



## Comparison of xylose fermentation by two high-performance engineered strains of *Saccharomyces cerevisiae*



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### ABSTRACT

Economical biofuel production from plant biomass requires the conversion of both cellulose and hemicellulose in the plant cell wall. The best industrial fermentation organism, the yeast *Saccharomyces cerevisiae*, has been developed to utilize xylose by heterologously expressing either a xylose reductase/xylitol dehydrogenase (XR/XDH) pathway or a xylose isomerase (XI) pathway. Although it has been proposed that the optimal means for fermenting xylose into biofuels would use XI instead of the XR/XDH pathway, no clear comparison of the best publicly-available yeast strains engineered to use XR/XDH or XI has been published. We therefore compared two of the best-performing engineered yeast strains in the public domain—one using the XR/XDH pathway and another using XI—in anaerobic xylose fermentations. We find that, regardless of conditions, the strain using XR/XDH has substantially higher productivity compared to the XI strain. By contrast, the XI strain has better yields in nearly all conditions tested.

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Xylose is the second most abundant sugar next to glucose in plant cell wall hydrolysates [10]. Therefore, efficient and rapid utilization of xylose along with glucose is essential for economic and sustainable production of fuels and chemicals from lignocellulosic biomass [16]. Although *Saccharomyces cerevisiae* has been exclusively used for producing bioethanol, this yeast cannot ferment xylose. In order to confer xylose-fermenting capabilities into *S. cerevisiae*, two metabolic pathways from other microorganisms have been introduced [4,18]. An oxidoreductase pathway consisting of xylose reductase (XR) and xylitol dehydrogenase (XDH) [10,17], produces reduced byproducts such as xylitol and glycerol during anaerobic xylose fermentation, likely due to the difference in cofactor specificities of XR and XDH [2,10]. XR uses NADPH, whereas XDH uses NAD<sup>+</sup>. Therefore, xylose fermentation facilitated by the XR/XDH pathway under anaerobic conditions can

lead to surplus NADH production [2,17], eliciting the production of glycerol and xylitol. As a solution of the redox imbalance problem, the second pathway involving xylose isomerase (XI) pathway was proposed [19]. Several XIs from anaerobic fungi and bacteria have been identified that are functionally expressed in *S. cerevisiae*, allowing construction of xylose-fermenting *S. cerevisiae* using the XI pathway [1,3,14,11].

In addition to the introduction and functional expression of two different metabolic pathways for xylose consumption (XR/XDH and XI), rational and evolutionary metabolic engineering strategies have been used to construct efficient xylose-fermenting *S. cerevisiae* strains [5,7,11,12]. An outstanding question in the field is whether one pathway or the other would be preferred for large-scale fermentation [18]. However, there has not been a direct comparison of the best performing strains with each pathway available in the public domain. Such a comparison would benefit future efforts to develop bioethanol production from lignocellulosic biomass.

Combined rational and evolutionary engineering efforts have led to the generation of strains SR8 [7] and SXA-R2P-E [12], two of the best-performing strains reported to utilize XR/XDH and XI pathway, respectively. SR8 contains two or more chromosomal

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copies of *Scheffersomyces stipitis* *XYL1* (XR), *XYL2* (XDH) and *XYL3* (xylulokinase, XK) each with optimized expression, along with deletion of acetaldehyde dehydrogenase, *ALD6*. It was evolved on xylose in the laboratory, which resulted in the loss of phosphatase Pho13 function. It was subsequently made auxotrophic for uracil (*ura3-52*) for additional engineering (SR8u) [7]. SXA-R2P-E, also evolved on xylose, has two copies of *Piromyces* sp. *xylA3\** (an improved XI mutant), *S. stipitis* *TAL1* (transaldolase) integrated, native *XKS1* (XK) overexpressed, and *GRE3* (aldose reductase) and *PHO13* deleted [12]. It has been recently reported that *PHO13* deletion leads to up-regulation of the pentose phosphate genes including *TAL1* [8].

