and *SpeI* to remove the wild-type *cdt-2* gene, and purified with a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) to get the linearized plasmid. Random mutagenesis of CDT-2 was performed with the GeneMorph II Random Mutagenesis Kit (Agilent, Santa Clara, CA). The conditions for PCR were set to create a low mutation rate (0-4.5 mutations/kb), following the manufacturer's directions. The linearized pRS426 plasmid, the mutated CDT-2 fragments, and pRS425-gh1-1(Galazka et al., 2010) were transformed using a high-efficiency yeast

transformation method (Gietz & Schiestl, 2007) to obtain transformants expressing both mutant CDT-2 and GH1-1. Approximately 30 µg of the mutated CDT-2 insert and 10 µg of the linearized pRS426 plasmids were transformed. After the heat shock, 0.1 % of the transformant culture was spread on SC agar medium containing 20 g/L of cellobiose to confirm the size of the library of the CDT-2 mutant. The rest of the transformants were used for fluorescence-activated cell sorting (FACS) experiments.

2.4. Fluorescence-activated cell sorting (FACS)-based library screening

Fluorescence-activated cell sorting was performed using a BD FACS ARIA II sorter in the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. The transformants expressing both mutant CDT-2 and GH1-1 were grown in SC medium containing 20 g/L of cellobiose as a sole carbon source. The culture medium was adjusted to pH 3 to sort the cells capable of consuming cellobiose efficiently under low pH conditions. Also, *S. cerevisiae* D452-2 expressing *cdt-2* and *gh1-1* without GFP fusion was used as a negative control of the FACS. GFP was excited at 488 nm and captured by a 530/30 nm bandpass filter. The transformants from the sorting were captured in SC medium and used for serial subcultures.

2.5. Serial subculture

The sorted transformants were grown in SC medium containing 80 g/L of cellobiose as the sole carbon source under oxygen-limited conditions, and the pH of the medium was adjusted to pH 3. The cellobiose fermentation was initiated with an initial optical density at 600 nm (OD_{600}) of 0.1, and transferred to fresh cellobiose media when

the cells were in the stationary phase. The transfers were repeatedly performed until there was no more improvement in the cellobiose consumption rate.

2.6. Structural analysis of transporter

The topology of CDT-2 was predicted by the Phyre2 web portal for protein modeling (Kelley et al., 2015). The amino acid sequence was analyzed using intensive mode, and 467 residues (89%) were modeled with >90% accuracy. The predicted result was visualized by UCSF Chimera (Pettersen et al., 2004).

2.7. Analytical methods

Cell growth was monitored by OD at 600 nm using a UV-visible spectrophotometer (Biomate 5, Thermo, NY). The glucose, cellobiose, xylose, xylitol, glycerol, acetate, and ethanol concentrations were determined by high performance liquid chromatography (HPLC, Agilent Technologies 1200 Series) equipped with a refractive index detector and a Rezex ROA-Organic Acid H^+ (8%) column (Phenomenex Inc., Torrance, CA). The column was eluted with 0.005 N of H_2SO_4 at a flow rate of 0.6 mL/min at 50˚C.

3. Results and discussion

3.1. Creation of CDT-2 mutant library by homologous recombination

Directed evolution was performed to isolate CDT-2 mutants that can transport cellobiose under acidic conditions. To construct the CDT-2 library, yeast was co-transformed with a linearized backbone plasmid with GFP fusion, mutated *cdt-2*

fragments, and a plasmid containing *gh1-1* (Du et al., 2012; Swers et al., 2004). The resulting transformants expressing GH1-1 and mutated CDT-2s utilized cellobiose as a sole carbon source. 0.1 % of the whole population of transformants was spread on agar plates containing SC medium and cellobiose and 1.9×10^5 transformants were obtained. To confirm the efficacy of the *in vivo* recombination for constructing the mutant library, ten colonies were randomly picked from the plates, and plasmids were isolated from the colonies for sequence confirmation of the mutation rate. As a result, five of ten plasmids contained between two and eight point mutations in *cdt-2*, indicating that the constructed mutant library of CDT-2 has a proper mutation rate.

