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PHYSIOLOGICAL RESPONSE TO FURAN DERIVATIVES STRESS BY *Kluyveromyces marxianus* SLP1 IN ETHANOL PRODUCTION

RESPUESTA FISIOLÓGICA AL ESTRÉS POR DERIVADOS DE FURANO EN *Kluyveromyces marxianus* SLP1 EN LA PRODUCCIÓN DE ETANOL

G. Flores-Cosio, M. Arellano-Plaza, A. Gschaedler, L. Amaya-Delgado*
Industrial Biotechnology, CIATEJ, Av. Normalistas 800, Guadalajara 44270, México.
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Abstract

The yeasts used in the production of second generation ethanol are affected by inhibitory compounds as 5-hydroxymethylfurfural (HMF) and furfural that are releasing during the hydrolysis step; these compounds affect the fermentative capacities of the yeast. To find new yeast strains with outstanding capacities to be used in the production of second generation ethanol; in this study, we evaluated the physiological response to furan derivatives stress by native yeast *Kluyveromyces marxianus* (SLP1), and compared it with the commercial yeast *Saccharomyces cerevisiae* ethanol red (ERD). We used a chemically defined medium added with HMF and furfural at different concentrations; a control condition without inhibitors, and four stressing conditions, HMF 7 gL^{-1}, furfural 3gL^{-1}, HMF 3.5 gL^{-1} with furfural 1.5 gL^{-1}, and HMF 7 gL^{-1} with furfural 3 gL^{-1}. *K. marxianus* exhibited a greater capacity to assimilate the inhibitory compounds in less time than *S. cerevisiae* ERD; also, *K. marxianus* SLP1 strain showed better behavior to produce ethanol on inhibitory conditions. Despite the effects provoked by the inhibitory compounds, the yeasts could produce ethanol over 80% of conversion. In conclusion, the yeast *K. marxianus* SLP1 can be an option to produce second generation ethanol at industrial level.
Keywords: physiological response, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, HMF, furfural, ethanol.

Resumen

Las levaduras utilizadas en la producción de etanol de segunda generación están afectadas por compuestos inhibidores como el 5-hidroximetilfurfural (HMF) y el furfural que se liberan durante la etapa de hidrólisis; estos compuestos afectan la capacidad fermentativa de la levadura. Para encontrar nuevas levaduras con una capacidad excepcional para ser utilizadas en la producción de etanol de segunda generación; en este estudio se evaluó la respuesta fisiológica al estrés por derivados de furano en la levadura nativa *Kluyveromyces marxianus* (SLP1), y se comparó con la levadura comercial *Saccharomyces cerevisiae* etanol red (ERD). Utilizamos un medio químicamente definido añadido con HMF y furfural a diferentes concentraciones; una condición de control sin inhibidores, y cuatro condiciones de estrés, HMF 7 gL^{-1}, furfural 3gL^{-1}, HMF 3,5 gL^{-1} con furfural 1,5 gL^{-1} y HMF 7 gL^{-1} con furfural 3 gL^{-1}. *K. marxianus* exhibió una mayor capacidad para asimilar los compuestos inhibidores en menor tiempo que *S. cerevisiae* ERD; también, *K. marxianus* SLP1 mostró un mejor comportamiento para producir etanol en condiciones de inhibición. A pesar de los efectos provocados por los compuestos inhibidores, las levaduras podrían producir etanol en un 80% de conversión. En conclusión, la levadura *K. marxianus* SLP1 puede ser una opción para producir etanol de segunda generación a nivel industrial.
Palabras clave: *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, HMF, furfural, etanol.

1 Introduction

Bioethanol is a source of renewable energy, and one of the alternatives to oil. Currently, most of the bioethanol is produced from the fermentation sugars in feedstocks such as sugar cane, sorghum, maize, wheat and constitute what is known as first generation biofuel (Taherzadeh and Karimi, 2007). Because first generation biofuels come from feedstocks directly related to human or animal feed and are considered not ethic, have led to the research of second generation biofuels which come from raw materials that are not food sources such as lignocellulosic material. All biomass residues

* *Corresponding author. E-mail*: lamaya@ciatej.mx
Tel.: +52-(33)-3345-5200 (ext. 1330)
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produced in agricultural and industrial activities, and even urban waste, have high concentrations of exploitable lignocellulosic materials. Regardless of the biomass used to produce bioethanol as fuel, the main objective is the substitution of petroleum derivatives, which allows to reduce the dependence of these fossil resources and to mitigate greenhouse gas (GHG) emissions. The main steps that are involved in the production of ethanol from lignocellulosic material are pretreatment, hydrolysis, and fermentation. The pretreatment of lignocellulosic biomass favors the release of monomeric sugars in the hydrolysis step, however, the high temperatures and acidic conditions in which the pretreatments are carried out causes the generation of compounds that are released into the medium during the hydrolysis and can strongly inhibit the fermentation stage. The three top groups of inhibitors formed are furan derivatives (HMF, furfural), organic acids, and (3) phenols (Almeida *et al*., 2007; Liu *et al*., 2008). HMF and furfural are derived from degradation of hexoses and pentoses, respectively (Lewkowski, 2001) and are considered to be the most potent and representative inhibitors of the yeast growth and fermentation. Those compounds reduce enzymatic and biological activities, damage DNA and membranes, and produce inhibition on protein and RNA synthesis (Lin *et al*., 2009; Modig *et al*., 2002; Allen *et al*., 2010). The stress caused by furans is reflected in a decrease of volumetric ethanol yield and productivity (Taherzadeh *et al*., 2000a). Some authors have been reported that the *S. cerevisiae* and *K. marxianus* strains has a prolonged lag phase during batch growth in the presence of aldehyde inhibitors such as furfural and HMF (Almeida *et al*., 2011; Ma and Liu, 2010). Furfural has been shown to inhibit the growth of *S. cerevisiae* at concentrations in the range of 0.5-2 g L^{-1} (Rumbold *et al*., 2009). HMF has been reported with negative effects on growth rate and fermentation rate (<1.0 g L^{-1} has been reported with negative effects on growth rate and fermentation rate (Taherzadeh *et al*., 2000b). The effects of HMF on ethanol production by *S. cerevisiae* have been deeply investigated in several studies (Laadan *et al*., 2009; Taherzadeh *et al*., 2000b). *S. cerevisiae* is the most used and preferred yeast in the industry, it can efficiently utilize hexose sugars as a carbon source but cannot use pentose sugars (such xylose) to produce ethanol. Xylose is the predominant sugar derived from hemicelluloses. The inability of the yeast to utilize xylose has limited its use in bioethanol applications. This has led the search and development of new yeast strains that can tolerate inhibitor and can assimilate hexoses and pentoses. Yeast that can efficiently utilize heterogeneous sugars and withstand stress conditions in the bioethanol process is key for lignocellulosic biomass conversion to ethanol. The yeast *K. marxianus* has advantageous potentials for application in ethanol production because can assimilate diverse sugars including xylose, arabinose, sucrose, raffinose, and inulin in addition to several hexoses (Lara-Hidalgo *et al*., 2017; Lertwattanasakul *et al*., 2011; Martínez-Corona *et al*., 2015; Pérez *et al*., 2013). Specifically, the ethanolic yeast *K. marxianus* SLP1 is a thermotolerant yeast isolated from mezcal process, and it can metabolize several inhibitors to cell growth as saponins, furan derivatives, and phenolics (Alcázar *et al*., 2017; Arellano-Plaza *et al*., 2013). Additionally, *K. marxianus* yeast can produce high-value compounds as esters, alcohols, carbonyls during alcoholic fermentation (Amaya-Delgado *et al*., 2013; Pérez *et al*., 2013). Therefore, the objective of this study was to study the stress response to HMF and furfural on *K. marxianus* (SLP1) during growth and fermentation, using a defined medium under controlled conditions. Yeast strains with improved tolerance to inhibitors, especially to furans, is a promising alternative to avoid detoxification steps to ferment lignocellulosic hydrolyze to obtain bioethanol.

