3 Biotech (2018) 8:119
https://doi.org/10.1007/s13205-018-1143-0

ORIGINAL ARTICLE

Single and combined effects of acetic acid, furfural, and sugars on the growth of the pentose-fermenting yeast *Meyerozyma guilliermondii*

Michelle dos Santos Cordeiro Perna[1] · Reinaldo Gaspar Bastos[1] · Sandra Regina Ceccato-Antonini[1]

Received: 3 November 2017 / Accepted: 30 January 2018 / Published online: 7 February 2018

Abstract
The tolerance of the pentose-fermenting yeast *Meyerozyma guilliermondii* to the inhibitors released after the biomass hydrolysis, such as acetic acid and furfural, was surveyed. We first verified the effects of acetic acid and cell concentrations and initial pH on the growth of a *M. guilliermondii* strain in a semi-synthetic medium containing acetic acid as the sole carbon source. Second, the single and combined effects of furfural, acetic acid, and sugars (xylose, arabinose, and glucose) on the sugar uptake, cell growth, and ethanol production were also analysed. Growth inhibition occurred in concentrations higher than 10.5 g l^{-1} acetic acid and initial pH 3.5. The maximum specific growth rate (μ) was 0.023 h^{-1} and the saturation constant (ks) was 0.75 g l^{-1} acetic acid. Initial cell concentration also influenced μ. Acetic acid (initial concentration 5 g l^{-1}) was co-consumed with sugars even in the presence of 20 mg l^{-1} furfural without inhibition to the yeast growth. The yeast grew and fermented sugars in a sugar-based medium with acetic acid and furfural in concentrations much higher than those usually found in hemicellulosic hydrolysates.

Keywords Biological detoxification · Cellulosic ethanol · Yeasts · Acetic acid · Pentoses · Furfural

Introduction

The shortage of energy has stimulated the research on the use of plant biomass for fuel production. Hemicelluloses are heteropolymers of pentoses and hexoses that can be fractionated to release these sugars for the production of the second-generation ethanol (Raele et al. 2014). However, not only sugars result from the hydrolysis but also toxic compounds as furan derivatives (furfural and hydroxymethylfurfural), phenolic compounds and weak organic acids (levulinic and acetic acids) are released. These by-products of the acid hydrolysis affect the microbial growth and the conversion of sugars to the value-added products as xylitol and ethanol (Palmqvist and Hahn-Hagerdal 2000; Mussatto and Teixeira 2010).

✉ Sandra Regina Ceccato-Antonini
antonini@ufscar.br

[1] Laboratory of Molecular and Agricultural Microbiology, Dept Tecnologia Agroindustrial e Sócio-Economia Rural, Centro de Ciências Agrárias, Universidade Federal de São Carlos, P.O. Box 153, Araras, São Paulo State 13600-970, Brazil

Acetic acid is a product of the deacetylation of the hemicellulose fraction and its toxicity is dependent on the pH. The undissociated form passively enters the yeast cell and the degree of dissociation depends on the intracellular pH. As a result, the transportation of protons across the cell membrane requires ATP (Verduyn et al. 1990). It causes negative effects on biomass production and ethanol yield (Bellissimi et al. 2009). One of the major problems to be solved to make the second-generation ethanol economically viable is the removal of acetic acid from the hydrolysates (Wei et al. 2013). The utilization of yeasts to promote the biological detoxification is a meaningful strategy towards the fermentation of lignocellulosic hydrolysates.

Furfural is a furaldehyde resulting from the degradation of five-carbon sugars. It is inhibitory to yeast growth and fermentation and its action is dose-dependent. It affects glycolytic activity and the tricarboxylic acid cycle, causing oxidative stress and decrease in enzyme activity of dehydrogenases mainly (Antal et al. 1991; Horvath et al. 2003; Almeida et al. 2007). Some yeast species can reduce furfural to furfuryl alcohol, a less toxic compound but a pathway that compromises the formation of NADH is utilized (Zhao et al. 2005). This important co-factor protects the cells against the damage

caused by reactive species as hydrogen peroxide, superoxides, and hydroxides anions, which can be accumulated and cause several damages to the cell (Perrone et al. 2008; Allen et al. 2010). Although furfural itself is a severe inhibitor of yeast metabolism, the presence of other inhibitor as acetic acid maximizes the negative effect (Palmqvist and Hahn-Hagerdal 2000).

The detoxification of acid hydrolysates by yeasts is restricted to a few species. *Issatchenkia occidentalis*, *Candida guilliermondii*, *Saccharomyces cerevisiae*, *Candida shehatae, Debaryomyces hansenii, Scheffersomyces stipitis, Zygosaccharomyces bailii,* and *Dekkera bruxellensis* have been reported to grow in media with inhibitors as acetic acid, furfural and 5-HMF (Sousa et al. 1996, 1998; Palmqvist et al. 1999; Carvalheiro et al. 2005; Rodrigues et al. 2006; Fonseca et al. 2011; Nogué et al. 2012; Gonçalves et al. 2013; Ma et al. 2013; Hanly and Henson 2014; Capusoni et al. 2016; Moktaduzzaman et al. 2016; Senatham et al. 2016).

Meyerozyma guilliermondii, an ascomycetous species belonging to the Saccharomycotina CTG clade, is the teleomorph of *Candida guilliermondii* and considered as *Pichia guilliermondii* until 2010, when another species name was assigned by Kurtzman and Suzuki (2010). A widely spread yeast in the environment, *M. guilliermondii* has displayed several characteristics of interest to biotechnology, such as production of riboflavin, ethanol, industrial enzymes, and xylitol, as reviewed by Papon et al. (2013). The potential use of *M. guilliermondii* isolates in the biological control of phytopathogens has emerged as an alternative to classical fungicide treatments (Zhang et al. 2011; Lima et al. 2012).

