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ORIGINAL RESEARCH PAPER

Homo- and heterofermentative lactobacilli are distinctly affected by furanic compounds

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Abstract
Purpose Second generation (2G) ethanol is produced using lignocellulosic biomass. However, the pre-treatment processes generate a variety of molecules (furanic compounds, phenolic compounds, and organic acids) that act as inhibitors of microbial metabolism, and thus, reduce the efficiency of the fermentation step in this process. In this context, the present study aimed to investigate the effect of furanic compounds on the physiology of lactic acid bacteria (LAB) strains that are potential contaminants in ethanol production.
Methodology Homofermentative and heterofermentative strains of laboratory LAB, and isolated from first generation ethanol fermentation, were used. LAB strains were challenged to grow in the presence of furfural and 5-hydroxymethyl furfural (HMF).
Results We determined that the effect of HMF and furfural on the growth rate of LAB is dependent on the metabolic type, and the growth kinetics in the presence of these compounds is enhanced for heterofermentative LAB, whereas they are inhibitory to homofermentative LAB. Sugar consumption and product formation were also enhanced in the presence of furanic compounds for heterofermentative LAB, who displayed effective depletion kinetics when compared to the homofermentative LAB.
Conclusion Homo- and heterofermentative LAB are affected differently by furanic compounds, in a way that the latter type is more resistant to the toxic effects of these inhibitors. This knowledge is important to understand the potential effects of bacterial contamination in 2G bioprocesses.

Keywords 2G ethanol · Biofuels · Fermentation · Lactic acid bacteria · Lignocellulosic inhibitors · Yeast

Introduction

Recent global economic development has enhanced the demand for alternative energy resources worldwide. This increased demand is due to the known drawbacks of fossil fuels, such as their high price, unsustainable nature, and contribution to global warming. However, 2G biofuels are potential candidates for alternative energy resources, and are produced from cheap and abundant plant biomass residues (Mood et al. 2013), including corn straw, corn

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cobs, wheat straw, rice husks, and sugarcane straw and bagasse. Furthermore, bioethanol from lignocellulosic materials can avoid the shortcomings of first-generation (1G) biofuels, by not utilizing edible feedstock resources (Aditiya et al. 2016).

Lignocellulosic biomass generally contains over 70% of carbohydrates in the form of cellulose and hemicelluloses, which may serve as a substrate for ethanol production (Klinke et al. 2004). Cellulose is a polymer formed by glucose units, whereas hemicellulose is a polymer composed of various units of xylose, arabinose, mannose, galactose, and glucose, and varies in composition depending on the biomass (Bobleter 1994; Fan et al. 1982). Associated with these carbohydrates, depending on the raw material source, is lignin in varying proportions. Lignin is an amorphous polyphenolic compound with an undefined molecular weight, composed predominantly of p-coumaryl alcohol, coniferyl alcohol, and synaphyl alcohol (Rubin 2008). Despite the large carbohydrate content, there are many chemical and physical barriers in the lignocellulosic biomass that makes it difficult for cellulose and hemicellulose to be available, requiring a pre-treatment stage to make the sugars easily fermentable by yeasts in the ethanol production stage (Alvira et al. 2010).

The main objectives of the pre-treatment stage are to alter the lignin-hemicellulose-cellulose complex. The deconstruction of the complex reduces the crystallinity and increases its porosity and surface area, thus, making it more accessible for the enzymatic hydrolysis reaction (Cardona et al. 2010). These treatments can be physical, chemical, physicochemical, or biological. Most of these pre-treatments applied for bioethanol production, due to their severity, generate large amounts of inhibitory compounds. The nature and concentration of these inhibitors are greatly affected by the adopted process and operating conditions, including, temperature, time, pressure, pH, and presence of catalysts (Klinke et al. 2004; van Maris et al. 2006). The inhibitors can be divided into 3 groups: furanic compounds, phenolic compounds, and organic acids. These compounds can severely affect the growth of microorganisms through DNA mutations, membrane disruption, intracellular pH decrease, among others (Chandel et al. 2013).

Furanic compounds (aldehyde inhibitors) are mainly formed during pre-treatments involving extremely acidic conditions, due to the degradation of pentoses (2-furfural) and hexoses (HMF) (Liu 2011; Dunlop 1948). The concentration of these aldehydes in lignocellulosic hydrolysates can vary from 0.1 to 11 g furfural l^{-1}, and from 0.1 to 8.6 g HMF l^{-1} (Vanmarcke et al. 2021). Aldehydes are chemically reactive and can form products with many biological molecule classes. Several potential mechanisms regarding the toxicity of aldehydes have been explored, including damage from chemical reactivity, direct inhibition of glycolysis and fermentation, and plasma membrane damage (Zaldivar et al. 1999). This class of inhibitors has been found to inactivate cell replication, therefore, reducing growth rate, biomass yield on ATP, and specific productivities.

Another common problem that may be present in industrial 2G ethanol production is contamination by lactic acid bacteria (Cola et al. 2020). In 1G ethanol plants, these contaminations are a serious concern, and they are reported to decrease ethanol yield by around 1–5% (Amorim et al. 2011). The genus *Lactobacillus* is the main contaminant (Lucena et al. 2010; Basso et al. 2014; Bonatelli et al. 2017), which results from difficulties in sterilizing large volumes of substrate and the successive recycling of yeast cells (Basso et al. 2014; Amorim et al. 2011). Albers et al. (2011) isolated microbial contaminants from a 2G ethanol plant in Sweden and found that the genus *Lactobacillus* was also the most abundant; they found more than 15 lactobacilli in sulfite liquor. Schell et al. (2007) also isolated more than 5 *Lactobacillus* species from a corn fiber 2G ethanol plant (Schell et al. 2007). More recently, Carvalho et al. (2021) studied the composition of the contaminating microbial population in a Brazilian 2G ethanol plant (Bioflex1, Granbio), which uses the hydrothermal pretreatment system over sugarcane straw to produce a lignocellulosic hydrolysate. Among the greatest relative abundances, they found significant representation of *Weissella, Klebsiella, Lactobacillus, Escherichia* and *Streptococcus* (Carvalho et al. 2021).

