Homo-and heterofermentative lactobacilli are differently affected by lignocellulosic inhibitory compounds

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Research Article

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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 (furan derivatives, 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 furan derivatives on the physiology of lactic acid bacteria (LAB) strains that are potential contaminants of 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 hydroxymethyylfurfural (HMF).

Results

We found that the effect of HMF and furfural on the growth rate of LAB is dependent of the metabolic type, and growth kinetics in the presence of these compounds is enhanced for heterofermentative LAB, whereas is inhibitory to homofermentative LAB. Sugar consumption and product formation were also enhanced in the presence of furaldehydes in heterofermentative LAB, that displayed effective detoxification kinetics when compared to the homofermentative LAB.

Conclusion

This knowledge is important because lactic acid bacteria can be explored within the scope of bio-detoxification, as well as to guide metabolic engineering strategies to yeast biocatalysts based on the mechanisms used by these bacteria.

Introduction

The recent global economic development enhanced the demand for alternative energy resources worldwide, majorly due to the known drawbacks of fossil fuels, such as its high price, unsustainable and non-renewable feedstock, and global warming. On the other hand, one of the possible candidates to provide alternative energy is second-generation biofuels, which are produced from cheap and abundant plant biomass residues (Mood et al., 2013; Basso et al., 2013). The second-generation (2G) biofuels are those produced through lignocellulosic materials, for instance, bioethanol, biodiesel, and biogas generated from these renewable materials. Among them, bioethanol is proving to be energetically and environmentally worthwhile when compared to the traditional first-generation ethanol. Furthermore,

bioethanol from lignocellulosic materials suits the shortcoming of the first-generation by not applying edible feedstock sources (Aditiya et al., 2016).

The lignocellulosic biomass generally contains over 70% of carbohydrates in the form of cellulose and hemicelluloses in its composition, which may serve as a substrate for ethanol production (Klinke, et al., 2004). Cellulose is a polymer formed of glucose units whereas hemicellulose is a polymer formed of various units of xylose, arabinose, mannose, galactose, and glucose, which varies in composition depending on biomass (Bobleter, 1994; Fan et al. 1982). Associated with these carbohydrates is lignin in varying proportions, depending on the raw material source. Lignin is an amorphous polyphenolic compound with undefined molecular weight, predominantly composed of p-coumaryl alcohol, coniferyl alcohol, and synaphyl alcohol (Rubin, et al. 2008). Although the large content of carbohydrates, there are many chemical and physical barriers in lignocellulosic biomass that make it difficult for cellulose and hemicellulose to be available, requiring a pre-treatment stage to make sugars easily fermentable by yeasts in 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 to the enzymatic hydrolysis reaction (Cardona, et al., 2010). These treatments can be physical, chemical, physicochemical, or biological. Most of these pre-treatments, due to their severity, generate large amounts of inhibitory compounds to the metabolism of microorganisms applied in bioethanol production. The nature and concentration of these inhibitors are extremely affected by the adopted process and its operating conditions, such as 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: furan derivatives, 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).

Furans are formed mainly during pre-treatments involving extremely acidic pH conditions due to the degradation of pentoses (2-furfural) and hexoses (5-hydroxymethylfurfural) (Dunlop, 1948). The concentration of furan aldehydes in lignocellulosic hydrolysates can range from 0.5 to 11 $g.L^{-1}$ (Almeida et al., 2007). Aldehydes are chemically reactive and can form products with many classes of biological molecules. Several potential mechanisms for the toxicity of aldehydes have been explored, including damage from chemical reactivity, direct inhibition of glycolysis and fermentation, and plasma membrane damage (Zaldivar and Martinez, 1999). This class of inhibitors has been found to inactivate the cell replication that reduces the growth rate and the cell mass yield on ATP, volumetric growth rate, and specific productivities. At low concentrations, furans can have a positive effect on cell growth, although these compounds inhibit both cell growth and ethanol production rate by decreasing membrane permeability at higher concentrations (Taherzadeh, et al., 1999).

Adaptation of microorganisms on high furfural concentration has been found a successful option to overcome the negative effect of furfural on growth (Chandel, et al., 2011). Several enzymes have also

been studied in yeast and bacterial cells, including NADH- and NADPH- dependent aldehyde reductases that can convert these compounds to the corresponding less inhibitory alcohols (Heer et al., 2009). Since the formation of inhibitory by-products is not easily prevented economically at an industrial scale, it is often preferred to remove inhibitors before fermentation. Several types of detoxification strategies, such as physical (membrane-mediated detoxification, evaporation), chemical (over liming, calcium hydroxide, neutralization, ion exchange resins, activated charcoal column, and extraction with ethyl acetate), biological (microbial and enzymatic), and in situ microbial detoxification, have been applied to remediate fermentation inhibitors. Biological methods of detoxification are in demand due to their simplicity, milder work conditions that avoid further use of chemicals, lower frequency of side reactions, and lower energy requirements (Parawira and Tekere, 2011). The main drawbacks of biological methods include slow reaction time, inhibitor specificity, and loss of fermentable sugars, which makes them unattractive to biorefineries (Tian et al., 2009).

