Supporting Information

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SI Text

SI Analysis. ***Improved ethanol yield of cofermentation when compared to single sugar fermentations (cellobiose or xylose).*** First, the mass balances between substrate (xylose, cellobiose, and a mixture of xylose and cellobiose) and fermentation products (cell mass and ethanol) explain the improved yield of cofermentation. In both cases of xylose (Fig. 3*A*) and cellobiose (Fig. 3*C*) fermentations, about 4.8 g/L of cells (OD ~ 16) were produced after consuming 40 g/L of sugars. In other words, the yields of biomass ($Y_{\text{Biomass/xylose}}$) from either xylose or cellobiose were about 0.12 g cell/g sugar. In cofermentation (Fig. 3*B*), the final cell density was only 6.2 g/L (OD ~ 22) even though twice amounts of sugars (total 80 g/L of sugars) were consumed. Therefore, the yield of biomass from the cofermentation was only 0.08 g/g sugars. These data explain how cofermentation results in higher ethanol yield than single sugar fermentation. Second, xylose fermentation by engineered *S. cerevisiae* requires oxygen-limited conditions for efficient ethanol production (1, 2). As shown in Fig. 3*A*, ethanol production from xylose begins only when the cell density is high enough to cause oxygen-limitation (after 12 h). However, in the case of cofermentation, yeast cells grew faster and oxygen-limitation started earlier than for single sugar fermentation conditions. As such, consumption of sugars during cofermentation can be less oxidative (or more fermentative) than single sugar fermentation, which resulted in more ethanol production. In addition, the slow release of glucose from intracellular hydrolysis of glucose may exert partial glucose repression, which brings about more fermentative sugar metabolism resulting improved ethanol production while xylose transport is not limited.

Prediction of sugar concentrations in cellulosic hydrolyzates. The composition of different lignocellulosic plants varies broadly. For instance, the US Department of Energy biomass database lists the composition of more than 150 biomass samples (http://www1.eere.energy.gov/biomass/m/feedstock_databases.html). The cellulose to hemicellulose ratios of these samples are between 1.4 and 19, and the average is 2.3. Energy crops typically have higher hemicellulose content than woody biomass. The average cellulose to hemicellulose ratios of sugarcane bagasse, corn stover, and sorghum are 2.0, 1.85, and 2.14, respectively. We therefore used a glucan/xylan ratio of 2 in our simulated sugar experiment design. The engineered yeast will likely be used in conjunction with traditional cellulase cocktails that are deficient in β-glucosidase activities for the biofuels production. The biomass hydrolysis process may result in small amounts of glucose in the lignocellulosic hydrolysates as 6–30% glucan-to-glucose conversions with incomplete cellulase cocktails were reported (3). Considering all the above factors, a sugar combination of 80 g/L cellobiose, 10 g/L glucose, and 40 g/L xylose was chosen in the simulated sugar experiments.

SI Discussion. ***Advantages of intracellular hydrolysis of cellobiose over extracellular hydrolysis.*** Our approach holds several advantages over the cell surface display strategy employed by Nakamura et al. (4). First, intracellular hydrolysis of cellobiose via the cellodextrin transport system (5) can reduce the glucose transport load of hexose/pentose sugar transporters. The transport of the extracellular glucose, generated from extracellular hydrolysis by a displayed enzyme on cell surface, may compete with xylose for cross-membrane transportation because glucose inhibits xylose transport competitively (2, 3, 5). In addition, the system presented in here exploits the higher affinity that cellodextrin transporter have for cellobiose ($K_M \approx 3$–4 μM) as compared to β-glucosidases [$K_M \approx 100$–$1{,}000$ μM (5)] and the *S. cerevisiae* hexose transporters' apparent affinity for glucose [$K_M \approx 1{,}000$–$10{,}000$ μM (5)]. The surface display of a β-glucosidase relies on the extracellular hydrolysis of cellobiose to glucose by a low-affinity beta-glucosidase followed by transport via low-affinity hexose transporters, and will be compromised at both steps. These inefficiencies will become particularly important during simultaneous saccharification and fermentation, when soluble sugars much be kept at a concentration that does not inhibit cellulases [19–410 μM (5)]. Second, expression levels of β-glucosidase on the cell surface needs careful optimization under given conditions in order to prevent excessive hydrolysis of cellobiose, as noted by Nakamura et al. (4). Excessive hydrolysis of cellobiose would result in glucose accumulation at high concentrations, which would impede cofermentation of xylose. In this sense, the cellodextrin transport system is more amenable for constructing cofermenting strain under various conditions. Third, the stability of intracellular β-glucosidase will be higher than a displayed β-glucosidase because the intracellular enzyme can be protected from harsh external environments. Intracellular expression could provide a significant benefit in fermentation of lignocellulosic hydrolyzates, which contain uncharacterized toxic or poisoning compounds to enzymes.