3.2. FACS-based screening and serial subculture for isolation of desired CDT-2 mutants

It was hypothesized that the mutations in CDT-2 might result in different expression levels or stabilities in the yeast membrane as well as changing cellobiose transporting activities. In order to identify a mutant CDT-2 which is well-expressed and stable in the yeast membrane under lower pH conditions, GFP was fused to the C-terminal region of the mutated CDT-2. With this design, FACS-based screening was applied to identify mutants with high fluorescence, and therefore high stability or expression, when cultured in conditions of low pH. In addition, the transformants with desirable CDT-2 mutants should grow better on cellobiose under low pH conditions. With the intention of using low pH cultures to differentiate desirable and undesirable mutants, different pH values of cellobiose-containing media were examined to evaluate the growth rate of a control strain expressing the wild-type CDT-2 and GH1-1. When

cultured for 120 hours, the control strain showed a gradual reduction in cell biomass as pH was reduced, which sharply dropped below pH 3 (Supplementary Fig. S1). If selection conditions are too harsh, many cells will cease to proliferate regardless of the presence of beneficial CDT-2 mutations. On the other hand, selection conditions which are too mild may not adequately differentiate between beneficial CDT-2 mutants and neutral mutants. Based on the cell growth of the control strain under various pH conditions, pH 3 was used for FACS-based screening.

After culturing the transformants harboring the CDT-2 mutant library in SC medium at pH 3 on cellobiose, the transformants showing high fluorescence intensities—having high GFP and CDT-2 in the membrane—were isolated through FACS. The top 0.15% (34/22728) of the whole population was isolated. As the FACS-isolated transformants were a mixture of CDT-2 mutants, serial subcultures on cellobiose with low pH were performed to select for the best-performing CDT-2 mutant within the population. It was hypothesized that the best CDT-2 mutant would proliferate to become the majority of the population if sufficient serial subcultures were performed under selective conditions. The serial subcultures were initiated with an optical density at 600 nm (OD_{600}) of 0.1, and the transfers were repeatedly performed until there was no further improvement in the cellobiose consumption rate. As the serial subcultures were repeated, the cellobiose consumption rates converged. The last culture showed 45% enhanced ethanol productivity from cellobiose than that by the first culture (Fig. 1). As a control, serial subcultures with the parental strain DBT2W expressing wild-type cellodextrin transporter (*cdt-2*) and β-glucosidase (*gh1-1*) under the same conditions were performed. As shown in Figure 1, the fermentation performance of the DBT2W

strain was much lower than those of the last subculture of the FACS-isolated transformants. Moreover, serial subcultures of DBT2W did not lead to significantly improved cellobiose fermentation. These results suggest two possible causes for the improved cellobiose fermentation by the last subculture of the FACS-isolated transformants: (1) unknown beneficial mutations or adaptations in the genome of the transformants improved cellobiose fermentation under low pH conditions, and/or (2) a desirable CDT-2 mutant enabled improved cellobiose under low pH conditions.

To confirm whether a CDT-2 mutation was the causal factor of the improved cellobiose fermentation during the serial subcultures, two transfers were performed in YP medium containing glucose to remove plasmids and thus isolate the host strains. After spreading the cells on a non-selective YP agar plate containing glucose (YPD), twenty colonies were picked and inoculated on a selective SC-Leu- Ura- agar plate to test for the presence of the plasmids. By comparing the colonies on the selective and non-selective plates, eight colonies that did not harbor any plasmids were isolated. After transforming wild-type cellodextrin transporter (*cdt-2*) and β-glucosidase (*gh1-1*) genes, these colonies showed no difference in phenotypes during fermentation of 80 g/L cellobiose at pH 3 as compared to the wild-type *S. cerevisiae* D452-2. This result indicates that the improved cellobiose fermentation under acidic conditions did not originate from the genetic perturbations in the genome of the host strain, but from CDT-2 mutants in the transformants. Second, the plasmids from the last subculture were isolated. The extracted plasmids were mixtures of the pRS425-*gh1-1* and pSR426-mutant *cdt-2*. By re-transforming the extracted plasmids into *E. coli* and confirming via PCR, ten plasmids containing the *cdt-2* gene were obtained. Three of ten had point

