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RNAi Assisted Genome Evolution Unveils Yeast Mutants with Improved Xylose Utilization

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Abstract

Xylose is a major component of lignocellulosic biomass, one of the most abundant feedstocks for biofuel production. Therefore, efficient and rapid conversion of xylose to ethanol is crucial in the viability of lignocellulosic biofuel plants. In this study, RNAi Assisted Genome Evolution (RAGE) was used to improve the xylose utilization rate in SR8, one of the most efficient publicly available xylose utilizing *Saccharomyces cerevisiae* strains. To identify gene targets for further improvement, we created a genome-scale library consisting of both genetic over-expression and down-regulation mutations in SR8. Followed by screening in media containing xylose as the sole carbon source, yeast mutants with 29% faster xylose utilization and 45% higher ethanol productivity were obtained relative to the parent strain. Two known and two new effector genes were identified in these mutant strains. Notably, down-regulation of *CDC11*, an essential gene, resulted in faster xylose utilization, and this gene target cannot be identified in genetic knock-out screens.

Introduction

Global warming and the instability of energy resources with fluctuating prices have resulted in an increasing interest in alternative energy resources (Asif and Muneer, 2007), with biofuels as one promising option. Each year, billions of gallons of bioethanol are produced in the United States alone, but they are primarily produced from food feedstocks such as corn starch and sugarcane, urging the exploration of alternative feedstock sources such as agricultural residues and non-food crops (Li et al., 2010; Ha et al., 2011; Jeffries and Jin, 2004; Lian et al., 2014; Wei et al., 2015). Lignocellulosic agricultural residues account for more than half of the agricultural phytomass in the world (Smil et al., 1999), and are considered a cost effective feedstock for biofuel production (Somerville et al., 2010). Other than incentives from the Environmental Protection Agency and Renewable Fuel Standard to move beyond corn biofuel, there are tremendous economic incentives to switch to low-cost lignocellulose.

Saccharomyces cerevisiae is one of the most widely used cell factories for the conversion of sugars to biofuels, thanks to its tolerance to harsh industrial conditions and resistance to phage contamination (Chen et al., 2013). However, *S. cerevisiae* cannot consume all lignocellulose-derived sugars, such as xylose, which may comprise up to 40% of the total sugars. Substantial improvement in xylose conversion may contribute to economically feasible production of lignocellulosic ethanol, and there have been many studies on engineering *S. cerevisiae* strains to improve xylose utilization (Jin et al., 2004a; Jin and Alper, 2005; Kuyper et al., 2005; Kwak and Jin, 2017; Lee et al., 2014; Zhou et al., 2012). Successful strategies include protein engineering of xylose transporters (Hahn-Hägerdal et al., 2001; Wang et al., 2016) and assimilation enzymes (Jeffries and Jin, 2004), as well as pathway engineering targeting flux optimization (Du et al., 2012; Feng and Zhao, 2013) and co-factor imbalance (Wei et al., 2013; Zhang et al., 2017), which

are all among the most powerful pathway optimization methods (Garcia-Ruiz et al., 2016). Despite these extensive efforts, however, low xylose utilization rate and biofuel productivity are still major hurdles to commercialize lignocellulosic biofuel production.

Resulted from both rational and evolutionary engineering, SR8 is one of the fastest growing and most efficient xylose utilizing strains ever reported in literature (Li et al., 2016). Serial subculturing in xylose media revealed a beneficial, loss-of-function mutation in *PHO13* (Kim et al., 2013). The mechanism of this improvement was later found to be, among others, transcriptional activation of genes in the pentose phosphate pathway (PPP) (Xu et al., 2016). The *ALD6* gene was also deleted to prevent acetate accumulation and improve the efficiency of xylose fermentation (Li et al., 2016). The present study aims to further optimize SR8 for enhanced xylose utilization rate and ethanol productivity. We hypothesize that even in an intensively engineered strain, iterative genome-scale screening may help to identify new genetic targets. RNA interference (RNAi) is a commonly used gene silencing mechanism in Eukaryotes and has been widely used in down-regulating genes in a wide range of organisms ranging from human cell lines (Hannon and Rossi, 2004) to yeast cells (Si et al., 2015a). RNAi can be used to perform genome-wide profiling by down-regulation of genes and then assess the effects of down-regulation on the phenotype of interest (Lee et al., 2002; Sönnichsen et al., 2005). We recently developed RNAi Assisted Genome Evolution (RAGE) for iterative genome-scale engineering in yeast (Si et al., 2014; Si et al., 2017) . In particular, a full-length cDNA library was constructed in the SR8 strain and processed into genome-wide modulation cassettes encoding both down-regulation and over-expression mutations. In each round of RAGE screening, modulation cassettes conferring the largest phenotypic improvement were integration to create new parent strains for the next round to identify

potential synergy between beneficial mutations (Figure 1). After three rounds of RAGE, the xylose utilization rate and ethanol productivity were improved by 29% and 45%, respectively.

