Supplemental Information

Strains, Media and Cultivation Conditions

Saccharomyces cerevisiae L2612 (*MATα leu2-3 leu2-112 ura3-52 trp1-298 can1 cyn1 gal*$^{+}$) was a gift from Professor Yong-su Jin.[1] *Escherichia coli* DH5α was used for recombinant DNA manipulation. Yeast strains were cultivated in synthetic dropout media to maintain plasmids (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.05% amino acid dropout mix). YPA medium (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate) with 2% D-glucose was used to grow yeast strains. *E. coli* strains were grown in Luria broth (Fisher Scientific, Pittsburgh, PA). *S. cerevisiae* strains were grown in un-baffled shake-flasks at 30 °C and 250 rpm for aerobic growth, and 30 °C and 100 rpm for oxygen limited condition. *E. coli* strains were grown at 37 °C and 250 rpm. All chemicals were purchased from Sigma Aldrich or Fisher Scientific.

Strain and Plasmid Construction

To integrate the D-xylose utilization pathway consisting of D-xylose reductase, xylitol dehydrogenase, and xylulokinase from *Pichia stipitis*, the genes and corresponding promoters and terminators (ADH1 promoter-xylose reductase-ADH1 terminator, pGK1 promoter-xylitol dehydrogenase-CYC1 terminator, pYK1 promoter-xylulokinase-ADH2 terminator) were PCR-amplified and cloned into the pRS416 plasmid using the DNA assembler method.[2] *BamH*I and *Hind*III were used to remove the DNA fragment encoding the D-xylose utilization pathway and then ligated to the pRS406 plasmid digested by the same two restriction enzymes. The resulting plasmid was then linearized by *Apa*I and integrated into the *URA3* locus on the chromosome of L2612, resulting in a recombinant xylose-utilizing yeast strain, HZ3001.

The pRS425 plasmid (New England Biolabs, Ipwich, MA) was used to co-express a cellobiose transporter gene and a β-glucosidase gene. As shown in Figure S1, the pRS425 plasmid was digested by *BamH*I and *Apa*I. The PYK1 promoter and the ADH1 terminator were added to the N-terminus and C-terminus of the cellobiose transporter, respectively, while the TEF1 promoter and the PGK1 terminator were added to the N-terminus and C-terminus of the β-glucosidase, respectively. These DNA fragments were assembled into the linearized pRS425 shuttle vector using the DNA assembler method.[2] Three cellobiose transporter genes *cdt-1* (GenBank Accession number XM_958708), *NCU00809* (GenBank Accession number XM_959259) and *cdt-2* (GenBank Accession number XM_958780) from *Neurospora crassa*[3] and two β-glucosidase genes *gh1-1* (GenBank Accession number XM_951090) from *N. crassa* and *BGL1* (GenBank Accession number D64088) from *Aspergillus aculeatus* were used. There are six combinations in total, each with one cellobiose transporter gene and one β-glucosidase gene.

Yeast plasmids were then transferred into *E. coli* DH5α, which were plated on LB plates containing 100 mg/L ampicillin. Single colonies of the *E. coli* transformants were then inoculated into LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN). These plasmids were transformed into the L2612 strain