mutations, and seven contained no mutation in the *cdt-2* gene. Interestingly, all three plasmids possessed the same two mutations (T287A/A1459G) in the *cdt-2* gene, resulting in amino acid mutations from isoleucine to asparagine at position 96 and from threonine to alanine at position 487 (I96N/T487A).

3.3. Comparison of wild-type CDT-2 and CDT-2 mutant I96N/T487A

The phenotypic changes by the CDT-2 mutant (I96N/T487A) were examined after re-transforming the isolated plasmid with the mutation into the *S. cerevisiae* D452-2 strain. The re-transformed strain (DBT2M) with the mutated CDT-2 (I96N/T487A) and a control strain (DBT2W) with the wild-type CDT-2 in addition to GH1-1 were compared for cellobiose fermentation under various conditions. First, both the DBT2W (wild-type CDT-2) and DBT2M (mutant CDT-2 I96N/T487A) strains were cultured in YP medium with 80 g/L cellobiose under oxygen-limited conditions. As shown in Figure 2A, both strains consumed 80 g/L of cellobiose within 72 hours. However, the DBT2M strain showed a 30 % higher cellobiose consumption rate at 30 hours (2.35 g/L·h) than the DBT2W strain (1.79 g/L·h). Also, the ethanol yield from cellobiose by the DBT2M strain (0.38 g ethanol/g cellobiose) was 31% higher than that by the DBT2W strain (0.29 g ethanol/g cellobiose). Next, the amount of stable CDT-2 protein in the membrane was determined by measuring the amount of GFP fluorescence per cell at a cell density equal to an OD of 5. However, there was no significant difference in GFP fluorescence per cell between wild-type and mutant *cdt-2* (Supplementary Fig. S2A). These results suggest that the improved cellobiose fermentation by the DBT2M strain in the neutral, complex (YP) medium might be caused by changes in the kinetic properties of the

mutant CDT-2 (I96N/T487A) rather than by better expression or stability of the mutant CDT-2 as compared to the wild-type CDT-2.

Second, differences in fermentation performance between the DBT2W and DBT2M strains in minimal (SC) medium with initial pH 3 and pH 6 were observed. While the performances of these strains in minimal medium were much poorer than in complex medium, the DBT2M strain again exhibited faster cellobiose consumption and more ethanol production as compared to the DBT2W strain (Fig. 2B and C). After culturing on cellobiose for 76 hours with an initial pH of 6, the DBT2W strain consumed 14 g/L of cellobiose and produced 2.5 g/L of ethanol, while the DBT2M strain consumed 25 g/L of cellobiose and produced 6.9 g/L of ethanol. When cultured at an initial pH of 3, both strains showed much lower cellobiose consumption rates and ethanol yields (Table 2). Also, the ratio of specific cellobiose fermentation rates between initial pH 3 and pH 6 differed according to the type of CDT-2 transporter. The ratio of specific cellobiose consumption rates under initial pH 3 and 6 for DBT2W was 0.863 (0.082 g/g cell/h/0.095 g/g cell/h), while that for DBT2M was 0.963 (0.155 g/g cell/h/0.161 g/g cell/h). Although the cellobiose consumption rates of the DBT2M strain were already higher than those of the DBT2W strain when cultured at an initial pH of either 6 or 3, the higher ratio of specific cellobiose consumption rate at initial pH 3 and 6 indicated that improved tolerance of the DBT2M strain under low pH was another factor improving cellobiose fermentation. The relative GFP level of the mutant CDT-2 under the pH 3 condition was approximately 3-fold higher than that of wild-type CDT-2 (Supplementary Fig. S2B). These results indicate that the mutations (I96N/T487A) in the mutant CDT-2 not only led to improved cellobiose consumption, but also to increased tolerance of low pH. Based