The previous works have showed that strains of *M. guilliermondii* were able to grow in xylose-containing media with acetic acid to produce ethanol and xylitol (Silveira 2014; Martini et al. 2016). However, the growth kinetics and the pattern of acetic acid consumption in the presence of sugars and other inhibitors were not evaluated yet. These characteristics are of capital importance for the context of the second-generation ethanol, because hemicellulosic hydrolysates contain sugars and other substances that may act as substrates or inhibitors. In this context, this work aimed to contribute to the study of factors that affect the acetic acid consumption (initial pH, initial cell concentration, acetic acid and furfural concentrations, and presence of sugars) and its conversion to biomass and ethanol in a strain of *M. guilliermondii* isolated from sugarcane juice.

Methods

Yeast strain and inoculum preparation

A strain of *M. guilliermondii* (CCT7783, deposited at Centro de Culturas Tropical—Fundação André Tosello, Campinas, São Paulo State, Brazil) was utilized in the experiments. It was isolated from sugarcane juice and identified according to the procedures described in Martini (2014). The yeast culture was maintained on YPD (10 g l^{-1} yeast extract, 20 g l^{-1} glucose, 20 g l^{-1} peptone, 20 g l^{-1} agar; for broth, agar was not added to the nutrient solution) slants at 4 °C with regular transfers to new medium. The inoculum was prepared by transferring two loops of cells to 50 mL of YPD broth and incubating overnight at 30 °C under agitation of 160 rpm. The cell mass was centrifuged at 580 g for 5 min, washed with sterile distilled water, and resuspended in YPD broth. The flasks were maintained under the same conditions described before, and the conditions of centrifugation, washing, and suspension were repeated twice.

Growth assays in semi-synthetic medium with acetic acid as the sole carbon source

The growth medium consisted of 5 g l^{-1} potassium dihydrogen phosphate, 1 g l^{-1} potassium chloride, 1.5 g l^{-1} ammonium chloride, 1 g l^{-1} magnesium sulphate heptahydrate, 6 g l^{-1} yeast extract (Martini et al. 2016), and acetic acid as the sole carbon source at concentrations of 1.5; 4.5; 10.5; 13.5; 16.5; and 19.5 g l^{-1}. The initial mass cell was about 0.2 g l^{-1} and it was determined by the conversion of absorbance values obtained at 600 nm (A_{600nm}) in a *Bio-Mate*® spectrophotometer utilizing a calibration curve 'absorbance versus dried biomass' (cell mass dried at 105 °C until a constant weight was achieved). The calibration curve was: Biomass (g l^{-1}) = A_{600nm} − 0.119/1.6927.

The initial pH was adjusted to 5.5 (with 12 mol l^{-1} NaOH solution) aseptically after the addition of the acetic acid to the Erlenmeyer flasks containing the nutrient solution. The inoculated flasks (500-mL Erlenmeyer flasks with a 100-mL final volume of medium, in duplicate) were maintained at 30 °C, 160 rpm, for 96 h. Samples were removed every 12 h for analysis.

Another set of experiments was performed to evaluate the effect of initial pH and initial cell concentration on the growth of the yeast. The growth medium was prepared as above, with 10.5 g acetic acid l^{-1} as the sole carbon source. Two initial pH values (3.5 and 5.5) and three initial cell concentrations (0.2, 0.5, and 2.5 g l^{-1}) were assayed in an incomplete factorial experiment 2 × 3. The yeast was cultivated under the same conditions of temperature and shaking described before. Samples were removed every 12 h for analysis.

Fermentation assays in semi-synthetic medium with sugars, acetic acid and furfural

The fermentation medium consisted of 5 g l^{-1} potassium dihydrogen phosphate, 1 g l^{-1} potassium chloride, 1.5 g l^{-1}

ammonium chloride, 1 g l^{-1} magnesium sulphate heptahydrate, 6 g l^{-1} yeast extract (Martini et al. 2016), 3 g l^{-1} glucose, 3 g l^{-1} arabinose, and 25 g l^{-1} xylose. Acetic acid and furfural were added to final concentrations of 5 and 10 g l^{-1}, and 20 and 40 mg l^{-1}, respectively. The experiments were performed in a factorial design of 3^3 in triplicates (also considering the treatments in which acetic acid and furfural were not added). The initial mass cell was about 2.5 g l^{-1} and it was determined by the conversion of absorbance values obtained at 600 nm as described before. The initial pH was adjusted to 5.5 (with 12 mol l^{-1} NaOH solution) aseptically after the addition of the acetic acid to the Erlenmeyer flasks containing the nutrient solution and furfural. The inoculated flasks (500 mL Erlenmeyer flasks with a 200 mL final volume of medium, in triplicate) were maintained at 30 °C, 160 rpm, for 96 h. Samples were removed every 12 h for analysis.

Analytical methods

Biomass (g l^{-1}) was determined by the conversion of absorbance values obtained at 600 nm as described before. Then, the samples were centrifuged at 580 g for 5 min and in the cell-free samples, pH was determined with a digital pH-meter, and reducing sugars was determined by the 3,5-dinitrosalycilic acid method (Miller 1959) using a calibration curve 'absorbance versus xylose concentration'. The calibration curve was: Reducing sugars (g l^{-1}) = Abs_{540nm} + 0.0490/0.8061. The xylose concentration was in the range of 0.15 a 1.5 g l^{-1}.

