Investigating Glucose and Xylose Metabolism in *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* via ^{13}C Metabolic Flux Analysis

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DOI 10.1002/aic.14182
Published online August 1, 2013 in Wiley Online Library (wileyonlinelibrary.com)

^{13}C metabolic flux analysis (^{13}C—MFA) has been extensively applied in studying the glucose metabolism of yeast strains such as Saccharomyces cerevisiae *and* Scheffersomyces stipitis. *Here, we tried to augment the previous fluxomic studies by applying ^{13}C—MFA to rigorously investigate the metabolic flux distributions in* S. stipitis *and the recombinant* S. cerevisiae *strains when xylose was utilized as the sole carbon substrate. It was found that less carbon fluxes were diverted into the TCA cycle in* S. stipitis *than the recombinant* S. cerevisiae *strains. Compared to single sugar utilization, the co-utilization of glucose and xylose by* S. stipitis *led to increased metabolic fluxes into the futile pathway and the TCA cycle, but did not improve sugar-based ethanol yield. In addition, heterologous expression of xylose pathway in engineered* S. cerevisiae *strains may affect the glucose utilization.* © 2013 American Institute of Chemical Engineers *AIChE J*, 59: 3195-3202, 2013

Keywords: ^{13}C—MFA, glucose metabolism, xylose metabolism, mixed sugar, yeasts, TCA cycle

Introduction

The sugar metabolism of yeast is a fundamentally important topic in both microbiology and bioengineering and has been studied for over a century. As one of the most physiologically reliable methods to describe the *in vivo* metabolic behaviors,[1] ^{13}C metabolic flux analysis (^{13}C—MFA) has been applied to investigate the glucose metabolism of *S. cerevisiae* and *S. stipitis* (Table 1). *S. cerevisiae* is a Crabtree positive yeast that can use the respiro-fermentative metabolism for ethanol production under aerobic condition. The impact of oxygen level,[2,3] temperature,[3] pH[3], and cultivation modes[4,5] on metabolic flux distributions of *S. cerevisiae* has been characterized by ^{13}C—MFA. In general, the TCA cycle activity of *S. cerevisiae* is affected by the oxygen level, pH, and the cultivation modes,[3,5] but not by the temperature.[3] Another yeast, *S. stipitis* (also known as *Pichia stipitis*), is a Crabtree negative yeast that has fully respiratory metabolism in aerobic condition. Recently, a comparative systems biology study on glucose metabolism of *S. stipitis* using ^{13}C—MFA, metabolomics analysis, RNAseq and *in silico* transcription factors analysis has been reported, which indicates similar metabolic flux distributions in both batch and chemostat cultivations.[6]

Previous fluxomic analyses of sugar metabolisms often focus on glucose utilization. However, several yeasts, such as *S. stipitis*, are well-known for their capability of using pentose sugars such as xylose as the carbon substrate.[6] A variety of recombinant *S. cerevisiae* strains have also been constructed to metabolize pentose sugars.[7–10] Therefore, a comprehensive study of xylose metabolism in yeast strains can be of great help in identifying the important metabolic responses to xylose utilization and improving the performance of the xylose fermentation. To augment the previous fluxomic studies, we applied ^{13}C—MFA for comparative investigation of the xylose metabolism in *S. stipitis* and two recombinant *S. cerevisiae* strains. The effects of the heterologous xylose pathway on glucose metabolism were also characterized by ^{13}C—MFA under glucose cultivation conditions. In addition, we have applied ^{13}C—MFA to decipher the metabolic rewiring during the co-utilization of xylose and glucose by *S. stipitis*. As uncovered by ^{13}C—MFA, the TCA cycle exhibited very different behaviors in xylose metabolism between *S. stipitis* and the recombinant *S. cerevisiae* strains. The heterologous expression of the xylose pathway affected not only the xylose metabolism but the glucose metabolism as well. Compared to single sugar utilization, the co-utilization of two sugars by *S. stipitis* did not improve sugar-based ethanol yield. To the best of our knowledge, this study presents the first quantitative readouts of

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Table 1. Summary of ^{13}C—MFA of Sugar Utilizations by *S. cerevisiae* and *S. stipites*

	^{13}C labeled sugar	Culture condition	Ref
S. cerevisiae	20% [U-^{13}C] and 80% unlabeled glucose, 5 g/L	Aerobic growth, with different temperatures and pH	3
	100% [1-^{13}C] glucose, 10 g/L 20% [U-^{13}C] and 80% unlabeled glucose, 10 g/L	Aerobic growth	12
	20% [U-^{13}C] and 80% unlabeled glucose, 5 g/L	Aerobic growth for different knock-out mutants	27
	10% [U-^{13}C] and 90% unlabeled glucose, 5 g/L (batch) 10% [U-^{13}C] and 90% unlabeled glucose, 3.6 g/L (chemostat)	Aerobic and anaerobic growth, batch and chemostat culture	4
	100% [1-^{13}C] glucose, 5 g/L (batch) 100% [1-^{13}C] glucose, 2 g/L (chemostat)	Aerobic growth, batch and chemostat culture	5
	10% [U-^{13}C] and 90% unlabeled glucose, 10 g/L	Growth with different O_2	2
	10% [U-^{13}C] and 90% unlabeled glucose, 5 g/L	Aerobic and anaerobic growth	16
S. stipitis	10% [U-^{13}C] and 90% unlabeled glucose, 5 g/L (batch) 10% [U-^{13}C] and 90% unlabeled glucose, 3.6 g/L (chemostat)	Aerobic growth, batch and chemostat culture	4
	100% [1-^{13}C] glucose, 5 g/L 100% [1-^{13}C] glucose, 2 g/L	Aerobic growth, batch and chemostat culture	6