Lactic acid bacteria (LAB) can be classified as homo- and heterofermentative. Homofermentative LAB use the Embden-Meyerhof Parnas pathway for glucose catabolism, and the pyruvate formed is reduced to lactic acid. Heterofermentative LAB converts glucose via the phosphoketolase pathway, resulting in an equimolar mixture of lactic acid and ethanol/acetic acid (Kandler 1983). *L. reuteri*, a heterofermentative LAB strain, can reduce furfural and

HMF, using these compounds as alternative electron acceptors, and enhancing regeneration of $NAD(P)^+$. This can result in increased bacterial growth, because of carbon rerouting towards acetate production, which is accompanied by an additional ATP formation (Van Niel et al. 2012).

Since the formation of inhibitory compounds is not easyly prevented on an industrial scale, tolerance to furanic compounds has been investigated (Liu 2011). Several enzymes have also been studied, including NADPH- dependent aldehyde reductases, which can convert these compounds into the corresponding and less inhibitory alcohols (Heer et al. 2009). In this context, the aim of this research was to study the effect of furanic compounds, generated from lignocellulosic hydrolysates, on the physiology of different lactic acid bacteria strains, divided into homo- and heterofermentative metabolism and laboratory and industrial strains. We believe this knowledge is important to understand the potential effects of bacterial contamination in the 2G bioprocesses, and also because LAB can be explored as a guide for metabolic engineering strategies applied to yeast biocatalysts, based on the mechanisms used by these bacteria.

Material and methods

Bacterial strains

The microorganisms used were 12 lactobacilli strains for the initial screening experiments, five homofermentative (*Lactiplantibacillus plantarum* CECT 221, *Lactobacillus delbrueckii, L. plantarum* E4, *Lacticaseibacillus paracasei* LAB 4, *L. paracasei* LAB 5) and seven heterofermentative (*Limosilactobacillus fermentum* DSM 20391, *Limosilactobacillus reuteri* ATCC 23272, *L. fermentum* E3, *L. fermentum* E5, *L. fermentum* 1L-6-MRS, *L. fermentum* 3L-2-M17, *L. paracasei* LAB 2). Strain codes and sources are provided in Table 1. For the co-cultivation experiments, *Saccharomyces cerevisiae* PE-2 was obtained from the Bioprocess Engineering Laboratory (BELa), University of São Paulo.

Culture and storage of microbial strains

Bacterial strains were grown in De Man, Rogosa & Sharpe medium (MRS) containing glucose (20 g l^{-1}), peptone (10 g l^{-1}), meat extract (10 g l^{-1}), yeast extract (5 g l^{-1}), K_2HPO_4 (2 g l^{-1}), sodium acetate (5 g l^{-1}), tri-ammonium citrate (2 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (200 mg l^{-1}), $MnSO_4 \cdot 4H_2O$ (50 mg l^{-1}), and Tween 80 (1 ml l^{-1}). The pH was adjusted to 6, and incubation temperature was 37 °C. Glycerol was then added at a concentration of 20% of the final volume obtained, and 2 mL aliquots were stored in a freezer at − 80 °C.

Inoculum preparation

The inoculum was prepared in 50 mL conical tubes containing 25 mL of MRS medium, where 200 µL of stock culture was added. The inoculum was grown for 24 h at 37 °C.

Table 1 LAB (Lactic Acid Bacteria) strains evaluated in the microplate experiment, and respective sampling locations

Code	Strain	Isolation
A	*Lactiplantibacillus plantarum CECT 221*	Food industry
B	*Lactobacillus delbrueckii*	Beer industry
C	*Lactiplantibacillus plantarum E4*	Sugar cane fermentation
D	*Lacticaseibacillus paracasei LAB 4*	Sugar cane fermentation
E	*Lacticaseibacillus paracasei LAB 5*	Sugar cane fermentation
F	*Limosilactobacillus fermentum DSM 20,391*	Human oral cavity
G	*Limosilactobacillus reuteri ATCC 23,272*	Rat intestine
H	*Limosilactobacillus fermentum E3*	Sugar cane fermentation
I	*Limosilactobacillus fermentum E5*	Sugar cane fermentation
J	*Limosilactobacillus fermentum 1L-6-MRS*	Sugar cane fermentation
K	*Limosilactobacillus fermentum 3L-2-M17*	Sugar cane fermentation
L	*Lacticaseibacillus paracasei LAB 2*	Sugar cane fermentation

MBL media supplemented with furanic compounds

To better simulate the stress response in the presence of furanic compounds, the experiments were performed in MBL medium containing glucose (20 g l^{-1}), yeast extract (5 g l^{-1}), peptone (5 g l^{-1}), K_2HPO_4 (2 g l^{-1}), $MgSO_4$ (0.2 g l^{-1}), and $MnSO_4$ (0.01 g l^{-1}) (Basso et al. 2014). The pH was adjusted to 6 and temperature conditions were controlled at 37 °C for 24 h. This medium was selected because it allows for composition alterations for further experiments (for example, the change in carbon source), since its composition is simpler than the MRS medium (usually applied in studies concerning lactobacilli).

Screening of bacterial growth with furanic compounds

Each of the strains were incubated in 96-well plates containing two concentrations of each of the furanic compounds studied. Furfural concentrations were 1.5 g furfural l^{-1} (15.6 mM) and 2.5 g furfural l^{-1} (26 mM), and HMF concentrations were 2 g HMF l^{-1} (15.85 mM) and 4 g HMF l^{-1} (31.7 mM). Control cultures were evaluated at the same time without the supplementation of inhibitory compounds. Triplicate cultures were carried out for each treatment. Concentrations were based on previously reported data from the literature (Cola et al. 2020; van der Pol et al. 2014). The growth evaluation was performed in MBL medium, where the OD_{600} was evaluated every 15 min with a mean value of five reads per well using the microplate reader, Tecan Infinite M200, from which the growth curve was calculated to estimate the specific maximum velocity (μ_{max}).

Kinetic assays

The kinetic assays were performed in 50 mL conical tubes with 30 mL of MBL medium and with the highest concentration for each inhibitor (4 g HMF l^{-1} and 2.5 g furfural l^{-1}). Control cultures were not supplemented with either inhibitor. Triplicate cultures were carried out for each treatment. The experiments were carried out at 37 °C until the sugar levels in the medium were depleted. Aliquots were taken from time to time to measure OD_{600} and to track sugar consumption and metabolite production.

