J Ind Microbiol Biotechnol (2012) 39:1453–1463
DOI 10.1007/s10295-012-1154-5

BIOENERGY/BIOFUELS/BIOCHEMICALS

Novel endophytic yeast *Rhodotorula mucilaginosa* strain PTD3 II: production of xylitol and ethanol in the presence of inhibitors

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Received: 30 March 2012 / Accepted: 28 May 2012 / Published online: 19 June 2012

Abstract A systematic study was conducted characterizing the effect of furfural, 5-hydroxymethylfurfural (5-HMF), and acetic acid concentration on the production of xylitol and ethanol by a novel endophytic yeast, *Rhodotorula mucilaginosa* strain PTD3. The influence of different inhibitor concentrations on the growth and fermentation abilities of PTD3 cultivated in synthetic nutrient media containing 30 g/l xylose or glucose were measured during liquid batch cultures. Concentrations of up to 5 g/l of furfural stimulated production of xylitol to 77 % of theoretical yield (10 % higher compared to the control) by PTD3. Xylitol yields produced by this yeast were not affected in the presence of 5-HMF at concentrations of up to 3 g/l. At higher concentrations of furfural and 5-HMF, xylitol and ethanol yields were negatively affected. The higher the concentration of acetic acid present in a media, the higher the ethanol yield approaching 99 % of theoretical yield (15 % higher compared to the control) was produced by the yeast. At all concentrations of acetic acid tested, xylitol yield was lowered. PTD3 was capable of metabolizing concentrations of 5, 15, and 5 g/l of furfural, 5-HMF, and acetic acid, respectively. This yeast would be a potent candidate for the bioconversion of lignocellulosic sugars to biochemicals given that in the presence of low concentrations of inhibitors, its xylitol and ethanol yields are stimulated, and it is capable of metabolizing pretreatment degradation products.

Keywords Acetic acid · Furfural · 5-hydroxymethyfurfural (5-HMF) · *Rhodotorula mucilaginosa* · Xylitol

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Introduction

Rhodotorula mucilaginosa strain PTD3 ferments xylose to xylitol, six carbon sugars (galactose, glucose, and mannose) to ethanol, and arabinose to arabitol as it was characterized by Bura [1]. It was shown that PTD3 is capable of rapid assimilation and catabolism of five and six carbon sugars as a single, double, and mixed carbon source. Bura [1] reported that this yeast performed best during fermentation of sugars coming from lignocellulosic hydrolysate, producing the highest yields of xylitol 76 % of theoretical yield (10 % higher compared to the synthetic sugar control) and ethanol 100 % of theoretical yield (16 % higher compared to the synthetic sugars control). This water-soluble fraction (hydrolysate) was collected from steam explosion of the mixture of hardwood (hybrid poplar) and softwood (Douglas-fir) chips with bark. The concentrations of fermentation inhibitors present in the hydrolysate were acetic acid (2.1 g/l), 5-hydroxymethyl furfural (1.2 g/l), and furfural (0.6 g/l). Additionally, PTD3 demonstrated a huge potential for bioconversion of lignocellulosic-rich urban waste into biochemicals [21]. The water-soluble fractions of mixed waste paper, yard waste, and food waste

were collected after pretreatment and assessed for their feasibility as media for effective fermentation to ethanol by PTD3 [21]. The ethanol yields from hexoses (glucose, mannose, and galactose) for all the sugar streams tested were close to 100 % of theoretical ethanol despite the presence of fermentation inhibitors [21]. Fermentation of the steam-pretreated lignocellulosic hydrolysates, municipal solid waste, low-grade mixed waste paper, and organic yard waste, served to illustrate PTD3's ability to utilize and ferment xylose in the presence of other sugars and to tolerate pretreatment degradation products. Since this is a novel yeast strain, very little is known about it, and its behavior during bioconversion of lignocellulosic sugars to ethanol and other co-products, especially in the presence of fermentation inhibitors that are generated during the pretreatment process of lignocellulosic biomass.

Lignocellulosic feedstock represents an abundant and inexpensive source of sugars that can be microbiologically converted to biochemicals. However, bioconversion of the sugars into biochemicals such as xylitol and ethanol using hydrolysates obtained after pretreatment of lignocellulosic materials is hindered by the presence of by-products liberated during the pretreatment process [15]. One problem associated with fermentation of such substrates is the presence of pretreatment-derived inhibitors, which adversely affect microbial growth and fermentation [10, 25]. The common degradation products are furfural, 5-hydroxymethylfurfural (5-HMF), weak acids such as acetic acid, and phenolic compounds that are lignin degradation products. All of these inhibitors significantly affect the sugar fermentation process [16]. The concentration of each degradation product in hydrolysates, obtained after pretreatment, depends on the severity of the pretreatment conditions and of the feedstock undergoing the pretreatment method [15].

The presence of these sugar degradation products in hydrolysates and their inhibitory effect on sugar fermentation processes have been studied intensively [10, 25]. However, the effects of inhibitors on xylose-to-xylitol bioconversion have not been deeply investigated [19]. Pereira [19] reported that acetic acid, syringaldehyde, and ferulic acid are compounds that adversely affected metabolism of *Candida guilliermondii* (mainly cell growth) during conversion of xylose to xylitol. For example, xylose consumption, xylitol production, and cell growth were reduced by 13, 18, and 30 %, respectively, by the presence of acetic acid at a concentration of 2.6 g/l compared to the control [19]. It was concluded that their toxic effect to *C. guilliermondii* was dependent on their concentration in the medium, with inhibition being more pronounced at higher concentrations. However, it was stated that employing any detoxification methods of the fermentation medium was not necessary to obtain efficient conversion of xylose to xylitol by *C. guilliermondii* [19]. This suggests that sugar degradation products being present at small concentrations in a medium might not have an inhibitory affect on the whole bioconversion process. The maximum tolerable concentration of each inhibitor that can be present in hydrolysate without affecting the efficiency of the fermentative process is dependent on the microorganism utilized and its degree of adaptation, the fermentation process employed, and the simultaneous presence of other inhibitors [10, 25]. Establishing all these parameters and utilizing an adequate microorganism for sugar fermentation is of a great importance for the whole bioconversion of lignocellulosic derived hydrolysates into various biochemicals.

