An Acad Bras Cienc (2020) 92(Suppl. 2): e20181030 DOI 10.1590/0001-3765202020181030
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

BIOLOGICAL SCIENCES

Second-generation ethanol production by *Wickerhamomyces anomalus* strain adapted to furfural, 5-hydroxymethylfurfural (HMF), and high osmotic pressure

NICOLE T. SEHNEM, ÂNGELA S. MACHADO, CARLA R. MATTE,
MARCOS ANTONIO DE MORAIS JR & MARCO ANTÔNIO Z. AYUB

Abstract: The aims of this work were to improve cell tolerance towards high concentrations of furfural and 5-hydroxymethylfurfural (HMF) of an osmotolerant strain of *Wickerhamomyces anomalus* by means of evolutionary engineering, and to determine its ethanol production under stress conditions. Cells were grown in the presence of furfural, HMF, either isolated or in combination, and under high osmotic pressure conditions. The most toxic condition for the parental strain was the combination of both furans, under which it was unable to grow and to produce ethanol. However, the tolerant adapted strain achieved a yield of ethanol of 0.43 g g^{-1}glucose in the presence of furfural and HMF, showing an alcohol dehydrogenase activity of 0.68 mU mg $protein^{-1}$. For this strain, osmotic pressure, did not affect its growth rate. These results suggest that *W. anomalus* WA-HF5.5strain shows potential to be used in second-generation ethanol production systems.

Key words: Second-generation ethanol, *Wickerhamomyces anomalus*, furaldehydes tolerance, osmotic pressure, lignocellulosic hydrolysates.

INTRODUCTION

There is a worldwide effort to ensure the production of energy from various sources of renewable raw materials. Second-generation ethanol, sometimes called *bioethanol*, is obtained from lignocellulosic biomass, especially from agriculture residues, which are abundant, inexpensive, and a desirable feedstock for the sustainable production of biofuels, among other things because these residues do not directly compete with food production for arable land, as is the case of ethanol from sugarcane and maize (Hasunuma et al. 2013). The production of ethanol from lignocellulosic feedstock requires pretreatments of biomass and hydrolyses steps in order to release sugars for yeast fermentation, process that could produce inhibitory compounds of yeast metabolism. Therefore, biomass hydrolysates pose a challenging to their use in bioprocess, since the operational costs of medium detoxification are, at present, prohibitive if second-generation ethanol must compete with first-generation sugarcane or starch-based ethanol.

In the research for second-generation ethanol production it is fundamentally important to consider the presence of pentoses and hexoses in the hydrolysates, which requires specific cell metabolisms for the total conversion of these sugars. *S. cerevisiae*, the most efficient yeast for conventional ethanol production, cannot assimilate pentoses, including xylose, usually present in great amounts in lignocellulosic

hydrolysates (da Cunha-Pereira et al. 2011, Cortivo et al. 2018, Sehnem et al. 2017). Therefore, it has been of great concern the research for isolating or engineering pentose-fermenting yeasts, with some reports showing pentose bioconversions by several non-recombinant strains of *Candida guilliermondii*, *Pichia stipitis*, *Pachysolen tannophylus*, *Spathaspora arborariae* and *Wickerhamomyces anomalus* (da Cunha-Pereira et al. 2011, Schirmer-Michel et al. 2008, Yadav et al. 2011, Zhao et al. 2008, Tao et al. 2011).

The ascomycetous yeast *W. anomalus* (formerly *Pichia anomala* and *Hansenula anomala*) has been isolated from many different habitats and shows a remarkable physiological robustness to cultivation stresses, such as extreme pH and low water activity. Compared to other yeast strains, *W. anomalus* is highly competitive in terms of growth and can inhibit cell growth of other microorganisms (Passoth et al. 2011). This species has been tested for several biotechnological applications, including food and beverage applications (as probiotics, sourdough fermentations, volatile aromas in wine), environmental bioremediation (sophorolipis as biosurfactants), biopharmaceuticals (production of aminobutiric acid), and biofuels (ethanol and isobutanol productions) (Walker 2011, Sehnem et al. 2017).

During ethanol fermentation, metabolism inhibition by substrate and product, besides medium osmotic pressure, are the most important adverse conditions (Zhao & Bai 2009, Sehnem et al. 2017, Hickert et al. 2014). In second-generation ethanol production, there is a further important problem, which is the degradation of sugars during hydrolyses of biomass and the consequent formation of toxic compounds (Almeida et al. 2008, Margeot et al. 2009, da Cunha-Pereira et al. 2011, Li et al. 2017, Guarnieri et al. 2017, Ling et al. 2014). The main inhibitors of yeast metabolism are 2-furaldehyde (furfural), which is formed by dehydration of pentoses, and 5-hydroxymethyl-2-furaldehyde (5-hydroxymethylfurfural - HMF), which is formed by dehydration of hexoses (Liu et al. 2009, 2008, Lenihan et al. 2010, Mansilla et al. 1998, Kim et al. 2015). Furaldehydes interfere with microbial growth by hampering glycolytic enzymes activities, as well as by impairing protein and RNA synthesis (Almeida et al. 2007, Luo et al. 2002, Modig et al. 2002, Kim et al. 2015), and negative synergistic effects of HMF and furfural have been demonstrated for *S. cerevisiae* and *S. arborariae* (Taherzadeh et al. 2000, da Cunha-Pereira et al. 2011). A common furaldehyde detoxification metabolism in yeasts is the reduction of the aldehydes into the less toxic corresponding alcohols. This reaction is catalyzed by alcohol dehydrogenases. These enzymes are NAD(P)H-dependent oxidoreductases that catalyze the reversible oxidation of alcohols to aldehydes or ketones (De Smidt et al. 2008, Liu et al. 2004, Almeida et al. 2007, Guarnieri et al. 2017).

