ACS SyntheticBiology

Research Article

pubs.acs.org/synthbio

Recycling Carbon Dioxide during Xylose Fermentation by Engineered *Saccharomyces cerevisiae*

Peng-Fei Xia,[†,‡] Guo-Chang Zhang,[§] Berkley Walker,[‡,∥] Seung-Oh Seo,[§] Suryang Kwak,[‡,§] Jing-Jing Liu,[‡] Heejin Kim,[‡,§] Donald R. Ort,[‡,∥,⊥] Shu-Guang Wang,[†] and Yong-Su Jin*[,‡,§]

[†]School of Environmental Science and Engineering, Shandong University, 27 Shanda Nanlu, Jinan 250100, People's Republic of China

[‡]Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana−Champaign, 1206 West Gregory Drive, Urbana, Illinois 61801, United States

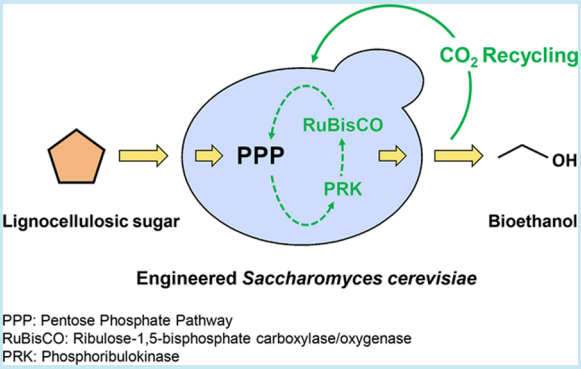
[§]Department of Food Science and Human Nutrition, University of Illinois at Urbana−Champaign, 905 South Goodwin Avenue, Urbana, Illinois 61801, United States

[∥]Photosynthetic Research Unit, United States Department of Agriculture, Urbana Illinois 61801, United States

[⊥]Department of Plant Biology, University of Illinois at Urbana−Champaign, Urbana, Illinois 61801, United States

Supporting Information

ABSTRACT: Global climate change caused by the emission of anthropogenic greenhouse gases (GHGs) is a grand challenge to humanity. To alleviate the trend, the consumption of fossil fuels needs to be largely reduced and alternative energy technologies capable of controlling GHG emissions are anticipated. In this study, we introduced a synthetic reductive pentose phosphate pathway (rPPP) into a xylose-fermenting *Saccharomyces cerevisiae* strain SR8 to achieve simultaneous lignocellulosic bioethanol production and carbon dioxide recycling. Specifically, ribulose-1,5-bisphosphate carboxylase/oxygenase from *Rhodospirillum rubrum* and phosphoribulokinase from *Spinacia oleracea* were introduced into the SR8 strain. The resulting strain with the synthetic rPPP was able to exhibit a higher yield of ethanol and lower yields of byproducts (xylitol and glycerol) than a control strain. In addition, the reduced release of carbon dioxide by the engineered strain was observed during xylose fermentation, suggesting that the carbon dioxide generated by pyruvate decarboxylase was partially reassimilated through the synthetic rPPP. These results demonstrated that recycling of carbon dioxide from the ethanol fermentation pathway in yeast can be achieved during lignocellulosic bioethanol production through a synthetic carbon conservative metabolic pathway. This strategy has a great potential to alleviate GHG emissions during the production of second-generation ethanol.



KEYWORDS: *CO_2 recycling, carbon conservation, bioethanol, Saccharomyces cerevisiae*

Global climate change caused by anthropogenic emission of greenhouse gases (GHGs) is one of the grand challenges jeopardizing human society. The consumption of fossil fuels needs to be limited to mitigate carbon dioxide (CO_2) emission and global warming.[1−4] Therefore, an alternative energy source is necessary to decrease reliance on fossil fuels. Biofuel has been considered a promising solution,[2] and bioethanol is currently the most widely produced and utilized biofuel at industrial scales.[5,6] Traditionally, bioethanol has been produced by an innate producer, *Saccharomyces cerevisiae*, *via* sugar fermentation using sugar cane and cornstarch as feedstock. However, this utilization of edible biomass for producing biofuel inevitably faces a dilemma between "food versus fuel".[7]

Lignocellulosic biomass, which is derived from agriculture and forestry residues or industrial wastes, is the most abundant organic resource on Earth, and it might be a better feedstock for bioethanol production without disturbing the food supply and arable land.[1,8,9] However, *S. cerevisiae* cannot ferment xylose, which is the second most abundant sugar in lignocellulosic biomass hydrolysates.[10−12] To solve this problem, two alternative heterologous xylose utilization pathways have been introduced into *S. cerevisiae*. One is the xylose reductase (XR)/xylitol dehydrogenase (XDH) pathway from *Scheffersomyces stipitis* (Figure 1),[13] and the other is the bacterial xylose utilization pathway in which xylose isomerase converts xylose to xylulose.[14,15] By introducing xylose utilization pathways together with up-regulation of the native pentose phosphate pathway (PPP), efficient and rapid fermentation of xylose into ethanol has been achieved.[10,11]

Received: June 12, 2016
Published: October 17, 2016

DOI: 10.1021/acssynbio.6b00167
ACS Synth. Biol. 2017, 6, 276−283

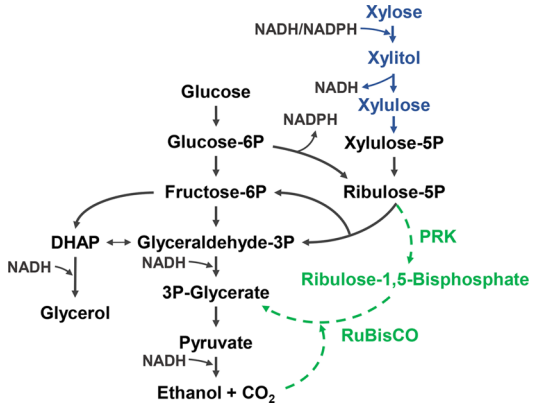


Figure 1. Overall scheme of CO_2 recycling during lignocellulosic bioethanol production. The heterologous XR/XDH pathway (blue), native glycolysis and PPP (black) in the xylose-fermenting strain *S. cerevisiae* SR8, plus the synthetic rPPP (green).