Among the biological options, *Lactobacillus* spp. arises as one possible candidate because of its ability to transform furfural and HMF in less toxic compounds of furfuryl alcohol and 2,5-bis(hydroxymethyl)furan, respectively. This process is also known as “in situ-detoxification” (Boopathy et al., 1993). Lactic 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. Furfural and HMF are able to inhibit important metabolic enzymes, including alcohol dehydrogenase, aldehyde dehydrogenase, and pyruvate dehydrogenase. Heterofermentative LAB converts glucose via the phosphoketolase pathway, resulting in an equimolar mixture of lactic acid and ethanol/acetic acid (Kandler, 1983). It has the ability to reduce furfural with NADH and NADPH using the furan derivatives as alternative electrons acceptors, enhancing regeneration of NAD^+. This can result in increased bacteria growth while blocking the production pathways of lactic acid and increasing the acetate/ethanol pathway because furfural and HMF are acting as NAD and NADP recyclers and the acetate/ethanol production route becomes energetically more advantageous.

In this context, the aim of this research was to study the effect of furaldehydes from lignocellulosic hydrolysates on the physiology of different strains of lactic acid bacteria, divided into homo and heterofermentative metabolism and laboratory and industrial. strains**.** We believe this knowledge is important because lactic acid bacteria can be explored within the scope of bio-detoxification, as well as to guide metabolic engineering strategies to yeast biocatalysts based on the mechanisms used by these bacteria.

Material And Methods

Strains

The microorganisms used were 12 lactobacilli strains in initial screening experiments, five homofermentative (*Lactobacillus plantarum* CECT 221*, Lactobacillus delbrueckii, Lactobacillus plantarum* ESALQ 4*, Lactobacillus paracasei* LAB 4*, Lactobacillus paracasei* LAB 5*)* and seven

heterofermentative (*Lactobacillus fermentum* DSM 20391, *Lactobacillus reuteri* ATCC 23272, *Lactobacillus fermentum* ESALQ 3, *Lactobacillus fermentum* ESALQ 5, *Lactobacillus fermentum* 1L-6-MRS, *Lactobacillus fermentum* 3L-2-M17, *Lactobacillus paracasei* LAB 2*).* Strain codes and sources are provided in Table 1. For the co-cultivation experiments, *Saccharomyces cerevisiae* PE-2 was obtained from stock banks of the Bioprocess Engineering Laboratory (BELa) isolated from the ethanol industry.

TABLE 1

Propagation and storage of microbial strains

Bacterial strains were grown in De Man, Rogosa & Sharpe medium (MRS) with 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 $g.L^{-1}$), $MgSO_4.7H_2O$ (200 $mg.L^{-1}$), $MnSO_4.4H_2O$ (50 $mg.L^{-1}$), Tween 80 (1 $mL.L^{-1}$), The pH was adjusted to 6 and temperature of cultivation to 37 °C. From the final volume obtained, 20% relative to it of glycerol was added and 2 mL aliquots were stored in a freezer at -80 °C.

Inoculum preparation

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

MBL media supplemented with furanic derivatives

To better evidence the stress response with 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.

Screening of bacterial growth with furanic derivatives

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

Kinetic assays

The kinetic assays were performed in 50 mL conical tubes with 30 mL of MBL medium with the highest concentration of each inhibitor (HMF 4 $g.L^{-1}$ and furfural 2.5 $g.L^{-1}$). Control cultures were not

supplemented with either inhibitor. Triplicate cultures were carried out for each treatment. The experiments were carried out at 37ºC until the sugar level in the medium was finished. Aliquots were taken from time to time to measure OD_{600} and to track the sugar consumption and the 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 present in ethanol fermentation, a kinetics of lactic acid bacteria in the presence of inhibitors and the yeast *S. cerevisiae* was performed. The tests were carried out in MBL medium with glucose as a sugar source with the addition of furfural (0.5 $g.L^{-1}$) and HMF (1.5 $g.L^{-1}$). 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 additive negative effect on the cultures. The microorganisms were inoculated in each experiment to start with an OD_{600} of 0.5 for the bacteria and 0.7 for yeast. 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, with aliquots taken every 4 h for monitoring the yeast population by viable cell count in Neubauer chamber. Samples were stained differentially with methylene blue. Viable cells are not stained while the non-viable ones are stained in blue, which allows 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, it was measured the sugar consumption and the extracellular metabolite production by HPLC.

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 to calculate the microbiological parameters (μ_{max} *and* λ) based on model parameters (A, b, and n) are given in Table 2.

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 fitting of the mathematical model to the experimental data was assessed in 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 as 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 to the experimental data.

Analytical methods

Metabolite samples were immediately centrifuged and stored at −20 °C until analysis. Sugar consumption and the metabolite production were analyzed using HPLC Prominence (Shimadzu) with ion exclusion column Aminex ®HPX-87H (300×7.8mm×9 µm) (Bio-RAD), isocratically eluted at 60 °C with 5 mM sulfuric acid flow rate of 0.6 $mL.min^{-1}$. The total run time was 50 min, refractive index detector was used.

