ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Directed Evolution of a Highly Efficient Cellobiose Utilizing Pathway in an Industrial *Saccharomyces cerevisiae* strain

Yongbo Yuan,[1] Huimin Zhao[1,2]

[1]Department of Chemical and Biomolecular Engineering, Institute for Genomic Biology, Urbana, Illinois, 61801; telephone: (217)-333-2631; fax: (217)-333-5052; e-mail: zhao5@illinois.edu

[2]Departments of Chemistry, Biochemistry, and Bioengineering, University of Illinois at Urbana-Champaign, Urbana, Illinois

ABSTRACT: Balancing and increasing the flux through an engineered heterologous pathway in a target organism to achieve high yield and productivity remains an overwhelming challenge in metabolic engineering. Here we report a novel strategy combining directed evolution and promoter engineering for rapid and efficient multi-gene pathway optimization. As proof of concept, this strategy was applied to optimize a cellobiose utilizing pathway in an industrial *Saccharomyces cerevisiae* strain for highly efficient cellulosic biofuels production. The resulting strain exhibited significantly higher cellobiose consumption rate (6.41-fold) and ethanol productivity (6.36-fold) compared to its parent strain. This study also showed that both the ratios and absolute values of the expression levels of the genes in the cellobiose utilizing pathway play an important role in cellobiose uptake, and β-glucose is likely one of the key factors affecting cellobiose metabolism.

Biotechnol. Bioeng. 2013;xxx: xx–xx.

KEYWORDS: directed evolution; promoter engineering; cellobiose utilization; pathway optimization; metabolic engineering

Introduction

Many applications in synthetic biology and metabolic engineering require the coordinated expression of multiple genes to balance the flux in a heterologous pathway (Anthony et al., 2009; Bond-Watts et al., 2011; Dueber et al., 2009; Luetke-Eversloh and Stephanopoulos, 2008; Pfleger et al., 2006; Salis et al., 2009; Shen et al., 2011). However, due to biological complexity and context-dependent issues, it is nearly impossible to rationally design an optimized pathway with balanced gene expression levels in a target organism, therefore necessitating the use of combinatorial or evolutionary design approaches (Pfleger et al., 2006). Recently, a number of combinatorial design strategies have been developed to balance the flux in a heterologous pathway through the engineering of ribosome binding sites (Salis et al., 2009), intergenic regions (Pfleger et al., 2006) or promoters (Alper et al., 2005; Lee et al., 2007). To date, however, no attempt has been made to combine directed evolution with promoter engineering. Here we show that the pathway optimization by the combination of directed evolution and promoter engineering represents a rapid yet efficient strategy for balancing and increasing the flux through a heterologous pathway. Directed evolution is a powerful approach for synthetic biology that enables rapid isolation of functionally improved or new biological entities from a large library of variants (Cobb et al., 2012a,b). Previously, most directed evolution studies were focused on single proteins and there are very few examples involving the use of directed evolution to engineer pathways (Johannes and Zhao, 2006; Schmidt-Dannert, 2001). To the best of our knowledge, there has been no report on the combined use of directed evolution and promoter engineering for pathway optimization.

This new strategy of balancing and increasing the flux through a heterologous pathway is shown in Figure 1. A pool of constitutive promoter mutants with varying strengths is generated for each promoter in the target pathway using error-prone PCR. The resulting promoter mutants are assembled with the corresponding structural genes and terminators to create a library of mutant pathways on a single copy plasmid in the target host using the DNA assembler method (Shao et al., 2009). A high throughput screening or selection method is used to identify the functionally improved pathways, which will serve as the parent pathway for the next round of directed evolution. This cycle of

No conflict of interest is declared.
Correspondence to: H. Zhao
Contract grant sponsor: Energy Biosciences Institute
Received 6 February 2013; Revision received 17 April 2013; Accepted 19 April 2013
Accepted manuscript online xx Month 2013;
Article first published online in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/bit.24946

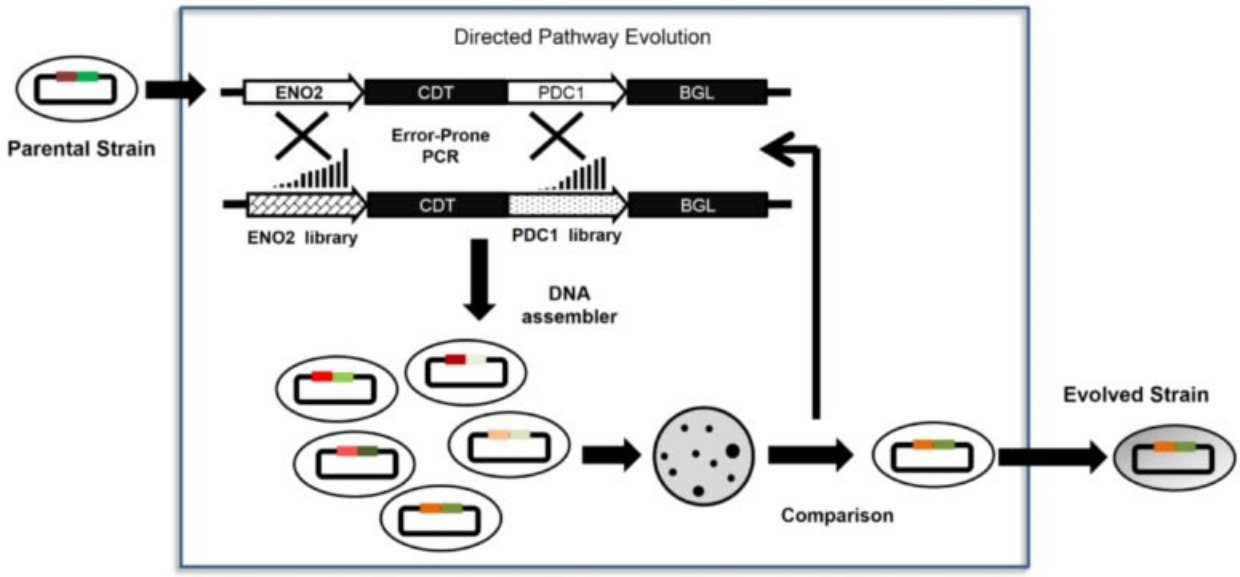

Figure 1. Scheme of the directed multi-gene pathway evolution strategy for strain development that combines directed evolution and promoter engineering.

