ACS SyntheticBiology

Letter

pubs.acs.org/synthbio

Simultaneous Utilization of Cellobiose, Xylose, and Acetic Acid from Lignocellulosic Biomass for Biofuel Production by an Engineered Yeast Platform

Na Wei,[†,#] Eun Joong Oh,[‡,§,#] Gyver Million,[§] Jamie H. D. Cate,[∥,⊥] and Yong-Su Jin*[,‡,§]

[†]Department of Civil and Environmental Engineering, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, United States

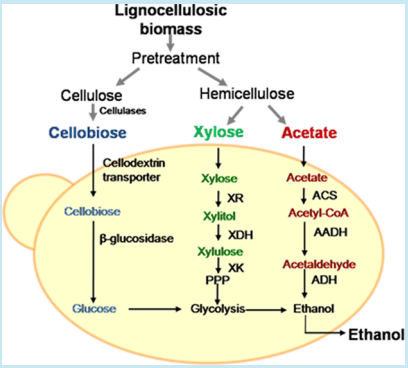
[‡]Department of Food Science and Human Nutrition and [§]Institute for Genomic Biology, University of Illinois at Urbana−Champaign, Urbana, Illinois 61801, United States

[∥]Departments of Molecular and Cell Biology and Chemistry, University of California at Berkeley, Berkeley, California 94720, United States

[⊥]Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, United States

Supporting Information

ABSTRACT: The inability of fermenting microorganisms to use mixed carbon components derived from lignocellulosic biomass is a major technical barrier that hinders the development of economically viable cellulosic biofuel production. In this study, we integrated the fermentation pathways of both hexose and pentose sugars and an acetic acid reduction pathway into one *Saccharomyces cerevisiae* strain for the first time using synthetic biology and metabolic engineering approaches. The engineered strain coutilized cellobiose, xylose, and acetic acid to produce ethanol with a substantially higher yield and productivity than the control strains, and the results showed the unique synergistic effects of pathway coexpression. The mixed substrate coutilization strategy is important for making complete and efficient use of cellulosic carbon and will contribute to the development of consolidated bioprocessing for cellulosic biofuel. The study also presents an innovative metabolic engineering approach whereby multiple substrate consumption pathways can be integrated in a synergistic way for enhanced bioconversion.



KEYWORDS: *cellulosic biofuels, cellobiose, xylose, acetic acid, Saccharomyces cerevisiae, fermentation*

Lignocellulosic biomass (e.g., agricultural and forest residues, municipal and industrial wastes, and energy crops) has been identified as the prime source for producing renewable biofuels and other value-added products due to its low cost, large-scale availability and potential for reducing greenhouse gas emissions.[1−4] Lignocellulosic biomass is composed of cellulose (40−55%), hemicellulose (25−50%), and lignin (10−40%).[5] In contrast to cellulose, which is composed of glucose, hemicellulose consists of both six and five-carbon sugars (e.g., xylose).[6] Thus, pretreatment and hydrolysis of lignocellulosic materials generate a mixture of hexose and pentose sugars.[5] Meanwhile, due to acetylation of hemicelluloses in lignocellulosic biomass, dilute-acid hydrolysates contain substantial amounts of acetic acid (1−15 g/L).[7] Complete and efficient utilization of the substrates in the hydrolysates is one of the prerequisites for making lignocellulosic biofuel processes economically competitive,[8−11] but it is still a major technical barrier.[8,12]

The yeast *Saccharomyces cerevisiae* has been widely used for sugar fermentation and is a preferred microorganism for metabolic engineering efforts to produce biofuels from cellulosic biomass because of its high carbon fluxes in central metabolic pathways, osmotolerance, and genetic tractability.[13] However, *S. cerevisiae* cannot naturally ferment xylose, the second most abundant component in lignocellulosic hydrolysates, due to the lack of a functional assimilation pathway.[14] Extensive studies have focused on engineering *S. cerevisiae* for efficient fermentation of xylose,[11,15−19] but xylose metabolism is highly repressed in the presence of glucose, which makes it difficult to realize continuous fermentation using hydrolysates that contain a xylose and glucose mixture. Meanwhile, the acetic acid ubiquitous in cellulosic hydrolysates can strongly diminish the bioconversion of sugar compounds due to its toxicity to fermenting microorganisms.[7,20−23] Many previous studies focused on developing methods to reduce the concentration of acetic acid in hydrolysates.[24] However, the detoxification strategies would require a separate process step and result in the loss of substantial amounts of carbon.[24]

A robust microbial system that efficiently utilizes mixed substrates derived from plant cell wall materials in toxic cellulosic hydrolysates is required for economically feasible production of lignocellulosic biofuels, but such a system has yet