One approach extensively used to select tolerant yeast strains to adverse environmental stresses, such as high temperature, ethanol inhibition, osmotic pressure, and inhibitory furaldehydesis the simple experiment of evolutionary engineering (Zhao & Bai 2009, Li et al. 2017).

In the present study, a strain of *W. anomalus* was submitted to evolutionary engineering in order to increase its resistance to furfural and HMF present in the culture medium. Both the parental and its tolerant-derived strain were evaluated in their abilities to grow and to produce ethanol, which were correlated to alcohol dehydrogenase enzymatic activity and to the reduction of the toxic furan concentrations in the medium. The ability of these strains to resist high osmotic pressures was also evaluated and compared.

MATERIALS AND METHODS

Microorganisms, cell maintenance and chemicals

The strain used in this work is part of the yeast collection of Bioteclab, named *W. anomalus* WA-001. This strain was isolated by this research group from piles of decomposing rice hulls deposited in the environment and it was identified comparing ITS1 and ITS4 amplicon sequences with GenBank database (www.ncbi.nlm.nih.gov/BLASTn). Stock cultures were kept frozen at -20 °C in medium composed of 20 % glycerol and 80 % of mid-exponential cell suspensions. All chemicals used in this research were of analytical grade and purchased from Sigma-Aldrich (St. Louis, USA).

Evolutionary engineering for HMF and furfural resistance

The evolutionary engineering approach was used aiming to increase furaldehydes resistance of *W. anomalus* WA-001. Cultivations were performed using YPD medium (composed of, in g L^{-1}: glucose, 20; yeast extract, 10; and peptone, 20), and were carried out in 125 mL Erlenmeyer flasks containing 30 mL of medium, at 28 °C and 150 rpm on a rotatory shaker. Cells were inoculated (10 % volume fraction of a cell suspension of OD 1.0, 600 nm) into YPD medium added of HMF and furfural (0.25 g L^{-1} each chemical), and incubated for 24 h. From this mother culture, successive batches were run by transferring 10 % (volume fraction) of cells (OD 1.0) to re-inoculate YPD medium with increasing concentrations of furaldehydes, up to 1 g L^{-1} each.

Kinetics of ethanol production in presence of furaldehydes

Cell cultivations were performed in YPD medium and supplemented with furaldehydes (see below). Cultures were carried out in 250 mL flasks filled with 60 mL of medium at 28 °C and 150 rpm on a rotatory shaker, which corresponds to an oxygen limitation condition. Inoculum was set as a cell suspension of 1.0 OD (600 nm). Strains were cultivated in absence (control) and presence of furfural (3 $g.L^{-1}$), HMF (3 $g.L^{-1}$), or the combination of both (1.5 $g.L^{-1}$ of each). Samples were taken at determined times during 48 h of cultivation for determination of glucose, ethanol, glycerol, furfural, HMF concentration, and for biomass formation.

Enzyme activities assays

To determine furfural and HMF reducing metabolic activities, samples were taken at 24 h of cultivation under conditions mentioned in the next item. Crude protein extracts were prepared by lysing cells with glass beads attrition. Cells were resuspended in 400 µL of 100 mM K_2HPO_4 buffer, pH 7.0, and 2 µL of a solution of 100 mM phenylmethylsulfonyl fluoride (PMSF) in a 2 mL Eppendorf tube, and added of an equal volume of glass beads (diameter of 500 µm). Cells were disrupted by six cycles of vortexing for 60 s, with samples cooled on ice for 60 s between each cycle. Protein extracts were collected after centrifugation at 13000 *g* (5 min at 4 °C) and its concentration was determined using the Lowry assay method. Alcohol dehydrogenase activity was performed by recording the decrease in absorbance at 340 nm using NADH as cofactor. NADPH was used as cofactor when HMF was used as substrate. The reaction mixture consisted of 10 mM HMF substrate, final concentration, and 100 µM of NADPH prepared in 100 mM potassium phosphate buffer, pH 7.0. All reagents were kept at 25 °C prior to use. Assays were performed in a volume of 1 mLat 30 °C for 1 min. The protein samples were kept on ice. To start the reaction, 25 µL of crude extract protein was added to the reaction mix. The NADPH molar absorption coefficient was 6.22 mM^{-1} cm^{-1}.

Osmotic pressure tolerance assay

Cultivations were performed in YPD medium and supplemented with varying concentrations of NaCl. Cultures were carried out in 250 mL flasks containing 60 mL of medium, incubated at 28 °C and 150 rpm on a rotatory shaker. Again, inoculum was set as a cell suspension of 1.0 OD (600 nm). When cells reached exponential phase (OD = 1.0), a pulse of NaCl was added into the medium to final concentrations of 0.25, 0.5, 0.75, 1, 1.25, and 1.5 M. Samples were taken at determined times during 12 h for determination of specific growth rate.

Analytical methods and calculation of kinetic parameters

Growth of yeast cells was estimated as optical density (OD) at 600 nm. Cultivations were run for 48 h, with sampling taken every 6 h. At the end of cultures, cells were centrifuged at 3500 *g*, and processed at once for analyses. Yeast biomass was determined using a standard curve correlating the OD and cell dry weight (g L^{-1}). Glucose, glycerol, ethanol, acetate, furfural, and HMF concentrations were determined according to Sehnem et al. (2013). Osmotic pressure (mOsm Kg^{-1}) was determined by vapor pressure measurement using an osmometer (Wescor, USA). Ethanol conversion yields ($Y_{P/S}$, g g^{-1}) was defined as the ratio of the concentration of ethanol produced and glucose consumed. The volumetric productivity (Q_p, g L^{-1} h^{-1}) was calculated using the maximal ethanol production in time interval to reach it. The specific growth rate of cells (μ, h^{-1}) was calculated using biomass formation in the time interval to reach it within the exponential growth phase.