Results

The effect of HMF and furfural on the growth rate of lactic acid bacteria is dependent of 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 in the microplate reader cultivations, it was observed that the inhibitory compounds affected differently 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 in the absence of the inhibitory compounds). In addition, a positive effect for both compounds in the elongation of the lag phase was also observed in the heterofermentative LAB, in which a decrease in the time required to reach the exponential phase was noticed as compared to the control condition. On the other hand, in the homofermentative LAB, the complete opposite effect was observed, since their growth rates were inhibited by the furan-derivatives as compared to the control, as 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.

FIG1

When comparing laboratory and industrial strains, it was observed that 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 in the industrial LAB strains. Comparing laboratory and industrial homofermentative LAB strains, it was not possible to observe a pattern for these two parameters that could be used to differentiate them. The only exception was the fact that in all conditions tested the decrease in the growth rate of the homofermentative laboratory strain was more pronounced than the industrial strains.

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

As a follow-up, the growth kinetics was further investigated in two representative LAB strains using flask cultures with the same MBL media supplemented with the two furan derivatives separately at the highest concentration tested. For that purpose, a representative homofermentative (*Lactobacillus plantarum* ESALQ 4) strain, and a representative heterofermentative (*Lactobacillus fermentum* ESALQ 3) strain were investigated in these conditions. The measurement of growth provides reliable and sensitive information

for the characterization of toxic compounds and conditions that adversely affect microbial cells (Franden et al., 2009)

As observed in the general growth screening described above in microplate cultures, the heterofermentative LAB showed faster growth kinetics and shorter lag phase in the presence of both inhibitors as compared to their absence (Fig. 2a). The exponential phase starts almost with 5 h of cultivation in the control kinetics and could be evidenced in 2 h in furfural presence. This effect is less pronounced in the presence of HMF. On the other hand, the growth kinetics in the presence of both inhibitors was inhibited as compared to the control condition (absence of furan derivatives) in homofermentative LAB. Apparently, in the concentrations tested, HMF seemed to be more detrimental to the homofermentative strain than furfural (Fig. 2b).

FIG2

Sugar consumption and product formation is enhanced in the presence of furaldehydes in heterofermentative lactic acid bacteria

Heterofermentative LAB normally present slow growth kinetics on glucose that is caused by the low activity of the ethanol pathway in the reoxidation of the extra two NADH (Maicas et al, 2002). In the presence of the furan inhibitors, we observed an enhanced sugar consumption rate, and we noticed a deviation towards the formation of acetate and ethanol with a concomitant decrease in lactate production when compared to the control condition (Fig. 3). Moreover, biomass yield was lower in the presence of both furan inhibitors as 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. In addition, as they do not need to use the ethanol route to reoxidize de NADH, the acetyl-P can be used by ATP synthesis and the acetate production route becomes energetically more advantageous (Ganzle, 2015).

FIG 3

TABLE 3

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

FIG 4

TABLE 4

Detoxification of furaldehydes 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 present another active pathway (Kandler and Weiss, 1986) and hexoses are converted to equimolar amounts of lactic acid, ethanol or acetate, and carbon dioxide, yielding 1 mol of ATP per mol of hexose fermented (Cogan and Jordan, 1994). With the conversion of acetyl phosphate to acetate instead of ethanol, an additional ATP can be produced. Then, the regeneration of surplus NAD^+ must be achieved through an alternative electron acceptor. In the heterofermentative LAB, it was possible to see the complete depletion of HMF and furfural which we hypothesized that were converted to furfuryl alcohol and 2,5- furandimethanol, respectively (Fig. 5). Previous studies indicate that yeast and bacteria strains were able to reduce furfural and HMF to their corresponding alcohols, as reported for *L. reuteri* (van Niel, 2012)*, S. cerevisiae (*Liu et al., 2011)*, and E. coli* (Jozefczuk, 2010) in which. These degradation products are of lower toxicity to microorganisms compared to their aldehyde precursors (Liu et al., 2011).

Heterofermentative *L. fermentum* seems to convert HMF at a slower rate as compared to furfural, which may be attributed to a lower cell membrane permeability of HMF when compared to furfural (Larsson et al., 1999). Therefore, under the oxygen-limited conditions that the experiments were performed, furan derivatives might have been reduced to their corresponding alcohols. In this way, such inhibitors seemed to be important co-substrates for heterofermentative lactobacilli, as opposed to homofermentative strains (Fig. 5).