The xylose fermentation performance of the two strains, SR8u and SXA-R2P-E, was compared in various anaerobic batch culture conditions (Table 1). Over a range of cell loadings (5–20 Optical Density (OD) at 600 nm), strain SR8u expressing XR/XDH always displayed higher rates of ethanol production when compared to strain SXA-R2P-E expressing XI (Fig. 1A). This result was also true for seed cultures prepared from mid-log cultures (Fig. 1B) and for fermentation carried out in both optimized Minimal Medium (oMM) and YSC media, which was used to evolve strain SXA-R2P-E (Fig. 2). While xylose consumption and ethanol production rates were higher in strain SR8u expressing the XR/XDH pathway, strain SXA-R2P-E had slightly higher ethanol yields and produced less xylitol as a byproduct (Table 1). Overall, strain SR8u showed 16–104% higher productivities (g ethanol/OD·h) and 5–19% lower yields (g ethanol/g xylose) than strain SXA-R2P-E in the tested xylose fermentation conditions.

Acetic acid is present in cellulosic hydrolysates due to the acetylation of hemicellulose in the plant cell wall [15]. However, its toxicity has inhibitory effects on fermentation [9,20]. We therefore compared xylose fermentation in the presence of 1 g/L of acetate, and found that strain SR8u consumed xylose and produced ethanol faster than strain SXA-R2P-E (Fig. 3). It is notable that no acetate reduction pathway was used in these experiments. This suggests that an acetate reduction pathway could be coupled with the XR/XDH pathway to improve ethanol productivity and yield even further by not only resolving the problematic redox cofactor imbalance but also using it to drive co-consumption of xylose and acetate [20].

Here we found that in two high-performance engineered yeast strains, the XR/XDH xylose utilization pathway was capable of higher xylose consumption and ethanol production rates compared to the XI pathway, despite formation of more xylitol as a byproduct. It is worth noting that the XR/XDH pathway has been

shown to produce much less xylitol or no xylitol when xylooligosaccharides are used rather than xylose in fermentations [13], or when undetoxified lignocellulose hydrolysate is used [6]. Thus, xylooligosaccharide consumption by means of the XR/XDH pathway could result in yeast strains with both high ethanol productivity and yield, without the drawback of xylitol byproduct formation. Here we used media conditions chosen previously in the development of these strains, to enable comparisons to previously published results. In the future, our results can serve as a baseline for optimizing industrially relevant media, and for in-depth metabolic engineering of industrial yeast strains. It will be important to continue benchmarking high-performance engineered yeast strains with these alternative xylose utilization pathways, to identify and remove remaining bottlenecks in yeast strains to be used in industrial applications.

## 1. Materials and methods

### 1.1. Anaerobic xylose fermentation

Yeast strains SR8u or SXA-R2P-E were pre-grown aerobically to mid-log or late-log phase in oMM or YSC medium containing 2% glucose, washed 3 times with water, and re-suspended in either oMM or YSC medium, depending on the batch fermentation. Media containing 4% w/v xylose (5% in Fig. 3) was inoculated with a range of starting OD values, as indicated in the figure panels, and then purged with N<sub>2</sub>. Anaerobic batch fermentations were carried out in 50 mL of oMM or YSC medium in 125 mL serum bottles, closed with butyl rubber stoppers, shaking at 220 rpm in a 30 °C shaker. At the indicated time points, 1 mL samples were removed with a syringe and pelleted. 5 µL supernatants were analyzed by ion-exclusion HPLC to determine xylose, xylitol, glycerol, and ethanol concentrations.

During the anaerobic fermentations, we collected OD values at every time point and used the average value of each time interval for the rate calculations. Xylose consumption rate (g xylose/OD·H) and ethanol production rate (g ethanol/OD·H) were calculated from the steepest slope of the curve using the average OD of that time interval. Ethanol yield (g ethanol/g xylose) was calculated using grams of xylose and ethanol directly measured at the initial and final time points.

