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BIOTECHNOLOGY AND INDUSTRIAL MICROBIOLOGY - RESEARCH PAPER

Variable and dose-dependent response of *Saccharomyces* and non-*Saccharomyces* yeasts toward lignocellulosic hydrolysate inhibitors

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Abstract

Lignocellulosic hydrolysates will also contain compounds that inhibit microbial metabolism, such as organic acids, furaldehydes, and phenolic compounds. Understanding the response of yeasts toward such inhibitors is important to the development of different bioprocesses. In this work, the growth capacity of 7 industrial *Saccharomyces cerevisiae* and 7 non-*Saccharomyces* yeasts was compared in the presence of 3 different concentrations of furaldehydes (furfural and 5-hydroxymetil-furfural), organic acids (acetic and formic acids), and phenolic compounds (vanillin, syringaldehyde, ferulic, and coumaric acids). Then, *Candida tropicalis* JA2, *Meyerozyma caribbica* JA9, *Wickerhamomyces anomalus* 740, *S. cerevisiae* JP1, B1.1, and G06 were selected for fermentation in presence of acetic acid, HMF, and vanillin because they proved to be most tolerant to the tested compounds, while *Spathaspora* sp. JA1 because its xylose consumption rate. The results obtained showed a dose-dependent response of the yeasts toward the eight different inhibitors. Among the compared yeasts, *S. cerevisiae* strains presented higher tolerance than non-*Saccharomyces*, 3 of them with the highest tolerance among all. Regarding the non-*Saccharomyces* yeasts, *C. tropicalis* JA2 and *W. anomalus* 740 appeared as the most tolerant, whereas *Spathaspora* strains appeared very sensitive to the different compounds.

Keywords Hydrolysate fermentation · Yeast comparison · Lignocellulose inhibitors · Vanillin, Furaldehyde, Organic acids

Introduction

Pretreatment and hydrolysis of the biomass are necessary to release sugars for microbial production of fuels and chemicals [1]. During the pretreatment and hydrolysis of biomass, it may occur the release or formation of compounds that inhibit microbial metabolism leading to negative effects on fermentative processes [2]. These inhibitors are generally classified as organic acids (e.g., acetic, formic, and levulinic acids), furaldehydes, mainly furfural and 5-hydroxymethyl-furfural (HMF), and phenolic compounds (e.g., vanillin, syringaldehyde, ferulic, and coumaric acids) and other compounds derived from lignin breakdown [2–5].

The source and inhibitory mechanisms of each group of inhibitors vary drastically. Organic acids are released from deacetylation of hemicellulose (for instance, acetic acid) and from breakdown of other furaldehydes (formic and levulinic acids). Organic acids can diffuse through the cytoplasmic membrane and then dissociate, causing intracellular pH reduction to levels poorly tolerated by microorganisms. Also, ATP production can be decoupled by the plasma membrane ATPase, which leads to increased ATP consumption, thus causing a reduction in biomass yield [2, 3, 6–8].

The furaldehydes HMF and furfural are formed by hexose and pentose dehydration, respectively. Furaldehydes act directly on the integrity of cellular membranes causing

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increased lag phase and decreased fermentation rates [9, 10]. The reduction of furaldehydes to their less toxic alcohol-derivatives is recognized as one of the main mechanisms to reduce their inhibitory effects on microbial metabolism [10, 11]. Phenolic compounds are generated from lignin breakdown during pretreatment of lignocellulosic biomass [12]. They lead to loss of membrane integrity, inactivation of enzymatic systems or essential enzymes and inactivation of the genetic material [2, 13, 14]. Phenolic compounds can also be converted to less toxic derivatives by yeast metabolism [15].

Yeasts have been extensively evaluated for the conversion of sugars into fuels and chemicals and understanding their microbial physiology becomes essential to the development of different bioprocesses [16]. *Saccharomyces cerevisiae* is widely used in industrial bioethanol production from sugarcane and corn, and also one of the most engineered yeasts for the production of renewable chemicals [17]. However, *S. cerevisiae* is not able to convert xylose, an abundant sugar in biomass hydrolysates. Thus, some species capable of utilizing xylose have been isolated and described in the literature. *Spathaspora passalidarum*, *S. arborariae*, and *Scheffersomyces stipitis* have shown potential for the production of ethanol from xylose [18–22], whereas species such as *Candida tropicalis*, *Meyerozyma caribbica*, *Spathaspora* sp. JA1, and *Wickerhamomyces anomalus* have shown potential for the production of xylitol [16, 23–27]. Although yeasts are recognized to have a higher tolerance to lignocellulosic inhibitors than filamentous fungi and bacteria, they are also sensitive to inhibitors [10, 28, 29]. In fact, tolerance varies greatly between different yeast species and even among the same species [30–33]. The effects of specific inhibitors have also been shown to be species and strain-dependent, with some strains being more tolerant to organic acids than furaldehydes [14]. However, few comparative studies are found, especially considering a systematic comparison of different strains of *S. cerevisiae* and species of non-*Saccharomyces* yeasts [16, 29, 34, 35].

Understanding the yeast response toward lignocellulosic hydrolysate inhibitors is essential to the development of biomass-based bioprocesses. Therefore, this study aimed to evaluate the tolerance of 7 *Saccharomyces cerevisiae* strains and 7 non-*Saccharomyces* yeasts against 8 inhibitors present in lignocellulosic hydrolysates. Initially, the growth capacity of all strains was evaluated in media supplemented with glucose. Then, the fermentative profile of selected *S. cerevisiae* strains was compared using glucose as a carbon source, while the non-*Saccharomyces* strains were compared in glucose and xylose. The results clearly demonstrate the effects of inhibitory compounds for different yeasts, as well as the yeast most tolerant to various compounds. In addition, the results highlight the higher tolerance of some yeasts to specific compounds.

Material and methods

Yeast strains

Fourteen yeast strains were employed in this work. The 7 *Saccharomyces* strains employed were *S. cerevisiae* CAT-1 (Catanduva—Ribeirão Preto, Brazil) and JP1 (Santa Rita—Paraíba, Brazil), both commercial strains employed in the bioethanol production in Brazil [36–38]. The other 5 *Saccharomyces* strains were previously isolated as contaminants of the industrial bioethanol production in Brazil, and they were selected due to their ability to grow in sugarcane bagasse hydrolysate (unpublished data): *S. cerevisiae* A11, A12, B1.1, G06, and G10. These strains are deposited in the "Collection of Microorganisms and Microalgae Applied to Agroenergy and Biorefining" from EMBRAPA Agroenergia (Brazilian Agricultural Research Corporation—Unit Agroenergy).