FIG 5

Homofermentative bacteria is more deleterious to yeast in the context of lignocellulose-based substrates

We finally performed co-cultivations with yeast and both homo- and heterofermentative bacteria, in the presence of furfural and HMF. After 24 h co-cultivation of yeast with LAB strains, it was possible to notice that when homofermentative LAB was the only strain, the viability of yeast cells was drastically reduced (50%, in terms of the fraction of viable cells) as compared to yeast monocultures in 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. 6), 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. 6A). Lactate accumulation was faster in the presence of the homofermentative strain and reached highest titters when compared to all conditions (Fig. 6B). Lactate accumulation in the presence of both bacteria (homo- and heterofermentative types) in the same fermentation flask was virtually the same as observed in cultivations with the heterofermentative strain alone. Acetate accumulation followed a similar trend as observed for lactate (Fig. 6C). Finally, ethanol accumulation was faster in the presence of the heterofermentative strain, but titters 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 6

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

FIG 7

Finally, furan detoxification 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 7h of cultivation. On the other hand, in the presence of the homofermentative strain, complete depletion of furfural only occurred after 24h of cultivation (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 together, the presence of heterofermentative LAB in co-cultures with yeasts in the context of lignocellulosic ethanol processes, seems to accelerate detoxification of furan derivatives, resulting in faster kinetics of ethanol production. However, the presence of heterofermentative bacterium still reduces ethanol titters in the end of the cultivation.

FIG 8

Discussion

According to the results it was observed that the two inhibitory furan-derivative compounds had a positive effect on growth of the heterofermentative LAB. Even when added in high concentrations to a culture in an early exponential growth phase, the compounds seem to enhance growth performance in the heterofermentative LAB. In this case, we hypothesized that the heterofermentative bacteria can reduce furfural with NADH and NADPH using furfural and HMF as alternative electrons 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 other several microorganisms like *S. cerevisiae* (Liu et al., 2011), *Escherichia coli* (Gutierrez et al., 2002) and other enteric bacteria (Boopathy et al., 1993). Also, it was observed the decreased in lag phase in heterofermentative LAB, this was also observed in *S. cerevisiae* by Liu (2009) because of the alcohol and aldehyde dehydrogenases upregulation that caused 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 to their corresponding alcohols (furfuryl alcohol and HMF alcohol). Therefore, in view of the enhanced

regeneration of NAD^+ in the presence of furans, glycolytic flux might have been enhanced in the cultures of the heterofermentative *L. fermentum* strain. The alcohol form reduced from the aldehyde form appeared to not affect bacteria fermentation and the accumulated detoxification products in the medium did not affect the final lactate production.

In homofermentative LAB many metabolic processes may be significantly altered and delayed in the presence of these inhibitors (Vertes et al., 2011). Furfural and HMF may inhibit glycolysis pathway and hexokinases responsible for phosphorylation of six-carbon sugars. Furfural is reported to cause cell membrane damage and to inhibit the activity of various glycolytic enzymes, such as hexokinase and glyceraldehyde-3-phosphate dehydrogenase (Almeida et al., 2007). Therefore, pyruvate production by this pathway would be depleted, leading consequently to a decrease in the lactic acid concentration in the homofermentative LAB. The deleterious effects observed on the growth kinetics in the homofermentative strains are sought to be due to enzyme inhibition and to damage in the cell membrane, that are 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 compound (alcohol, aldehyde, and carboxylic acid) should be carefully monitored to establish whether the furanic aldehydes are actually metabolized or only transformed into a less toxic form (Wierckx et al. 2010; Wierckx et al., 2011).

The presence of the homofermantative LAB was more harmful to the yeast than the heterofermentative LAB. These data agree with the 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%, due to high concentrations of lactic acid produced also indicated by the drop in pH of 4.94 with only yeast to 3.53 in co-cultivation with homofermentative LAB.

In the co-cultivations, after the yeast adapted to the presence of HMF and furfural, it was possible to observe an accelerated consumption of glucose, a fact also reported by Taherzadeh et al, (2000) and Liu et al., (2004). As the tolerant yeast can perform in situ detoxification, more $NADP^+$ is generated which accelerates the biosynthesis and cell growth (Liu, 2021). In addition, the alcohol dehydrogenase is favoured to convert acetaldehyde to ethanol, since the detoxification generates a supply of NAD^+ for this, factors that contribute to the accelerated consumption of glucose (Liu, 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 that is directly related. with the redox balance, as alcoholic fermentation is a neutral redox balance process, and to maintain that balance, the entire NADH formed in oxidation reactions (from biomass production and acid formation organic) must be consumed in reduction reactions, coupled with the production of glycerol (Blomberg, Adler, 1992; Van-Dijken, Scheffers, 1986).

Additionally, the glycerol is an osmoregulatory metabolite, and its formation is increased when there is a high osmotic pressure in the medium, protecting the cells from stress osmotic (Guo et al, 2011). The hypothesis suggested by Lino (2021) in homofermentative LAB, like *L. amylovorus* tested, is that a smaller of glycerol production by the yeast because it uses the acetaldehyde produced by bacteria to reoxidize NADH, no longer using 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 of its viability because its route redox balancing through glycerol has been suppressed. Another hypothesis of what may be happening is that, in the presence of heterofermentative, the bacterium is promoting the detoxification of the 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.