1. Jin YS, Laplaza JM, Jeffries TW (2004) *Saccharomyces cerevisiae* engineered for xylose metabolism exhibits a respiratory response. *Appl Environ Microbiol* 70:6816–6825.
2. Souto-Maior AM, Runquist D, Hahn-Hägerdal B (2009) Crabtree-negative characteristics of recombinant xylose-utilizing *Saccharomyces cerevisiae*. *J Biotechnol* 143:119–123.
3. Medve J, Karlsson J, Lee D, Tjerneld F (1998) Hydrolysis of microcrystalline cellulose by cellobiohydrolase I and endoglucanase II from *Trichoderma reesei*: adsorption, sugar production pattern, and synergism of the enzymes. *Biotechnol Bioeng* 59:621–634.
4. Nakamura N, et al. (2008) Effective xylose/cellobiose cofermentation and ethanol production by xylose-assimilating *S. cerevisiae* via expression of beta-glucosidase on its cell surface. *Enz Microb Technol.* 43:233–236.
5. Galazka JM, et al. (2010) Cellodextrin transport in yeast for improved biofuel production. *Science* 330:84–86.

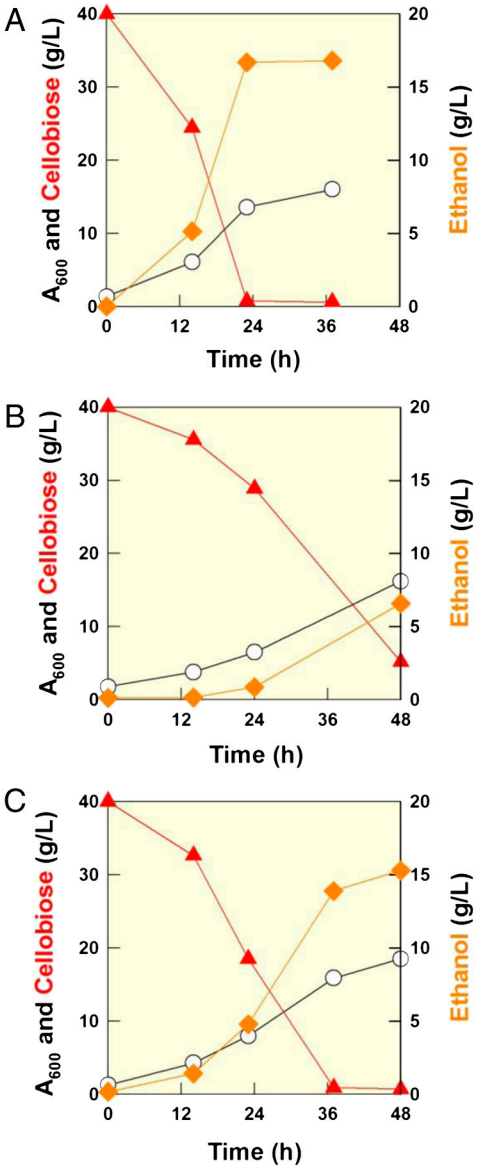


Fig. S1. Comparison of cellobiose utilization by yeast strains expressing one of three cellobiose transporters and a β-glucosidase (*gh1-1*). Figure shows utlization by strains expressing (*A*) *cdt-1*, (*B*) NCU00809, and (*C*) *cdt-2*. Symbols: cellobiose (red triangle), ethanol (orange diamond), and OD (open circle).