As a carbon-neutral process, lignocellulosic bioethanol production can contribute to the reduction of CO_2 emission, but it will be ideal if the fermentation process becomes carbon-conservative *via* reassimilating the carbon from the carbon dioxide released from the decarboxylation step of pyruvate into acetaldehyde in the ethanol-producing pathway. Bioethanol production from phototrophic microorganisms offers a promising future to capture the lost carbon through direct fixation of atmospheric CO_2. However, the titers of usable biofuels produced from the engineered autotrophic microorganisms have so far failed to match the industrial requirements.[16−19] In the meanwhile, the feasibility of introducing CO_2 conservative pathways to heterotrophic hosts has been demonstrated. For instance, the CO_2 fixation pathways including the reductive pentose phosphate pathway (rPPP)[20−22] and the 3-hydroxypropionate pathway,[23] as well as synthetic pathways like the nonoxidative glycolysis[24] and methanol condensation pathways,[25] have been introduced into either *Escherichia coli* or *S. cerevisiae*. These achievements exhibited the possibilities to combine the advantages of lignocellulosic bioethanol production and direct CO_2 conservation.

In this study, we introduced two key enzymes driving the rPPP, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and phosphoribulokinase (PRK), into an engineered *S. cerevisiae* strain SR8 harboring the XR/XDH pathway and up-regulated PPP[11] to enable CO_2 recycling through a synthetic rPPP during xylose fermentation (Figure 1). Particularly, RuBisCO converts one ribulose-1,5-bisphosphate (RuBP) and CO_2 to two molecules of glycerate-3-phosphate (G3P), and PRK catalyzes ribulose-5-phosphate (R5P) to RuBP. As the XR/XDH pathway generates surplus nicotinamide adenine dinucleotide (NADH) during xylose fermentation under anaerobic conditions,[26] the synthetic rPPP can exploit the surplus NADH for recycling CO_2. In addition, the robust PPP of the SR8 strain allows substrates to initiate the synthetic pathway. The feasibility of this strategy is demonstrated in this study. To our knowledge, it is the first study investigating and achieving CO_2 recycling during lignocellulosic bioethanol production.

RESULTS AND DISCUSSION

Functional Expression of *Rhodospirillum rubrum* RuBisCO in *S. cerevisiae*. The first step of installing the rPPP for reassimilating carbon dioxide is to functionally express RuBisCO in yeast. In this study, we chose form-II RuBisCO from *R. rubrum*, which consists of only large subunits and forms a dimer.[27,28] The *R. rubrum* RuBisCO has been reported to be functionally expressed in *E. coli* with the help of group-I HSP60 chaperonins, GroEL and GroES.[28] As the cytosolic HSP60 chaperonins in eukaryotic cytosols are different from the group-I chaperonins,[29,30] the *E. coli* GroEL and GroES chaperonins (denoted together as GroE) were introduced into *S. cerevisiae* together with *R. rubrum* RuBisCO. All three genes were integrated *via* CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins) based genome modification into the genome of *S. cerevisiae* SR8, which is capable of fermenting xylose rapidly.[11]

The first copy of *cbbM* (coding for the *R. rubrum* RuBisCO) under the control of a constitutive promoter P_{TDH3} and the *groE* cassette (consisting of *groL* and *groS* under the control of P_{TDH3} and P_{TEF1}, respectively) were integrated into the *ALD6* and *PHO13* loci to generate the SR8C strain. The *ALD6* and *PHO13* genes, beneficial knockout targets for enhanced xylose fermentation, were already deactivated during construction of the SR8 strain[11] so that the integrations of *cbbM* and *groE* expression cassettes into the loci might not cause unintended phenotype changes. As we expected, the RuBisCO was active in the SR8C strain when the *cbbM* was coexpressed with the GroE chaperonins (Figure 2). The RuBisCO activity in the crude

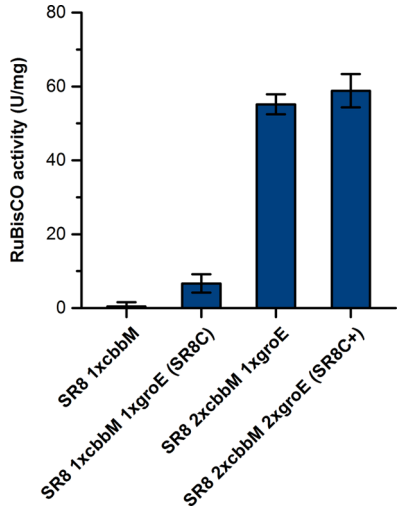


Figure 2. RuBisCO activity of engineered *S. cerevisiae*. The *cbbM* gene together with the *groE* cassette were integrated into the genome of SR8 using CRISPR/Cas9. The activity was measured by monitoring the change of NADH spectrophotometrically *via* coupled enzymatic reaction.

extract of the SR8C strain was similar to that reported in a previous study expressing *cbbM* from a different microorganism.[20] As RuBisCO is a notoriously inefficient enzyme, we examined if we can further increase the RuBisCO activity in yeast by gene dosage. Another copy of *cbbM* under the control of P_{TDH3} was integrated into the intergenic site (CS6) of the SR8C strain. We observed a significant increase of RuBisCO activity in the SR8C strain with the extra copy of *cbbM* (SR8C-*cbbM*). The RuBisCO activity in the SR8C-*cbbM* strain was 8-fold higher than that in the SR8C strain. A second copy of the *groE* cassette was then integrated into another intergenic site (CS8) of the SR8C-*cbbM* strain (with two copies of *cbbM* and

DOI: 10.1021/acssynbio.6b00167
ACS Synth. Biol. 2017, 6, 276−283

one copy of *groE*) to generate a stable platform strain SR8C+, which has two copies of *cbbM* plus two copies of *groE*. As a result, the highest RuBisCO activity was achieved in the SR8C+ strain and reached 58.8 ± 4.5 U/mg (Figure 2). The fermentation profiles of all engineered strains were examined (Figure S1), and no significant differences were observed, indicating that functional expression of *R. rubrum* RuBisCO and additional expression of *groE* have no significant influence on the fermentation properties of the engineered yeast strains.