Co-fermentation of contaminating bacteria with yeast in the presence of furanic inhibitors

To investigate the effects of inhibitors in the presence of the main microorganisms in ethanol fermentation, the kinetics of lactic acid bacteria in the presence of inhibitors and *S. cerevisiae* was evaluated. The tests were carried out in MBL medium, with glucose as a sugar source, and with the addition of furfural (0.5 g furfural l^{-1}) and HMF (1.5 g HMF l^{-1}). Inhibitor concentrations were reduced from the screening stage because it was considered that there could be a synergistic effect between the two inhibitors, causing an added negative effect on the cultures. The microorganisms were inoculated to start with an OD_{600} of 0.5 for the bacteria and 0.7 for yeast in each experiment. The kinetics experiment was conducted in 50 mL conical tubes, containing 30 mL of the MBL medium with inhibitors, in triplicate at 32 °C without agitation; aliquots were taken every 4 h for monitoring the yeast population through viable cell counts in a Neubauer chamber. Samples were differentially stained with methylene blue. Viable cells are not stained, while the non-viable ones are stained blue, which allows for the visualization and differentiation of dead cells from living cells. The viability was expressed as a percentage, depending on the proportion of living cells by total cells (viable plus non-viable cells). In addition, the sugar consumption, and the extracellular metabolite production by HPLC was also measured.

Maximum specific growth rate and lag phase estimation

The maximum specific growth rate (μ_{max}) and the lag phase (λ) were determined by carefully applying the empirical sigmoidal model of Morgan–Mercer–Flodin (Eq. 1) (Tjørve 2003) to the natural logarithm of the bacterial count [y = ln(N)], determined as OD_{600} reads. The corresponding equations used to calculate the microbiological parameters (μ_{max} *and* λ) based on model parameters (A, b, and n) are given in Table 2.

The complete mathematical approach and theoretical background are described in detail by Longhi et al. (2017). The parameters of the sigmoidal model

are the upper asymptote parameter (A) and the shape parameters (b and n). The fit of the mathematical model to the experimental data was assessed using the optimization toolbox of MATLAB R2015b software (MathWorks, Natick, USA). The *lsqcurvefit* function was applied using a non-linear least-squares method, and the trust-region reflective Newton algorithm with the initial value of parameters selected by experimental data observation. The Adjusted Coefficient of Determination (R^2_{adj}) and the square sum of the residual were used to evaluate the quality of the fitting procedure on the experimental data.

Analytical methods

Metabolite samples were immediately centrifuged and stored at − 20 °C until further analysis. Glucose, HMF, lactic acid, acetate, glycerol, and ethanol were analyzed using a HPLC Prominence (Shimadzu) with an ion exclusion column, Aminex ®HPX-87H (300 × 7.8 mm × 9 µm) (Bio-RAD), and isocratically eluted at 60 °C with 5 mM sulfuric acid at a flow rate of 0.6 ml min^{-1}. The total run time was 50 min, and a refractive index detector was used.

Furfural and furfuryl alcohol were analyzed using a HPLC Prominence (Shimadzu) system with a Shim-pack CLC-ODS (M)® C18 column (250 mm × 4.6 mm) (Shimadzu); and isocratically eluted at 30 °C with a mobile phase consisting of acetonitrile/H_2O (15/85, v/v), at a flow rate at 1 ml min^{-1}, using a UV detector at 220 nm.

Statistical analyses

The statistical analyses were performed using the software GraphPad Prism. For comparing product yield among different condition, multiple t tests (statistical significance analysis with alpha value of 0.05) were performed.

Table 2 Equations used to calculate microbiological parameters: maximum specific growth rate (μ_{max}) and the lag phase (λ), based on the parameters (A, b, and n) of the Morgan–Mercer–Flodin model

Morgan–Mercer–Flodin model	
$y(t) = y_0 + \frac{A.t^n}{b+t^n}$	(Eq. 1)
$\mu_{máx} = \frac{A.(n-1)^{\left(\frac{n-1}{n}\right)}.(n+1)^{\left(\frac{n+1}{n}\right)}}{4.n.\sqrt[n]{b}}$	(Eq. 2)
$\lambda = \sqrt[n]{b}.\left(\frac{n-1}{n+1}\right)^{\left(\frac{n+1}{n}\right)}$	(Eq. 3)

Results

The effect of HMF and furfural on the growth rate of lactic acid bacteria is dependent on the metabolic type

The effect of HMF and furfural was studied in lactic acid bacteria (LAB) displaying homo- and heterofermentative metabolism. According to the results obtained from the microplate reader cultivations, the inhibitory compounds displayed different effects between the two groups of LAB. The two inhibitory furan-derivative compounds had a positive effect on the growth of the heterofermentative LAB (Fig. 1), increasing its maximum specific growth rate by up to 2.4 times, in comparison to the control condition (cultivation media with the absence of inhibitory compounds). In addition, a positive effect for both compounds during the elongation of the lag phase was observed in heterofermentative LAB, wherein a decrease in the required time to achieve the exponential phase was noticed, as compared to the control condition (Supplementary Fig. 1). On the other hand, in homofermentative LAB, the complete opposite effect was observed, since their growth rates were inhibited by furanic compounds when compared to the control, shown in Fig. 1. Likewise, homofermentative LAB also had a negative effect on the lag phase duration when cells were cultivated in the presence of the two inhibitors.

When comparing laboratory and industrial strains, the growth performance (evaluated by the maximum specific growth rate and the elongation of the lag phase) of the laboratory heterofermentative LAB strains were less stimulated or partially inhibited than the industrial LAB strains. However, when comparing laboratory and industrial homofermentative LAB strains, a pattern for these two parameters that could be used to differentiate between them could not be determined. The only exception was the fact that under all test conditions, the decrease in growth rate of the homofermentative laboratory strain was more pronounced than the industrial strains.

