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BIOENERGY/BIOFUELS/BIOCHEMICALS

Novel endophytic yeast *Rhodotorula mucilaginosa* strain PTD3 I: production of xylitol and ethanol

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Abstract An endophytic yeast, *Rhodotorula mucilaginosa* strain PTD3, that was isolated from stems of hybrid poplar was found to be capable of production of xylitol from xylose, of ethanol from glucose, galactose, and mannose, and of arabitol from arabinose. The utilization of 30 g/L of each of the five sugars during fermentation by PTD3 was studied in liquid batch cultures. Glucose-acclimated PTD3 produced enhanced yields of xylitol (67% of theoretical yield) from xylose and of ethanol (84, 86, and 94% of theoretical yield, respectively) from glucose, galactose, and mannose. Additionally, this yeast was capable of metabolizing high concentrations of mixed sugars (150 g/L), with high yields of xylitol (61% of theoretical yield) and ethanol (83% of theoretical yield). A 1:1 glucose:xylose ratio with 30 g/L of each during double sugar fermentation did not affect PTD3's ability to produce high yields of xylitol (65% of theoretical yield) and ethanol (92% of theoretical yield). Surprisingly, the highest yields of xylitol (76% of theoretical yield) and ethanol (100% of theoretical yield) were observed during fermentation of sugars present in the lignocellulosic hydrolysate obtained after steam pretreatment of a mixture of hybrid poplar and Douglas fir. PTD3 demonstrated an exceptional ability to ferment the hydrolysate, overcome hexose repression of xylose utilization with a short lag period of 10 h, and tolerate sugar degradation products. In direct comparison, PTD3 had higher xylitol yields from the mixed sugar hydrolysate compared with the widely studied and used xylitol producer *Candida guilliermondii*.

Keywords Xylitol · Ethanol · Xylose · *Rhodotorula mucilaginosa* · *Candida guilliermondii*

Introduction

Different types of lignocellulosic biomass including agricultural, hardwood, and softwood residues can potentially be converted into various value-added products including biofuels and biochemicals. One of the products which can be obtained from biomass is xylitol. For over 30 years, considerable efforts have been focused on microbial production of xylitol from xylose [17, 35].

Xylitol is a five-carbon sugar alcohol with an established commercial history as an alternative sweetener. It has recently drawn the attention of food and drink manufacturers due to its low caloric value and thus the possibility of its use to reduce or control weight, leading to applications as a sweetener in chewing gums, mints, sweets, and toothpaste [9]. It has also been utilized in the pharmaceutical industry due to its role in reduction of dental cavities [28]. Although xylitol is currently produced chemically by catalytic reduction of xylose, various microorganisms can convert xylose to xylitol by biological means. Several xylose-fermenting yeasts which reduce xylose to xylitol by NAD(P)H-dependent xylose reductase (XR) such as *Candida*, *Pachysolen*, and *Debaryomyces* strains have been

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tested [8, 10, 28, 35]. *Candida* yeasts in particular have been extensively studied with regards to their biotechnological application in production of xylitol. Xylitol yields as high as 0.77 (g/g) for *C. guilliermondii* and 0.85 (g/g) for *C. tropicalis* have been reported by Barbosa [1] and Kwon [16], respectively. However, the yields depend on the type of microorganism employed and conditions for fermentation (nutrients, oxygen, pH, and temperature) used during the conversion of sugars to xylitol.

Establishing all experimental parameters and utilizing an appropriate microorganism for sugar fermentation are of great importance for complete bioconversion of sugars into various biochemicals. One yeast which was identified in our laboratories as being capable of rapid assimilation and catabolism of five- and six-carbon sugars (arabinose, galactose, glucose, xylose, and mannose) is *Rhodotorula mucilaginosa* strain PTD3, an endophytic yeast of hybrid poplar *Populus trichocarpa* × *deltoides* [4, 36]. Since this is a novel, newly discovered yeast, very little is known about it and its behavior during bioconversion of lignocellulosic sugars to xylitol, ethanol, and other co-products. Although studies have been done with other members of this yeast species for production of carotenoid pigments [20], lipid accumulation [13], and esterase activity [19], there has not yet been a detailed study conducted about *Rhodotorula mucilaginosa*'s ability to utilize a variety of sugars to produce xylitol and ethanol.