Effects of PRK on Sugar Metabolism in *S. cerevisiae*. PRK was known to be functionally expressed in either bacterial or eukaryotic cells, but merely expressing PRK was toxic to the host.[20,22] It has been reported that even the native host of PRK, *R. rubrum*, cannot grow well when a mutation occurs in RuBisCO and RuBP becomes an end-product inside of the cell.[31] In this study, the PRK was overexpressed in the SR8 strain using a multicopy plasmid carrying the *prk* gene (coding for PRK) from *Spinacia oleracea* under the control of P_{TEF1}. As expected, the toxic effect of PRK overexpression in the SR8 strain was observed when xylose was the carbon source (Figures 3a and S2c) in both yeast extract-peptone (YP) and synthetic complete (SC) media. However, the toxicity was not observed when glucose was the carbon source (Figure S2a,b).

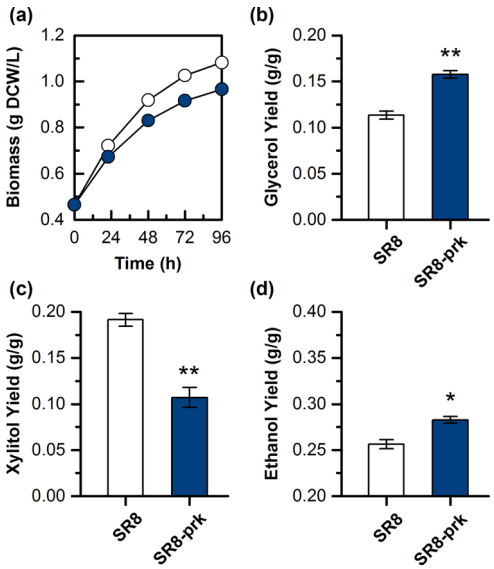


Figure 3. Growth and fermentation profiles of SR8 expressing PRK. Engineered SR8 with PRK overexpression was cultivated in SC medium using 20 g/L xylose as the carbon source. Anaerobic fermentation was conducted at 30 °C and 100 rpm. (a) Growth profiles; (b) glycerol, (c) xylitol, and (d) ethanol yield.

Surprisingly, we found that overexpression of *prk* alone in the SR8 strain influenced the production of byproducts and increased the ethanol yield in xylose fermentation (Figures 3 and S3 and Table 1). In both YP and SC media, the variations of byproducts shared similar trends, and the differences were more outstanding in SC media. In SC media (Figure 3), the glycerol yield of the SR8 strain with PRK overexpression (SR8-*prk*) increased 38% from 0.114 ± 0.004 to 0.158 ± 0.004 g/g, while the xylitol yield decreased 44% from 0.191 ± 0.007 to 0.107 ± 0.011 g/g compared with the control strain (SR8 with the empty plasmid). The ethanol yield of the SR8-*prk* strain increased 10% from 0.256 ± 0.005 to 0.283 ± 0.004 g/g. Both the toxicity and the variations of byproducts demonstrated the functional expression of PRK in the SR8 strain. The toxicity and the effects of PRK on xylose fermentation can also be regarded as indicators that the heterologous PRK is interacting with the endogenous metabolic network in yeast, especially when xylose is used as a carbon source.

Completing the Synthetic rPPP in Engineered *S. cerevisiae*. To install a synthetic rPPP capable of reassimilating carbon dioxide, we overexpressed the *prk* in the SR8C and SR8C+ strains exhibiting different RuBisCO activities (Figure 2). Anaerobic fermentation in the SC medium with 20 g/L xylose was conducted to investigate whether or not recycling of CO_2 can be achieved by synthetic rPPP during xylose fermentation. First, the rescue of growth defects of cells expressing *prk* only was observed when overexpressing *prk* in the SR8C and SR8C+ strains (Figure 4a). The SR8C or SR8C+ strains with the overexpression of *prk* (denoted as SR8C-*prk* and SR8C+-*prk*, respectively) grew better than SR8-*prk* (SR8 strain with *prk* overexpression). Moreover, the SR8C+-*prk* strain reached a higher cell density even than the control strain (SR8 with the empty plasmid), indicating that the active PRK and RuBisCO may consume the RuBP and complete the designed pathway.

In xylose fermentation, the yields of glycerol and xylitol, which indicated redox imbalance during anaerobic xylose fermentation, of each engineered strain were analyzed (Figure 4b,c), and the fermentation data at 96 h are listed in Table SIV. The results showed that the glycerol yield of SR8C-*prk* increased 20% from 0.114 ± 0.004 to 0.137 ± 0.007 g/g and the xylitol yield decreased 26% from 0.191 ± 0.007 to 0.141 ± 0.005 g/g compared to the control strain. The xylitol yield of SR8C+-*prk* decreased 23% from 0.191 ± 0.007 to 0.147 ± 0.003 g/g, while the glycerol yield was similar to the control strain. The time course of the fermentation profiles further evidenced the results (Figure S6). These results implied that when the RuBisCO was functionally overexpressed the engineered strain was able to recover from the toxic effects

Table 1. Summary of Fermentation Results by Engineered *S. cerevisiae*[a]

	xylitol yield		glycerol yield		xylitol + glycerol	ethanol yield (g/g)	
strains	g/g	mol/mol	g/g	mol/mol	(mol/mol)	overall[c]	net
SR8	0.191 ± 0.007	0.189 ± 0.007	0.114 ± 0.004	0.185 ± 0.007	0.374 ± 0.003	0.256 ± 0.005	0.324 ± 0.004
SR8-*prk*[b]	0.107 ± 0.011	0.106 ± 0.011	0.158 ± 0.004	0.257 ± 0.006	0.363 ± 0.004	0.283 ± 0.004	0.315 ± 0.003
SR8C-*prk*	0.141 ± 0.005	0.140 ± 0.005	0.137 ± 0.007	0.224 ± 0.012	0.363 ± 0.016	0.283 ± 0.004	0.330 ± 0.005
SR8C+-*prk*	0.147 ± 0.003	0.145 ± 0.003	0.117 ± 0.003	0.191 ± 0.005	0.337 ± 0.003	0.283 ± 0.002	0.336 ± 0.004

[a]Fermentation was conducted in SC media using xylose as the carbon source. [b]"-*prk*" indicates that the *prk* gene was overexpressed in the engineered strain. [c]The overall ethanol yield was calculated based on consumed xylose, while the net ethanol yield was calculated based on the metabolized carbon *via* deducting the produced xylitol.