Materials and Methods

Strains, media and cultivation conditions

S. cerevisiae strain SR8 (Kim et al., 2013) was a generous gift from Dr. Yong-Su Jin at the University of Illinois at Urbana-Champaign. Top10 chemical competent *E. coli* and NEB 10β electrocompetent *E. coli* (New England Biolabs, Ipswich, MA) cells were used for plasmid amplification and library construction, respectively. *S. cerevisiae* strains were cultivated in synthetic complete (SC) media with the appropriate dropout (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.083% amino acid dropout mix, 0.5% ammonium sulfate, 0.01% adenine hemisulfate and 2% glucose or 4% xylose as appropriate) or YPD medium (1% yeast extract, 2% peptone, and 2% glucose) for seed culture preparation, and plasmid amplification and isolation as appropriate. YPX 4% (1% yeast extract, 2% peptone, and 4% xylose) was used as the fermentation media and the cells were grown in oxygen limiting conditions (30°C, 100 rpm in unbaffled flasks) to assess the xylose utilization rate and ethanol productivity. Growth media was supplemented with 200 μg/mL of hygromycin B, or 200 μg/mL of G418 to select mutants harboring the plasmid or integrated cassettes as needed. The LiAc/SS DNA/PEG method was used for yeast transformation (Gietz and Schiestl, 2007) and the cells were recovered for 4 hours after heat shock in YPD media when antibiotics were used as the selection marker. *E. coli* cells were grown in Luria Broth (LB) medium (Fisher Scientific, Pittsburgh, PA) with 100 μg/mL of ampicillin or 25 μg/mL kanamycin to maintain the plasmid as appropriate. All restriction enzymes, Q5 polymerase, and the *E. coli* - *S. cerevisiae* shuttle vectors were purchased from New

England Biolabs (Ipswich, MA) and all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. A summary of all strains used in this study is shown in Table 1.

DNA manipulation and plasmid construction

Zymoprep II yeast plasmid isolation kit (Zymo Research, Irvine, CA) was used to isolate plasmids from overnight *S. cerevisiae* cell cultures and QIAGEN Plasmid Mini Kit (QIAGEN, Valencia, CA) was used to isolate plasmids from *E. coli* cells. RNA was extracted using FastRNA™ SPIN Kit for Yeast (MP Biomedicals, Santa Ana, CA).

The pITy3 plasmid designed for integration in the delta sites in *S. cerevisiae* using G418 as the marker was obtained from Dane Wittrup's lab and was described elsewhere (Parekh et al., 1996). Since the G418 marker was used for integration of the RNAi machinery, pITy3 was modified and the kanamycin marker was replaced with an ampicillin marker and the hygromycin resistance gene was inserted using the Gibson assembly method (Gibson et al., 2009), allowing the construction of the new pITyH-amp plasmid. The best mutants from the first round of screening were amplified and inserted into the pITyH-amp plasmid using the DNA assembler method (Shao et al., 2008). The best mutants from the second round were inserted in the pRS406 plasmid using the DNA assembler method and integrated into the genome and grown on SC-URA agar plates as the selection pressure. Since both down-regulation and over-expression libraries were constructed on pRS416, the Uracil marker on the integrated strain was recovered by amplifying the *URA3* gene from *S. cerevisiae* CEN.PK strain genomic DNA and transforming it into the best mutant of the second round and plating the cells on SC-complete supplemented with 1 mg/mL of 5-fluoroorotic acid (5-FOA) to eliminate all the cells containing the functional URA marker (Widlund and Davis,

2005). All the plasmids used and cloned in this study are summarized in Table 1 and all the primers are summarized in Table S1.

Analytical methods

Cell growth was determined by measuring the absorbance at 600nm from a Biotek Synergy 2 Multi-Mode Microplate Reader (Winooski, VT). Xylose and ethanol concentrations were measured using a Shimadzu HPLC (Columbia, MD) with an Aminex HPX-87 column (Bio-Rad, Hercules, CA) and Shimadzu RID-10A refractive index detector. The column temperature was 65°C and the mobile phase for HPLC was 0.5 mM sulfuric acid solution with the flow rate of 0.6 mL/min. All data points shown were the mean of at least two replicates and data points for ethanol fermentation and xylose consumption were performed in three replicates unless noted otherwise.