This is the first report on the bioconversion of xylose to xylitol, of six-carbon sugars to ethanol, and of arabinose to arabitol by the newly discovered yeast *Rhodotorula mucilaginosa* strain PTD3 during synthetic single, double, and mixed sugar fermentation. The objective of this work is to characterize the novel yeast for utilization of sugars for xylitol and ethanol production in single, double, and mixed sugar fermentation media and to test the PTD3 strain in fermentation of hydrolysate from pretreated lignocellulosic biomass in order to reveal its unique properties. The ultimate goal of our research regarding xylose utilization is to establish fermentation processes using both hexose and pentose fractions of hydrolysates obtained after steam pretreatment of lignocellulosic biomass to improve the feasibility of the bioconversion process.

Materials and methods

Yeast strains

Rhodotorula mucilaginosa strain PTD3, a pink yeast strain, was isolated from stems of hybrid poplar clone 184–402 (*Populus trichocarpa* × *P. deltoides*) from a greenhouse at Oregon State University, Corvallis [36]. *Candida guilliermondii* FTI-20037 (NRC 5578) was obtained from the American Type Culture Collection (ATCC), a nonprofit biological resource center (BRC), Manassas, VA.

These strains were 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 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 concurrent transfer to fresh medium performed every 24 h. *C. guilliermondii* was pregrown in a similar way, but instead of glucose, xylose was utilized.

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 and alcohols

Synthetic sugars (glucose, xylose, galactose, mannose, and arabinose) were obtained from Supelco (Bellefonte, PA). Ethanol 4 mg/mL, xylitol 5 mg/mL, arabitol, and glycerol were obtained from Sigma–Aldrich (St. Louis, MO).

Fermentations

Synthetic sugars

All fermentation experiments were performed three times with the appropriate controls that consisted of media lacking 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 to fermentation media.

Single sugar fermentations were performed in foam-plugged 125-mL Erlenmeyer flasks (semi-aerobic) containing 1% (w/v) yeast extract, 1× Murashige and Skoog medium [21], and 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 initial pH value of ~6.0.

Double and mixed sugar fermentations were performed in a similar manner as single sugar fermentation with the following modification: for double sugar fermentations, media consisted of 3% (w/v) each of glucose and xylose, while mixed sugar fermentation media contained 3% (w/v) of each sugar (arabinose, galactose, glucose, xylose, and mannose). Sampling was aseptically performed at 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, which 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.

Water-soluble fraction (hydrolysate) fermentation

A mixture of hardwood (hybrid poplar) and softwood (Douglas fir) chips (size 3/4 × 3/4/× 1/5 inch^3) with bark (60.0% moisture content) was obtained from a University of Washington waste facility and stored at 4°C until use. The mixture was pre-pretreated by soaking in water overnight prior to SO_2-catalyzed steam explosion. The detailed procedure of steam explosion experiments has been described previously by Ewanick [5]. Briefly, samples of 300 g oven-dried weight (ODW) soaked chips were impregnated overnight with anhydrous SO_2 in plastic bags. The samples were then loaded, in 50 g batches, into a preheated 2-L steam gun in Gresham, OR and exploded at temperature of 210°C, time 10 min, and 3% (w/w) SO_2 concentration.

The water-soluble fraction (hydrolysate) from steam explosion of the mixture of hardwoods and softwoods was recovered by filtration and kept at 4°C until use. The fermentation was performed in a similar manner to other fermentation experiments described earlier. The initial concentration of sugars present in the hydrolysate was arabinose (1.8 g/L), galactose (2.7 g/L), glucose (9 g/L), xylose (7.6 g/L), and mannose (9.2 g/L), and the concentration of fermentation inhibitors was acetic acid (2.1 g/L), 5-hydroxymethyl furfural (1.2 g/L), and furfural (0.6 g/L). A 1% (w/v) yeast extract and 1× Murashige and Skoog medium [21] were added to the hydrolysate fermented by PTD3, while 0.1% (w/v) yeast extract, 0.17% (w/v) yeast nitrogen base without amino acids, and 0.5% (w/v) urea were added to the hydrolysate fermented by *C. guilliermondii*. The initial pH of the hydrolysate was adjusted to pH 6 prior to fermentation. The controls consisted of synthetic sugars at the same concentration as measured in the hydrolysate.