directed evolution will be repeated until no further improvement is observed. The new directed pathway evolution strategy allows simple and rapid optimization of a heterologous pathway in a target host without detailed knowledge of the pathway and the host. Moreover, the optimized pathway can be directly integrated into the chromosome to create a practically relevant production host without the use of an expensive inducer.

As proof of concept, we sought to use this strategy to create a highly efficient cellobiose utilizing industrial *S. cerevisiae* strain for cellulosic biofuels production. Cellulosic biomass is one of the most abundant renewable resources that can serve as an alternative to traditional fossil fuels. Due to the high cost of the conversion from cellulose to glucose, large-scale production of cellulosic biofuels remains economically unattractive (Lynd et al., 1991). Among the various strategies for cellulosic biofuels production, simultaneous saccharification and fermentation (SSF) by *S. cerevisiae* which is a highly useful platform organism for production of fuels and chemicals (Nevoigt, 2008), is considered one of the most promising strategies (Philippidis et al., 1993). However, due to accumulation of cellobiose (Ghosh et al., 1982), which not only inhibits cellulases but also is non-fermentable in *S. cerevisiae*, addition of β-glucosidase (BGL; EC 3.2.1.21) is required to eliminate the cellobiose. To this end, *S. cerevisiae* was engineered to secrete or display a BGL (Wen et al., 2010) or introduce a lactose permease to import cellobiose for intracellular phosphorolysis (Sadie et al., 2011) to convert cellobiose to glucose. Recently, a heterologous cellobiose utilizing pathway consisting of a cellobiose transporter (CDT) and a BGL was discovered (Galazka et al., 2010) and successfully introduced into a laboratory *S. cerevisiae* strain (Ha et al., 2011; Li et al., 2010). However, this heterologous pathway was not optimized in the new hosts since the cellobiose consumption rate, the ethanol yield and productivity in the cellobiose fermentation were not as high as those in glucose fermentation. Moreover, compared to laboratory strains, industrial strains often show a higher growth rate and a higher tolerance to low pH, higher ethanol and sugar concentrations. Therefore, engineering of an industrial *S. cerevisiae* strain by introducing and optimizing the heterologous cellobiose utilization pathway is highly desirable.

In this work, we demonstrated the effectiveness of the above-mentioned directed pathway evolution strategy in pathway optimization by creating a cellobiose utilizing industrial *S. cerevisiae* strain that exhibited significantly higher cellobiose consumption rate (6.41-fold) and ethanol productivity (6.36-fold) compared to its parent strain. In addition, we investigated the mechanisms of cellobiose metabolism in *S. cerevisiae*.

Materials and Methods

Strains, Media, and Cell Cultivation

Escherichia coli DH5α (Cell Media Facility, University of Illinois at Urbana-Champaign, Urbana, IL) was used for recombinant DNA manipulation. The industrial *S. cerevisiae* strain Still Spirits (Classic) Turbo Distiller's Yeast was purchased from Homebrew Heaven (Everett, WA). Yeast strains were cultivated in YPA medium (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate) supplemented with glucose (YPAD), cellobiose (YPAC), or maltose (YPAM). *E. coli* strains were cultured in Luria broth (LB) (Fisher Scientific,

Pittsburgh, PA) at 37°C and 250 rpm. *S. cerevisiae* strains were cultured at 30°C and 250 rpm (for aerobic growth) or 100 rpm (for oxygen limited conditions). All restriction enzymes and plasmid pRS414 were purchased from New England Biolabs (Ipswich, MA). All restriction enzymes and plasmid pRS414 were purchased from New England Biolabs (Ipswich, MA). All chemicals were purchased from Sigma–Aldrich (St Louis, MO) or Fisher Scientific.

Plasmid and Strain Construction

For the construction of the cellobiose utilizing pathway, a fragment containing a CDT gene *cdt-1* (GenBank Accession number XM_958708) from *Neurospora crassa* and a PGK1 terminator, and a fragment containing a BGL gene *gh1-1* (GenBank Accession number XM_951090) from *N. crassa* and an ADH1 terminator, were PCR-amplified from a previously constructed plasmid pRS425-nc801 (Li et al., 2010). In order to create the promoter libraries with large dynamic range, two strong constitutive promoters (Sun et al., 2012), ENO2 (for 5′-CGTTGTAAAACGACGGCCAGTGAG CGCGCGTAATACG-ACTCACTATAGGGCGAATTG-3′, rev 5′-CTCGGTGCTGG-CCCCGTCATGGGA GCCGTGAGACGACATTATTATTGT-ATGTTATAG-3′) and PDC1 (for 5′-GTACTCTTTAGATCCA GTATAGTGTATTCTTCCTGCATGCGACTGGGTGAGCAT-ATG -3′, rev 5′-CAGTAGCGAAGCCCC AGAGGAAATCCT-TAGGAAGAGACATTTTGATTGATTTGACTGTG-3′), were PCR-amplified and flanked with regions homologous to the transporter gene and BGL gene, respectively. All these four fragments were then transformed together with *Sal*I–*Not*I digested pRS-KanMX plasmid into the industrial strain Classic Turbo Yeast using the DNA assembler method (Shao et al., 2009). Transformants were then spread on the YPAD plates supplemented with 200 mg/L G418. The Classic Turbo Yeast strain containing the correct plasmid harboring a CDT gene driven by the original ENO2 promoter and a BGL gene under the control of the original PDC1 promoter was named as the parent strain.