Among the non-*Saccharomyces*, 7 yeast strains with efficient xylose metabolism were chosen. Three with respiratory metabolism—*Candida tropicalis* JA2 [26], *Spathaspora* sp. JA1, and *Meyerozyma caribbica* JA9 [27], and 4 with fermentative metabolism—*S. arborariae* Y-48658 [22], *Wickerhamomyces anomalus* 740 [25], and *S. passalidarum* NRRL Y-27907 [21], *Scheffersomyces stipitis* NRRL Y-7124—these last two strains kindly provided by the ARS-NRRL culture collection (Peoria, USA).

Media

Yeast Peptone Dextrose (YPD) synthetic medium (yeast extract 1% w/v, peptone 2% w/v, and glucose 2% w/v) was the standard culture medium for comparisons between *Saccharomyces* and non-*Saccharomyces* yeasts. In addition, YPX (yeast extract 1% w/v, peptone 2% w/v, and xylose 2% w/v) was used in evaluation of non-*Saccharomyces* yeasts. When appropriate, the YPD and YPX medium were supplemented with the inhibitory compounds for evaluation.

Growth assays and analysis

Growth of the 14 yeast strains was evaluated in 200 μL of YPD, pH 5, supplemented or not with lignocellulosic derived-inhibitors as described below. For this, yeasts were recovered from −80 °C stocks, streaked in YPD plates, and used to prepare a pre-culture. The yeasts from a pre-culture grown in 10 mL YPD, at 28 °C, for 16 h were inoculated in 96-well flat-bottomed ELISA microtiter plates to an optical density (OD_{600nm}) of 0.1. Then, the plates were incubated at 28 °C in an oven, without shaking. The yeast growth was followed by regular reading (usually 4 h) of absorbance for 72 h at a wavelength of 600 nm with Epoch 2 equipment (BioTek microplate reader). The experiments were carried out in biological

duplicates and technical triplicates, with positive control (culture medium without the inhibitor) and negative control (culture medium without yeast).

The inhibitors used were organic acids—acetic and formic acids (AA and FA) in concentrations of 1, 5, and 10 g/L; furaldehydes—furfural (FU) 0.5, 1, and 3 g/L and 5-hydroxymethyl-furfural (HMF) in concentrations of 0.5, 1, and 5 g/L; and phenolic compounds—vanillin (VA), syringaldehyde (SE), ferulic acid (FEA), and coumaric acid (CA) in concentrations of 0.5, 0.75, and 1.5 g/L. All compounds were from SIGMA ALDRICH. The concentrations of inhibitors were based in the lowest and highest concentrations reported in different hydrolysates described in the literature, and a third average concentration for comparison purposes [3, 6, 9, 10, 12, 34, 39].

To compare the growth performance of the yeasts in the presence of the inhibitors, a heat map was constructed. Averages and standard deviations of absorbance values were calculated for each yeast in the presence and absence of inhibitors. The OD values obtained in the media without inhibitor were set to 100% (no inhibition) OD $_{600nm}$~1.5 and the growth values measured in the presence of inhibitors were expressed in terms of percentile, e.g., 0% OD $_{600nm}$ < 0.5 (inhibited). The time point of 24 h was used to express the data, as it was the point at which the majority of strains appeared to have overcome the inhibitory effects and/or reaching the final exponential phase.

Fermentation kinetics

Based on the growth assays results, selected yeast strains were collected from agar plates, inoculated in 50 mL of YPD or YPX in Erlenmeyer flasks, and incubated in a rotary shaker (Brand: Thermo Fisher Scientific, model: MAXQ6000) at 28°C and 200 rpm. Cells from precultures in the exponential phase were harvested by centrifugation at 10,000 g for 10 min (Brand: Thermo Fisher Scientific, model: Heraeus Megafuge 16R Centrifuge), collected, and used to start the experiment. For this, 50 mL of YPD or YPX medium, pH adjusted to 5.0, in Erlenmeyer flask of 250 mL was inoculated with cells to an initial OD 600_{nm} ~5. The fermentation experiments were carried out in absence of inhibitors and in the presence of acetic acid 5 g/L, HMF 3 g/L, and vanillin 1.5 g/L. HMF 3 g/L was selected for fermentation because at this concentration, it is possible to observe the inhibitory effects without affecting the total growth of yeasts as occurred in the growth curve on HMF 5 g/L. All fermentation assays were performed in duplicate. Culture samples were also collected for metabolite analysis by ultra/high-performance liquid chromatography (UHPLC/HPLC).

Analytical methods

After fermentation, the samples collected were centrifuged for 5 min at 10,000 g and 250 μL was transferred to a vials tube containing 500 μL of ultrapure water. Extracellular metabolites were quantified by HPLC, using a Waters System (Brand: Waters Acquity UPLC, model: H Class, USA), equipped with an Animex HPX-87H column (300 × 7.8 mm, 9 μm, Bio-rad) coupled to an RI detector to quantify organic compounds; the mobile phase was 5 mM H_2SO_4 used at a flow rate of 0.6 mL/min, at a column temperature of 45 °C for 40 min, with an injection volume of 10 μL [27]. The calibration curves were used to quantify the following metabolites: glucose, xylose, xylitol, glycerol, acetic acid, HMF, vanillin, and ethanol.

The UHPLC 1290 Agilent equipped with Acquity UPLC HSS T3 column (2.1 × 150 mm, 1.8 μm Acquity) coupled to the 280 nm DAD detector for vanillin quantification. The injection volume was 1 μL. The mobile phase A = 0.1% formic acid and B = acetonitrile. Gradient: 0 min (90% A and 10% B), 5 min (80% A and 20% B), 7.5 min (75% A and 25% B), and 12.5, min (55% A and 45% B), at a flow rate of 0.4 mL/min, at a column temperature of 40 °C for 20 min [40].

Cell densities were determined by absorbance measurements at 600 nm and correlated with the dry cell weight. For cell dry weight measurements, the cell suspension samples were centrifuged at 10,000 g for 5 min (Brand: Eppendorf, model: Mini Spin Plus), the supernatant was discarded, and the pellet was washed, dried, and weighed for biomass calculation.

Results

Growth performance of yeasts in the presence of inhibitors

To compare the tolerance of *Saccharomyces* and non-*Saccharomyces* yeasts to 8 different lignocellulose derived-inhibitors, the growth capacity of 14 yeast strains was assayed in three different concentrations of each inhibitor. The concentrations for acetic and formic acids, furfural, HMF, vanillin, syringaldehyde, ferulic, and coumaric acids were based on the lowest and highest concentrations reported in different hydrolysates described in the literature, and a third average concentration for comparison purposes. 7 *S. cerevisiae* strains (*S. cerevisiae* CAT-1, JP1, A11, A12, B1.1, G06, G10) used in or isolated from industrial bioethanol production mills and 7 non-*Saccharomyces* yeasts (*C. tropicalis* JA2, *Spathaspora* sp. JA1, *M. caribbica* JA9, *W. anomalus* 740, *S. stipitis*, *S. passalidarum*, and *S. arborariae*) were chosen due to their biotechnological potential. The *S. cerevisiae* strains are not

able to grow in xylose, so glucose was chosen as the sole carbon source for comparison of all strains evaluated.