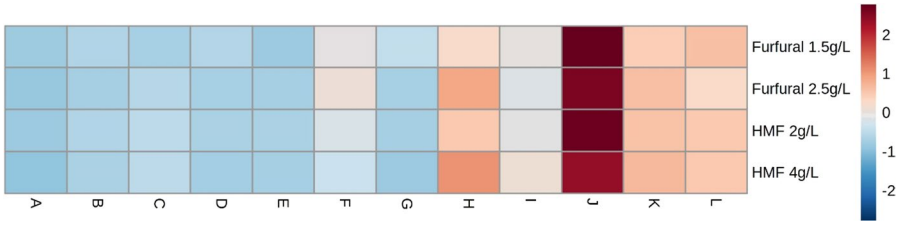

Fig. 1 Normalized maximum specific growth rates (relative to the control condition) of homo- and heterofermentative lactic acid bacteria strains cultured in the presence of HMF and furfural supplemented, semi-defined media. Values with a red trend indicate higher growth rates as compared to the control (without inhibitors). Values with a blue trend, indicate lower growth rates as compared to the control (without inhibitors). Homofermentative lactic acid bacteria: A (laboratory), B, C, D and E (industrial). Heterofermentative lactic acid bacteria: F, G (laboratory), H, I, J, K and L (industrial)

Growth kinetics in the presence of HMF and furfural is only enhanced for heterofermentative lactic acid bacteria

As a follow-up, the growth kinetics were further investigated in two representative LAB strains, using flask cultures with the same MBL media supplemented separately with the two furanic compounds, at the highest concentrations. For this purpose, a representative homofermentative (*L. plantarum* E4) strain, and a representative heterofermentative (*L. fermentum* E3) strain were evaluated under these conditions. Measuring growth provides reliable and accurate information regarding the characterization of toxic compounds, and the conditions that adversely affect microbial cells (Franden et al. 2009).

As observed in the general growth evaluation in microplate cultures, the heterofermentative LAB displayed faster growth kinetics and a shorter lag phase in the presence of both inhibitors as opposed to their absence (Fig. 2a). The exponential phase commenced after almost 5 h of cultivation in the control kinetics, whereas in the presence of furfural, the exponential phase commenced after 2 h. This effect is less pronounced in the presence of HMF. However, growth kinetics in the presence of both inhibitors was inhibited, when compared to the control (absence of furanic compounds) for homofermentative LAB. Apparently, under the tested concentrations, HMF appeared to be more detrimental to the homofermentative strain than furfural (Fig. 2b).

Heterofermentative lactic acid bacteria sugar consumption and product formation is enhanced in the presence of furanic compounds

Heterofermentative LAB normally presents slow growth kinetics on glucose, caused by the decreased activity of the ethanol pathway in the reoxidation of the extra two NADH molecules (Maicas et al. 2002). In the presence of the furan inhibitors, we observed an enhanced sugar consumption rate, and noticed a deviation towards the formation of acetate and ethanol with a concomitant decrease in lactate production, when compared to the control (Fig. 3). Moreover, biomass yield was lower in the presence of both furan inhibitors when also compared to the control, and major conversion yields (glucose to lactate, acetate, and ethanol) showed a deviation towards acetate and ethanol formation, with a concomitant decrease in lactate (Table 3). It seems this observation is caused by the fact that furfural and HMF are promoting the reoxidization NAD^+ and $NADP^+$, respectively (van Niel et al. 2012). In addition, as they do not need to use the ethanol route to reoxidize the NADH, the acetyl-P can be used for ATP synthesis and the acetate production route becomes energetically more advantageous (Ganzle 2015).

In homofermentative LAB cultures, the opposite behaviour was observed, sugar consumption rate decreased in the presence of inhibitors, along with a noticeable decrease in lactate production kinetics (Fig. 4) and a slightly decreased biomass yield (Table 3).

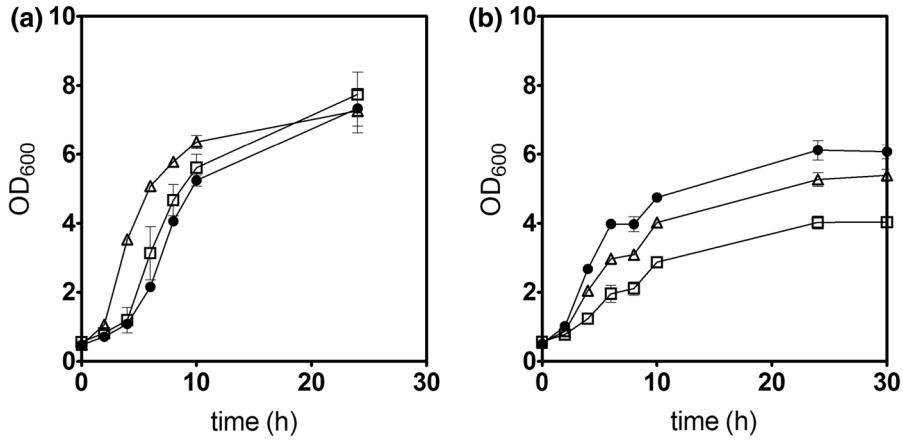

Fig. 2 Effect of inhibitory compounds on the growth kinetics of industrial **a** heterofermentative and **b** homofermentative lactic acid bacteria. Cultures were performed using MBL medium supplemented with 4 g HMF l^{-1} (open squares), 2.5 g furfural l^{-1} (open triangles), or not supplemented (closed circles). Results are given as average values from triplicate experiments, and error bars represent the standard deviation