RESULTS AND DISCUSSION

Evolutionary engineering for furaldehyde tolerance

The principle of evolutionary engineering was applied in order to select a strain resistant to furfural and HMF. It was possible to increase the tolerance of WA-001 strain to 5.5 g L^{-1} towards furaldehydes (2.25 g of furfural and HMF, each) after 22 days of successive cultivations with increasing concentrations of furfural and HMF (results not shown). Therefore, it was possible to further increase furaldehydes tolerance of WA-001 strain by 80 % in relation to its parental strain. This resistant strain was named WA-HF5.5 and it was used in the following experiments. Evolutionary engineering has been used as a powerful tool for the optimization and the introduction of new cellular processes. For instance, this strategy has been efficiently applied to obtain several interesting resistant strains of *S. cerevisiae,* as well as for strains showing improved gluconate assimilation in wine fermentation (Cadière et al. 2011), and improved xylose-glucose co-assimilation by recombinant xylose-fermentingstrains (Kuyper et al. 2005), improved arabinose consumption by recombinant arabinose-fermenting strains (Wisselink et al. 2007), and improved HMF tolerance of an industrial strain of *S. cerevisiae* (Sehnem et al. 2013). During the processes of hydrolysis of lignocellulosic biomass, the production of furfural and HMF is unavoidable; the toxic synergistic effects of these compounds have been investigated and demonstrated in previous studies for *S. cerevisiae* and *S. arborariae* (Taherzadeh et al. 2000, da Cunha-Pereira et al. 2011). Thus, improved tolerance towards both furfural and HMF is an important strategy for second-generation ethanol production.

Culture kinetics behavior in response to furfural and HMF concentration

Physiological analyses of parental and resistant strains were carried out under oxygen limitations. High sensibility of WA-001 strain to furfural and to the synergic effect when using furfural and HMF resulted in practically no metabolic activity, as shown in Figure 1, where no formation of biomass, ethanol, or glycerol can be observed, with only small alcohol dehydrogenase activity (Figure 2). This blocked metabolic activity is a direct consequence of the inability to reduce furfural and HMF under synergic conditions, since the control culture (without furaldehydes) grew normally, producing 7.79 g L^{-1} of biomass (Table I). For the parental strain, results show that HMF is depleted in 24 h of cultivation, while furfural concentration did not decrease in 48 h of cultivation (Figure 2a), and when both toxics were added, even the metabolism of HMF was repressed. This result is an evidence of the negative synergic effects of furaldehydes on WA-001 strain. Similar effects were observed for cultivations of *S. cerevisiae* CBS 8066, where the specific ethanol productivity rate was highly affected under the synergic combination of furaldehydes (Taherzadeh et al. 2000). *S. arborariae* NRRL Y-48658 also showed high sensitivity to toxics in synergic conditions (da Cunha-Pereira et al. 2011). Synergic effects can be