Construction of genome-wide cDNA over-expression and down-regulation libraries

The cDNA library was prepared using the In-Fusion SMARTer Directional cDNA Library Construction Kit (Clontech Laboratories, Mountain View, CA), following the manufacturer's instructions and our previous work (Si et al., 2017) with some modifications. In short, total RNA was extracted from the SR8 strain while grown on YPX4% media and harvested in the mid-log phase, the cDNA was reverse transcribed from the RNA and in the process, two 15 bp adaptor sequences of 5'-AAGCAGTGGTATCAA-3' and 5'-CGGGGTACGATGAGA-3' were added to the different ends of the cDNA molecules to control the directionality of expression cassettes in the expression plasmids. The cDNA library was then cloned in two directions in two separate pRS416 plasmids under the expression of a P_{TEF1} promoter and a T_{PGK1} terminator using In-Fusion cloning kit (Clontech Laboratories, Mountain View, CA). The assembled plasmid was then

transformed into NEB 10β electrocompetent *E. coli* (New England Biolabs, Ipswich, MA) and recovered for 1 hour in SOC outgrowth media. Following the cell recovery, all the cells were plated on 50 LB+Amp agar plates for amplification. Solid media was chosen for amplification to prevent the possible bias resulting from the different growth rates in liquid media due to size difference among the cDNA inserts. Roughly 10^6 independent colony forming units (CFU) were observed, resulting in roughly 100-fold coverage of yeast genes. The colonies were scraped off the plates and the plasmids were isolated using QIAGEN Plasmid Midi Kit (QIAGEN, Valencia, CA).

To test the diversity of the libraries, both over-expression and down-regulation libraries were transformed into *E. coli* cells. Five colonies were picked from each of the over-expression and down-regulation libraries and the harboring plasmids were amplified and purified. The isolated plasmids were sequenced by ACGT, Inc. (Wheeling, IL). Each of the 10 sequenced plasmids contained a different insert, suggesting efficient assembly and satisfactory diversity.

The RNAi machinery constructed and reported in our previous studies (Si et al., 2014; Si et al., 2017; Xiao and Zhao, 2014) was integrated into the SR8 genome with G418 selection. The genomic DNA from the positive colonies was extracted using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) and the integration of *AGO1* and *DCR1* was confirmed by PCR amplification.

Library screening

The down-regulation and over-expression libraries were transformed into the SR8 strain on selective synthetic media with xylose as the sole carbon source (SCX4%-URA). After 3 days, the 80 largest colonies were visually chosen and picked from each library of ~10^7 colonies and were grown in SC media with glucose. The overnight cultures were then used to inoculate 3 mL

SC 4% xylose media with an initial optical density at 600 nm (OD_{600}) of 0.1 and the growth was monitored using OD_{600} measurements. The fastest growers from both libraries were picked and the harboring plasmids were re-transformed into fresh SR8 cells to isolate the effect of the genes from possible adaptation and confirm the positive effect of manipulation of the genes on cell growth. The top mutants were then identified and the over-expression or down-regulation gene cassettes responsible for the superior growth were integrated into the SR8 genome. The cells were then inoculated in SCD media with the appropriate drop-out or antibiotic selection. The overnight cultures were then inoculated in 10 mL YPX 4% xylose media with an initial OD_{600} of 1 without any antibiotic supplementation and the ethanol production and xylose utilization were measured every 12 hours. Since antibiotic supplementation hinders the growth, and since the genes were already integrated in the genome, it was assumed that the integration was stable enough for the short period of cell growth given the stable nature of the integrated cassette (Bai Flagfeldt et al., 2009).

Estimation of down-regulation and over-expression efficiencies

To estimate the down-regulation and over-expression efficiency, quantitative qPCR was performed. First, the total RNA was extracted from the mutant strains and control SR8 strain grown on YPX4% at mid-log phase using RNeasy mini kit (QIAGEN, Valencia, CA). Reverse transcription was performed on 1ug of RNA to produce first strand cDNA using iScript cDNA synthesis kit (BioRAD, Hercules, CA). For each cDNA sample, the primers for the target gene as well as *ALG9* gene were included as internal control in six replicates for each. Quantitative PCR reaction was performed using QuantStudio 7 Flex Real-Time PCR System with Power SYBR

Green PCR Master Mix (ThermoFisher Scientific). Primers for qPCR are shown in Table S1 as "qPCR-gene_name-F" and "qPCR-gene_name-R".

Results and discussion

Screening of down-regulation and over-expression libraries

The over-expression and down-regulation libraries were separately used to transform the SR8 strain. The transformed cells were screened on media with xylose as the sole carbon source. The best performing mutants from the first round of RAGE were identified. Over-expression of Vacuolar Protein Sorting 13 (*VPS13*) and down-regulation of Cell Division Cycle 11 (*CDC11*) were found to be the most beneficial changes for improved xylose utilization and ethanol productivity. Since VPS13 was superior to *CDC11* in improving the xylose utilization and ethanol productivity, the SR8 with *VPS13* integrated in its genome (named MH1 from this point) was chosen for further optimization.