The response of each yeast varied drastically according to each inhibitor evaluated, but, commonly, higher concentrations of inhibitors lead to stronger impact on yeast growth (Figs. 1, 2, and 3). All strains were able to grow in the YPD media without inhibitors and entered the stationary phase after 24 h of incubation. Figures 1 and 2 exemplify, respectively, the growth pattern for *Saccharomyces* and non-*Saccharomyces* strains in the presence of acetic acid, whereas Fig. 3 resumes the impact of each inhibitor on yeast growth. For instance, growth of *Saccharomyces* strains was reduced in the presence of 5 g/L of acetic acid and completely abolished at concentration of 10 g/L, whereas non-*Saccharomyces* were sensitive even to the lowest concentration of acetic acid (1 g/L) (Figs. 1 and 2).

Among the three groups of inhibitors, the organic acids (acetic and formic acid) were the most toxic under the evaluated conditions (Fig. 3). Even in the lowest concentration evaluated (1 g/L), both acids partially or completely abolished the growth of some yeasts. From the group of non-*Saccharomyces*, only *C. tropicalis* JA2, *M. caribbica* JA9,

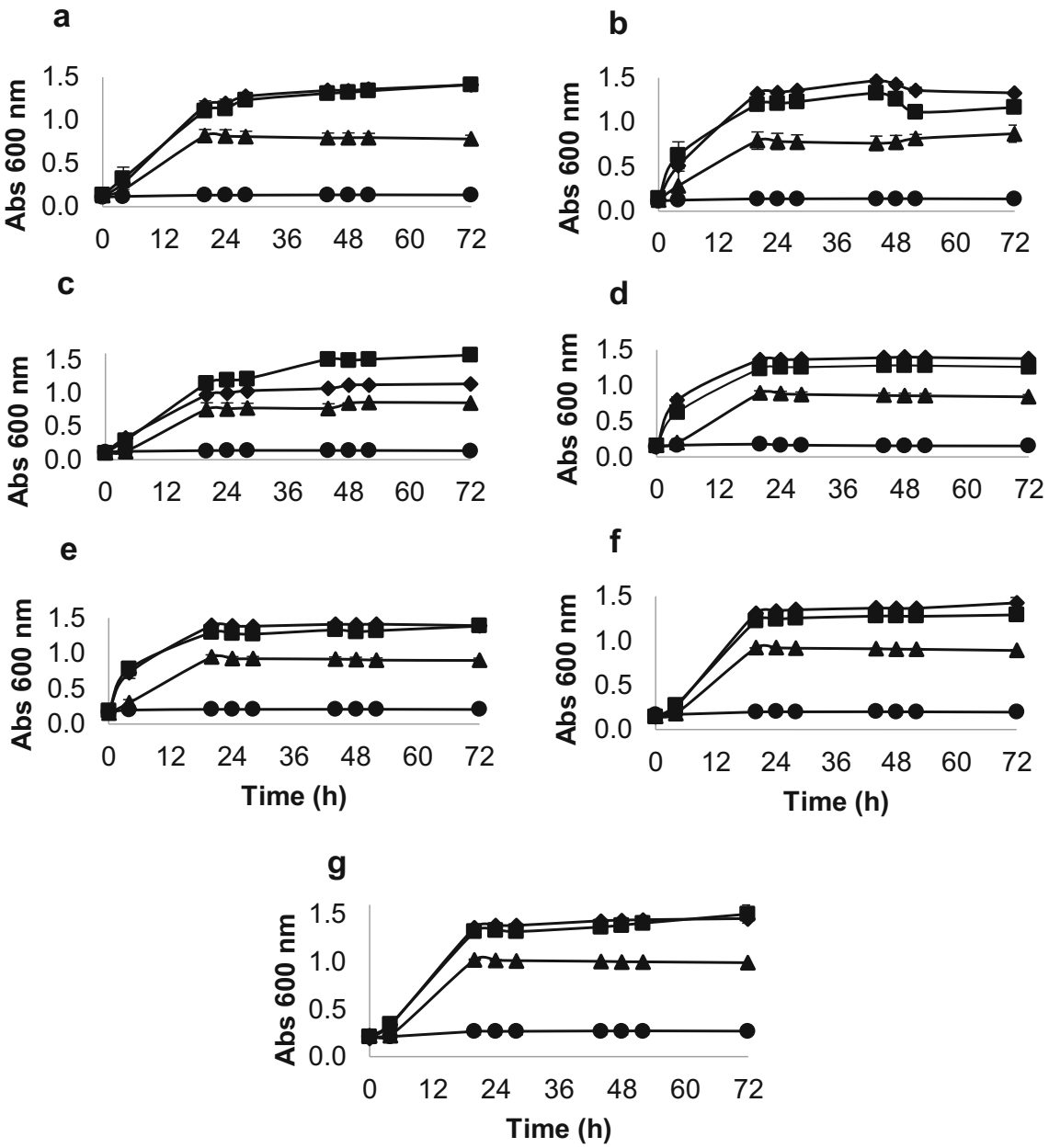

Fig. 1 Growth of *S. cerevisiae* strains in media with and without acetic acid. Growth of **a** *S. cerevisiae* A11, **b** *S. cerevisiae* A12, **c** *S. cerevisiae* JP1, **d** *S. cerevisiae* CAT-1, **e** *S. cerevisiae* B1.1, **f** *S. cerevisiae* G06, **g** *S. cerevisiae* G10, in YPD with or without acetic acid. Acetic acid 1 g/L (■), 5 g/L (▲), and 10 g/L (●), without acetic acid (♦). Results are shown as mean and standard deviation of a minimum of six replicates