The glucose concentration was analysed by the enzymatic kit GOD-PAP Laborlab® based on the glucose oxidase reaction. The concentration of pentoses (xylose + arabinose) was determined by the difference between the reducing sugar concentration and glucose concentration.

The samples were filtered using a membrane filter porosity 0.45 µm (Analitica®). Ethanol and acetic acid were quantified in a gas chromatograph (GC 2010 Plus, Shimadzu®) equipped with a flame ionization detector (FID), using a Restek Stabilwax-DA column ($30 \times 0.25 \times 0.25$ mm^3) in the following conditions: injector temperature: 220 °C; carrier gas: helium; linear rate of the carrier gas: 20 cm s^{-1}; split rate: without split; FID temperature: 280 °C; column temperature: 30 °C for 5 min, increment of 12 °C per min up to 100 °C, following increment of 15 °C per min up to 165 °C, and increment of 80 °C up to 230 °C for 6 min.

Calculation of kinetics parameters

Ethanol and biomass yields (g g^{-1}) were calculated based on the ratio of the product concentration (maximum concentration along 96 h for ethanol; and variation after 96 h for biomass) to the substrate consumed (the sum of reducing sugars and acetic acid). Maximum specific growth rate (h^{-1}) was the slope by plotting 'Ln biomass vs. time' in the exponential phase of the yeast growth. Using the initial concentration of acetic acid (g l^{-1}) as substrate plotted against the correspondent specific growth rates (h^{-1}), the substrate saturation constant (k_S) and the maximum specific growth rate (h^{-1}) were calculated, applying the linearized Monod equation (Rao 2010). The rate of acetic acid consumption was calculated by the variation in the concentrations of the acetic acid along the time with and without furfural by utilizing regression analysis.

Results

Growth assays in semi-synthetic medium with acetic acid as the sole carbon source

The growth of *M. guilliermondii* in semi-synthetic medium containing acetic acid as the sole carbon source is demonstrated in Fig. 1. The increase in acetic acid concentration favoured the biomass production until 10.5 g l^{-1}; after that, the biomass accumulation was stable (Fig. 1a). The low growth in the highest concentrations did not alter the medium pH, but it increased significantly in concentrations ranging from 1.5 to 10.5 g l^{-1} (Fig. 1b) due to the total acetic acid consumption (Fig. 1c), which decreased further. The highest specific growth rates were observed in concentrations ranged from 4.5 to 19.5 g l^{-1} (Fig. 1d).

The maximum specific growth rate and the saturation constant (ks) were defined by Monod Equation as 0.023 h^{-1} and 0.75 g l^{-1}, respectively (Fig. 1d).

The effects of initial pH and cell concentration are depicted in Table 1. The decrease of pH from 5.5 to 3.5 inhibited totally the yeast growth both with 0.5 and 2.5 g l^{-1} initial cell concentration, but there was acetic acid consumption. With initial pH 5.5, the maximum specific growth rate was much higher with 0.5 g initial cell concentration l^{-1} than with 0.2 or 2.5 g l^{-1} cell concentration, with almost acetic acid depletion (Table 1).

Maximum ethanol production (1.47 g l^{-1}) was obtained in conditions of 10.5 g l^{-1} acetic acid, pH 5.5, 2.5 g l^{-1} initial cell concentration, and 96 h of incubation. In the other conditions, no measurable ethanol was detected.

Fermentation assays in semi-synthetic medium with sugars, acetic acid, and furfural

The effects of acetic acid on the growth and fermentation of *M. guilliermondii* were also evaluated in medium with xylose, arabinose and glucose, in the presence of furfural, at pH 5.5 and 2.5 g l^{-1} initial cell concentration. The addition of acetic acid decreased the ethanol yield. The biomass yield

