METABOLIC KEYS FOR EFFICIENT XYLOSE FERMENTATION AND INHIBITOR TOLERANCE IN ENGINEERED YEAST STRAINS

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ABSTRACT

Lignocellulosic hydrolysates are used for the microbial conversion of biomass sugars into bioproducts, but they contain a variety of inhibitors that limit fermentative fitness. Through long-term evolution using acetic acid and xylose as evolutionary selective pressure, we created an efficient xylose-fermenting strain tolerant to the inhibitor and investigated the molecular mechanisms behind its superior xylose consumption and inhibitor tolerance. Here, we show that the evolved strain exhibited a decrease in the copy number of *xylA* gene, suggesting that fine-tunning xylose isomerase expression is crucial for achieving optimal metabolic load. Additionally, we describe the role of mutations in the *ZWF1* and *CLN3* genes in improving C5 sugar consumption, identifying a novel target for engineering xylose metabolism in XI strains. Furthermore, we developed a diploid strain capable of fermenting hydrolysates from various lignocellulose biomasses with high ethanol yield and productivity. In summary, our results demonstrate different metabolic strategies that can enhance the conversion of sugars derived from lignocellulosic biomass into valuable bioproducts.

Keywords: Xylose. Acetic Acid. *Saccharomyces cerevisiae*. ALE. *CLN3*. *ZWF1*.

1 INTRODUCTION

Lignocellulosic biomass is a renewable alternative to fossil resources, with the potential to promote a sustainable and circular economy by producing a range of bioproducts via fermentation of its sugars. However, biomass pretreatment and hydrolysis are required to release the carbohydrates used for the microbial bioconversion, which leads to the production of inhibitory compounds for the microorganism and therefore limits the fermentation process[1]. Industrial strains of the baker's yeast *Saccharomyces cerevisiae* are typically the primary microorganisms used in Brazilian distilleries, especially due to their robustness against the harsh industrial environment of bioethanol plants, as the presence of high ethanol concentration and low pH[2]. Besides a high tolerance to inhibitors found in lignocellulosic hydrolysates, the strains used must be able to convert all sugars present in the lignocellulosic biomass for a feasible process – including the second most abundant sugar xylose, a pentose that is not naturally consumed by the yeast. This has led to the development of genetically engineered *S. cerevisiae* strains for xylose consumption over the years. However, the strains developed are still beyond the desired fermentation rate, which is reflected in the small fraction of the global bioethanol production that uses lignocellulosic biomass as feedstock[3]. This shows the necessity of efficient xylose-fermenting and inhibitor-resistant strains for the process viability.

In a previous study of our research group, a haploid cell derived from the industrial PE-2 strain was genetically engineered for xylose consumption, with the heterologous expression of the xylose isomerase gene (*xylA*) and overexpression of endogenous genes from the pentose phosphate pathway and the xylulokinase[4]. This strain was evolved using xylose as the sole carbon, generating the fast xylose-consuming strain LVY34.4[4]. Here, we used adaptive laboratory evolution (ALE) in acetic acid, one of the major inhibitors present in the lignocellulosic hydrolysates[5], to identify potential new targets to increase xylose consumption in the presence of this inhibitor.

2 MATERIAL & METHODS

The evolved xylose-fermenting strain LVY34.4[4] was used for the ALE in complete medium containing 50 g/L of xylose (YPX 5%) with the addition of acetic acid, in semi-aerobic conditions. The acetic acid concentration used increased along the successive batch cultures, ranging from 4 g/L to 8 g/L, without pH adjustment. Resistant cells were isolated from the evolved pool in xylose minimal medium (YNBX) with 5 g/L of acetic acid and the most resistant isolate, EvAA.14, was selected after screening in YPX 5% with 5 g/L of acetic acid.