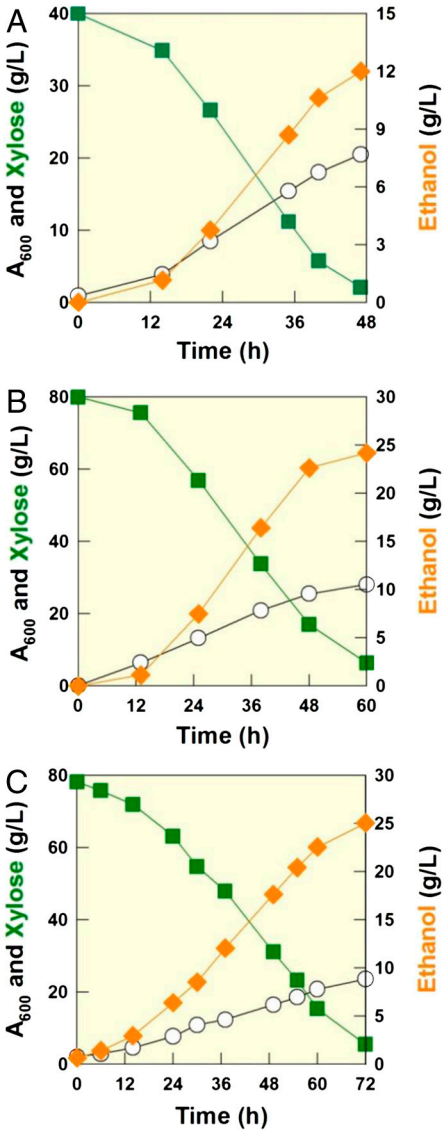
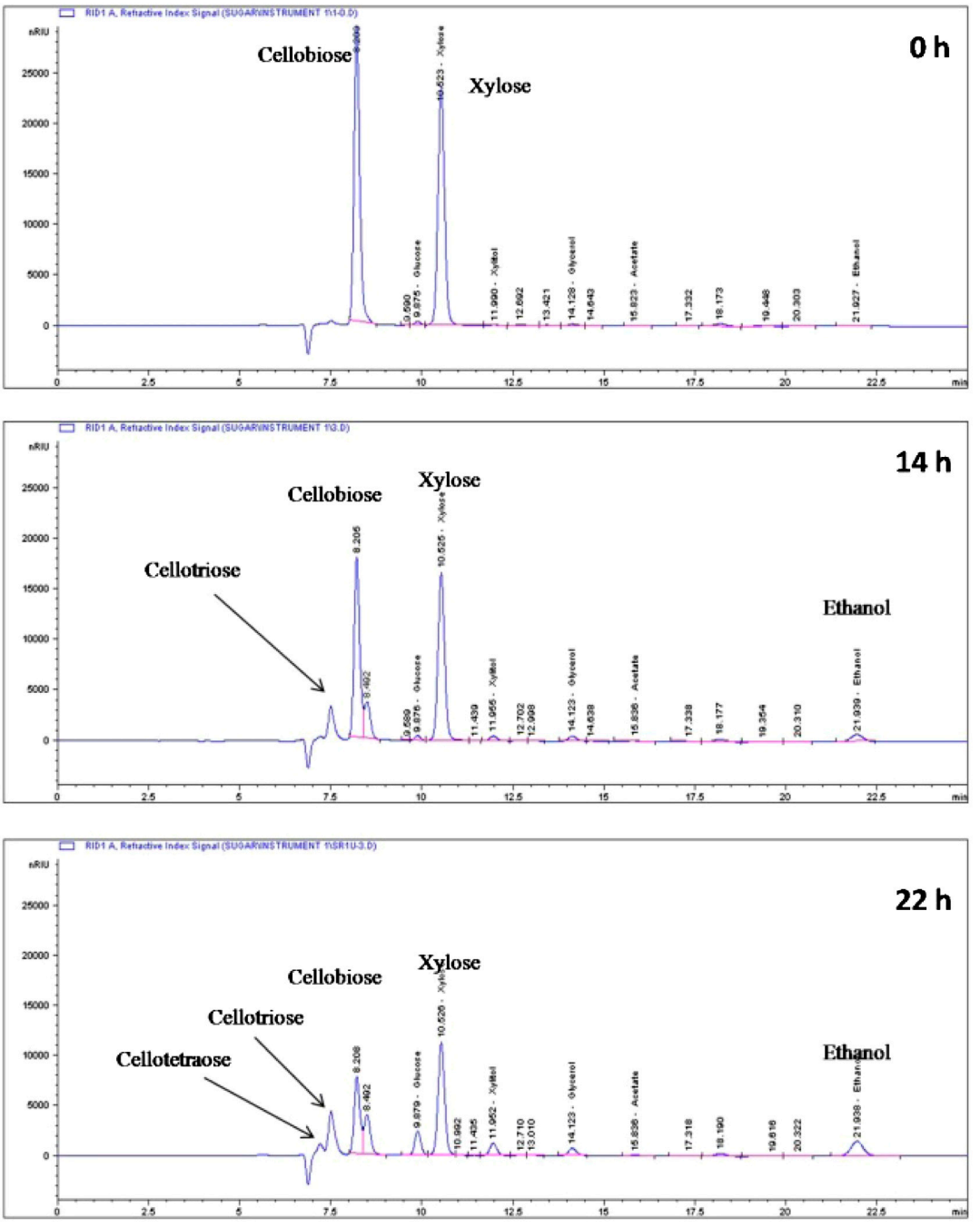