Received: October 28, 2014
Published: January 14, 2015

DOI: 10.1021/sb500364q
ACS Synth. Biol. 2015, 4, 707−713

to be developed. In our previous study, intracellular hydrolysis of cellobiose (a dimer of glucose) minimized the glucose repression problem described above and enabled cofermentation of xylose and cellobiose by engineered *S. cerevisiae*.[25] On the other hand, to address the acetic acid toxicity problem, we developed a strategy that converts acetic acid to ethanol in anaerobic xylose fermentation by engineered *S. cerevisiae* strains.[26] The strategy, in contrast to getting rid of acetic acid, utilizes it and improves the yield and productivity of ethanol while allowing *in situ* detoxification of the cellulosic hydrolysates.[26] While the two approaches have been demonstrated separately, ideally, one strain would be able to coferment all three of the substrates—cellobiose, xylose, and acetic acid—under industrially relevant anaerobic conditions. However, the possibility of simultaneous utilization of all the three components in engineered yeast has not been demonstrated. Therefore, the objective of this study was to integrate the heterologous cellobiose assimilation pathway, xylose fermentation pathway, and acetic acid reduction pathway into a single yeast strain and demonstrate a strategy for making complete and efficient use of cellulosic carbons. The study demonstrated for the first time successful integration of the three distinct heterologous pathways into one microbial platform using synthetic biology approaches and the pathway integration brought unique synergistic effects in enhancing biofuel production.

The metabolic pathways for the coconsumption of cellobiose, xylose and acetic acid are illustrated in Figure 1. A

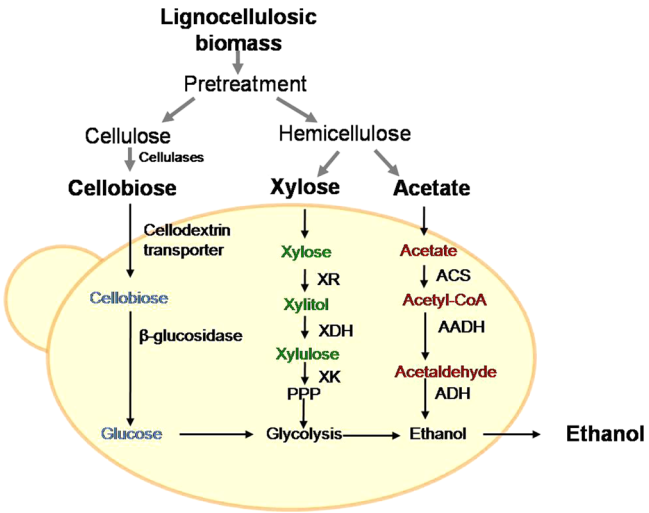


Figure 1. Schematic overview of biofuel production through coutilization of cellobiose, xylose, and acetic acid from lignocellulosic biomass by engineered yeast. XR: xylose reductase. XDH: xylitol dehydrogenase. XK: xylulose kinase. ACS: acetyl-CoA synthetase. AADH: acetylating acetaldehyde dehydrogenase. ADH: alcohol dehydrogenase.

cellodextrin transporter (*cdt-1*) and intracellular *β*-glucosidase (*gh1-1*) from the cellulolytic fungi *Neurospora crassa* can be expressed in *S. cerevisiae* to enable cellobiose consumption[25,27] and bypass glucose repression problem. The xylose assimilation pathway can be implemented by expressing NAD(P)H-linked xylose reductase (XR) and NAD^+-linked xylitol dehydrogenase (XDH) genes from *Scheffersomyces stipitis*.[10,28] The yeast *S. cerevisiae* does not have an acetate reduction pathway, so an NADH-consuming acetylating acetaldehyde dehydrogenase (AADH) pathway was introduced by expressing *adhE* from *Escherichia coli* to enable the reduction of acetate to ethanol. This pathway couples to the XR-XDH pathway to use surplus NADH as a driving force for acetate reduction and regenerates NAD^+ to improve the xylose assimilation pathway.[26]

The yeast strain SR8, capable of efficient xylose fermentation, was constructed in our lab[10] through optimization in *S. cerevisiae* D452-2 of the expression levels of *XYL1* (coding for XR), *XYL2* (coding for XDH), and *XYL3* (coding for xylulose kinase (XK)) from *S. stipitis*, and through its laboratory evolution on xylose. The *ALD6* gene coding for acetaldehyde dehydrogenase was deleted to minimize acetate accumulation. The SR8 strain is able to ferment and grow on xylose under strictly anaerobic conditions as well as oxygen-limited conditions, with the fermentation performance comparable to the best performing engineered xylose-fermenting *S. cerevisiae* strains reported previously.[10] Using the strain SR8, we then aimed to implement an efficient cellobiose fermentation pathway and an acetic acid reduction pathway to achieve coconversion of the three substrates to ethanol by one strain. First, we introduced the *cdt-1* and *gh1-1* gene expression cassettes (Table 1) into the genome of *S. cerevisiae* for stable expression, resulting in the strain EJ3, which showed a low ethanol yield ($Y_{Ethanol/Cellobiose}$ = 0.32 g/g) and productivity ($P_{Ethanol/Cellobiose}$ = 0.42 g/L·h) from cellobiose under oxygen-limited conditions (Figure 2a).