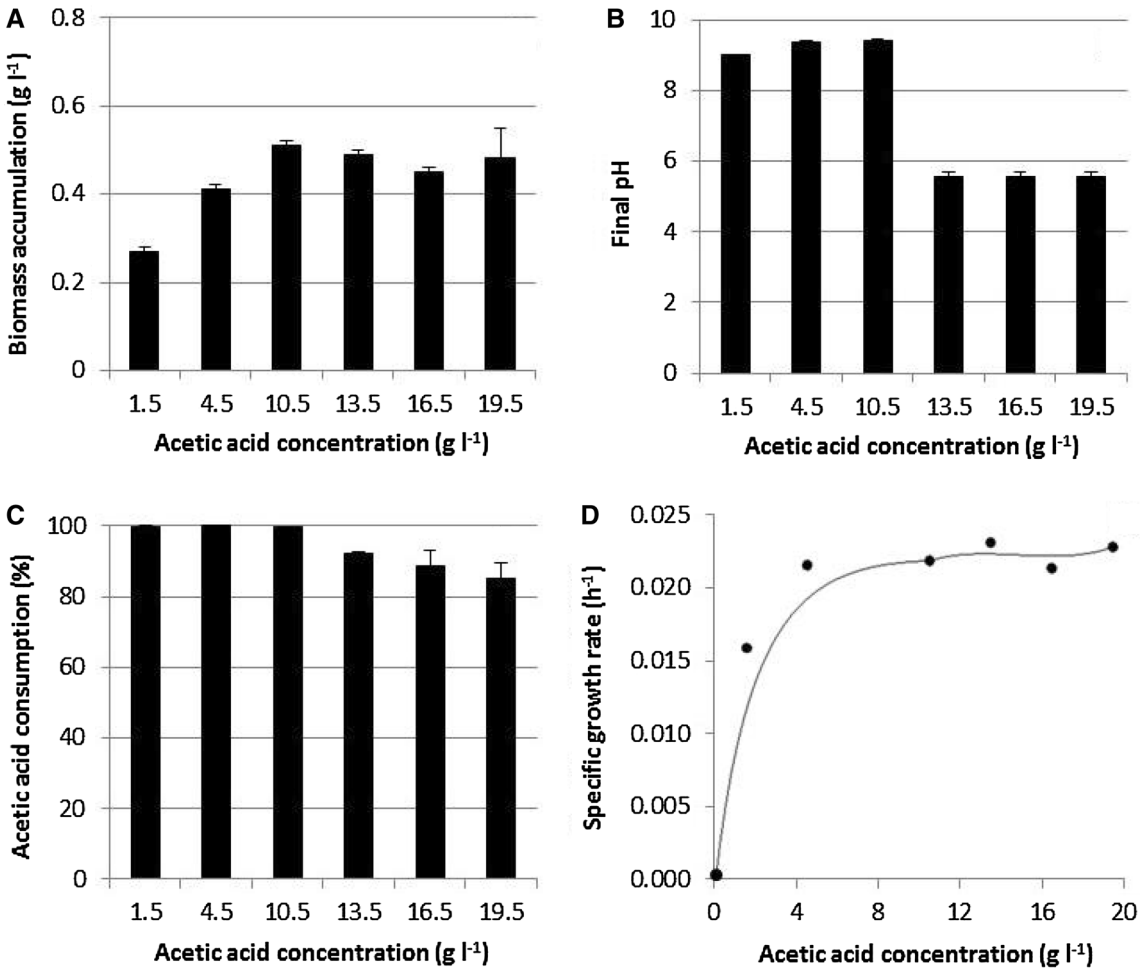

Fig. 1 Effects of acetic acid concentration on the growth of *M. guilliermondii* (**a**), final pH (**b**), acetic acid consumption (**c**), and specific growth rate (**d**) in semi-synthetic medium containing acetic acid as the sole carbon source in concentrations ranging from 1.5 to 19.5 g l^{-1}, at 30 °C, 160 rpm, initial pH 5.5, and initial cell concentration 0.2 g l^{-1}, after 96 h

Table 1 Effects of the initial pH and initial cell concentration on the growth of *M. guilliermondii* in semi-synthetic medium containing 10.5 g acetic acid l^{-1} as the sole carbon source, at 30 °C, 160 rpm, initial pH 3.5 or 5.5, and initial cell concentration of 0.2, 0.5, and 2.5 g l^{-1}, after 96 h

Initial pH	Initial cell concentration (g l^{-1})	Biomass accumulation (g l^{-1})[1]	Maximum specific growth rate (μ, h^{-1})	Final pH	Acetic acid consumption (%)
3.5	0.5	0	0	3.47 ± 0.08	79.1 ± 1.6
	2.5	0	0	3.40 ± 0.03	75.7 ± 8.7
5.5	0.2	0.51 ± 0.01	0.021 ± 0.001	9.42 ± 0.02	99.6 ± 0.2
	0.5	3.72 ± 0.27	0.046 ± 0.001	9.74 ± 0.01	96.8 ± 1.1
	2.5	4.55 ± 0.23	0.013 ± 0.001	9.65 ± 0.14	99.7 ± 0.1

[1] Difference between the biomass values at 96 h and 0 h of incubation

and specific growth rate were also reduced when the concentration increased from 5 to 10 g l^{-1} acetic acid. When furfural was concomitantly added to the fermentation medium with sugars, the decrease in yields and growth rate was even higher. The increase in the acetic acid concentration did not result in a higher ethanol yield (Table 2).

The substrate consumption and ethanol titers are demonstrated in Fig. 2. The consumption of glucose was almost completed by 24 h of cultivation regardless the acetic acid concentration and with 20 mg l^{-1} furfural (Fig. 2b, e, and h). However, with 40 mg l^{-1} furfural, glucose consumption was slower even when no acetic acid was added to the medium.

Table 2 Effects of acetic acid and furfural on the growth and yields of *M. guilliermondii* in semi-synthetic medium containing glucose (3 g l^{-1}), arabinose (3 g l^{-1}), xylose (25 g l^{-1}), acetic acid (0, 5 or 10 g l^{-1}) and furfural (0, 20 and 40 mg l^{-1}), at 30 °C, 160 rpm, initial pH 5.5, and initial cell concentration 2.5 g l^{-1}, for 96 h

Acetic acid (g l^{-1})	Furfural (mg l^{-1})	Biomass accumulation (g l^{-1})[a]	Specific growth rate (μ, h^{-1})	Final pH[b]	$Y_{p/s}$ (g g^{-1})[c]	$Y_{x/s}$ (g g^{-1})[d]
0	0	5.44 ± 0.91	0.011 ± 0.001	5.30 ± 0.22	0.14 ± 0.01	0.22 ± 0.04
	20	6.90 ± 0.21	0.012 ± 0.001	5.46 ± 0.20	0.06 ± 0.01	0.28 ± 0.01
	40	3.22 ± 0.33	0.008 ± 0.001	4.80 ± 0.06	0.33 ± 0.14	0.16 ± 0.02
5	0	6.38 ± 0.40	0.012 ± 0.001	6.72 ± 0.13	0.04 ± 0.01	0.23 ± 0.01
	20	5.08 ± 0.61	0.011 ± 0.001	6.81 ± 0.13	0.04 ± 0.01	0.19 ± 0.02
	40	2.86 ± 0.60	0.009 ± 0.002	6.77 ± 0.01	0.08 ± 0.02	0.11 ± 0.02
10	0	2.78 ± 0.14	0.009 ± 0.001	5.21 ± 0.01	0.07 ± 0.02	0.09 ± 0.01
	20	2.53 ± 0.25	0.008 ± 0.001	5.48 ± 0.01	0.06 ± 0.02	0.08 ± 0.01
	40	2.79 ± 0.03	0.009 ± 0.001	4.89 ± 0.01	0.15 ± 0.04	0.09 ± 0.01