DOI: 10.1021/acssynbio.6b00167
ACS Synth. Biol. 2017, 6, 276−283

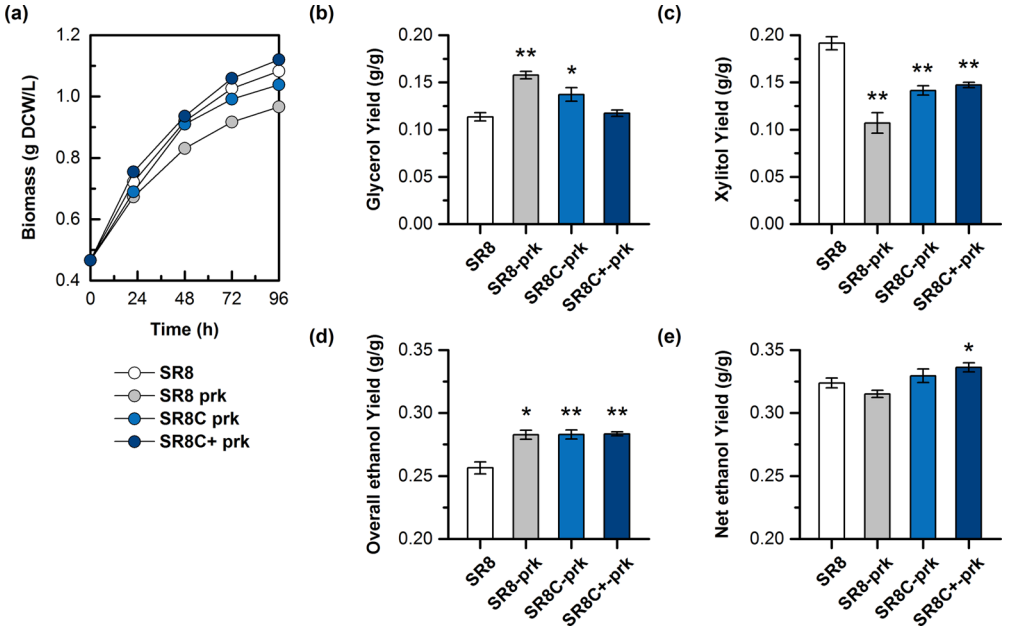


Figure 4. Fermentation properties of the engineered SR8. Fermentation was conducted anaerobically at 30 °C and 100 rpm in SC media with 20 g/L xylose. The overall ethanol yield was calculated based on consumed xylose, while the net ethanol yield was calculated based on the metabolized carbon *via* deducting the produced xylitol. (a) Growth profiles; (b) glycerol, (c) xylitol, (d) overall ethanol yield, and (e) net ethanol yield.

by PRK *via* the metabolism of RuBP by a complete synthetic rPPP.

Interestingly, similar increases in overall ethanol yield were observed in all strains overexpressing *prk* (Figure 4d), but the extra amounts of ethanol were not necessarily produced from the recycled CO_2. We calculated the net ethanol yield based on the metabolized carbon that entered into the PPP *via* deducting the produced xylitol from consumed xylose. The results showed that, even though the overall ethanol yield of SR8-*prk* (SR8 with *prk* overexpression) increased, the net ethanol yield of SR8-*prk* decreased (Figure 4e and Table 1). These results indicate that the extra amounts of ethanol originated from the decreased amounts of byproducts rather than the recycled CO_2. The net ethanol yield of SR8C-*prk* increased from 0.324 ± 0.004 to 0.330 ± 0.005 g/g but is not statistically significant. However, a significant increase of net ethanol yield was identified in the SR8C+ strain with *prk* overexpression, and the net ethanol yield increased from 0.324 ± 0.004 to 0.336 ± 0.004 g/g, demonstrating that the consumed carbon diverted more to produce ethanol and the extra carbon might come from the produced CO_2.

In this study, the host *S. cerevisiae* strain SR8 has been developed for efficient and rapid fermentation of xylose as well as glucose through a combined rational and evolutionary approach.[11] The *XYL1*, *XYL2*, and *XYL3* genes from *S. stipitis*, which code for the XR, XDH, and xylulokinase, respectively, were integrated into the genome of the SR8 strain to achieve xylose utilization. Additionally, the *PHO13* gene was deleted to up-regulate the overall PPP,[32,33] and the *ALD6* gene was deleted to eliminate the production of acetic acid, which is known as an inhibitor of xylose fermentation.[34,35] The resulting robust PPP of the SR8 strain supplies enough R5P to drive the first reaction, and the efficient utilization of xylose offers enough CO_2 for the RuBisCO from the decarboxylation reaction of pyruvate to acetaldehyde during ethanol fermentation. These benefits were more distinguishable when comparing different carbon sources. Because of the repression effect of glucose,[36] little carbon flux was diverted to the PPP when glucose was the carbon source. The overexpression of *prk* in SR8 did not show a toxic effect nor influence the byproducts (Figure S2 and S4), and the SR8C+ stain with *prk* overexpression displayed similar fermentation profiles to the control strain when glucose was the carbon source (Figure S5). When xylose was the carbon source and a robust PPP was achieved, the differences of fermentation profiles between the SR8 or SR8C+ with *prk* overexpression and the control stains were quite clear. We observed a significant increase of ethanol yield and a decrease of byproducts under this condition (Figure 4). Considering all of the data above, we believe that the recycling of CO_2 was achieved during xylose fermentation by introducing the synthetic rPPP.