2 Materials and methods

2.1 Strain, medium, cultivation condition

The commercial strain *Saccharomyces cerevisiae* (ERD) and *Kluyveromyces marxianus* strain (SLP1) were used in this study. SLP1 is part of the collection of CIATEJ strains and was isolated from the process of mezcal production in San Luis Potosi, Mexico. The strains were stored on YPD agar medium at 4 ºC. Pre-inoculum was prepared in YPD medium and incubated at 30°C, pH 4.5 at 250 rpm for 12 hours. After this time, the cells were recovered by centrifugation (13,000 rpm for 10 min.) and resuspended in sterile physiological solution (NaCl 0.8%) to wash and obtain a suspension of the cell (without traces of the previous medium), which was used as inoculum.

2.2 Treatments with inhibitory compounds

To evaluate the physiological parameters of yeasts, the kinetics experiments with inhibitors were carried out

in mineral medium (250 ml Erlenmeyer flasks) and inoculated with 10×10^6 cel mL^{-1} (pre-inoculum). The flasks were incubated in a rotary shaker at 30 °C, pH 4.5. For aerobic conditions, 250 rpm were used and 100 rpm for anaerobic conditions. Mineral medium was composed of 1.49 g L^{-1} Na_2HPO_4, $2H_2O$; 3 g L^{-1} K_2HPO_4; 3 g L^{-1} (NH_4), $2SO_4$, 1 g L^{-1} glutamic acid; glucose as only carbon source (20 g L^{-1} for aerobic conditions and 40 g L^{-1} for anaerobic conditions); oligoelements (1 mL L^{-1}) and vitamins (2.5 mL L^{-1}). The composition of oligoelements and vitamins is as follows: oligoelements (0.41 mg L^{-1} of $MgCl_2$, $6H_2O$; 0.0192 mg L^{-1} $ZnCl_2$; 0.0006115 mg L^{-1} of $CuCl_2$, $2H_2O$; 0.004449 mg L^{-1} of $MnCl_2$, $4H_2O$; 0.0005 mg L^{-1} of $CoCl_2$, $6H_2O$; 0.017365 mg L^{-1} of $CaCl_2$; 0.011661 mg L^{-1} of $FeCl_2$, $4H_2O$; 0.00036 mg L^{-1} of (NH_4), $6Mo_7O_{24}$, $4H_2O$; 0.003 mg L^{-1} of H_3BO_3) and vitamins (0.001 mg L^{-1} aminobenzoic acid; 0.125 mg L^{-1} mesoinositol; 0.005 nicotinic acid; 0.005 mg L^{-1} pantothenic acid; 0.005 mg L^{-1} pyridoxine; 0.005 mg L^{-1} thiamine HCl; 0.000012 mg L^{-1} biotin). Sterilized HMF and furfural were added to the medium after the inoculation at the final concentrations as shows following: 7 g L^{-1} HMF (stress condition 2); 3 g L^{-1} furfural (stress condition 3); 3.5 g L^{-1} HMF + 1.5 g L^{-1} furfural (stress condition 4) and, 7 g L^{-1} HMF + 3 g L^{-1} furfural 3 g L^{-1} (stress condition 5). Oligoelements, vitamins, and inhibitors were sterilized by filtration (0.20 μm). Physiological parameters such as specific growth rate (μ), substrate consumption rate (Rs) HMF consumption rate (Rsh) and furfural consumption rate (Rsf) were calculated in the exponential phase. Biomass yield on substrate (Yx/s), specific substrate consumption rate (qs), volumetric substrate uptake rate (Qs), ethanol yield on substrate (Yp/s), specific ethanol productivity (qp) and volumetric ethanol productivity (Qp) were calculated in the stationary phase.

2.3 *Analytical methods*

Glucose and organic acids were measured using an Agilent HPLC (1220 Infinity) equipped with a refractive index detector (IR) and a Bio-Rad Aminex HPX-87H column (300 mm x 7.8 mm, 9 μm). The column was maintained at 50 °C, and as phase mobile was used 5 mM H_2SO_4 at 0.5 mL min^{-1} during 30 min. Organic acids were quantified by wavelength UV detector at 210 nm. Furans (HMF and furfural) compounds were also determined by HPLC, using a UV detector (at 262 nm, 275.5 nm, 295.5 nm and 342.5 nm) and a Zorbax Eclipse Plus C18 column (4.6 mm × 250 mm, 5 μm) at room temperature. The mobile phase consisted of A) 2.5% formic acid in water B) 100% methanol. A gradient was performed for 55 min. from 0-48% B at a flow of 0.8 mL min^{-1}. Cell biomass, expressed as cell dry weight (CDW), was obtained from cell pellet in 5 mL of culture and dried in an oven at 60 °C to a constant weight.