HPLC analysis

Monomeric sugars

The concentration of monomeric sugars (arabinose, galactose, glucose, xylose, and mannose) was measured on a Dionex (Sunnyvale, CA) high-performance liquid chromatography (HPLC, ICS-3000) system equipped with an AS autosampler, ED electrochemical detector, dual pumps, and anion exchange column (Dionex, CarboPac PA1). Deionized water at 1 mL/min was used as eluent, and post-column addition of 0.2 M NaOH at flow rate of 0.5 mL/min ensured optimization of baseline stability and detector sensitivity. After each analysis, the column was reconditioned with 0.25 M NaOH. Twenty microliters of each sample was injected after filtration through a 0.22-μm syringe filter (Restek Corp., Bellefonte, PA, USA). Standards were prepared containing sufficient arabinose, galactose, glucose, xylose, and mannose to encompass the same range of concentrations as the samples. Fucose (0.2 g/L) was added to all samples and standards as an internal standard. The specific consumption rates were calculated based on the log-mean cell density

$$q_s = \frac{(S_0 - S)\ln\left(\frac{X}{X_0}\right)}{(X - X_0)\Delta t},$$

where S is the substrate or product, X is dry cell weight, and t is time [14].

Ethanol, xylitol, and arabitol analysis

Ethanol, xylitol, arabitol, and glycerol were measured using refractive index detection on a Shimadzu Prominence LC. Separation of these compounds was achieved by an anion exchange column [REZEX RHM-Mono saccharide H+ (8%); Phenomenex, Inc., Torrance, CA, USA] with an isocratic mobile phase that consisted of 5 mM H_2SO_4 at flow rate of 0.6 mL/min. The column oven was maintained at constant temperature of 63°C. Twenty microliters 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 [23]. Ethanol yields and percent theoretical yields were calculated using the equations formulated by Keating [15]. The theoretical yield for xylitol production from glucose used was 0.91 g xylitol g^{-1} xylose [35].

Separation of arabinose, arabitol, and xylitol was achieved by an anion exchange column [REZEX RCM-Mono saccharide Ca+ (8%); Phenomenex, Inc., and

Torrance, CA, USA] with isocratic mobile phase that consisted of HPLC-grade water at flow rate of 0.6 mL min^{-1}. The column oven temperature was maintained at 82°C. Twenty microliters of each sample was injected after being appropriately diluted in deionized water and filtered through a 0.22-mm syringe filter (Restek Corp., Bellefonte, PA, USA).

It was assumed that all xylitol formed during the growth phase of the mixed sugar fermentations was derived from xylose, and all arabitol formed during the same fermentation process was derived from arabinose. Cumulative xylitol (Yxylitol; g xylitol produced g^{-1} total xylose consumed) and arabitol (YArabitol; g arabitol produced g^{-1} total arabinose consumed) yields were calculated during and at the end-point of the fermentations. The specific production rates of xylitol from xylose, of ethanol from galactose, glucose, and mannose, and of arabitol from arabinose were calculated as described in the previous section.

Results and discussion

Single synthetic sugar fermentation

The utilizations of five single sugars (xylose, glucose, arabinose, galactose, and mannose) by *Rhodotorula mucilaginosa* strain PTD3 were studied in liquid batch cultures. In addition, the fermentation conditions for PTD3 (nitrogen, temperature, pH requirements, and inoculum size) had been tested previously as part of the preliminary investigation (data not shown). This is the first report on utilization of xylose to produce xylitol and six-carbon sugars to produce ethanol by *R. mucilaginosa* strain PTD3.

Previous research with other xylose utilizers such as *Pachysolen tannophilus* and *Pichia stipitis* has shown that the activities of xylose reductase (XR) and xylitol dehydrogenase (XDH), two key enzymes in xylitol production, are induced in xylose-pregrown but not glucose-pregrown yeasts [2]. Since *R. mucilaginosa* strain PTD3 has never been studied for production of xylitol from xylose and of ethanol from six-carbon sugars, during fermentation of xylose and glucose we tested yeast pregrown on glucose or xylose. It was shown that *R. mucilaginosa* pregrown on glucose converted xylose to xylitol and glucose to ethanol more efficiently (67 and 84% of theoretical yield, respectively) than when pregrown on xylose (59 and 64% of theoretical yield, respectively), with consumption and production rates being approximately twice as low (Tables 1, 2, 3). Contrary to previous research [2], pregrowing on xylose did not stimulate xylose consumption and improve *R. mucilaginosa* xylitol fermentations (Table 3). Therefore, to test the utilization of galactose, mannose, and arabinose, *R. mucilaginosa* was acclimated to glucose prior to fermentations. *R. mucilaginosa* exhibited varying responses to mannose, galactose, and arabinose following acclimation to glucose. Ethanol yields of 94% and 86% of theoretical and arabitol yields of 29% of theoretical yield were observed, respectively (Tables 1,2). Of these sugars, utilization of mannose was the most rapid, where complete consumption of 30 g/L of this sugar required 26 h (data not shown). This was followed by galactose (~50 h). Only 29% of theoretical yield conversion of arabinose to arabitol occurred within 100 h of fermentation (data not shown). The highest sugar consumption rate when PTD3 was pregrown on glucose was observed in glucose (0.53 g g^{-1} h^{-1}) and the lowest in arabinose (0.06 g g^{-1} h^{-1}) (Table 3). Among hexoses, the ethanol production rate was the highest from glucose (0.27 g g^{-1} h^{-1}) and the lowest from galactose (0.08 g g^{-1} h^{-1}) (Table 3).