Ratio between Accumulated CO_2 and Ethanol Reveals Recycling of CO_2. Then, we designed an additional experiment to provide evidence whether the CO_2 was indeed recycled by the synthetic rPPP. Because one pyruvate molecule will release one CO_2 molecule before generating one molecule of ethanol (Figure 1), the ratio between accumulated CO_2 and ethanol ($r_{C/E}$) should be constant for a certain strain in the same fermentation system. A lower $r_{C/E}$ indicates less CO_2 released per unit of ethanol. Thus, we measured both the accumulated CO_2 and ethanol concentration at different sampling points during fermentation. As shown in Figure 5a,b, the quantitative relationships between concentrations of ethanol and the pressure of CO_2 were calculated. The correlations were well-defined using the linear regression model, indicating that the assumption that a certain strain has a constant $r_{C/E}$ was valid. In particular, the $r_{C/E}$ of the SR8 strain during xylose fermentation was 1.34, while the $r_{C/E}$ of SR8C+-*prk* with the synthetic rPPP under the same conditions was 1.12. The ratios of accumulated CO_2 and ethanol from all sampling points were further collected and compared (Figure 5c). The statistical analysis also showed a significant difference (P = 0.037) between the SR8C+-*prk* strain and the control strain. Similar conclusions could be determined *via* a

DOI: 10.1021/acssynbio.6b00167
ACS Synth. Biol. 2017, 6, 276−283

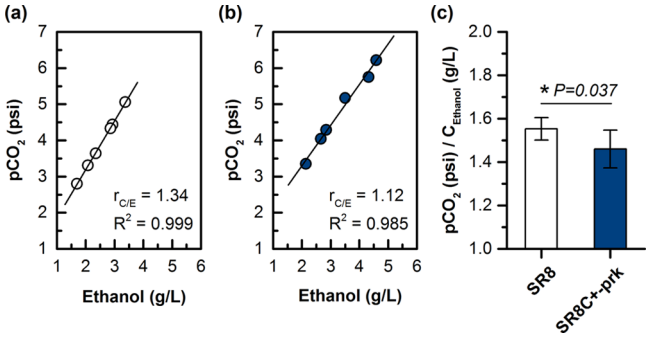


Figure 5. Analysis of the ratio between accumulated CO_2 and produced ethanol ($r_{C/E}$) during xylose fermentation in SC medium. The accumulated pressure of CO_2 and the ethanol concentration of the control strain are plotted in (a), while those of SR8C+ with PRK are plotted in (b). Linear regression was applied, and the slope was calculated to determine $r_{C/E}$. (c) Student's *t* test of all data from different sampling points.

preliminary analysis of carbon balance. The ratios between carbon in CO_2 and ethanol ($r_{C/E\ in\ C}$) of SR8 and SR8C+-*prk* strains were calculated from the carbon balance, and the $r_{C/E\ in\ C}$ of SR8 was 0.752 mol/mol while that of SR8C+-*prk* was 0.708 mol/mol, indicating the reduced release of CO_2 (detailed calculation is included in Supporting Information and Table SI). All of these data demonstrated that the engineered *S. cerevisiae* carrying the synthetic rPPP releases less CO_2 than the control strain during xylose fermentation.

Establishing the synthetic rPPP in a xylose-fermenting strain creates a win−win situation for xylose consumption and CO_2 recycling. One major problem of the xylose-fermenting strain harboring the XR/XDH pathway is the redox imbalance caused by the cofactor differences between XR and XDH. XR uses both NADH and nicotinamide adenine dinucleotide phosphate (NADPH), whereas XDH uses NAD^+ only.[37,26] This cofactor difference results in redox imbalance and leads to production of surplus NADH and xylitol accumulation during anaerobic fermentation. Moreover, the glycerol production also occurs to compensate for the excessive NADH produced from both xylose metabolism and native biosynthetic reactions.[34,35] Therefore, the production of xylitol and glycerol represents the redox cofactor imbalance in xylose-fermenting strains with the XR/XDH pathway. By installing the rPPP, the CO_2 can be used as an alternative electron acceptor from the surplus NADH, decreasing the production of reduced byproducts (xylitol and glycerol) as well as increasing the carbon flux to ethanol. As shown in Table 1, the SR8C+ with *prk* overexpression was able to reduce the xylitol and glycerol yield from 0.374 ± 0.003 to 0.337 ± 0.003 mol/mol, mitigating 10% of the total redox cofactor imbalance.

RuBisCO is the most abundant enzyme in nature, but it is also known as inefficient in regards to carboxylase activity.[38,39] As a bifunctional enzyme, the oxygenase occupies one out of four total reactions of RuBisCO in the presence of atmospheric O_2, thus competing against the carboxylase activity. Fortunately, oxygen concentration during anaerobic fermentation by *S. cerevisiae* is minimal, and CO_2 concentration is high relative to atmospheric conditions, resulting in negligible oxygenase activity and maximal carboxylase activity by RuBisCO. Therefore, the oxygenase activity would be neglected when RuBisCO is overexpressed in engineered yeast during xylose fermentation, thus making RuBisCO work as a single-function carboxylase and increasing the efficiency of RuBisCO. Furthermore, the amount of CO_2 in the system accumulates during fermentation, and this will be an operating advantage for this synthetic rPPP. The accumulated CO_2 can increase the substrate availability for RuBisCO and be regarded as compensation for the missing carbon concentration mechanism in heterotrophic hosts.

Global climate change has been a serious issue for all human beings. To mitigate the trend, a 2 °C global warming obligation was set out in the Copenhagen Accord. As estimated, the use of fossil fuel should be significantly reduced to control the emission of GHGs and achieve the obligation by the end of this century.[40,4] Lignocellulosic bioethanol is a promising substitute for fossil fuels. First, bioethanol production by *S. cerevisiae* is currently the largest industrial fermentation process. Second and most importantly, it is a carbon-neutral process because the lignocellulosic biomass comes from CO_2 fixation through photosynthesis without disturbing the food supply and arable land. In our study, this carbon-neutral process was successfully transformed to a carbon-conservative process *via* introducing a synthetic rPPP. The synthetic rPPP can enable the recycling of CO_2 produced during fermentation. The reduced emission of CO_2 during fermentation was verified by the decreased ratio between accumulated CO_2 and ethanol ($r_{C/E}$), which indicated less CO_2 release when one unit of ethanol was produced (Figure 5). Therefore, our process has better capability to contribute to the reduction of GHGs as well as to compensate or substitute for the demand on fossil fuels.

■ CONCLUSIONS

In this study, we introduced RuBisCO and PRK into engineered *S. cerevisiae* capable of fermenting xylose to construct a synthetic rPPP and enable CO_2 recycling during bioethanol production. The feasibility of this strategy was demonstrated *via* the fermentation profiles as well as the decreased ratio between accumulated CO_2 and ethanol. We successfully converted the lignocellulosic biofuel production into a carbon-conservative process *via* combining the advantages of a heterotrophic biofuel producer and autotrophic CO_2 utilizer. Ultimately, engineered yeast capable of fermenting cellulosic hydrolysates with CO_2 recycling can be employed for producing second-generation ethanol. This strategy has great potential to alleviate GHG emission during the production of second-generation ethanol and will inspire research on combing the benefits of autotrophic and heterotrophic microorganisms.