[a]Difference between the biomass values at 96 h and 0 h of incubation

[b]After 96 h

[c]Ethanol yield

[d]Biomass yield

With 5 g l^{-1} acetic acid, the consumption rate increased but with 10 g l^{-1}, only a slight variation in glucose concentration was detected until 72 h of cultivation (Fig. 2c, f, and i). Pentose consumption did not differ substantially in any concentration of acetic acid and furfural, except for the treatment when no acetic acid was added but 20 mg l^{-1} furfural was (Fig. 2b). Acetic acid was consumed regardless of furfural concentration, but slowly when 40 mg l^{-1} was added (Figs. 2f and i).

In summary, the acetic acid did not influence the sugar consumption in concentrations of 5 and 10 g l^{-1}, even in the presence of 20 mg l^{-1} furfural. On the other hand, furfural did affect the sugar consumption by *M. guilliermondii* in concentration of 40 mg l^{-1} even in the absence of acetic acid. The same result was observed for ethanol titers.

The variation in medium pH was considerably high in the first set of experiments, in which acetic acid was the sole carbon source in concentrations until 10.5 g l^{-1} (Fig. 1). However, lower variation of pH was observed in semi-synthetic medium with pentoses and glucose, observing alkalinization only when 5 g l^{-1} acetic acid was utilized, regardless of the furfural concentration (Table 2).

The rates of acetic acid consumption were calculated by fitting the values to a zero-order kinetic with high values of correlation coefficients (R^2). Rates of 0.034 to 0.036 g l^{-1} h^{-1} and of 0.060 to 0.068 g l^{-1} h^{-1} were obtained for 5 g l^{-1} and 10 g l^{-1} acetic acid, respectively (Table 3).

Discussion

The ability of *M. guilliermondii* to utilize acetic acid as substrate is an important characteristic in the context of the second-generation ethanol, because this acid is commonly found in acid hydrolysates of lignocellulosic biomass (Ceccato-Antonini et al. 2017). Concentrations of 3.2 to 5.1 g acetic acid l^{-1} were observed in hydrolysates utilizing diluted sulfuric acid as catalyst (Aguilar et al. 2002; Noronha et al. 2010; Martini et al. 2016). In our work, much higher concentrations of acetic acid were assayed and no decrease in specific growth rate was observed from 4.5 to 19.5 g l^{-1} of acetic acid. A high affinity of *M. guilliermondii* to acetic acid (very low ks to the range of concentration) was verified, which was below the lowest concentration assayed (1.5 g l^{-1}). Values of ks were not found for acetic acid, since this molecule is typically treated as an inhibitor rather than a substrate (Converti et al. 2000).

The growth of fermenting microorganisms as *Zymomonas mobilis* and *S. cerevisiae* is found to be inhibited by acetic acid in concentrations above 2 g l^{-1} (Luján-Rhenals et al. 2014). A decrease of 20–30% in cell concentrations of immobilized *S. cerevisiae* was verified when the acetic acid concentration increases from 2.5 to 20 g l^{-1}. The authors attributed the effects to the cell death by stress (Ylitervo et al. 2014). With a recombinant *S. cerevisiae* strain (LNH-ST 424A), concentrations up to 6.2 g l^{-1} initial acetic acid did not affect the fermentation, since the pH was properly adjusted in poplar hydrolysates (Lu et al. 2009). However, in concentrations of 7.5 and 15 g l^{-1} acetic acid, the effects on the cell biomass concentration, glucose, and xylose consumption rates were observed, when the same strain of recombinant *S. cerevisiae* as above was utilized (Casey et al. 2010). For another strain of *M. guilliermondii* (UFV-1), concentration of 4 g l^{-1} of acetic acid resulted in a growth inhibition as high as 86% compared to the medium (YP + xylose) without acetic acid; however, it was able to grow in spite of acetic acid which is not very common in xylose-fermenting yeasts (Silveira 2014).