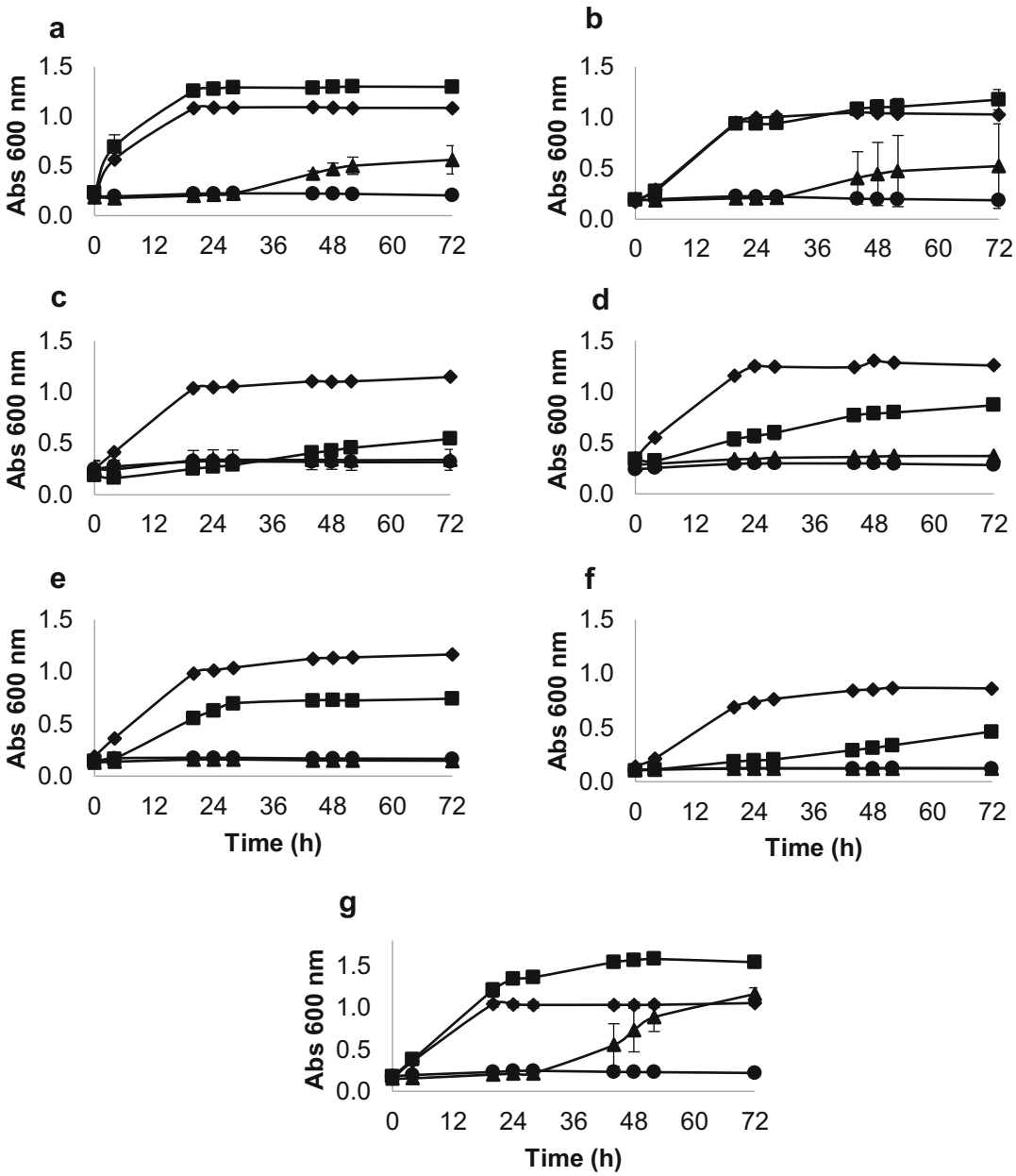

Fig. 2 Growth of non-*Saccharomyces* yeasts in media with and without acetic acid. Growth of **a** *C. tropicalis* JA2, **b** *M. caribbica* JA9, **c** *S. arborariae*, **d** *S. passalidarum*, **e** *S. stipitis*, **f** *Spathaspora* sp. JA1, **g** *W. anomalus* 740, in YPD with and without acetic acid. Acetic acid 1 g/L (■), 5 g/L (▲), and 10 g/L (●), without acetic acid (♦). Results are shown as mean and standard deviation of a minimum of six replicates

and *W. anomalus* 740 did not undergo growth inhibition at the lowest concentration of both acids. All yeasts were strongly inhibited in the presence of the higher concentrations of acids (5 and 10 g/L), except for *Saccharomyces* strains that showed some growth in 5 g/L of acetic acid (Fig. 3). The formic acid (1 g/L equal to × 0.021 mol/L) was more inhibitory than acetic acid (1 g/L equal to × 0.016 mol/L) for all yeasts (Fig. 3).

Furaldehydes showed the lowest impact on yeast growth under the concentrations evaluated (Fig. 3). However, yeasts showed considerable reduced growth (above 80% when compared with the media without inhibitor) when HMF and furfural were present in the highest concentrations of 5 g/L and 3 g/L respectively. Similarly to organic acids, furaldehydes were more inhibitory to non-*Saccharomyces* than to *Saccharomyces* yeasts (Fig. 3). Among the former ones, *C. tropicalis* JA2, *M. caribbica* JA9, and *W. anomalus* 740 were more resistant toward furaldehydes. Among *Saccharomyces* strains, *S. cerevisiae* JP1 was the most resistant.

The group of phenolic compounds showed intermediate inhibitory effects when compared with organic acids and furaldehydes (Fig. 3). Vanillin and syringaldehyde reduced

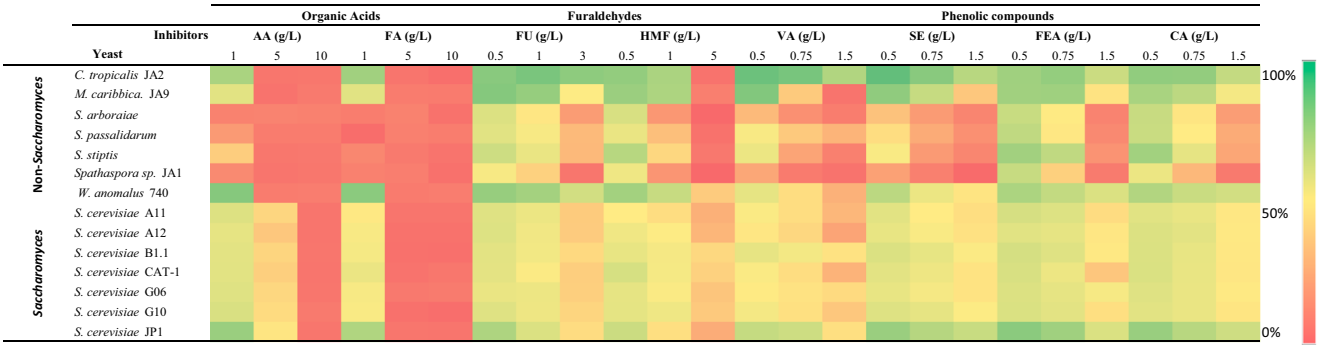

Fig. 3 Heat map showing the inhibitory score of furaldehydes, organic acids, and phenolic compounds on yeast growth. The effect of eight inhibitors at three different concentrations (g/L) on the growth of 14 yeast strains was scored on defined medium supplemented with glucose after 24 h of incubation. All the values were normalized with the growth in media without inhibitor (OD_{600} 24 h–OD_{600} 0 h). ex. 100% (green) is equal to OD 600~1.5 (not inhibited) while 0% (red) is equal to equal to OD 600 < 0.5 (inhibited) AA, acetic acid; FA, formic acid; FU, furfural; HMF, 5-hidroxymetil-furfural; VA, vanillin; SE, syringaldehyde; FEA, ferulic acid; CA, coumaric acid

