CO-FERMENTATION OF XYLOSE/SUCROSE WITH DIFFERENT INITIAL SUGAR CONCENTRATIONS BY RECOMBINANT *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

This study sought to increase the efficiency of ethanol production by optimizing the co-fermentation of first generation (1G) and second generation (2G) sugars (sucrose and xylose, respectively) using a recombinant xylose fermenting industrial *S. cerevisiae* strain (MP-G1) that consumes sucrose directly through its active transport and intracellular hydrolysis, and consequently not producing glucose or fructose that will compete with xylose for uptake by the yeast cells. By evaluating different initial concentrations of sugars (from 80 to 220 g L^{-1}) in a mixture with approximately 30% xylose with 70% sucrose, our results indicate that 130 g L^{-1} of sugars showed the best xylose consumption rates, ethanol yields, and global ethanol volumetric productivities by strain MP-G1, even when compared with other recombinant *S. cerevisiae* yeasts. Our results highlight the potential of genetic engineering strategies to improve the efficiency of bioethanol production, especially in a 1G/2G integrated process.

Keywords: Sucrose. Xylose. *Saccharomyces cerevisiae*. Bioethanol. Co-fermentation.

1 INTRODUCTION

The substitution of fossil fuels with bioethanol has a lengthy and successful track record in Brazil.[1] Since the oil crisis in 1973, Brazil has been seeking alternative fuel sources, leading to the inception of the National Alcohol Program.[2,3] Although bioethanol is available in the market, the majority of global energy demand is still met by non-renewable sources, representing 90.57% of energy usage in the transportation sector. Fossil fuels have a significant role in industry but pose several environmental concerns.[4]

Bioethanol leads as the most utilized biofuel in the market, accounting for 62%, followed by biodiesel, with 26%.[5] In Brazil, only in the year 2022, about 31.27 billion liters were produced from sugarcane.[6] Molasses, rich in sucrose and obtained from sugarcane juice, is crucial in the fermentation for first-generation ethanol (1G) production. *S. cerevisiae* can utilize sucrose through extracellular hydrolysis, or through active transport with subsequent intracellular hydrolysis, and genetic modifications involving constitutive expression of the intracellular form of invertase (*iSUC2*) and the non-expression of the extracellular invertase (*suc2*Δ), with overexpression of the high-affinity sucrose transporter encoded by the *AGT1* gene, can improve the efficiency of sucrose fermentation.[7,8] Additionally, sugarcane bagasse and straw, residues rich in cellulose, hemicellulose, and lignin, are utilized for second-generation ethanol (2G) production.[3,9] Hemicellulose consists mainly of xylan, a polysaccharide made of xylose monomers.[10] However, although *S. cerevisiae* has the enzymes needed to consume xylose, their expression is not induced by this pentose and consequently this yeast does not ferment xylose.[11] One strategy to overcome this limitation is to express the xylose oxido-reductive pathway by overexpressing the genes *XYL1* (xylose reductase, XR), *XYL2* (xylitol dehydrogenase, XDH) from *Scheffersomyces stipitis*, and *XKS1* (xylulokinase, XK) from *S. cerevisiae*.[12,13]

In this study, with the objective to integrate and enhance the production of 1G/2G ethanol, we used a recombinant *S. cerevisiae* industrial strain capable of transporting sucrose directly and promoting its intracellular fermentation[8], and further modified to consume xylose[12,13], evaluating different concentrations of total sugars in sucrose and xylose co-fermentations.

2 MATERIAL & METHODS

The strain used in this study is MP-G1, an isogenic strain to the diploid industrial CAT-1 strain[14] but *suc2*Δ::LoxP-Ble^R-LoxP, LoxP-KanMX-LoxP-P_{ADH1}::*iSUC2* P_{GPD}::*AGT1* and *AUR1*::pAUR-XKXDHXR. A cryotube of yeasts (kept at −80ºC) was inoculated in Petri dishes containing YP medium (1% yeast extract, 2% peptone) with 2% glucose and 1.5% agar and incubated at 30ºC for 48 h. After growth, a colony was inoculated into YP medium containing 2% glucose, and after 12 h of growth the cells were used to inoculate 50 mL of YP medium containing 1% sucrose and 1% xylose (pH 5.0) with an initial cell density adjusted to an absorbance at 600 nm of 0.1. The cells were incubated at 30ºC and 180 rpm in a shaker (New Brunswick-Innova 44) for 16 h, when the cells were centrifuged, and the initial cell density in the fermentation medium was adjusted to an absorbance at 600 nm of 40 (~10 g/L of dry cell weight). The anaerobic batch fermentations were performed at 30ºC in closed 50 mL bottles with a magnetic stir bar (2 cm) to allow mild agitation The total sugar concentrations tested were 80, 130, 180, and 220 g L^{-1}. The medium composition consisted of a mixture of 62.5% sucrose with 37.5% xylose, supplemented with 3 g L^{-1} yeast extract, 2.3 g L^{-1} urea, and 1 g L^{-1} $MgSO_4.7H_2O$.[15] At different time medium samples were aseptically harvested from the fermentation, centrifuged, and the supernatants stored at -20ºC. Sucrose, glucose, fructose, xylose, ethanol, xylitol, and glycerol were determined by high performance liquid chromatography (HPLC) equipped with a refractive index detector (Prominence LC-20A, Shimadzu, Japan)

using an Aminex HPX-87H column at 35ºC using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml min^{-1} and 0.02 ml injection volume.