Construction and Screening of a Library of Cellobiose Utilizing Pathways

The ENO2 and PDC1 promoter libraries were created by error-prone PCR using the same primers used in the construction of the wild type cellobiose utilizing pathway as described elsewhere (Du et al., 2012). The pathway library was then created by transforming the two promoter libraries together with the DNA fragment containing the CDT gene, the DNA fragment containing the BGL gene, and the *Sal*I–*Not*I digested pRS-KanMX plasmid into the industrial strain Classic Turbo Yeast. Transformants were first recovered in the YPAD medium for 5 h and then a small amount of transformants (1%) were spread on an YPAD plate supplemented with G418 to determine the library size. Ten random colonies were cultured and the corresponding plasmids were extracted for DNA sequencing to determine the mutagenic rate. When 200 ng of each DNA fragment was used for pathway construction, a library of 10^3–10^4 transformants was obtained.

The rest of the transformants were diluted in an appropriate cell density, spread on YPAC agar plates, and incubated at 30°C for 48 h until the colonies were large enough to distinguish the larger ones from the smaller ones. Subsequently, the top 20 large colonies were inoculated into 2 mL of YPAD liquid medium supplemented with 200 mg/L G418 in a culture tube and shaken at 30°C with 250 rpm. This step was carried out to avoid the host adaption to cellobiose and collect seed cells for the subsequent cultivation. After 12 h, cells were washed once by YPAC medium and transferred into 4 mL YPAC liquid medium in a culture tube with a starting OD_{600} of 1 and shaken at 30°C with 250 rpm. Samples were taken twice at the late exponential phase. OD_{600} was measured immediately by a Cary 300 UV–Vis spectrophotometer (Agilent Technologies, Santa Clara, CA) at a wavelength of 600 nm after a proper dilution and ethanol concentrations were analyzed by HPLC. The top five mutant strains with the highest ethanol concentrations were selected and inoculated into 2 mL of YPAD liquid medium supplemented with 200 mg/L G418 in the culture tube. After 12 h, cells were washed once by YPAC medium once and transferred into 10 mL YPAC liquid medium in 50 mL un-baffled shake flasks with a starting OD_{600} of 1 and shaken at 30°C and 100 rpm. Samples were taken twice at the late exponential phase, both OD_{600} and ethanol concentrations were measured as described above. The mutant strain with the highest ethanol concentration was selected for further characterization by repeating the same procedure except for using 50 mL YPAC liquid medium in 250 mL un-baffled shake flasks. Samples were taken during the entire growth period, OD_{600}, concentrations of cellobiose, glucose, and ethanol were measured by UV–Vis spectrophotometer and HPLC. The sugar consumption rate and ethanol productivity were compared between the selected mutant strain and the parent strain. If the selected mutant strain was better than the parent strain, then another round of directed evolution would be carried out until no further improvement is observed. After each round, the selected plasmids were always transformed into the fresh host to avoid any adaptation of the host.

Fermentation and HPLC Analysis

Samples from fermentation were first centrifuged at 13,500*g* for 5 min and the supernatants were diluted 10 or 20 times before HPLC analysis. The HPLC system equipped with an HPX-87H column (BioRad, Hercules, CA) and a refractive index detector (Shimadzu Scientific Instruments, Columbia, MD). Cellobiose, glucose, and ethanol in the samples were analyzed following the manufacturer's instructions using 5 mM sulfuric acid as the mobile phase. The LC solution Software (Shimadzu Scientific Instruments) was used for the analysis of HPLC chromatograms.

Quantitative PCR (qPCR)

Cells were cultured in YPAD or YPAC medium and harvested at the exponential phase. Immediately, the total RNA was

isolated using the RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's instruction. Isolated RNA samples were first cleaned using TURBO DNA-free Kit (Ambion, Inc., Austin, TX). The cleaned RNA samples were then reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) with oligo-dT primer following the manufacturer's instructions. Promoters for ENO2 (For 5′- AATCCCCTCGCTTCCTATTTG-3′, Rev 5′-TCCTGATACCGTCCCTCATC-3′). Primers for PDC1 (For 5′-CTTTGGAAAGTACCCCGACTC-3′, Rev 5′-AGTTGGCTGTGTAGTGGTTC-3′). The qPCR was performed using LightCycler 480 SYBR Green Master (Roche) on a Roche Light Cycler® 480 System (Roche, Indianapolis, IN) following the manufacturer's instructions. The relative abundance of the mRNA levels for the target genes was normalized to the asparagine-linked glycosylation 9 gene (ALG9) expression to determine expression levels (Morrison et al., 1998).