■ METHODS

Strains and Media. *S. cerevisiae* strains used in this study are summarized in Table 2. Yeast cells were cultured in a synthetic complete (SC) medium (1.7 g/L yeast nitrogen base with 5 g/L ammonium sulfate and amino acids) or a yeast extract-peptone (YP) medium (20 g/L peptone and 10 g/L yeast extract) with antibiotics and buffer (50 mM potassium phthalate buffer, pH 6.0). The antibiotics were added to select and maintain the plasmids carrying antibiotic resistance markers, and the working concentrations of G418, hygromycin, and clonNAT were 300, 300, and 120 μg/mL, respectively. The potassium phthalate buffer (50 mM) was added to maintain a pH of 6.0. *D*-glucose or *D*-xylose (20 g/L) was added separately into the media as a single carbon source. *E. coli* TOP10 were used for gene cloning and manipulation. *E. coli* cells were cultivated in Luria−Bertani media (10 g/L tryptone,

DOI: 10.1021/acssynbio.6b00167
ACS Synth. Biol. 2017, 6, 276−283

Table 2. Engineered *S. cerevisiae* Strains Used in This Study

strains	description	ref
SR8	evolved from D452-2, two copies of *XYL1*, *XYL2*, and *XYL3*, Δ*PHO13*, Δ*ALD6*	11
SR8-*cbbM*[a]	SR8, *TDH3p-cbbM-CYCt*	this study
SR8C	SR8-*cbbM*, *TDH3p-groL-CYCt-TEF1p-groS-ADHt*	this study
SR8C-*cbbM*	SR8C, *TDH3p-cbbM-CYCt*	this study
SR8C+	SR8C-*cbbM*, *TDH3p-groL-CYCt-TEF 1p-groS-ADHt*	this study

[a]"-*cbbM*" indicates that an additional copy of the *cbbM* gene was integrated into the genome.

5 g/L yeast extract, and 10 g/L NaCl), and 100 μg/mL ampicillin was added to select and maintain the plasmids.

Plasmid Construction. The plasmids and primers used in this study are summarized in Tables SII and SIII. All plasmids were constructed based on the pRS series vectors with antibiotic markers.[41] The rPPP was introduced *via* the overexpression of RuBisCO and PRK. The DNA sequence codings for RuBisCO[27] from *R. rubrum* (EC 4.1.1.39) and PRK[42] from *S. oleracea* (EC 2.7.1.19) were codon-optimized and synthesized by Integrated DNA Technologies, Inc., Coralville, USA. To functionally express the RuBisCO, the *groL* and *groS* genes, coding for the groEL and groES chaperonins, respectively, were amplified from the *E. coli* BL21 genome DNA. The PCR products of these genes were then inserted into the multiple cloning sites of the vectors using restriction enzyme digestion and ligation. The yeast transformation was conducted by the standard lithium acetate transformation method.[43]

CRISPR/Cas-Based Genome Modification. The yeast genome editing in this study was conducted *via* the type-II CRISPR system based technique, as previously established.[44,45] Briefly, the plasmid carrying Cas9 was first introduced into *S. cerevisiae*, and the plasmid expressing the guide RNA together with the double-strand repairing donor DNA was transformed into the transformants carrying the Cas9 plasmid. After being selected on proper antibiotic plates, the surviving colonies were verified by colony PCR, and the strains with correct construction were stored for later use. The plasmids carrying the constructed cassettes and the empty backbones were used as templates of donor DNA.

Enzyme Activity Assay. *S. cerevisiae* was grown to the exponential phase in YP medium with 20 g/L glucose and was harvested by centrifugation for 10 min at 4000 rpm at 4 °C. The cells were then washed twice and suspended in the extraction buffer (100 mM phosphate buffer, 15 mM $MgCl_2$, 15 mM $NaHCO_3$, and protease inhibitor (Sigma-Aldrich, USA), pH 7.5). The cells were broken down by the glass bead method,[10] and after removing the glass beads and cell debris, the supernatant was regarded as the crude extract for enzyme activity assay. The protein concentration was measured by a BCA protein assay kit (Pierce Biotechnology, USA).

The RuBisCO activity was determined spectrophotometrically from the enzymatically coupled conversion of NADH to NAD^+.[46,47] The crude extracts were first activated in 15 mM $MgCl_2$ and 15 mM $NaHCO_3$ for 10 min before being placed in assay buffer (100 mM EPPS-NaOH pH 8.0, 20 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, 5 mM creatine phosphate, 20 mM $NaHCO_3$, 0.2 mM NADH, and coupling enzymes). The activity was determined from the first minute following initiation of the reaction with 0.5 mM RuBP. One unit of the RuBisCO activity was defined as the amount of enzyme required to produce 1 nM product per minute.

Anaerobic Fermentation Experiments. *S. cerevisiae* cells were cultured until stationary phase and inoculated to YP or SC media with an initial OD_{600} of 1.0 in serum bottles sealed with butyl rubber stoppers. Xylose and glucose were added as carbon sources, respectively. The fermentations were conducted at 30 °C, shaking at 100 rpm. G418 (200 μg/mL) was added to maintain the engineered strains carrying the plasmid with a kanamycin resistance marker. The growth profiles were recorded at 600 nm, and the sugars, ethanol, and any byproduct concentrations were measured by high-performance liquid chromatography (HPLC, Agilent, CA, USA) equipped with an RID detector, as described previously.[11,26]

Analysis of the Ratio between Accumulated CO_2 and Ethanol ($r_{C/E}$). To analyze the ratio between accumulated CO_2 and ethanol ($r_{C/E}$), fermentations were conducted as described above, except that each bottle was equipped with a gas analyzer (ANKOM Technology, Macedon, U.S.), which records the accumulated pressure of produced CO_2. Installation of the gas analyzer into the fermentation bottle was done under strict anaerobic conditions in an anaerobic chamber. At each sampling point, the accumulated pressure was recorded, and the sample was analyzed by HPLC for ethanol concentration. The data were plotted with the concentration of ethanol along the *X*-axis and the pressure of CO_2 along the *Y*-axis. The linear regression model was applied, and the slope was denoted as $r_{C/E}$, indicating the ratio between the pressure of CO_2 and the concentration of ethanol. The value of $r_{C/E}$ indicates the CO_2 generated by a particular strain when producing one unit of ethanol in the same system.