The detoxification of furan aldehydes to the corresponding alcohols occurs through the reoxidation of NADH by the enzyme ADH1 or from NADPH, through the enzymes ADH6, ADH7, FFR and XR (Liu, 2009). Liu et al, 2011 identified that the overexpression of enzymes such as ADH6 and ADH7 increased the capacity reductive of yeast to HMF and furfural, showing aldehyde reductase activity almost 100x more intense than its oxidative activity, indicating the possibility of that its primary metabolic function is more like aldehyde reductase than alcohol dehydrogenase, when in the presence of inhibitors (Liu, 2009). Mutants with the ADH1 overexpression also showed high aldehyde reductase activity (Almeida et al, 2008).

Conclusions

The heterofermentative bacterium presented the ability to decrease the concentrations of furfural and HMF in the fermentation medium, with simultaneous lactic acid production. Low concentrations of these compounds present in the sugarcane bagasse hemicellulosic liquor did not have inhibitory effects on the lactic acid production. The approach of bio-detoxification of the fermentation broth dispenses a hemicellulosic liquor detoxification process prior to fermentation and expands the possibility for lactic acid production from second-generation feedstock. Co-culture studies of lactic acid bacteria with yeast in the presence of HMF and furfural were also carried out; indicating that heterofermentative LAB detoxifies the medium more quickly, allowing the yeast to ferment sugars to ethanol. The homofermentative LAB represented another source of stress for the yeast, since in addition to not being able to detoxify the environment; they make the environment more hostile due to the production of lactic acid. A better understanding of the genetic mechanisms and biochemical pathways responsible for inhibitor response in bacteria may allow the development of genetically engineered novel strains to withstand major inhibitors generated from biomass pre-treatment.

Mathematical Notation

μ_{max} - maximum specific growth rate

λ - lag phase

Ysx: biomass conversion yield from substrate

Ysl: lactate conversion yield from substrate

Yse: ethanol conversion yield from substrate

Ysa: acetate conversion yield from substrate

Declarations

Author contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Thamiris G. Giacon, Gabriel C. Gois e Cunha, and Kevy P. Eliodório. Supervision and resources were provided by Ricardo P. S. Oliveira and Thiago O. Basso. The first draft of the manuscript was written by Thamiris G. Giacon and Thiago O. Basso, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Tables

Table 1. LAB (Lactic Acid Bacteria) strains cultivated the in microplate experiment and respective locations from which they were isolated.

Code	Strain	Isolation
A	*Lactobacillus plantarum* CECT 221*	Food industry
B	*Lactobacillus delbrueckii*	Beer industry
C	*Lactobacillus plantarum* ESALQ 4*	Sugar cane fermentation
D	*Lactobacillus paracasei* LAB 4*	Sugar cane fermentation
E	*Lactobacillus paracasei* LAB 5*	Sugar cane fermentation
F	*Lactobacillus fermentum* DSM 20391*	Human oral cavity
G	*Lactobacillus reuteri* ATCC 23272*	Rat intestine
H	*Lactobacillus fermentum* ESALQ 3*	Sugar cane fermentation
I	*Lactobacillus fermentum* ESALQ 5*	Sugar cane fermentation
J	*Lactobacillus fermentum* 1L-6-MRS*	Sugar cane fermentation
K	*Lactobacillus fermentum* 3L-2-M17*	Sugar cane fermentation
L	*Lactobacillus paracasei* LAB 2*	Sugar cane fermentation

**Lactobacillus plantarum* (now *Lactiplantibacillus plantarum*), *Lactobacillus paracasei* (now *Lacticaseibacillus paracasei*), *Lactobacillus fermentum* (now *Limosilactobacillus fermentum*), *Lactobacillus reuteri* (now *Limosilactobacillus reuteri*)

Table 2. Equations to calculate the microbiological parameters: maximum specific growth rate (μ_{max}) *and* the lag phase (λ) *based on* parameters (A, b, and n) of 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)

Table 3. Conversion yields (in $g.g^{-1}$) homofermentative LAB strain (*L. plantarum*) cultivated in media containing furfural and HMF compared with control treatment (without furanic compounds). Where Ysx: biomass conversion yield from substrate; Ysl: lactate conversion yield from substrate. Values are expressed as means (n = 3). Different letters in the same column indicate significant difference ($P \leq 0.05$).

Condition	*Ysx*	*Ysl*
Control	0.100±0.004 (A)	1.148±0.039 (A)
HMF (4.0 g L^{-1})	0.089±0.005 (B)	1.156±0.038 (A)
Furfural (2.5 g L^{-1})	0.093±0.002 (AB)	1.132±0.009 (A)

Table 4. Conversion yields (in $g.g^{-1}$) heterofermentative LAB strain (*L. fermentum*) cultivated in media containing furfural and HMF compared with control treatment (without furanic compounds). Where Ysx: biomass conversion yield from substrate; Ysl: lactate conversion yield from substrate, Yse: ethanol conversion yield from substrate and Ysa: acetate conversion yield from substrate. Values are expressed as means (n = 3). Different letters in the same column indicate significant difference ($P \leq 0.05$).

