Title: Gene Duplication and Single Nucleotide Polymorphisms (SNPs) via Adaptive Laboratory Evolution (ALE) of Engineered *Yarrowia lipolytica* Enabled the Efficient Utilization of Sugars in Lignocellulosic Hydrolysate

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Project Goals:

1. To enable rapid and efficient utilization of xylose by *Y. lipolytica*
2. To understand the limiting factors in xylose metabolism by *Y. lipolytica* and to identify critical genes capable of alleviating the limiting factors.
3. To enable the ample production of cytosolic acetyl-CoA and achieve the production of acetyl-CoA derived products from lignocellulosic hydrolysates.

Abstract

Y. lipolytica has received extensive attention for converting cellulosic hydrolysates due to its potential to produce acetyl-CoA-derived products, such as TAL, lipids, and polyketides [1]. However, *Y. lipolytica* cannot metabolize xylose, the second abundant sugar in cellulosic hydrolysates. Therefore, metabolic engineering to confer xylose metabolism into *Y. lipolytica* is necessary. While *Y. lipolytica* harbors the endogenous xylose utilizing enzymes, namely xylose reductase (ylXR), xylitol dehydrogenase (ylXDH), and xylulokinase (ylXK), the activities of those enzymes are not active enough to facilitate xylose metabolism in *Y. lipolytica* [2]. Therefore, previous researchers introduced heterologous oxidoreductase and isomerase pathways to enable xylose utilization in *Y. lipolytica* but conducted adaptive laboratory evolution to improve lipid accumulation [3]. As such, the causality of the identified mutations on xylose fermentation has not been proved yet. In this study, we introduced a heterologous XR, XDH, and XK from *Pichia stipitis* into *Y. lipolytica* PO1f, and the resulting transformant was evolved under xylose conditions. Evolved strains capable of consuming xylose rapidly were isolated, and their genome sequences were determined to identify genetic perturbations related to the improved xylose utilization phenotypes. Based on the read-depth analysis, RT-PCR, and enzymatic analysis, we confirmed that rapid xylose assimilation by the evolved strain was enabled by the amplification of heterologous XR, XDH, and XK. Several genetic variants in YALI0B12100g

coding for GTPase-activating protein, YALI0B18282g coding for cAMP-independent regulatory protein, YALI0F05346g coding for cutinase gene palindrome-binding protein, YALI0F32065g coding for CCR4 transcriptional subunit, YALI0E18117g coding for E3 ubiquitin-protein ligase, YALIE18700g coding for CTP_transf_like domain-containing protein, YALI0E23474g coding for cytochrome P450 were also identified. Among the mutations, we confirmed the positive effects of YALIB12100g S409F on xylose assimilation via Cas9-based genome editing. When lignocellulosic hydrolysates were used, the lipid titer of the evolved strains was 2.3-times higher (0.83 g/L → 1.94 g/L) than a control strain (before the ALE). Overall, this study provides a better understanding of xylose metabolism in *Y. lipolytica* and beneficial mutations to achieve economic conversion from cellulosic sugar to value-added products.

References/Publications

1. Lee, J. W., Yook, S., Koh, H., Rao, C. V., & Jin, Y. S. (2021). Engineering xylose metabolism in yeasts to produce biofuels and chemicals. Current Opinion in Biotechnology, 67, 15–25. https://doi.org/10.1016/j.copbio.2020.10.012
2. Ledesma-Amaro, R., Lazar, Z., Rakicka, M., Guo, Z., Fouchard, F., Coq, A. M. C. L., & Nicaud, J. M. (2016). Metabolic engineering of *Yarrowia lipolytica* to produce chemicals and fuels from xylose. Metabolic Engineering, 38, 115–124. https://doi.org/10.1016/j.ymben.2016.07.001
3. Rodriguez, G. M., Hussain, M. S., Gambill, L., Gao, D., Yaguchi, A., & Blenner, M. (2016). Engineering xylose utilization in *Yarrowia lipolytica* by understanding its cryptic xylose pathway. Biotechnology for Biofuels, 9(1). https://doi.org/10.1186/s13068-016-0562-6

Funding Statement: *This work was funded by the DOE Center for Advanced Bioenergy and Bioproducts Innovation (U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research under Award Number DE-SC0018420). Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the U.S. Department of Energy.*