Quantitative Measurements of Intracellular Metabolites

Quantitative measurement of intracellular metabolites was performed via the methanol quenching method (Villas-Boas and Bruheim, 2007; Villas-Boas et al., 2005). Cells were cultured in YPAC medium to the exponential growth phase. 1 mL sample was taken and immediately injected into 10 mL 60% (v/v) buffered methanol (−58°C in acetone–dry ice mixture, 10 mM HEPES) (Bolten and Wittmann, 2008), mixed and centrifuged for 5 min at 13,500*g* at −19°C (rotor precooled to −40°C). The cells were quickly washed four times at −19°C to ensure complete removal of the medium. Intracellular metabolites were extracted using the boiling ethanol extraction method (Canelas et al., 2009). Boiling ethanol (75% ethanol, 95°C) was quickly poured over the cell pellet, vortexed immediately, and incubated at 95°C. After 3 min, the sample was centrifuged 5 min at 13,500*g*. The supernatant was lyophilized and stored at −80°C until use.

Results

Construction of a Cellobiose Utilizing Industrial *S. cerevisiae* Strain

A heterologous cellobiose utilizing pathway consisting of the wild type ENO2 promoter and the wild type PDC1 promoter was introduced into an industrial *S. cerevisiae* strain (Du et al., 2012). The resulting strain, named parent strain, exhibited a lag phase as long as 100 h and very low cellobiose consumption rate (0.388 g/L/h) and ethanol productivity (0.137 g/L/h; Fig. 2, Table I).

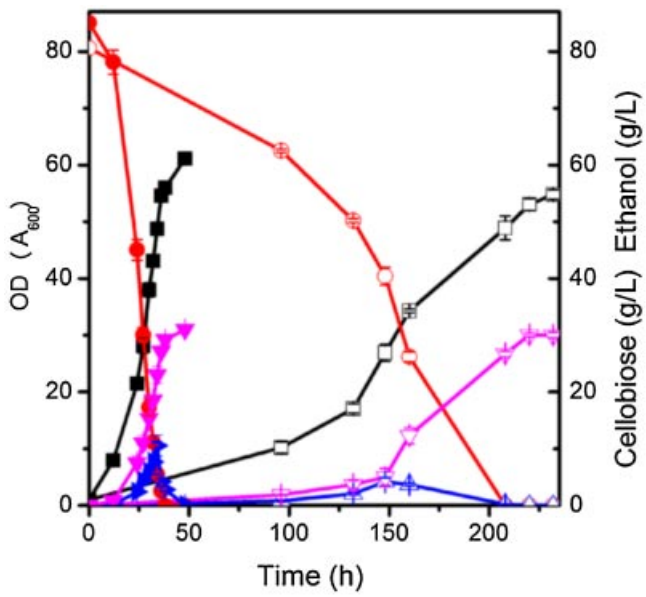

Figure 2. Comparison of fermentation performance between the parent strain and strain #9. Open symbol: the parent strain with the original heterologous pathway. Solid symbol: #9 strain with the evolved heterologous pathway after the first round of directed pathway evolution. Circle: cellobiose, square: OD (A_{600}), down triangle: ethanol, up triangle: glucose.

Directed Pathway Evolution

To improve the efficiency of the cellobiose utilization pathway, a library of ENO2 promoter mutants with varying strengths and a library of PDC1 promoter mutants with varying strengths were created by error-prone PCR as described in Materials and Methods Section. For each promoter, a total of 10 promoter mutants were randomly selected for DNA sequencing. An average mutation rate of 12 nucleotide substitutions per kb for the ENO2 promoter and an average mutation rate of 16 nucleotide substitutions per kb for the PDC1 promoter were obtained. These two promoter libraries, together with the CDT gene, the BGL gene, and the pRS-KanMX backbone, were transformed into the Classic Turbo Yeast strain to generate a library of cellobiose utilizing pathways via the DNA assembler method (Shao et al., 2009). The resulting strains were analyzed using a colony-size

Table I. Comparison of the fermentation profiles of the evolved strains and the reported best cellobiose fermenting strain in the literature.

	Parent	#9	#9-1	#9-1-18	Ha et al. (2011)[a]
Cellobiose consumption (g cellobiose/L/h)	0.39 ± 0.13	2.24 ± 0.11	2.50 ± 0.12	2.50 ± 0.21	1.67
Ethanol productivity (g ethanol/L/h)	0.14 ± 0.04	0.77 ± 0.02	0.81 ± 0.02	0.89 ± 0.01	0.70
Yield (g ethanol/g cellobiose)	0.37 ± 0.02	0.36 ± 0.01	0.36 ± 0.01	0.37 ± 0.01	0.42

[a]Estimated based on the data shown in (Ha et al., 2011).

based high throughput screening method (Du et al., 2012). After screening a total of 2×10^5 strains, one particular strain, named as #9, exhibited a 5.7-fold higher cellobiose consumption rate and a 5.5-fold higher ethanol productivity of 0.77 g/L/h compared to the parent strain (Fig. 2, Table I). A second round of directed evolution was performed using the plasmid isolated from strain #9 as template for error-prone PCR, which resulted in strain #9-1. This strain showed 11.6% higher cellobiose consumption rate (2.5 g/L/h) and 5.2% higher ethanol productivity (0.81 g/L/h) compared to strain #9 (Fig. 3b and d, Table I). A third round of directed evolution using the plasmid isolated from strain #9-1 as template led to strain #9-1-18 with fermentation ability similar to strain #9-1 (Fig. 3, Table I). Since the selected plasmids were always transformed into the fresh host after each round of evolution, all of above improvements were due to the optimized pathway rather than strain adaptation.