Condition	*Ysx*	*Ysl*	*Yse*	*Ysa*
Control	0.093±0.007 (A)	0.854±0.013 (A)	0.113±0.004 (A)	0.032±0.0007 (A)
HMF (4.0 g L^{-1})	0.087±0.007 (AB)	0.592±0.016 (B)	0.175±0.015 (B)	0.101±0.008 (B)
Furfural (2.5 g L^{-1})	0.076±0.004 (B)	0.579±0.007 (B)	0.221±0.004 (C)	0.068±0.0004 (C)

Figures

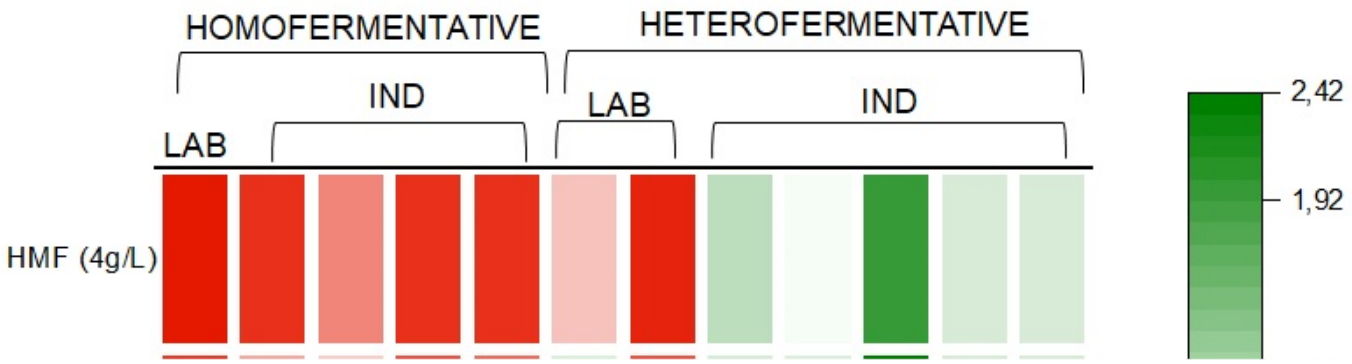

Figure 1

Normalized growth rate (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 green tendency mean tests where the maximum specific speed was benefited by the presence of the inhibitors. Values with a tendency to red, indicate tests where the presence of the inhibitors was harmful to the cultivation. 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)