In order to further improve the cellobiose fermentation rates of the strain EJ3, we performed serial subcultures in medium with 80 g/L of cellobiose as the sole carbon source. We hypothesized that serial subcultures on cellobiose would enable the selection of evolved strains with rapid cellobiose-fermenting capability. The culture was initiated with an initial optical density at 600 nm (OD_{600}) of 1.0 and transferred to fresh cellobiose media when the cells were in the stationary phase. The transfers were performed repeatedly until there was no more improvement of the cellobiose consumption rate. The EJ3 strain showed gradual improvement in its cellobiose consumption rate and ethanol production as we transferred the cells (Supporting Information (SI) Figure S1). The last subculture exhibited a higher cellobiose consumption rate (2.32 g/L·h) and ethanol production than the first one after 36 h. Then, we isolated colonies from the last subculture, evaluated their cellobiose fermentation performance, and identified the strain with the best cellobiose fermentation properties, called EJ4 (Table 1). As shown in Figure 2b, the EJ4 strain consumed cellobiose at nearly a 200% higher rate than the EJ3 strain. Interestingly, the sequenced the genome of the EJ4 strain did not contain any mutations related to the rapid cellobiose fermenting phenotype, except for increased copy numbers of *gh1-1* and *cdt-1* in the genome of the strain. Quantitative PCR analysis results showed that strain EJ4 had substantially higher copy numbers of *gh1-1* (18 copies) and *cdt-1* (4 copies) than the strain EJ3 (6 copies of *gh1-1* and 2 copies of *cdt-1*) (Figure 3a). Also, an enzymatic activity assay showed that the EJ4 strain had a higher *β*-glucosidase activity, which corresponded well with its increased cellobiose consumption rate and increased copy number of *gh1-1* (Figure 3b). With the improvement in cellobiose fermentation ability due to amplification of *cdt-1* and *gh1-1*, the EJ4 strain was able to coconsume cellobiose and xylose simultaneously (SI Figure S2).

The strain EJ4 was then transformed with a plasmid containing an *E. coli adhE* overexpression cassette for an acetate reduction pathway. The resulting strain coexpressing

DOI: 10.1021/sb500364q
ACS Synth. Biol. 2015, 4, 707−713

Table 1. Strains and Plasmids Used in This Study

strains or plasmids	description	ref
	strains	
D452-2	*MATa, leu2, his3, ura3, can1*	34
SR8	D452-2 expressing *XYL1, XYL2,* and *XKS1* through integration (two copies for each gene), evolutionary engineering in xylose-containing media, and *ALD6* deletion; point mutation Gly253Asp in *PHO13* gene	10
EJ3	SR8 *leu2*::*LEU2* pRS405-gh1-1*ura3*::*URA3* pRS406-cdt-1	
EJ4	Evolved strain of EJ3 by repeated transferring in cellobiose-containing media	this study
EJ4-a	EJ4 expressing *E. coli adhE* in a multicopy plasmid (pRS425-*kanMX-adhE*)	this study
EJ4-c	EJ4 containing a multicopy plasmid without insert (pRS425-*kanMX*), as a control	this study
plasmids		
pRS405	integration plasmid, *LEU2*	34
pRS406	integration plasmid, *URA3*	34
pRS425	Multicopy plasmid, 2 μm origin, *LEU2*	34
pRS425-gh1-1	pRS425 *PGK 1p-gh1-1-CYC1t*	27
pRS426-cdt-1	pRS426 *PGK 1p-cdt-1-CYC1t*	27
pRS405-gh1-1	pRS405 *PGK 1p-gh1-1-CYC1t*	this study
pRS406-cdt-1	pRS406 *PGK 1p-cdt-1-CYC1t*	this study
pRS425-*kanMX*	pRS425 with *kanMX*	26
pRS425-*kanMX-adhE*	*E. coli adhE* under the control of *GPD* promoter and *CYC1* terminator in pRS425-*kanMX*	26

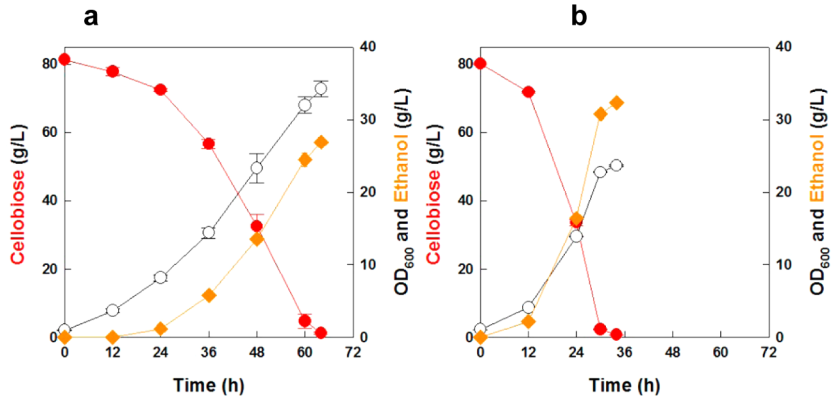


Figure 2. Fermentation of cellobiose under oxygen-limited conditions by *S. cerevisiae* strain EJ3 (a) and EJ4 (b). Results are the mean of duplicate experiments; error bars indicate standard deviations and are not visible when smaller than the symbol size. Symbols: cell growth (open circle), cellobiose (red circle), and ethanol (orange diamond).