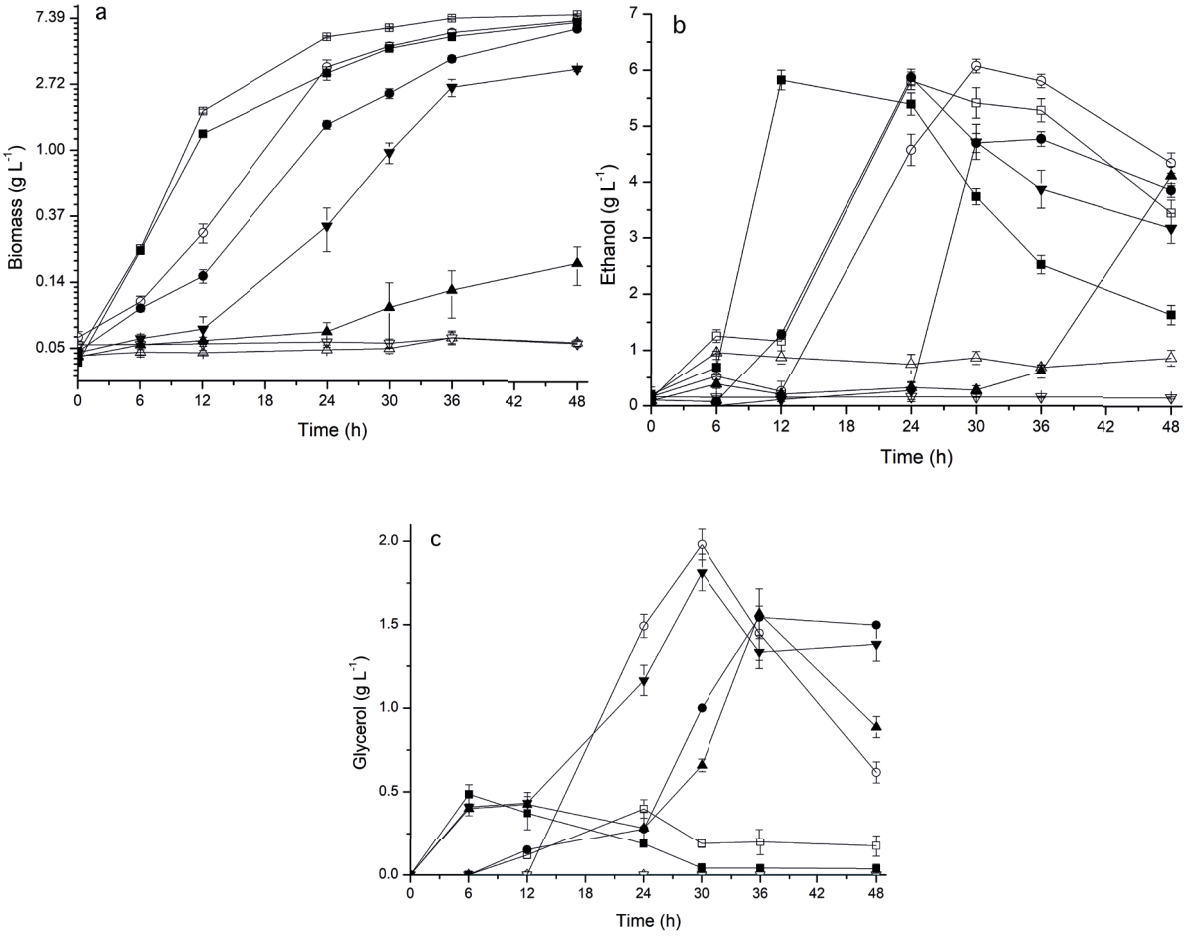

Figure 1. Physiological analysis of *W. anomalus* strains WA-001 (open symbols) and WA-HF5.5 (filled symbols) cultivated in YPD (□, ■), YPD containing 3 g L^{-1} HMF (○, ●), YPD containing 3 g L^{-1} furfural (△, ▲), or YPD containing 1.5 g L^{-1} of both furaldehydes (▽, ▼). Biomass formation (a), ethanol (b) and glycerol concentrations (c). Results represent the mean of triplicate.

related to the immediate furfural effects on the glycolytic and TCA pathways, which are involved in the energy metabolism of yeasts. Furfural decreases activity of ADH and the glycolytic enzymes hexokinase and glyceraldehyde-3-phosphate dehydrogenase, also inducing reactive oxygen species to accumulate, vacuole, mitochondrial membranes, chromatin, and actin damage (Almeida et al. 2007). When HMF is also present, the low conversion rate of this compound also contributes to increase furfural toxicity of yeast cells (Taherzadeh et al. 2000).

Culture kinetics of resistant WA-HF5.5 strain showed the formation of biomass under all conditions of furaldehydes concentrations. Under synergic conditions, lag phase lasted 12 h, and biomass production was more affected in the presence of 3 g L^{-1} of furfural (Figure 1a). Interesting results were obtained concerning ethanol formation relative to the addition of furaldehydes to culture medium. Although final ethanol concentrations achieved for both strains under control and HMF conditions were similar, productivity was higher for the adapted strain under both conditions. Moreover, when growth was carried out in the presence of furfural, the parental strain lacked the ability to form ethanol, while strain WA-HF5.5 was able to produce