on the results of GFP fluorescence and cellobiose fermentation, the two point mutations in the *cdt-2* gene resulted in enhanced cellobiose fermentation under neutral pH conditions as well as improved expression or stability of the mutated cellodextrin transporter under acidic conditions (pH 3). Low pH conditions affect not only the activity of proteins located in the plasma membrane such as H^+-ATPase and transporters, but also the level of mRNA from genes related to the plasma membrane (Carmelo et al., 1996). In addition, the yeast cell wall structure can be changed by low external pH conditions (Kapteyn et al., 2001). For these reasons, stable expression of the CDT-2 transporter under acidic conditions might be limited. Notably, the mutations in the *cdt-2* gene improved its cellobiose uptake activity and tolerance to low pH conditions.

3.4. Improved cellobiose fermentation under the presence of toxic levels of acetic acid by the mutant CDT-2 I96N/T487A

An un-dissociated form of acetic acid drastically increases when the pH of the fermentation media is lower than the pK_a (4.85, at 30 ˚C) of acetic acid and the un-dissociated form can enter the cell and lower the intracellular pH (i Nogué et al., 2013). To keep the intracellular pH neutral, the cell has to pump out excess protons using ATP (Pampulha & Loureiro-Dias, 2000). While the CDT-2 mutant (I96N/T487A) is a facilitator which does not require ATP, the active transporter of cellobiose CDT-1 requires ATP. Therefore, the mutant CDT-2 should allow better cellobiose fermentation as compared to CDT-1 under ATP-limiting conditions. It was hypothesized that the engineered yeast with the CDT-2 mutant (I96N/T487A) might ferment cellobiose better in minimal medium with low pH than wild-type CDT-1 and CDT-2. To test this hypothesis, the cellobiose

fermentation profiles of the DBT1W, DBT2W, and DBT2M strains using 20 g/L cellobiose with initial pH 3 were compared. As shown in Figure 3A, DBT2M showed a 33% higher cellobiose consumption rate than that by DBT2W. Nonetheless, the specific cellobiose consumption rate and ethanol productivity by the DBT2W strain were lower than those by the DBT1W strain. Kim and coworkers reported that a CDT-1 expressing strain showed higher ethanol yields and productivities than a CDT-2 expressing strain regardless of complex or minimal media (Kim et al., 2014). In this study, wild-type CDT-1 performed the best among transporters, even in minimal medium adjusted to pH 3, which is consistent with the previous results. Next, fermentation performances were examined using a minimal medium with initial acetic acid, which is more toxic than just a low pH on its own (pH 3). Because no cell could grow in minimal medium containing 3 g/L of acetic acid, 2 g/L of acetic acid was used. Without any adjustment, the initial pH of the minimal medium containing 2 g/L of acetic acid was 3.3. Interestingly, the cellobiose fermentation with 2 g/L of acetic acid by the DBT2M was much higher than those of DBT1W (Fig. 3B). These results indicate that the energy-saving advantage of CDT-2, which does not require ATP to uptake extracellular cellobiose, plays an important role in its cellobiose fermentation under ATP-limiting conditions.

To predict the structure of the CDT-2 mutant (I96N/T487A), the Phyre2 web portal for protein modeling (Kelley et al., 2015) was used. According to the visualized topology of the mutant CDT-2 (I96N/T487A), I96 was located in the transmembrane helix, and T487 was located in the intracellular loop structure (Supplementary Fig. S3). Previous reports of phenotype-altering mutations in transporters have varied in location from transmembrane helix domains to loop structures (Eriksen et al., 2013; Lian et al.,