xylose metabolism and mixed sugar metabolism in *S. stipitis*, and provides fluxomic characterization for diverse sugar metabolism in yeasts.

Material and Methods

Strains, media, and culture conditions

S. stipitis strain was obtained from NRRL (ARS Culture Collection, Peoria, IL). The parent *Saccharomyces cerevisiae* strain INVSc1 (*MATa his3Δ1 leu2 trp1–289 ura3–52 MATα his3Δ1 leu2 trp1–289 ura3–52*) was purchased from Invitrogen (Life Technologies, Grand Island, NY). Two xylose pathways (i.e., the wild-type and optimized xylose pathways) were constructed in pRS416 plasmid and transformed into the parent strain INVSc1 to make recombinant *S. cerevisiae* strains for xylose metabolism. Such xylose pathway constructs consist of a xylose reductase gene from *Candida shehatae* flanked with a PDC1 promoter and an ADH1 terminator, followed by a xylitol dehydrogenase gene from *Candida tropicalis* flanked with a TEF1 promoter and a CYC1 terminator, and a xylulokinase gene from *Pichia pastoris* flanked with an ENO2 promoter and an ADH2 terminator (i.e., PDC1p-csXR-ADH1t-TEF1p-ctXDH—CYC1t-ENO2p-ppXKS—ADH2t). For the wild-type xylose pathway, all of the promoters remain intact, while for the optimized xylose pathway, the promoter strengths of PDC1p, TEF1p and ENO2p were optimized using the COMPACTER approach.[11] All yeast strains were stored in 25% glycerol at −80°C.

To cultivate *S. cerevisiae* and *S. stipitis* strains, seed cultures were grown in YPAD media (1% yeast extract, % peptone, 100 mg/L adenine hemisulfate, 2% glucose) at 30°C overnight. The seed cultures were then inoculated (0.1%, v/v) into the defined minimal medium as described elsewhere.[3,12] For xylose metabolism of yeasts, 5 g/L xylose was used as the sole carbon source. For glucose metabolism of yeasts, 5 g/L glucose was used as the sole carbon source. For mixed sugar metabolism of yeasts, 2.5 g/L xylose and 2.5 g/L glucose were used as the sole carbon source. Due to the lack of *de novo* synthesis of certain amino acids, histidine (12 mg/L), tryptophan (8 mg/L), and leucine (50 mg/L) were supplemented into the minimal medium when culturing the recombinant *S. cerevisiae* strains, while histidine (12 mg/L), tryptophan (8 mg/L), leucine (50 mg/L), and uracil (50 mg/L) were supplemented into the minimal medium when culturing the parent *S. cerevisiae* strain. The yeast strains were grown in triplicates at 30°C, 250 rpm in capped culture tubes (14 mL) containing 6 mL defined minimal medium to achieve the oxygen limited condition. Cell growth was monitored at OD_{600}. In the exponential phase, two samples were taken for growth rate calculation and extracellular metabolites measurement.

For ^{13}C tracer experiments in defined minimal medium, the same culture conditions were used to cultivate yeast strains, except that the carbon source was replaced with ^{13}C labeled sugars. For xylose metabolism of yeasts, [1-^{13}C] xylose (5 g/L) was used as the sole carbon source. For glucose metabolism of yeasts, 80% (w/w) [1-^{13}C] and 20% (w/w) [U-^{13}C] glucose (5 g/L) was used as the sole carbon source. For mixed sugar metabolism of yeasts, [1-^{13}C] xylose (2.5 g/L) and 80% (w/w) [1-^{13}C] and 20% (w/w) [U-^{13}C] glucose (2.5 g/L) were used as the sole carbon source. All of the ^{13}C labeled sugars were purchased from Cambridge Isotope Laboratories. For all ^{13}C tracer experiments, the yeast strains were grown in duplicates, and the cells (i.e., biomass) were harvested at the mid-log phase based on the growth curve previously determined using non-labeled sugar substrates in defined minimal medium (see online for Additional Supporting Information for Figures S1 and S2).

The concentrations of extracellular metabolites, including glucose, xylose, xylitol and ethanol, were analyzed by HPLC equipped with a refractive-index detector (Shimadzu Scientific Instruments, Columbia, MD) and an HPX-87H column (BioRad, Hercules, CA). The flow rate was 0.6 mL/min at 65 °C using 5 mM sulfuric acid as the mobile phase.