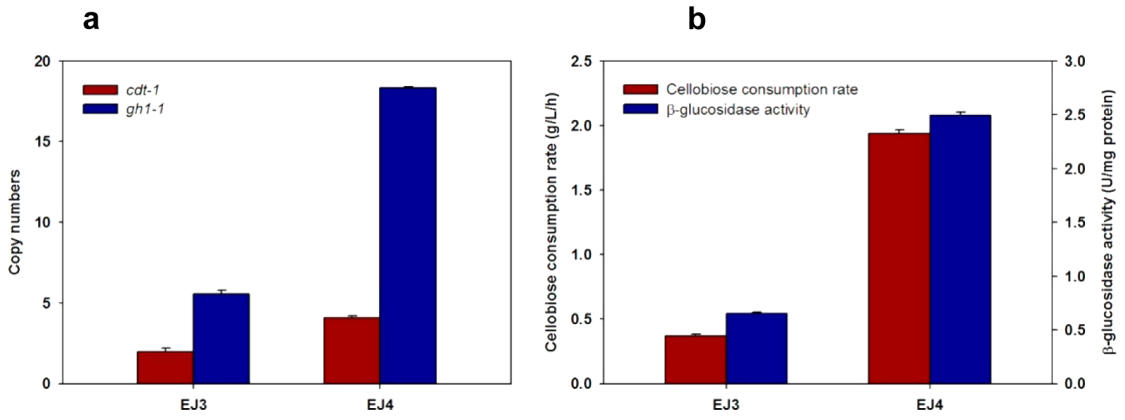


Figure 3. Copy numbers of the *cdt-1* and *gh1-1* genes (a), and cellobiose consumption rates and β-glucosidase activities (b) in the strains EJ3 and EJ4 in medium containing 20 g/L cellobiose. Results are the mean of duplicate experiments and error bars indicate standard deviations.

three heterologous pathways to coconsume xylose, cellobiose, and acetic acid was named EJ4-a, and the EJ4 strain with an empty (control) plasmid was named EJ4-c (Table 1). Batch fermentation experiments under anaerobic conditions were performed to examine the ability of the engineered strains for simultaneous utilization of the three target substrates and to determine the effect of acetate reduction on the anaerobic fermentation of xylose and cellobiose.

Simultaneous coconsumption of cellobiose, xylose, and acetic acid was achieved by the strain EJ4-a under strictly anaerobic conditions (Figure 4a). All the cellobiose added (40 g/L) and nearly 40 g/L xylose were consumed within 120 h and 1.4 g/L

DOI: 10.1021/sb500364q
ACS Synth. Biol. 2015, 4, 707−713

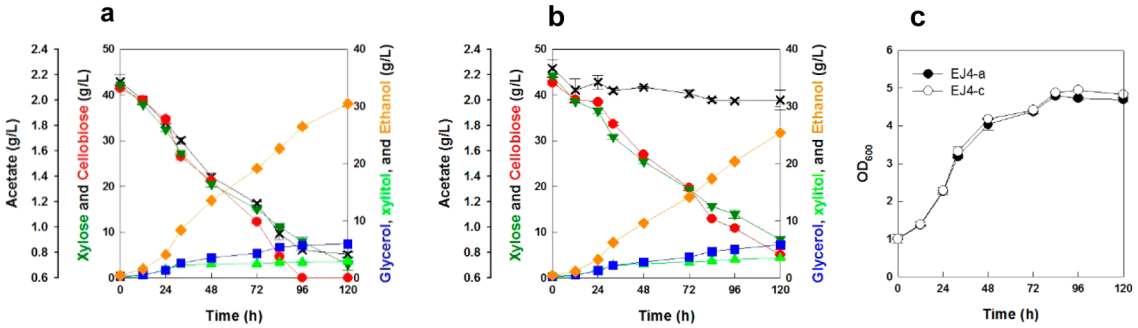


Figure 4. Anaerobic fermentation of cellobiose and xylose with simultaneous reduction of acetate by *S. cerevisiae* strain EJ4-a (a) and by the strain EJ4-c (b). (c) Cell growth (OD_{600}) comparison for EJ4-a and EJ4-c. Results are the mean of duplicate experiments; error bars indicate standard deviations and are not visible when smaller than the symbol size. Symbols: cell growth (open and closed circle), cellobiose (red circle), xylose (green triangle down), acetate (black x), glycerol (blue square), xylitol (green triangle up), and ethanol (orange diamond).

