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ORIGINAL PAPER

Overcoming inhibitors in a hemicellulosic hydrolysate: improving fermentability by feedstock detoxification and adaptation of *Pichia stipitis*

Rosanna M. Stoutenburg · Joseph A. Perrotta · James P. Nakas

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Abstract In order to improve the fermentative efficiency of sugar maple hemicellulosic hydrolysates for fuel ethanol production, various methods to mitigate the effects of inhibitory compounds were employed. These methods included detoxification treatments utilizing activated charcoal, anion exchange resin, overliming, and ethyl acetate extraction. Results demonstrated the greatest fermentative improvement of 50% wood hydrolysate (v/v) by *Pichia stipitis* with activated charcoal treatment. Another method employed to reduce inhibition was an adaptation procedure to produce *P. stipitis* stains more tolerant of inhibitory compounds. This adaptation resulted in yeast variants capable of improved fermentation of 75% untreated wood hydrolysate (v/v), one of which produced 9.8 g/l ± 0.6 ethanol, whereas the parent strain produced 0.0 g/l ± 0.0 within the first 24 h. Adapted strains RS01, RS02, and RS03 were analyzed for glucose and xylose utilization and results demonstrated increased glucose and decreased xylose utilization rates in comparison to the wild type. These changes in carbohydrate utilization may be indicative of detoxification or tolerance activities related to proteins involved in glucose and xylose metabolism.

Keywords Hemicellulosic · Hydrolysate · Detoxification · Ethanol · Adaptation

R. M. Stoutenburg · J. A. Perrotta · J. P. Nakas (✉)
Department of Environmental and Forest Biology,
SUNY College of Environmental Science and Forestry,
1 Forestry Drive, Syracuse, NY 13210, USA
e-mail: jpnakas@esf.edu

Introduction

Blending ethanol with gasoline has been widely employed for fuel oxygenation and to minimize consumption of non-renewable petroleum; however, the majority of ethanol produced for fuel utilizes corn-based feedstocks which are of questionable sustainability, and therefore, investigations for the efficient fermentation of other renewable feedstocks are of paramount importance [25]. As a result, production of ethanol from lignocellulosic hydrolysates for biofuel has been of great interest, but has faced issues due to the presence of hydrolysis-produced microbial inhibitors such as furfural, 5-hydroxymethylfurfural, acetic acid, and phenolics including vanillin and syringaldehyde [9, 11].

Investigators have utilized various methods of hydrolysate treatment in an attempt to reduce these inhibitory compounds in hydrolysate feedstocks [4, 7, 8, 10, 15, 20]. However, methods of post-hydrolysis treatment which optimize detoxification for improved fermentation will vary depending on the hydrolysate and the fermenting microorganism [21]. In addition, inhibitor removal techniques may also result in the removal of fermentable carbohydrates [10]. Therefore, comparisons of different treatments are warranted in order to maximize inhibitor removal and minimize reduction of carbohydrate concentrations.

Another alternative to post-hydrolysis treatment is use of inhibitor-tolerant microorganisms enabling the efficient fermentation of xylose, glucose, and other sugars to ethanol [18, 24]. Utilization of a microorganism with the ability to tolerate inhibitory compounds would reduce overall production costs for hemicellulose-derived ethanol because post-hydrolysis detoxification procedures could be eliminated or minimized.

In this study, post-hydrolysis detoxification treatments were assessed for *Pichia stipitis* (NRRL Y-7124) fermentation

of a sugar maple hemicellulosic hydrolysate. In addition, an adaptation protocol is described by which nine adapted *P. stipitis* variants were produced that are capable of growth on undiluted and untreated hydrolysate. Three of these variants (*P. stipitis* strains RS01, RS02, and RS03) were subsequently examined for rates of xylose and glucose utilization in comparison to the wild type.