The second round of RAGE was then performed where the down-regulation and over-expression libraries were transformed into the SR8 with VPS13 and the same screening protocol was repeated. Down-regulation of *CDC11* and over-expression of Cytochrome c Oxidase (*COX5A*) and malate dehydrogenase 1 (*MDH1*) genes were found to improve xylose utilization and ethanol productivity the most. It was notable that down-regulation of *CDC11* was found in both the first and second rounds of RAGE, showing its significance in xylose utilization. The third round of RAGE was performed with the strain harboring *VPS13* and *CDC11* (MH2), but after re-transformation, none of the mutants were superior in xylose utilization and therefore no further rounds of screening were performed.

Analysis of the best mutant

After three rounds of RAGE, the genes responsible for faster xylose utilization were identified and further analyzed. The mean of three replicates was used for comparison of xylose utilization and ethanol productivity between the SR8 and the best mutant of each round (Figure 2). Ethanol productivity and xylose utilization rate in the best mutant were improved by 45% and 29%, respectively, compared with the SR8 strain with no modifications.

To confirm the effectiveness of down-regulation and over-expression, the expression level of the mutants was estimated using quantitative PCR (Figure 3). It was observed that the down-regulation and over-expression was successful. The expression level was considerably reduced in strains with down-regulation cassette and increased with strains with over-expression cassettes integrated.

Characterization of the improved mutants

To examine the dependence of growth on the carbon source, the growth of the same strains was tested in glucose and galactose conditions and it was found that when grown on glucose, all the mutants showed a similar growth rate. However, all the other mutants grew faster than SR8 on galactose media (Figure 4). *S. cerevisiae* was evolved to grow on glucose and it is expected that these genetic modifications were unable to improve its growth in glucose media. However, the improved growth on galactose media may be indicative of glucose repression and regulation that has been observed repeatedly in the literature (Escalante-Chong et al., 2015; Newcomb et al., 2003; Salusjärvi et al., 2008).

To observe its effect on growth, each of the genes from the second round were integrated into the genome and their phenotypes were compared to that of SR8 with no modifications (Figure

5). All of them showed improved xylose utilization and ethanol productivity, but when co-expressed with *VPS13*, they showed faster growth, which suggests a synergistic or additive effect between these genes.

A one-tailed, two-sample unequal-variance t-test was applied, and it was observed that the improved xylose utilization rate (29%) and ethanol productivity (45%) ~~as well as the single mutants shown in Figure 5~~ were statistically significant with a p-value < 0.01 for six biological replicates from two experiments. The change in the ethanol yield was not statistically significant, suggesting that these mutants grew faster but did not necessarily yield more ethanol. This was not unexpected since the screening was based on the growth rate rather than final ethanol production. The comparison between the SR8 strain and the best mutant in the first round and top 3 mutants in the second round is shown in Table 2. The statistical significance of the improvements of best mutants from the second round over SR8 strain (Figure 5) was evaluated and summarized in Table S2 using a two-sample unequal-variance t-test with most of p-values <0.01 and some p-values <0.05.

Discussion

In this study, RAGE was used to improve an extensively engineered yeast strain for xylose assimilation. Using a genome-wide library encoding both over-expression and down-regulation mutations, four gene targets were found to improve the xylose utilization rate in *S. cerevisiae*. Combining these genetic manipulations, 29% and 45% improvement in xylose utilization rates and ethanol productivity were observed, respectively. It was found that over-expression of *VPS13*, *MDH1,* and *COX5A,* as well as down-regulation of *CDC11* improved xylose utilization. These findings provide valuable insights on the bottlenecks of xylose utilization.

Although the exact reason for these genes to be beneficial to xylose utilization rate remains to be elucidated, it is possible to speculate the mechanism resulting in the favorable changes of this phenotype from functions previously described in the literature. The function of these genes are briefly described in Table 3. Jin and co-workers did a genome-wide expression analysis under different carbon sources and oxygen level conditions and found that growth in xylose resulted in more than two-fold up-regulation of many genes in the TCA cycle and respiration pathways (Jin et al., 2004a). Two of the over-expression genes found in our study (*COX5A* and *MDH1*) are in those categories. We speculate that since these genes are over-expressed in xylose media, they may have an important role in xylose utilization which makes them reasonable targets for our genome wide library screening. It was shown that the *MDH1* gene, for example, was expressed twice as much under xylose assimilating conditions as when it was grown on glucose. We hypothesize that the over-expression of *MDH1* is improving xylose utilization by bypassing pyruvate carboxylase and conserving one ATP in the process.