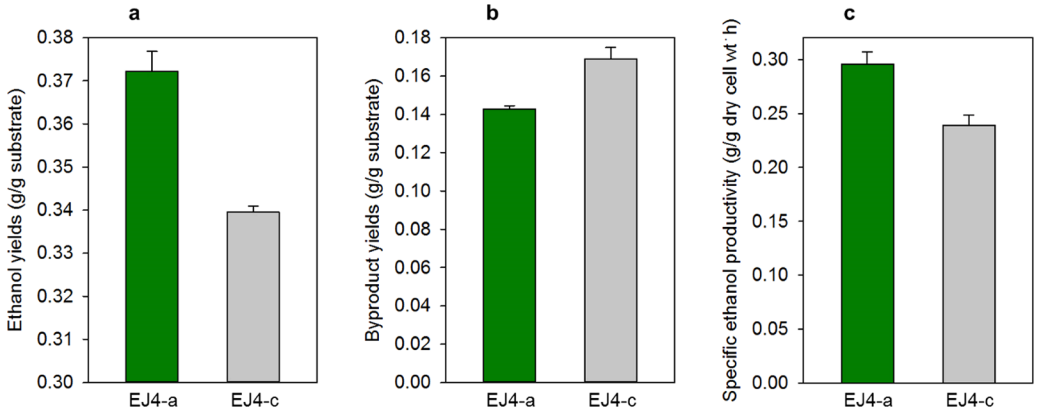


Figure 5. Ethanol yields (a), byproduct yields (b), and specific ethanol productivity (c) during anaerobic fermentation by strains EJ4-a and EJ4-c in medium containing 40 g/L cellobiose, 40 g/L xylose, and 2 g/L acetate. Results are the mean of duplicate experiments and error bars indicate standard deviations.

acetate was consumed, demonstrating functional coexpression of the three heterologous pathways in the strain EJ4-a. In comparison, the control strain EJ4-c without the acetate reduction pathway was unable to consume acetate (Figure 4b). The final ethanol concentration in the fermentation by EJ4-a was above 30 g/L (Figure 4a). In contrast, fermentation by the EJ4-c strain had substantial amounts of cellobiose and xylose remaining even after 120 h and only 25 g/L ethanol was produced (Figure 4b). The results demonstrated that a single engineered yeast platform could integrate the pathways for coutilization of three important cellulosic biomass components (cellobiose, xylose, and acetic acid) for ethanol production under industrially relevant anaerobic conditions.

Close comparisons of the fermentation performance by EJ4-a and the control EJ4-c strains revealed synergistic effects of the pathway integration (Figure 5). Co-fermentation of the three substrates by EJ4-a led to a substantially higher ethanol yield (Figure 5a) and a lower accumulation of byproducts (Figure 5b) than the control without acetate consumption. Acetate reduction to ethanol by the strain EJ4-a could directly contribute to the improved ethanol yields meanwhile, reoxidization of NADH to NAD^+ through the AADH reaction could alleviate the redox cofactor imbalance from the xylose fermentation and thus allow more carbon flux to ethanol instead of byproducts.[26] It is worthy of mentioning that the surplus NADH from the XR-XDH pathway was beneficial here in terms of providing the reducing equivalent required for acetate reduction to ethanol. Additionally, the specific ethanol productivity by the strain EJ4-a was 24% higher than that by EJ4-c (Figure 5c). The results show the benefits in increasing both ethanol yield and productivity by combining the acetate reduction pathway with mixed sugar fermentation. Noticeably, the coutilization of xylose and acetic acid in our previous study[26] had a specific ethanol productivity of only 0.12 g/(g dry cell wt·h), while the coconsumption of cellobiose together with xylose and acetic acid by the EJ4-a strain in this study under the same conditions had much higher specific ethanol productivity of 0.3 g/(g dry cell wt·h) (Figure 5c), demonstrating the synergistic effects of simultaneous fermentation of mixed sugars.

It is noteworthy that the growth curves of the strains EJ4-a and EJ4-c were similar (Figure 4c), even though the acetate reduction pathway in the strain EJ4-a consumed ATP (Figure 1). Furthermore, the EJ4-a strain had an even higher sugar consumption rate than the EJ4-c strain (Figure 4a and b). The results are consistent with our previous observations[26] and suggest that the ATP supply might not be a limiting factor and that the beneficial effects of implementing the acetate reduction pathway might overcome the potential negative effect of additional ATP consumption. In addition, the biomass yield of the strain EJ4-a (0.0112 ± 0.0002) was lower than that of EJ4-c (0.0130 ± 0.0002), suggesting that implementing the ATP-consuming acetate reduction pathway could have advantage in reducing the carbon flux toward biomass.[29−31]

The study demonstrated for the first time the coconversion of three major carbon components (xylose, cellobiose, and

DOI: 10.1021/sb500364q
ACS Synth. Biol. 2015, 4, 707−713

acetic acid) derived from cellulosic feedstocks to ethanol in a single engineered microbial cell platform. The economically feasible production of cellulosic biofuels requires a microbial system capable of using the mixed substrates derived from cellulosic biomass for high-yield and rapid production of fuel compounds, but natural organisms able to metabolize multiple cellulosic substrates simultaneously have yet to be discovered. The metabolic engineering strategy reported here integrated three heterologous pathways into one yeast strain to solve challenges in developing efficient and cost-effective bioconversion processes for cellulosic biofuel production and shows the unique value of designing metabolic pathways in a networked way and exploring the synergy between individual pathways. Development of the strain EJ4-a presents an innovative metabolic engineering approach whereby multiple substrate consumption pathways can be integrated in a synergistic way for enhanced bioconversion.