3 RESULTS & DISCUSSION

Given that high sugar concentrations and thus high ethanol titers are necessary for industrial fermentations in order to process and distill the ethanol efficiently[16], we used the recombinant industrial yeast MP-G1 for fermentation tests with high sugar concentrations. Since in this yeast strain sucrose is actively transported into the cells, producing very low levels of glucose or fructose in the medium[8], the lack of these monosaccharides outside the cells will favor the uptake of xylose, as glucose and xylose compete for the same *HXT* transporters in *S. cerevisiae*.[17] In a previous work, using another xylose fermenting strain (MP-C5H1) derived from the industrial CAT-1 strain (transformed with the same integrative pAUR-XKXDHXR plasmid, and with stabilization of the *HXT1* permease at the plasma membrane), it was found that a mixture containing approximately 30% xylose with 70% sucrose resulted in optimal sugar consumption and fermentation.[12] Thus, we used a mixture of 62.5% sucrose with 37.5% xylose to analyze the fermentation of different concentrations of total initial sugar (from 80 to 220 g L^{-1}) by our MP-G1 strain (Figure 1).

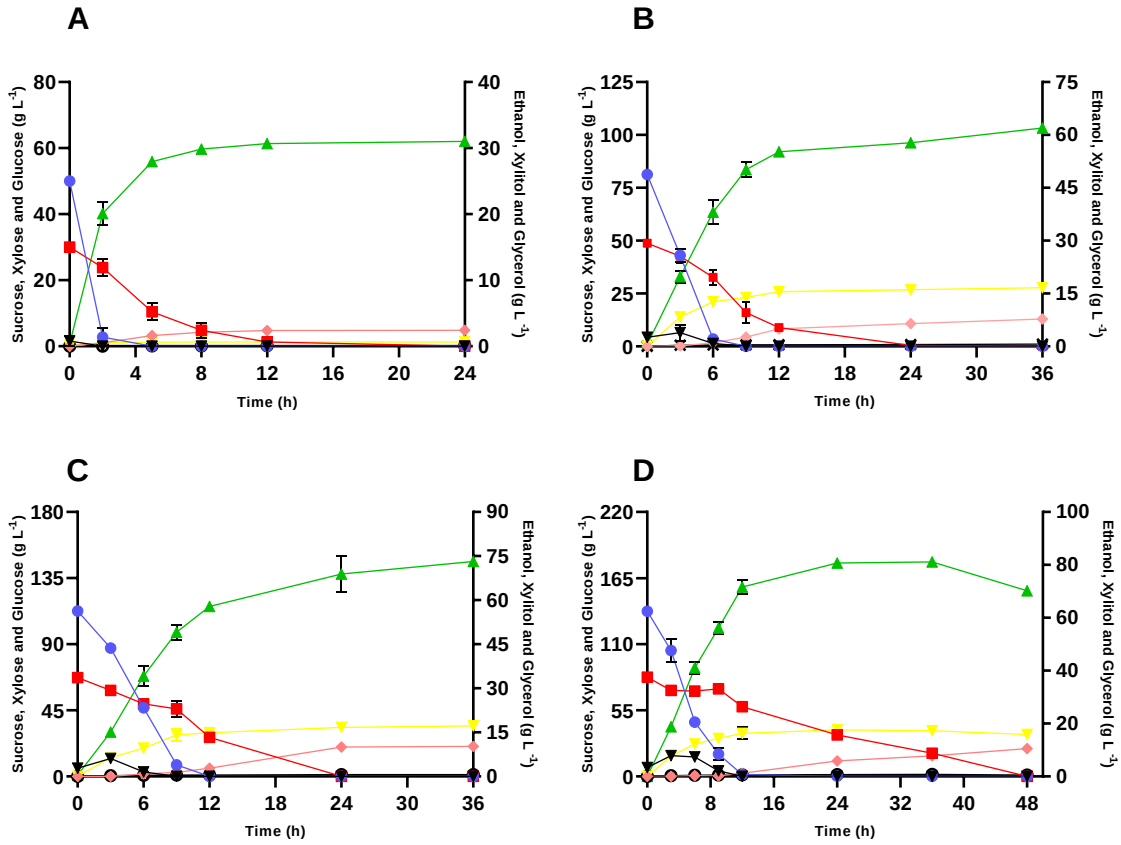

Figure 1 Fermentation of a 64.5 % sucrose and 37.5% xylose mixture by the recombinant MP-G1 strain in flasks with different initial total sugar concentration of (g L^{-1}): 80 (A); 130 (B); 180 (C); and 220 (D). At the indicated time points, the concentrations of xylose (red), sucrose (blue), glucose (black), xylitol (pink), glycerol (yellow), and ethanol (green) were determined.