Statistical Analysis. All experiments were conducted at least in duplicate, and the error bars denote the standard deviation from the means of independent experiments. The differences between data were evaluated using Student's *t* test with $P < 0.05$ as a significant difference and $P < 0.001$ as a highly significant difference.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00167.

Results of carbon analysis, plasmids and primers used in this study, fermentation data of the engineered strains, figures supporting the main results, information of the intergenic sites (CS6 and CS8), calculation of the overall and net yield, as well as preliminary analysis of the carbon balance (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: ysjin@illinois.edu.

Author Contributions

Y.-S.J., S.-G.W, and P.-F.X developed the idea of this work. Y.-S.J., D.R.O., P.-F.X., and G.-C.Z designed the experiments. P.-F.X., G.-C.Z, B.W., S.-O.S., S.K., J.-J.L., and H.K. performed the experiments. Y.-S.J. and P.-F.X. wrote the manuscript.

Notes

The authors declare no competing financial interest.

DOI: 10.1021/acssynbio.6b00167
ACS Synth. Biol. 2017, 6, 276−283

■ ACKNOWLEDGMENTS

This work was supported by funding from the Energy Biosciences Institute and the National Natural Science Foundation of China (No. 21476130). The authors thank Timothy L. Turner for valuable suggestions and proofreading of the text. P.-F.X. would like to thank the China Scholarship Council for financial support.

■ REFERENCES

(1) Peralta-Yahya, P. P., Zhang, F., del Cardayre, S. B., and Keasling, J. D. (2012) Microbial engineering for the production of advanced biofuels. *Nature 488*, 320−328.
(2) Caspeta, L., Buijs, N. A., and Nielsen, J. (2013) The role of biofuels in the future energy supply. *Energy Environ. Sci. 6*, 1077−1082.
(3) Ho, S.-H., Ye, X., Hasunuma, T., Chang, J.-S., and Kondo, A. (2014) Perspectives on engineering strategies for improving biofuel production from microalgae - A critical review. *Biotechnol. Adv. 32*, 1448−1459.
(4) Philp, J. (2015) Balancing the bioeconomy: supporting biofuels and bio-based materials in public policy. *Energy Environ. Sci. 8*, 3063−3068.
(5) 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.
(6) Rabinovitch-Deere, C. A., Oliver, J. W., Rodriguez, G. M., and Atsumi, S. (2013) Synthetic biology and metabolic engineering approaches to produce biofuels. *Chem. Rev. 113*, 4611−4632.
(7) Tilman, D., Socolow, R., Foley, J. A., Hill, J., Larson, E., Lynd, L., et al. (2009) Beneficial biofuels - the food, energy, and environment trilemma. *Science 325*, 270−271.
(8) Brethauer, S., and Studer, M. H. (2014) Consolidated bioprocessing of lignocellulose by a microbial consortium. *Energy Environ. Sci. 7*, 1446−1453.
(9) Zhang, G. C., Liu, J. J., Kong, II, Kwak, S., and Jin, Y. S. (2015) Combining C6 and C5 sugar metabolism for enhancing microbial bioconversion. *Curr. Opin. Chem. Biol. 29*, 49−57.
(10) 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.
(11) 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.
(12) Wei, N., Quarterman, J., and Jin, Y. S. (2013) Marine macroalgae: an untapped resource for producing fuels and chemicals. *Trends Biotechnol. 31*, 70−77.
(13) Hahn-Hägerdal, B., Karhumaa, K., Jeppsson, M., and Gorwa-Grauslund, M. F. (2007) Metabolic Engineering for Pentose Utilization in *Saccharomyces cerevisiae*. In *Biofuels* (Olsson, L., Ed.), pp 147−177, Springer, Berlin, Heidelberg, Germany.
(14) Kuyper, M., Harhangi, H. R., Stave, A. K., Winkler, A. A., Jetten, M. S. M., de Laat, W. T. A. M., et al. (2003) High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? *FEMS Yeast Res. 4*, 69−78.
(15) Kuyper, M., Hartog, M. M. P., Toirkens, M. J., Almering, M. J. H., Winkler, A. A., van Dijken, J. P., and Pronk, J. T. (2005) Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation. *FEMS Yeast Res. 5*, 399−409.
(16) Larkum, A. W. D., Ross, I. L., Kruse, O., and Hankamer, B. (2012) Selection, breeding and engineering of microalgae for bioenergy and biofuel production. *Trends Biotechnol. 30*, 198−205.
(17) Oliver, J. W., and Atsumi, S. (2014) Metabolic design for cyanobacterial chemical synthesis. *Photosynth. Res. 120*, 249−261.
(18) Sarsekeyeva, F., Zayadan, B. K., Usserbaeva, A., Bedbenov, V. S., Sinetova, M. A., and Los, D. A. (2015) Cyanofuels: biofuels from cyanobacteria. Reality and perspectives. *Photosynth. Res. 125*, 329−340.
(19) Savakis, P., and Hellingwerf, K. J. (2015) Engineering cyanobacteria for direct biofuel production from CO_2. *Curr. Opin. Biotechnol. 33*, 8−14.
(20) Guadalupe-Medina, V., Wisselink, H. W., Luttik, M. A., de Hulster, E., Daran, J.-M., Pronk, J. T., and van Maris, A. J. (2013) Carbon dioxide fixation by Calvin-Cycle enzymes improves ethanol yield in yeast. *Biotechnol. Biofuels 6*, 125.
(21) Zhuang, Z. Y., and Li, S. Y. (2013) Rubisco-based engineered *Escherichia coli* for in situ carbon dioxide recycling. *Bioresour. Technol. 150*, 79−88.
(22) Gong, F., Liu, G., Zhai, X., Zhou, J., Cai, Z., and Li, Y. (2015) Quantitative analysis of an engineered CO_2-fixing Escherichia coli reveals great potential of heterotrophic CO_2 fixation. *Biotechnol. Biofuels 8*, 1.
(23) Mattozzi, M. d., Ziesack, M., Voges, M. J., Silver, P. A., and Way, J. C. (2013) Expression of the sub-pathways of the *Chloroflexus aurantiacus* 3-hydroxypropionate carbon fixation bicycle in *E. coli*: Toward horizontal transfer of autotrophic growth. *Metab. Eng. 16*, 130−139.
(24) Bogorad, I. W., Lin, T.-S., and Liao, J. C. (2013) Synthetic non-oxidative glycolysis enables complete carbon conservation. *Nature 502*, 693−697.
(25) Bogorad, I. W., Chen, C.-T., Theisen, M. K., Wu, T.-Y., Schlenz, A. R., Lam, A. T., and Liao, J. C. (2014) Building carbon−carbon bonds using a biocatalytic methanol condensation cycle. *Proc. Natl. Acad. Sci. U. S. A. 111*, 15928−15933.
(26) Wei, N., Quarterman, J., Kim, S. R., Cate, J. H., and Jin, Y. S. (2013) Enhanced biofuel production through coupled acetic acid and xylose consumption by engineered yeast. *Nat. Commun. 4*, 2580.
(27) Somerville, C. R., and Somerville, S. C. (1984) Cloning and expression of the Rhodospirillum rubrum ribulosebisphosphate carboxylase gene in *E. coli*. *Mol. Gen. Genet. 193*, 214−219.
(28) Goloubinoff, P., Gatenby, A. A., and Lorimer, G. H. (1989) GroE heat-shock proteins promote assembly of foreign prokaryotic ribulose bisphosphate carboxylase oligomers in *Escherichia coli*. *Nature 337*, 44−47.
(29) Saibil, H. (2013) Chaperone machines for protein folding, unfolding and disaggregation. *Nat. Rev. Mol. Cell Biol. 14*, 630−642.
(30) Xia, P. F., Zhang, G. C., Liu, J. J., Kwak, S., Tsai, C. S., Kong, I. I., et al. (2016) GroE chaperonins assisted functional expression of bacterial enzymes in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng. 113*, 2149−2155.
(31) Wang, D., Zhang, Y., Pohlmann, E. L., Li, J., and Roberts, G. P. (2011) The poor growth of *Rhodospirillum rubrum* mutants lacking RubisCO is due to the accumulation of ribulose-1, 5-bisphosphate. *J. Bacteriol. 193*, 3293−3303.
(32) Kim, S. R., Xu, H., Lesmana, A., Kuzmanovic, U., Au, M., Florencia, C., et al. (2015) Deletion of *PHO13*, Encoding Haloacid Dehalogenase Type IIA Phosphatase, Results in Upregulation of the Pentose Phosphate Pathway in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol. 81*, 1601−1609.
(33) Xu, H., Kim, S., Sorek, H., Lee, Y., Jeong, D., Kim, J., et al. (2016) *PHO13* deletion-induced transcriptional activation prevents sedoheptulose accumulation during xylose metabolism in engineered *Saccharomyces cerevisiae*. *Metab. Eng. 34*, 88−96.
(34) van Dijken, J. P., and Scheffers, W. A. (1986) Redox balances in the metabolism of sugars by yeasts. *FEMS Microbiol. Lett. 32*, 199−224.
(35) Medina, V. G., Almering, M. J., van Maris, A. J., and Pronk, J. T. (2010) Elimination of glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use acetic acid as an electron acceptor. *Appl. Environ. Microbiol. 76*, 190−195.
(36) Johnston, M. (1999) Feasting, fasting and fermenting: glucose sensing in yeast and other cells. *Trends Genet. 15*, 29−33.
(37) Walfridsson, M., Anderlund, M., Bao, X., and Hahn-Hagerdal, B. (1997) Expression of different levels of enzymes from the *Pichia stipitis XYL1* and *XYL2* genes in *Saccharomyces cerevisiae* and its effects on