The substrate coutilization approach described here not only increases the ethanol yield and productivity but can also contribute to the development of consolidated bioprocessing (CBP) for cellulosic biofuels. CBP has received increased attention and research effort due to the economic benefits of process integration.[32] Its ultimate goal is to combine all of the processes involved in cellulosic biofuel production into one cost-effective step. One critical challenge and technical obstacle to process integration is the development of a robust CBP-enabling microorganism, which ideally has inhibitor tolerance, ability to utilize hexose and pentose sugars simultaneously at high efficiency, and ability to depolymerize lignocelluloses.[1,4] The strain EJ4-a shows several traits that are beneficial for CBP: the simultaneous utilization of various major substrates from lignocellulosic biomass increases productivity and yield and reduces the overall fermentation time; reduction of the fermentation inhibitor acetic acid allows conversion of the previously unused carbon fraction to ethanol as well as in situ detoxification; and the assimilation of cellobiose and intracellular hydrolysis using β-glucosidase produced by strain reduces the requirement for exogenous enzymes. Future work will focus on strain optimization to improve the above-mentioned traits and thus advance the development of economically viable cellulosic biofuels.

METHODS

Strains, Media, and Culture Conditions. All strains used in this study are summarized in Table 1. *Escherichia coli* DH5 (*F− recA1 endA1 hsdR17 [rK− mK+] supE44 thi-1 gyrA relA1*) (Invitrogen, Gaithersburg, MD) was used for gene cloning and manipulation. *E. coli* was grown in Luria−Bertani medium; 50 μg/mL of ampicillin was added to the medium when required. Yeast strains were routinely cultivated at 30 °C in YP medium (10 g/L of yeast extract and 20 g/L of peptone) with 20 g/L of glucose or 20 g/L of cellobiose and/or xylose.

Strains and Plasmid Construction. All plasmids are listed in Table 1. In order to construct integrative plasmids expressing β-glucosidase (*gh1-1*) and cellodextrin transporter (*cdt-1*), the expression cassettes of β-glucosidase (*gh1-1*) and cellodextrin transporter (*cdt-1*), which are both under the control of a *PGK1* promoter and *CYC1* terminator, were amplified by PCR with T3 and T7 sites respectively from previously developed multicopy yeast plasmids.[25] Linearized vectors were prepared by PCR amplifying the region between the T3 and T7 sites of empty plasmids pRS405 and pRS406. Auxotrophic markers and replicons are the backbone of the region. Expression cassettes with T3/T7 sites were cloned to the linearized vectors by using CloneEZ PCR cloning kit (Genscript, Piscataway, NJ). Recombination using homologous T3/T7 sites was performed according to the manufacturer's protocol. The resulting plasmids pRS405-gh1-1 and pRS406-cdt-1 were genome-integrated at the *LEU2* locus and *URA3* locus of the SR8 strain, respectively, yielding the strain EJ3. Because SR8 did not have any auxotrophic markers, the strain EJ3 was selected on yeast synthetic complete (YSC) agar medium containing 20 g/L of cellobiose as the sole carbon source.

The plasmid containing the adhE expression cassette and control plasmid was constructed in our lab, as described previously.[26] Transformation of the plasmid constructs into the strain EJ4 was performed using the yeast EZ-Transformation kit (BIO 101, Vista, CA). YP agar plate containing 20 g/L glucose and 200 μg/mL G418 was used to select positive yeast transformants using the *KanMX* resistance gene marker. The resulting transformants were named EJ4-a and EJ4-c.

Fermentation Experiments. Yeast cells were grown in YP medium containing 20 g/L of glucose or cellobiose with 200 μg/mL G418 when needed to prepare inoculums for ethanol production fermentation experiments. Cells were harvested at midexponential phase and inoculated after washing twice with sterilized water with an initial optical density (OD) of 1.0 at 600 nm. Flask fermentation experiments under oxygen-limited conditions were performed using 25 mL of YP medium containing appropriate sugars in a 125 mL flask at 30 °C and 100 rpm. Anaerobic cofermentation experiments using mixed substrates were performed in serum bottles containing YP medium and appropriate substrates flushed with nitrogen and sealed with butyl rubber stoppers at 30 °C and 100 rpm. Ergosterol and Tween 80 were added to final concentrations of 0.01 g/L and 0.42 g/L, respectively, for anaerobic fermentation.[33]

Enzymatic Assay of β-Glucosidase Activity. Yeast cells grown to mid log phase at 30 °C in YP medium with 20 g/L of cellobiose were harvested by centrifugation at 3000*g* for 5 min, and the cell pellet was washed and suspended in Y-PER solution (Pierce, Rockford, IL). A crude cell extract was prepared following the manufacturer's instructions. The β-glucosidase activity of the crude cell extract was measured according to previous reports.[27] Briefly, the reaction solution contained 50 mM sodium acetate buffer (pH 4.8), 6.7 mM para-nitrophenyl β-D-glucopyranoside (*p*NPG), and the crude cell extract. The release of *p*-nitrophenol (NP) was monitored by a microplate reader (Synergy 2; Biotek, Winooski, VT) at 405 nm during 1 h of incubation at 30 °C. One unit of enzyme activity is defined as the amount of enzyme that catalyzes 1 μmol of substrate per min at 30 °C. Protein concentration was determined by the BCA method (Pierce, Rockford, IL).