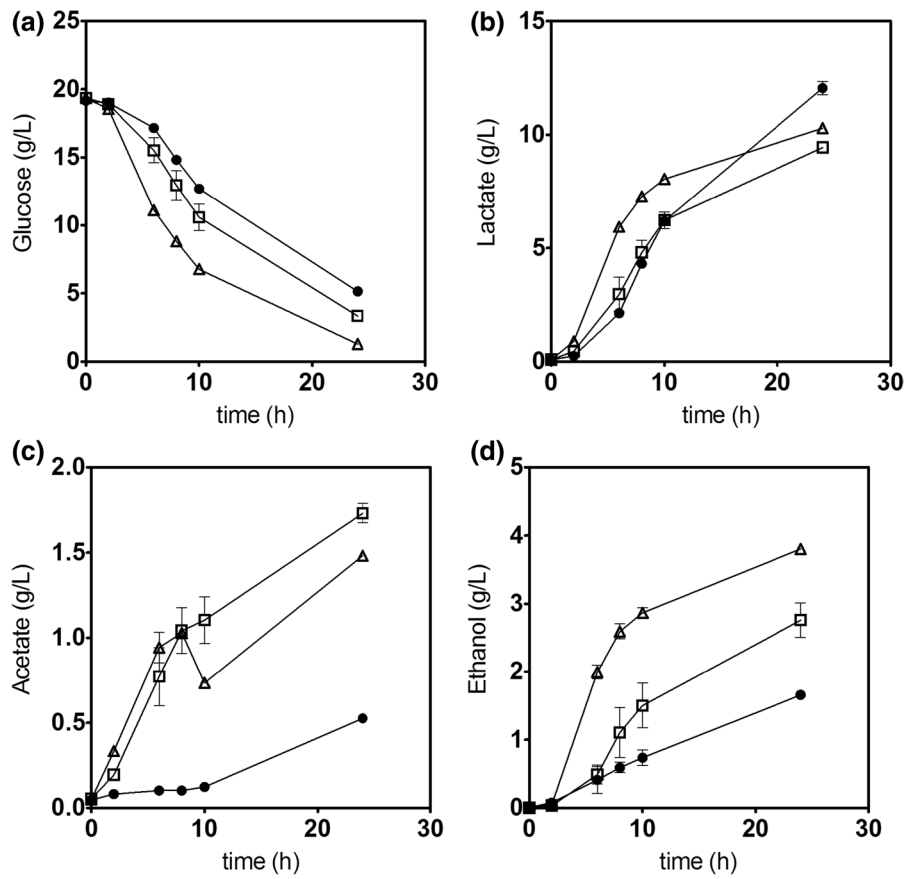

Fig. 3 Heterofermentative LAB **a** glucose consumption and production of **b** lactate, **c** acetate and **d** ethanol. Cultures were performed using semi-defined medium supplemented with 4 g HMF l^{-1} (open squares), 2.5 g furfural l^{-1} (open triangles), or not supplemented (closed circles). Results are given as average values from triplicate experiments, and error bars represent the standard deviation

Table 3 Product yields (in g product g glucose^{-1}) of the homofermentative (*L. plantarum*) and heterofermentative (*L. fermentum*) LAB strains cultured on media containing furfural and HMF compared to the control treatment (without furanic compounds)

	Biomass	Lactate	Ethanol	Acetate
L. plantarum				
Control	0.10 ± 0.00 (A)	1.15 ± 0.04 (A)	0	0
HMF (4 g l^{-1})	0.09 ± 0.01 (B)	1.16 ± 0.04 (A)	0	0
Furfural (2.5 g l^{-1})	0.09 ± 0.00 (AB)	1.13 ± 0.01 (A)	0	0
L. fermentum				
Control	0.09 ± 0.01 (A)	0.85 ± 0.01 (A)	0.11 ± 0.00 (C)	0.03 ± 0.00 (C)
HMF (4 g l^{-1})	0.09 ± 0.01 (AB)	0.59 ± 0.02 (B)	0.17 ± 0.01 (B)	0.10 ± 0.01 (A)
Furfural (2.5 g l^{-1})	0.09 ± 0.00 (B)	0.58 ± 0.01 (B)	0.22 ± 0.00 (A)	0.07 ± 0.00 (B)

Values represent the average ± standard deviation from triplicate experiments. Letters indicate if averages are statistically similar (equal letters) or different (different letters) for each strain

Depletion of furanic compounds is very effective in heterofermentative lactic acid bacteria

Homofermentative bacteria dissimilate hexoses through glycolysis, where fermentation of 1 mol of hexose results in the formation of 2 mol of lactic acid and 2 mol of ATP. In comparison, heterofermentative bacteria presents another active pathway (Kandler and Weiss 1986), where hexoses are converted into equimolar amounts of lactic acid, ethanol or acetate, and carbon dioxide, yielding 1 mol of ATP per mol of fermented hexose (Cogan and Jordan 1994). With the conversion of acetyl phosphate to acetate instead of ethanol, an additional ATP can be produced. Therefore, the regeneration of surplus NAD^+ must be achieved through an alternative electron acceptor. In cultures of heterofermentative LAB, we observed a complete depletion of furfural from the medium, with a concomitant conversion into furfuryl alcohol (Fig. 5). Therefore, we hypothesize that HMF is also converted into its corresponding alcohol (2,5-furandimethanol). Previous studies indicate that yeast and bacteria strains were able to reduce furfural and HMF into their corresponding alcohols, as reported for *L. reuteri* (van Niel et al. 2012), *S. cerevisiae* (Liu 2011), *and E. coli* (Jozefczuk et al. 2010). These degradation products are lower in toxicity to microorganisms when compared to their aldehyde precursors (Liu 2011).

Heterofermentative *L. fermentum* seems to convert HMF at a slower rate compared to furfural, which may be attributed to lower cell membrane permeability of HMF, when compared to furfural (Larsson et al. 1999). Therefore, under the oxygen-limited conditions in which experiments were performed, furanic compounds might have been reduced to their corresponding alcohols. In this way, such inhibitors seem to be

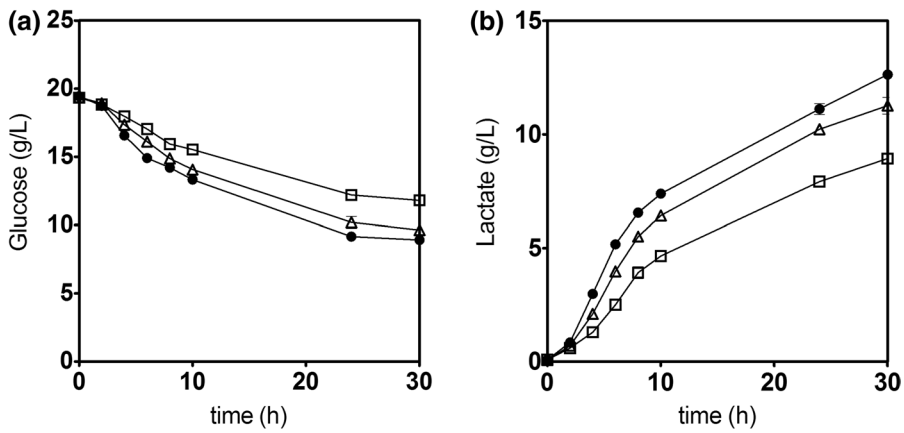

Fig. 4 Homofermentative LAB. **a** Glucose consumption and production of **b** lactate. Cultures were performed using semi-defined medium supplemented with 4 g HMF l^{-1} (open squares), 2.5 g furfural l^{-1} (open triangles), or not supplemented (closed circles). Results are given as average values from triplicate experiments and error bars represent the standard deviation