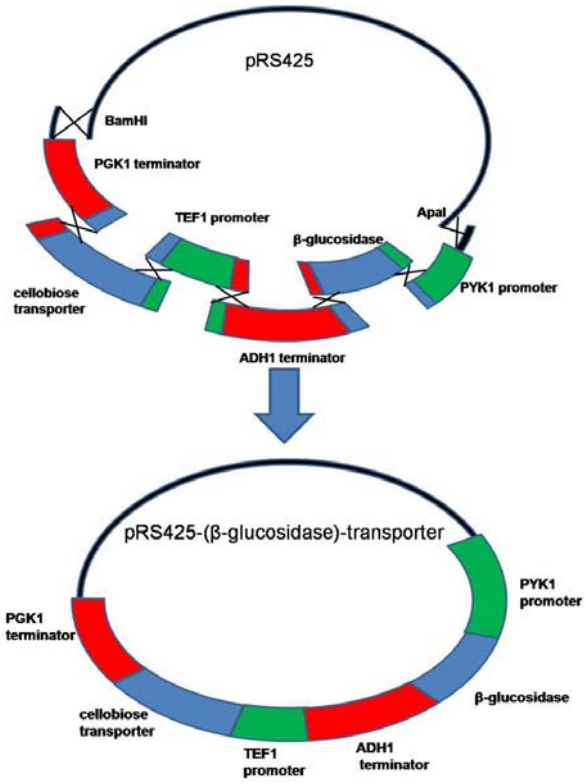

Fig. S1 Scheme for plasmid construction.

individually to yield the following strains: SL01 (containing the plasmid harboring the *cdt-1* cellobiose transporter gene and the *gh1-1* β-glucosidase gene from *N. crassa*), SL02 (containing the plasmid harboring the *NCU00809* cellobiose transporter gene and the *gh1-1* β-glucosidase gene from *N. crassa*), SL03 (containing the plasmid harboring the NCU08114 cellobiose transporter gene and the *gh1-1* β-glucosidase gene from *N. crassa*), SL04 (containing the plasmid harboring the *cdt-1* cellobiose transporter gene from *N. crassa* and the *BGL1* gene from *A. aculeatus*), SL05 (containing the plasmid harboring the *NCU00809* cellobiose transporter gene and the *BGL1* gene from *A. aculeatus*), and SL06 (containing the plasmid harboring the *cdt-2* cellobiose transporter gene from *N. crassa* and the *BGL1* gene from *A. aculeatus*). The empty pRS425 plasmid was transformed into the HZ3001 strain to yield the SL00 strain as a negative control. Yeast transformation was carried out using the standard lithium acetate method.[4] The resulting transformation mixtures were plated on SC-Ura-Leu medium supplemented with 2% glucose.

To confirm the proper construction of plasmids using the DNA assembler method, plasmids were isolated from yeast cells using the Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research, Orange, CA) and then transformed into *E. coli* DH5α cells. The resulting cells were spread on LB plates containing 100 mg/L ampicillin. Single *E. coli* colonies were inoculated into LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and checked by diagnostic PCR or restriction digestion using *Cla*I and *Hind*III. All restriction enzymes were obtained from New England Biolabs (Ipwich, MA).

Mixed Sugar Fermentation in Shake-flasks

For each yeast strain, a single colony was first grown up in 2 mL SC-Ura-Leu medium plus 20 g L^{-1} glucose, and then inoculated into 50 mL of the same medium in a 250 mL shake flask to obtain enough cells for mixed sugar fermentation studies. After one day of growth, cells were spun down and inoculated into 50 mL of YPA medium supplemented with 40 g L^{-1} cellobiose and 50 g L^{-1} D-xylose; 40 g L^{-1} cellobiose, 50 g L^{-1} D-xylose, and 5 g L^{-1} glucose; or 40 g L^{-1} cellobiose, 50 g L^{-1} D-xylose, and 10 g L^{-1} glucose in a 250 mL unbaffled shake-flask. YPA media supplemented with 10 g L^{-1} glucose and 40 g L^{-1} cellobiose or solely 10 g L^{-1} glucose were also used to determine the ethanol production efficiency in the presence of cellobiose. Starting from an initial $OD_{600} \approx 1$, cell cultures were grown at 30 °C at 100 rpm for fermentation under oxygen limited conditions. OD_{600} readings and cell culture samples were taken at various time points. Dry cell weight was measured gravimetrically using an aluminum foil weighing dish after evaporating under 65 °C for approximate 72 hours. Sugars and ethanol concentrations were determined using Shimadzu HPLC equipped with a Bio-Rad HPX-87H column (Bio-Rad Laboratories, Hercules, CA) and Shimadzu RID-10A refractive index detector following the manufacturer's protocol. The HPX-87H column was kept at 65 °C using a Shimadzu CTO-20AC column oven. 0.5mM sulfuric acid solution was used as mobile phase at a constant flow rate of 0.6 mL/min. 10 μL of filtered sample was injected into the HPLC system with a Shimadzu SIL-20AC HT auto sampler, and each run was stopped at 25 minutes after the injection. The concentration of the sugars and ethanol were determined using a standard curve generated using a series of external standards. Each data point represented the mean of triplicate samples. The mixed sugar fermentation results for the strains ranging from SL00 to SL06 are shown in Figure S2. The best strain SL01 was selected for further characterization.