2014; Lu & Bush, 1998; Young et al., 2012). Amino acid I96 in the transmembrane helix might be involved with interactions between cellobiose and the transmembrane associated with binding cellobiose. Single amino acid substitution can change not only the activity but also the optimum pH of protein because of the shift of pK_a (Joshi et al., 2000). Similarly, the shift of pK_a from isoleucine to asparagine (I96N) in the helix might induce a change in charge of the structure, and improve cellobiose uptake under low pH conditions. In addition, mutations in the intracellular loop of transporters also occurred in previous studies (Lian et al., 2014; Young et al., 2012). When the mutant CDT-2 homology model was structurally aligned to XylE from *E. coli*, T487A was adjacent to the conserved PETK motif at C-terminus of transmembrane 12. This motif is one of the motif residues involved in the interactions between the transmembrane segments and the intracellular domain (Sun et al., 2012). The single point mutation near the signature motif might play an essential role in transporter activity.

Although two mutations in the mutated CDT-2 (I96N/T487A) contributed to the improved cellobiose fermentation under low pH conditions, future assessment of the effect of each mutation (I96N and T487A) on the catalytic properties of the CDT-2 mutants can provide more information about how the point mutations caused the phenotype changes. Also, the fermentation performances by the mutant CDT-2 (I96N/T487A) indicate that it is necessary to further improve its cellobiose consumption rate for enhanced ethanol production. More rounds of directed evolution of the mutant CDT-2 can be used to obtain other desired mutations eliciting further improvement of cellobiose fermentation. Furthermore, directed evolution of various fungal cellobiose transporters such as *Penicillium chrysogenum* sugar transporter might uncover

structural and functional mechanisms for desired phenotypes in engineered *S. cerevisiae* (Bae et al., 2014).

The present study demonstrated that directed evolution of cellodextrin transporter CDT-2 can be performed to improve cellobiose fermentation by engineered yeast under lower pH conditions, resulting in obtaining the CDT-2 mutant (I96N/T487A) with enhanced stability as compared to the wild-type CDT-2. Continued improvement in CDT-2 could expand the capabilities of the engineered yeast to produce cellulosic biofuels and chemicals. As the mutant CDT-2 provides better cellobiose uptake activity at lower pH conditions, it might be suitable for a continuous fermentation process using lignocellulosic hydrolysates (Ha et al., 2013). In addition, the improved tolerance of the mutant cellodextrin transporter against lower pH conditions can contribute to the production of diversified chemicals beyond bioethanol as shown in the examples of succinic acid production at low pH (Yan et al., 2014; Yuzbashev et al., 2010).

4. Conclusions

Cellodextrin transporter CDT-2 was engineered to improve its tolerance against low pH conditions. Transporter engineering was accomplished by a combination of directed evolution, FACS isolation, and serial subculture. The identified mutations in the *cdt-2* gene (I96N/T487A) contributed to the improvement of both kinetic properties and tolerance to low pH conditions. The strategy presented here could be applied to transporter engineering for improving tolerance to fermentation inhibitors derived from lignocellulosic hydrolysates such as HMF and furfural.

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522 pH by a recombinant strain of the aerobic yeast *Yarrowia lipolytica*. Biotechnol. Bioeng.,
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525 **Figure legends**

526

527 Figure 1. Serial subculture of the engineered *S. cerevisiae* strains under low pH
528 conditions. The DBT2W strain expressing cellodextrin transporter (*cdt-2*) and β-
529 glucosidase (*gh1-1*) and the FACS-isolated transformants were cultured in SC medium
530 with 80 g/L of cellobiose, and the initial pH of the medium was adjusted to 3. The
531 subcultures were performed under oxygen-limited conditions (100 rpm), and the initial
532 cell density of each subculture was adjusted to 0.029 g/L (OD_{600} = 0.1).

533

534 Figure 2. Comparison of fermentation profiles of cellodextrin transporters between the
535 engineered *S. cerevisiae* DBT2W, containing *gh1-1* and wild-type *cdt-2* (solid symbols),
536 and DBT2M, containing *gh1-1* and mutant *cdt-2* (open symbols) strains. Fermentations
537 were performed in YP medium (A), SC medium with initial pH values of 6 (B), and 3 (C)
538 containing cellobiose (80 g/L and 40 g/L) under oxygen-limited conditions. The initial cell
539 density was adjusted to 0.29 g/L (OD_{600} = 1). Cellobiose is shown by squares and
540 ethanol by diamonds. The results are the mean of duplicate experiments, and the error
541 bars indicate the standard deviations.