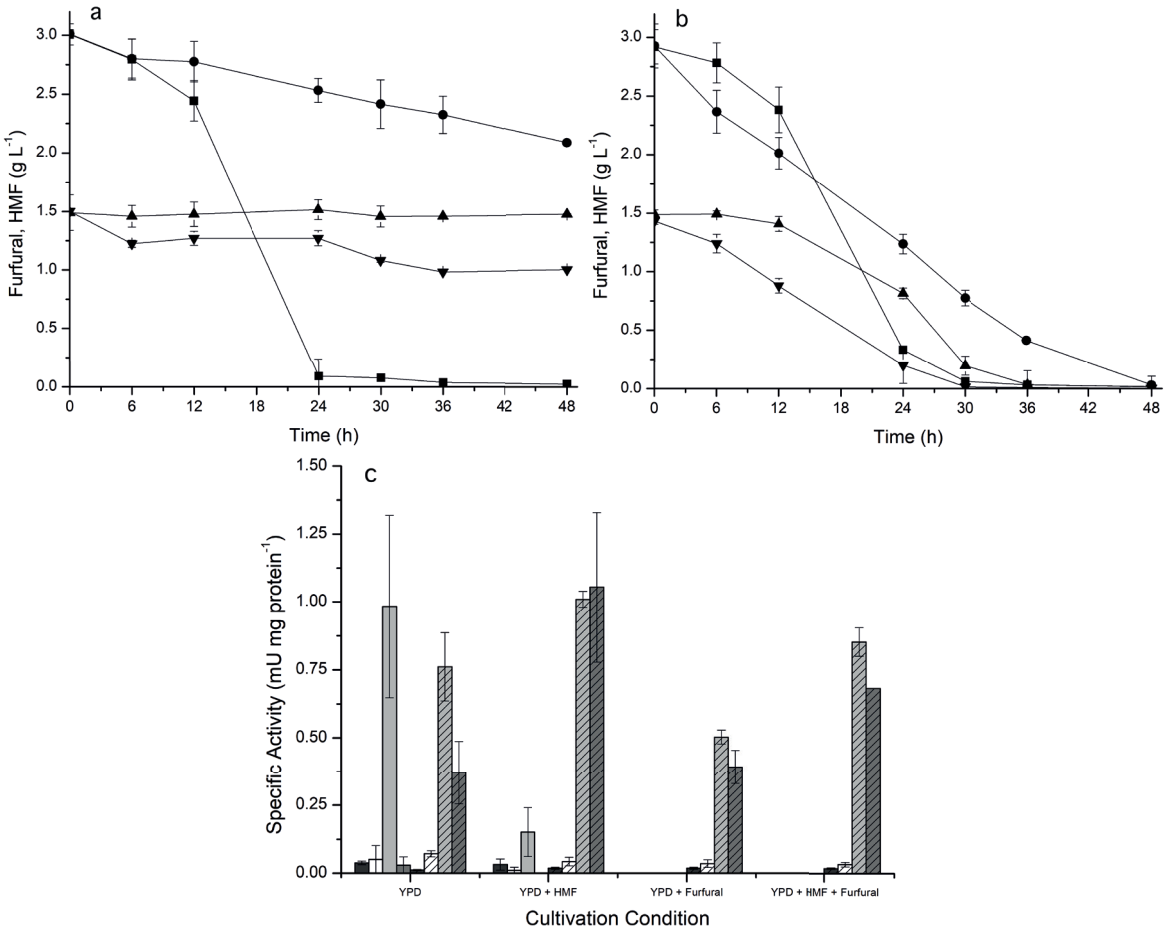

Figure 2. Furfural and HMF reduction during growth of *W. anomalus* strains WA-001 (a) and WA-HF5.5 (b). YPD containing 3 g L^{-1} HMF (■), YPD containing 3 g L^{-1} furfural (●), or YPD containing 1.5 g L^{-1}furfural (▼) plus HMF at 1.5 g L^{-1}(▲). Comparative analysis of NAD(P)H-dependent reduction activity (c) using as substrates: HMF and NADPH (dark gray bars); HMF and NADH (white bars), furfural and NADH (light gray bars), or HMF and furfural and NADH (dark gray bars) in 24 h of cultivation, solid bars, strain WA-001; hatched bars, strain WA-HF5.5. Results represent the mean of duplicate.

ethanol to reasonable amounts (Table I). These results show that evolutionary engineering was able to improve the ethanol production in strain WA-HF5.5, even when compared to the control cultivation (Figure 1b). Strain WA-HF5.5 showed a better metabolism when both furaldehydes were present in the medium than when only furfural was present (Figure 2b). For strain WA-HF5.5, furfural was reduced in an earlier stage than HMF, as shown in Figure 2b. HMF contains hydroxymethyl groups, being less hydrophobic, reducing cell membrane permeability, thus causing a low conversion rate of this compound (Taherzadeh et al. 2000).

In this study, the NADH-dependent reduction activity of furaldehydes was strongly related to the alcohol dehydrogenase activity, as can be seen in Figure 2c. The furfural reducing activity showed higher levels only for the control condition in the parental strain, indicating that it was sensitive to furaldehydes. The enzymatic activity on WA-HF5.5 strain showed different profiles. Higher levels of enzymatic activity were induced in YPD medium with HMF, followed by YPD added of both toxic compounds. These results show that the reducing enzymes are produced in higher levels in the presence of furaldehydes. Alcohol dehydrogenases (ADHs) are enzymes able to catalyze this reaction. This class of enzymes is well characterized for *S. cerevisiae*, constituting a large family of enzymes responsible for the reversible oxidation of alcohols to aldehydes, with the concomitant reduction of NAD^+ or $NADP^+$. Physiologically, the ADH reaction in *S. cerevisiae*, and in related yeast species, plays an important role in sugar metabolism because specific ADH isozyme serves to regenerate the glycolytic NAD^+, thereby restoring the redoxbalance through the reduction of acetaldehyde to ethanol (De Smidt et al. 2008).

Table I. Comparison of biomass formation, maximum specific growth rate (μ_{max}), glycerol concentration, ethanol concentration and yields ($Y_{P/S}$), and ethanol productivity (Q_p) for *W. anomalus* strains WA-001 and WA-HF5.5. Cultures in YPD medium containing 3 g L^{-1} HMF, 3 g L^{-1} furfural, or both furans (1.5 g $L^{-1}$of each). Results are the mean of triplicate.

	Strains											
Condition	**WA-001**	**WA-HF5.5**	**WA-001**	**WA-HF5.5**	**WA-001**	**WA-HF5.5**	**WA-001**	**WA-HF5.5**	**WA-001**	**WA-HF5.5**	**WA-001**	**WA-HF5.5**
	Biomass (g L^{-1})		μ_{max} (h^{-1})		Ethanol (g L^{-1})		$Y_{P/S}$ (g g^{-1})		Q_p(g L^{-1} h^{-1})		Glycerol (g L^{-1})	
Control	7.79 ± 0.10	6.93 ± 0.22	0.35	0.30	5.81 ± 0.15	5.82 ± 0.17	0.30	0.35	0.24	0.47	0.40 ± 0.05	0.48 ± 0.05
HMF 3 g L^{-1}	7.12 ± 0.26	6.29 ± 0.08	0.21	0.19	6.07 ± 0.12	5.87 ± 0.14	0.27	0.30	0.18	0.24	1.98 ± 0.09	1.54 ± 0.02
Furfural 3 g L^{-1}	0.05 ± 0.00	0.18 ± 0.05	0.00	0.06	0.51 ± 0.03	4.1 ± 0.17	0.00	0.22	0.00	0.08	0.00 ± 0.00	1.56 ± 0.14
Furfuraland HMF	0.00 ± 0.00	3.41 ± 0.12	0.00	0.19	0.00 ± 0.00	4.7 ± 0.31	0.00	0.43	0.00	0.16	0.00 ± 0.00	1.81 ± 0.11

Concerning *S. cerevisiae*, enzymes responsible for furaldehydes reducing activities have preference for NADH and NADPH cofactors (Liu 2011b). However, some enzymes have preference only for NADPH, such as Adh6p (Petersson et al. 2006), or NADH for Adh1p (Laadan et al. 2008). In the present work, it can be observed that HMF, when it is used as substrate, produces low activity with NADH or NADPH as cofactors (Figure 2c). Unlike HMF, furfural is the enzymatic substrate producing higher activity with reducing enzymes present in the crude extract, and similar results were obtained using the mixture of both furaldehydes as substrate. The results in this work suggest that for WA-HF5.5 enzymes catalyzing furaldehydes reduction appear to be more active for NADH. These results are consistent to *in vitro* and in *vivo* assays using several *S. cerevisiae* strains, suggesting that HMF and furfural reductions are coupled with NADPH and NADH oxidation, respectively (Almeida et al. 2007). HMF is mainly reduced by NADPH-dependent Adh6p and Adh7p (Petersson et al. 2006, Sehnem et al. 2013), whereas furfural reduction is catalyzed by NADH-dependent Ald4p, and Gre3p in *S. cerevisiae* (Liu et al. 2008).