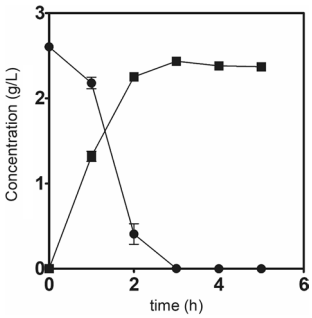

Fig. 5 Conversion kinetics of furfural (circles) into furfuryl alcohol (squares) by heterofermentative lactic acid bacteria, *L. fermentum* E3. Results are given as average values from triplicate experiments, and error bars represent the standard deviation

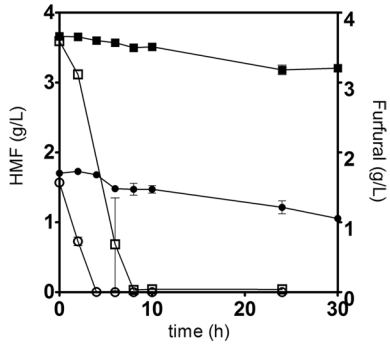

Fig. 6 Depletion kinetics of HMF (squares) and furfural (circles) by hetero- (open symbols) and homofermentative (closed symbols) lactic acid bacteria. Results are given as average values from triplicate experiments, and error bars represent the standard deviation

important co-substrates for heterofermentative lactobacilli, as opposed to homofermentative strains (Fig. 6).

Homofermentative bacteria are more deleterious to yeast in the context of lignocellulose-based bioprocesses

Finally, co-cultures with yeast and both homo- and heterofermentative bacteria were performed in the presence of furfural and HMF. After 24 h of yeast with LAB strains co-cultivation, the viability of yeast cells was drastically reduced (50%, in terms of the fraction of viable cells) when homofermentative LAB was the only strain, when compared to yeast monocultures on the same medium (95%). On the other hand, in the treatment with the heterofermentative strain, viability was at an intermediate value (71%).

Regarding substrate and product kinetics (Fig. 7), glucose consumption was faster in co-cultures than in yeast monocultures. Yet, the presence of heterofermentative bacteria resulted in a faster consumption of glucose when compared to the homofermentative strain (Fig. 7a). Lactate accumulation was faster in the presence of the homofermentative strain and achieved the highest titers when compared to all conditions (Fig. 7b). Lactate accumulation in the presence of both bacterial strains (homo- and heterofermentative) in the same fermentation flask was virtually the same as observed in cultures with only the heterofermentative strain. Acetate accumulation followed a similar trend observed for lactate (Fig. 7c). Finally, ethanol accumulation was faster in the presence of the heterofermentative strain, but titers were higher when yeast was cultured alone. It seems that the higher lactic acid accumulation by homofermentative strains represents an additional source of inhibition to yeast cells, leading to decreased ethanol titters (Fig. 7d), and lower ethanol yield as compared to the heterofermentative strain (Table 4).

There was also an increase in glycerol titers for cultures in the presence of heterofermentative bacteria, when compared to yeast monocultures. This observation is in accordance with results published by Meikle et al. (1988). On the other hand, in cultures with homofermentative bacteria and yeast cells, but without the presence of heterofermentative cells, glycerol titers were much lower (Fig. 7e).

Finally, furan depletion by yeast cells is slower than by heterofermentative LAB (Fig. 8). When in the presence of the heterofermentative bacteria, both HMF and furfural concentrations displayed a significant depletion after only 7 h of culturing. However, in the presence of the homofermentative strain, complete depletion of furfural only occurred after 24 h (Fig. 8a). As for HMF, more than half of what was initially available in the culture medium remained untouched at the end of the cultivation (Fig. 8b). Taking all this into account, the presence of heterofermentative LAB in co-cultures