In addition, both SL00 and SL01 were cultivated using the YPA media supplemented with a mixture of 10 g L^{-1} glucose and 40 g L^{-1} cellobiose or solely 1% glucose (Figure S3). For SL01, with solely 10 g L^{-1} glucose, 91.3% glucose was consumed and the maximum ethanol productivity and yield reached 0.40 g L^{-1} h^{-1} and 0.32 g per g sugar, respectively, at 9 hours. After that, ethanol was gradually consumed (Fig. S3b). In comparison, with 10 g L^{-1} glucose and 40 g L^{-1} cellobiose, the ethanol productivity and yield were 0.38 g L^{-1} h^{-1} and 0.28 g per g sugar, respectively, at 9 hours, and reached the maximum level (0.44 g L^{-1} h^{-1} and 0.30 g per g sugar, respectively) at 24 hours (Fig. S3a). Thus, the effect of cellobiose on the maximum ethanol yield and productivity was insignificant. For SL00, with 10 g L^{-1} glucose and 40 g L^{-1} cellobiose, no cellobiose consumption was observed (Fig. S3c), while with solely 10 g L^{-1} glucose, the profile of glucose consumption and ethanol production was almost identical to that of SL01 (Fig. S3d).

Mixed Sugar Fermentation in Bioreactors

The Multifors system (Infors-HT, Bottmingen, Switzerland) was used for mixed sugar fermentation. Each vessel has a total capacity volume of 750 mL. For each vessel, there was one set of a pO_2 sensor, air sparger, exit gas cooler, temperature sensor, inoculation port, spare port, dip tube, antifoam sensor, pH sensor, drive shaft, heater block, rotameter, and peristaltic pump system. The whole bioreactor system was equipped with a ThermoFlex900

cooling system (Thermo Scientific, Waltham, MA).

Single colonies of NC801 and L34 were first grown up in 2 mL SC-Ura-Leu medium plus 20 g L^{-1} glucose, and then inoculated into 50 mL of the same medium in a 250 mL shake flask to obtain enough cells for mixed sugar fermentation studies. After one day of growth, 10 mL saturated culture were inoculated in 500 mL YPA medium supplemented with 40 g L^{-1} cellobiose and 50 g L^{-1} D-xylose; 40 g L^{-1} cellobiose, 50 g L^{-1} D-xylose, and 5 g L^{-1} glucose; or 40 g L^{-1} cellobiose, 50 g L^{-1} D-xylose, and 10 g L^{-1} glucose. The temperature was maintained at 30 °C and the pH was maintained at 5.5, adjusted by addition of either 2N H_2SO_4 or 4N NaOH. In the first 48 hours, the air flow rate was maintained at 0.5 L min^{-1}, with the impeller speed at 250 rpm. Afterwards, the air flow rate was adjusted to 0.2 L min^{-1} to achieve high ethanol production under oxygen limited conditions. Triplicate samples were taken at various time points and the OD_{600}, sugar concentration, and ethanol concentration were determined as described above.

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2. Shao, Z. Y.; Zhao, H.; Zhao, H. M., DNA assembler, an in vivo genetic method for rapid construction of biochemical pathways. *Nucleic Acids Res.* **2009,** *37* (2).
3. Galazka, J. M.; Tian, C.; Beeson, W. T.; Martinez, B.; Glass, N. L.; Cate, J. H. D., Cellodextrin transport in yeast for improved biofuel production. **2010**, submitted.
4. Gietz, R. D.; Schiestl, R. H.; Willems, A. R.; Woods, R. A., Studies on the transformation of intact yeast cells by the LiAc/s-DNA/PEG procedure. *Yeast* **1995,** *11* (4), 355-360.