For this microorganism, with glucose, ethanol was the major fermentation product and glycerol concentration was negligible (0.2 g/L) (data not shown). Similarly, during metabolism of xylose by PTD3, xylitol was the main product and no ethanol was accumulated. Corresponding sugar utilization patterns and lower xylitol (55% of theoretical) yield were reported previously for *C. guilliermondii* by Lee [17]. During single sugar fermentation, utilization of glucose was the most rapid; complete consumption of 20 g/L of this sugar required 12 h. This was followed by mannose (25 h), xylose (36 h), and galactose (42 h) with similarity to PTD3's ethanol yields [17]. These results indicate PTD3's great ability to metabolize each available substrate with high xylitol and ethanol yields.

Double synthetic sugar fermentation

The ability of *R. mucilaginosa* to utilize and ferment concurrently glucose and xylose was studied. Since single sugar fermentation demonstrated that product yields were affected by acclimation conditions, during double sugar fermentation *R. mucilaginosa* was pregrown on glucose. For *R. mucilaginosa*, when glucose was present in the medium with xylose, a sequential pattern of utilization was observed, with glucose being consumed ahead of xylose (Fig. 1). Utilization of glucose was not affected by xylose and commenced immediately (Fig. 1). However, xylose consumption was clearly affected by glucose and proceeded after lag period of 10 h (Fig. 1). This indicates the existence of a threshold above which glucose repression occurs as previously observed for *C. guilliermondii* and other yeast strains [17]. Double sugar fermentations indicated that *R. mucilaginosa* utilized xylose more slowly (75 h) compared with single sugar fermentation (56 h) (Fig. 1). Indicative of the preference for glucose, the

Table 1 Maximum xylitol yields [product per unit substrate (YP/S) and percent theoretical (Y%T)], arabitol yields, and biomass accumulation during single sugar fermentation by *R. mucilaginosa* (*Rm*) pregrown on glucose or xylose

	[XOH/AOH] YP/S (g/g)*	[XOH/AOH] Y%T (%)*	[Dry cell] max (g/L)
Xyl (Glu-grown)	0.3 ± 0	67 ± 0.3	13.6 ± 0.1
Xyl (Xyl-grown)	0.3 ± 0	59 ± 0.1	11.8 ± 0.1
Ara (Glu-grown)	0.2 ± 0	29 ± 0.3	7.8 ± 0.2

Standard deviation is indicated

* Xylitol (XOH) is the product of xylose, and arabitol (AOH) is the product of arabinose fermentation

Table 2 Maximum ethanol yields [product per unit substrate (YP/S) and percent theoretical (Y%T)] and biomass accumulation during single sugar fermentation by *R. mucilaginosa* (*Rm*) pregrown on glucose or xylose

	EOH YP/S (g/g)	EOH Y%T (%)	[Dry cell] max (g/L)
Glu (Glu-grown)	0.5 ± 0	84 ± 1	18.3 ± 0.1
Glu (Xyl-grown)	0.3 ± 0	64 ± 1	13.4 ± 0.2
Gal (Glu-grown)	0.4 ± 0	86 ± 1	16.2 ± 0.1
Man (Glu-grown)	0.5 ± 0	94 ± 1	16.1 ± 0.1

Standard deviation is indicated

Table 3 Specific rates of sugar consumption and XOH, EOH, and AOH production from synthetic sugars by *Rhodotorula mucilaginosa* (Rm) during single, double, and mixed sugar fermentation