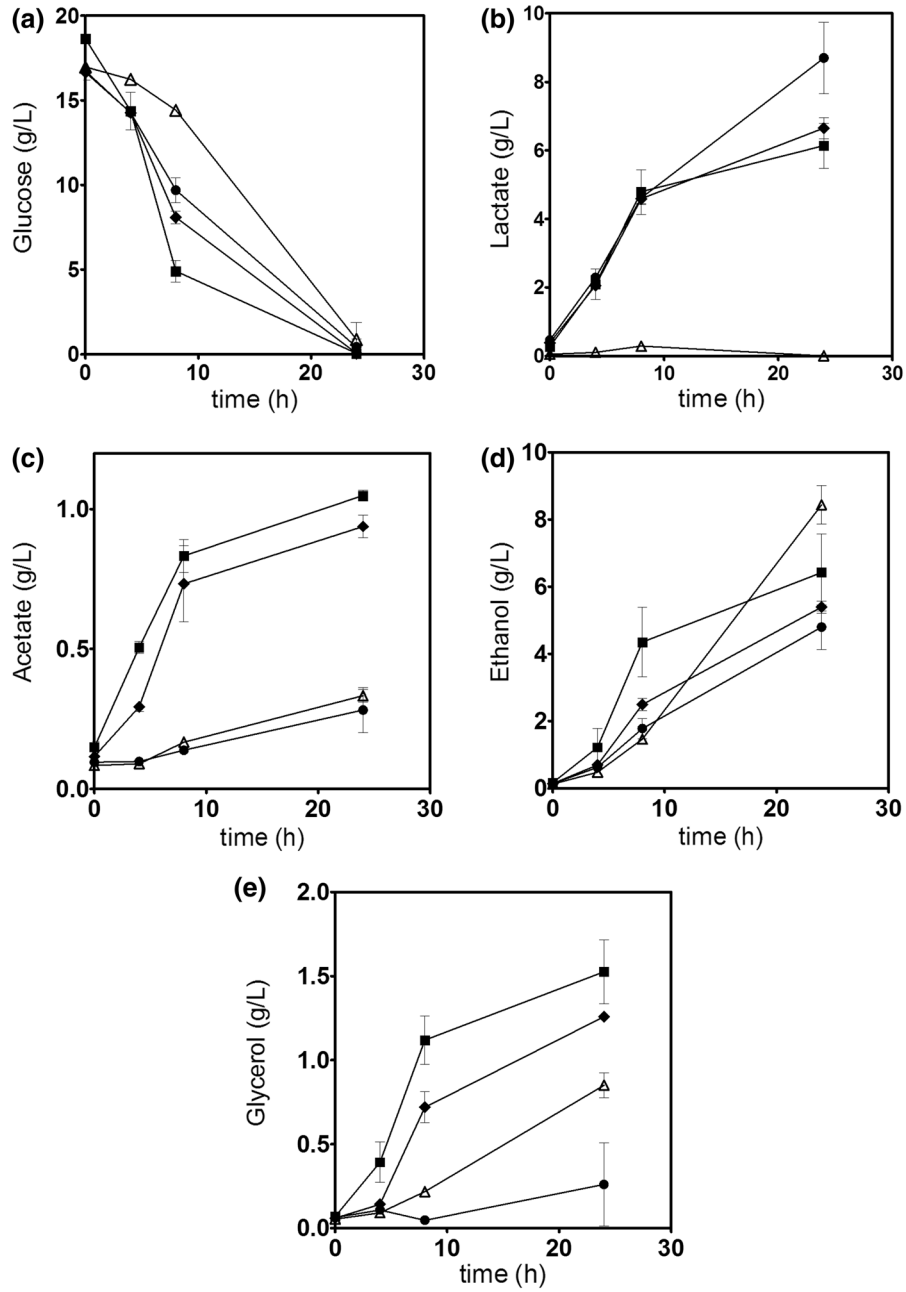

Fig. 7 Kinetics of **a** glucose consumption and **b** lactate, **c** acetate and **d** ethanol, and **e** glycerol production for co-cultures of *L. fermentum* E3 + *S. cerevisiae* (squares), *L. plantarum* E4 + *S. cerevisiae* (circles), *L. fermentum* E3 + *L. plantarum* E4 + *S. cerevisiae* (triangles), and *S. cerevisiae* monoculture (crosses) in the presence of inhibitors. Results are given as average values from triplicate experiments, and error bars represent the standard deviation

with yeasts in the context of lignocellulosic ethanol processes, seems to accelerate depletion of furanic compounds, resulting in faster kinetics of ethanol production. However, the presence of heterofermentative bacterium still reduces ethanol titers by the end of cultivation.

Discussion

According to the results, the two inhibitory furan-derivative compounds had a positive effect on the growth of heterofermentative LAB. This is also the case when the compounds are added at high concentrations to a culture in the early exponential growth

Table 4 Product yields (in g product g glucose^{-1}) for co-cultures of *L. fermentum* E3 (heterofermentative) + *S. cerevisiae*, *L. plantarum* E4 (homofermentative) + *S. cerevisiae*, *L. fermentum* E3 + *L. plantarum* E4 + *S. cerevisiae*, and *S. cerevisiae* monoculture in the presence of inhibitors

	Ethanol	Lactate	Acetate	Glycerol
L. fermentum E3 + *S. cerevisiae*	0.34 ± 0.01 (B)	0.31 ± 0.01 (B)	0.05 ± 0.01 (A)	0.08 ± 0.00 (B)
L. plantarum E4 + *S. cerevisiae*	0.27 ± 0.01 (D)	0.49 ± 0.07 (A)	0.01 ± 0.00 (B)	0.00 ± 0.00 (C)
L. fermentum E3 + *L. plantarum* E4 + *S. cerevisiae*	0.30 ± 0.02 (C)	0.37 ± 0.02 (B)	0.05 ± 0.00 (A)	0.07 ± 0.00 (A)
S. cerevisiae monoculture	0.52 ± 0.01 (A)	0	0.02 ± 0.00 (B)	0.05 ± 0.00 (B)

Values represent the average ± standard deviation from triplicate experiments. Letters indicate if averages are statistically similar (equal letters) or different (different letters) for each condition

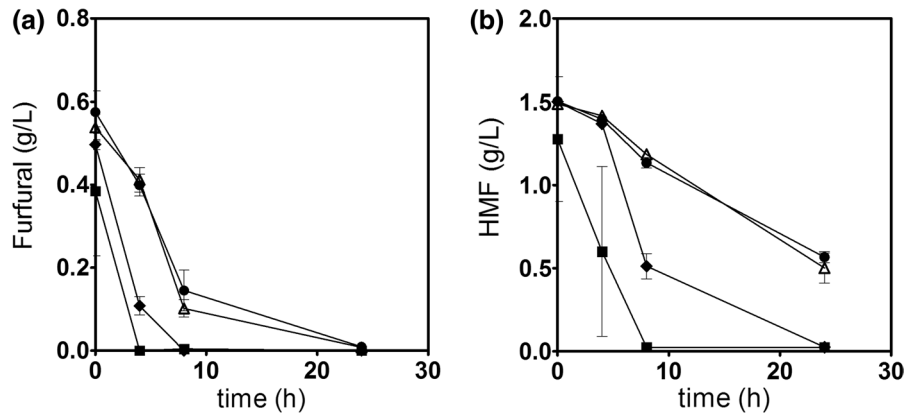

Fig. 8 Depletion kinetics of **a** furfural and **b** HMF in co-cultures of *L. fermentum* E3 + *S. cerevisiae* (squares), *L. plantarum* E4 + *S. cerevisiae* (circles), *L. fermentum* E3 + *L. plantarum* E4 + *S. cerevisiae* (triangles), and *S. cerevisiae* monoculture (crosses) in the presence of inhibitors. Results are given as average values from triplicate experiments, and error bars represent the standard deviation

phase, the compounds seem to enhance growth performance in heterofermentative LAB. In this case, we hypothesized that the heterofermentative bacteria can reduce furfural with NADH and NADPH, using furfural and HMF as alternative electron acceptors. The reduction of furfural is preferentially dependent on NADH, and the reduction of HMF has been mainly associated with the consumption of NADPH (Wahlbom and Hahn-Hägerdal 2002). This behavior was also observed in *L. reuteri* by van Niel (2012), and in several other microorganisms like *S. cerevisiae* (Liu 2011), *Escherichia coli* (Gutierrez et al. 2002) and other enteric bacteria (Boopathy et al. 1993). As described by Carvalho et al. (2021): *Klebsiella, Lactobacillus, Escherichia* and *Streptococcus* are some of the most representative bacterial genera contaminants in 2G ethanol fermentation; and the fact that these bacteria are also able to make the presence of furans something positive, can give the yeast a disadvantage and hinder contamination control methodologies, causing the yeasts to suffer from the consequences caused by these bacteria (Carvalho et al. 2021).