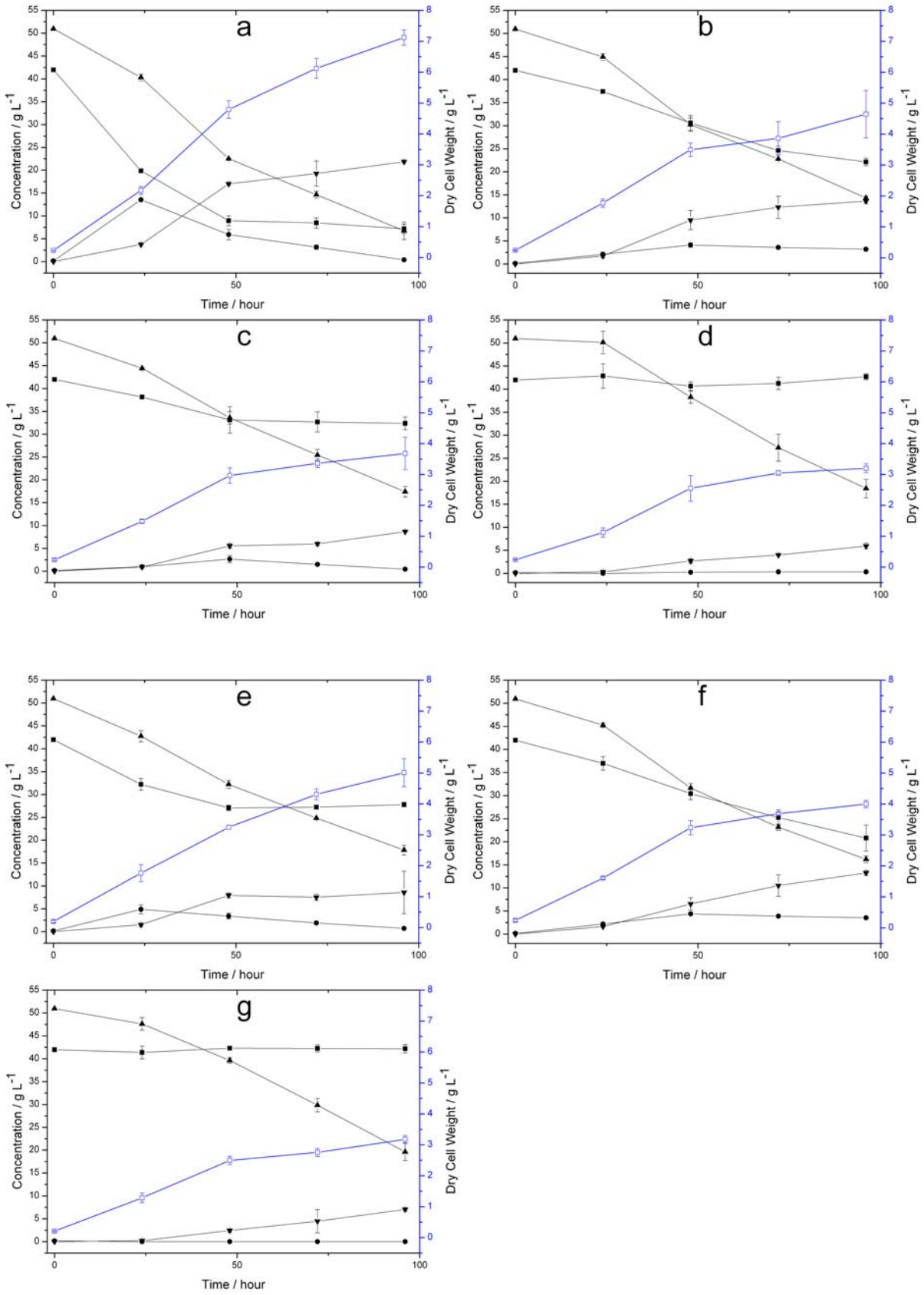

Fig. S2 Concentrations of cellobiose (■), glucose (●), D-xylose (▲), ethanol (▼), and dry cell weight (□) in the co-fermentation of 40 g L^{-1} cellobiose and 50 g L^{-1} D-xylose of SL01(a), SL02 (c), , SL03(e), SL04 (b), SL05(d), SL06(f), SL00(g), plotted as a function of time. Error-bars indicate standard deviations of triplicate samples.