Fig. S2. Xylose fermentation by strain DA24 under various conditions. (*A*) 40 g/L of xylose in a shaker flask, (*B*) 80 g/L of xylose in a flask, (*C*) 80 g/L of xylose in a bioreactor. Symbols: xylose (green square), ethanol (orange diamond), and OD (open circle).


0 h
Cellobiose
Xylose
14 h
Cellobiose
Xylose
Cellotriose
Ethanol
22 h
Cellobiose
Xylose
Cellotriose
Cellotetraose
Ethanol

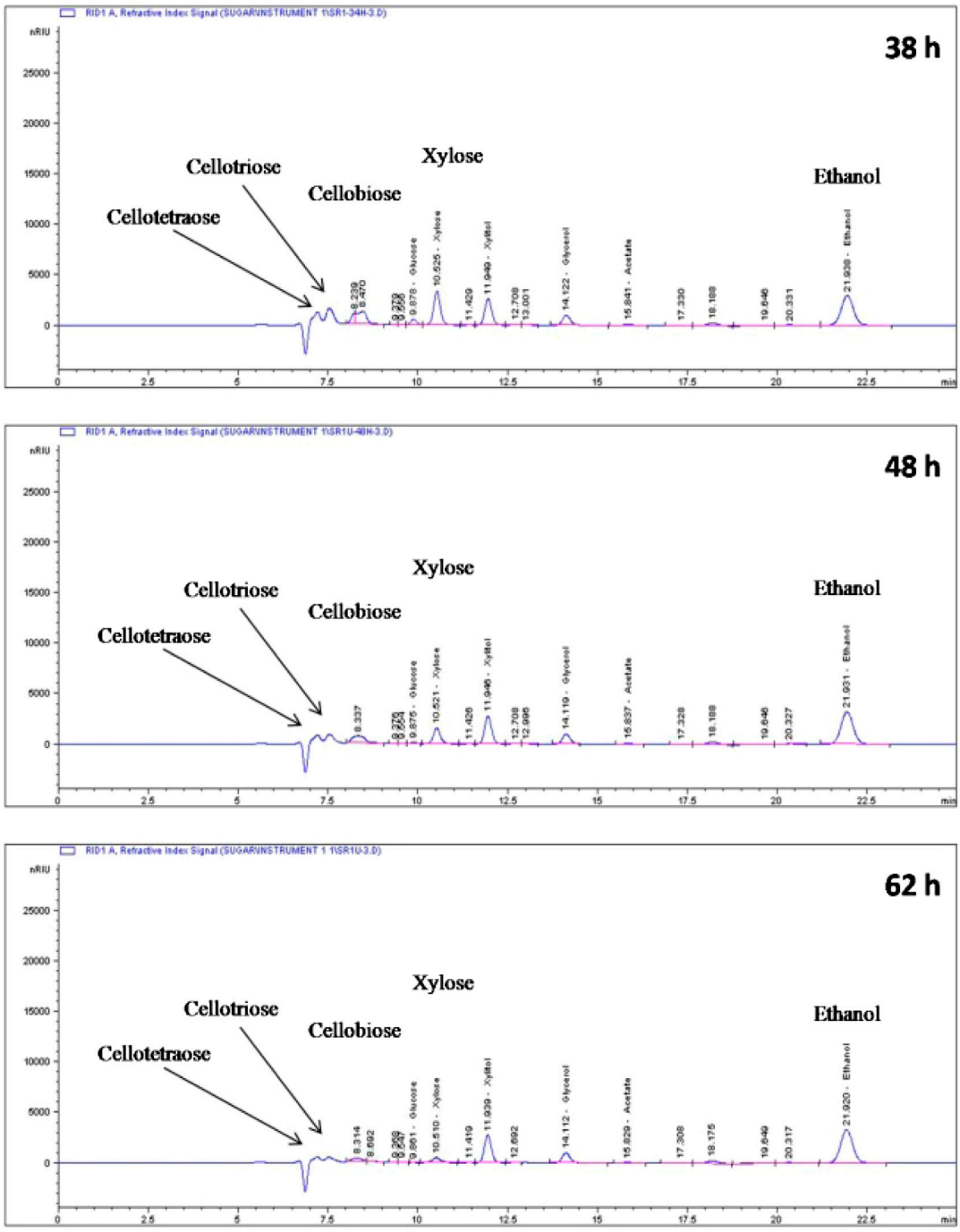


Fig. S3. HPLC chromatograms from each time point suggesting cellotriose and cellotetraose accumulation during cofermentation of cellobiose and xylose by strain DA24-16BT3.

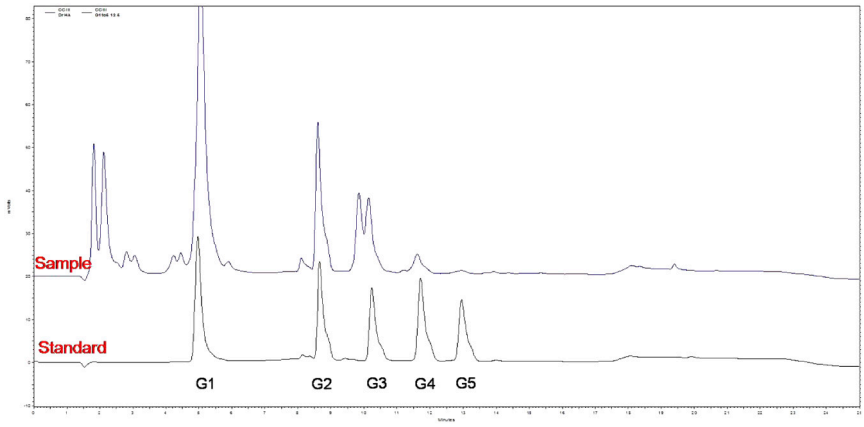