the yeast growth more strongly than ferulic and coumaric acids for both *Saccharomyces* and non-*Saccharomyces* strains (Fig. 3). The *Saccharomyces* showed to be more tolerant to the four phenolic compounds tested. Non-*Saccharomyces* yeasts showed reduced growth at the three concentrations of vanillin and syringaldehyde evaluated, showing some tolerance only to coumaric and ferulic acids.

In general, the *Saccharomyces* showed more tolerance than non-*Saccharomyces* for all compounds evaluated (Fig. 3), but strains among each group could be differentiated. When comparing the non-*Saccharomyces*, the most tolerant were *C. tropicalis* JA2, *M. caribbica* JA9, *W. anomalus* 740. These yeasts were able to grow in the presence of furfural and HMF (except at the highest concentrations), and at all concentrations of coumaric and ferulic acids and syringaldehyde. The least tolerant yeasts were *S. arborariae*, *S. passalidarum*, *Spathaspora* sp. JA1, and *S. Stipitis* which showed reduced growth at all concentrations of organic acids, at high concentrations of furfural and HMF, and at high concentrations of ferulic and coumaric acids, and at low concentrations of vanillin and syringaldehyde.

Among the *S. cerevisiae*, the strains B1.1, G06, and JP1 appeared as more tolerant, since these yeasts had none or low reduction in growth in the presence of the four phenolic compounds at the three concentrations tested. They were affected only in the presence of high concentrations of acetic acid (10 g/L), formic acid (5 g/L), furfural (3 g/L), and HMF (5g/L). On the other hand, those considered less tolerant were the *S. cerevisiae* A11, A12, G10, and CAT-1 strains that showed sensibility to 1.5 g/L vanillin.

Seven yeasts were chosen for further characterization in terms of tolerance toward three inhibitors: acetic acid, HMF, and vanillin. Acetic acid and HMF were chosen because they are commonly found in the lignocellulosic biomass hydrolysates [10]. The concentration chosen for the fermentation with acetic acid (5 g/L) and HMF (3 g/L) because at these values, they already cause inhibition in both groups of yeasts, but it does not completely stop their performance. Vanillin (1.5 g/L) was chosen because it was the most toxic compound in the phenolic group, and at this concentration, it similarly affected the two groups of yeasts. The six most tolerant yeasts were selected for characterization of the fermentative profile under oxygen-limited conditions: three *Saccharomyces* (*S. cerevisiae* JP1, B1.1, and G06) and three non-*Saccharomyces* (*M. caribbica* JA9, *C. tropicalis* JA2, and *W. anomalus* 740). Finally, the yeast *Spathaspora* sp. JA1 was selected because it was reported as good xylitol producer in sugarcane bagasse hydrolysate [27].

Fermentative performance of *S. cerevisiae* strains in the presence of inhibitors

The fermentative profiles of the three selected strains, *S. cerevisiae* B1.1, JP1, and G06 in the presence of acetic acid (AA), HMF, and vanillin (VA) were evaluated under oxygen-limited conditions in YPD. In the presence of 5 g/L of AA, the three strains slightly increased the ethanol production to around 8.5 g/L, whereas decreased the biomass production in 24 h of fermentation. *S. cerevisiae* G06 and JP1 showed a slightly better glucose consumption than B1.1. After 8 h of fermentation, they consumed around 50 % of the available glucose, while the B1.1 consumed only 23% of the available glucose. Anyway, the strains G06 and JP1 were able to completely consume all the glucose in about 24 h of fermentation while strain B1.1 have glucose residual (Fig. 4).

The three *Saccharomyces* strains aforementioned showed similar fermentative profiles in the presence of HMF (Fig. 4). Likewise, in the presence of AA, the sugar consumption was delayed in the first 8 h of fermentation, but it was completely consumed after 24 h. The production of biomass and ethanol was not modified, but a slight production of glycerol was observed (Fig. 4). The glycerol produced during the initial 8 h fermentation for *S. cerevisiae* G06 was 1.88 g/L compared to 2.4 g/L and 3.5 g/L for JP1 and B1.1, respectively. All these