Determination of Genomic Copy Numbers of *gh1-1* and *cdt-1* by Quantitative PCR. The genomic DNA of the strains to test was prepared with the YeaStar Genomic DNA kit (Zymo Research, Orange, CA) and quantified by NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE). Real-time quantitative PCR was performed on a Lightcycler 480 instrument (Roche Applied Science, Indianapolis, IN) with SYBR Green I Master (Roche) following the manufacturer's introductions. As shown in SI Table S1, primers were designed to detect the *gh1-1* and *cdt-1* genes. A standard curve was generated by a*gh1-1* gene fragment purified from pRS425-gh1-1 and another curve by a *cdt-1* fragment purified from pRS426-cdt-1. As described by Kim *et al.*,[10] the genomic copy numbers

DOI: 10.1021/sb500364q
ACS Synth. Biol. 2015, 4, 707−713

(X) of the gene in the genomic DNA samples were estimated by the equation $AX/B = C/D$, where A is the size of the *gh1-1* (1.43 kb) or *cdt-1* (1.74 kb) gene fragment, B is the size of the whole genome of *S. cerevisiae* (12000 kb), C is the gene concentration calculated by quantitative PCR (ng/μL), and D is the concentration of the genomic DNA samples (ng/μL).

Analytical Methods. Cell growth was monitored by optical density (OD) at 600 nm using a UV−visible Spectrophotometer (Biomate 5, Thermo, NY). The cellobiose, xylose, glucose, glycerol, xylitol, acetate, and ethanol concentrations were determined by a high performance liquid chromatography (HPLC, Agilent Technologies 1200 Series) equipped with a refractive index detector as described before.[26]

■ ASSOCIATED CONTENT

Supporting Information

Evolution of the EJ3 strain; co-fermentation of cellobiose and xylose under oxygen-limited conditions; primers used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ysjin@illinois.edu.

Author Contributions

#N.W. and E.J.O. contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by funding from the Energy Biosciences Institute.

■ REFERENCES

(1) Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M. F., Liden, G., and Zacchi, G. (2006) Bio-ethanol—The fuel of tomorrow from the residues of today. *Trends Biotechnol. 24*, 549−556.

(2) Tilman, D., Socolow, R., Foley, J. A., Hill, J., Larson, E., Lynd, L., Pacala, S., Reilly, J., Searchinger, T., Somerville, C., et al. (2009) Beneficial biofuels—The food, energy, and environment trilemma. *Science 325*, 270−271.

(3) Somerville, C. (2006) The billion-ton biofuels vision. *Science 312*, 1277−1277.

(4) Lynd, L. R., van Zyl, W. H., McBride, J. E., and Laser, M. (2005) Consolidated bioprocessing of cellulosic biomass: An update. *Curr. Opin. Biotechnol. 16*, 577−583.

(5) Sun, Y., and Cheng, J. (2002) Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresour. Technol. 83*, 1.

(6) Saha, B. C. (2003) Hemicellulose bioconversion. *J. Ind. Microbiol. Biotechnol. 30*, 279−291.

(7) Klinke, H. B., Thomsen, A. B., and Ahring, B. K. (2004) Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Appl. Microbiol. Biotechnol. 66*, 10−26.

(8) Weber, C., Farwick, A., Benisch, F., Brat, D., Dietz, H., Subtil, T., and Boles, E. (2010) Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels. *Appl. Microbiol. Biotechnol. 87*, 1303−1315.

(9) Kim, S. R., Ha, S. J., Wei, N., Oh, E. J., and Jin, Y. S. (2012) Simultaneous co-fermentation of mixed sugars: A promising strategy for producing cellulosic ethanol. *Trends Biotechnol. 30*, 274−282.

(10) Kim, S. R., Skerker, J. M., Kang, W., Lesmana, A., Wei, N., Arkin, A. P., and Jin, Y. S. (2013) Rational and evolutionary engineering approaches uncover a small set of genetic changes efficient for rapid xylose fermentation in *Saccharomyces cerevisiae*. *PLoS One 8*, e57048.

(11) Hahn- Hägerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I., and Gorwa-Grauslund, M. F. (2007) Towards industrial pentose-fermenting yeast strains. *Appl. Microbiol. Biotechnol. 74*, 937−953.

(12) Nielsen, J., Larsson, C., van Maris, A., and Pronk, J. (2013) Metabolic engineering of yeast for production of fuels and chemicals. *Curr. Opin. Biotechnol. 24*, 398−404.

(13) Hong, K. K., and Nielsen, J. (2012) Metabolic engineering of *Saccharomyces cerevisiae*: A key cell factory platform for future biorefineries. *Cell. Mol. Life Sci. 69*, 2671−2690.