Fermentation parameters	Single fermentation		Double fermentation		Mixed fermentation	
	Consumption ($g\ g^{-1}\ h^{-1}$)[a]	Production ($g\ g^{-1}\ h^{-1}$)[b]	Consumption ($g\ g^{-1}\ h^{-1}$)[a]	Production ($g\ g^{-1}\ h^{-1}$)[b]	Consumption ($g\ g^{-1}\ h^{-1}$)[a]	Production ($g\ g^{-1}\ h^{-1}$)[b]
Xylose (Xyl[c])	0.20 (0.12[c])	0.09 (0.06[c])	0.16	0.10	0.16	0.07
Glucose (Xyl[c])	0.53 (0.37[c])	0.27 (0.12[c])	0.51	0.24	0.47	NC
Galactose	0.18	0.08	NA	NA	0.16	NC
Mannose	0.34	0.17	NA	NA	0.46	NC
Arabinose	0.06	0.02	NA	NA	0.01	0

The reported results are the average of triplicate studies with deviation $\leq 2\%$

NA not applicable, *NC* not calculated. The ethanol production rate from each of glucose, galactose, and mannose was not calculated due to the difficulty of knowing the exact concentration of ethanol produced from each sugar during mixed sugar fermentation

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

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

[c] Production and consumption rates of PTD3 when pregrown on xylose; otherwise, PTD3 was always pregrown on glucose

specific glucose consumption rate was 3 times higher ($0.51\ g\ g^{-1}\ h^{-1}$) compared with the xylose rate ($0.16\ g\ g^{-1}\ h^{-1}$) (Table 3). Xylose and glucose specific consumption and production rates for double sugar fermentation were lower compared with single sugar fermentation (Table 3). The complete xylose consumption by *R. mucilaginosa* yielded almost identical xylitol yields of 65% of theoretical yield, compared with single sugar fermentation where 67% conversion of theoretical yield was achieved (Table 1; Fig. 1). The complete consumption of glucose for single and double sugar fermentations occurred in 20 h (Fig. 1). However, the ethanol yield for double sugar fermentation (92% of theoretical yield) was 12% higher compared with single sugar fermentation (Table 2; Fig. 1). The higher ethanol yield could be explained by the fact that PTD3 cell biomass was higher (3 g/L more compared with the single sugar fermentation, data not shown) in double sugar fermentation. It is assumed that xylose was utilized for cell growth rather than for xylitol production.

In comparison, the other xylose-fermenting and xylitol-producing microbe *Candida guilliermondii* during double sugar fermentation of xylose and glucose had a xylitol yield of about 38% of theoretical yield and metabolized 20 g/L of xylose within 70 h [17]. Metabolism of 20 g/L glucose started immediately and was completed in 10 h

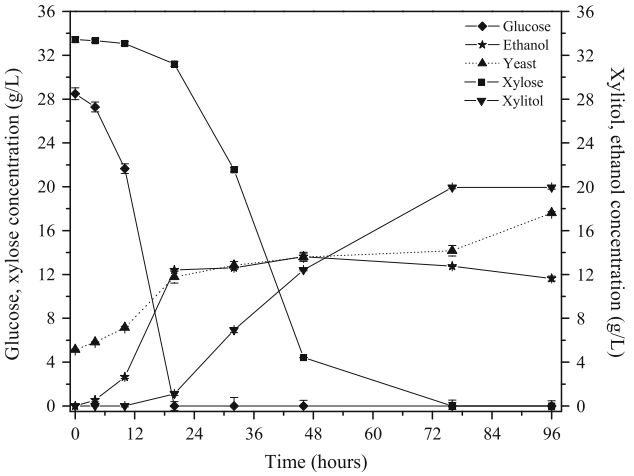

Fig. 1 Sugar consumption and xylitol and ethanol production during double (glucose and xylose) fermentation by *R. mucilaginosa* following acclimation to glucose. The *error bars* indicate standard deviation

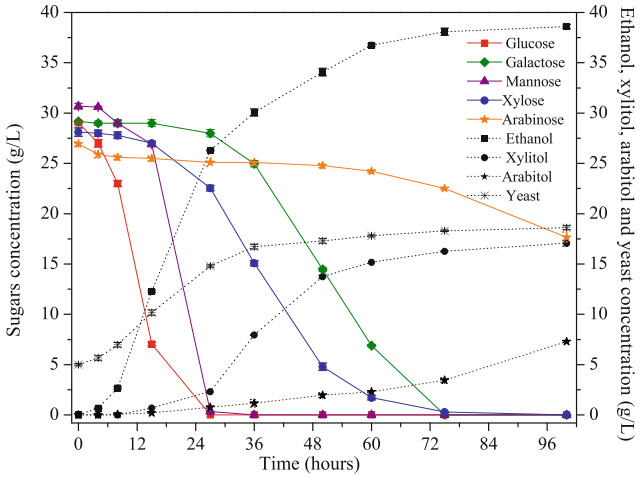

Fig. 2 Sugar consumption and xylitol and ethanol production during mixed (arabinose, galactose, glucose, mannose, and xylose) fermentation by *R. mucilaginosa* following acclimation to glucose. The *error bars* indicate standard deviation