Compared to the wild type ENO2 promoter, the ENO2 promoter mutants from strains #9, #9-1, and #9-1-18 had 6, 9, and 11 nucleotide substitutions, respectively. There were five substitutions between the ENO2 promoter mutants from strains #9 and #9-1, but only two substitutions between the ENO2 promoter mutants from strains #9-1 and #9-1-18. In contrast, more substitutions were found in the PDC1 promoter mutants with 20, 26, and 25 nucleotide substitutions for those corresponding strains #9, #9-1, and #9-1-18 compared to the wild type PDC1 promoter. Six nucleotide substitutions were found between the PDC1 promoter mutants from strains #9 and #9-1, but only one nucleotide substitution between the PDC1 promoter mutants from

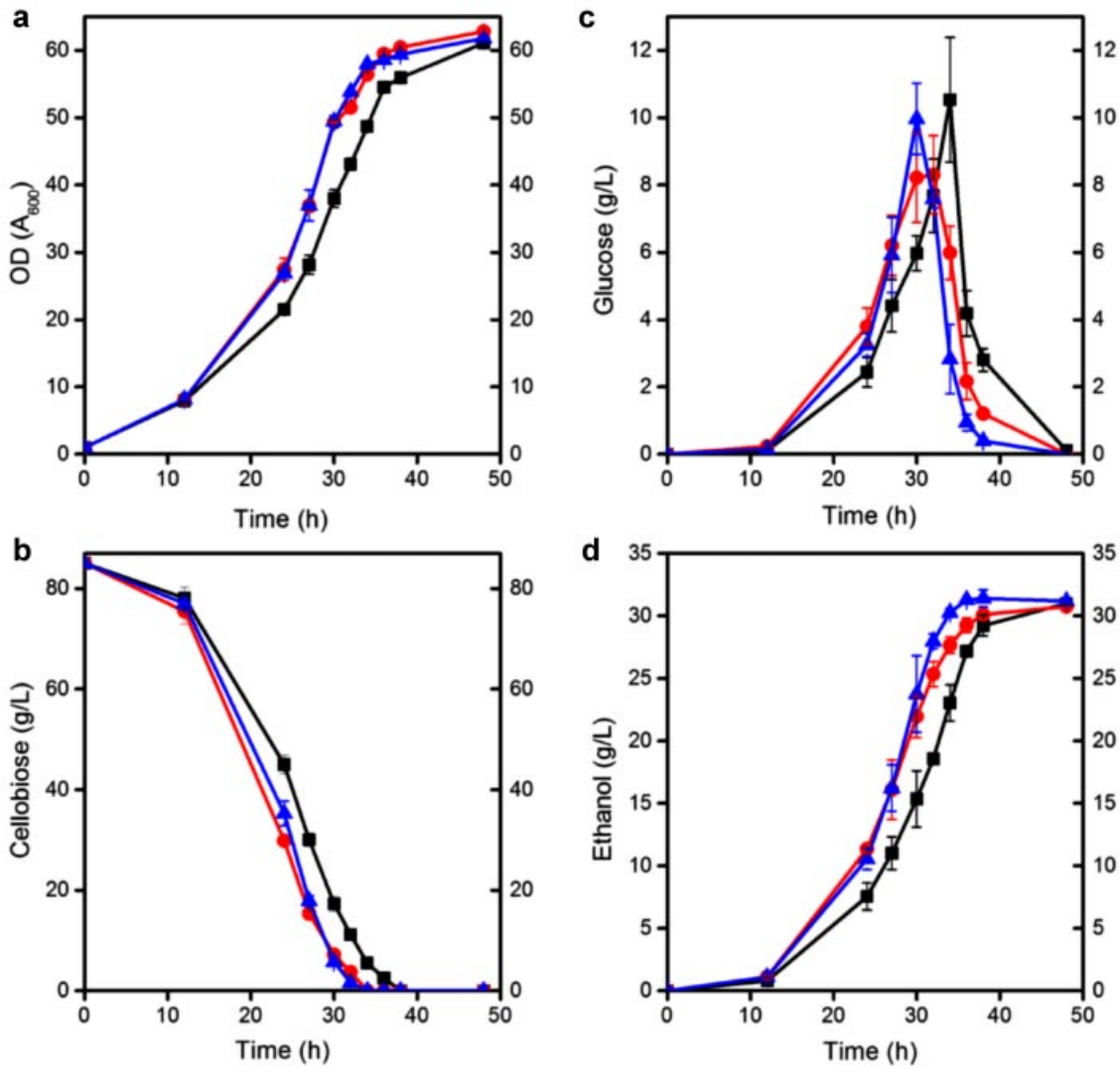

Figure 3. Directed evolution of a highly efficient cellobiose utilizing industrial *S. cerevisiae* strain. Solid symbols for **a**–**d** (black square: strain #9, red circle: strain #9-1, blue up triangle: strain #9-1-18). a: Comparison of OD_{600} between evolved strains. b: Comparison of cellobiose consumption between evolved strains. c: Comparison of extracellular glucose concentration between evolved strains. d: Comparison of ethanol concentration between evolved strains.

strains #9-1 and #9-1-18 (Supplementary Fig. 1). No mutant was found in CDT and BGL genes and terminators.

Characterization of the Evolved Strains

The expression levels of the CDT and BGL in the evolved cellobiose utilizing strains #9, #9-1, #9-1-18 and the parent cellobiose utilizing strain were determined by quantitative PCR in cellobiose cultivation. Both genes exhibited dramatically higher expression levels in the evolved strains compared to the parent strain. The CDT expression levels of #9, #9-1, and #9-1-18 were increased by 13.89-fold, 16.05-fold and 24.40-fold, respectively. The BGL expression levels were increased by 2.56-, 3.24-, and 3.00-fold, respectively (Fig. 4a). However, all the three evolved strains, #9, #9-1, and #9-1-18, showed similar BGL/CDT ratios ranging from 1.7 to 2.8, which were significantly lower than the ratio in the parent strain (13.8; Fig. 4a). In contrast, compared to the parent strain, CDT expression levels of #9, #9-1, and #9-1-18 dropped 4.8-, 5.6-, 3.9-fold in glucose cultivation (Fig. 4b).

