Growth characterization of adapted *Rhodosporidium toruloides* in sugarcane biomass hemicellulosic hydrolysate

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1. Introduction

In the last decade researchers have made a great effort aiming the valorization of agro-industrial waste, and lignocellulosic biomass has emerged as a potential raw material because of its availability, and capacity to be processed to obtain value-added products such as: biofuels, functional ingredients, renewable chemicals; with the behoof of not competing with the food industry (Liu et al. 2021a). Lignocellulosic biomass is composed primarily of cellulose, hemicellulose, and lignin, the first two components have been successfully employed as a carbon source for lipids production from non-conventional yeasts in the production of lipids, highlighting the potential showed by *Rhodosporidium toruloides* (Monteiro de Oliveira et al. 2021). Due to the complex structure of lignocellulosic biomass, there is the need for pretreatment or severe hydrolysis to open this structure, however these processes release inhibitors compounds, which are essentially furan derivates, aliphatic acids and phenolic compounds (Liu et al. 2021a). Such inhibitors directly affect the lipids production yield from oleaginous yeasts, to overcome this obstacle researchers are resorting to adaptative strategies to improve yeast tolerance to inhibitors, resulting in an evolved yeast with a smaller lag phase, enriched lipids accumulation and a better growth performance (Liu et al. 2021b). Therefore, the aim of this study was improved the lipid production by *R. toruloides* employing adaptative strategy in hemicellulosic hydrolysate.

2. Material and methods

R. toruloides UFMG-CM-Y2781 and *R. toruloides* UFMG-CM-Y2882 were obtained from Collection of Microorganisms of UFMG-Brazil. The C5-sugars enriched hemicellulosic hydrolysate, here named as C5-HBB, was produced via diluted acid hydrolysis from mixture 1:1 of bagasse and straw sugarcane, provided by Usina São Martinho (São Paulo, Brazil). The total amounts of sugars and inhibitory compounds were (g/L): glucose, 3.8; xylose, 26.3; acetic acid, 3.77; and (mg/L): vanillin, 1.09; furfural, 4.9. The pH of the hydrolysate was adjusted to 5.5 with NaOH. The yeast was cultivated in mimetic medium (25g/L xylose, 4g/L glucose, 4g/L acetic acid), and supplemented with 2g/L yeast extract, 1g/L $MgSO_4$, 2g/L NH_4SO_4, 3g/L KH_2PO_4, in shaking flasks at 28ºC, 250 rpm for 96 h. Afterward, the cells were collected by centrifugation.

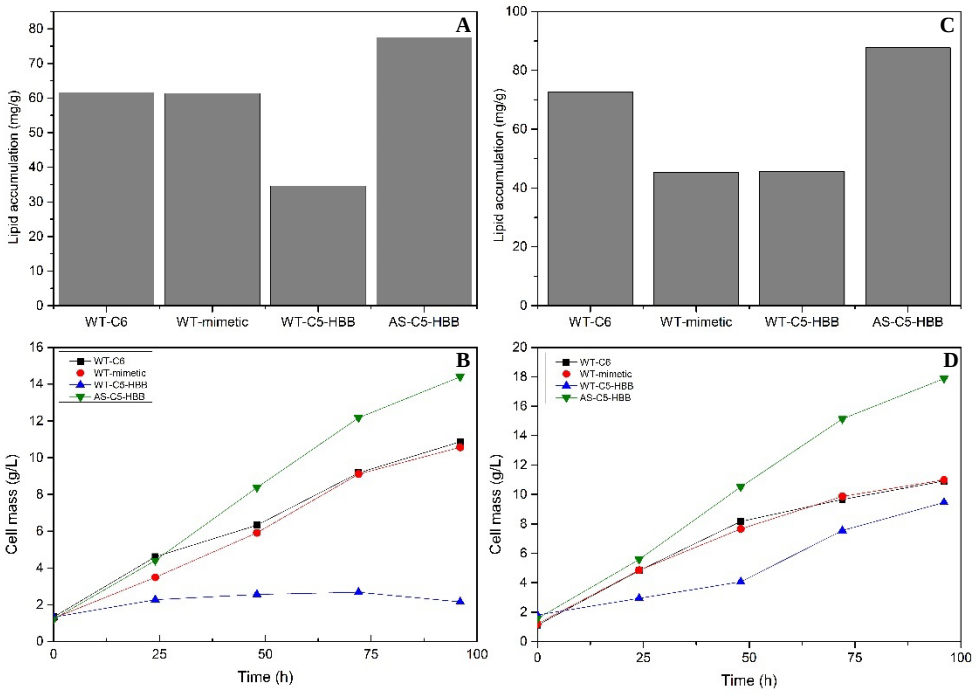
For the adaptation strategy *R. toruloides* 2882 and *R. toruloides* 2781 were cultivated in a blend of mimetic medium and C5-HBB, starting with 10% of C5-HHB until 100% C5-HBB. After, 100 µL of broth was spread on YPD agar plates and incubated, obtaining a stable yeast strain. Finally, the non-adapted and adapted yeasts were cultivated in the C5-HBB supplemented with (g/L): $MgSO_4.7H_2O$, 1; $(NH_4)_2SO_4$, 2; KH_2PO_4. For all experiments (in duplicate), samples were taken regularly and analyzing the cell growth, substrates consumption, and metabolites quantification by HPLC. After 96 h broth were withdrawn for lipid extraction and quantification as described in Folch *et al.*, (1957).