Materials and methods

Microorganism and culture media

Pichia stipitis Y-7124 was obtained from the Agricultural Research Service (Northern Regional Research Laboratory, USDA, Peoria, IL), cultured in a nutrient medium with 3% xylose, and subsequently stored in 25% glycerol (v/v) at –85°C. All cultures were supplemented with 10 g/l yeast extract and 2 g/l KH_2PO_4, adjusted to pH 5.5, and maintained at 30°C and 150 rpm.

Hemicellulosic hydrolysate

Sugar maple hydrolysate was produced at SUNY ESF, Syracuse, NY, as described in Stoutenburg et al. [26]. Briefly, acid hydrolysis with 1–1.5% H_2SO_4 was followed by processing with a membrane purification system (diafiltration) to remove low and high molecular weight organics to preserve high carbohydrate concentrations and remove inhibitory compounds. However, adaptation and the subsequent fermentation with the adapted variants on 75% hydrolysate (v/v) were conducted utilizing a hydrolysate in which acid hydrolysis employed 1–1.5% 1:1 H_2SO_4/HNO_3 without diafiltration treatment.

Post-hydrolysis detoxification

Treated hydrolysates were adjusted to pH 5.5 with NaOH and sterilized at 70°C and 15–17 psi for 90 min. This method of sterilization (low temperature) was found to prevent pH reduction resulting from typical autoclaving conditions (Stoutenburg, unpublished). For a final concentration of 50% hydrolysate (v/v), 50 ml of 2× nutrient medium (as described above) was autoclaved in 250-ml flasks and the sterilized and treated hydrolysates were added. One treatment of 50% hydrolysate (v/v) pH 5.5 was filter-sterilized without other treatment to assess if autoclave conditions have an effect on fermentation. Additionally, a no treatment control of hydrolysate was autoclaved without alteration. All treatments were inoculated with 2 ml of a 3-day culture of *P. stipitis* grown on 3% xylose nutrient medium.

Activated charcoal

In 125-ml flasks, 3 and 5% activated charcoal powder (w/v) were added to 50 ml hemicellulosic hydrolysate. Covered flasks were subjected to treatment for 24 h at 150 rpm and 4°C to prevent microbial growth. The hydrolysate was subsequently filtered with Whatman no. 1 filter paper [17].

Anion exchange resin

Bio-Rad (Richmond, CA) analytical grade anion exchange resin AG 1-2X, chloride form, was washed with distilled water and 8 g was added to 50 ml of hydrolysate in 125-ml flasks (pH 3.46). Shake flasks were maintained at 150 rpm and 30°C for 1 h prior to filtration with Whatman no. 1filter paper [20].

Overliming

The pH of the wood hydrolysate was adjusted to 10.5 using $Ca(OH)_2$. These solutions were subsequently filtered through a cellulose acetate filter (0.22 μm) and adjusted to pH 5.5 prior to autoclaving [23].

Ethyl acetate extraction

Hydrolysate was adjusted to pH 6.5 with NaOH solution and 50 ml ethyl acetate was added (1:1). The hydrolysate/ethyl acetate mixture was maintained at 150 rpm and 30°C for 45 min. Following removal with a separatory funnel, residual ethyl acetate was removed by overnight evaporation [8].

Adaptation

In Pyrex 20 × 125-mm round-bottom screw-top test tubes, 10 ml of 10–100% (in increments of 10) and 25% hydrolysate (v/v) with nutrient medium components were autoclaved utilizing low temperature sterilization as described above. Hydrolysate agar plates were composed of 3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 30 g/l agar, and 1% hydrolysate (v/v) to maintain selective pressure. Figure 1 illustrates the methodology employed to adapt *P. stipitis* which was modeled after a procedure originally described by Yomano et al. [28]. Each transfer to a greater hydrolysate concentration was executed when vigorous growth was observed judged by gas production in the culture (3–8 days). The resulting nine adapted variants were each cultured in 25% hydrolysate (v/v) and subsequently stored in a 25% glycerol solution at –85°C.