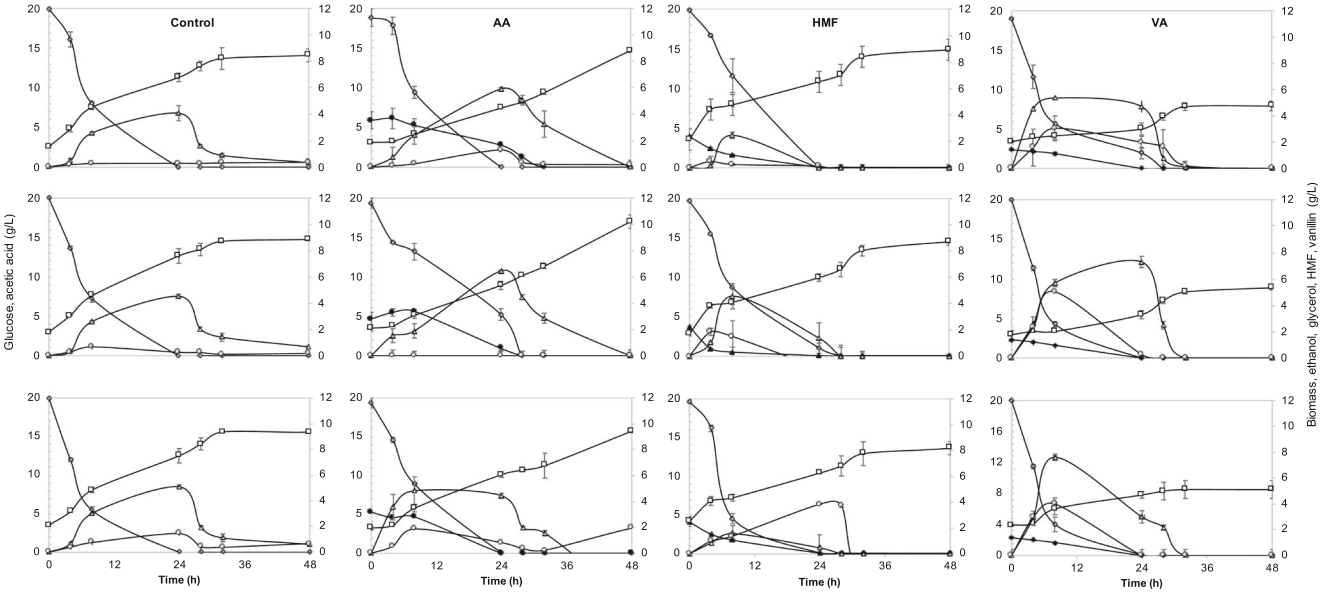

Fig. 4 The fermentative profile of industrial *S. cerevisiae* strains in the presence of inhibitors under aerobic conditions in YPD. Top line: *S. cerevisiae* JP1; middle line: *S. cerevisiae* B1.1; bottom line: *S. cerevisiae* G06. From left to right: first column: control (no inhibitor present in the medium); second column: acetic acid 5 g/L; third column: HMF 3 g/L; fourth column: vanillin 1.5 g/L. Glucose (◊); biomass (□); glycerol (○); ethanol (Δ); HMF (▲); vanillin (♦); acetic acid (●). Results are shown as mean and standard deviation of biological duplicate

three *S. cerevisiae* strains can convert all HMF present in the medium within about 8 h of fermentation, presenting similar tolerance to this compound (Fig. 4).

The vanillin reduced sugar consumption and final biomass production for all three strains (Fig. 4). However, the ethanol production was less affected by the inhibitor, with the *S. cerevisiae* G06, JP1, and B1.1 producing 2.9 g/L, 5.3 g/L, and 5.2 of ethanol in 8 h fermentation. The 3 strains were able to convert all VA present in the medium and produce ethanol in 24 h (Fig. 4).

Fermentative performance of non-*Saccharomyces* strains in the presence of inhibitors

The fermentation performance of non-*Saccharomyces C. tropicalis* JA2, *M. caribbica* JA9, and *Spathaspora* sp. JA1 *e W. anomalus* 740 was evaluated in the presence of AA 5 g/L, HMF 3 g/L, and VA 1.5 g/L using glucose as the sole carbon source. In the absence of inhibitors, non-*Saccharomyces* yeasts consumed all glucose in the media and produced mainly biomass, ethanol, and glycerol. The presence of AA inhibited sugar metabolism and growth of all yeast strains. However, *C. tropicalis* JA2 and *W. anomalus* 740 were able to present glucose consumption and growth after 8 h of incubation (Fig. 5). These two strains were capable of consuming all glucose and the AA present in the medium within the first 48 h of fermentation. Despite the reduced sugar consumption rate, both strains showed similar production of metabolites when compared with fermentation without inhibitors. On the other hand, *Spathaspora* sp. JA1 and *M. caribbica* JA9 showed a very slow sugar consumption, and they were not able to consume the glucose even after 72 h of fermentation (Fig. 5).

In the presence of HMF, all non-*Saccharomyces* yeasts have shown a detoxification capacity as they converted all the HMF within approximately 8 h of fermentation (Fig. 5). Like *Saccharomyces* yeasts, non-*Saccharomyces* yeasts were not inhibited by 3 g/L HMF under the conditions tested. The aerobic growth of the yeasts at the beginning of the fermentation might have helped the fast detoxification of this compound. Thus, sugar consumption and product formation were not affected by HMF (Fig. 5).

When in the presence of VA, only *C. tropicalis* JA2 did not show reduced biomass formation (Fig. 5). The reduced biomass formation of yeasts *Spathaspora* sp. JA1, *M. caribbica* JA9, and *W. anomalus* 740 was correlated with the delay in glucose consumption, which took about 36 h. Even if the VA was converted within approximately 12 h of fermentation, only *C. tropicalis* JA2 was capable to maintain ethanol production and biomass formation at levels comparable to the control (Fig. 5).

The fermentation performances of *C. tropicalis* JA2, *M. caribbica* JA9, *Spathaspora* sp. JA1, and *W. anomalus* 740 in the presence of AA 5 g/L, HMF 3 g/L, and VA 1.5 g/L were also evaluated using xylose as the sole carbon source. Similarly, to what was observed when using glucose as carbon source, acetic acid strongly inhibited the yeasts, but not HMF and vanillin. In the presence of AA, only *C. tropicalis* JA2 showed xylose consumption and produced mainly biomass and xylitol during the cultivation, whereas the yeasts