The quantification of ethanol and volatiles was performed by gas chromatography (GS) on an Agilent chromatograph (model 7890B) with a flame ionization detector coupled to a Head-space (model 7697A). An HP Innowax column (60 m x 0.32 mm x 0.25 μm) was used with a pressure of 23.79 psi and a flow rate of 1.3 mL min^{-1} for a velocity of 24.50 cm sec^{-1}. The oven heating ramp started at 45 °C for 8 min and was then brought to 80 °C for 0 minutes at a rate of 2 °C min^{-1}, then the temperature was increased 5 ° C min^{-1} to 160 °C for 0 minutes to finally reach 220 °C at a rate of 25 °C min^{-1} for 4 min. The temperature of the detector was 250 °C; the gas flows were: Helium 40 mL min^{-1}, air 400 mL min^{-1} and Nitrogen 30 mL min^{-1}. The head-space was programmed under the following conditions: Temperature of the vial of 90 °C for 5 min, temperature of the loop of 110 °C, temperature of the transfer line 115 °C, time of equilibrium 5 min, time of injection 0.5 min, cycle time 60 min.

3 Results and discussion

3.1 *Physiological response in aerobic conditions*

In order to know the tolerance of *K. marxianus* SLP1 and *S. cerevisiae* ERD, various concentrations of furfural and HMF were tested to evaluate their effect on the physiological response of the yeasts in aerobic fermentation. The physiological response was related with the yeasts growth kinetic (lag phase, specific growth rate μ and biomass substrate yield coefficient Yx/s) and the sugar and furan derivatives assimilation rates (splain each Rs, Rsh, Rsf, qs, and Qs). In aerobic conditions controls for both strains reached a stationary state and consumed all glucose present in the media at around 8 hours (Figure 1 A-1, B-1). In the presence of inhibitors, SLP1 showed a lag growth phase; the same behavior presented *S. cerevisae* ERD which was used as control. However, both strains showed the ability to reduce the furans and deplete its toxicity only if the concentration of the inhibitors

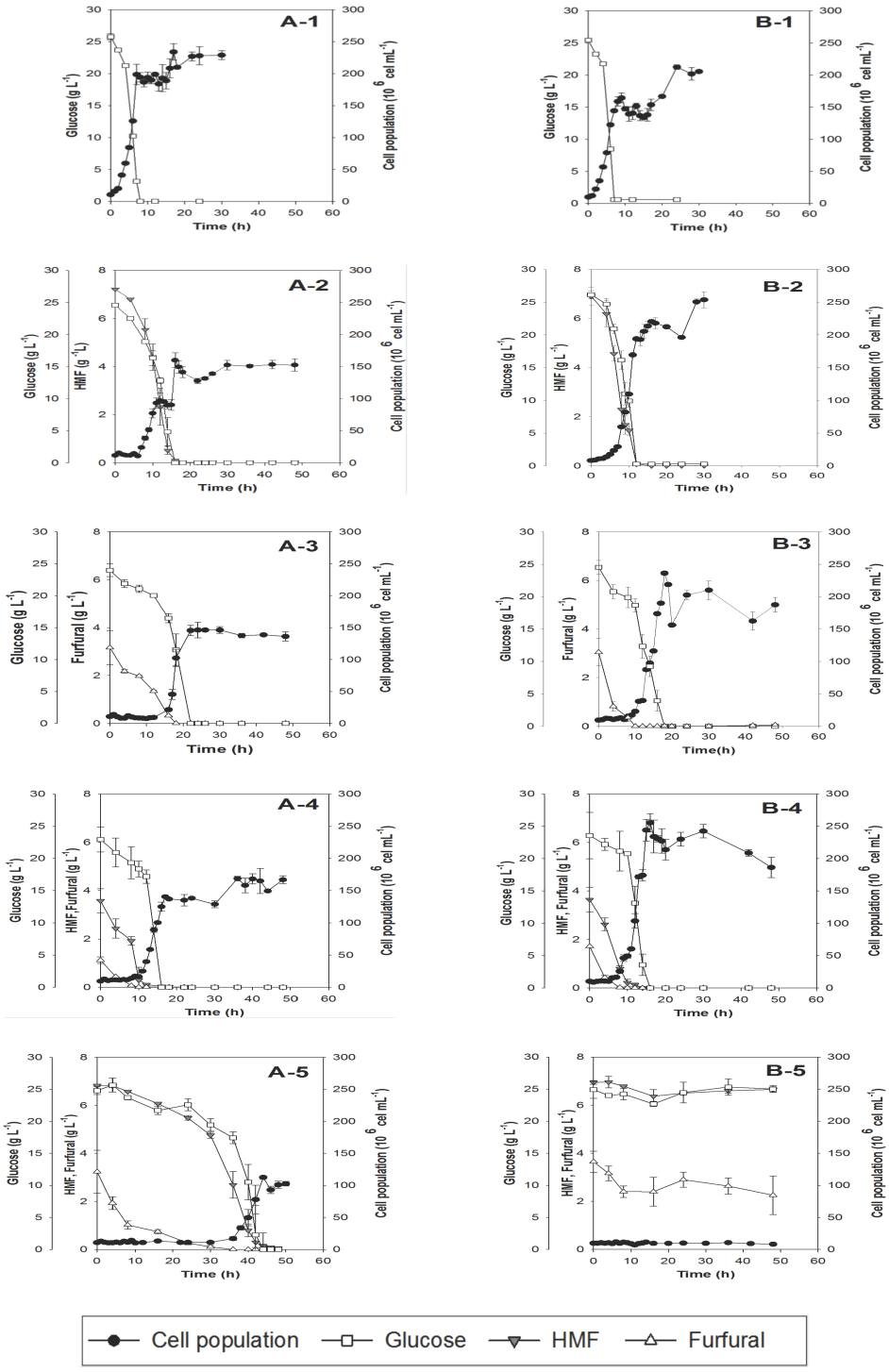

Fig. 1. Growth kinetics and glucose and furans consumption in mineral medium (250 rpm y 30 ºC). A: ERD (*S. cerevisiae*). B: SLP1 (*K. marxianus*). 1= control, 2= HMF (7 g L^{-1}), 3= Furfural (3 g L^{-1}), 4= HMF (3.5 g L^{-1}) + Furfural (1.5 g L^{-1}), 5= HMF (g L^{-1}) + Furfural (3 g L^{-1}).