with ethanol yield of 25% of theoretical yield. PTD3 had higher yields of both xylitol and ethanol compared with *C. guilliermondii*. However, PTD3 consumed xylose more slowly than *Candida guilliermondii*. This is possibly due to the presence of a higher concentration of xylose (30 g/L) compared with the media (20 g/L) that was fermented by *C. guilliermondii*. Xylose is needed for induction of xylose reductase and xylitol dehydrogenase, and thus high xylose concentration favors higher xylitol formation in yeasts [17, 35]. PTD3's higher xylitol yield could be due to the concentration of yeast used and nutrients added to the fermentation media that were different for *Candida guilliermondii*. Ultimately, after assessing PTD3's co-fermentability of xylose with glucose with high yields, the next step was to study PTD3's performance in mixed sugar fermentation composed of all five sugars that are naturally present in lignocellulosic hydrolysates.

Mixed synthetic sugar fermentation

For *R. mucilaginosa*, in the mixed sugar fermentation (arabinose, galactose, glucose, mannose, and xylose), a sequential pattern of utilization was observed, with glucose being consumed ahead of mannose, xylose, galactose, and arabinose (Fig. 2). Mixed sugar fermentations indicated that, for *R. mucilaginosa*, xylose was utilized as fast as for double sugar fermentation (75 h) and slower compared with single sugar fermentation (56 h) (Fig. 2). The complete xylose consumption by *R. mucilaginosa* yielded similar xylitol yields of 61% of theoretical compared with double (65%) and single sugar fermentation, where 67% conversion of theoretical yield was achieved (Table 1; Fig. 1).

The complete consumption of glucose from mixed sugar fermentation for PTD3 occurred in 26 h, of mannose in 36 h, and of galactose in 74 h. It took 6 h longer for complete glucose metabolism for mixed sugar fermentation compared with single and double sugar fermentations (20 h for both) (Table 1; Figs. 1 and 2). However, the ethanol yield (83%) for all the six-carbon sugars from mixed sugar fermentation was similar to or smaller than for single and double sugar fermentation (84 and 92% of theoretical yield, respectively) (Table 2; Figs. 1,2).

The utilization of mannose was not decreased but rather improved by the presence of the other sugars, while arabinose, galactose, glucose, and xylose were affected by being mixed together during mixed sugar fermentation compared with when they were the sole carbon source. The specific consumption rates of arabinose, galactose, glucose, and xylose (0.01, 0.16, 0.47, and 0.16 g g^{-1} h^{-1}, respectively) were smaller compared with those found when they were the sole carbon source (0.06, 0.18, 0.53, and 0.20 g g^{-1} h^{-1}, respectively) (Table 3). During mixed sugar fermentation, PTD3 fully metabolized mannose within the same time period (27 h) as during single sugar fermentation. Galactose was consumed completely, although at a rate slower (76 h) than when it was the sole carbon source (50 h), while arabinose was not completely metabolized within 100 h compared with single sugar fermentation (100 h).

The ability of *Rhodotorula mucilaginosa* strain PTD3 to utilize and ferment xylose in the presence of other sugars showed similar behavior to *Candida tropicalis* with a xylitol yield of 69% of theoretical yield [29]. When glucose, mannose, and galactose were present in the medium mixed with xylose, a specific pattern of consumption was

observed, with six-carbon sugars being consumed ahead of xylose and arabinose. In culture of PTD3, the presence of xylose did not affect hexose utilization. The assimilation of glucose and mannose commenced immediately, while that of xylose proceeded with a lag period of 12 h, similar to double sugar fermentation. This indicates the existence of a threshold above which hexose repression occurs. This behavior has been seen with other yeast strains [2, 27]. Also, galactose consumption lagged by 24 h. Although traditional microorganisms employed in ethanol fermentation exhibit preferences for hexose sugars, the mixture of glucose, galactose, and mannose presents a metabolic obstacle to efficient production of ethanol. This can be related to catabolite repression in which substrates are fermented sequentially; for example, galactose utilization markedly lags behind glucose and mannose consumption in the yeast *Saccharomyces cerevisiae* because of catabolite repression [7]. After assessing PTD3's fermentability of synthetic single, double, and mixed sugars, it was necessary to test its ability to ferment streams collected after steam pretreatment of lignocellulosic substrates.