(14) Jeffries, T. W., and Shi, N. Q. (1999) Genetic engineering for improved xylose fermentation by yeasts. *Adv. Biochem. Eng. Biotechnol. 65*, 117.

(15) Kim, S. R., Ha, S.-J., Kong, I. I., and Jin, Y.-S. (2012) High expression of XYL2 coding for xylitol dehydrogenase is necessary for efficient xylose fermentation by engineered *Saccharomyces cerevisiae*. *Metab. Eng. 14*, 336−343.

(16) Matsushika, A., Inoue, H., Kodaki, T., and Sawayama, S. (2009) Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: Current state and perspectives. *Appl. Microbiol. Biotechnol. 84*, 37−53.

(17) Jeffries, T. W., and Jin, Y. S. (2004) Metabolic engineering for improved fermentation of pentoses by yeasts. *Appl. Microbiol. Biotechnol. 63*, 495−509.

(18) Zhou, H., Cheng, J. S., Wang, B. L., Fink, G. R., and Stephanopoulos, G. (2012) Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. *Metab. Eng. 14*, 611−622.

(19) van Maris, A. J. A., Winkler, A. A., Kuyper, M., and Pronk, J. T. (2007) *Biofuels 108*, 179−204.

(20) Almeida, J. R. M., Modig, T., Petersson, A., Hahn-Hägerdal, B., Liden, G., and Gorwa-Grauslund, M. F. (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *J. Chem. Technol. Biotechnol. 82*, 340−349.

(21) Pampulha, M. E., and Loureirodias, M. C. (1989) Combined effect of acetic-acid, pH And ethanol on intracellular pH of fermenting yeast. *Appl. Microbiol. Biotechnol. 31*, 547−550.

(22) Bellissimi, E., van Dijken, J. P., Pronk, J. T., and van Maris, A. J. A. (2009) Effects of acetic acid on the kinetics of xylose fermentation by an engineered, xylose-isomerase-based *Saccharomyces cerevisiae* strain. *FEMS Yeast Res. 9*, 358−364.

(23) Palmqvist, E., and Hahn-Hägerdal, B. (2000) Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. *Bioresour. Technol. 74*, 17−24.

(24) Alves Silva, J. P., Carneiro, L. M., and Roberto, I. C. (2013) Treatment of rice straw hemicellulosic hydrolysates with advanced oxidative processes: A new and promising detoxification method to improve the bioconversion process. *Biotechnol. Biofuels*, 6.

(25) Ha, S. J., Galazka, J. M., Kim, S. R., Choi, J. H., Yang, X., Seo, J. H., Glass, N. L., Cate, J. H. D., and Jin, Y.-S. (2011) Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. *Proc. Natl. Acad. Sci. U.S.A. 108*, 504−509.

(26) Wei, N., Quarterman, J., Kim, S. R., Cate, J. H. D., and Jin, Y. S. (2013) Enhanced biofuel production through coupled acetic acid and xylose consumption by engineered yeast. *Nature Commun.*, 4.

(27) Galazka, J. M., Tian, C., Beeson, W. T., Martinez, B., Glass, N. L., and Cate, J. H. D. (2010) Cellodextrin transport in yeast for improved biofuel production. *Science 330*, 84−86.

(28) Jeffries, T. W. (2006) Engineering yeasts for xylose metabolism. *Curr. Opin. Biotechnol. 17*, 320.

(29) de Kok, S., Yilmaz, D., Suir, E., Pronk, J. T., Daran, J. M., and van Maris, A. J. A. (2011) Increasing free-energy (ATP) conservation in maltose-grown *Saccharomyces cerevisiae* by expression of a heterologous maltose phosphorylase. *Metab. Eng. 13*, 518−526.

(30) Ha, S. J., Galazka, J. M., Oh, E. J., Kordic, V., Kim, H., Jin, Y. S., and Cate, J. H. D. (2013) Energetic benefits and rapid cellobiose fermentation by *Saccharomyces cerevisiae* expressing cellobiose

DOI: 10.1021/sb500364q
ACS Synth. Biol. 2015, 4, 707−713

phosphorylase and mutant cellodextrin transporters. *Metab. Eng. 15*, 134−143.
(31) Semkiv, M. V., Dmytruk, K. V., Abbas, C. A., and Sibirny, A. A. (2014) Increased ethanol accumulation from glucose via reduction of ATP level in a recombinant strain of *Saccharomyces cerevisiae* overexpressing alkaline phosphatase. *BMC Biotechnol. 14*, 42−42.
(32) Olson, D. G., McBride, J. E., Shaw, A. J., and Lynd, L. R. (2012) Recent progress in consolidated bioprocessing. *Curr. Opin. Biotechnol. 23*, 396−405.
(33) Eliasson, A., Christensson, C., Wahlbom, C. F., and Hahn-Hägerdal, B. (2000) Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying XYL1, XYL2, and XKS1 in mineral medium chemostat cultures. *Appl. Environ. Microbiol. 66*, 3381−3386.
(34) Mumberg, D., Muller, R., and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene 156*, 119−122.

DOI: 10.1021/sb500364q
ACS Synth. Biol. 2015, 4, 707−713