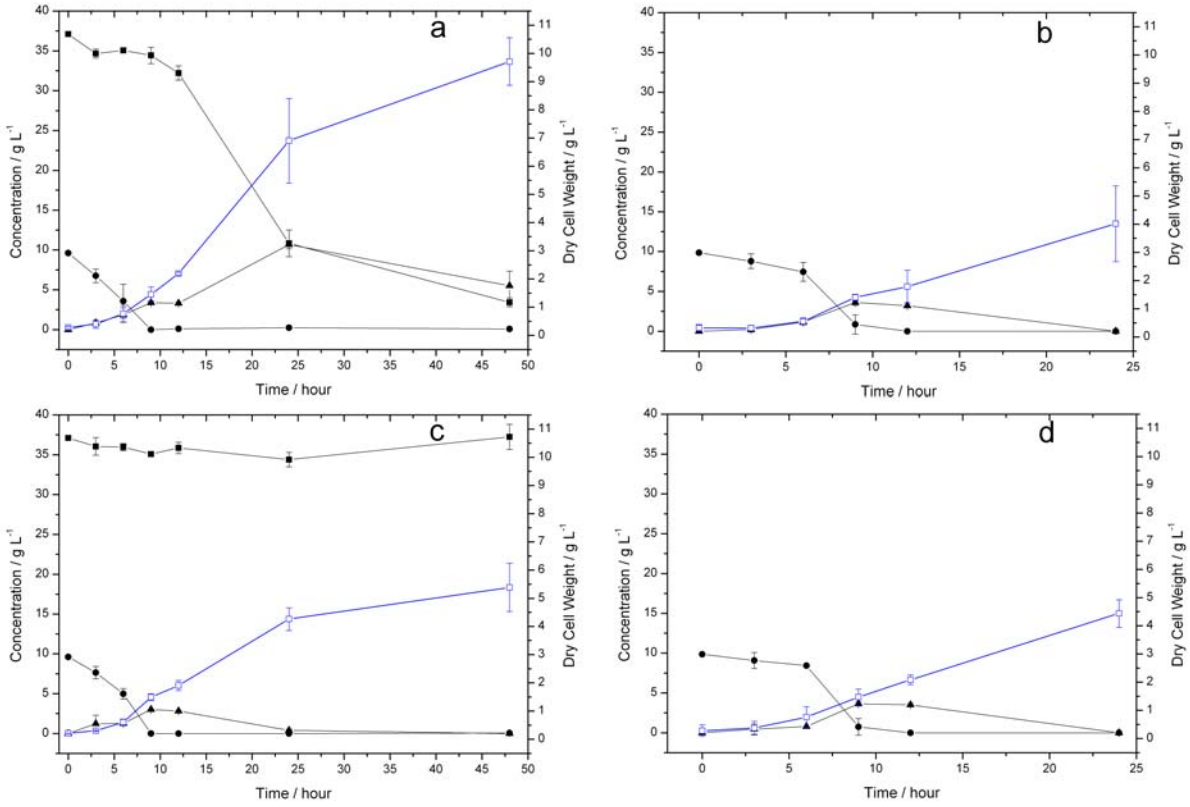

Fig. S3 Concentrations of cellobiose (■), glucose (●), ethanol (▲), and dry cell weight (□) of SL01 (a, b) and SL00 (c,d) in the co-fermentation of 40 g L^{-1} cellobiose and 10 g L^{-1} glucose (a, c), or 10 g L^{-1} glucose (b, d), plotted as a function of time. Error-bars indicate standard deviations of triplicate samples.