Knowledge regarding inhibitors and how to minimize their effects is of a great importance for a successful fermentation. Ultimately, after assessing PTD3's co-fermentability of xylose, the six carbon sugars, and sugar degradation products with high xylitol and ethanol yields, the next step was to study further PTD3's tolerance of higher concentrations of sugar degradation products. It was shown that PTD3 is capable of assimilating and fermenting xylose, glucose, galactose, mannose, and arabinose as a single and as well as mixed carbon source. Remarkably, this yeast performed best during fermentation of sugars coming from lignocellulosic hydrolysates despite the presence of fermentation inhibitors. Since PTD3 is novel yeast, not much is known about its fermentation abilities, especially in the presence of inhibitors. Therefore, in order to learn the full potential of *R. mucilaginosa* strain PTD3 for bioconversion of lignocellulosic hydrolysates to biochemicals, the objective of this research was a systematic study of the effect of acetic acid, furfural and 5-HMF on the fermentation of both xylose and glucose to xylitol and ethanol, respectively, by this yeast.

Materials and methods

Yeast strains

Rhodotorula mucilaginosa strain PTD3, a pink yeast strain, was isolated from stems of hybrid poplar 184-402 (*Populus trichocarpa* × *P. deltoides*) grown in greenhouse at the Oregon State University, Corvallis [26]. Throughout all fermentation experiments, the *R. mucilaginosa* strain PTD3 was used. The strain was taken from −80 °C stocks and maintained on YPG solid medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose, and 18 g/l agar, Difco, Becton–Dickinson, MD) at 4 °C and transferred to fresh plates on a weekly basis.

Culture media conditions

Cells were grown to high cell density in foam-plugged 1-l Erlenmeyer flasks containing 500 ml of YP-sugar liquid

media (10 g/l yeast extract and 10 g/l peptone, supplemented with 10 g/l glucose) in an orbital shaker for 2 days at 30 °C and 150 rpm, with a transfer to fresh medium performed every 24 h.

After 48 h of growth, cell cultures were harvested, centrifuged, and decanted to yield cell pellets. Pellets were then washed three times with sterile distilled water and subsequently adjusted with sterile distilled water to a calculated concentration of 5 g dry cell weight (DCW) per liter on a spectrophotometer (Shimadzu UV-1700, Columbia, MD) via standard curves relating 600-nm absorbance to $DCWL^{-1}$ (dry cell weight (DCW) per liter) concentration.

Carbohydrates, inhibitors, and alcohols

Synthetic sugars (glucose, xylose, galactose, mannose, and arabinose), furfural, 5-HMF and acetic acid were obtained from Supelco, (Bellefonte, PA). Ethanol 4 mg/ml, xylitol 5 mg/ml, and glycerol were obtained from Sigma-Aldrich (St. Louis, MO).

Fermentations

All fermentation experiments were performed three times with the appropriate controls that consisted of a media lacking the microorganism. Within each experiment, tests were conducted in triplicate in separate flasks. All media were sterilized by autoclaving. Solutions with sugars were filter-sterilized separately, and appropriate quantities added aseptically to the desired concentration in the fermentation media. Single sugar fermentations were performed in foam-plugged 125-ml Erlenmeyer flasks (semi-aerobic) containing yeast extract-MS Murashige and Skoog medium [14] (1 % (w/v) yeast extract, 1X MS, 3 % (w/v) glucose or xylose) with 50 ml total volume. All fermentations were incubated at 30 °C and maintained with continuous agitation (175 rpm), and pH value of ~6.0. Sampling was aseptically performed at the time of inoculation and at specific time points thereafter. One-milliliter aliquots were immediately centrifuged (14,000 rpm) for 4 min at 4 °C to yield cell-free supernatants. These samples were then decanted and the supernatant was filtered by using a 0.22-μm syringe filter (Restek Corp., Bellefonte, PA, USA) and then stored at −20 °C until analysis. For media requirement analysis, 1 % (w/v) Bacto-peptone was used along with MS with or without yeast extract. Inhibitor-supplemented media was augmented with different concentrations of furfural (1, 1.5, 3, 5, 10, and 20 g/l), 5-HMF (1, 1.5, 3, 5, 10, and 15 g/l) or acetic acid (5, 10, and 20 g/l). The acetic acid-augmented media were adjusted to pH 6.0 prior to inoculation.

HPLC analysis

Monomeric sugars, inhibitors, ethanol, and xylitol analysis

The concentration of xylose, glucose, ethanol, xylitol, glycerol, acetic acid, furfural, and 5-HMF were measured using high-performance liquid chromatography refractive index detection on a Shimadzu Prominence LC [21]. Separation of those compounds was achieved by an anion exchange column (REZEX RHM-Mono saccharide H^+ (8 %), Phenomenex, Inc., Torrance, CA, USA) with isocratic mobile phase that consisted of 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}. The column oven temperature was maintained at a constant 63 °C [21]. Samples were defrosted from −20 °C and 20 μl of each sample was injected after being appropriately diluted in deionized water and filtered through a 0.22-μm syringe filter (Restek Corp., Bellefonte, PA, USA). Standards were prepared and used to quantify the unknown samples.