To account for variations from the stated initial xylose concentration of 40 g/L (50 g/L in Fig. 3), rate and yield calculations in Table 1 and the text used values measured by HPLC, including values measured for the time = 0 h time point. This addressed some

**Table 1**  
Fermentation performance of the two xylose-utilizing *S. cerevisiae* strains.

Medium <sup>a</sup>	Seed condition	Strain <sup>b</sup>	Xylose consumption rate <sup>c</sup> (g xylose OD <sup>-1</sup> H <sup>-1</sup> ) [OD] <sup>e</sup>	Ethanol production rate <sup>c</sup> (g ethanol OD <sup>-1</sup> H <sup>-1</sup> ) [OD] <sup>e</sup>	Ethanol yield <sup>d</sup> (g ethanol·g xylose <sup>-1</sup> )
oMM	late-log phase, 5 OD	SR8u	0.127 [5.9]	0.063 [5.9]	0.369
oMM	late-log phase, 5 OD	SXA-R2P-E	0.072 [8.3]	0.038 [6.6]	0.402
oMM	late-log phase, 10 OD	SR8u	0.112 [12.6]	0.052 [12.6]	0.378
oMM	late-log phase, 10 OD	SXA-R2P-E	0.085 [14.4]	0.045 [14.8]	0.419
oMM	late-log phase, 20 OD	SR8u	0.102 [24.4]	0.044 [24.4]	0.390
oMM	late-log phase, 20 OD	SXA-R2P-E	0.046 [26.0]	0.022 [26.0]	0.430
oMM	mid-log phase, 20 OD	SR8u	0.097 [23.7]	0.049 [23.7]	0.392
oMM	mid-log phase, 20 OD	SXA-R2P-E	0.052 [21.8]	0.024 [24.6]	0.412
YSC	late-log phase, 10 OD	SR8u	0.129 [14.7]	0.046 [14.7]	0.378
YSC	late-log phase, 10 OD	SXA-R2P-E	0.077 [15.7]	0.033 [15.7]	0.453
oMM+ 0.1% Ac	late-log phase, 10 OD	SR8u	0.087 [11.0]	0.042 [11.0]	0.374
oMM+ 0.1% Ac	late-log phase, 10 OD	SXA-R2P-E	0.062 [14.6]	0.031 [14.6]	0.403

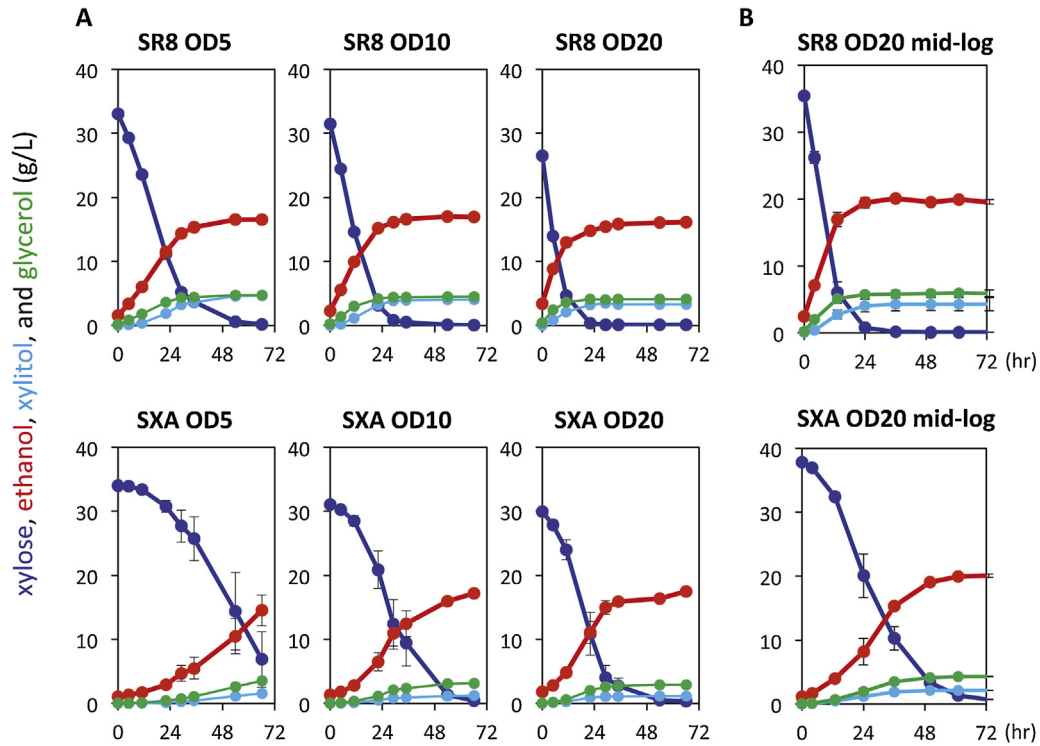
<sup>a</sup> Fermentations were carried out anaerobically in batch conditions using serum bottles and 40 g/L xylose.