Many reports have suggested that aeration and oxygen supply can greatly affect xylose consumption (Jeffries, 2006; Jeffries and Jin, 2004; Jin et al., 2004a; Kastner et al., 1999; Kim et al., 2010). Fundamental differences in oxygen requirements between growth in xylose and glucose have been observed in fungi and oxygen starvation studies showed induction of cell death on xylose but not glucose in the yeast strain *Candida shehatae* (Kastner et al., 1999). These results emphasized the importance of aeration and electron chain transport in xylose utilization and hence showed the relevance of *COX5A*. In another study, it was also observed that *COX5A* was expressed 3.5 times more under xylose utilizing conditions (Jeffries and Jin, 2004). With ample evidence on the importance of aeration found in literature, it is not unexpected that over-expression of *COX5A* resulted in improved xylose utilization.

To the best of our knowledge, there has been no report associating *CDC11* and *VPS13* to better growth on xylose. However, *CDC11* was observed to be expressed less in media with xylose as the carbon source compared to glucose (Jin et al., 2004b). In *S. cerevisiae*, CDC11 is one of the main septins that by introducing a diffusion barrier between the mother and daughter cells in the bud neck, recruits other proteins to the site of cytokinesis (Finnigan et al., 2015). *CDC11* deficient cells are usually inviable or have a very slow growth rate (Versele et al., 2004) and *ΔCDC11* strains were shown to be inviable on glucose medium at room temperature whereas they were able to be recovered on galactose medium (McMurray et al., 2011). This shows the carbon source dependence of the effect of this gene in the cell and may be related to its effect on xylose utilization rate. Detecting *CDC11* as a target also emphasizes the power of down-regulation over knock-out since *CDC11* is an essential gene and the knock-out strain would not have been viable and thus not found in the screening.

The exact mechanism of over-expression of *VPS13* contributing to xylose utilization is unknown. However, *VPS13* was recently shown to be involved in vacuole–mitochondria interface interaction which could be one possible explanation for improved cell growth (Peter et al., 2017). Both *VPS13* and *CDC11* were reported to affect morphology and cell growth (Finnigan et al., 2015; Park and Neiman, 2012). Since the growth rate in glucose is different from xylose, tuning these genes may be beneficial for xylose utilization since they play a role in the timing of cell division. *VPS13* was also reported to be important for the delivery of proteins to and from the vacuole (Park and Neiman, 2012), which may explain why this gene effects xylose utilization.

It is noteworthy that the SR8 strain has previously been extensively engineered and optimized and therefore is difficult to further improve as it harbors many of the already known beneficial genetic manipulations. RAGE can be a tool for improving the phenotype even when all

other tools have been exhausted. The growth rate did not improve after 5 rounds of adaptation on SR8 Figure S1, but RAGE could still improve the xylose utilization rate by 29%.

Competing interest

The authors declare no competing interest.

Authors' contribution

MH, JL and HZ have contributed in designing the experiments and writing the manuscript. MH, JL and HL have performed the experiments and all authors have approved the final manuscript.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version.

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Figure 1. Overall workflow of RNAi Assisted Genome Engineering. mRNA from SR8 strain is extracted and reverse transcribed to cDNA library. The library is then cloned in forward and reverse directions to generate over-expression and down-regulation libraries respectively. The libraries are then transformed in SR8 strain and screened on solid media with xylose as the sole carbon source. The largest colonies were identified and the improvement was confirmed and the best cassette was integrated into the genome of SR8. The new, improved strain was then used for the next round of RAGE and this cycle was repeated until no further improvements were observed.

Figure 2. Xylose utilization and ethanol production in **a**) SR8; **b**) MH1; **c**) MH2.

Figure 3. Quantitative PCR analysis of the down-regulated and over-expressed gene targets. The expression levels compared are CDC11 in SR8 and MH4, COX5A in SR8 and MH2, MDH1 in SR8 and MH3 and VPS13 in all the strains, respectively.

Figure 4. Growth curve of the best mutants in media with **a)** galactose and **b)** glucose as the sole carbon source

Figure 5. Xylose utilization **(a)** and ethanol production **(b)** in SR8 strain and all the mutants identified in the second round of RAGE.