Extra- and intra-cellular quantification of cellobiose were performed to determine and compare cellobiose concentrations among the evolved strains and the parent strain. No significant variation of intracellular cellobiose concentrations was observed (Supplementary Fig. 4). To further investigate the potential toxicity of intracellular cellobiose to the cell growth, the parent strain was cultivated in the YPA medium supplemented with two different sugar substrates, 4% glucose and a mixture of 4% glucose and 4% cellobiose. In the first 14 h, they showed a very similar growth curve (Supplementary Fig. 3a), suggesting that cellobiose does not inhibit glucose metabolism. In parallel, the potential toxicity of the intracellular cellobiose was also evaluated in xylose fermentation. A plasmid containing both a xylose utilizing pathway and a cellobiose utilizing pathway (the same cellobiose utilizing pathway as in the parent strain in this study) was constructed for this investigation. The cultivation of the strain harboring both pathways was performed in the YPA medium supplemented with three different sugars: 4% cellobiose, 4% xylose, and a mixture of 4% cellobiose and 4% xylose. The cell growth rate in xylose fermentation was the same as that in co-fermentation of xylose and cellobiose before the start of cellobiose consumption (Supplementary Fig. 3b).

Discussion

Microbial synthesis of fuels and chemicals often requires the introduction of heterologous pathway(s) into host organisms (Du et al., 2011; Keasling, 2008). However, it remains a key challenge to balance the flux of newly imported pathway(s). In this study, we developed a novel directed pathway evolution strategy to create a highly efficient cellobiose utilizing industrial yeast strain for ethanol production. The final evolved strain showed 6.41- and 6.35-fold higher cellobiose consumption rate and ethanol productivity, respectively, than the parent strain (Table I).

Balancing the flux of the cellobiose utilizing pathway for ethanol production was achieved by continuously fine-tuning the expression levels of the CDT gene and the BGL gene. It was observed that the expression levels of both genes could dramatically affect the cell growth rate (particularly the lag phase) in cellobiose cultivation (Supplementary Fig. 2, all

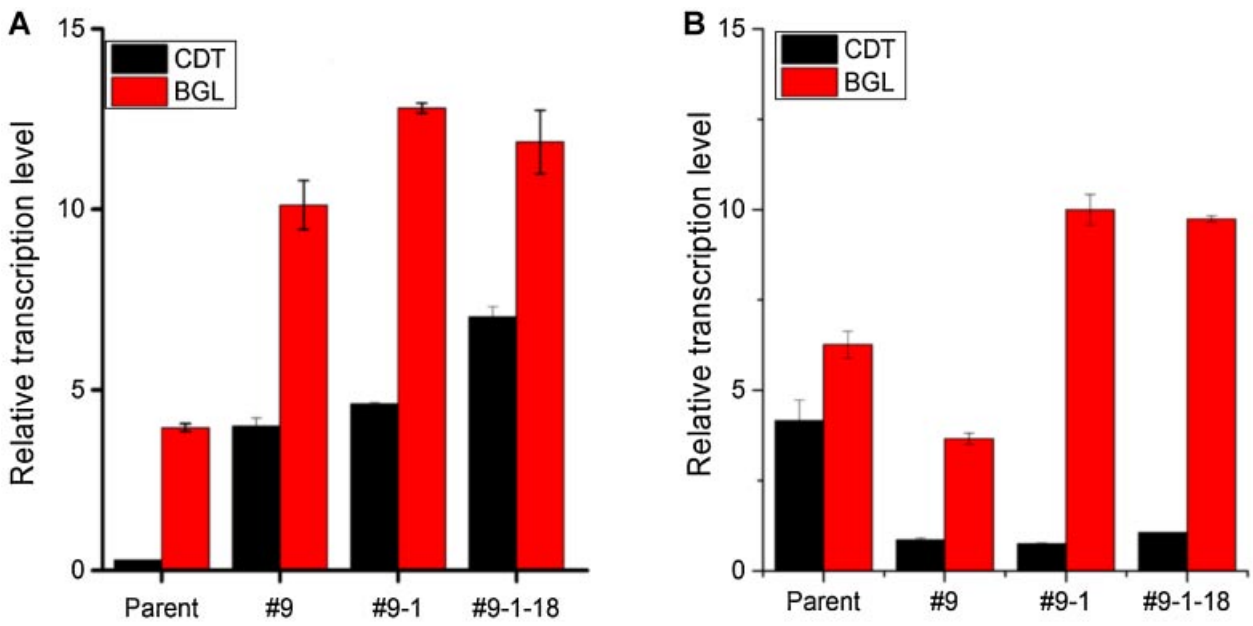

Figure 4. Relative transcription levels of the cellobiose transporter (CDT) gene and the β-glucosidase (BGL) gene in the evolved strains measured by qPCR during cellobiose cultivation (**a**) and glucose cultivation (**b**).

