XYLOSE METABOLISM AFFECTS LIGNOCELLULOSE INHIBITORS' IMPACT ON INDUSTRIAL *Saccharomyces cerevisiae*

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ABSTRACT

Second generation ethanol production faces bottlenecks such as the incapacity of non-modified *Saccharomyces cerevisiae* strains to ferment pentoses, and yeast growth inhibition caused by products of biomass pretreatment, known as lignocellulose inhibitors. Different groups of inhibitors act differently on the microorganism. Understanding the mechanism of action of these compounds assists the development of tolerant strains. Regarding those two bottlenecks, genetically modified industrial strains of *S. cerevisiae* for xylose metabolization harboring the Xylose-Reductase/Xylitol dehydrogenase (XR-XDH) and the Xylose Isomerase (XI) pathways were evaluated under lignocellulose inhibitors challenge in different conditions. In industrial hydrolysate, XI strain can ferment glucose and xylose, but the XR-XDH strain cannot. The combination of different synthetic media for XR-XDH strain revealed the acids as main inhibitor, and aldehydes can improve ethanol yield by modification in the redox imbalance and lower growth ratio. Surprisingly, the XI strain presented an improvement of xylose consumption in the presence of acids.

Keywords: *Saccharomyces cerevisiae*. Second-generation ethanol. Lignocellulose inhibitors. Xylose consumption.

1 INTRODUCTION

Lignocellulose inhibitors are one of the main concerns regarding second generation (2G) ethanol production since they affect ethanol yield in *Saccharomyces cerevisiae* due changes in yeast metabolism and cell growth [1,2]. Within sugarcane ethanol industry, sugarcane straw hydrolysate contains different classes of inhibitors, which can be generated as products of sugars degradation, mainly weak acids such as formic and acetic acid and aldehydes such as furfural and hydroxymethyl-furfural (HMF) [3,4].

Furfural and HMF have a carbonyl group (C=O) at the chain end, what characterizes the aldehydes class of inhibitors, and for yeast are considered the most important inhibitors in a hydrolysate [5]. Furfural is formed from the degradation of xylose and arabinose, pentoses presented in the hemicellulose fraction of biomass, while HMF is generated through hexoses (glucose, mannose and galactose) dehydration [6]. Posterior decomposition of furfural and HMF generates formic acid which is present in lower amount than acetic acid, however, as its pKa is smaller, formic acid has a greater negative effect on yeast during ethanol fermentation [7,8]. Acetic acid is formed by the deacetylation of acetyl groups of hemicellulose during solubilization and hydrolysis of this structure; this acid is found in significant concentrations, varying from 0 to more than 17 g/L and its toxicity is increased at lower pH [1,6]. These different classes of inhibitors act differently on the microorganism, and understanding the mechanism of action of these compounds helps in the development of more tolerant yeast strains.

Another challenge in the 2G ethanol process is the conversion of pentoses, such as xylose, into ethanol, as *S. cerevisiae*, the most common yeast used in ethanol production, is only capable of fermenting hexoses [9]. Although this yeast has the potential to consume and ferment glucose at high rates, xylose is the most abundant pentose in hemicellulose, reaching values above 80% [10], comprising more than 25% of total sugar content in a hydrolysate [11]. Several studies for the insertion of pentose assimilation pathways in *S. cerevisiae* have been carried out, such as the insertion of the Xylose-Reductase/Xylitol Dehydrogenase pathway (XR-XDH) and the Xylose Isomerase pathway (XI) [12]. The XR-XDH pathway is carried out by the action of two enzymes dependent on the cofactors NAD(P)H and NAD^+ while the XI pathway is based on the reversible conversion of xylose to xylulose in just one step, without the need for cofactors, which avoids redox imbalance and the consequent production of by-products of metabolism [9,13].