Isotopic analysis

For GC—MS measurement of isotopomer labeling patterns in proteinogenic amino acids, the biomass was harvested by centrifugation and hydrolyzed using 6 M HCl (24 h at 100°C). The amino acids were derivatized in 50 μL of tetrahydrofuran and 50 μL of N—(*tert*-butyl dimethylsilyl)—N-methyl-trifluoroacetamide (Sigma-Aldrich, St. Louis, MO). A gas chromatograph (GC2010, Shimadzu, Kyoto, Japan) equipped with a DB5-MS column (J&W Scientific, Folsom, CA) and a mass spectrometer (QP2010, Shimadzu, Kyoto, Japan) were used for analyzing metabolite labeling profiles. Three types of charged fragments were detected by GC—MS for Ala, Gly, Ser, Asp, Glu, Phe, and Val (supplemental Text

Table 2. Fermentation Behaviors of Yeast Strains with Diverse Sugar Utilizations

		PS	Sc-Xyl-WT	Sc-Xyl-Opt	Sc-WT
Glucose (5 g/L)	μ (h^{-1})	0.19 ± 0.03	0.25 ± 0.01	0.22 ± 0.01	0.24 ± 0.02
	$q_{glucose}$ (mmol/g DCW/h)	13.48 ± 2.11	43.95 ± 2.11	39.63 ± 2.02	20.40 ± 3.34
	Y_{EtOH} (g/g)	0.21 ± 0.02	0.28 ± 0.01	0.28 ± 0.01	0.33 ± 0.03
	$Y_{glycerol}$ (g/g)	ND	0.01 ± 0.00	0.01 ± 0.00	0.05 ± 0.01
Xylose (5 g/L)	μ (h^{-1})	0.23 ± 0.04	0.01 ± 0.00	0.03 ± 0.01	NA[a]
	q_{xylose} (mmol/g DCW/h)	16.53 ± 5.43	1.31 ± 0.29	2.07 ± 0.25	NA
	Y_{EtOH} (g/g)	0.11 ± 0.03	ND[a]	ND	NA
Glucose (2.5 g/L) + Xylose (2.5 g/L)	μ (h^{-1})	0.15 ± 0.01	NA	NA	NA
	q_{xylose} (mmol/g DCW/h)	19.47 ± 2.23	NA	NA	NA
	$q_{glucose}$ (mmol/g DCW/h)	14.24 ± 1.67	NA	NA	NA
	Y_{EtOH} (g/g)	0.08 ± 0.01	NA	NA	NA

[a]ND: Not detected; NA: Not available

S1): the $[M-57]^+$ group (containing unfragmented amino acids); and the $[M-159]^+$ or $[M-85]^+$ group (containing amino acids that had lost an α-carboxyl group). For each type of fragments, the labeling patterns were represented by M0, M1, M2, etc., as the fractions of unlabeled, singly labeled, and doubly labeled amino acids, etc. The effects of natural isotopes on isotopomer labeling patterns were corrected by previously reported algorithms.[13]

^{13}C metabolic flux analysis

The central metabolic pathway map of recombinant *S. cerevisiae* and *S. stipitis* strains was generated based on genome annotation from the KEGG (Kyoto Encyclopedia of Genes and Genomes) database and previous fluxomics studies.[6,12,14–16] The simplified pathway map includes the fungal xylose pathway (for xylose metabolism and mixed sugar metabolism), the oxidative and reductive pentose phosphate pathway, glycolysis, the futile pathways, the transport pathways between cytosol and mitochondria, and the TCA cycle. As discussed before,[17] to develop a pseudo-steady-state flux model for glucose or xylose metabolism, the sugar uptake rates were measured at the mid-log phase and normalized to 100 units. For the mixed sugar metabolism in *S. stipitis*, the xylose uptake rate was normalized to 100 units. The biomass compositions of *S. cerevisiae* and *S. stipitis* were reported in previous studies,[6,12] and used as loose constraints (i.e., allowed to have variations of up to 20%) for the fluxes into building block synthesis. To determine the remaining unknown fluxes of the metabolic network, a nonlinear optimization problem was formulated to iteratively search a set of fluxes that can satisfy the reaction stoichiometry and minimize the objective function[17] $\varepsilon(v_n)=\sum_{i=1}^{a}\left(\frac{M_i-N_i(v_n)}{\delta_i}\right)^2$, where v_n are the unknown fluxes to be optimized in the program, M_i are the measured isotopomer labeling patterns of proteinogenic amino acids, N_i are the model-simulated isotopomer labeling patterns of proteinogenic amino acids using isotopomer mapping matrices (IMM), and δ_i are corresponding standard deviations of the GC—MS data from two biological replicates. The nonlinear optimization was finished by using the "fmincon" command in MATLAB (MathWorks, Natick, MA). The optimization was run 100 times with different initial guesses to search for a likely global solution for the unknown fluxes. The observed and simulated isotopomer labeling patterns of proteinogenic amino acids were compared in supplemental Figure S3. The confidence intervals of the calculated fluxes (Supplementary text S2) were generated via a Monte Carlo approach as described previously.[17] In short, the isotopomer labeling patterns of the proteinogenic amino acids were randomly perturbed for 100 times within the standard derivation. For each perturbed dataset, the ^{13}C—MFA was applied and the flux distributions were calculated. Then, the standard derivations of each flux were derived from such 100 simulations. The cofactor balancing for NADPH was not included in the mass balancing of ^{13}C—MFA since the relative activities of isoenzymes cannot be decided. Only the glycolysis pathway and the TCA cycle (without considering isocitrate dehydrogenase) were taken into the calculation for the NADH production. The NADH produced was assumed to be used in two ways: generating fermentation products (e.g., ethanol and glycerol), and producing ATP via oxidative phosphorylation.