542

543 Figure 3. Fermentation performances of engineered *S. cerevisiae* according to
544 cellodextrin transporters (wild-type *cdt-1*, *cdt-2*, and mutant *cdt-2*). Fermentations were
545 performed in SC medium containing 20 g/L cellobiose under oxygen-limited conditions.
546 To evaluate tolerance to acidic conditions, SC medium with the initial pH 3 (A) or SC
547 medium containing 2 g/L acetic acid with the initial pH 3.3 (B) was used. The initial cell

548 density was adjusted to 0.29 g/L (OD_{600} = 1). The results are the mean of duplicate
549 experiments, and the error bars indicate the standard deviations.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
D452-2	*MATα, leu2, his3, ura3, and can1*	(Hosaka et al., 1992)
DBT1W	D452-2/pRS425-gh1-1/pRS426-cdt-1	This study
DBT2W	D452-2/pRS425-gh1-1/pRS426-cdt-2	This study
DBT2M	D452-2/pRS425-gh1-1/pRS426-cdt-2m	This study
Plasmids		
pRS425	2 µm origin, *LEU2*	(Christianson et al., 1992)
pRS426	2 µm origin, *URA3*	(Christianson et al., 1992)
pRS425-gh1-1	pRS425 *PGK1p-gh1-1-CYC1t*	(Galazka et al., 2010)
pRS426-cdt-1	pRS426 *PGK1p-cdt-1-CYC1t*	(Galazka et al., 2010)
pRS426-cdt-2	pRS426 *PGK1p-cdt-2-CYC1t*	(Galazka et al., 2010)
pRS426-cdt-2m	pRS426 containing the CDT-2 (I96N/T487A) with *PGK1p* and *CYC1t*	This study

563 Table 2. Fermentation parameters[1] of DBT2W and DBT2M under various conditions

Media[2]	Strains	$r_{Cellobiose}$	$r_{Cellobiose}$*	$P_{Ethanol}$	$P_{Ethanol}$*	$Y_{Ethanol}$
YPC80 (at 36 h)	DBT2W	1.865 ± 0.042	0.031 ± 0.001	0.483 ± 0.007	0.096 ± 0.002	0.259 ± 0.002
	DBT2M	2.136 ± 0.011	0.033 ± 0.000	0.738 ± 0.033	0.136 ± 0.005	0.346 ± 0.014
SCC40 (pH 6, at 76 h)	DBT2W	0.185 ± 0.003	0.095 ± 0.003	0.033 ± 0.002	0.017 ± 0.001	0.177 ± 0.014
	DBT2M	0.329 ± 0.006	0.161 ± 0.002	0.090 ± 0.000	0.044 ± 0.000	0.274 ± 0.005
SCC40 (pH 3, at 76 h)	DBT2W	0.136 ± 0.000	0.082 ± 0.000	0.016 ± 0.000	0.010 ± 0.000	0.121 ± 0.001
	DBT2M	0.261 ± 0.007	0.155 ± 0.003	0.064 ± 0.001	0.038 ± 0.000	0.244 ± 0.003