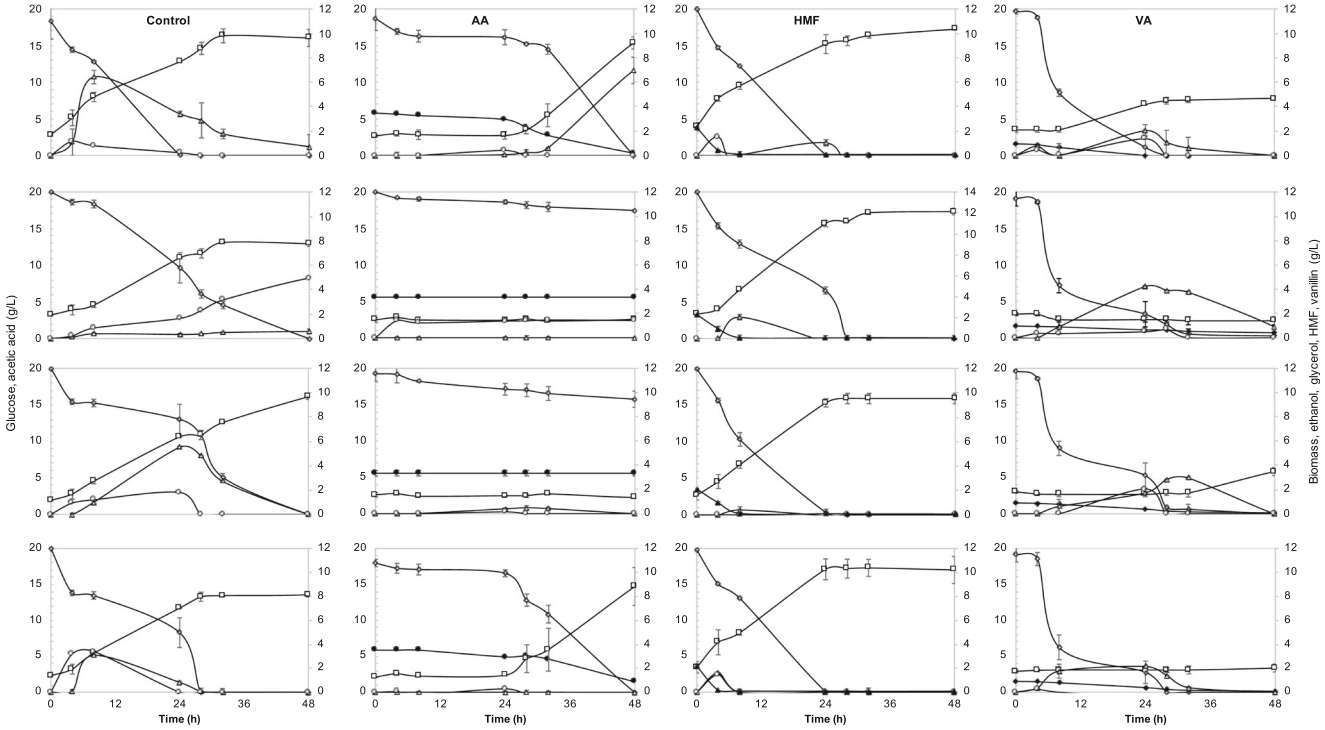

Fig. 5 The fermentative profile of non-*Saccharomyces* yeasts in the presence of inhibitors using glucose as sole carbon source. First line (from top to bottom): *C. tropicalis* JA2; middle line: *Spathaspora* sp. JA1; third line: *M. caribbica* JA9; fourth line: *W. anomalus* 740. First column: control (no inhibitor present in the medium); second column: acetic acid 5 g/L; third column: HMF 3 g/L; fourth column: vanillin 1.5 g/L. Glucose (◊); biomass (□); glycerol (○); ethanol (Δ); HMF (▲); vanillin (♦); acetic acid (●). Results are shown as mean and standard deviation of biological duplicate

Spathaspora sp. JA1, *M. caribbica* JA9, and *W. anomalus* 740 were not able to consume xylose (Fig. 6), even when the incubation was performed for 48 h (data not shown). In presence of HMF and vanillin, the non-*Saccharomyces* yeasts were able to convert the inhibitors present in the medium and showed similar xylose consumption profile to the fermentation without inhibitor (Fig. 6). Similar to the cultivation without inhibitors, *C. tropicalis* JA2 was able to complete consume the xylose in around 26 h, whereas *Spathaspora* sp. JA1, *M. caribbica* JA9, and *W. anomalus* 740 were not able to complete xylose consumption at the same time. In all cases, biomass and xylitol were the main products of all cultivations on xylose (Fig. 6).

Discussions

The isolated effects of organic acids (acetic and formic acids), furaldehydes (HMF and furfural), and phenolic compounds (vanillin, syringaldehyde, ferulic, and coumaric acids) present in the lignocellulosic hydrolysates on the physiology of seven *S. cerevisiae* and seven non-*Saccharomyces* yeasts were compared for the first time in this study. The results clearly demonstrated a dose-dependent response of the 7 *S. cerevisiae* strains (*S. cerevisiae* CAT-1, JP1, A11, A12, B1.1, G06, G10) and the 7 non-*Saccharomyces* yeasts (*C. tropicalis* JA2, *Spathaspora* sp. JA1, *M. caribbica* JA9, *W. anomalus* 740, *S. stipitis*, *S. passalidarum*, and *S. arborariae*) to the inhibitors, and the higher tolerance of *Saccharomyces* strains against the eight inhibitors evaluated. The dose-dependent response of yeasts toward lignocellulosic inhibitors has been demonstrated especially for *S. cerevisiae* and *S. stipitis* strains [10, 33]. However, few studies have evaluated and compared the tolerance of other yeasts. When Pandey and co-workers compared tolerance of several strains of *S. cerevisiae*, *K. marxianus*, *S. stipitis*, *C. sheatae*, *C. lusitaniae*, *C. albicans*, *W. anomalus*, *O. thermophile*, *C. glabrata*, *P. kudriavzevii*, *C. dubliniensis*, and *C. tropicalis* toward furfural, HMF, acetic acid, and ethanol, a strain of *S. cerevisiae* stood out as the most promising for fermentation of lignocellulosic biomass [29]. These results confirmed our own reported in the present study, which showed the stronger capacity of *Saccharomyces* strains to withstand lignocellulosic hydrolysate inhibitors. Other studies compared the tolerance of one or few yeast strains to specific inhibitors or to lignocellulosic hydrolysates [10, 32, 33, 39, 41–43].

Even though *Saccharomyces* showed higher tolerance toward inhibitors than non-*Saccharomyces*, there were significant differences among the strains evaluated in this study. Species-specific variations in tolerance to acetic acid, furaldehydes, and hydrolysates have been shown previously [10, 44]. The *Saccharomyces* strains *S. cerevisiae* G06, B1.1,