Results

Physiology of sugar metabolism in S. cerevisiae and S. stipitis

When using glucose as the sole carbon substrate and culturing in oxygen limited conditions, all of the *S. cerevisiae* strains, including the parent strain (i.e., Sc_WT), the recombinant strain with the wild-type xylose pathway (i.e., Sc_WT_Xyl), and the recombinant strain with the optimized xylose pathway (i.e., Sc_OPT_Xyl), demonstrated higher growth rates (0.22–0.24 h^{-1}, Table 2), ethanol yields (0.28–0.33 g/g) and glucose uptake rates (20.40–43.95 mmol/g/h) than those of *S. stipitis* (0.19 h^{-1}, 0.21 g/g and 13.48 mmol/g/h, respectively). The heterologous expression of the xylose pathway in *S. cerevisiae* changed the physiology in the recombinant strains with increased glucose uptake rates (i.e., 39.63 and 43.95 mmol/g/h in Sc_OPT_Xyl and Sc_WT_Xyl, respectively), and slightly decreased ethanol yields (0.28 g/g) compared to the parent strain (i.e., 20.40 mmol/g/h and 0.33 g/g in Sc_WT). However, the growth rates of the recombinant strains were not significantly affected.

For xylose metabolism under oxygen limited conditions, *S. stipitis* had superior fermentation performance compared to the recombinant *S. cerevisiae* strains. In general, the growth rate and xylose uptake rate of *S. stipitis* (0.23 h^{-1} and 16.53 mmol/g/h, respectively) were one order of magnitude higher than those of *S. cerevisiae* strains (0.01–0.03 h^{-1} and 1.31–2.07 mmol/g/h, respectively). Compared to the xylose fermentation by *S. stipitis* in which the ethanol yield can reach 0.11 g/g, no ethanol can be detected in fermentations by *S. cerevisiae* strains. The absence of ethanol production and low growth rate indicated that the xylose cannot yet be efficiently used by recombinant *S. cerevisiae* strains and most of the carbons might be lost as CO_2.

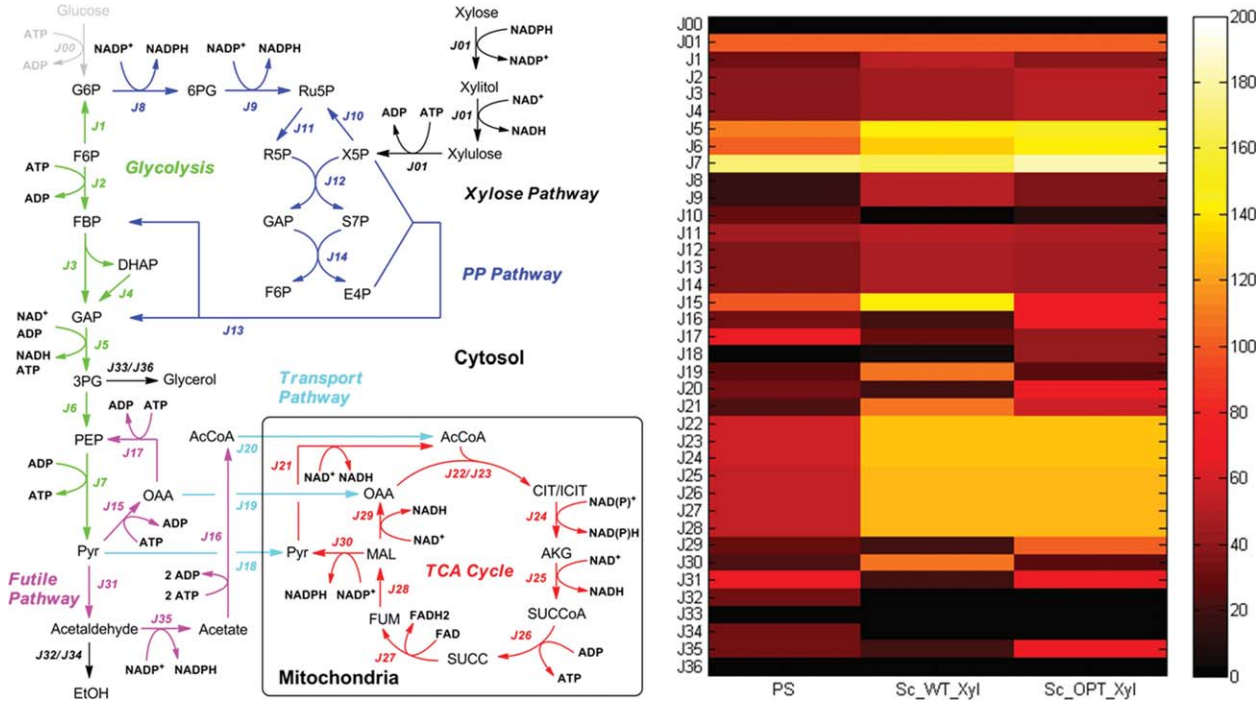

Figure 1. Heatmap of metabolic flux distributions of yeast strains in xylose metabolism.