Table 1. Strains and plasmids used in this study

Strains or plasmids	Name	Characteristics	Reference or source
Strains			
SR8		SR7e3 ald6::AUR1-C pAUR_d_ALD6	(Kim et al., 2013)
SR8+VPS13u	MH1	SR8 pITy3H-Amp $TEF1_p$-VPS13-$PGK1_t$	This study
SR8+VPS13u+COX5Au	MH2	SR8 pITy3H-Amp $TEF1_p$-VPS13-$PGK1_t$ - pRS406- $TEF1_p$- COX5A-$PGK1_t$	This study
SR8+VPS13u+MDH1u	MH3	SR8 pITy3H-Amp $TEF1_p$-VPS13-$PGK1_t$ - pRS406- $TEF1_p$- MDH1-$PGK1_t$	This study
SR8+VPS13u+CDC11d	MH4	SR8 pITy3H-Amp $TEF1_p$-VPS13-$PGK1_t$ - pRS406- $TEF1_p$- $CDC11_{Reverse}$ - $PGK1_t$	This study
SR8+ COX5Au	MH5	SR8 pITy3H-Amp $TEF1_p$-VPS13-$PGK1_t$	This study
SR8+ MDH1u	MH6	SR8 pITy3H-Amp $TEF1_p$- MDH1-$PGK1_t$	This study
SR8+ CDC11d	MH7	SR8 pITy3H-Amp $TEF1_p$- $CDC11_{Reverse}$-$PGK1_t$	This study
Plasmids			
pRS416-RAGE-FWD		pRS416-$TEF1_p$-ccdB-$PGK1_t$	(Si et al., 2015b)
pRS416-RAGE-REV		pRS416-$TEF1_p$- ccdB -$PGK1_t$	
pRS416-over-expression		pRS416-$TEF1_p$-cDNA-$PGK1_t$	This study
pRS416-down-regulation		pRS416-$TEF1_p$-$cDNA_{reverse}$-$PGK1_t$	This study
RNAi Integration		CEN.Ago.Dcr	(Si et al., 2015b)
pITy3			(Parekh et al., 1996)
pITyH-Amp		pITy3-Hygro-Amp	This study

Table 2. Fermentation profiles of SR8 and SR8 with over-expression and down-regulation of beneficial genes found in this study. The fermentation was performed in YPX 4% and SCX4% in oxygen limited condition (100 rpm). Parameters in this table are: r_{xylose}, xylose consumption rate (g/L/h), r_{xylose}^{*}, specific xylose consumption rate (g/g cell/h), $Y_{ethanol}$, ethanol yield (g ethanol/g sugars) and $P_{ethanol}$, ethanol productivity (g ethanol/L/h), $P_{ethanol}^{*}$, specific ethanol productivity (g ethanol/g cell/h).

Growth Media		SR8	MH1	MH2	MH4	MH3
YPX 4%	r_{xylose}	1.16±0.05	1.37±0.03	1.40±0.01	1.50±0.03	1.46±0.02
	r_{xylose}^{*}	0.73±0.09	0.85±0.07	0.88±0.05	0.93±0.03	0.92±0.02
	$Y_{ethanol}$	0.29±0.03	0.29±0.04	0.29±0.05	0.32±0.06	0.32±0.04
	$P_{ethanol}$	0.33±0.02	0.40±0.03	0.42±0.02	0.48±0.04	0.44±0.03
	$P_{ethanol}^{*}$	0.22±0.03	0.27±0.02	0.28±0.03	0.32±0.02	0.29±0.03
SCX 4%	r_{xylose}^{*}	0.56±0.03	0.66±0.04	0.69±0.02	0.76±0.03	0.75±0.04
	$Y_{ethanol}$	0.29±0.01	0.30±0.02	0.28±0.04	0.33±0.06	0.31±0.04
	$P_{ethanol}^{*}$	0.17±0.01	0.22±0.03	0.24±0.03	0.26±0.04	0.24±0.02

Table 3. Known gene functions for beneficial over-expression and down-regulation targets

Gene	Description (www.yeastgenome.com)
VPS13	Protein involved in prospore membrane morphogenesis; peripheral membrane protein that localizes to the prospore membrane and at numerous membrane contact sites; involved in sporulation, vacuolar protein sorting, prospore membrane formation during sporulation, and protein-Golgi retention; required for mitochondrial integrity; contains a PH-like domain; homologous to human CHAC and COH1 which are involved in Chorea-acanthocytosis and Cohen syndrome, respectively
CDC11	Component of the septin ring that is required for cytokinesis; present at the ends of rod-like septin hetero-oligomers; C-terminal extension is important for recruitment of Bni5p to the mother-bud neck, which in turn is required for Myo1p recruitment and cytokinesis; septin rings at the mother-bud neck act as scaffolds for recruiting cell division factors and as barriers to prevent diffusion of specific proteins between mother and daughter cells
COX5A	Subunit Va of cytochrome c oxidase; cytochrome c oxidase is the terminal member of the mitochondrial inner membrane electron transport chain; Cox5Ap is predominantly expressed during aerobic growth while its isoform Vb (Cox5Bp) is expressed during anaerobic growth; COX5A has a paralog, COX5B, that arose from the whole genome duplication
MDH1	Mitochondrial malate dehydrogenase; catalyzes interconversion of malate and oxaloacetate; involved in the tricarboxylic acid (TCA) cycle; phosphorylated