<sup>b</sup> Strain characteristics: (1) SR8u: *XYL1*, *XYL2*, *XYL3*,  $\Delta$ *ald6*, *ura3-52*, evolved, (2) SXA-R2P-E: *xylA3\**, *XKS1*, *TAL1*,  $\Delta$ *gre3*,  $\Delta$ *pho13*, evolved.

<sup>c</sup> Maximal values in g of xylose consumed or ethanol produced per OD per hour.

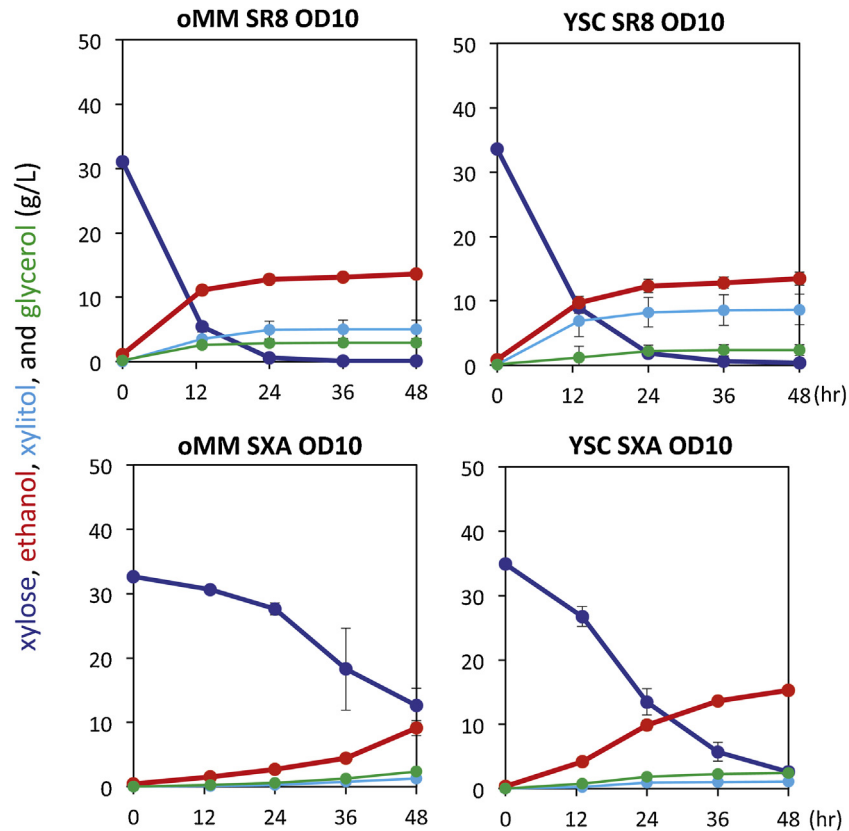
<sup>d</sup> Grams of ethanol yield per g of xylose.

<sup>e</sup> OD value at the maximum rate of xylose consumption or ethanol production.



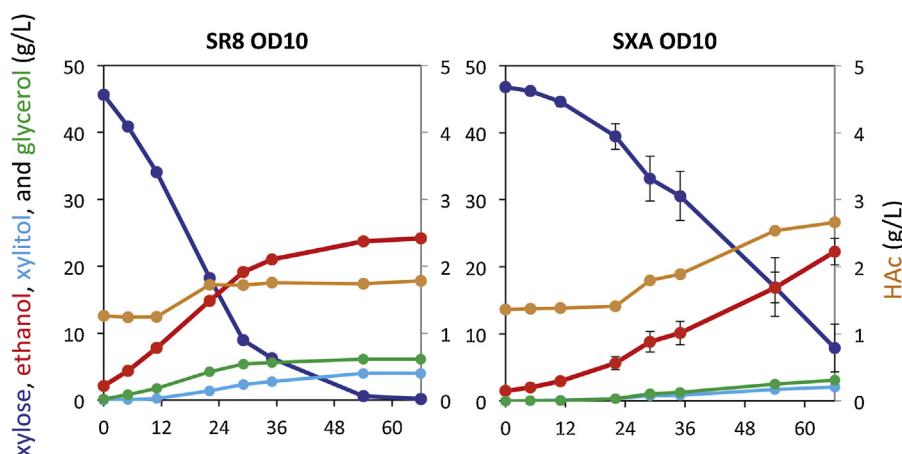
**Fig. 1.** Comparison of XI and XR/XDH pathway fermentation performance.