Table 1. Physiological parameters of *Saccharomyces cerevisiae* ERD and *Kluyveromyces marxianus* SLP1 under different inhibitor conditions in mineral medium (at 250 rpm, 30 ºC, pH 4.5).

Stress condition	Lag phase	μ	Rs	Rsh	Rsf	Yx/s	qs	Qs
				ERD				
Control	0	0.38	5.10	-	-	0.32	1.18	1.07
HMF (7 g l^{-1})	6	0.20	3.25	0.46	-	0.16	1.26	1.14
Furfural (3 g l^{-1})	12	0.29	3.95	-	0.23	0.16	1.78	1.20
HMF (3.5 g l^{-1}) + Furfural (1.5 g l^{-1})	10	0.31	2.63	0.25	0.11	0.17	1.75	0.95
HMF (7 g l^{-1}) + Furfural (3 g l^{-1})	36	0.23	2.01	0.11	0.31	0.09	2.39	1.01
				SLP1				
Control	0	0.42	3.49	-	-	0.27	1.54	1.13
HMF (7 g l^{-1})	5	0.36	2.16	0.61	-	0.20	1.83	1.08
Furfural (3 g l^{-1})	10	0.30	2.38	-	0.30	0.14	2.09	1.05
HMF (3.5 g l^{-1}) + Furfural (1.5 g l^{-1})	8	0.31	2.05	0.34	0.21	0.15	2.02	0.98
HMF (7 g l^{-1}) + Furfural (3 g l^{-1})	-	-	-	-	-	-	-	-

Lag phase (h); μ specific growth rate (h^{-1}); Rs substrate consumption rate (g l^{-1} h^{-1}); Rsh HMF consumption rate (g l^{-1} h^{-1}); Rsf furfural consumption rate (g l^{-1} h^{-1}); Yx/s biomass substrate yield (g dry cell weight g substrate utilized-1); qs specific substrate consumption rate (g substrate consumed g dry cell weight^{-1} h^{-1}); Qs volumetric substrate uptake rate (g substrate consumed l^{-1} h^{-1}).

is tolerable for the yeasts. The lag phases for both strains were similar in each condition, except when was used both inhibitors at maximum concentrations. Once ERD and SLP1 detoxified the media, the cell growth was restored and could consume glucose. Although cell growth was restored after reduction of the inhibitors, but it was not possible to achieve the levels to a control culture (Figure 1).

In treatment using 7 g L^{-1} of HMF, it was observed that its reduction occurred in parallel to glucose consumption for the two strains. ERD showed a greater decrease in cell growth compared to control conditions (32.25% decrease) (Figure 1 A-2, B-2). Treatments using furfural at 3 g L^{-1} showed an extended lag growth phase compared with HMF. It is important to note that only when furfural is completely reduced from the medium, the cell growth is resumed, this demonstrated that furfural is more suppressive to cell growth (Figure 1 A-3, B-3). For both strains, the metabolism rate of furfural was lower than HMF (Table 1). Combined treatment of both inhibitors at intermediate concentrations showed a lower inhibitory effect on cell growth compared to the individual treatment of furfural. ERD and SLP1 had a cell growth inhibition of about 28% about the control conditions (Figure 1 A-4, B-4). When both inhibitors were applied in combination at maximum concentrations, the lag phase considerably was prolonged and an inhibition of cell growth were observed. Until 36 hours of growth, ERD reduced the inhibitors and started the cell growth. On the other hand, SLP1 was completely inhibited; no cell growth or inhibitor reduction was observed after 72 h (Figure 1 A-5, B-5).

Table 1 summarizes the physiological parameters of ERD and SLP1 in aerobic conditions. For both strains, the HMF consumption rate was higher than furfural consumption rate by a factor of approximately 2. However, SLP1 shown a better capacity to assimilate HMF and furfural, because it showed higher Rsh and Rsf than ERD.

The toxic effect of furans appears to be because aldehydes are chemically reactive compounds that have the potential to act as external electron receptors in metabolism and can form compounds with certain biological molecules such as lipids, proteins, and nucleic acids. Furfural and HMF are furan derivatives having a furan ring and an aldehyde functional group; Liu *et al*. (2008) mention that apparently, the aldehyde functional group in furfural and HMF is toxic to yeast but not the furan ring or associated alcohol functional groups. Any potential further reduction or degradation of the furan ring or alcohol groups may not play a significant role in the detoxification of furfural and HMF by the yeast.

The inhibition behavior in the cell growth in the presence of furans (HMF, furfural) has already been observed previously. Some authors have already reported that concentrations of furan and phenolic compounds from 0.3 g L^{-1} causes adverse effects and inhibition of growth during the fermentation process

(LU *et al*., 2007). Previous studies by Huang *et al*. (2011) indicate that a concentration above 4 g L^{-1} of furans (HMF, furfural) and acids (acetic, formic) are already critical for the growth and inhibition of ethanol production in *S. cerevisiae*, however that depends on the yeast strain. Hawkins & Doran-Peterson (2011) worked with several *Saccharomyces* yeasts strains and found complete growth inhibition at 5.76 g L^{-1} of furfural and partial inhibition at 2.88 g L^{-1}; using HMF they observed various degrees of partial inhibition at a concentration of 3.8 g L^{-1}, and a complete or partial inhibition at 7.6 g L^{-1} depending on the strain. Liu *et al*. (2004) made several experiments at different concentrations of HMF and furfural with *S. cerevisiae* (ATCC 211239); Allen *et al*. (2010) also found a lag phase in growth in *S. cerevisiae* using furfural at 2.4 g L^{-1} (25 mM) they observed a lag phase of 24 hours, whereas at 4.8 g L^{-1} (50 mM) no cell growth was observed. Yang & Tian (2013) reported a lag phase of 8 hours at 0.96 g L^{-1} (10 mM) furfural for *P. stipites*, and also at 2.88 g L^{-1} (30 mM) furfural the lag phase extended to 24 hours and under exposure to 3.84 g L^{-1} (40 mM) furfural they did not observe a substantial cell growth.