The theoretical yield for ethanol production from glucose is 0.51 g ethanol g^{-1} glucose [15]. Ethanol yields and percent theoretical yields were calculated using the equations formulated by [8]. The theoretical yield for xylitol production from xylose used was 0.91 g xylitol g^{-1} xylose [25]. The specific consumption and production rates were calculated based on the log-mean cell density,

$q_6 = \frac{(S_0 - S)\ln(\frac{X}{X_0})}{(X - X_0)\Delta t}$, where S is the substrate or product, X is dry cell weight, and t is time [6]. Since within each experiment tests were conducted in triplicate in separate flasks, the standard deviation was calculated between three samples using Microsoft Excel's statistical function.

Results and discussion

Furfural-supplemented fermentation

The effect of furfural at concentrations from 1 to 20 g/l on the fermentation of xylose to xylitol and glucose to ethanol by *R. mucilaginosa* strain PTD3 was studied (Fig. 1, Table 1a). Surprisingly, the higher the concentration of furfural up to 5 g/l, the higher the xylitol yield (10 % higher compared to the furfural-free control, 67 % of theoretical) that was produced by PTD3 (Fig. 1e, Table 1). Based on this, we conclude that furfural at concentrations up to 5 g/l stimulated xylitol yield and to our knowledge this has not been reported in the literature. Kelly [9] found that in the presence of up to 3 g/l furfural, xylitol production rate by *C. guilliermondii* was reduced 43 % of theoretical compared to the control. Not only can PTD3 produce a higher concentration of xylitol in presence of furfural at up to 5 g/l, but it also metabolized all the

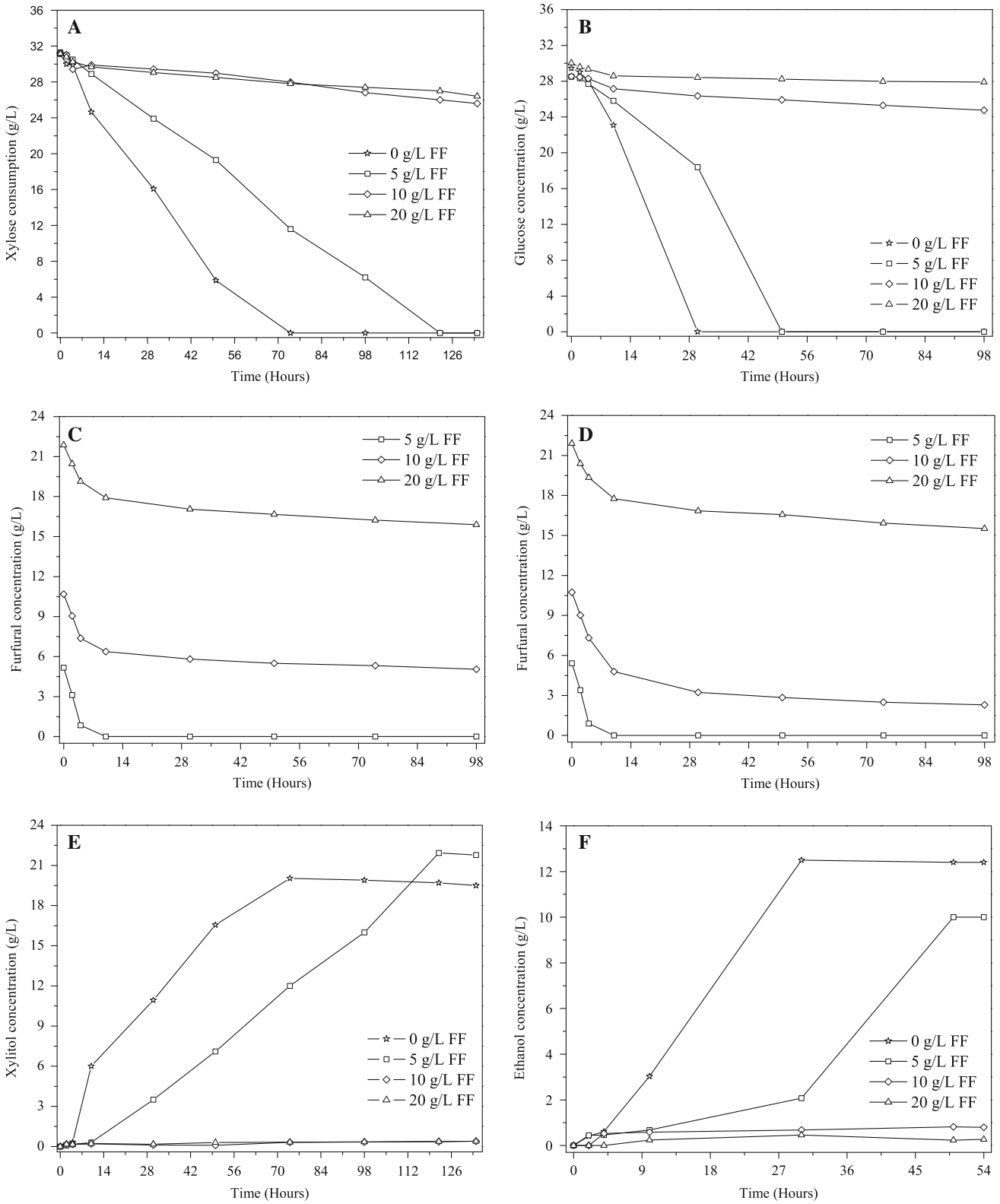

Fig. 1 Xylose consumption (**a**), glucose consumption (**b**), furfural consumption in presence of xylose (**c**), furfural consumption in presence of glucose (**d**), xylitol production (**e**), and ethanol production (**f**) in furfural-augmented fermentation medium by *R. mucilaginosa* strain PTD3