promoter strengths were determined in glucose cultivation). The concentrations of intracellular cellobiose and glucose were determined by the cellobiose uptake rate and the cellobiose hydrolysis rate. By carefully adjusting these two rates, the intracellular concentrations of both cellobiose and glucose inside cells will be at an optimized condition which benefits the cell growth rate and ethanol productivity. After the first round of directed pathway evolution, the cellobiose consumption rate and ethanol productivity of the evolved strain (#9) were significantly improved, which is consistent with the fact that the ENO2 and PDC1 promoters in strain #9 exhibited much higher expression levels in cellobiose fermentation compared to the parent strain (Figs. 2 and 4a). The improved CDT and BGL expressions may accelerate the cellobiose uptake rate and the corresponding hydrolysis rate of cellobiose to glucose. In contrast, both genes showed much lower expression during glucose fermentation in strain #9 than that in the parent strain (Fig. 4b), which indicates that this strategy enables tailor-made optimization of a target pathway. This result is actually consistent with the first principle of directed evolution, "you get what you screen for" (Cobb et al., 2012a). In the meantime, the BGL/CDT ratio was changed dramatically from 13.8 (parent strain) to 2.5 (strain #9), which demonstrates that the expression levels of both CDT and BGL genes in the pathway were optimized simultaneously. Taken together, these results indicate that both the absolute expression levels of the CDT and BGL genes and the BGL/CDT ratio are critical to cellobiose utilization.

It should be noted that our directed evolution strategy is more advantageous than the traditional approach of simply changing promoters. First, our strategy is capable to perform fine tuning of the transcription levels of all pathway genes simultaneously. However, it will be difficult to obtain an optimized pathway by simply changing promoters because there are a limited number of native constitutive promoters available and the dynamic ranges of the strengths of these promoters are limited. Second, our strategy is more amenable to construct a pathway library with large size and good diversity. In our strategy, a promoter library containing mutants with varying strengths but same length and nearly identical sequence identity is used for each pathway gene. As such, each promoter variant will have the same chance to be incorporated into a variant pathway through homologous recombination. In comparison, if different native promoters are used for each pathway gene, due to their varying lengths and low sequence similarity, they will have different chances to be incorporated into a variant pathway through homologous recombination, resulting in poor library diversity and limited library size.

Despite the significant improvement in cellobiose utilization, the cellobiose consumption rate in the final evolved strain (#9-1-18) was still not as fast as that in glucose fermentation (80 g/L glucose is consumed in 10 h, data not shown). Moreover, a large amount of glucose was accumulated in the evolved strains (Fig. 3c). We first hypothesized that the overexpression of the CDT may be toxic to the cells because it is a membrane protein. However, the comparison of the glucose fermentation profiles between an evolved strain with the cellobiose utilizing pathway and the same host without the cellobiose utilizing pathway indicated that the overexpression of both the CDT and the BGL had very little effect on cell growth (data not shown). Secondly, we hypothesized that the transported intracellular cellobiose might have caused the lag phase by repressing certain enzymes in glucose metabolism. Both extracellular and intracellular cellobiose were quantitatively measured and compared between the evolved strains (Supplementary Fig. 4). To avoid the contamination of the intracellular cellobiose from extracellular cellobiose, the quenched cells were washed with cold water–methanol (60% v/v) four times. No intracellular glucose was detected, indicating that the contamination from extracellular metabolites was negligible (Supplementary Fig. 4). The intracellular cellobiose levels were similar among the three evolved strains even though they had totally different growth rates. Moreover, co-fermentation of cellobiose with glucose and xylose showed that the presence of cellobiose did not significantly affect the glucose and xylose consumption rates compared to the control without cellobiose (Supplementary Fig. 3). Therefore, the intracellular cellobiose itself was not toxic to the cell growth and not the key factor affecting the growth rate.

Since the intracellular cellobiose and the CDT did not show toxicity to the cell growth, the intermediate in the cellobiose utilizing pathway, β-glucose, is very likely one of the key factors affecting glucose metabolism. Cellobiose consists of two glucose molecules linked by a $\beta(1 \rightarrow 4)$ bond and it can be broken down into two β-glucose molecules by enzymatic hydrolysis. Although β-glucose can be easily converted to α-glucose in the media, the efficiency of this conversion might be limited under the intracellular environment. In this study, we observed that the extracellular glucose levels in the evolved strains (10 g/L) were higher than the parent strain (4 g/L; Fig. 2), which indicates that the subsequent β-glucose metabolism might be the rate-limiting step in cellobiose fermentation in yeast.

Conclusions

In summary, we developed a new strategy for pathway optimization which consists of directed evolution and promoter engineering. As a demonstration, this strategy was used to create an industrial *S. cerevisiae* strain that exhibited significantly improved cellobiose consumption rate and ethanol productivity. Further characterization of the evolved strains provided new insights on the mechanisms of cellobiose metabolism. It was demonstrated that the absolute expression levels of the CDT gene and the BGL gene, the BGL/CDT ratio, and the intermediates in cellobiose catabolism are all key factors influencing cellobiose utilization in yeast. This simple yet efficient evolutionary strategy should allow rapid development of microbial systems for production of useful biofuels, chemicals, pharmaceuticals, and other value-added compounds.

This work was supported by Energy Biosciences Institute.

References

Alper H, Fischer C, Nevoigt E, Stephanopoulos G. 2005. Tuning genetic control through promoter engineering. Proc Natl Acad Sci USA 102:12678–12683.

Anthony JR, Anthony LC, Nowroozi F, Kwon G, Newman JD, Keasling JD. 2009. Optimization of the mevalonate-based isoprenoid biosynthetic pathway in *Escherichia coli* for production of the anti-malarial drug precursor amorpha-4,11-diene. Metab Eng 11:13–19.

Bolten CJ, Wittmann C. 2008. Appropriate sampling for intracellular amino acid analysis in five phylogenetically different yeasts. Biotechnol Lett 30:1993–2000.