We observed that tolerable concentrations of inhibitors allowed yeasts to recover despite from a lag phase during the initial part of the incubation. In several investigations, have already determined that yeasts be able to convert HMF and furfural into less toxic compounds, furan-2,5-dimethanol (FDM) and furanmethanol (FM) respectively (Liu *et al*., 2005, 2004; Z. L. Liu *et al*., 2008; Liu and Slininger, 2006). These researches have evidence that reduction of HMF and/or furfural coincided with the formation of FDM/FM and therefore with the restore of the cell growth, suggesting that reduction of furans in their respective alcohols is a primary mechanism of the tolerance for yeast strains. Our results agree with the previous observations, indicating that reduction of HMF and furfural to less toxic compounds is an important aspect of the survival of yeasts.

This behavior is mainly accomplished via the activity of functional reductase and numerous enzymes possessing NADH and NADPH-dependent aldehyde reduction activities (alcohol dehydrogenases). Previous studies have found that the presence of furans causes a shortage of NADH (Liu *et al*., 2008). The reduction of furfural and HMF competes for cofactor NADH and interferes with cell glycolysis during regeneration of NAD^+. In normal cell growth, NAD^+ needs to be regenerated from NADH to enable the functioning of glycolysis. When furfural and or HMF are at higher concentrations, they can gain the competition for NADH. As a result, the glycolysis is delayed, and acetaldehyde is accumulated (which causes a delay of acetate and ethanol production). Glycolysis and pentose phosphate pathway are the main routes for glucose metabolisms that provide energy and important intermediate metabolites for biosynthesis and ethanol production. Essential enzymes of glycolysis are inhibited by furans affecting the glucose consumption. Since cofactors are involved in biosynthesis pathways, their simultaneous competition during the reduction of inhibitors adds extra stress to cell growth and maintenance. This can also disturb and delay the metabolic process and cause redox imbalance. Furans decrease amount of free available energy since their detoxification generates a redirection of yeast energy to repair the damage caused, these high demands of energy cannot be used for growth and glucose consumption.

In the presence of furans, *K. marxianus* SLP1 showed a better stress resistance. In comparison to ERD, SLP1 showed a slight decrease in the cellular growth and higher reduction rates of inhibitors. One of the reasons for these differences can be attributed to the own characteristics of the species and the origin isolation of the strains (suggesting that could have a greater expression of some functional proteins or enzymes that help it against stress caused by furans). The SLP1 strain was isolated from the fermentation process of mezcal, which is carried out under hostile environmental conditions (high concentrations of initial sugars, high or low temperatures and the presence of growth inhibitors such as saponins). Being in stress condition, the yeasts responds quickly synthesizing molecules that allow it to attenuate or repair the damage caused by stress, these adaptations are multiple and involve adaptation at genetic, physiological and molecular levels. Studies on the effect of saponins in *S. cerevisiae* and *K. marxianus* carried out by Alcázar *et al*. (2017) demonstrated that saponin exhibited a stronger growth inhibitory effect in both yeast strains; however, in *K. marxianus* growth recovery was observed, because this strain showed saponinase activity that performed the saponin hydrolysis (contributing to the reduction of the inhibitory effect of saponin extracts). Some strains can be adapted to the metabolites present in their source of isolation (inhibitory compounds) or possesses enzymes that can hydrolyze these, this characteristic may be an important point in the inhibition response to cellular growth found in SLP1

in the presence of furans. Another point about the difference found between ERD and SLP1 may be related to the composition of its cell wall and cell membrane. Alcázar *et al*. (2017) also found changes in the cell wall composition in *S. cerevisiae* and *K. marxianus* in the presence of saponins. They observed that saponin extracts strongly affected the *S. cerevisiae* cell wall, due to that break down of their cell wall was observed 10 min after the addition of the saponin extract and at the same amount of time the structure of the *K. marxianus* cell wall solely presented slight invagination.

The adaptive response to stress requires the synthesis of new proteins, indicating that changes in gene expression are critical and fundamental. Liu, (2006) indicated that the yeast adaptation to furfural and HMF is a continued dynamic process involving multiple genes at the genome level. In transcriptional analysis research Gao *et al*. (2015) reported that in comparison with *S. cerevisiae*, *K. marxianus* Y179 had a very high transcriptional level of heat shot protein 26 (HSP), up to 53%. The HSP family is a family of chaperones that assist proteins to fold correctly and maintain activities under some strict environmental stresses. Gao *et al*. (2017) cloned the gene KmTPX1 from *K. marxianus* and overexpressed in *S. cerevisiae*. They proved that the overexpression of KmTPX1 gen regulates the intracellular levels of reactive oxygen species (ROS), which correspondingly increased the tolerance of *S. cerevisiae* to both oxidative stress and multiple lignocellulose derived inhibitors formic acid, acetic acid, and furfural). KmTPX1 gene belongs to a large and highly conserved peroxiredoxin family and is homologous to one of the five peroxiredoxins (Prxs) in *S. cerevisiae* (TSA1/TPX1). Tas1p has an antioxidant role which allows it to protects cells from DNA damage and cell death (Cui *et al*., 2015; Iraqui *et al*., 2009). It has been reported that Tas1p in *S. cerevisiae* participate in detoxification of reactive oxygen species (ROS) (Rhee, 2016).