Table 1 The specific rates of sugar consumption and XtOH and EtOH production from synthetic sugars by *R. mucilaginosa* strain PTD3 in the presence of furfural (a), 5-HMF (b), acetic acid (c) during fermentation

Fermentation parameters	Consumption[a]/ production[b] ($gg^{-1}h^{-1}$)	Consumption[a]/ production[b] ($gg^{-1}h^{-1}$)	Consumption[a]/ production[b] ($gg^{-1}h^{-1}$)	Consumption[a]/ production[b] ($gg^{-1}h^{-1}$)	Consumption[a]/ production[b] ($gg^{-1}h^{-1}$)	Consumption[a]/ production[b] ($gg^{-1}h^{-1}$)	Consumption[a]/ production[b] ($gg^{-1}h^{-1}$)
Concentration	0 g/l	1 g/l	1.5 g/l	3 g/l	5 g/l	10 g/l	20 g/l
Furfural fermentation (a)							
Xylose	0.05	0.05	0.05	0.05	0.05	0	0
XtOH	0.03 (67 %)*	0.03 (66 %)*	0.03 (69 %)*	0.03 (74 %)*	0.02 (77 %)*	0 (1 %)*	0 (1 %)*
Inhibitor	–	0.08	0.12	0.24	0.09	0.01	0.01
Glucose	0.13	0.12	0.09	0.08	0.07	0.01	0
EtOH	0.06 (84 %)*	0.04 (74 %)*	0.03 (71 %)*	0.03 (68 %)*	0.02 (67 %)*	0 (5 %)*	0 (3 %)*
Inhibitor	–	0.06	0.09	0.18	0.08	0.02	0.01
Concentration	0 g/l	1 g/l	1.5 g/l	3 g/l	5 g/l	10 g/l	15 g/l
5-HMF fermentation (b)							
Xylose	0.05	0.06	0.05	0.05	0.04	0.04	0.01
XtOH	0.03 (67 %)*	0.05 (67 %)*	0.04 (68 %)*	0.03 (70 %)*	0.02 (63 %)*	0.02 (45 %)*	0 (6 %)*
Inhibitor	–	0.1	0.09	0.08	0.08	0.05	0.03
Glucose	0.13	0.12	0.11	0.1	0.06	0.06	0.07
EtOH	0.06 (84 %)*	0.05 (80 %)*	0.04 (78 %)*	0.04 (73 %)*	0.04 (63 %)*	0.02 (58 %)*	0.02 (55 %)*
Inhibitor	–	0.1	0.1	0.07	0.06	0.14	0.07
Concentration	0 g/l	5 g/l	10 g/l	20 g/l			
Acetic acid fermentation (c)							
Xylose	0.05	0.03	0.03	0.03			
XtOH	0.03 (67 %)*	0.02 (41 %)*	0.01 (40 %)*	0.01 (32 %)*			
Inhibitor	–	0.01	0.01	0.01			
Glucose	0.13	0.11	0.12	0.13			
EtOH	0.06 (84 %)*	0.05 (85 %)*	0.06 (88 %)*	0.03 (99 %)*			
Inhibitor	–	0.01	0.01	0.01			

The reported results are the average of triplicate studies with a deviation of ≤ 2 %

* The theoretical yield for xylitol and ethanol production from xylose and glucose, respectively, for tested concentrations of furfural

[a] The specific rates of sugar consumption were calculated based on the log-mean dry cell density and the Δsubstrate and Δtime

[b] The specific rates of xylitol from xylose and ethanol from glucose, production were calculated based on the log-mean dry cell density and the product concentration and Δtime

furfural (5 g/l) in solution within 12 h (Fig. 1c). The specific xylose consumption rate (0.05 $gg^{-1}h^{-1}$) was the highest for up to 5 g/l of furfural (Table 1). In the present study, the 30 g/l of xylose was completely consumed by PTD3 within 120 h for concentrations of up to 5 g/l of furfural tested (Fig. 1a). However, at the higher concentrations of furfural (10 and 20 g/l), slower xylose consumption was observed compared to the control (Fig. 1a). The specific production rate for xylitol was the highest and the same for up to 3 g/l of furfural (Table 1). Surprisingly enough, the specific consumption rate of furfural increased as the concentration of furfural increased from 1 to 3 g/l and was the highest at 3 g/l. However, at concentrations above 3 g/l furfural, the specific consumption rate of furfural decreased significantly. This suggest that concentrations of up to 3 g/l furfural have rather a positive influence on conversion of xylose to xylitol by PTD3 while the higher concentrations adversely affect xylose metabolism and xylitol yield.

Indicative of the negative effect of higher concentrations of 10–20 g/l furfural, xylitol production was delayed and reduced to 1.2 % of theoretical (Fig. 1d) compared to control (Fig. 1e, Table 1). The consumption of furfural was also reduced as the concentration of furfural increased: 53 % of 10 g/l and 27 % of 20 g/l (Fig. 1c). It is understood that yeast, during bioconversion of six carbon sugars to ethanol, metabolizes furfural to furfural alcohol [16]. NADH-dependent alcohol dehydrogenase is thought to be responsible for this reduction, causing the attenuated xylitol and ethanol yields in our study. Since all NADH generated is used for furfural reduction, the glucose-to-ethanol and likely also the xylose-to-xylitol process are

greatly affected. This is because there is an increased acetaldehyde accumulation inside the cell caused by an insufficient amount of NADH-dependent alcohol dehydrogenase available in order to reduce acetaldehyde to ethanol. Intracellular acetaldehyde accumulation is then considered to be the reason for the lag-phase in growth and ultimately lower yields produced at the higher concentration of this inhibitor [9, 16].