The genome of isolate EvAA.14 was sequenced by Illumina/HiSEQ 2500 platform and the assembled genome was aligned to the parental strain LVY34.4 for identification of copy number variation (CNV) and single nucleotide polymorphisms (SNPs). The functional analysis of the point mutation was performed through reverse genetic engineering using the EasyGuide CRISPR/Cas9 system[6], carried out in the strain LVY59[4] as the background. Knock-out and SNP mutants were confirmed by PCR and Sanger sequencing. The effect of loss of function and point mutation was assessed in fermentations with YPX 5% with the mutant strains.

The diploid EvAA.14^{2n} was developed by transitional expression of the endonuclease *HO* and was used for fermentation in hydrolysates from eucalyptus chips, sugarcane straw, sugar cane bagasse, and energy cane. The hydrolysates were prepared by steam explosion pretreatment and hydrolysis with the Novozymes Cellic® CTec3 commercial cocktail. The pH was adjusted to 6 with KOH and 1g/L of urea was added prior to fermentation.

3 RESULTS & DISCUSSION

Aiming to increase the robustness of the evolved xylose-fermenting strain LVY34.4 against hydrolysate inhibitors, the strain was submitted to ALE in the presence of acetic acid, an inhibitor present in high concentrations in the lignocellulosic hydrolysates[5]. The ALE experiment was performed in YPX 5% with an initial concentration of 4 g/L of acetic acid, without pH adjustment, to increase the selective pressure with the dissociated form of the weak acid in low pH[7]. As the tolerance to the inhibitor increased through the successive batches, the acetic acid concentration was elevated, up to a final concentration of 8 g/L.

The evolved pool, named EvAA, showed an increased tolerance to the inhibitor compared to the parental strain LVY34.4 and 50 colonies were isolated from the pool and tested in the presence of acetic acid. Among the isolated colonies, the EvAA.14 was the one with the best overall fermentation profile. When compared to the parental strain in YPX 5% in the presence of 4 g/L of acetic acid, the EvAA.14 showed a 3.7-fold increase in productivity (Figure 1). This strain was then selected for further analysis through genome sequencing to identify the molecular bases responsible for the fermentation improvement.
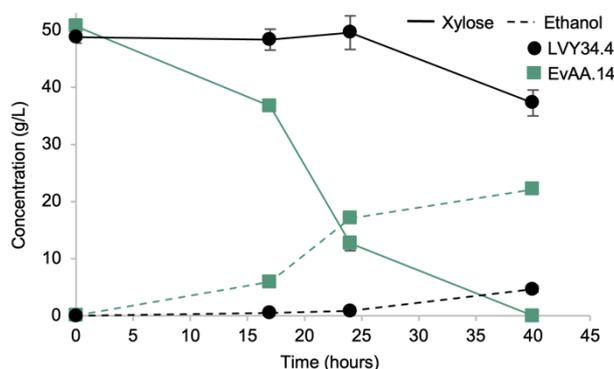

Figure 1 | Fermentation of the isolated strain EvAA.14 and the parental LVY34.4 in YPX 5% with 4 g/L of acetic acid.

The analysis of CNV of EvAA.14 revealed a decrease in the copy number of the gene encoding the xylose isomerase, *xylA*, when compared to the parental strain LVY34.4. The *xylA* gene was *in tandem* amplified during the xylose evolution process of LVY34.4, and the reduction of its copy number associated with the increased xylose performance observed in EvAA.14 may indicate a mechanism to evade the metabolic burden generated by the high copy number of this heterologous protein[8]. In a recent ALE experiment performed by our research group, around 16x amplification of *xylA* was achieved in 10 different populations (personal communication), suggesting that an optimal copy number is necessary to balance effective xylose fermentation and cellular metabolic load.