Fermentation of the water-soluble fraction (hydrosylate) obtained after steam pretreatment of hardwood and softwood mixture

To characterize *Rhodotorula mucilaginosa* strain PTD3's fermentability of lignocellulosic hydrolysates, direct comparison with the well-known xylitol and ethanol producer *Candida guilliermondii* [11, 18, 32] was carried out.

Hydrolysate obtained from a steam-pretreated hardwood and softwood mixture was used as medium for production of xylitol and ethanol by *Rhodotorula mucilaginosa* strain PTD3. The comparison of *Rhodotorula mucilaginosa* and *Candida guilliermondii* was done using the same lignocellulosic hydrolysate and parameters for both microorganisms. However, the lignocellulosic hydrolysate prepared for PTD3 was supplemented with different nutrients compared with the hydrolysate fermented by *C. guilliermondii* (as described in "Methods" section). The synthetic mixed sugar controls were prepared using concentrations of each sugar found within the hydrolysate.

It was shown that PTD3 consumed xylose within 24 h, and a high xylitol yield of 78% of theoretical yield was obtained (Fig. 3a), higher compared with synthetic sugar control (65% of theoretical yield, data not shown). *Candida guilliermondii* consumed xylose within 9 h, and a high xylitol yield of 73% of theoretical yield was obtained (Fig. 3b), higher compared with control (64%, data not shown). Xylose consumption commenced immediately by both microorganisms, and no lag phase existed. The xylitol specific production rate for PTD3 was higher (0.04 g g^{-1} h^{-1}) compared with *C. guilliermondii* (0.03 g g^{-1} h^{-1}) (data not shown), and based on the xylitol theoretical yields, the xylitol production for PTD3 was more pronounced.

During fermentation of the hydrolysate, glucose was consumed within 9 h, and a high ethanol yield from all the six-carbon sugars was obtained of 100% of theoretical yield (Fig. 3a), higher compared with the control (83%, data not shown). For *C. guilliermondii*, glucose was consumed within 4 h, and a high ethanol yield of 100% was obtained (Fig. 3b), higher compared with control (66%, data not shown). The results from lignocellulosic hydrolysate were comparable to those attained in a synthetic medium and showed that lignocellulosic hydrolysate can be converted into xylitol and ethanol with approximately 15% higher yields. It is important to mention that the results were obtained without employing any detoxification methods such as yeast adaptation, neutralization and overliming, evaporation, solvent extraction, charcoal

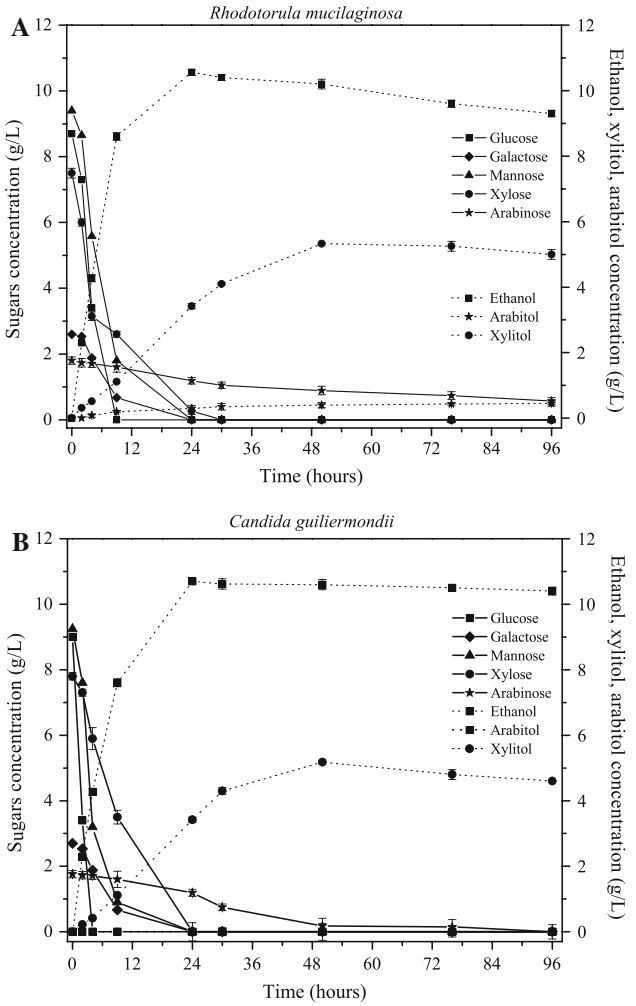

Fig. 3 Sugar consumption and xylitol and ethanol production during fermentation of hydrolysates obtained during steam explosion of the mixture of softwoods and hardwoods by: **a** *R. mucilaginosa* following acclimation to glucose, **b** *C. guilliermondii* following acclimation to xylose. The *error bars* indicate standard deviation