Xylose fermentations using strain SR8u (XR/XDH pathway) and strain SXA-R2P-E (XI pathway) with (A) different starting cell loadings (5–20 OD<sub>600</sub>), (B) mid-log phase cultures as fermentation seeds. The strain and starting OD<sub>600</sub> values are indicated above each panel. The medium used in these fermentations was oMM. Error bars represent the standard deviation of biological triplicates. Xylose fermentation samples were resolved as previously described [13].



**Fig. 2.** Xylose fermentation performance of strains SR8u (XR/XDH) and SXA-R2P-E (XI), using either oMM or YSC medium.

The media, strain, and starting OD<sub>600</sub> values are given above each panel. Error bars represent the standard deviation of biological triplicates.



**Fig. 3.** Xylose fermentation performance of strains SR8u (XR/XDH) and SXA-R2P-E (XI) in the presence of acetate.

The strain and starting OD600 values are given above each panel. The medium used in these fermentations was oMM. Error bars represent the standard deviation of biological triplicates.

variation that occurred due to the time needed to set up fermentations in parallel. Experiments to directly compare SR8u and SXA-R2P-E in a given condition and with a given OD were prepared from the same batches of reagents.

In the experiment in which strains were pre-grown to mid-log phase (Fig. 1B), we observed ethanol production over the theoretical maximum. We believe this violation likely arose from cell loading of glucose, as the cells were pre-grown on media containing glucose.

Optimal minimal medium (oMM) contained 1.7 g/L YNB (Y1251, Sigma, Saint Louis, MO, USA), two-fold complete CSM, 10 g/L  $(\text{NH}_4)_2\text{SO}_4$ , 1 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 6 g/L  $\text{KH}_2\text{PO}_4$ , 100 mg/L adenine hemisulfate, 10 mg/L inositol, 100 mg/L glutamic acid, 20 mg/L lysine, 375 mg/L serine and 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) pH 6.0. Yeast synthetic complete (YSC) medium contained 6.7 g/L YNB and appropriate CSM dropout mixture.