564

565 [1]Fermentation experiments were performed under oxygen-limited conditions (100 rpm)
566 and an initial cell density was adjusted to 0.29 g/L (OD_{600} = 1). The results are the
567 means of duplicate experiments.
568
569 [2]YPC80: YP medium containing 80 g/L of cellobiose, SCC40: SC medium containing 40
570 g/L of cellobiose.
571
572 Parameters: $r_{cellobiose}$, cellobiose consumption rate (g/L·h); $r_{cellobiose}$*, specific cellobiose
573 consumption rate (g/g cell/h); $P_{ethanol}$, volumetric ethanol productivity (g/L·h); $P_{ethanol}$*,
574 specific ethanol productivity (g/g cell/h); $Y_{ethanol}$, ethanol yield (g/g sugars)
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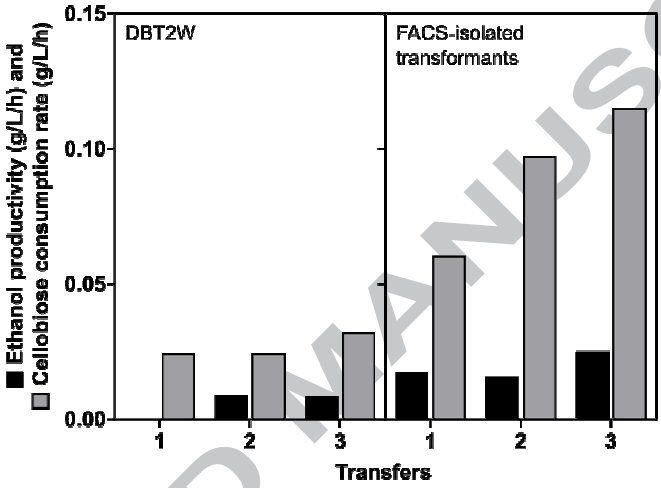
588 **Figure 1.**