adsorption, biological treatment, or use of ion-exchange resin [3, 22, 24, 34]. PTD3 demonstrated an ability to metabolize a variety of sugars coming even from lignocellulosic hydrolysates and producing higher xylitol yields compared with those of *Candida guilliermondii* reported in other studies. Conversion of 66% of theoretical yield of xylose to xylitol was shown by Silva [31] using *C. guilliermondii* from acid-hydrolyzed hemicellulosic fractions of sugarcane bagasse and rice straw. Using the same strain, Felipe [6] reported xylitol yields of 29% of theoretical yield from hemicellulosic hydrolysate that was obtained by acid hydrolysis of eucalyptus chips.

It is noteworthy that PTD3 had a higher ethanol yield (100% of theoretical yield) from steam-pretreated hardwood and softwood mixture compared with *Saccharomyces cerevisiae*, Tembec Ltd. strain, which had an ethanol yield of 74% of theoretical yield from steam-pretreated Douglas fir with 10% bark [30]. The total concentrations of six-carbon sugars (31 g/L) and xylose (3.4 g/L) in hydrolysates that Robinson [30] tested were similar to the concentrations (21 g/L and 7.8 g/L, respectively) found in the hydrolysate tested in our study. The concentrations of fermentation inhibitors furfural and HMF that Robinson [30] reported were 0.3 and 1.4 g/L, respectively, while in the water-soluble stream we tested, concentrations of acetic acid (2.1 g/L), furfural (0.6 g/L), and HMF(1.2 g/L) were measured. The pretreatment conditions for both biomass types were similar, causing reduced generation of process-derived fermentation inhibitors. However, the difference was in the presence of 2.1 g/L of acetic acid in the hydrolysate that was tested in our study. This is due to the added presence of hardwoods in our feedstock. Hardwood hemicellulose is highly acetylated [25, 33], and thus acetic acid is produced by lignocellulose degradation.

One possible explanation for the higher ethanol yield obtained by PTD3 could be that PTD3 is a more robust, wild yeast compared with *S. cerevisiae* Tembec. Another is that the acetic acid present in the hydrolysate improved ethanol yields due to the yeast's need to maintain a neutral intracellular pH which is crucial for cell viability [12]. Low concentrations of acetic acid have been shown to have a stimulating effect on ethanol production by *S. cerevisiae* [26]. Thus, this could enhance the potential of this yeast for fermentation of hexose sugars in hydrolysates of lignocellulosic substrates.

In this study *R. mucilaginosa* strain PTD3 demonstrated the ability to assimilate all five sugars that are naturally present in lignocellulosic biomass and behaved similarly to the widely studied and used xylitol producer *C. guilliermondii*. Although *R. mucilaginosa* strain PTD3 is a novel yeast, it demonstrated great potential for future studies of bioconversion of lignocellulosic hydrolysates to biochemicals.

Conclusions

R. mucilaginosa strain PTD3 was found capable of assimilating and fermenting xylose, glucose, galactose, mannose, and arabinose as single as well as mixed carbon source. This strain produced not only xylitol from xylose but also ethanol and arabitol from hexoses and arabinose, respectively. When pregrown on glucose, PTD3's ability to metabolize sugars and produce xylitol and ethanol is enhanced. Xylitol and ethanol yields were not affected by a 1:1 ratio of xylose to glucose, resulting in repeated high theoretical yields (65 and 92%, respectively). Furthermore, the yeast exhibited the ability to ferment high concentrations of mixed sugars (150 g/L). Additionally, the specific xylitol production rate was highest for double sugar (0.10 g g^{-1} h^{-1}), and for ethanol was highest for single sugar fermentation (0.27 g g^{-1} h^{-1}). Remarkably, this yeast performed best during fermentation of sugars coming from lignocellulosic hydrolysate, producing the highest yields of xylitol (76% of theoretical yield) and ethanol (100% of theoretical yield). Fermentation of the steam-pretreated lignocellulosic hydrolysate served to illustrate PTD3's ability to utilize and ferment xylose in the presence of other sugars and to tolerate pretreatment degradation products.

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