3.2 Physiological response in anaerobic conditions

The effects of HMF and furfural on growth, ethanol fermentation in *S. cerevisiae* ERD and *K. marxianus* SLP1 were further examined in anaerobic conditions. Under anaerobic conditions, the lag phase times for both strains were lower in comparison with the aerobic conditions, except for SLP1 at 7 g L^{-1} which presented twice of the adaptation time (Figure 2). When cell growth started after furans inhibition the ethanol production and other metabolites (glycerol, acetate, acetic acid and other volatile compounds like esters, and other alcohols) started. In treatment using HMF at 7 g L^{-1} SLP1 showed a lower inhibition of cell growth (20% of inhibition) respect ERD (67% of inhibition) (Figure 2 A-2, B-2) and a higher HMF consumption rate (Table 2). Compared with the control conditions, ERD showed a decrease in the ethanol substrate yield (Yp/s 9%) and a slight reduction in volumetric productivity (Qp). On the other hand, the ethanol yield for SLP1 increased by 7%, and their Qp slight increase (Table 2). It was also found that HMF increased the glycerol and acetic acid production (Table 3). In general, after reduction of the inhibitors acetic acid was detected in the medium. HMF also reduces the amyl alcohols production for SLP1. When furfural was used at 3 g L^{-1} ERD shown higher cell growth inhibition compared to SLP1; however, the volumetric productivity for ERD was not affected (Table 2). Furfural consumption rates were similar for both strains. Compared to HMF, furfural reduces the formation of glycerol. The concentration of acetaldehyde for ERD was higher by a factor of approximately 5. For ERD the accumulation of acetaldehyde was also observed in the other conditions of inhibitors; however, the highest concentration was found using furfural (Table 3). When inhibitors were combine at intermediate concentrations, SLP1 showed a greater reduction in cellular growth (51%) than with individual inhibitors; the inhibitors consumption rates also were reduced. The inhibition of cellular growth for ERD was like that found using furfural (47%). In this condition, SLP1 obtained the highest ethanol yield (22% increase). For volatile compounds, the ethyl acetate and 1-propanol production were not affected at tolerable concentrations of inhibitors in SLP1, while isobutanol was affected in all stress conditions. Using both inhibitors at maximum concentrations, the damages were lethal. No ethanol or other fermentation products could be detected. Also, at this condition, ERD and SLP1 shown a drastic decrease on their fermentative capacities and in the metabolites production.

The response of the yeasts to the presence of furans is a continuing dynamic process. In addition to the perturbations in cell growth, ethanol yield and volumetric productivity are other of the parameters that are also affected by furans (Table 3). In this study, slight changes in volumetric productivity were observed, but in other studies, have been reported greater effects. Sehnem *et al*. (2013a) found that *S. cerevisiae* in presence with HMF (at 5 g L^{-1}) shows a

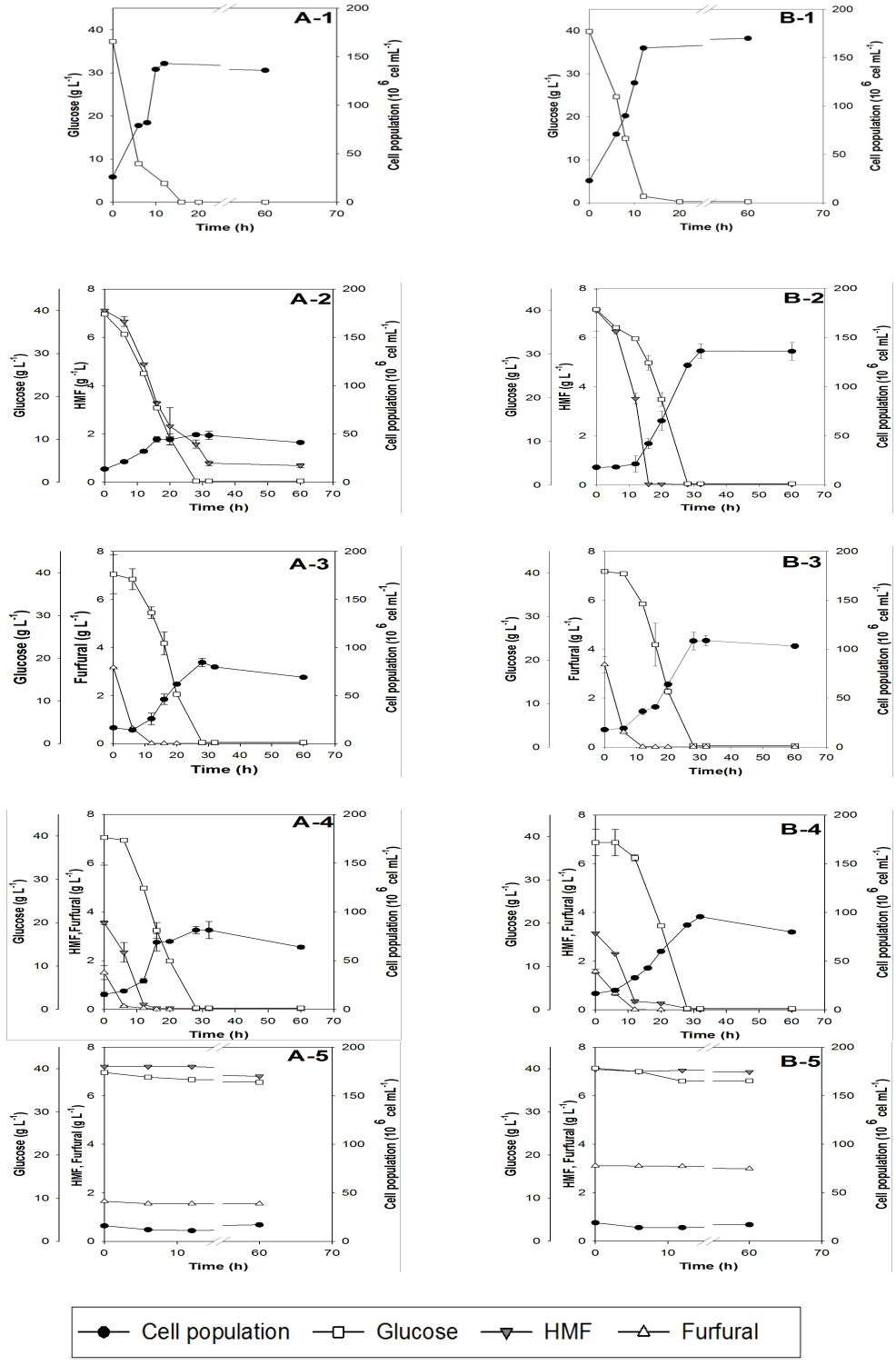

Fig. 2. Growth kinetics and glucose and furans consumption in mineral medium (100 rpm y 30 ºC). A: ERD (*S. cerevisiae*). B: SLP1 (*K. marxianus*). 1= control, 2= HMF (7 g L^{-1}), 3= Furfural (3 g L^{-1}), 4= HMF (3.5 g L^{-1}) + Furfural (1.5 g L^{-1}), 5= HMF (g L^{-1}) + Furfural (3 g L^{-1}).