Fig. S4. HPAEC analysis showing cellodextrin accumulation in the fermentation medium after 22 h fermentation by strain DA24-16BT3 during cofermentation of cellobiose and xylose. (G1: glucose; G2: cellobiose; G3: cellotriose; G4: cellotetraose; and G5:cellopentaose).

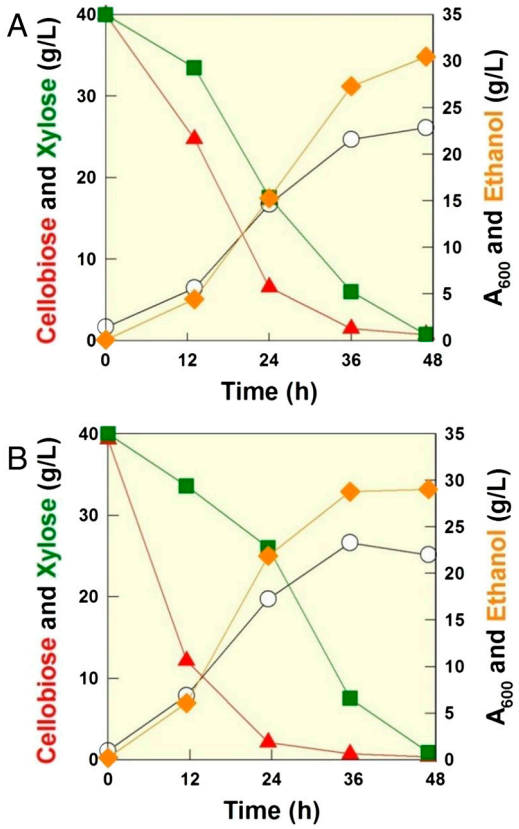


Fig. S5. Comparison of sugar utilization patterns by transformants with an integrated copy of *cdt-1* (*A*), and *cdt-1* on a multicopy plasmid (*B*) during co-fermentation of 40 g/L of cellobiose and 40 g/L of xylose mixture. Symbols: cellobiose (red triangle), xylose (green square), ethanol (orange diamond), and OD (open circle).