Figure 1

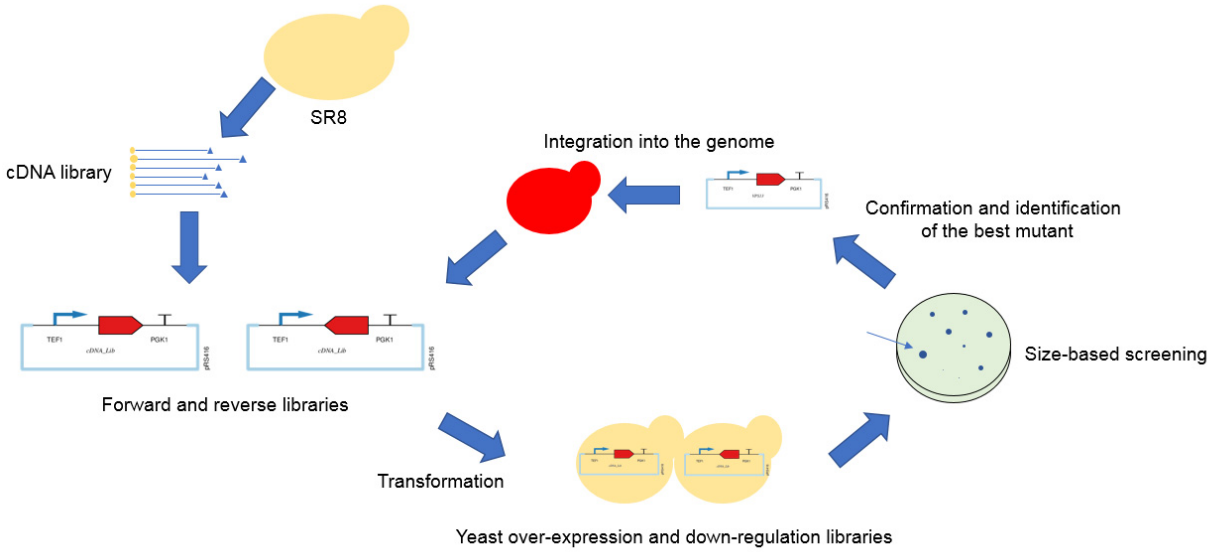
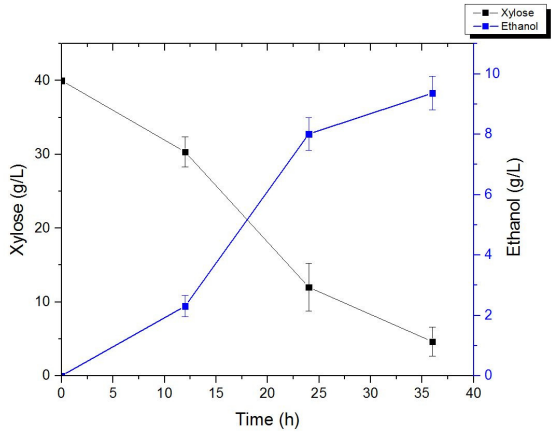
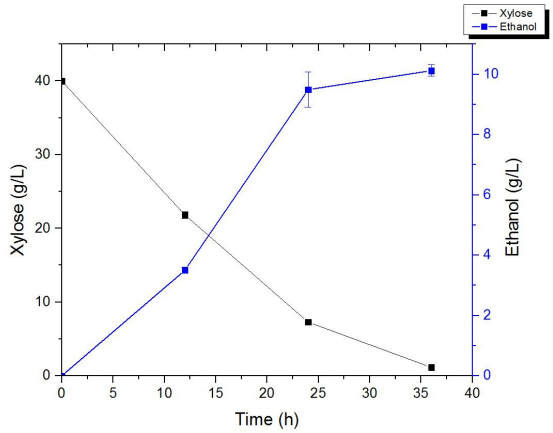

SR8
cDNA library
Integration into the genome
Confirmation and identification of the best mutant
Size-based screening
Forward and reverse libraries
Transformation
Yeast over-expression and down-regulation libraries

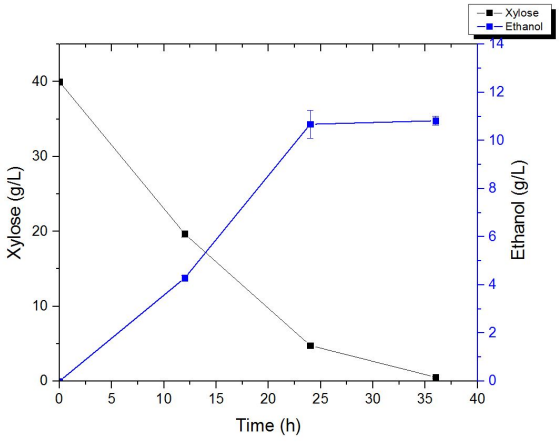
Figure 2


Xylose
Ethanol
Xylose (g/L)
Ethanol (g/L)
Time (h)