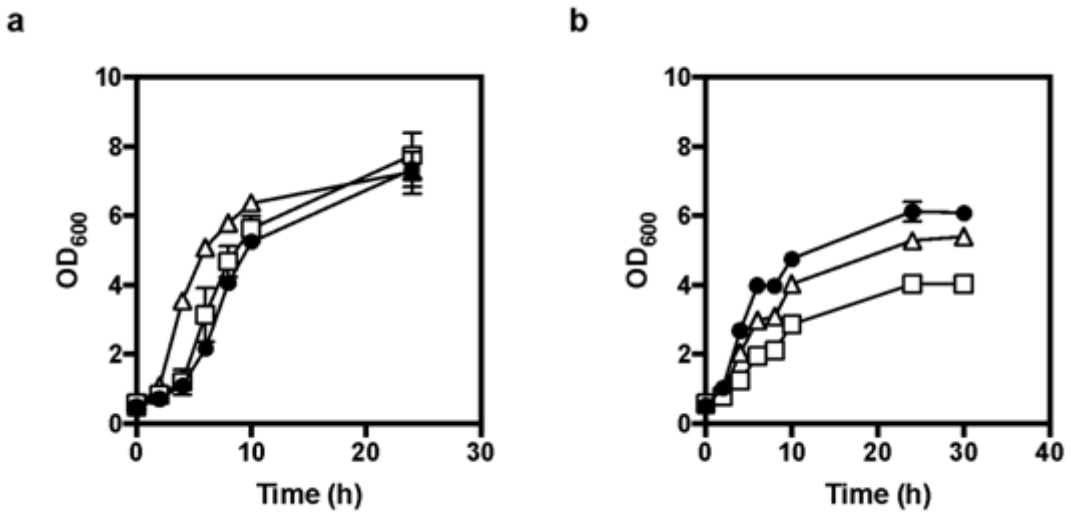

Figure 2

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

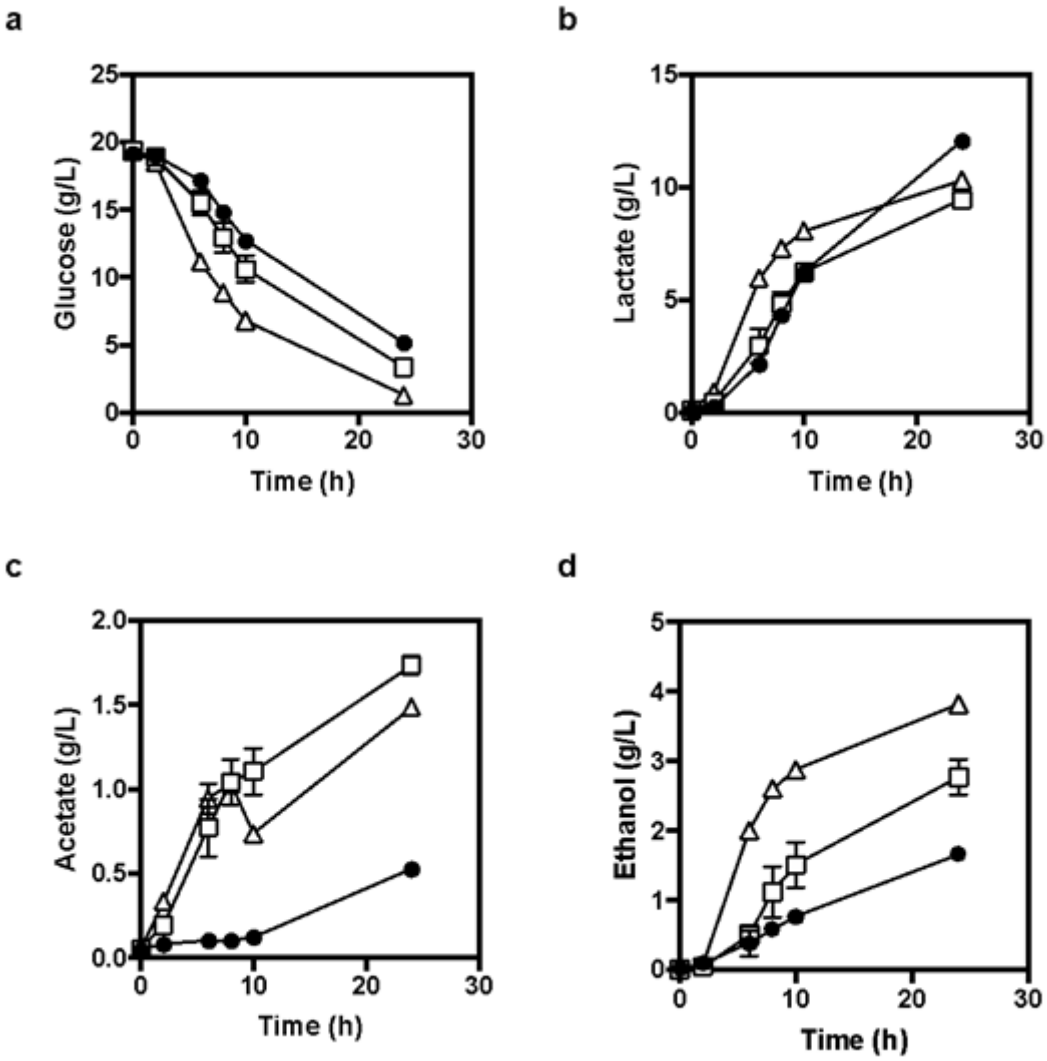

Figure 3

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

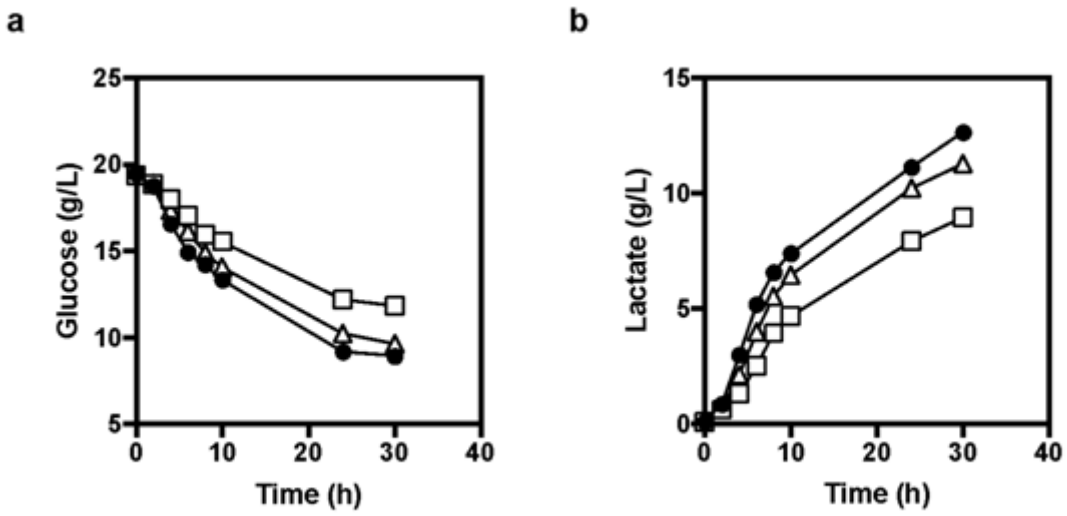

Figure 4

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

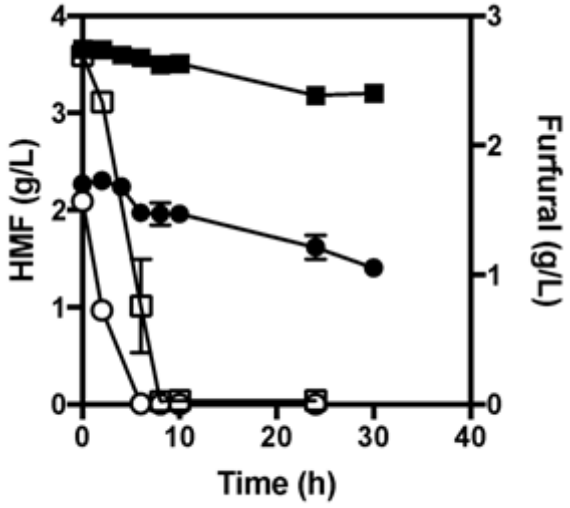

Figure 5