Table S1. Comparison of fermentation parameters by DA24 and DA24-16 under two different sugar conditions

Carbon source	Strains	Produced ethanol (g/L)	Sugar consumption rate (g/L·h)	Yield (g/g)	Productivity (g/L·h)
Xylose (80 g/L)	DA24	24.2	1.16	0.34	0.40
	DA24-16	27.9	1.32	0.35	0.47
Glucose (70 g/L) and xylose (40 g/L)	DA24	34.8	1.45	0.39	0.74
	DA24-16	45.1	1.78	0.42	0.96

Table S2. Comparison of fermentation parameters of DA24 and DA24-16 with other engineered *S. cerevisiae* strains

Strain	Relevant genotype/phenotype	Specific xylose consumption rate (g xylose/g cell · h)	Ethanol yield (g/g)	Xylitol yield (g/g)	Reference
DA24	*XYL1, a mutant XYL1, XYL2,* and *XKS1*	0.53	0.32	0.08	This study
DA24-16	Evolved isolate from DA24	0.71	0.35	0.04	This study
H1693	*XYL1* and *XYL2*	0.09	0.04	0.47	(10)
H1691	*XYL1*, *XYL2*, and *XKS1*	0.2	0.12	0.41	(10)
TMB3399	*XYL1*, *XYL2*, and *XKS1*	NA	0.05	0.59	(11)
TMB3400	Chemical mutant of TMB3399	NA	0.18	0.25	(11)
C1	Evolved isolate from TMB3001	0.56	0.24	0.32	(12)
H2674 (control)	*XYL1*, *XYL2*, and *XKS1*	0.07	0.14	0.56	(13)
H2673 (GPD1)	*XYL1*, *XYL2*, *XKS1*, and *GPD1* overexpression	0.06	0.17	0.49	(13)
H2723 (Δ*zwf1*)	*XYL1*, *XYL2*, *XKS1*, and Δ*zwf1*	0.05	0.18	0.29	(13)
H2684 (*GPD1*Δ*zwf1*)	*XYL1*, *XYL2*, *XKS1*, *GPD1* overexpression, and Δ*zwf1*	0.06	0.31	0.35	(13)
RWB202-AFX	*XI, evolved isolate*	0.21	0.42	0.02	(14)
RWB217	*XI, XK*, Δ*GRE3,* and overexpressed pentose phosphate pathway (PPP) enzymes	NA	0.43	0.003	(15)
RWB218	*XI, XK*, Δ*GRE3*, overexpressed PPP, and selected for enhanced glucose uptake	NA	0.41	0.001	(16)
H2490-4	*XYL1*, *XYL2*, *XKS1*, and *adaptation*	0.58	0.14	0.82	(17)
TMB3001	*XYL1*, *XYL2*, and *XKS1*	0.15	0.31	0.29	(18)
TMB3255	*XYL1*, *XYL2*, *XKS1*, and Δ*zwf1*	0.02	0.41	0.05	(18)
TMB3008	*XYL1*, *XYL2*, *XKS1*, and Δ*gnd1*	0.08	0.38	0.13	(18)
TMB3250	*XYL1*, *XYL2*, *XKS1*	0.1	0.3	0.3	(18)
TMB3251	*XYL1*, *XYL2*, *XKS1*, and attenuated PGI	0.07	0.34	0.21	(18)
TMB3256	*XYL1*, *XYL2*, *XKS1*, and *ZWF1*	0.06	0.36	0.13	(19)
TMB3037	*XYL1*, *XYL2*, *XKS1*, and YRP13-*ZWF1*	0.11	0.34	0.19	(19)
TMB3260	*XYL1*, *XYL2*, *XKS1*, and high XR activity	0.25	0.3	0.13	(20)
TMB3253 control	*XYL1*, *XYL2*, *XKS1*, strain for TMB3254	0.16	0.28	0.34	(19)
TMB3254	*XYL1*, *XYL2*, *XKS1*, overproducing transhydrogenase	0.16	0.28	0.3	(19)

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Jeppsson M, Träff K, Johansson B, Hahn-Hägerdal B, Gorwa-Grauslund MF (2003) Effect of enhanced xylose reductase activity on xylose consumption and product distribution in xylose-fermenting recombinant *Saccharomyces cerevisiae*. *FEMS Yeast Res* 3:167–175.