Unlike xylitol yields, the presence of furfural negatively affected ethanol yields by PTD3 at all concentration tested. As the concentration of furfural in media increased (from 0 to 20 g/l), glucose-to-ethanol yields by this strain decreased and even at the lowest concentration of furfural (1 g/l) tested, the ethanol yield (74 % of theoretical) was already negatively affected compared to the furfural-free control (84 % of theoretical) (Fig. 1f, Table 1). The specific glucose consumption and ethanol production rates (0.12 and 0.04 $gg^{-1}h^{-1}$, respectively) were highest at 1 g/l of furfural (Table 1). The yeast metabolized 30 g/l glucose in media with up to 5 g/l furfural within 50 h but was slower compared to the control, while at 10 and 20 g/l furfural glucose was not completely consumed (Fig. 1b). Similar to xylose-augmented media, in glucose-supplemented media, PTD3 was able to completely metabolize up to 5 g/l of furfural within 12 h, whereas consumption of 79 % of 10 g/l and 29 % of 20 g/l was observed (Fig. 1d). The specific consumption rate of furfural was the highest at 3 g/l, while afterwards was decreased significantly (Table 1). A 5 g/l furfural resulted in a 72 and 50 % inhibition of cellular growth of the microorganism in xylose and the glucose-supplemented fermentation media, respectively, compared to furfural-free media (data not shown). However, at concentrations above 5 g/l furfural no cellular growth was noted. Duarte [2] found that 0.5 g/l furfural decreased the specific growth rate of *Debaryomyces hansenii* CCMI 941, and at concentrations above 3.5 g/l furfural no cellular growth was observed. Olsson [15] reported that 2 g/l furfural resulted in a 90 and 99 % inhibition of growth of *Saccharomyces cerevisiae* and *Pichia stipitis*, respectively.

Unlike observed before, it was shown that furfural boosted the xylitol yields at lower concentrations of furfural with constant xylitol production and xylose consumption rates but negatively affected ethanol yields, glucose consumption, and ethanol production rates by PTD3.

5-HMF-supplemented fermentation

Xylitol yield by *R. mucilaginosa* strain PTD3 was not affected for concentrations of up to 3 g/l of 5-HMF and was similar to the 5-HMF-free control (67 % of theoretical) (Fig. 2e, Table 1). However, clearly xylitol yield was impaired by the presence of 5-HMF at higher concentrations (from 5 to 15 g/l) tested (Fig. 2e). Unlike with PTD3, Sanchez et al. [20] reported that in the presence of up to 2 g/l of 5-HMF, growth inhibition of *C. guilliermondii* was noted, subsequently with drastically reduced xylitol yields compared to their controls. Note that the 30 g/l of xylose was completely metabolized by PTD3 at concentrations of up to 5 g/l 5-HMF, whereas consumption of 83 and 21 % of the sugar at 10 and 20 g/l of 5-HMF, respectively, was observed (Fig. 2a). Consequently, it resulted in lower xylitol yields by the yeast (63, 45, and 6 %, respectively) (Fig. 2e). The specific xylose consumption and xylitol production rates decreased as the concentration of 5-HMF increased (Table 1). Although, PTD3 completely metabolized 5-HMF at all concentrations tested in xylose-supplemented media (Fig. 3c), the highest 5-HMF consumption rate (0.06 $gg^{-1}h^{-1}$) was for the lowest 5-HMF concentration tested 1 g/l (Table 1). Similarly to the inhibition mechanism by furfural, during bioconversion of six carbon sugars to ethanol, yeast metabolizes 5-HMF to HMF-alcohol [9, 16]. NADPH-dependent alcohol dehydrogenase is understood to be responsible for this reduction. As such, the reduction of 5-HMF does not regenerate NAD^+, and thus carbon is allocated to glycerol production (to produce NAD^+ and thus maintain overall redox balance) [16]. Since all NADPH generated is used for 5-HMF reduction, it is believed that the xylose-to-xylitol and glucose-to-ethanol yields are adversely affected in our study.

As with xylose, 5-HMF had a similarly unfavorable effect on the glucose consumption and ethanol yields by the yeast. As the 5-HMF concentration increased from 1 to 15 g/l, the corresponding ethanol yields by PTD3 decreased (80, 78, 73, 63, 58, and 55 % of theoretical, respectively) (Fig. 2f, Table 1). Similarly, Keating [7] noted that ethanol yield by *S. cerevisiae* was substantially lowered at a concentration of 4 g/l 5-HMF. In the presence of 5-HMF at all concentrations tested, 100 % of glucose (30 g/l) was consumed by PTD3 (Fig. 2b). However, the specific consumption rates of glucose and production rates of ethanol decreased as the concentration of 5-HMF tested increased (Table 1). Additionally, the highest specific consumption rate of glucose and production rate of ethanol (0.12 and 0.05 $gg^{-1}h^{-1}$) by this microorganism were at 1 g/l of 5-HMF and decreased as the concentration of 5-HMF present in media increased (Table 1). PTD3 completely metabolized 5-HMF at all concentrations tested in this media (Fig. 3d). However, the specific 5-HMF consumption rate of 10 g/l 5-HMF in glucose media was the highest (Table 1). 5-HMF caused a concentration-dependent decrease in yeast growth for both media types. A 15 g/l 5-HMF resulted in a 70 and 40 % inhibition of cellular growth of PTD3 in xylose and the glucose-supplemented fermentation media, respectively, compared to 5-HMF-free media (data not shown).