Conversion 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.

Figure 6

Kinetics of A) glucose consumption and production of B) lactate, C) acetate and D) ethanol 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

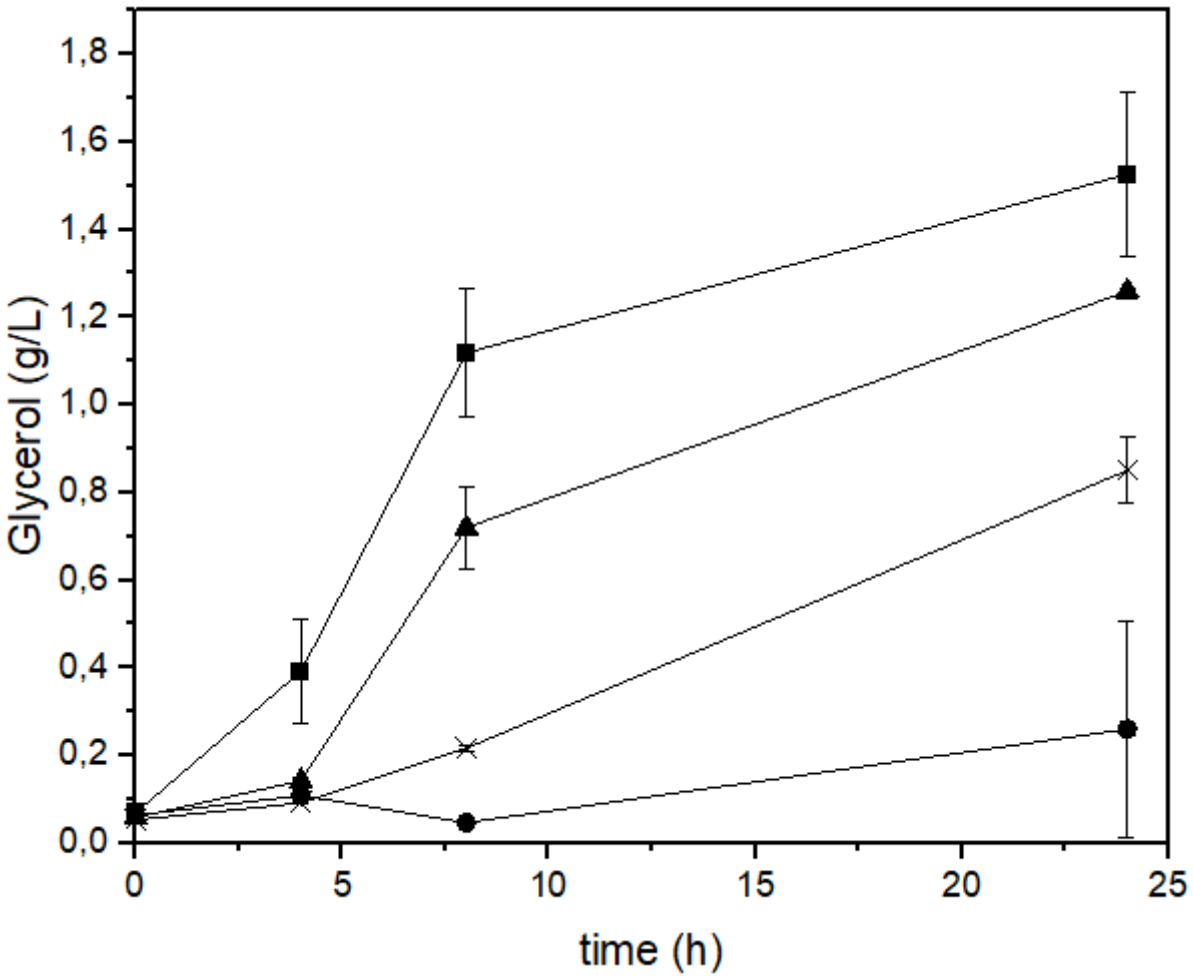

Figure 7

Kinetics of glycerol production 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

Figure 8

Detoxification 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