Glycerol production was also increased in the presence of furaldehydes for WA-HF5.5, whereas for the parental strain glycerol production was increased only when HMF was added to cultures (Figure 1c). Under anaerobic conditions, glycerol is normally produced to regenerate excess NADH formed during biosynthetic processes (Palmqvist & Hahn-Hägerdal 2000). Production of glycerol is also associated with responses to several stress conditions. In *S. cerevisiae*, changes in internal metabolic fluxes that lead to the production of glycerol help to counter cell stress or adapt to stressful conditions such as osmotic pressure, high ethanol and CO_2 concentrations, among others (Pandey et al. 2007). Glycerol production in response to environmental stress was also reported for *W. anomalus*, as this yeast survives in media at low water activity resulting from increasing NaCl concentrations in the culture medium by producing compatible solutes, like glycerol, arabitol, and trehalose (Djelal et al. 2012).

Analysis of cell osmotic pressure tolerance

In order to access the effects of medium osmotic pressure on the parental and resistant strains, cells were exposed to varying concentrations of NaCl. This test is important because second-generation ethanol is based on high osmotic pressure media such as hydrolyzed lignocellulosic materials. Several yeast species have been studied in cultures under conditions of low water activity and high osmotic pressure media in our group, such as *S. cerevisiae*, *C. guilliermondii*, *S. arborariae*, and *C. shehatae* (Schirmer-Michel et al. 2008, da Cunha-Pereira et al. 2011, Hickert et al. 2013), and it would be interesting to know the response of *W. anomalus* to these conditions. Results of NaCl growing concentration exposition for both strains are shown in Table II. The parental strain showed sensibility to increasing osmotic pressures, showing total lack of growth at concentration of 1.5 M NaCl, whereas the WA-HF5.5 strainhad its specific growth rate hardly affected. Previous studies using *S. cerevisiae* have shown that the increase in medium osmotic pressure up to 20 % (volumetric fraction) of sorbitol decreased cell viability and growth, and fermentation performance (Pratt et al. 2003). In this work, the evolutionary engineering for furans tolerance was also positive towards improving osmotic pressure resistance of *W. anomalus*. One possible explanation for this acquired trait is that the analyses of gene expression in response to furfural and HMF in *S. cerevisiae* showed that the responses are distributed across a wide range of functional categories and pathways, which

Table II. Comparison between specific growth rate of *W. anomalus* strains WA-001, and WA-HF5.5, in hyperosmotic stress cultivation. Results represent the mean of duplicate.

Condition	Osmolarity (mM kg^{-1})	Specific Growth Rate μ (h^{-1})	
		WA-001	WA-HF5.5
YPD	209	0.31	0.28
YPD + 0.25 M NaCl	661	0.29	0.29
YPD + 0.50 M NaCl	1024	0.27	0.27
YPD + 0.75 M NaCl	1350	0.24	0.28
YPD + 1.00 M NaCl	1653	0.21	0.25
YPD + 1.25 M NaCl	1969	0.18	0.23
YPD + 1.50 M NaCl	2060	0.08	0.21

includes the stress-related high-osmolarity glycerol (HOG) pathway and heat shock protein genes (Liu 2011a). Probably, the furan inhibitors induce similar stress responses in *W. anomalus* compared to those previously observed for *S. cerevisiae*, where the HOG pathway is induced.

CONCLUSION

An osmotolerant *W. Anomalus* resistants train WA-HF5.5 was obtained by evolutionary engineering, possessing high HMF and furfural tolerance. The resistant strain showed better physiological performances than its parental strain in media containing furfural, HMF, or both, where concentrations of these furans were simulating lignocellulosic biomass hydrolysates. Moreover, the resistant strain was also able to tolerate high media osmotic pressures, which is another interesting trait for industrial applications. Results presented show that the evolutionary engineering that conferred HMF and furfural tolerance improved yeast fermentative capacity, and ethanol productivity, suggesting that this strain can be further studied in the research for second-generation ethanol production.

Acknowledgments

The authors wish to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação do Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) for their financial support (funding) for the project and scholarships.
Compliance with Ethical Standards & Conflict of interests: All authors of this work declare that they have no conflicts of interests.

REFERENCES

ALMEIDA JR, MODIG T, PETERSSON A, HÄHN-HÄGERDAL B, LIDÉN G & GORWA-GRAUSLUND MF. 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. J Chem Technol Biotechnol 82: 340-349.

ALMEIDA JRM, RÖDER A, MODIG T, LAADAN B, LIDÉN G & GORWA-GRAUSLUND M-F. 2008. NADH- vs NADPH-coupled reduction of 5-hydroxymethyl furfural (HMF) and its implications on product distribution in *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 78: 939-945.

CADIÈRE A, ORTIZ-JULIEN A, CAMARASA C & DEQUIN S. 2011. Evolutionary engineered *Saccharomyces cerevisiae* wine yeast strains with increased *in vivo* flux through the pentose phosphate pathway. Metabolic Eng 13: 263-271.

CORTIVO PRD, HICKERT LR, HECTOR R & AYUB MAZ. 2018. Fermentation of oat and soybean hull hydrolysates into ethanol and xylitol by recombinant industrial strains

of *Saccharomyces cerevisiae* under diverse oxygen environments. Ind Crops Prod 113: 10-18.