3. Results and discussion

Figure 1 shows the effect of adaptative strategy of *R. toruloides* in cell growth and lipids production. Both adapted strains cultivated in C5-HBB showed a xylose consumption of 81 and 93% for *R. toruloides* 2781 and *R. toruloides* 2882; respectively. While consumption of the non-adapted cells was 8% (*R. toruloides* 2781*)* and 31% (*R. toruloides* 2882). The growth pattern of the strains was partially inhibited by the presence of toxic compounds derived from the degradation of lignocellulose, since the biomass concentration increased from 2.16 to 14.39g/L for the *R. toruloides* 2781 strain and from 9.45 to 17.87 g/L for the strain *R. toruloides* 2882 after 96 hours of cultivation, when comparing non-adapted and adapted strains (Fig. 1B e 1D), moreover about 92% and 69% of the initial concentration of sugars remained in the fermentation broth at the end of the experiment (non-adapted strain); respectively. Both strains showed the same pattern for sugars consumption, with xylose assimilation only started after most of available glucose was consumed. The sequential consumption of sugars by oleaginous yeasts agrees with reported by Liu et al. (2021b) and could be attributed to a carbon catabolite repression (Monteiro et al., 2021). This behavior can also be associated to the presence of inhibitory compounds in the C5-HBB, such acetic acid, phenolic compounds, and furfural, which can be toxic to microorganisms acting independently or with synergistic effect. However, acetic acid in low concentration (lower than 5 g/L) can be used as a carbon source mainly for the adapted strains, resulting in increasing pH of fermentation medium (pH = 7.5 at the end of fermentation 7.5). Although the non-adapted strains (mainly *R. toruloides* 2781 strain) grown in C5-HBB presented a significant lag phase, fact that probably is due to the presence of high level of phenolic compounds, both adapted strains showed a reduction in the lag phase, comparable to the use of mimetic media (Fig. 1B e 1D), confirming the application of adaptative strategy can be an effective tool to select a strain with desired

characteristics caused by selection pressure. Besides, the smaller the lag phase the higher advantage in terms of industrial application, reducing the global energy demand of the bioprocess plus to a better tolerance to toxic compounds present in the medium. Comparing cell growth and lipid accumulation (Fig. 1), for both yeast (*R. toruloides* 2781 and *R. toruloides* 2882) it is possible to observe that adapted strain cultivated resulted in higher cell growth and the higher lipid production, while non-adapted yeasts resulted in the lower cell growth and the lower lipid accumulation, both cultivated in C5-HBB. The accumulation of lipids for *R. toruloides* 2781 was 77.56 mg/g, and *R. toruloides* 2882 at 87.71 mg/g, confirming the positive effect over accumulation of lipids when cells were adapted in the hydrolysate (Fig. 1A and 1C). In general, lipid accumulation is not associated with biomass accumulation, there must be a balance between this factor and the rate of lipid biosynthesis, so that the majority carbon is deflected to lipid synthesis and a minimal amount to other metabolic pathways, therefore, an adequate C/N ratio must be found to favors the bioprocess, which is usually close to 100 (Ageitos et al., 2011).

Fig. 1. Lipid accumulation in mg/g biomass by the wild-type (WT) (medium C6, mimetic, and C5-HBB), adapted strains (AS) (C5-HBB) of *R. toruloides* 2781 (A) and *R. toruloides* 2882 (B). Growth profile of *R. toruloides* 2781 (WT and AS) (B) and *R. toruloides* 2882 (WT and AS) (D) cultivated in medium C6, mimetic, C5-HBB supplemented.



Conclusion

Production of lipids by oleaginous yeasts from lignocellulosic hydrolysate is considered an attractive and sustainable approach. Understanding the growth pattern of these yeasts, influence of toxic compounds from the pre-treatment steps is essential to overcoming this obstacle and enable these bioprocesses. The use of adaptative strategy proves to be an advantage for obtaining stronger strains, making them capable of overcoming the inhibition of toxic compounds present in hydrolysed biomass due to its simplicity and effectiveness. In this study, the adapted strain of *R. toruloides* showed better performance to grow in non-detoxified hemicellulosic hydrolysate, showing better capacity to accumulate lipids.

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