Hydrolysate fermentation by adapted variants

The nine adapted *P. stipitis* variants and the wild-type strain were cultured in 100 ml of 75% untreated hydroly-

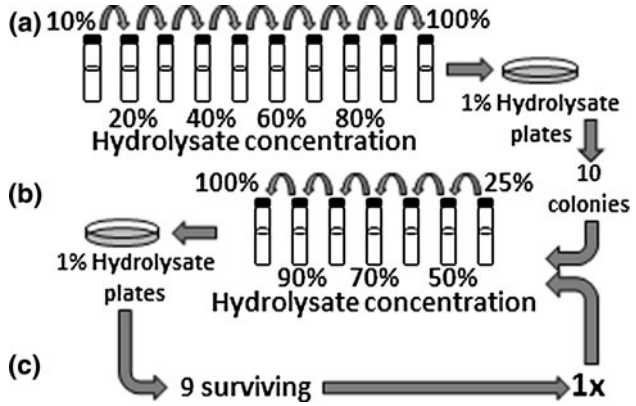

Fig. 1 Process by which adapted variants of *P. stipitis* were created. **a** *P. stipitis* was cultured in an untreated hydrolysate with concentrations from 10 to 100% (v/v). Each transfer to the next highest hydrolysate concentration was 1 ml into 10 ml of hydrolysate media. Subsequently, the 100% hydrolysate culture was streaked onto 1% untreated hydrolysate agar plates to maintain the presence of inhibitors present in the hydrolysate. **b** From the hydrolysate agar plates, 10 colonies were selected and each was separately cultured through the adaptation process from 25 (v/v) to 100% untreated hydrolysate. From the 100% hydrolysate cultures, 100-µl volumes were spread plated on hydrolysate plates. **c** Following **b**, nine variants were successfully cultured on hydrolysate plates. Each of the nine variants was cultured in media containing 25–100% (v/v) hydrolysate as described in **b**

Table 1 Results from hydrolysate detoxification treatments

	Sugar loss[a] (g/l)	Ethanol[b] (g/l)	Ethanol yield[c] (g ethanol/g sugar)	Phenolics[d] (% decr.)
3% Charcoal	4.8 ± 1.0	6.9 ± 0.4	0.21 ± 0.00	70
5% Charcoal	13.2 ± 0.6	6.5 ± 0.5	0.20 ± 0.02	70
Ethyl acetate	13.7 ± 1.6	4.8 ± 0.2	0.15 ± 0.01	25
Overliming	9.6 ± 1.2	3.9 ± 1.0	0.12 ± 0.02	2
Anion exchange	16.6 ± 2.8	3.2 ± 0.7	0.14 ± 0.03	85
Filter sterilize	–	0.9 ± 0.1	0.05 ± 0.01	0
No treatment	–	0.4 ± 0.0	0.03 ± 0.00	0

[a] Concentration of total sugars (g/l) lost resulting from the detoxification treatment (46.1 ± 1.7 g/l initial total sugar concentration)

[b] Ethanol concentrations (g/l) at 96 h

[c] g ethanol produced/g total sugar consumed at 96 h

[d] Percent decrease in phenolics after detoxification treatment

sate (v/v) supplemented with nutrient medium components (in 250-ml Erlenmeyer flasks). Utilization of a 75% hydrolysate concentration allowed for growth yet provided strong inhibitor pressure [26]. Hydrolysate precipitate was removed presterilization via centrifugation at 7,000 × *g* for 10 min.

Carbohydrate utilization by adapted strains

Adapted *P. stipitis* strains, RS01, RS02, and RS03, and the wild type were cultured on 2% xylose, 2% xylose plus 2% glucose, and 2% glucose nutrient media. Culture volumes were 100 ml in 250-ml Erlenmeyer flasks.

Analyses

Carbohydrate and ethanol concentrations were measured by high-performance liquid chromatography (HPLC) and gas chromatography, respectively, as previously described [26]. Phenolics were measured spectrophotometrically at 279 nm and a dilution of 1:100. Furans, which may increase absorbance readings at the aforementioned wavelength, were determined to be negligible by HPLC utilizing a C18 column.