DA CUNHA-PEREIRA F, HICKERT LR, SEHNEM NT, DE SOUZA-CRUZ PB, ROSA CA & AYUB MAZ. 2011. Conversion of sugars present in rice hull hydrolysates into ethanol by *Spathaspora arborariae*, *Saccharomyces cerevisiae*, and their co-fermentations. Bioresour Technol 102: 4218-4225.

DE SMIDT O, DU PREEZ JC & ALBERTYN J. 2008. The alcohol dehydrogenases of *Saccharomyces cerevisiae*: a comprehensive review. FEMS Yeast Res 8: 967-978.

DJELAL H, LARHER F, MARTIN G & AMRANE A. 2012. Continuous culture for the bioproduction of glycerol and ethanol by *Hansenula anomala* growing under salt stress conditions. Ann Microbiol 62: 49-54.

GUARNIERI MT, ANN FRANDEN M, JOHNSON CW & BECKHAM GT. 2017. Conversion and assimilation of furfural and 5-(hydroxymethyl) furfural by *Pseudomonas putida* KT2440. Metab Eng 4: 22-28.

HASUNUMA T, OKAZAKI F, OKAI N, HARA KY, ISHII J & KONDO A. 2013. A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology. Bioresor Technol 135: 513-522.

HICKERT LR, CRUZ MM, DILLON AJP, FONTANA RC, ROSA CA & AYUB MAZ. 2014. Fermentation kinetics of acid–enzymatic soybean hull hydrolysate in immobilized-cell bioreactors of *Saccharomyces cerevisiae*, *Candida shehatae*, *Spathaspora arborariae*, and their co-cultivations. Biochem Eng J 88: 61-67.

HICKERT LR, DA CUNHA-PEREIRA F, DE SOUZA-CRUZ PB, ROSA CA & AYUB MAZ. 2013. Ethanogenic fermentation of co-cultures of *Candida shehatae* HM 52.2 and *Saccharomyces cerevisiae* ICV D254 in synthetic medium and rice hull hydrolysate. Bioresour Technol 131: 508-514.

KIM S-K, JIN Y-S, CHOI I-G, PARK Y-C & SEO J-H. 2015. Enhanced tolerance of *Saccharomyces cerevisiae* to multiple lignocellulose-derived inhibitors through modulation of spermidine contents. Metab Eng 29: 46-55.

KUYPER M, TOIRKENS MJ, DIDERICH JA, WINKLER AA, DIJKEN JP & PRONK JT. 2005. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. FEMS Yeast Res 5: 925-934.

LAADAN B, ALMEIDA JRM, RÅDSTRÖM P, HAHN-HÄGERDAL B & GORWA-GRAUSLUND M. 2008. Identification of an NADH-dependent 5-hydroxymethylfurfural-reducing alcohol dehydrogenase in *Saccharomyces cerevisiae*. Yeast 25: 191-198.

LENIHAN P, OROZCO A, O'NEILL E, AHMAD MNM, ROONEY DW & WALKER GM. 2010. Dilute acid hydrolysis of lignocellulosic biomass. Chem Eng J 156: 395-403.

LI Y-C, GOU Z-X, ZHANG Y, XIA Z-Y, TANG Y-Q & KIDA K. 2017. Inhibitor tolerance of a recombinant flocculating industrial *Saccharomyces cerevisiae* strain during glucose and xylose co-fermentation. Braz J Microbiol 48: 791-800.

LING H, TEO W, CHEN B, LEONG SSJ & CHANG MW. 2014. Microbial tolerance engineering toward biochemical production: from lignocellulose to products. Curr Opin Biotechnol 29: 99-106.

LIU ZL. 2011a. Genomics of yeast tolerance and in situ detoxification. In: Liu Z (Ed). Microbial stress tolerance for biofuels, Berlin: Springer, p. 1-28.

LIU ZL. 2011b. Molecular mechanisms of yeast tolerance and *in situ* detoxification of lignocellulose hydrolysates. App Microbiol Biotechnol 90: 809-825.

LIU ZL, MA M & SONG M. 2009. Evolutionarily engineered ethanologenic yeast detoxifies lignocellulosic biomass conversion inhibitors by reprogrammed pathways. Mol Genet Genomics 282: 233-244.

LIU ZL, MOON J, ANDERSH BJ, SLININGER PJ & WEBER S. 2008. Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxymethylfurfural by *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 81: 743-753.

LIU ZL, SLININGER PJ, DIEN BS, BERHOW MA, KURTZMAN CP & GORSICH SW. 2004. Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-bis-hydroxymethylfuran. J Ind Microbiol Biotechnol 31: 345-352.

LIU ZL, WANG X & WEBER SA. 2018. Tolerant industrial yeast *Saccharomyces cerevisiae* posses a more robust cell wall integrity signaling pathway against 2-furaldehyde and 5-(hydroxymethyl)-2-furaldehyde. J Biotechnol 276-277: 15-24.

LUO C, BRINK DL & BLANCH HW. 2002. Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol. Biomass & Bioenergy 22: 125-138.

MANSILLA HD, BAEZA J, URZÚA S, MATURANA G, VILLASEÑOR J & DURÁN N. 1998. Acid-catalysed hydrolysis of rice hull: Evaluation of furfural production. Bioresour Technol 66: 189-193.

MARGEOT A, HAHN-HAGERDAL B, EDLUND M, SLADE R & MONOT F. 2009. New improvements for lignocellulosic ethanol. Curr Op Biotechnol 20: 372-380.

MODIG T, LIDÉN G & TAHERZADEH MJ. 2002. Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. Biochem J 363: 769-776.