Within this context, several studies have explored the impacts of lignocellulose inhibitors on yeast, but few have specifically investigated their effects on *S. cerevisiae* genetically modified for xylose consumption and, to date, no article has compared both routes of xylose consumption using industrial yeasts strains and industrial hydrolysate. [3,7,8,14–16]. In this work, we investigated the associative effects of the main lignocellulose inhibitors (furfural, HMF, acetic acid and formic acid) on two modified industrial *S. cerevisiae* strains containing the XR-XDH pathway (XR-XDH strain) and the XI pathway (XI strain). We evaluated the performance of both strains in an industrial hydrolysate of sugarcane straw pretreated by steam explosion and in synthetic media mimicking the concentrations of sugars and different combination of inhibitors present in the hydrolysate. For the XR-XDH strain, the presence of organic acids hindered the consumption of xylose and decreased cell growth. Nevertheless, the presence of HMF and furfural reduced the production of xylitol and glycerol, resulting in an increase in ethanol yield. These results were detailed

from flow balance analysis (pFBA) simulations. Surprisingly, in XI strain, organic acids increased xylose consumption by 1.49-fold in comparison with the absence of acids.

2 MATERIAL & METHODS

Yeast strains and growth conditions: The *S. cerevisiae* strains with the XI or XR-XDH pathways used in this work were developed in a previous study in our group [9,12]. Yeast sporulation was made in petri plates with a YPD solid medium, containing 1% of yeast extract, 2% of peptone, 2% of D-glucose and 2% of agar. The plates were incubated in 30ºC for 2-3 days and one colony was used for inoculum preparation. Yeast cells for inoculation were grown in 100 mL of liquid YPD medium containing 1% of yeast extract, 2% of peptone and 2% of D-glucose at 30ºC for 15 hours, with orbital shaking at 200 rpm. The optical density (OD) was measured by spectrophotometry (600 nm), and an amount corresponding to an OD = 1.0 was centrifuged for 5 minutes (18.0 G, 4ºC); the supernatant was discarded and the pellet was resuspended in sterilized distilled water and centrifuged using the same parameters. The supernatant was again discarded and the yeasts cells were resuspended in the correspondent fermentation media.

Production of sugarcane straw hydrolysate and synthetic media: The pre-treated sugarcane straw was provided by the company GranBio S.A. after steam explosion process. For the hydrolysis it was added 6% of w_{enzyme}/w_{glucan} of Cellic® CTEC3, by Novozymes in 17% of solids. The pH was adjusted to 5 with NH_4OH. The reaction was submitted to the parameters of 200 rpm, 50 ºC, for 72 hours at orbital shaker. For the synthetic media, it was evaluated a YNB medium (sugars and 6.7 g/L of Yeast Nitrogen Base without amino acids), with or without inhibitors. The same amount of sugars and inhibitors was added to each media based on its concentrations present in the sugarcane straw hydrolysate (see results for details).

Fermentation assays: All fermentations were carried out under the parameters of 30 ºC and 150 rpm for 120 hours, with an initial OD_{600} at 1.0 for both XR-XDH and XI strains. The sugarcane straw hydrolysate and synthetic media were used to evaluate the yeast's performance. All media used for fermentation assays had their pH adjusted to 5 using NH_4OH.

Analytical procedures: Cellular growth was measured by optical density (OD = 600 nm). Glucose, xylose, xylitol, glycerol, acetic acid, formic acid, HMF, furfural and ethanol concentrations were quantified by high-performance liquid chromatography (HPLC), using the Alliance® HPLC System from Waters with the refractive index detector (Waters 2414) at 410 nm and phothodiode array detector (Waters 2998) at 280 nm. It was used the ion exclusion Aminex HPX-87H column (300 mm × 7.8 mm, BioRad®) at 50 °C. Samples were diluted (1:10) in Milli-Q® water and filtered through a 0.2 µm filter (Millipore) before analysis. A solution of 5 mM of H_2SO_4 were used as mobile phase at a flow of 0.6 mL/min.

Parsimonious Flux Balance Analysis simulation: The COBRApy [17] was used in Google Colab for in silico studies, with GLPK as the solver. The iMM904 model [18] representing yeast metabolism, was utilized with flux calculations based on fermentation data collected from 0 to 8 hours.