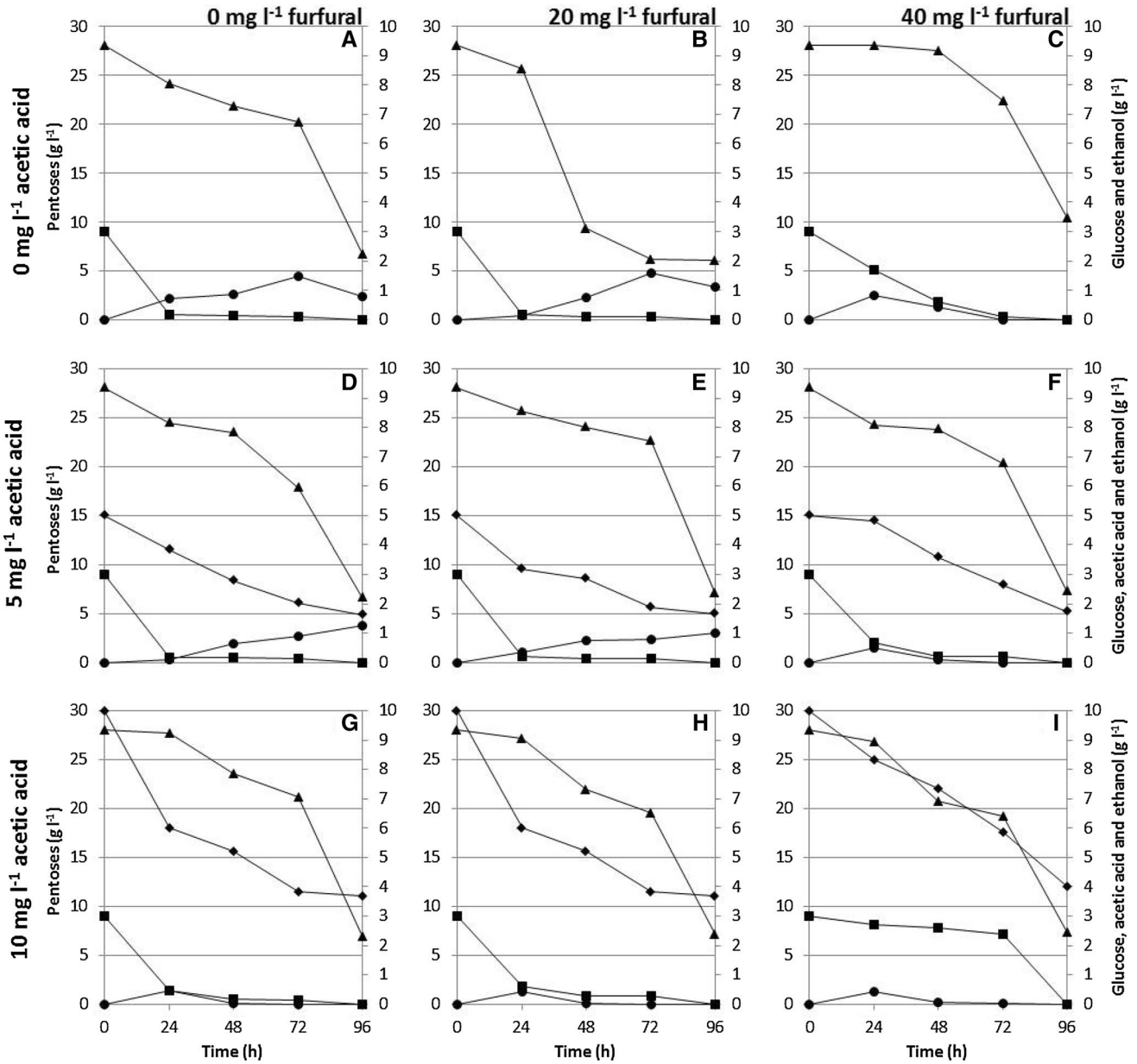

Fig. 2 Glucose (filled square), pentoses (filled triangle), acetic acid (filled diamond), and ethanol (filled circle) concentrations in the cultivations of *M. guilliermondii* in semi-synthetic medium containing glucose (3 g l^{-1}), arabinose (3 g l^{-1}), xylose (25 g l^{-1}), acetic acid (0, 5 or 10 g l^{-1}), and furfural (0, 20 and 40 mg l^{-1}), at 30 °C, 160 rpm, pH 5.5, and initial cell concentration of 2.5 g l^{-1}, for 96 h

A strain of *I. occidentalis* was able to assimilate only 6.1% of initial acetic acid (3.3 g l^{-1}) in a concentrated medium consisted of sugarcane bagasse hydrolysate (Fonseca et al. 2011). In our work, the strain of *M. guilliermondii* consumed 85% of the initial acetic acid in the highest concentration evaluated (19.5 g l^{-1}) when the acid was the sole carbon source. In semi-synthetic medium with pentoses (arabinose and xylose) and glucose, the lowest acetic acid consumption was 60%, in the condition of 10 g acetic acid l^{-1} and 40 mg furfural l^{-1}, in 96 h of cultivation.

Rodrigues et al. (2006) verified an expressive consumption of acetic acid by *C. guilliermondii* during fermentation of acid hydrolysates of sugarcane bagasse. For *S. cerevisiae* in defined medium with acetic acid, the same result was observed (Pereira et al. 2011). However, only a small variation in pH was observed in both reports due to the medium buffering. This result was also verified when *M. guilliermondii* was grown in semi-synthetic medium with acetic acid as the sole carbon source in concentrations of 13.5, 16.5, and 19.5 g l^{-1}. The buffering occurred in the treatments in

Table 3 Acetic acid consumption rate (g l^{-1} h^{-1}) of *M. guilliermondii* in semi-synthetic medium containing glucose (3 g l^{-1}), arabinose (3 g l^{-1}), xylose (25 g l^{-1}), acetic acid (5 or 10 g l^{-1}), and furfural (0, 20, and 40 mg l^{-1}), at 30 °C, 160 rpm, initial pH 5.5, and initial cell concentration of 2.5 g l^{-1}, after 96 h

Acetic acid (g l^{-1})	Furfural (mg l^{-1})	Acetic acid consumption (g l^{-1} h^{-1})	R^2
5	0	0.036	0.9659
	20	0.034	0.9056
	40	0.036	0.9677
10	0	0.062	0.8318
	20	0.068	0.9018
	40	0.060	0.9907

which the cell growth was less expressive or totally inhibited. The partial dissociation of aliphatic acids may favour the buffering in values close to their pKa range, which is 4.75 for acetic acid (Mollapour and Piper 2008). Conversely, the intense medium alkalinization verified in the first set of experiments (when acetic acid was the sole carbon source) is probably regarded to the high acetic acid consumption; that is, higher pH values were coincident with higher acetic acid consumption (Sene et al. 2000).