Table 2. Physiological parameters of *Saccharomyces cerevisiae* ERD and *Kluyveromyces marxianus* SLP1 under different inhibitor conditions in mineral medium (at 100 rpm, 30 ºC, pH 4.5).

Stress condition	Lag phase	μ	Rs	Rsh	Rsf	Yx/s	qs	Qs	Yp/s	qp	Qp
					ERD						
Control	0	0.14	3.83	NA	NA	0.059	2.47	1.55	0.49	1.21	0.30
HMF (7 g l^{-1})	0	0.07	1.74	0.21	NA	0.043	1.67	1.62	0.45	0.75	0.29
Furfural (3 g l^{-1})	6	0.08	1.87	NA	0.26	0.047	1.76	1.65	0.46	0.82	0.30
HMF (3.5 g l^{-1}) + Furfural (1.5 g l^{-1})	6	0.12	1.52	0.27	0.22	0.045	2.78	1.65	0.46	1.29	0.30
HMF (7 g l^{-1}) + Furfural (3 g l^{-1})	-	-	-	-	-	-	-	-	-	-	-
					SLP1						
Control	0	0.16	3.20	NA	NA	0.053	3.03	1.66	0.40	1.22	0.26
HMF (7 g l^{-1})	12	0.10	1.32	0.43	NA	0.049	2.11	1.59	0.43	0.91	0.27
Furfural (3 g l^{-1})	6	0.07	1.75	NA	0.28	0.048	1.60	1.55	0.48	0.78	0.30
HMF (3.5 g l^{-1}) + Furfural (1.5 g l^{-1})	6	0.06	1.25	0.23	0.13	0.046	3.46	1.48	0.49	1.73	0.29
HMF (7 g l^{-1}) + Furfural (3 g l^{-1})	-	-	-	-	-	-	-	-	-	-	-

Lag phase (h); μ specific growth rate (h^{-1}); Rs substrate consumption rate (g l^{-1} h^{-1}); Rsh HMF consumption rate (g l^{-1} h^{-1}); Rsf Furfural consumption rate (g l^{-1} h^{-1}); Yx/s biomass substrate yield (g dry cell weight g substrate utilized^{-1}); qs specific substrate consumption rate (g substrate consumed g dry cell weight^{-1} h^{-1}); Qs volumetric substrate uptake rate (g substrate consumed l^{-1} h^{-1}); Yp/s ethanol yield on substrate (g ethanol produced g substrate utilized^{-1}); qp specific ethanol productivity (g ethanol produced g dry cell weight^{-1} h^{-1}); Qp volumetric ethanol productivity (g ethanol produced l^{-1} h^{-1}).

reduction in the yield of ethanol (Yp/s) and volumetric productivity (Qp) compared to control without HMF (obtaining about 10 % of Yp/s and Qp). Experiments performed by Tofighi *et al*. (2010) with different concentrations of furfural in *S. cerevisiae* observed a reduction of 4.8%, 3.7% and 1.4% in ethanol production at 4, 5 and 6 of g L^{-1}. The consequent increase in the concentration of inhibitors can cause a greater decrease in the productivity of ethanol. In *Pichia stipites*, Silva *et al*. (2016) reported a decrease in ethanol productivity with furfural and HMF. Assays carried out in hydrolysates (from lignocellulosic biomass) have also observed a decrease in volumetric productivity (Cheng *et al*., 2008; Tian *et al*., 2011).

The increases in ethanol yield observed in the presence of furans it has already been observed in other studies. Palmqvist *et al*. (1999) found that furfural decreased cell replication without a proportional effect on cell metabolism and had a double effect on the kinetics of glucose metabolism in *S. cerevisiae*, at a nonlethal concentration of furfural the glucose metabolism rate was inhibited but the final ethanol yield was slightly increased. Therefore, furfural reduction caused more glucose to be available for ethanol production. Also, by decreasing cell replication without inhibiting cell activity, furfural caused a larger proportion of glucose to be used for ethanol production. Horváth *et al*. (2003) observed an increase of 12% in the specific ethanol production rate and 9% in the ethanol yield using a *S. cerevisiae* when furfural was present in the medium at a 5.8 g L^{-1}. In researches carried out by LU *et al*. (2007) found that ethanol yields were not influenced apparently when furfural concentration was increased, and it has no significant differences in different furfural concentration treatment. Determinations made in *S. cerevisiae* with the addition of pulses of HMF (at 2 g L^{-1}) showed that HMF causes the ATP demand for biomass production to increase, thus giving a slightly increased ethanol yield. Also, the yields of acetate, pyruvate, and glycerol were affected (Taherzadeh *et al*., 2000b).

We also observed that under anaerobic conditions the production of glycerol was affected by the presence of furfural, several authors have already reported this behavior (Table 4). Taherzadeh *et al*. (2001) found a significant difference in the glycerol yield because of the addition of furfural. Lin *et al*, (2009) also observed that furfural severely inhibits glycerol formation. Ylitervo *et al*. (2013) reported that pulses addition of furfural in *S. cerevisiae* reduces glycerol production (especially when large amounts of furfural were added). Glycerol is produced by yeasts during fermentation of glucose to ethanol to maintain the redox balance. Yeast can be used as carbon and energy source and to protect against environmental factors (such as temperature, aeration, sugar concentration and osmotic stress). Under anaerobic conditions intracellular cofactor NADH is generated from reduction of NAD^+ in glycolysis and biosynthetic reactions such as amino acid synthesis. The reduction of acetaldehyde to ethanol maintain the redox balance is thereby regenerating NAD^+ (Albers *et al*., 1996).

Table 3. Metabolites generated by *S. cerevisiae* (ERD) and *K. marxianus* (SLP1) under different inhibitor conditions in anaerobic fermentation.