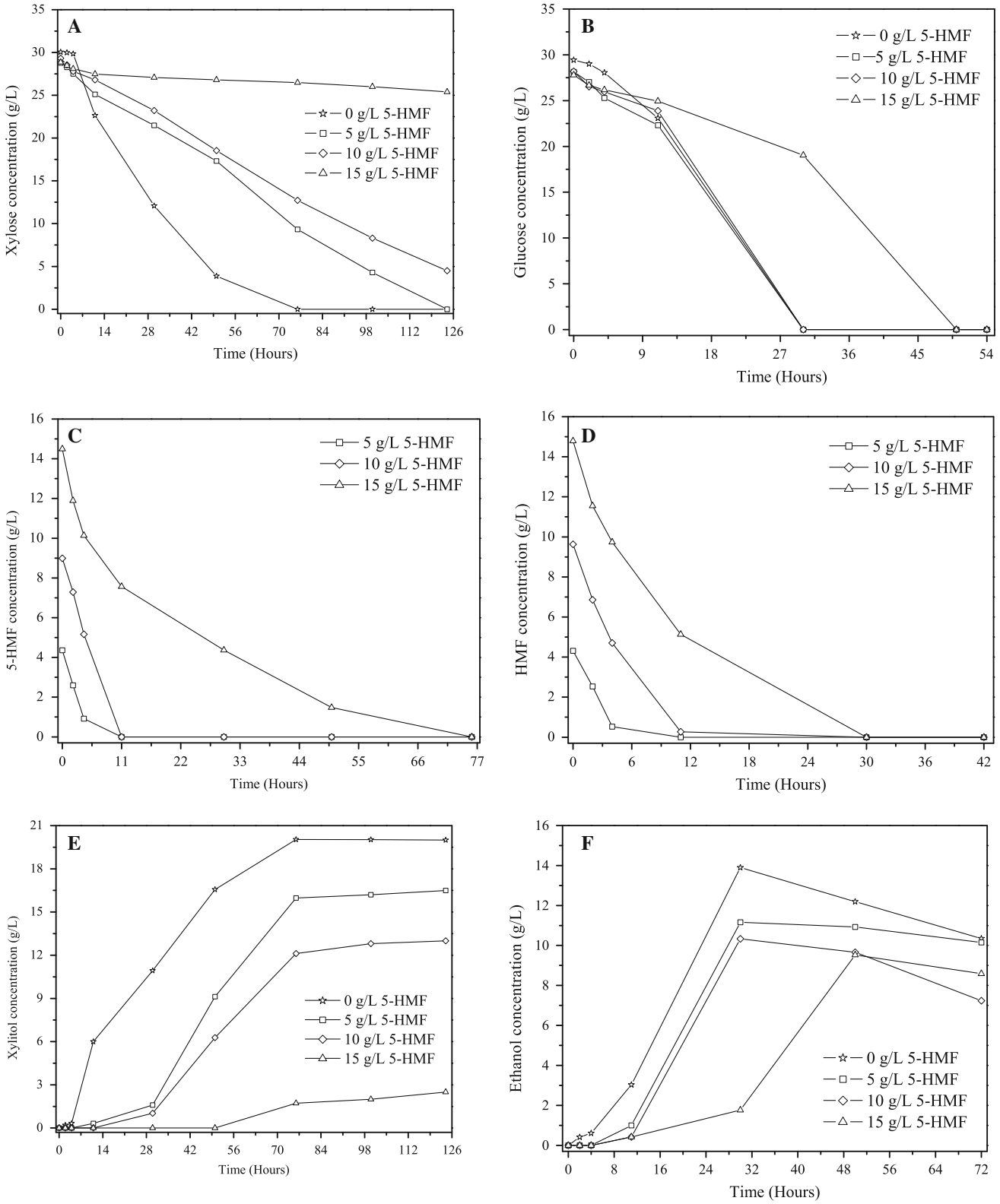

Fig. 2 Xylose consumption (**a**), glucose consumption (**b**), 5-HMF consumption in presence of xylose (**c**), 5-HMF consumption in presence of glucose (**d**), xylitol production (**e**), and ethanol production (**f**) in 5-HMF-augmented fermentation medium by *R. mucilaginosa* strain PTD3