The xylose uptake flux in each of the yeast strains was normalized as 100 units. Sc_OPT_Xyl: *S. cerevisiae* with optimized xylose pathway; Sc_WT_Xyl: *S. cerevisiae* with wild-type xylose pathway; PS: *Scheffersomyces stipites*. Abbreviations: G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; F6P, fructose-6-phosphate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; X5P, xylulose-5- phosphate; E4P, erythrose-4-phosphate; S7P, seduheptulose-7-phosphate; GAP, glyceraldehyde-3-phosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; EtOH, ethanol; AcCoA: acetyl-CoA; ACD: acetaldehyde; CIT/ICIT, citrate/isocitrate; AKG, oxoglutarate; SUCC, succinate; FUM, fumarate; MAL, malate; OAA, oxaloacetate.

The mixed sugar metabolism of *S. stipitis* using 2.5 g/L glucose and 2.5 g/L xylose showed that the uptake rate of xylose (19.47 mmol/g/h, Table 2 and Figure S2) was slightly faster than that of glucose (14.24 mmol/g/h). Compared to the sugar metabolism using either glucose or xylose as the sole carbon substrate, the mixed sugar metabolism in *S. stipitis* led to the similar sugar uptake take rate but lower growth rate and ethanol yield.

Flux analysis of xylose metabolism in S. cerevisiae and S. stipitis

The metabolic flux distributions of xylose metabolism in recombinant *S. cerevisiae* strains and *S. stipitis* were determined by feeding 5 g/L [1-^{13}C] xylose and tracing the isotopomer labeling patterns in proteinogenic amino acids. By normalizing the xylose uptake into 100 units, the flux ratios of key metabolic pathways can hereby be compared among different yeasts. Several pathways, especially the TCA cycle, were found to have significantly different behaviors. Basically, the fluxes into the TCA cycle were much lower in *S. stipitis* (56.7 units, Figure 1) than those in recombinant *S. cerevisiae* strains (128.7–128.8 units), while the glycolytic fluxes were similar among different yeasts (164.3–182.0 units). Under oxygen limited conditions, *S. stipitis* demonstrated respiro-fermentative metabolism (with an active TCA cycle and an ethanol fermentative pathway) while recombinant *S. cerevisiae* strains showed respiratory metabolism (with an active TCA cycle and an inactive ethanol fermentative pathway).

Glycolysis and the TCA cycle in xylose metabolism of yeasts generate great amounts of NADH for either producing fermentation products such as ethanol or converting to ATP through oxidative phosphorylation. Compared to that in recombinant *S. cerevisiae* strains, the NADH produced for oxidative phosphorylation in *S. stipitis* was smaller since the TCA cycle activity was lower and part of the NADH pool was diverted for ethanol production. In other words, the ATP requirement by *S. stipitis* was not as high as that of the recombinant *S. cerevisiae* strains, which could save some of the NADH for ethanol production.

Flux analysis of glucose metabolism in S. cerevisiae and S. stipitis

The most remarkable difference in glucose metabolism among yeasts was found in the TCA cycle (Figure 2). In general, the TCA cycle activity in *S. stipitis* (65.0 units) was ~93% higher than that in wild type *S. cerevisiae* strains (33.7 units as in Sc_WT). Such a gap reflects the different metabolic regulations of glucose metabolism in Crabtree positive and Crabtree negative yeasts. In Crabtree positive yeasts such as *S. cerevisiae*, the high concentration of glucose (i.e., over 0.1 g/L) diverted the metabolic flux into the ethanol fermentative pathway instead of the TCA cycle. On the other hand, in Crabtree negative yeasts such as *S. stipitis*, the respiratory metabolism was strong even under the oxygen limited conditions, which led to the higher TCA cycle flux than that in Crabtree positive yeasts.