3 RESULTS & DISCUSSION

The industrial sugarcane straw hydrolysate generated about 78 g/L of sugars (50 g/L of glucose and 28 g/L of xylose) and 7.3 g/L of acetic acid, 1.9 g/L of formic acid, 0.7 g/L of HMF and 1.0 g/L of furfural. The XR-XDH strain and the XI strain had different performances in the industrial hydrolysate (Figure 1). As both strains share a common background but with individual xylose consumption metabolism, it becomes evident that these two metabolisms are being differentially influenced by the inhibitors in the medium.

It is crucial to assess the distinct impacts of inhibitor groups (organic acids and aldehydes) on the strains to identify the most influential group affecting sugar consumption and, consequently, ethanol production. Thus, as seen in Figure 1, the class of inhibitors that is most affecting the XR-XDH strain are the acids, since in the presence of these compounds, xylose metabolism was severely impacted, while in their absence, the strain was able to consume this sugar at better rates. These results indicate that the presence of aldehydes does not totally hinder xylose metabolism for this strain, probably due to the aldehyde detoxification mechanism that regenerates $NAD(P)^+$ during the aldehyde consumption [5,19]. To evaluate the increase in ethanol yield in the presence of aldehydes, a parsimonious Flux Balance Analysis (pFBA) simulation was conducted using the genome-scale model (GEM) iMM904 [18] with detoxification reactions for HMF and furfural added. Comparing the absence and the presence of aldehydes, the pFBA simulations revealed a redirection through the G6PDH (*ZWF1*) and *GND1* (YHR183W) reactions in the presence of aldehydes. Another significant modification revealed by the model was observed in the *RPE1* reaction, which adjusted its flux to favor the production of xylulose-5-phosphate in the presence of aldehydes. This redirection of carbon diverts it to the non-oxidative phase of the PPP. It was also observed an increase in the flux of the *XYL2* reaction that corroborates the need for the regeneration of the NADH cofactor, which is necessary in the presence of aldehyde, for detoxification. This gene leads to the oxidation of xylitol to xylulose by xylitol dehydrogenase. Consequently, there was a reduction in the concentration of xylitol in culture medium at the end of the fermentation in the presence of aldehyde (data not shown).

For the XI strain, and contrary to what was observed for the XR-XDH strain, the presence of organic acids was responsible for the enhancement of consumption of xylose (Figure 1). An enhancement in ethanol production and decrease on cell growth in the presence of low concentrations of acetic acid has been documented in previous studies [1,16,20]. Nevertheless, in those studies, acetic acid exhibited negative effects on xylose metabolism [1,16]. In contrast, the current study demonstrated a 1.49-fold improvement in xylose consumption in the presence of organic acids in relation to the control without inhibitors. As organic acid consumption was not observed in the XI strain (data not shown), this suggests that the presence of these acids enables xylose consumption by the Xylose Isomerase pathway.

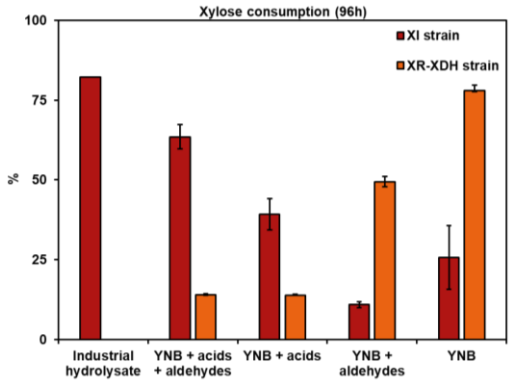

Figure 1 Xylose consumption in 96 hours of XR-XDH strain and XI strain in different media.

4 CONCLUSION

The XR-XDH strain is more susceptible to organic acids, while the presence of aldehydes in low quantities triggers a complex restructuring of yeast metabolism, enhancing ethanol yield and efficiency. This phenomenon corroborates the results found previously and explained by in-silico simulation through pFBA. For XI strain, surprisingly, the presence of organic acids promoted an improvement in xylose consumption and ethanol production. Furthermore, additional analyzes at molecular level, such as transcriptomic, will likely expand knowledge about this phenomenon. These insights could pave the way for more targeted strategies to optimize xylose metabolism in engineered strains for enhanced bioethanol production from lignocellulose.

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