The concentration of undissociated acids is very dependent on pH. Lu et al. (2009) and Casey et al. (2010) verified that the acetic acid has reduced inhibitory effect in increasing media pH, which corroborates the fact that the undissociated form of this acid is, indeed, the inhibitory form of the molecule. At low external pH, there is an accumulation of anions inside the cell in *S. cerevisiae*, causing toxicity. It has been shown to increase 10–1000 times when external pH fell from 6.0 to 3.5 (Casal et al. 1996). That may be the reason why there was no *M. guilliermondii* growth at pH 3.5, even with the initial cell concentration as high as 2.5 g l^{-1}. However, we evaluated the effect of such low pH over the yeast growth to assess the viability of using this yeast in acid hydrolysates with lower cost of alkalis to raise the pH. However, it did not work out.

Besides pH, the toxicity of inhibitors is quite dependent on the inoculum size (Palmqvist and Hahn-Hagerdal 2000; Chandel et al. 2011), which could be relieved by high cell densities. There was a decrease in the specific growth rate at the highest cell concentration evaluated here (2.5 g l^{-1}); however, no effect on the acetic acid consumption was observed. Furthermore, ethanol was detected only at this cell concentration. The initial cell concentration of 2.5 g l^{-1} was then chosen for the next set of experiments in semi-synthetic medium with sugars and inhibitors.

In spite of the high acetic acid consumption, the maximum specific growth rate was very low (0.023 h^{-1}) when compared with *D. bruxellensis* that had a specific growth rate of 0.07 h^{-1} in YPD supplemented with 120 mM of acetic acid (7.2 g l^{-1}), in the study by Moktaduzzaman et al. (2016). The specific growth rate fell about 61% with the addition of acetic acid and the 29 strains examined by the authors could not metabolise acetic acid in the presence of glucose. A glucose repression mechanism on the acetyl-CoA synthetase activity would be responsible for this defect.

Interestingly, *M. guilliermondii* metabolised acetic acid in the presence of both pentoses (xylose and arabinose) and glucose, even simultaneously, and did not interfere with the sugar uptake as *Z. bailii* did (Sousa et al. 1996, 1998). In our experiments, we utilized a semi-synthetic medium that resembled mostly the proportion of xylose:glucose:arabinose of acid hydrolysates (approximately 10:1:1, respectively), as reported by Cadete et al. (2009) and Martini et al. (2016). Yeasts as *S. cerevisiae, Candida utilis, Torulaspora delbrueckii,* and *Dekkera anomala* did not metabolise acetic acid in the presence of glucose, because the inducible active transport of acetate is repressed by glucose (Geros et al. 2000; Rodrigues et al. 2012).

However, the maximum specific growth rate of *M. guilliermondii* was lower in semi-synthetic medium with sugars and acetic acid (0.012 h^{-1}) than in semi-synthetic medium with acetic acid as the sole carbon source (0.023 h^{-1}) probably due to the high affinity for acetic acid (low ks). In semi-synthetic medium with sugars, the effect of increasing acetic acid concentration on the specific growth rate was less prominent (from 0.011 to 0.009 h^{-1}, in 5 and 10 g l^{-1} acetic acid). A strain of *M. guilliermondii* isolated from Amazonian termites showed a specific growth rate of 0.0369 h^{-1} in sugarcane bagasse hydrolysate, although the acetic acid concentration was not reported (Matos et al. 2014). Using YNB medium at pH 5.0 with glucose and acetic acid (6 and 12 g l^{-1}), Nogué et al. (2012) verified a very low effect of the acetic acid on the specific growth rate in three strains of *S. cerevisiae*, although the rate values were much higher (0.25 to 0.45 h^{-1}), depending on the yeast strain.

The increase in acetic acid concentration from 5 to 10 g l^{-1} resulted in about 60% lower biomass yield, probably because energy was deviated to compensate the effects of high acetic acid concentration outside the cell. The acetic acid is transported across the plasma membrane by passive diffusion in its undissociated form and once inside the cell, the dissociation occurs due to its pK_a of 4.75 and the higher intracellular pH. It causes intracellular acidification. Protons have to be pumped out of the cells by the plasma membrane to counteract the raise in internal cell pH, at the expense of ATP (Pampulha and Loureiro-Dias 1989). This also may explain the acetic acid consumption (76–79%) by *M. guilliermondii* at initial pH 3.5, although no growth and no variation in the medium pH were observed.

Arneborg et al. (1995) verified a decrease of 28% in biomass yield of *S. cerevisiae* when 2 g l^{-1} acetic acid was added to a glucose-based medium. No change in the values

of biomass yield of *M. guilliermondii* was observed when 5 g acetic acid l^{-1} was added to the pentose-glucose medium, featuring the important characteristic of this yeast to tolerate high concentrations of acetic acid.