*Metabolites	*Saccharomyces cerevisiae* ERD Stress conditions				*Kluyveromyces marxianus* SLP1 Stress conditions			
	1	2	3	4	1	2	3	4
Organic acids (mg L^{-1})								
Malic acid	1.31 ± 0.00	1.54 ± 0.12	1.35 ± 0.02	1.48 ± 0.11	1.08 ± 0.00	2.56 ± 0.36	1.94 ± 0.00	2.42 ± 0.00
Succinic acid	0.26 ± 0.00	0.26 ± 0.13	0.26 ± 0.00	0.28 ± 0.00	0.26 ± 0.00	0.22 ± 0.04	0.18 ± 0.00	0.25 ± 0.00
Acetic acid	-	5.18 ± 0.21	1.46 ± 0.13	4.02 ± 0.24	-	8.88 ± 1.27	2.31 ± 0.24	4.50 ± 0.29
Propionic acid	1.14 ± 0.00	0.68 ± 0.01	1.24 ± 0.01	0.99 ± 0.03	1.28 ± 0.00	0.98 ± 0.17	1.00 ± 0.02	1.04 ± 0.03
Aldehydes (mg L^{-1})								
Acetaldehyde	14.40 ± 0.00	39.97 ± 2.70	62.72 ± 0.92	61.51 ± 3.03	14.89 ± 0.00	7.13 ± 0.14	11.37 ± 0.76	10.16 ± 0.19
Esters (mg L^{-1})								
Ethyl acetate	1.43 ± 0.00	0.96 ± 0.07	1.03 ± 0.03	1.03 ± 0.03	36.90 ± 0.00	73.50 ± 1.82	49.20 ± 1.34	75.59 ± 7.14
Alcohols (mg L^{-1})								
Ethanol	18220 ± 0.00	17600 ± 0.82	18590 ± 0.30	18440 ± 0.21	16040 ± 0.00	16410 ± 1.29	18200 ± 0.09	17780 ± 0.00
1-Propanol	15.59 ± 0.00	18.58 ± 1.46	13.91 ± 0.09	14.47 ± 0.03	19.61 ± 0.00	20.15 ± 0.16	20.87 ± 0.05	19.15± 0.04
Isobutanol	7.35 ± 0.00	15.26 ± 1.53	8.89 ± 0.16	8.37 ± 0.20	29.38 ± 0.00	14.16 ± 2.38	22.64 ± 0.97	18.79± 0.24
Amyl alcohols	22.43 ± 0.00	18.19 ± 1.74	26.78 ± 0.42	26.32 ± 0.20	43.01 ± 0.00	33.10 ± 5.4	53.26 ± 0.46	44.91± 0.22
Glycerol	3.09 ± 0.00	10.09 ± 0.16	2.14 ± 0.06	5.17 ± 0.26	3.28 ± 0.00	11.16 ± 2.00	2.37 ± 0.05	6.10 ± 0.24

*The quantification of metabolites was taken at 12 h for control condition (1); and at 32 h for stress conditions HMF 7 g L^{-1} (2), Furfural 3 g L^{-1} (3), HMF 3.5 g L^{-1} + Furfural 1.5 g L^{-1} (4), HMF 7 g L^{-1} + Furfural 3 g L^{-1} (5).

The NADH generated in biosynthetic pathways is reoxidized by the formation of glycerol (van Dijken and Scheffers., 1986). However, NADH is also required for reduction of furfural; therefore, his competition causes a reduction in the production of glycerol. NADH/NADPH are used in numerous metabolic processes, perturbations in the levels of these cofactors can have a big impact on metabolism. With HMF we observed a notable increased in glycerol production (up to 3 time more than in control strain). Petersson *et al*. (2006) found an increased glycerol yield (under anaerobic and aerobic conditions) in HMF containing media with recombinant *S. cerevisiae* strains. In later experiments with the same strains, Almeida *et al*. (2008) and Ishii *et al*. (2013) observed this behavior and attributed it to the regeneration of NAD^+ by HMF reduction. Sehnem *et al*. (2013b) also observed that glycerol production was greatly induced by HMF in industrial strain *S. cerevisiae* JP1 and the HMF-tolerant P6H9 strain.

High production of acetic acid was observed in stress conditions seems to be another compound associated with the reduction of the inhibitors (Table 4). Allen *et al*. (2010) observed an accumulation of acetic acid in *S. cerevisiae* during a stress condition by furfural and attributed their production to compensate the lack of cofactor (NADPH). Acetic acid can be produced from acetaldehyde and is catalyzed by aldehyde dehydrogenases (AlDHs). The oxidation of acetaldehyde generates NADH, which requires reoxidation to maintain the redox balance of the cell. The detoxification of inhibitors requires a high demand for cofactors (NADH/NADPH), so there is a need to regenerate NADH, in this case by oxidizing acetaldehyde. Therefore, acetic acid can be produced to regenerate reducing equivalents in the cytoplasm.

Conclusions

In the current study, we observed that the addition of furans to the cultures inhibited cell growth, glucose consumption and fermentative capacities of *S. cerevisiae* ERD and *K. marxianus* SLP1. The strains used resist higher concentrations of inhibitors compared to others previously reported. The most pronounced inhibition was observed when furfural and HMF were combined at high concentrations, this condition produced no cellular growth and, also metabolic activity was not detected in anaerobic fermentation. This demonstrated a clear dose dependent inhibition of yeasts to furfural and HMF and indicated that these inhibitors act negatively in a synergic way. An important aspect for yeast survival is the ability to convert the furans to less inhibitory compounds. The reduction of both HMF and furfural can be carried out aerobically and anaerobically. Since conversion rates were much lower for furfural and in a combination of furans (at intermediate and maximum concentration) it seems that their reduction depends on an active metabolism. The prolonged lag phase before the recovery of the cell growth suggests a major shift in the physiology of the cells. Glycerol and acetic acid were one of the main metabolites generated in stress with inhibitors. The different adaptive response between both strains shows that *K. marxianus* SLP1

has more effective mechanisms to withstand these furan compounds than *S. cerevisiae* ERD. This allowed it to have slightly better ethanol yield and volumetric ethanol productivity in the presence of furan derivatives, also SLP1 may be a promising strain in the industry due to the production of byproducts (like 1-propanol, isobutanol, ethyl acetate, amyl alcohols) despite being in high concentrations of inhibitors. The implementation of bio-refineries could be economically viable if the conversion of byproducts generated during biofuel production were considered. The search of new yeasts or adaptation of the strains with greater inhibitor tolerance is a promising alternative to obtain more efficient processes during conversion of lignocellulosic biomass to ethanol. The lack of understanding of mechanisms stresses tolerance in yeasts has made more difficult their study, therefore further research is needed to elucidate their adaptation mechanisms.

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