Table S3. Cofermentation of cellobiose and xylose using mixtures containing various concentrations of cellobiose and xylose by DA24-16BT3 strain

Cellobiose/xylose (g/L)	Produced ethanol (g/L)	Sugar consumption rate (g/L·h)	Yield (g/g)	Productivity (g/L·h)
20/20	14.8	1.12	0.37	0.41
30/30	23.3	1.33	0.37	0.50
40/40	30.5	1.67	0.39	0.65

Table S4. Cofermentation of glucose, cellobiose, and xylose (10 g/L, 80 g/L, and 40 g/L, respectively) using bioreactor by DA24-16BT3 strain using different inoculums

Target initial OD (A_{600})	Produced ethanol (g/L)	Sugar consumption rate (g/L·h)	Ethanol yield (g/g)	Productivity (g/L·h)
~1 (1.2)	47.9	1.93	0.37	0.71
~10 (10.2)	48.1	2.18	0.37	0.82

Table S5. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
Strains		
D452-2	*MATa, leu2, his3,ura3,can1*	Hosaka et al. (6)
D801-130	D452-2 expressing β-glucosidase (*gh1-1*) and *cdt-1* (NCU00801)	In this study
D809-130	D452-2 expressing β-glucosidase (*gh1-1*) and NCU00809	In this study
D8114-130	D452-2 expressing β-glucosidase (*gh1-1*) and *cdt-2* (NCU08114)	In this study
DA24	D452-2 expressing *XYL1*, *mXYL1*, *XYL2*, and *XKS1* (Isogenic of D452-2 except for *leu2* :: $TDH3_P$-*XYL1*-$TDH3_T$, *ura3* :: *URA3*-PGK_P-*mXYL1*-PGK_T-PGK_P-*XYL2*-PGK_T, *Ty3* :: *neo*-TDH_P-*XKS1*-TDH_T)	In this study
DA24-16	Evolved strain of DA24 in xylose containing media	In this study
DA24-16BT3	DA24-16 expressing β-glucosidase (*gh1-1*) in a multicopy plasmid and *cdt-1* (NCU00801) though single-copy integration	In this study
DA24-16BT-M	DA24-16 expressing β-glucosidase (*gh1-1*) and *cdt-1* (NCU00801) in multicopy plasmids	In this study
P. stipitis CBS 6054	NRRL Y-11545 = ATCC58785 = IFO10063	Jeffries et al. (7)
Plasmids		
pRS425	*LEU2*, a multicopy plasmid	Christianson et al. (8)
pRS426	*URA3*, a multicopy plasmid	Christianson et al. (8)
pRS403	*HIS3*, anintegrative plasmid	Sikorski et al. (9)
pRS405	*URA3*, an integrative plasmid	Sikorski et al. (9)
pRS425-β-glucosidase	β-glucosidase (*gh1-1*) under the control of PGK promoter in pRS425	Galazka et al. (5)
pRS426-*cdt-1*	*cdt-1* under the control of PGK promoter in pRS426	Galazka et al. (5)
pRS426-*cdt-2*	*cdt-2* under the control of PGK promoter in pRS426	Galazka et al. (5)
pRS426-NCU00809	NCU00809 under the control of PGK promoter in pRS426	Galazka et al. (5)
pRS403-*cdt-1*	*cdt-1* under the control of PGK promoter in pRS403	In this study

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Jeffries TW, et al. (2007) Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. *Nat Biotechnol* 25:319–326.
Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene* 110:119–122.
Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122:19–27.

Table S6. Synthetic oligonucleotides used in this study

Name	Sequences
NCU00801-F	ATGGATCCAAAAATGTCGTCTCACGGCTCC
NCU00801-R	ATGAATTCCTACAAATCTTCTTCAGAAATCAATTTTTGTTCAGCAACGATAGCTTCGGAC
NCU08114-F	ATACTAGTAAAAATGGGCATCTTCAACAAGAAGC
NCU08114-R	GCATATCGATCTACAAATCTTCTTCAGAAATCAATTTTTGTTCAGCAACAGACTTGCCCTCATG
NCU00130-F	GCATACTAGTAAAAATGTCTCTTCCTAAGGATTTCCTCT
NCU00130-R	ATACTGCAGTTAATGATGATGATGATGATGGTCCTTCTTGATCAAAGAGTCA AAG