Results and discussion

Post-hydrolysis detoxification

All post-hydrolysis detoxification treatments improved the fermentability of the hydrolysate by *P. stipitis* (conducted in triplicate), as shown by the ethanol yields (g ethanol/g sugar) and concentrations (g/l) at 96 h in comparison to the filter-sterilized and no treatment control (Table 1). In Fig. 2, ethanol concentrations for each treated hydrolysate further demonstrated improvements in ethanol yields and in some cases a reduced lag phase by *P. stipitis* on 50% sugar

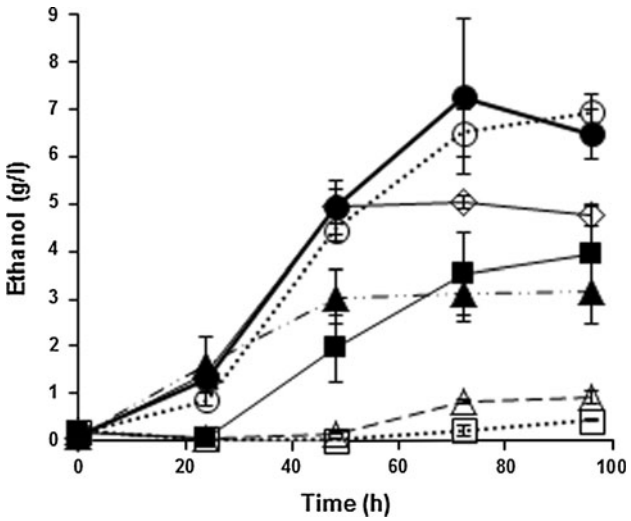

Fig. 2 Ethanol concentrations of *P. stipitis* grown on 50% (v/v) wood hydrolysate detoxified by treatment with 5% activated charcoal (*filled circles*), 3% activated charcoal (*open circles*), ethyl acetate extraction (*open diamonds*), overliming (*filled squares*), anion exchange resin (*filled triangles*), filter sterilization (*open triangles*), and the no treatment control (*open squares*)

maple hydrolysate (v/v). Treatment with activated charcoal produced the most ethanol (7.26 g/l ± 1.64 at 72 h and 6.9 g/l ± 0.4 at 96 h for 5% and 3% activated charcoal (w/v), respectively), utilized the most sugar, and exhibited the greatest conversion efficiency (Table 1). Treatment with 3% charcoal removed 8.4 g/l less sugars than 5% charcoal thereby preserving a greater possible ethanol yield. Processing wood hydrolysates with activated charcoals has been found to remove a number of inhibitory compounds including phenolics, aromatics, furans, coniferyl aldehyde, vanillin, vanillic acid, *p*-hydroxybenzoic acid, and acetic acid. This ability to remove a diversity of inhibitors likely contributed to the greatest improvement in ethanol yields among the detoxification treatments [6, 17].

Although the decrease in phenolics was minimal as a result of the ethyl acetate extraction (25%) in comparison to anion exchange (85%) or activated charcoal (70%) treatments, this detoxification method resulted in relatively high ethanol production (in comparison to the other treatments and controls), which suggests that phenolics, as an aggregate, may not be the primary source of inhibition for *P. stipitis*. This result is indicative of substantial inhibition of *P. stipitis* by either specific phenolic types or other inhibitory compounds, such as acetic acid or furans [21]. In fact, Delgenes et al. [9] reported that 2 g/l furfural or vanillin resulted in significant reduction of ethanol production by *P. stipitis*, whereas 1 g/l hydroxymethylfurfural did not.

Despite the improvement in ethanol production as a result of these detoxification techniques, ethanol concentrations remained below 8 g/l, even though total sugar utilization for treatments such as activated charcoal and ethyl acetate extraction were 80–100%. For 3% activated charcoal, ethanol yields were only 42% of the theoretical maximum (0.5 g ethanol/g sugar) [26]. It is possible that *P. stipitis* requires carbohydrate-derived metabolic energy for detoxification processes and survival mechanisms in the presence of the remaining inhibitors [22].