On the other hand, furfural was, indeed, inhibitory in concentrations of 20 and 40 mg l^{-1} but in the presence of acetic acid. Individually, furfural inhibited yeast growth only in concentration of 40 mg l^{-1}. Slower sugar uptake and lower ethanol titers were also verified. It may be related to the production of furfuryl alcohol resulting from the transformation of furfural, which is less toxic to cells (Heer et al. 2009). The reduction of furfural can act as an alternative redox sink (NADH pool) favouring furfuryl alcohol formation and resulting in a decrease in glycerol yield and increase in biomass formation (Horvath et al. 2003; Greetham 2014). Although furfuryl alcohol was not analysed in our experiments, furfural concentration was found to decrease substantially along the time (data not shown). In the treatment without acetic acid, the addition of 20 mg l^{-1} furfural had a strong effect in sugar consumption and, consequently, the highest biomass yield was observed. Furfural concentration as high as 40 mg l^{-1}, however, inhibited yeast growth and ethanol production. This inhibition was more pronounced in the presence of acetic acid. Palmqvist et al. (1999) verified that furfural concentrations up to 2 g l^{-1} (50 times higher than the highest concentration here utilized) stimulated biomass yield when acetic acid was not added. A recombinant strain of *S. cerevisiae* (LNH-ST 424A) was much more tolerant to furfural than *M. guilliermondii,* because only at concentrations of 10–15 g l^{-1}, the inhibition of glucose and xylose consumption rates was significant in YEPDX (Lu et al. 2009).

The specific growth rate was not influenced by the addition of acetic acid and furfural in concentrations of 5 g l^{-1} and 20 mg l^{-1}, respectively, even when both were present in the medium. Palmqvist et al. (1999) and Ding et al. (2011) verified a synergistic effect of both acetic acid and furfural on the specific growth rate of yeasts, but the effect was dependent on the concentration of the inhibitors.

Furfural can also affect the assimilation pathways for the pentose-fermenting yeasts. This inhibitor was more toxic for xylose fermentation than acetic acid and methanol (Noronha et al. 2010); however, the concentrations of furfural (0.1–0.7 g l^{-1}) were much higher than those utilized here (20 and 40 mg l^{-1}), and for acetic acid, much lower concentrations were utilized by the authors (0.2–1.3 g l^{-1}) when compared with our work (5 and 10 g l^{-1}). At 40 mg furfural l^{-1}, the sugar uptake was slower regardless of the addition of acetic acid. However, *M. guilliermondii* was able to consume 5.97 and 6.30 g acetic acid l^{-1} (initial concentration of 10 g l^{-1}) in the presence of 40 and 20 mg l^{-1} of furfural, respectively, much faster with 20 mg l^{-1}. The yeast *I. occidentalis* removed only 1.99 g l^{-1} of acetic acid in the presence of furfural and hydroxymethylfurfural (Fonseca et al. 2011). Only 30% of the pentoses were consumed by *I. occidentalis* growing in hemicellulosic hydrolysates (Gonçalves et al. 2013), while *M. guilliermondii* assimilated 80% of the pentoses. This characteristic of our strain is quite interesting, because it shows that the presence of inhibitors does not affect the sugar uptake substantially.

Undoubtedly, acetic acid was consumed as substrate even in the presence of furfural and sugars. The results of acetic acid consumption rates showed that there is a linear consumption proportional to its concentration for all the treatments. High correlation coefficients were obtained for the zero-order equations, as depicted in Table 3, and it suggests non-limiting conditions of substrate consumption. This result is extremely important to obtain in a natural yeast strain. A strain of *S. cerevisiae* was genetically modified by combining an NADH-consuming acetate consumption pathway and an NADH-producing xylose utilization pathway to convert five- and six-carbon sugars along with acetic acid in ethanol under anaerobic conditions (Wei et al. 2013). The overexpression of *WHI2* and *PSR1* resulted in improved acetic acid resistance in a strain of *S. cerevisiae* providing a new strategy to engineer yeast strains for the second-generation ethanol (Chen et al. 2016).

However, the assimilation of acetic acid by *M. guilliermondii* did not result in a higher ethanol yield. There was a decrease in ethanol yield from 0 to 10 g l^{-1} acetic acid without addition of furfural. On the other hand, furfural increased ethanol yield especially in the concentration of 40 mg l^{-1} regardless of the presence of acetic acid. This result was also observed by Palmqvist et al. (1999) but in the absence of acetic acid. Xylitol was not analysed in the present work, but its production is commonly associated with this yeast species in acid hydrolysates (Silveira 2014; Martini et al. 2016) and it is most likely that xylitol was produced in the conditions of the present work.

When the same strain of *M. guilliermondii* was grown in sugarcane bagasse acid hydrolysate (non-detoxified) in the same cultural conditions, higher biomass accumulation (6.1 g l^{-1}), ethanol yield (0.14 g l^{-1}), and ethanol titer (around 3.5 g l^{-1}) were observed, however, in a longer fermentation time (144 h). This comparison was made in relation to the results obtained in the present work in semi-synthetic medium most resembling the hydrolysate composition (20 mg l^{-1} furfural, 5 g l^{-1} acetic acid, 3 g l^{-1} glucose, 25 g l^{-1} xylose, and 3 g l^{-1} arabinose).

We could then prove that acetic acid is a substrate for *M. guilliermondii* and is co-consumed with sugars (xylose, arabinose, and glucose) even when furfural, a powerful growth inhibitor, is present. Further optimization of ethanol production using this yeast would make it an attractive organism for biotechnological route for the second-generation ethanol utilizing acid hydrolysates containing acetic acid and furfural.

Author contributions M.S.C. Perna planned and performed the experiments, analysed, and interpreted the data, drafted, and commented the manuscript. R.G. Bastos analysed and interpreted the data and revised critically the manuscript. S.R. Ceccato-Antonini made the study conception and design, analysed and interpreted the data, and revised critically the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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