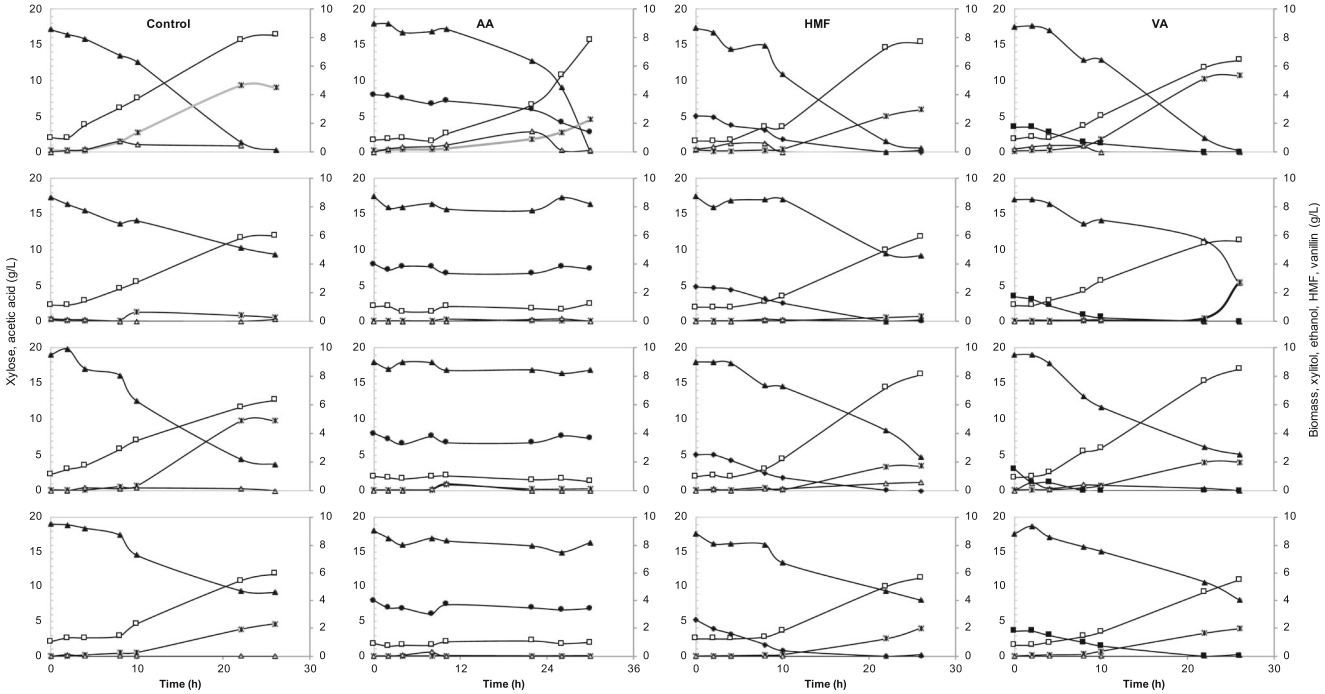

Fig. 6 The fermentative profile of non-*Saccharomyces* yeasts in the presence of inhibitors using xylose as sole carbon source. First line (from top to bottom): *C. tropicalis* JA2; middle line: *Spathaspora* sp. JA1; third line: *M. caribbica* JA9; fourth line: *W. anomalus* 740. First column: control (no inhibitor present in the medium); second column: acetic acid 5 g/L; third column: HMF 3 g/L; fourth column: vanillin 1.5 g/L. Glucose (▲); biomass (□); xylitol (x), ethanol (Δ); acetic acid (●), HMF (♦); vanillin (■). Results are shown as mean and standard deviation of biological duplicate

and JP1 showed the best tolerance in relation to the other four ones evaluated. These three strains showed no or low reduction in growth in the presence of the four phenolic compounds at the three concentrations tested, and relatively low impact with acetic acid and furaldehydes. *S. cerevisiae* JP1 is an industrial strain commonly used in the bioethanol production in Brazil [37], and the tolerance required under these conditions—including acid wash, pH variance, contaminant competitors, and natural defense with glycerol production [37, 45]—may favor the cross tolerance to other stresses, like the ones imposed by lignocellulosic-derived inhibitors. Since *S. cerevisiae* G06 and B1.1 strains were isolated as contaminants in industrial mills, they may also have been selected for robustness. Indeed, these three strains showed very similar response to the inhibitors evaluated (Fig. 3). Similar results have been shown for *Saccharomyces* isolated from adapted strains in the presence of acetic acid, showing variable tolerances, including strains capable to withstand concentrations of acetic acid as high as 9 g/L [44].

Among the non-*Saccharomyces* yeasts, *C. tropicalis* JA2, *M. caribbica* JA9, and *W. anomalus* 740 have shown the better growth performance in microtiter evaluation. However, when the fermentative performances of these three strains were evaluated, *M. caribbica* JA9 showed reduced rates of glucose consumption and product formation in the presence of acetic acid, contrary to the other two. These results may be explained by the isolation and selection of *C. tropicalis* JA2 and *W. anomalus* 740 based on growth in lignocellulosic hydrolysate rich in acetic acid, whereas *M. caribbica* JA9 was selected by growth in synthetic media [25–27, 29]. Despite the similar performance of *C. tropicalis* JA2 and *W. anomalus* 740 on glucose in presence of acetic acid, when cultivated on xylose, only *C. tropicalis* was able to consume this sugar and to grow. These results corroborate the efficiency of xylose consumption by *C. tropicalis* even in presence acetic and previous observations that xylose uptake is more inhibited than glucose uptake in presence of inhibitors [26, 29, 46].

Scheffersomyces stipitis, *Spat. passalidarum*, *Spat. arborariae*, and *Spathaspora* sp. JA1 were more affected by the inhibitors, showing little growth in microtiter plates. Despite these results are in good agreement with previous reports that showed strong inhibition of *Spat. passalidarum* and *Schef. stipitis* by acetic acid and furfural [46], other reports have indicated that *S. stipitis* and *S. passalidarum* have good fermentative performances in hydrolysates and single inhibitor compounds [41, 42, 47, 48], but direct comparisons were not performed. As experimental variations, like inoculum size, sugar, and inhibitor concentrations, cause direct interferences in the performance of the yeasts [3, 46, 49], the unique comparison carried out in this study highlighted the robustness of *C. tropicalis* JA2 toward lignocellulosic-derived inhibitors.

Considering the concentrations of inhibitors usually found in biomass hydrolysates and used in this study, among the

eight inhibitors evaluated, for *Saccharomyces* yeasts, acetic and formic acids were the most toxic ones, while coumaric and ferulic acids were the least toxic. In addition to acetic and formic acids, non-*Saccharomyces* yeasts also showed some sensibility to HMF and vanillin. These results corroborate previous observations that *Candida tropicalis* and *Pichia stipitis* are more tolerant to furaldehydes (furfural) than to formic and acetic acid [50]. HMF is known to prolong lag phase of yeast and reduce fermentation rate cultivation [49], but under the conditions employed in this work (relatively initial high cell density and oxygen limited conditions), all the yeasts were able to convert this inhibitor and restore the fermentation rate that is in good agreement with the physiological effects of yeast in presence of HMF [49]. Despite the fact that non-equimolar concentrations of inhibitors were employed in this study, these results indicated important thresholds for yeast performance for each inhibitor. As the composition of hydrolysates are variable and dependent of the biomass and processes employed in their pretreatments and hydrolysis [3], these results may indicate desirable chemical profile of hydrolysates to be obtained.

Conclusion

This work showed the dose-dependent response of yeasts toward eight different lignocellulosic-derived inhibitors. Among the fourteen yeasts compared, *S. cerevisiae* strains showed higher tolerance, than non-*Saccharomyces*, but even among them, some strains with the highest tolerances could be identified. Among seven species of non-*Saccharomyces*, *C. tropicalis* JA2 and *W. anomalus* 740 appeared as the most tolerant, whereas *Spathaspora* strains appeared very sensitive to the different compounds. These results may be related with the processes of isolation and selection of such yeasts. A careful comparison of the mechanistic responses to the inhibitors should be carried out in the future to explain the performances obtained in this study.

Code availability Not applicable

Author contribution All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by Carlos E. V. F. Soares and Jessica C. Bergamann. The first draft of the manuscript was written by Carlos Soares and João R. M. Almeida and all authors commented on the previous of the manuscript. All authors read and approved the final manuscript.

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Data Availability Not applicable

Declarations

Ethics approval and consent to participate This article does not contain any studies with human participants or animals performed by any of the authors

Consent to participate Not applicable.

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Competing interests The authors declare no competing interests.

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