Adaptation

Following the adaptation procedure depicted in Fig. 1a, ten isolates of *P. stipitis* were selected and nine of these survived the subsequent adaptation. Growth of these nine adapted variants on 75% untreated wood hydrolysate (v/v), replicated in triplicate, revealed a significant improvement in ethanol production rates compared to the wild type (Fig. 3). *P. stipitis* variants 4, 8, and 9 continued to exhibit a significant lag period similar to the wild type, whereas 1, 2, 3, 5, 6, and 7 produced a significant quantity of ethanol (6.4–9.8 g/l) within the first 24 h (Fig. 3). Maximum ethanol concentrations were comparable for all the variants and the wild type; however, the rate at which ethanol production occurred varied considerably (Table 2). The wild type was strongly inhibited in the 75% hydrolysate as evidenced by ethanol concentrations of 0.0 g/l ± 0.0 at 24 h, whereas *P. stipitis* variant 1 reached 9.8 g/l ± 0.6 ethanol within 24 h. All adapted variants demonstrated higher ethanol concentrations in comparison to the wild type at 24 h through reduction or elimination of the initial lag period (Fig. 3). Similarly, Nigam [18] conducted adaptation experiments in which *P. stipitis* was adapted to tolerate 60% of a red oak acid prehydrolysate (v/v) and found that the adapted culture demonstrated no lag period, whereas the parent culture was incapable of ethanol production until after 50 h (30% prehydrolysate) or 65 h (60% prehydrolysate) of growth. Other studies have also demonstrated improved fermentability as a result of *P. stipitis* adaptation on wheat straw and corn cob hemicellulosic hydrolysates [3, 19]. Adapted *P. stipitis* variants 1, 3, and 5 (subsequently renamed RS01, RS02, and RS03, respectively) exhibited great potential for efficient ethanol production from sugar maple hydrolysate because they were capable of generating 8.1–9.8 g/l ethanol within 24 h on 75% (v/v) untreated hydrolysate and demonstrated high conversion efficiencies of sugar to ethanol (Table 2).

An increased rate of ethanol production has a significant impact when considering industrial production costs. Longer fermentations incur additional expense because more energy is required to maintain conditions (temperature, aeration, agitation, etc.) in a bioreactor. Therefore, production of comparable ethanol concentrations within

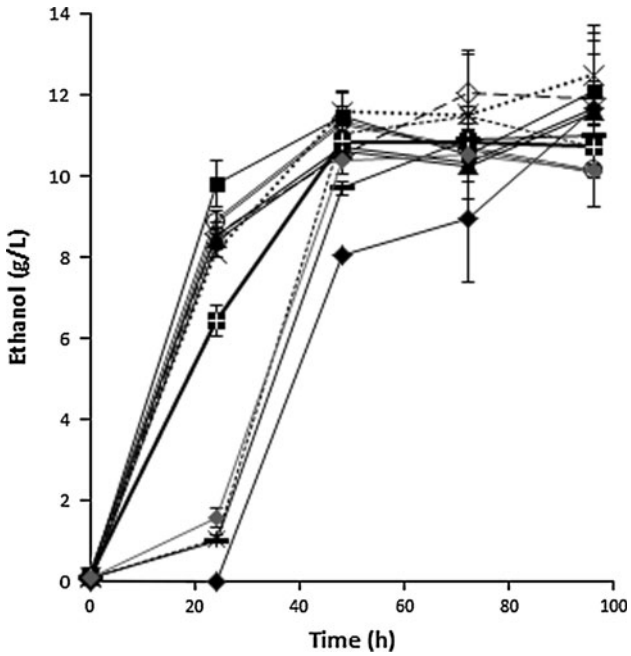

Fig. 3 Ethanol concentrations of adapted *P. stipitis* variants 1 (*filled squares*), 2 (*filled triangles*), 3 (*multiplication symbols*), 4 (*asterisks*), 5 (*open circles*), 6 (*plus symbols*), 7 (*open diamonds*), 8 (*filled rectangles*), 9 (*gray shaded diamonds*), and the wild type (*filled diamonds*) on 75% (v/v) hydrolysate