DOI: 10.1021/acssynbio.6b00167
ACS Synth. Biol. 2017, 6, 276−283

product formation during xylose utilisation. *Appl. Microbiol. Biotechnol. 48*, 218−224.
(38) Parry, M. A., Andralojc, P. J., Scales, J. C., Salvucci, M. E., Carmo-Silva, A. E., Alonso, H., and Whitney, S. M. (2013) Rubisco activity and regulation as targets for crop improvement. *J. Exp. Bot. 64*, 717−730.
(39) Walker, B. J., VanLoocke, A., Bernacchi, C. J., and Ort, D. R. (2016) The Costs of Photorespiration to Food Production Now and in the Future. *Annu. Rev. Plant Biol. 67*, 107−129.
(40) McGlade, C., and Ekins, P. (2015) The geographical distribution of fossil fuels unused when limiting global warming to 2 degrees C. *Nature 517*, 187−190.
(41) 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.
(42) Milanez, S., and Mural, R. J. (1988) Cloning and sequencing of cDNA encoding the mature form of phosphoribulokinase from spinach. *Gene 66*, 55−63.
(43) Gietz, R. D., and Schiestl, R. H. (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc. 2*, 31−34.
(44) Zhang, G.-C., Kong, I. I., Kim, H., Liu, J.-J., Cate, J. H., and Jin, Y.-S. (2014) Construction of a Quadruple Auxotrophic Mutant of an Industrial Polyploid *Saccharomyces cerevisiae* Strain by Using RNA-Guided Cas9 Nuclease. *Appl. Environ. Microbiol. 80*, 7694−7701.
(45) Tsai, C. S., Kong, I. I., Lesmana, A., Million, G., Zhang, G. C., Kim, S. R., and Jin, Y. S. (2015) Rapid and marker-free Cas9/CRISPR refactoring yields equivalent xylose-utilization performance in yeast. *Biotechnol. Bioeng. 112*, 2406−2411.
(46) Ruuska, S. A., Badger, M. R., Andrews, T. J., and von Caemmerer, S. (2000) Photosynthetic electron sinks in transgenic tobacco with reduced amounts of Rubisco: little evidence for significant Mehler reaction. *J. Exp. Bot. 51*, 357−368.
(47) Yamori, W., and von Caemmerer, S. (2009) Effect of Rubisco activase deficiency on the temperature response of CO_2 assimilation rate and Rubisco activation state: insights from transgenic tobacco with reduced amounts of Rubisco activase. *Plant Physiol. 151*, 2073−2082.

DOI: 10.1021/acssynbio.6b00167
ACS Synth. Biol. 2017, 6, 276−283