Also, a decrease in the lag phase of heterofermentative LAB was observed, this was also reported in *S. cerevisiae* by Liu et al. (2009), due to alcohol and aldehyde dehydrogenase upregulation, which causes this decrease in the lag phase elongation and an increase in furan tolerance.

It is possible that the presence of HMF or furfural in the medium may have enhanced glycolysis, via regeneration of NAD^+, because NADH may be involved in the reduction of these furans into their corresponding alcohols (furfuryl alcohol and HMF alcohol). Therefore, in view of the enhanced NAD^+ regeneration in the presence of furans, glycolytic flux might have been enhanced in cultures of the heterofermentative *L. fermentum* strain. The alcohol form reduced from the aldehyde form appeared to not affect bacterial fermentation, and the accumulated detoxification products in the medium did not affect the final lactate production.

In homofermentative LAB, several metabolic processes may be significantly altered and delayed in the presence of these inhibitors (Vertes et al. 2011). Furfural and HMF may inhibit the glycolysis pathway and the hexokinases responsible for phosphorylation of six-carbon sugars. Furfural is reported to cause cell membrane damage and inhibit the activity of various glycolytic enzymes, such as hexokinase and glyceraldehyde-3-phosphate dehydrogenase (Almeida et al. 2007). Therefore, pyruvate production through this pathway would be depleted, consequently, leading to a decrease in the lactic acid concentration of the homofermentative LAB. The deleterious effects observed on the growth kinetics in the homofermentative strains are thought to be due to enzyme inhibition and damage to the cell membrane; this is exacerbated by the fact that these compounds are not metabolized by the homofermentative strain (Taherzadeh and Karimi 2011).

Nevertheless, it is unclear to which extent furanic compounds are truly metabolized. In several reports, it is merely established that the furanic aldehydes have disappeared, without mention of the metabolic pathways of the corresponding alcohols or carboxylic acids. Therefore, all the different forms of the furanic compounds (alcohol, aldehyde, and carboxylic acid) should be carefully monitored, to establish whether the furanic aldehydes are metabolized or only transformed into a less toxic form (Wierckx et al. 2011).

The presence of homofermantative LAB was more harmful to the yeast than the heterofermentative LAB. This data corroborates with results obtained by Basso et al. (2014); where it was observed that when bacteria and yeast were inoculated at equal concentrations, the homofermentative LAB was more harmful, reducing yeast viability to 65%. This was due to the high concentrations of lactic acid produced, and indicated by a drop in pH; from 4.9 with only yeast to 3.5 in co-culture with homofermentative LAB.

In the co-cultures, after the yeast had adapted to the presence of HMF and furfural, an accelerated consumption of glucose was observed, a fact that was also reported by Taherzadeh et al. (1999) and Liu et al. (2004). The tolerant yeast can perform in situ detoxification, where more $NADP^+$ is generated and accelerates biosynthesis and cell growth (Liu 2011). In addition, alcohol dehydrogenase is favored to convert acetaldehyde to ethanol, since detoxification generates a supply of NAD^+ for this; factors that contribute to the accelerated consumption of glucose (Liu et al. 2009).

It was possible to notice an increase in glycerol production by the yeast when in the presence of bacteria. Glycerol is a yeast by-product produced to maintain the redox balance, and its production consumes excess NADH that is formed from oxidation reactions, such as from biomass production and organic acid formation (Blomberg 1992; Van-Dijken, Scheffers, 1986). Additionally, glycerol is an osmoregulatory metabolite, and its formation is increased when there is a high osmotic pressure in the medium, protecting the cells from osmotic stress (Guo et al. 2011). The hypothesis suggested by Lino (2021) regarding homofermentative LAB, such as the evaluated *L. amylovorus*, is that smaller glycerol production by yeast results from the acetaldehyde produced by bacteria to reoxidize NADH, and no longer uses the glycerol route. In this way, the bacterium takes advantage of an exclusive metabolic niche created by the yeast, which may have led to the drop in its viability because of its route redox balancing through glycerol has been suppressed. Another hypothesis of what may be happening is that, in the presence of heterofermentative LAB, the bacterium promotes the detoxification of furanic aldehydes. In this case, the yeast produces glycerol to re-establish the internal redox balance. In the presence of homofermentative bacteria alone, as it is not able to carry out detoxification, the yeast itself is promoting the reduction of furanic aldehydes. Thus, it no longer needs to use the glycerol route for the redox balance, resulting in less glycerol production. In one the few studies on LAB contamination in the context of 2G-ethanol production, Collograi et al. (2019) found that *L. fermentum* was not able to grow in a defined medium lacking vitamins and aminoacids (containing glucose, xylose, urea, K_2PO_4, $MgSO_4$. H_2O and trace elements), in coculture with *Spathaspora passalidarum*, using a cell recycling system. Under such conditions, no flocculation was observed, and bacterium did not affect fermentative performance nor cell viability of the yeast, probably due to the fastidious nature of these contaminants.

Conclusions

In general, heterofermentative strains presented a higher resistance to furfural and HMF when compared to homofermentative strains. In addition, sugar consumption and product formation are enhanced in the presence of furanic compounds in the heterofermentative strain. Co-culture studies of LAB with yeast in the presence of HMF and furfural indicated that homofermentative bacterium seem to be more deleterious to yeast, in the context of lignocellulose-based bioprocesses, probably because heterofermentative strains convert furanic compounds into its corresponding alcohols faster than homofermentative strains.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on request.

Declarations

Conflict of interest The authors declare that they are no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals.

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