Table 2 Results for *P. stipitis* adapted variants and wild type on 75% hydrolysate cultures (with 51.0 g/l ± 1.5 initial sugar concentration)

Variant[a]	24 h		Maximum ethanol[c]		
	g ethanol/g sugar[b]	g ethanol/h	Time (h)	Ethanol[d]	g ethanol/g sugar[b]
Wild type	0.00 ± 0.00	0.00 ± 0.00	96	11.6 ± 1.7	0.40 ± 0.02
1 (RS01)	0.48 ± 0.02	0.41 ± 0.02	48	11.5 ± 0.2	0.46 ± 0.03
2	0.46 ± 0.02	0.35 ± 0.01	48	10.7 ± 0.2	0.48 ± 0.01
3 (RS02)	0.41 ± 0.02	0.34 ± 0.01	48	11.6 ± 0.5	0.49 ± 0.01
4	0.10 ± 0.01	0.04 ± 0.01	48	11.1 ± 0.2	0.48 ± 0.02
5 (RS03)	0.45 ± 0.02	0.37 ± 0.01	48	11.3 ± 0.7	0.46 ± 0.02
6	0.40 ± 0.08	0.27 ± 0.01	48	10.8 ± 0.2	0.45 ± 0.04
7	0.41 ± 0.02	0.04 ± 0.01	72	12.0 ± 1.0	0.40 ± 0.02
8	0.15 ± 0.04	0.04 ± 0.01	72	10.9 ± 0.2	0.36 ± 0.01
9	0.27 ± 0.03	0.07 ± 0.01	48	10.4 ± 0.3	0.43 ± 0.02

[a] The numbers 1–9 indicate the number assigned to a particular *P. stipitis* variant. Subsequently, variants 1, 3, and 5 were renamed RS01, RS02, and RS03, respectively

[b] g ethanol produced/g total sugar consumed

[c] Maximum ethanol data provided are for the highest ethanol concentrations reached at the earliest time point that are not significantly different (within standard deviations) from later concentrations

[d] Ethanol concentrations are given in g/l

48 h by the adapted variants (compared to 96 h in the wild type) is of considerable importance in order to obtain a more marketable product. Additionally, it would be more economical to utilize a microorganism with high tolerance to inhibitors rather than undertaking costly processing of the hydrolysate such as the aforementioned detoxification treatments. Further hydrolysate processing for inhibitor removal would require more inputs (activated charcoal, ethyl acetate, etc.) and thereby increase the overall cost of the product. Therefore, identification of tolerant strains and investigations into the factors contributing to such tolerances are of the utmost importance for reducing production costs of hemicellulosic hydrolysate-derived fuel ethanol.

Carbohydrate utilization of adapted strains

Carbohydrate consumption for *P. stipitis* RS01, RS02, RS03, and the parent strain (wild type) grown on 2% xylose, 2% xylose plus 2% glucose, and 2% glucose (in triplicate) revealed that the adapted strains utilized glucose more rapidly and consumed xylose at a reduced rate in comparison to the wild type (Fig. 4). In the 2% glucose cultures and 2% glucose plus 2% xylose cultures, the adapted strains completely depleted glucose by 24 h and produced maximum ethanol concentrations of 7.8 g/l ± 0.4 (RS02) (24 h) and 9.4 g/l ± 0.6 (RS03) (48 h), respectively (Fig. 4b, c). However, the wild-type (WT) strain exhibited 8.5 g/l ± 0.4 glucose remaining after 96 h in 2% glucose plus 2% xylose cultures but consumed all glucose by 72 h in 2% glucose cultures (Fig. 4b, c). It is possible that glucose utilization was reduced in the wild type because sugar transporters in *P. stipitis* are generalized and largely will transport both glucose and xylose, and therefore, transport competition and cellular carbohydrate concentrations may have inhibited glucose transport into cells [14, 27]. A change in this activity to preferentially transport glucose in the adapted strains may explain the observed increases in glucose utilization.