Although EvAA.14 showed an improved xylose fermentation and acetic acid tolerance compared to LVY34.4, only 2 point mutations identified in the sequencing are potentially linked to the EvAA.14 phenotype: the frameshift variant of *CLN3* gene (*cln3*^{T556fs}) and the amino acid change of the product of *ZWF1* gene (*zwf1*^{E192D}). These point mutations were assessed in the control strain LVY59[4], which harbors all genetic modifications necessary for xylose consumption without any other mutation on its genome. The fermentation results of the strains with deletion and point mutation of *ZWF1* and *CLN3* genes showed the benefit of these mutations for xylose consumption (Figure 2). Deletion of the glucose-6-phosphate dehydrogenase *ZWF1* had been shown to improve xylose consumption in strains carrying the oxido-reductive pathway[9,10], and only recently had been also reported for a strain with the xylose isomerase[11]. On the other hand, the G1 cyclin *CLN3* is a novel target that, to our knowledge, had not been described for xylose utilization. Further analysis of *ZWF1* and *CLN3* mutants is being performed in xylose medium with acetic acid to investigate the possible effects of the mutations in the increased inhibitor tolerance shown by the evolved strain EvAA.14.
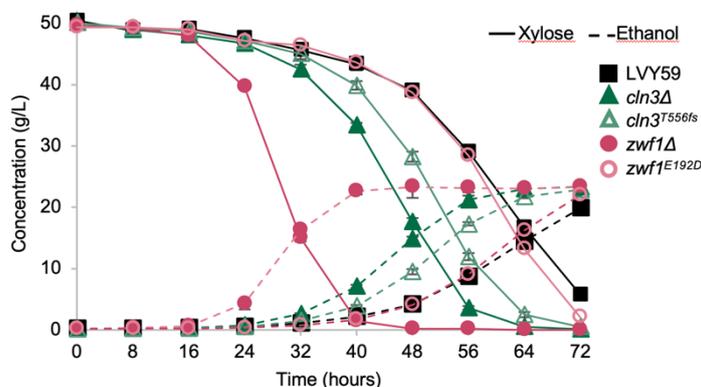

Figure 2 | Fermentative performance of *CLN3* and *ZWF1* mutants in YPX 5% compared to the control LVY59.

In order to evaluate the performance of EvAA.14 in the presence of inhibitors from different hydrolysates, we generated the diploid strain EvAA.14^{2n} through self-crossing and used it for fermentation of hydrolysates produced from sugar cane straw, sugar cane bagasse, energy cane bagasse, and eucalyptus chips. The diploid strain fermented all four hydrolysates and generated a high

ethanol yield even in the presence of acetic acid, whose concentration reached almost 6.5 g/L in the eucalyptus hydrolysate (Table 1). The fermentation of sugar cane bagasse hydrolysate was the one that achieved the highest ethanol volumetric productivity.

Table 1 | EvAA.14^{2n} performance in hydrolysates from different biomass.

Hydrolysate	Acetic acid initial concentration (g/L)			Ethanol yield (g/g)			Max. Ethanol volumetric productivity (g/L h^{-1})		
Sugar cane straw	4.18	±	0.16	0.42	±	0.01	1.574	±	0.050
Sugar cane bagasse	4.53	±	0.20	0.42	±	0.01	1.856	±	0.043
Energy cane bagasse	3.24	±	0.04	0.40	±	0.01	1.600	±	0.058
Eucalyptus chips	6.48	±	0.09	0.41	±	0.01	1.466	±	0.040

4 CONCLUSION

In this study, an efficient xylose-fermenting strain tolerant to acetic acid was generated through ALE experiments. Our results suggest that, in order to survive the imposed inhibitory condition, the cells alleviated the metabolic burden generated by the xylose isomerase expression through the reduction of *xylA* copy number, balancing efficient xylose fermentation and cellular metabolism. We also explored the point mutations found in the best isolate strain EvAA.14, showing that the mutants of *CLN3* and *ZWF1* genes were able to increase xylose metabolism and are potential gene targets for metabolic engineering. In addition, we performed a fermentation in different hydrolysates using the diploid strain carrying mutations found in this study, showing that the industrial strain developed is a good platform for use in a variety of 2G biorefineries. Altogether, our findings reveal a variety of adaptive strategies developed by the yeast during the ALE process to withstand the inhibitory conditions and shed light on genetic targets and approaches for improving the fermentation of different lignocellulosic hydrolysates used in industry.

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ACKNOWLEDGEMENTS