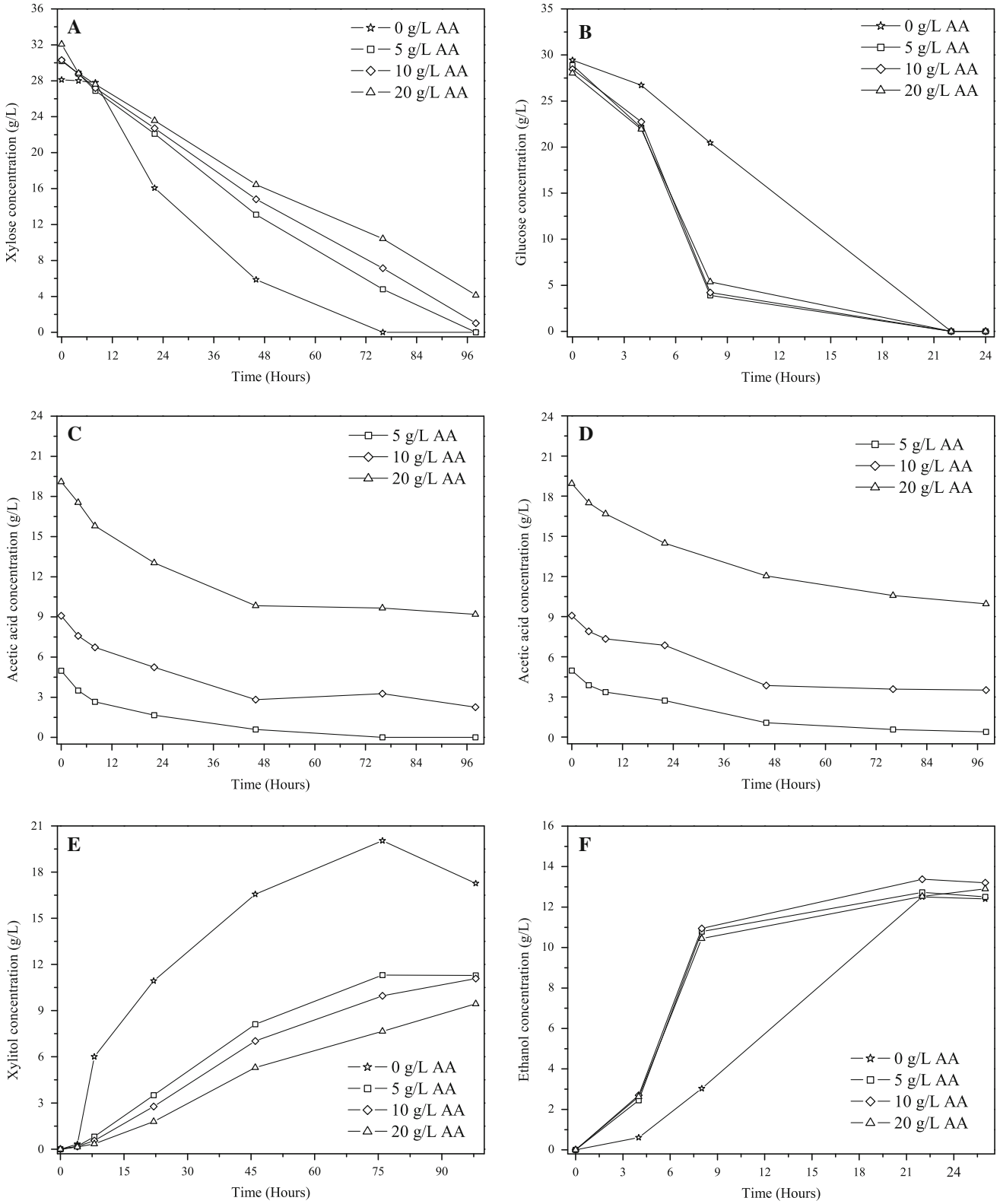

Fig. 3 Xylose consumption (**a**), glucose consumption (**b**), acetic acid consumption in presence of xylose (**c**), acetic acid consumption in presence of glucose (**d**), xylitol production (**e**), and ethanol production (**f**) in acetic acid-augmented fermentation medium by *R. mucilaginosa* strain PTD3

Based on these data, we can conclude that PTD3 is capable of metabolizing 15 g/l 5-HMF and that at concentrations at up to 3 g/l 5-HMF does not affect xylitol yield, whereas the ethanol yield is inhibited at all concentrations of 5-HMF tested.

Acetic acid-supplemented fermentation

In the presence of 5–20 g/l, acetic acid negatively affected xylitol production from xylose by *R. mucilaginosa* strain PTD3, whereas its presence enhanced glucose-to-ethanol bioconversion by this strain. It was shown that acetic acid enhanced production of ethanol by the yeast for all the concentrations tested. As the concentration of acetic acid in the media with xylose increased from 5 to 20 g/l, xylitol yields produced by PTD3 (lowest yield 32 % of theoretical at 20 g/l acetic acid) decreased compared to the fermentation lacking acetic acid (67 % of theoretical) (Fig. 3e). The highest xylitol production rate (0.06 $gg^{-1}h^{-1}$) was at a concentration of 5 g/l of acetic acid (Table 1). For PTD3 it took longer to consume 100 % of 30 g/l xylose as the concentration of acetic acid increased (Fig. 3a). The xylose consumption rate was the same (0.03 $gg^{-1}h^{-1}$) for all the concentrations of acetic acid tested (Table 1). In xylose-supplemented fermentation, PTD3 metabolized completely only 5 g/l of acetic acid within 76 h while it metabolized 72 % of 10 g/l and 45 % of 20 g/l of this inhibitor (Fig. 3c). The exact mechanism of acetic acid inhibition of xylose-to-xylitol bioconversion has not been thoroughly investigated [19]. It is believed that at high concentrations, acetic acid either jeopardizes the availability of ATP that is available for cell growth or interferes with xylose transport across the plasma membrane. Acetic acid was characterized as a powerful inhibitor of xylose metabolism of yeast cells but its inhibition to *C. guilliermondii* depended on its concentration [3, 4]. The negative effect of high concentrations of acetic acid on xylitol production by *C. guilliermondii* was also shown by Lima [12] and Silva [22]. Silva [22] found that *C. guilliermondii* in the presence of a concentration as low as 1 g/l of acetic acid favors conversion of xylose to xylitol while at concentrations higher than 3 g/l, xylose consumption and xylitol formation are inhibited. Lima [12] found that xylitol production by *C. guilliermondii* was not affected by the presence of acetic acid until the concentration tested reached 10 g/l. The lowest concentration of acetic acid used in this study was 5 g/l, therefore improvements of xylitol yields by PTD3 at low acetic acid concentrations were not observed.