Xylose utilization by the adapted strains was reduced in comparison to the wild type when grown in the presence of 2% xylose (Fig. 4a). At 48 h, the adapted cultures (in 2% xylose) exhibited concentrations of 13.4–16.3 g/l xylose, whereas the wild type exhibited an average concentration of 1.0 g/l ± 0.4 xylose. All xylose was consumed by the wild type in 72 h but the adapted strains exhibited concentrations of 3.2–4.9 g/l xylose at 96 h. Ethanol production by the adapted strains was greatly reduced having concentrations less than 1 g/l (Fig. 4a). In contrast, the wild type yielded a maximum ethanol concentration of 4.6 g/l ± 0.1 in 48 h.

It is likely that the observed changes in carbohydrate utilization rates are linked with mechanisms enabling the adapted strains to better tolerate inhibitory compounds present in the hydrolysate to which they were adapted. Previous studies have indicated that yeast tolerance to inhibitors such as furfural, 5-hydroxymethylfurural, and ethanol are likely conveyed by multiple genes [1, 16]. Such genes have been known to include functional and regulatory genes which act in concert to convey improved growth in the presence of inhibitors [16].

In *P. stipitis*, xylose is metabolized via the pentose phosphate pathway (PPP) which then feeds into glycolysis [13].

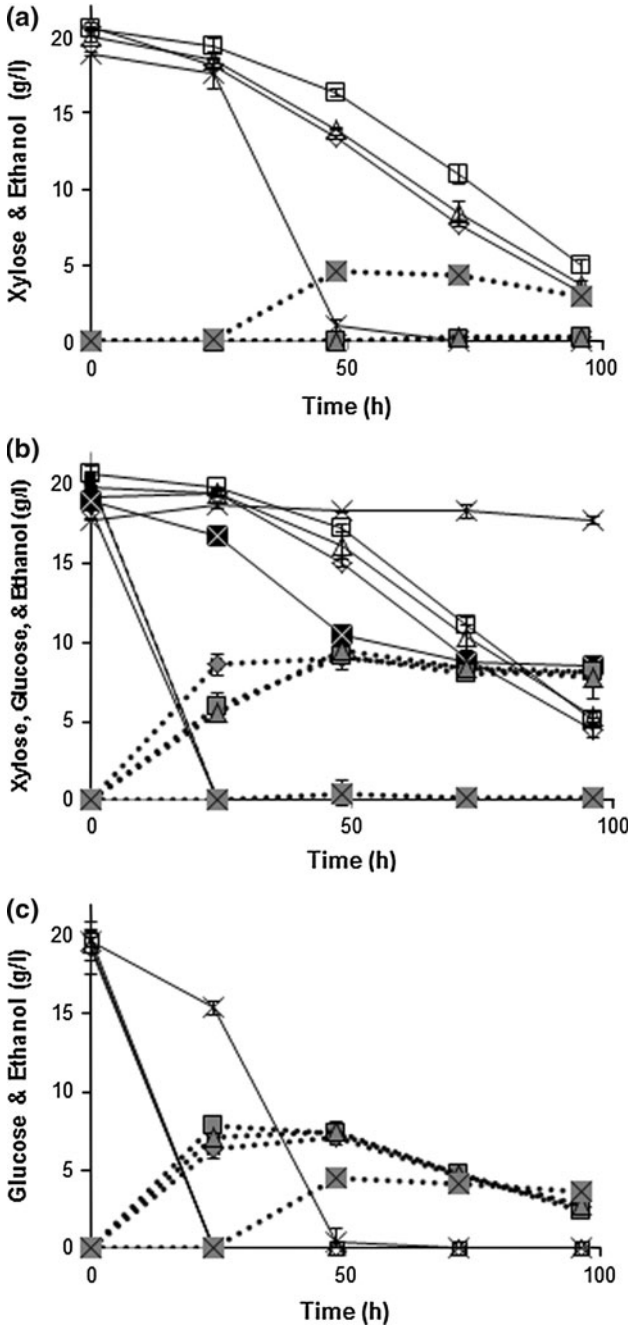

Fig. 4 Xylose concentrations for RS01 (*open diamonds*), RS02 (*open squares*), RS03 (*open triangles*), and WT (*multiplication symbols*); glucose concentrations for RS01 (*filled diamonds*), RS02 (*filled squares*), RS03 (*filled triangles*), and WT (*filled multiplication symbols*); and ethanol concentrations for RS01 (*gray shaded diamonds*), RS02 (*gray shaded squares*), RS03 (*gray shaded triangles*), and WT (*gray shaded multiplication symbols*) on **a** 2% xylose, **b** 2% xylose plus 2% glucose, and **c** 2% glucose cultures. In **b** and **c**, glucose concentrations for RS01, RS02, and RS03 are 0 g/l at 24 h

Because glucose metabolism was increased significantly in the adapted strains, and xylose metabolism was reduced, it is not likely that there was a change in the glycolytic enzymes or their expression. Therefore, the change in glucose metabolism observed in this study is likely linked with the initial glycolytic reactions upstream of PPP additions into glycolysis [27].

Table 3 Rates of carbohydrate consumption (g sugar/h[a]) for 2% xylose, 2% xylose plus 2% glucose, and 2% glucose cultures of *P. stipitis* RS01, RS02, RS03, and the parent strain

	2% Xylose	2% Xylose + 2% Glucose		2% Glucose
		Xylose	Glucose	
Wild type	0.31	0.00	0.15	0.30
RS01	0.19	0.21	0.77	0.80