Interestingly, the activity of the TCA cycle was not the same even among different *S. cerevisiae* strains. The ones that expressed heterologous proteins (i.e., Sc_WT_Xyl and Sc_OPT_Xyl) had higher TCA cycle activities (30–38%) than the parent strain (i.e., Sc_WT). It has been confirmed by several studies that the metabolic burden caused by heterologous protein expression often triggers the elevated

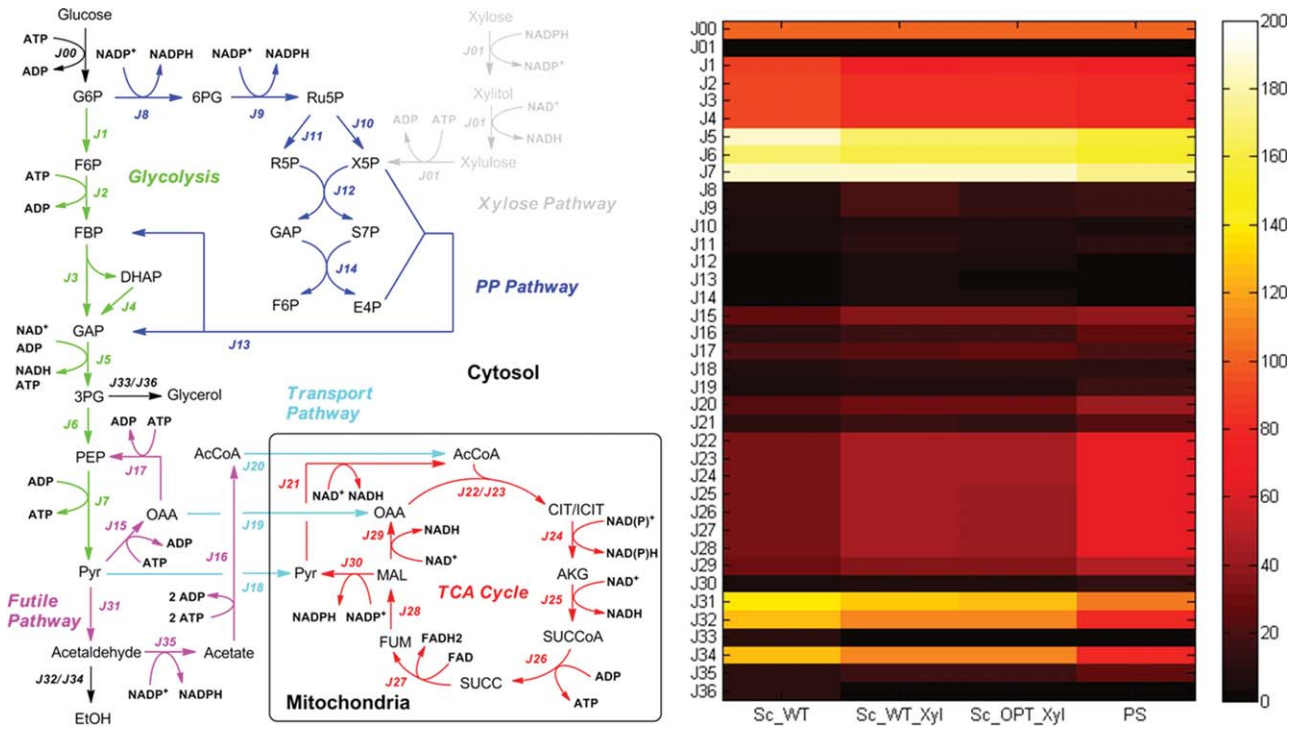

Figure 2. Heatmap of metabolic flux distributions of yeast strains in glucose metabolism.

The glucose uptake flux in each of the yeast strains was normalized as 100 units. Sc_OPT_Xyl: *S. cerevisiae* with the optimized xylose pathway; Sc_WT_Xyl: *S. cerevisiae* with wild-type xylose pathway; Sc_WT: *S. cerevisiae* without the xylose pathway; PS: *S. stipites*.

TCA cycle activity to provide extra maintenance energy as required by protein folding and other energy costs.[18–20] Therefore, the increased TCA cycle activity in recombinant *S. cerevisiae* strains indicates that the metabolic engineering of xylose metabolism may not only affect the xylose utilization but other sugar metabolism as well due to the metabolic burden introduced by the heterologous pathway.

Flux analysis of mixed sugar metabolisms in S. stipitis

During the co-utilization of glucose and xylose by *S. stipitis*, the utilization of xylose mainly contributed to the flux into the reductive pentose phosphate pathway (Figure 3) because the flux into the reductive pentose phosphate pathway (37.9 units) in mixed sugar metabolism was similar to that in the xylose metabolism (36.2 units). Glucose was converted to glucose-6-phosphate to enter the metabolic network. The metabolic fluxes from glucose utilization then split to glycolysis and the oxidative pentose phosphate pathway in the ratio of 4:1. The flux into the oxidative pentose phosphate pathway was slightly smaller (14.5 units) than that in glucose metabolism (15.8 units) or xylose metabolism (16.4 units). The fluxes into glycolysis and the reductive pentose phosphate pathway merged at the metabolite node at glyceraldehyde 3-phosphate and formed a large flux toward phosphoenolpyruvate production.