As shown in Figure 1, the yeast cells consumed and fermented sucrose rapidly, and xylose was consumed and fermented even when sucrose was still present in the media (the only exception was with 220 g L^{-1} total sugar concentration, where xylose was consumed only after all sucrose was depleted, Fig. 1D). There was practically no glucose (Fig. 1) or fructose (data not shown) been produced in the medium during sucrose consumption, and low concentrations (2-10 g L^{-1}) of xylitol were produced during the fermentations. Regarding glycerol production, very low levels of this compound were produced at low total sugar concentration (Fig. 1A), but as the concentrations of sugars increased, the amount of glycerol in the medium increased (Fig. 1B-D). The very low levels of monosaccharides in the medium resulted in high xylose consumption rates and high ethanol yields, especially when using 130 g L of total initial sugars (Table 1).

Table 1 Data on ethanol production, xylose consumption rate, substrate-to-product conversion factor ($Y_{P/S}$), global ethanol volumetric productivity (Qp) and overall yield (Ƞ) for the recombinant yeast MP-G1 at different initial substrate concentrations.

Assay	Total sugar concentration (g L^{-1})	Ethanol produced (g L^{-1})	Xylose consumption rate (g L^{-1} h^{-1})	$Y_{P/S}$ (gP gS^{-1})	Qp (gP L^{-1} h^{-1})	η (%)
A	80	31.6	2.71	0.40	1.27	78.2
B	130	61.5	3.40	0.47	2.07	92.0
C	180	72.4	3.10	0.40	2.00	78.2
D	220	81.1	1.53	0.37	2.19	72.5

In our previous publication the xylose-fermenting recombinant strain MP-C5H1 (derived from the same background, the industrial CAT-1 strain), when consuming 90 g L^{-1} of a 70% sucrose plus 30% xylose mixture, presented a $Y_{P/S}$ of ~0.35 gP gS^{-1} (the best yield obtained by this strain in different sucrose/xylose mixtures), and a Qp of 1.23 (gP L^{-1} h^{-1})[12]. The data shown in Table 1 indicates that the new recombinant strain MP-G1, when compared with strain MP-C5H1, consumed xylose more rapidly and produced more ethanol, although the experimental conditions were not exactly the same. For example, with 130 g L^{-1} (see Table 1), strain MP-G1 had a higher yield ($Y_{P/S}$ of ~0.47 gP gS^{-1}) and global ethanol volumetric productivity (Qp of 2.07 gP L^{-1} h^{-1}), than strain MP-C5H1 fermenting 130 g L^{-1} of total sugar ($Y_{P/S}$ of ~0.21 gP gS^{-1} and Qp of 0.41 gP L^{-1} h^{-1})[12]. This highlights that our present strategy, an industrial strain that consumes sucrose directly by its active transport and intracellular hydrolysis, producing low levels of monosaccharides in the medium, is a promising approach to promote sucrose/xylose co-fermentations by recombinant *S. cerevisiae* strains, opening opportunities for the integration of 1G/2G ethanol production processes.

4 CONCLUSION

This study investigated the optimization of ethanol production through the co-fermentation of first generation (1G) and second generation (2G) sugars (sucrose and xylose, respectively) using a recombinant industrial xylose-fermenting *S. cerevisiae* strain (strain MP-G1) that ferments sucrose through its active transport and intracellular hydrolysis. By evaluating different initial concentrations of sugar in a mixture comprising approximately 30% xylose and 70% sucrose, our results indicated that optimal xylose consumption rates and global ethanol titers by strain MP-G1 were obtained with a total initial sugar concentration of 130 g L^{-1}, ensuring complete fermentation in less than 24 h. These results can be used to develop fed-batch fermentation processes that mimic industrial practices currently used in Brazil, which will allow high and efficient bioethanol production from the integration of 1G/2G ethanol production processes.

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ACKNOWLEDGEMENTS

The authors thank the National Council for Scientific and Technological Development (CNPq, # 305173/2022-7 and 309047/2023-4) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for scholarships. This work is part of the project “INCT Yeasts: Biodiversity, preservation and biotechnological innovation,” supported by CNPq (# 406564/ 2022-1).