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## References

- [1] D. Brat, E. Boles, B. Wiedemann, Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*, *Appl. Environ. Microbiol.* 75 (2009) 2304–2311.
- [2] P.M. Bruinenberg, P.H.M. Debot, J.P. Vandijken, W.A. Scheffers, The role of redox balances in the anaerobic fermentation of xylose by yeasts, *Eur. J. Appl. Microbiol.* 18 (1983) 287–292.
- [3] S.J. Ha, S.R. Kim, J.-H. Choi, M. Park, Y.S. Jin, Xylitol does not inhibit xylose fermentation by engineered *Saccharomyces cerevisiae* expressing *xylA* as severely as it inhibits xylose isomerase reaction in vitro, *Appl. Microbiol. Biotechnol.* 92 (2011) 77–84.
- [4] B. Hahn-Hägerdal, K. Karhumaa, M. Jeppsson, M.F. Gorwa-Grauslund, Metabolic engineering for pentose utilization in *Saccharomyces cerevisiae*, *Adv. Biochem. Eng. Biotechnol.* 108 (2007) 147–177.
- [5] Y.S. Jin, H. Alper, Y.T. Yang, G. Stephanopoulos, Improvement of xylose uptake and ethanol production in recombinant *Saccharomyces cerevisiae* through an inverse metabolic engineering approach, *Appl. Environ. Microbiol.* 71 (2005) 8249–8256.
- [6] K. Karhumaa, R.G. Sanchez, B. Hahn-Hägerdal, M.F. Gorwa-Grauslund, Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*, *Microb. Cell Fact.* 6 (5) (2007) 1–10.
- [7] S.R. Kim, J.M. Skerker, W. Kang, A. Lesmana, N. Wei, A.P. Arkin, Y.S. Jin, Rational and evolutionary engineering approaches uncover a small set of genetic changes efficient for rapid xylose fermentation in *Saccharomyces cerevisiae*, *PLoS One* (2013) 8.
- [8] S.R. Kim, H. Xu, A. Lesmana, U. Kuzmanovic, M. Au, C. Florencia, E.J. Oh, G. Zhang, K.H. Kim, Y.S. Jin, Deletion of *PHO13* encoding haloacid dehalogenase type IIA phosphatase, results in upregulation of the pentose phosphate pathway in *Saccharomyces cerevisiae*, *Appl. Environ. Microbiol.* 81 (2015) 1601–1609.
- [9] H.B. Klinker, A.B. Thomsen, B.K. Ahring, Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass, *Appl. Microbiol. Biotechnol.* 66 (2004) 10–26.
- [10] P. Kotter, M. Ciriacy, Xylose fermentation by *Saccharomyces cerevisiae*, *Appl. Microbiol. Biotechnol.* 38 (1993) 776–783.
- [11] M. Kuyper, M.M.P. Hartog, M.J. Toirkens, M.J.H. Almering, A.A. Winkler, J.P. Van Dijken, J.T. Pronk, Metabolic engineering of a xylose-isomerase-expressing *Saccharomyces cerevisiae* strain for rapid anaerobic xylose fermentation, *FEMS Yeast Res.* 5 (2005) 399–409.
- [12] S.-M. Lee, T. Jellison, H. Alper, Systematic and evolutionary engineering of a xylose isomerase-based pathway in *Saccharomyces cerevisiae* for efficient conversion yields, *Biotechnol. Biofuels* 7 (2014) 122.
- [13] X. Li, V.Y. Yu, Y. Lin, K. Chomvong, R. Estrela, A. Park, J.M. Liang, E.A. Znameroski, J. Feehan, S.R. Kim, Y.S. Jin, N.L. Glass, J.H.D. Cate, Expanding xylose metabolism in yeast for plant cell wall conversion to biofuels, *eLife* (2015) 4.
- [14] A. Madhavan, S. Tamalampudi, K. Ushida, D. Kanai, S. Katahira, A. Srivastava, H. Fukuda, V.S. Bisaria, A. Kondo, Xylose isomerase from polycentric fungus *Orpinomyces*: gene sequencing cloning, and expression in *Saccharomyces cerevisiae* for bioconversion of xylose to ethanol, *Appl. Microbiol. Biotechnol.* 82 (2009) 1067–1078.
- [15] E. Palmqvist, B. Hahn-Hägerdal, Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition, *Bioresour. Technol.* 74 (2000) 25–33.
- [16] G. Stephanopoulos, Challenges in engineering microbes for biofuels production, *Science* 315 (2007) 801–804.
- [17] M. Tantirungki, N. Nakashima, T. Seki, T. Yoshida, Construction of xylose-assimilating *Saccharomyces cerevisiae*, *J. Ferment. Bioeng.* 75 (1993) 83–88.
- [18] A.J. van Maris, A.A. Winkler, M. Kuyper, W.T. de Laat, J.P. van Dijken, J.T. Pronk, Development of efficient xylose fermentation *Saccharomyces cerevisiae*: xylose isomerase as a key component, *Adv. Biochem. Eng. Biotechnol.* 108 (2007) 179–204.
- [19] M. Walfridsson, X. Bao, M. Anderlund, G. Lilius, L. Bulow, B. Hahn-Hägerdal, Ethanol fermentation of xylose with *Saccharomyces cerevisiae* harboring the *Thermus thermophilus xylA* gene, which expresses an active isomerase, *Appl. Environ. Microbiol.* 62 (1996) 4648–4651.
- [20] N. Wei, J. Quarterman, S.R. Kim, J.H. Cate, Y.S. Jin, Enhanced biofuel production through coupled acetic acid and xylose consumption by engineered yeast, *Nat. Commun.* 4 (2013) 2580.