The activities of the futile pathways and the TCA cycle were significantly elevated in the mixed sugar metabolism. The fluxes through pyruvate carboxylase and phosphoenolpyruvate carboxykinase in the mixed sugar metabolism were 2.8–7.1 fold and 3.2–10.8 fold of those in xylose or glucose metabolism alone, which could serve as "metabolic buffer" for the strong glycolytic flux. The TCA cycle flux was increased by nearly threefold in the mixed sugar metabolism. Considering the lower growth rate and ethanol yield but similar sugar uptake rates during co-utilization of glucose and xylose, the maintenance energy requirement for mixed sugar metabolism could be much higher than that in glucose or xylose metabolism, which may need a TCA cycle with higher activity to generate enough ATP. On the other hand, since too much flux went into the futile pathway and the TCA cycle, the fluxes into the fermentative pathway for ethanol production decreased and less ethanol was produced during the mixed sugar metabolism.

Discussion

The xylose-based ethanol production has been found to be optimal under oxygen limited conditions for both *S. cerevisiae* and *S. stipitis* strains.[6,21] Knowing the insight about the metabolic rewiring during yeast cultivation at oxygen limited conditions can be of great interests to the cellulosic biofuel production. Despite thorough research on cell physiology of *S. cerevisiae* and *S. stipitis* strains under aerobic conditions, little is known about the intracellular flux distributions of yeast strains under oxygen limited conditions. The ^{13}C—MFA applied in this study aimed at overcoming such knowledge gap by revealing a cluster of unique metabolic features of *S. cerevisiae* and *S. stipitis* strains that are usually difficult to characterize by measuring the fermentation profiles. We have chosen xylose utilization, glucose utilization, and glucose-xylose co-utilization for fluxomics profiling. It is worthy noticing that the co-utilization of glucose and xylose in *S. cerevisiae* was not included in this study since it has not yet

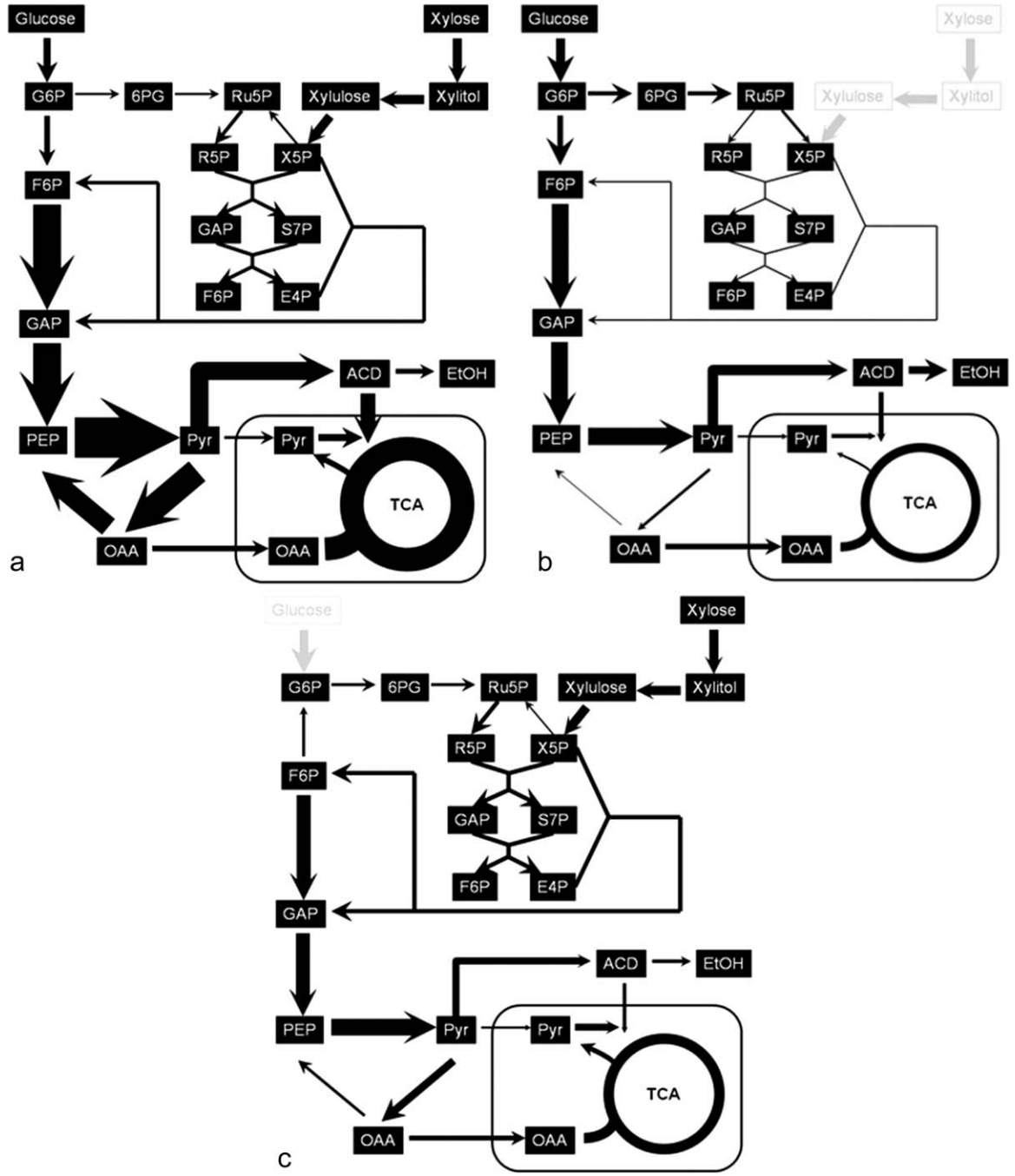

Figure 3. Metabolic flux distributions of *S. stipitis* in (A) mixed sugar metabolism, (B) glucose metabolism, and (C) xylose metabolism.