PALMQVIST E & HAHN-HÄGERDAL B. 2000. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour Technol 74: 25-33.

PANDEY G, YOSHIKAWA K, HIRASAWA T, NAGAHISA K, KATAKURA Y, FURUSAWA C, SHIMIZU H & SHIOYA S. 2007. Extracting the hidden features in saline osmotic tolerance in *Saccharomyces cerevisiae* from DNA microarray data using the self-organizing map: biosynthesis of amino acids. Appl Microbiol Biotechnol 75: 415-426.

PASSOTH V, OLSTORPE M & SCHNÜRER J. 2011. Past, present and future research directions with *Pichia anomala*. Ant Van Leeuw 99: 121-125.

PETERSSON A, ALMEIDA JRM, MODIG T, KARHUMAA K, HAHN-HÄGERDAL B, GORWA-GRAUSLUND MF & LIDÉN G. 2006. A 5-hydroxymethyl furfural reducing enzyme encoded by the *Saccharomyces cerevisiae* ADH6 gene conveys HMF tolerance. Yeast 23: 455-464.

PRATT PL, BRYCE JH & STEWART GG. 2003. The effects of osmotic pressure and ethanol on yeast viability and morphology. J Inst Brew 109: 218-228.

SCHIRMER-MICHEL ÂC, FLÔRES SH, HERTZ PF, MATOS GS & AYUB MAZ. 2008. Production of ethanol from soybean hull hydrolysate by osmotolerant *Candida guilliermondii* NRRL Y-2075. Bioresour Technol 99: 2898-2904.

SEHNEM NT, DA SILVA MACHADO A, LEITE FCB, DE BARROS PITA W, DE MORAIS MA & AYUB MAZ. 2013. 5-Hydroxymethylfurfural induces ADH7 and ARI1 expression in tolerant industrial *Saccharomyces cerevisiae* strain P6H9 during bioethanol production. Bioresour Technol 133: 190-196.

SEHNEM NT, HICKERT LR, DA CUNHA-PEREIRA F, DE MORAIS MA & AYUB MAZ. 2017. Bioconversion of soybean and rice hull hydrolysates into ethanol and xylitol by furaldehyde-tolerant strains of *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, and their cofermentations. Biomass Convers Bior 7: 199-206.

TAHERZADEH M, GUSTAFSSON L, NIKLASSON C & LIDEN G. 2000. Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. App Microbiol Biotechnol 53: 701-708.

TAO N, GAO Y & LIU Y. 2011. Isolation and characterization of a *Pichia anomala* strain: a promising candidate for bioethanol production. Braz J Microbiol 42: 668-675.

WALKER GM. 2011. *Pichia anomala:* cell physiology and biotechnology relative to other yeasts. Anton Van Leeuw 99: 25-34.

WISSELINK HW, TOIRKENS MJ, DEL ROSARIO FRANCO BERRIEL M, WINKLER AA, VAN DIJKEN JP, PRONK JT & VAN MARIS AJA. 2007. Engineering of *Saccharomyces cerevisiae* for Efficient Anaerobic Alcoholic Fermentation of l-Arabinose. Appl Env Microbiol 73: 4881-4891.

YADAV KS, NASEERUDDIN S, PRASHANTHI GS, SATEESH L & RAO LV. 2011. Bioethanol fermentation of concentrated rice straw hydrolysate using co-culture of *Saccharomyces cerevisiae* and *Pichia stipitis*. Bioresour Technol 102: 6473-6478.

ZHAO L, ZHANG X & TAN T. 2008. Influence of various glucose/xylose mixtures on ethanol production by *Pachysolen tannophilus*. Biomass & Bioenergy 32: 1156-1161.

ZHAO XQ & BAI FW. 2009. Mechanisms of yeast stress tolerance and its manipulation for efficient fuel ethanol production. J Biotechnol 144: 23-30.

How to cite

SHNEM NT, MACHADO AS, MATTE CR, MORAIS JR MA & AYUB MAS. 2020. Second-generation ethanol production by *Wickerhamomyces anomalus* strain adapted to furfural, 5-hydroxymethylfurfural (HMF), and high osmotic pressure. An Acad Bras Cienc 92: e20181030. DOI 10.1590/0001-3765202020181030.

Manuscript received on September 3, 2018;
accepted for publication on April 8, 2019

NICOLE T. SEHNEM[1]
https://orcid.org/0000-0001-6195-1529

ÂNGELA S. MACHADO[1]
https://orcid.org/0000-0003-2948-8567

CARLA R. MATTE[1]
https://orcid.org/0000-0003-1739-6729

MARCOS ANTONIO DE MORAIS JR[2]
https://orcid.org/0000-0002-9727-0300

MARCO ANTÔNIO Z. AYUB[1]
https://orcid.org/0000-0003-4410-6336

[1]Universidade Federal do Rio Grande do Sul, Departamento de Tecnologia de Alimentos, Av. Bento Gonçalves, 9500, Campus do Vale, 91501-970 Porto Alegre, RS, Brazil

[2]Universidade Federal de Pernambuco, Departamento de Genética, Av. Moraes Rego, 1235, Cidade Universitária, 50670901 Recife, PE, Brazil

Correspondence to: **Marco Antônio Záchia Ayub**
E-mail: mazayub@ufrgs.br

Author contributions

Nicole Teixeira Sehnem: Investigation, Conceptualization, Methodology, Writing original draft. Ângela da Silva Machado: Investigation, Conceptualization, Methodology. Carla Roberta Matte: Methodology, Writing original draft. Marcos Antonio de Morais Jr: Resources, Conceptualization. Marco Antônio Záchia Ayub: Resources, Conceptualization, Methodology, Writing - Review & Editing, Funding acquisition, Project administration, Supervision.