Bond-Watts BB, Bellerose RJ, Chang MCY. 2011. Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways. Nat Chem Biol 7:222–227.

Canelas AB, ten Pierick A, Ras C, Seifar RM, van Dam JC, van Gulik WM, Heijnen JJ. 2009. Quantitative evaluation of intracellular metabolite extraction techniques for yeast metabolomics. Anal Chem 81:7379–7389.

Cobb RE, Si T, Zhao H. 2012a. Directed evolution: An evolving and enabling synthetic biology tool. Curr Opin Chem Biol 16:285–291.

Cobb RE, Sun N, Zhao H. 2013. Directed evolution as a powerful synthetic biology tool. Methods 60:81–90.

Du J, Shao Z, Zhao H. 2011. Engineering microbial factories for synthesis of value-added products. J Ind Microbiol Biotechnol 38:873–890.

Du J, Yuan Y, Si T, Lian J, Zhao H. 2012. Customized optimization of metabolic pathways by combinatorial transcriptional engineering. Nucleic Acids Res 40:e142.

Dueber JE, Wu GC, Malmirchegini GR, Moon TS, Petzold CJ, Ullal AV, Prather KL, Keasling JD. 2009. Synthetic protein scaffolds provide modular control over metabolic flux. Nat Biotechnol 27:753–759.

Galazka JM, Tian C, Beeson WT, Martinez B, Glass NL, Cate JH. 2010. Cellodextrin transport in yeast for improved biofuel production. Science 330:84–86.

Ghosh P, Pamment NB, Martin WRB. 1982. Simultaneous saccharification and fermentation of cellulose: Effect of β-D-glucosidase activity and ethanol inhibition of cellulases. Enzyme Microb Technol 4:425–430.

Ha SJ, Galazka JM, Kim SR, Choi JH, Yang X, Seo JH, Glass NL, Cate JH, Jin YS. 2011. Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. Proc Natl Acad Sci USA 108:504–509.

Johannes TW, Zhao H. 2006. Directed evolution of enzymes and biosynthetic pathways. Curr Opin Microbiol 9:261–267.

Keasling JD. 2008. Synthetic biology for synthetic chemistry. ACS Chem Biol 3:64–76.

Lee SK, Chou HH, Pfleger BF, Newman JD, Yoshikuni Y, Keasling JD. 2007. Directed evolution of AraC for improved compatibility of arabinose- and lactose-inducible promoters. Appl Environ Microbiol 73:5711–5715.

Li SJ, Du J, Sun J, Galazka JM, Glass NL, Cate JHD, Yang XM, Zhao HM. 2010. Overcoming glucose repression in mixed sugar fermentation by co-expressing a cellobiose transporter and a beta-glucosidase in *Saccharomyces cerevisiae*. Mol BioSyst 6:2129–2132.

Luetke-Eversloh T, Stephanopoulos G. 2008. Combinatorial pathway analysis for improved L-tyrosine production in *Escherichia coli*: Identification of enzymatic bottlenecks by systematic gene overexpression. Metab Eng 10:69–77.

Lynd LR, Cushman JH, Nichols RJ, Wyman CE. 1991. Fuel ethanol from cellulosic biomass. Science 251:1318–1323.

Morrison TB, Weis JJ, Wittwer CT. 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 24:954–958; 960–962.

Nevoigt E. 2008. Progress in metabolic engineering of *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 72:379–412.

Pfleger BF, Pitera DJ, Smolke CD, Keasling JD. 2006. Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. Nat Biotechnol 24:1027–1032.

Philippidis GP, Smith TK, Wyman CE. 1993. Study of the enzymatic hydrolysis of cellulose for production of fuel ethanol by the simultaneous saccharification and fermentation process. Biotechnol Bioeng 41:846–853.

Sadie CJ, Rose SH, den Haan R, van Zyl WH. 2011. Co-expression of a cellobiose phosphorylase and lactose permease enables intracellular cellobiose utilisation by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 90:1373–1380.

Salis HM, Mirsky EA, Voigt CA. 2009. Automated design of synthetic ribosome binding sites to control protein expression. Nat Biotechnol 27:946–950.

Schmidt-Dannert C. 2001. Directed evolution of single proteins, metabolic pathways, and viruses. Biochemistry 40:13125–13136.

Shao Z, Zhao H, Zhao H. 2009. DNA assembler, an in vivo genetic method for rapid construction of biochemical pathways. Nucleic Acids Res 37:e16.

Shen CR, Lan EI, Dekishima Y, Baez A, Cho KM, Liao JC. 2011. Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. Appl Environ Microb 77:2905–2915.

Sun J, Shao Z, Zhao H, Nair N, Wen F, Xu JH, Zhao H. 2012. Cloning and characterization of a panel of constitutive promoters for applications in pathway engineering in *Saccharomyces cerevisiae*. Biotechnol Bioeng 109:2082–2092.

Villas-Boas SG, Bruheim P. 2007. Cold glycerol-saline: The promising quenching solution for accurate intracellular metabolite analysis of microbial cells. Anal Biochem 370:87–97.

Villas-Boas SG, Hojer-Pedersen J, Akesson M, Smedsgaard J, Nielsen J. 2005. Global metabolite analysis of yeast: Evaluation of sample preparation methods. Yeast 22:1155–1169.

Wen F, Sun J, Zhao H. 2010. Yeast surface display of trifunctional minicellulosomes for simultaneous saccharification and fermentation of cellulose to ethanol. Appl Environ Microbiol 76:1251–1260.

Supporting Information

Additional Supporting information may be found in the online version of this article at the publisher's web-site.