The apparent lack of effect on the rate of glucose consumption and ethanol yield by PTD3 was surprising, especially with acetic acid concentrations of 10.0 and 15.0 g/l, since [13] reported inhibition of cellular processes in *S. cerevisiae* at concentrations ranging from 0.5 to 9.0 g/l. Unlike xylitol production, ethanol production from 30 g/l glucose in the presence of acetic acid (from 5 to 20 g/l) had theoretical yields of (85, 88, and 99 % of theoretical, respectively) by this strain that were identical or higher compared to the fermentation lacking acetic acid (84 % of theoretical) (Fig. 3f). All 30 g/l of glucose was consumed by PTD3 between 14 and 20 h for all the acetic acid concentrations tested (Fig. 3b). During fermentation of glucose with acetic acid, only 5 g/l acetic acid was completely metabolized by PTD3 within 100 h while consumption of 62 % of 10 g/l and 41 % of 20 g/l was observed (Fig. 3d). The highest sugar consumption rate (0.13 $gg^{-1}h^{-1}$) was noted when PTD3 was fermenting glucose supplemented with 20 g/l acetic acid (Table 1c). The ethanol production rate (0.06 $gg^{-1}h^{-1}$) was the highest in glucose supplemented with 10 g/l acetic acid (Table 1). Similar to the other inhibitors, as the acetic acid concentration increased in fermentation media, the cellular growth of *R. mucilaginosa* strain PTD3 in both xylose and glucose-augmented media decreased. The highest concentration of acetic acid tested, 20 g/l, resulted in a 33 and 22 % inhibition of cellular growth of the yeast in xylose and the glucose-augmented fermentation media, respectively, compared to acetic acid-free media (data not shown). Silva et al. [22] also observed that with the presence of acetic acid in the media, cell growth decreases. Its presence affects cell growth by increasing the adaptation lag time and decreasing or altering the growth rate [17]. For both glucose and xylose supplemented media, the consumption rate of acetic acid was the same (0.01 $gg^{-1}h^{-1}$) for all the concentrations tested (Table 1, Fig. 3c, d).

The improved ethanol yields in the presence of acetic acid could be explained by the yeast's need to maintain a neutral intracellular pH, which is crucial for cell viability [5]. In the presence of acetic acid, intracellular pH drops by dissociation of acetic acid into lipophobic acetate and protons, resulting in the drop in intracellular pH [11]. The pH is then neutralized at the expense of ATP hydrolysis by the plasma membrane ATPase. In order to maintain the intracellular pH, additional ATP must be generated and under anaerobic conditions this is accomplished by increased ethanol production at the expense of cellular growth [24]. The enhanced ethanol yields for the concentration of acetic acid up to 20 g/l suggest the possibility that the carbon normally diverted from the glycolytic intermediate dihydroxyacetone phosphate toward glycerol production was instead available for ethanol production, compensating for any inhibition of alcohol dehydrogenase (or other glycolytic enzyme) activity [16, 17]. Low concentrations of acetic acids have been shown to have a stimulating effect on ethanol production by *S. cerevisiae* [18]. It was shown previously that acetic acid at concentrations up to 10 g/l can increase ethanol yield during

fermentation of glucose by *S. cerevisiae* whereas higher concentrations of this compound decreased ethanol yields [17]. We have also demonstrated that even glucose to ethanol conversion by PTD3 was enhanced by the presence of a concentration as high as 20 g/l acetic acid.

In the presence of acetic acid at all concentrations, PTD3 was affected to the same extent positively for glucose to ethanol conversion and negatively for xylose to xylitol conversion. It was noted that the fermentation products ethanol and xylitol were also assimilated by PTD3 in all experiments conducted when the carbon source (glucose or xylose) was depleted and thus concurred with previous findings [23]. Although this strain of the *R. mucilaginosa* strain PTD3 is a novel, not fully understood yeast, it demonstrated a great potential for future studies assessing its suitability in the bioconversion of lignocellulosic hydrolysates to biochemicals.

Conclusions

In this study, we investigated the effect of inhibitors on the production of xylitol and ethanol by *R. mucilaginosa* strain PTD3. Contrary to previous observations, it was shown that furfural boosted the xylitol yields at up to 5 g/l of furfural with constant xylitol production and xylose consumption rates, however, furfural negatively affected ethanol yields, glucose consumption and ethanol production rates by PTD3. 5-HMF at concentrations lower than 5 g/l increased or did not affect production of xylitol but lowered ethanol yields by this microorganism. Acetic acid, even at 20 g/l, stimulated ethanol yields for PTD3 while the opposite was observed for xylitol. PTD3 demonstrated the ability to tolerate higher concentrations of inhibitors during xylitol and ethanol production compared to other yeasts described in the literature. The use of PTD3 can been proposed to selectively remove inhibitors from lignocellulosic hydrolysates to improve the fermentability, since it was capable of metabolizing concentrations of 5, 15, and 5 g/l of furfural, 5-HMF, and acetic acid, respectively. PTD3 demonstrated an exceptional ability to ferment the sugars in presence of sugar degradation products, and to tolerate and metabolize furfural, 5-HMF, and acetic acid. The implications of this work cannot be understated. By simply fermenting media containing a certain concentration of fermentation inhibitors, the yield of xylitol and ethanol can be increased. PTD3 is a robust microorganism that is capable of fermenting lignocellulosic hydrolysates with the higher xylitol and ethanol yields compared to the control. This represents a promising means of increasing commercial ethanol and xylitol yields through simply monitoring and altering concentrations of fermentation inhibitors as they enter the process. Also, unlike reported before, this means that a detoxification step during bioconversion process is not necessary and PTD3 can be employed as detoxification agent due to its ability to metabolize the inhibitors. These findings have the potential to improve reproducibility of bench-scale research and reduce costs at the industrial scale by not needing the detoxification step during bioconversion process and improving xylitol and ethanol yields.

Acknowledgments This research was partially supported with funding from NSF DGE award 0654252, IGERT program on Bioresource Based Energy for Sustainable Societies, Consortium for Plant Biotechnology Research grant # EPA82947901, and Washington State Department of Ecology, Beyond Waste Organics Waste to Resources (OWR) project. We would like to thank Shannon Ewanick for all of her invaluable insights and help in the lab. We also thank Denman Professorship in Bioresource Science Engineering, University of Washington, for their financial support.

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