RS02	0.17	0.21	0.84	0.80
RS03	0.18	0.20	0.84	0.80

[a] Rates were determined using best fit slopes during active carbohydrate consumption

Additionally, genes involved in the PPP may be responsible for the reduced xylose utilization observed in the adapted strains [13]. Gorsich et al. [12] presented evidence that some PPP genes are connected with furfural tolerance in *Saccharomyces cerevisiae* and therefore are of particular interest for tolerance activities in these adapted *P. stipitis* strains.

The xylose reductase has previously been established as an enzyme involved in detoxification processes in *P. stipitis* and, therefore, may be related to changes observed in the adapted strains [2]. However, because there are multiple copies of the gene cluster encoding the xylose reductase gene (aldo/keto reductase) only a regulatory change would have resulted in the observed decrease in xylose utilization [13]. If this were the case, then this gene cluster would be downregulated because the adapted strains have a reduced consumption of xylose. However, given that xylose reductase has been demonstrated in aiding detoxification processes, it is unlikely that downregulation would be advantageous for *P. stipitis* survival in a concentrated wood hydrolysate [2]. Therefore, it is doubtful that xylose reductase caused the observed change in xylose metabolism in *P. stipitis* RS01, RS02, and RS03.

P. stipitis will preferentially consume glucose prior to xylose because xylose reductase and xylitol dehydrogenase induction is inhibited in the presence of glucose; therefore, catabolite repression and slow glucose utilization in the glucose/xylose medium prevented any xylose consumption by the wild type [5]. As the adapted strains rapidly consumed all the glucose allowing for xylose utilization, this is an important improvement in *P. stipitis* activity because hemicellulosic hydrolysates contain some glucose. Thus this novel ability by the adapted *P. stipitis* strains enables more efficient ethanol production in a mixed carbohydrate feedstock. The glucose/xylose mixture improved the rates of xylose consumption for the adapted strains over those of xylose alone (Table 3).

Much can be learned about the mechanisms by which *P. stipitis* will tolerate inhibitors through analyzing these adapted strains. An analysis of inhibitor tolerances in addition to investigations into detoxification genes, which may be upregulated or altered resulting in improved survival and fermentation, may reveal information vital to improving fermentation of hemicellulosic hydrolysates.

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