a


Xylose
Ethanol
Xylose (g/L)
Ethanol (g/L)
Time (h)

b


Xylose
Ethanol
Xylose (g/L)
Ethanol (g/L)
Time (h)

c

Figure 3

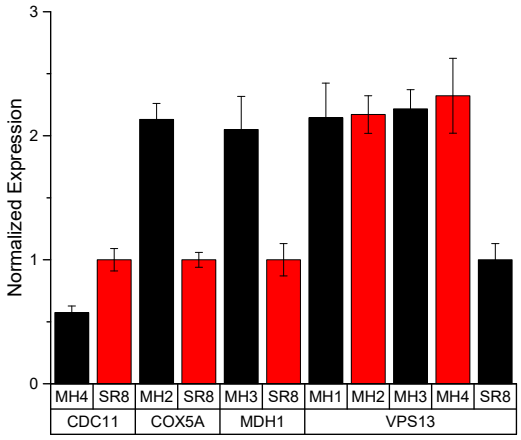
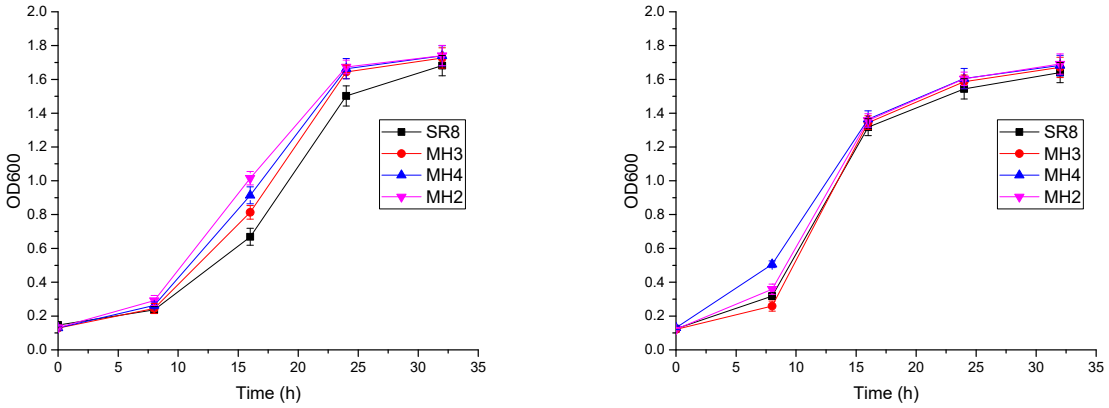
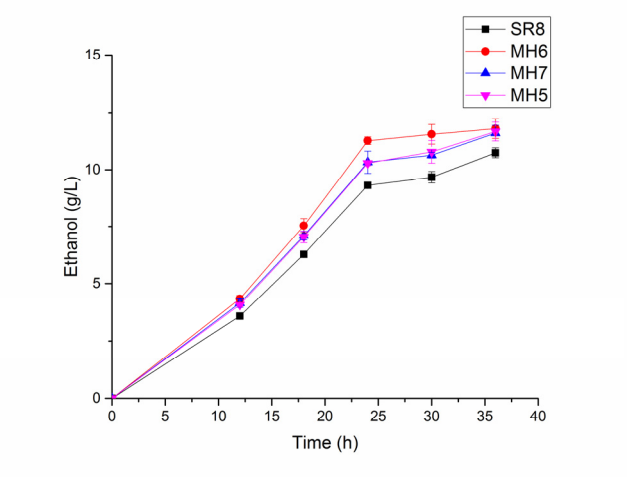


Figure 4

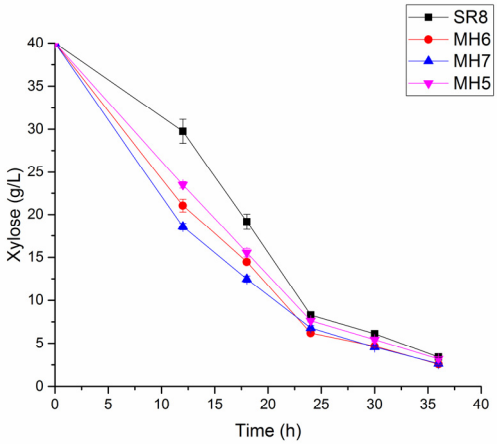


a b

Figure 5


SR8
MH6
MH7
MH5
Ethanol (g/L)
Time (h)
0
5
10
15
20
25
30
35
40

a


SR8
MH6
MH7
MH5
Xylose (g/L)
Time (h)
0
5
10
15
20
25
30
35
40

b