The thickness of each arrow is proportional to the relative flux value. For (A) and (C), the xylose uptake flux was normalized as 100 units. For (B), the glucose uptake flux was normalized as 100 units.

been efficiently achieved due to multiple reasons, such as competition for hexose transporter and the glucose repression of xylose metabolism. For flux analysis of xylose metabolism, we found that more fluxes were diverted into the TCA cycle in the recombinant *S. cerevisiae* strains. Therefore, metabolic engineering approaches that can decrease the TCA cycle activities, such as disrupting the oxidative phosphorylation, could be helpful to improve xylose utilization. For flux analysis of glucose metabolism, we found that the recombinant *S. cerevisiae* strains have modified physiology compared to the wild-type strain. Further investigation by ^{13}C—MFA revealed that the TCA cycle was elevated when expressing the heterologous pathways. For the glucose and xylose co-utilization by *S. stipitis*, the futile pathway was identified by ^{13}C—MFA as another pathway beside the TCA cycle that was significantly regulated.

The ^{13}C—MFA achieved high quality in this study, as indicated by the small discrepancy between the measured and simulated isotopomer labeling patterns of proteinogenic amino acids (Figure S3). The residuals of all the

isotopomer data were smaller than the errors (including both machine errors and the biological variations from duplicate samples) when calculating flux distributions in glucose metabolism of *S. cerevisiae* and *S. stipitis* strains, and xylose metabolism of *S. cerevisiae* strains, which indicates high accuracy of the flux calculations. As for the flux calculation of *S. stipitis* strains under xylose utilization and glucose-xylose co-utilization conditions, the essence of metabolic flux distributions were captured since the high-quality fitting was achieved for most of the amino acids. The simulations of two amino acids, phenylalanine and glutamate, have relatively large residuals and the simulated isotopomer labeling patterns have 6–10% derivation from the experimental observations. This indicates that certain novel features in the pentose phosphate pathway and the TCA cycle (e.g., novel pathways, flux channeling) may get involved in xylose and mixed sugar utilization. According to the recently reconstructed genome-scale metabolic models of *S. stipitis* strains,[22,23] a large number of reactions (100–200 reactions) in the metabolic network were unique compared to those of *S. cerevisiae* strains. Till now, the most comprehensive yeast metabolic model covers no more than 20% of the total genes.[22,23] Since we assumed that the similar central metabolic pathways were used by both *S. cerevisiae* and *S. stipitis* strains in this study, the isotopomer fitting can be further improved by taking into consideration of such unique metabolic features in flux calculations in the future. Another possible reason for the imperfect flux calculation of pentose phosphate pathways could be the flexible reversibility of transketolase and transaldolase in the pentose phosphate pathway, which, as previously reported in another ^{13}C—MFA of glucose metabolism in *S. cerevisiae,*[24] could lead to huge confidence intervals when estimating the fluxes into the pentose phosphate pathways. To solve this problem, applying parallel tracing experiments[25,26] in future ^{13}C—MFA experiments could reveal more information about the pathway usage and hence raise the possibility to improve the flux estimation in the pentose phosphate pathway.

Conclusions

In this study, ^{13}C—MFA has been systematically applied to decode diverse sugar metabolisms of two yeasts, *S. cerevisiae* and *S. stipitis*. In xylose metabolism under oxygen limited conditions, *S. stipitis* demonstrated the respiro-fermentative metabolism but *S. cerevisiae* demonstrated only respiratory metabolism. The fluxes into the TCA cycle were smaller in xylose metabolism of *S. stipitis* than those in the recombinant *S. cerevisiae* strains. The heterologous expression of the xylose pathway in *S. cerevisiae* affected not only the xylose metabolism but the glucose metabolism as well, since the TCA cycle flux was increased in the recombinant strains compared to the parent strain. In addition, during the co-utilization of xylose and glucose by *S. stipitis*, the *in vivo* activities of the futile pathway and the TCA cycle were significantly enhanced, which led to smaller flux into the fermentative pathway for ethanol production. As indicated by ^{13}C—MFA, the TCA cycle could be the target for metabolic engineering of *S. cerevisiae* to improve the xylose utilization, while the futile cycle and the TCA cycle could be the target for metabolic engineering of *S. stipitis* to improve the glucose-xylose co-utilization.

Acknowledgments

This work was supported by Energy Biosciences Institute. XF thanks the Shen postdoc fellowship from the Department of Chemical and Biomolecular Engineering at the University of Illinois at Urbana-Champaign.

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Manuscript received Mar. 12, 2013, and revision received Jun. 14, 2013.