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604 **Figure 2.**

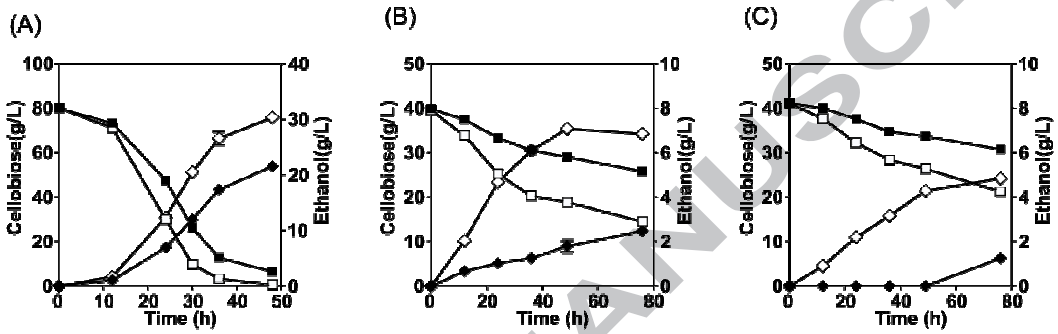

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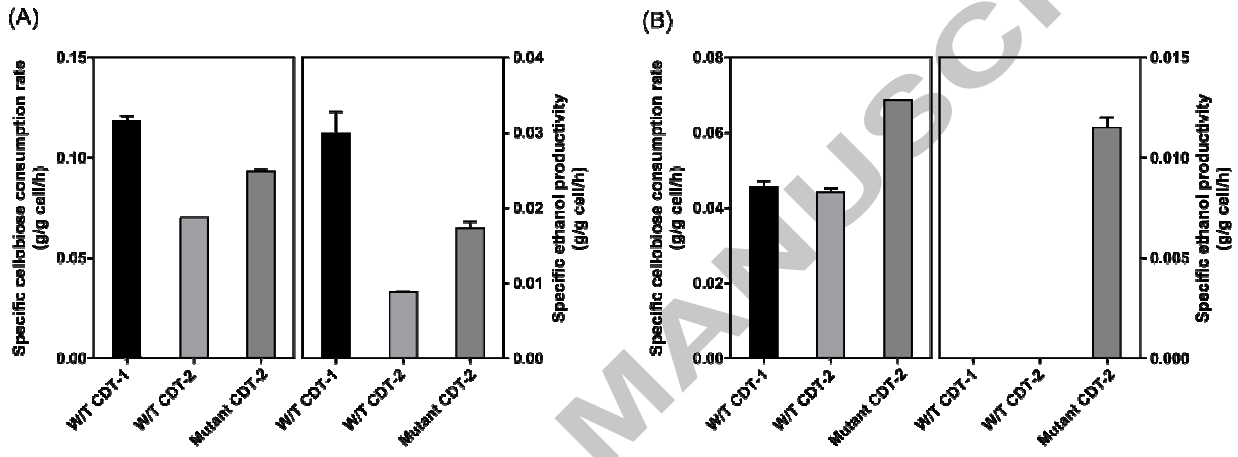
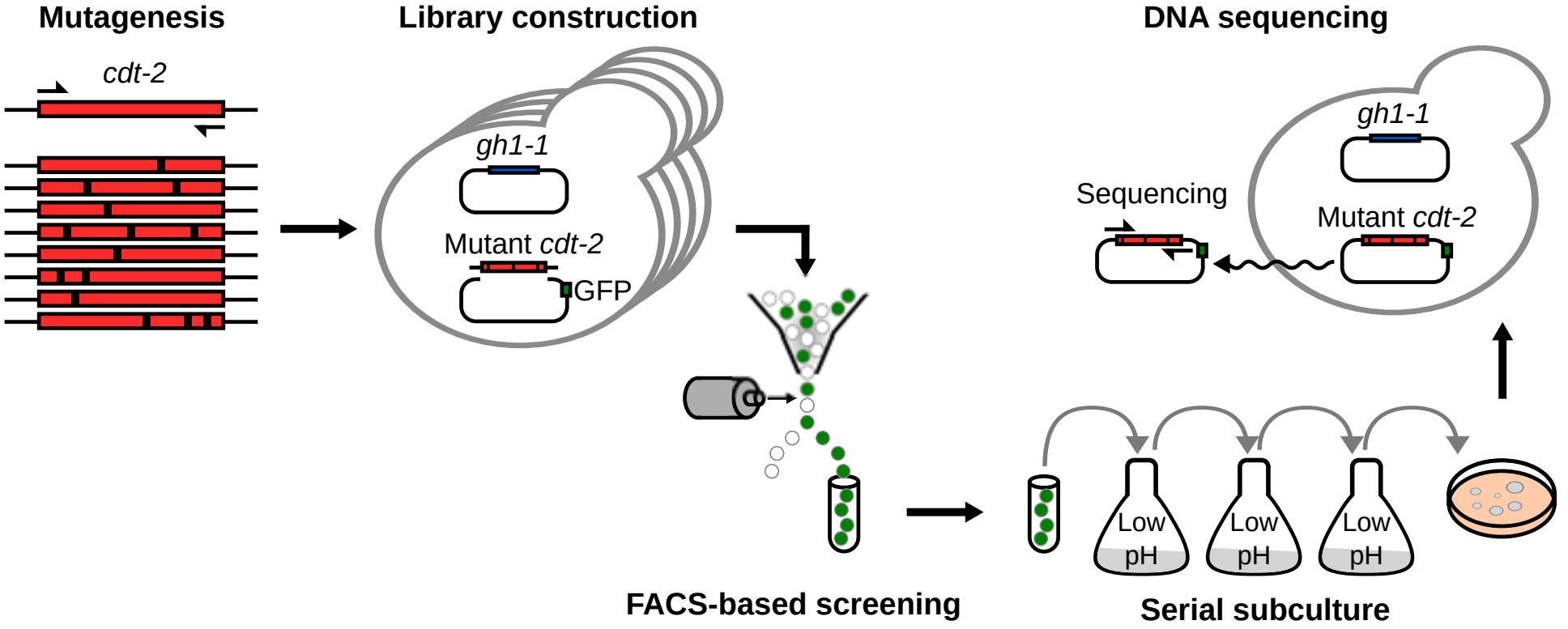
Figure 3.




Mutagenesis
Library construction
DNA sequencing
cdt-2
gh1-1
Mutant cdt-2
GFP
Sequencing
gh1-1
Mutant cdt-2
Low pH
Low pH
Low pH
FACS-based screening
Serial subculture