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Mutants of the Pentose-Fermenting Yeast *Pichia stipitis* With Improved Tolerance to Inhibitors in Hardwood Spent Sulfite Liquor

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ABSTRACT: Mutants of *Pichia stipitis* NRRL Y-7124 able to tolerate and produce ethanol from hardwood spent sulfite liquor (HW SSL) were obtained by UV mutagenesis. *P. stipitis* cells were subjected to three successive rounds of UV mutagenesis, each followed by screening first on HW SSL gradient plates and then in diluted liquid HW SSL. Six third generation mutants with greater tolerance to HW SSL as compared to the wild type (WT) were isolated. The WT strain could not grow in HW SSL unless it was diluted to 65% (v/v). In contrast, the third generation mutants were able to grow in HW SSL diluted to 75% (v/v). Mutants PS301 and PS302 survived even in 80% (v/v) HW SSL, although there was no increase in cell number. All the third generation mutants exhibited higher growth rates but significantly lower growth yields on xylose or glucose compared to the WT. The mutants fermented 4% (w/v) glucose as efficiently as the WT and fermented 4% (w/v) xylose more efficiently with a higher ethanol yield than the WT. In a medium containing 4% (w/v) each of xylose and glucose, all the third generation mutants utilized glucose as efficiently and xylose more efficiently than the WT. This resulted in higher ethanol yield by the mutants. The mutants retained the ability to utilize galactose and mannose and ferment them to ethanol. Arabinose was consumed slowly by both the mutants and WT with no ethanol production. In 60% (v/v) HW SSL, the mutants utilized and fermented glucose, mannose, galactose and xylose while the WT could not ferment any of these sugars.

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KEYWORDS: biomass; ethanol; fermentation; lignocellulose; mutants; *Pichia stipitis*; spent sulfite liquor (SSL); tolerance; yeast

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Introduction

Lignocellulosic residues, composed of cellulose, hemicellulose, and lignin, are the most abundant renewable resource available for the production of ethanol. One of the major barriers of lignocellulose conversion to ethanol is the presence of pretreatment-derived chemical inhibitors which adversely affect yeast growth and fermentation (Klinke et al., 2004; Lohmeier-Vogel et al., 1998). *Saccharomyces cerevisiae*, commonly used for industrial ethanol fermentation, has a high tolerance to the pretreatment derived inhibitory compounds (Olsson and Hahn-Hägerdal, 1993). However, wild-type (WT) strains of this yeast cannot utilize or ferment the pentoses in lignocellulosic hydrolysates to ethanol. Naturally occurring pentose-fermenting yeasts such as *Pichia stipitis*, *Candida shehatae*, and *Pachysolen tannophilus* ferment both glucose and xylose to ethanol (Bicho et al., 1988; Du Preez and Prior, 1985). In particular, strains of *P. stipitis* can convert xylose almost exclusively to ethanol with no other significant byproducts (Slininger et al., 1985). However, pentose-fermenting yeasts generally suffer from hexose repression (Bicho et al., 1988), low ethanol tolerance (Barbosa et al., 1990) and they perform poorly in the presence of hydrolysate inhibitors (Lohmeier-Vogel et al., 1998).

Among the lignocellulosic hydrolysates, spent sulfite liquor (SSL) is produced by the acid bisulfite pulping of wood. The sugar content of SSL ranges from 3% to 4% (w/v), and varies depending upon the source of the wood being pulped. Sulfite pulping of hardwoods produces SSL with higher pentose content (50–70% of total sugars) than softwoods (26% of total sugars) (Helle et al., 2004). SSL also contains inhibitory compounds such as acetic acid, hydroxymethyl furfural and furfural (Klinke et al., 2004; Nigam, 2001a). SSL typically has a high BOD load and presents a disposal problem. To alleviate the pollution

problem, some sulfite pulp mills such as Tembec in Témiscaming (Québec, Canada) use SSL as a substrate for alcohol production. *S. cerevisiae*, commonly used in SSL fermentation, is unable to ferment pentoses to ethanol. Some researchers have investigated the use of pentose-fermenting yeasts such as *P. stipitis* (Nigam, 2001b) or *C. shehatae* (Yu et al., 1987) for SSL fermentation. To alleviate the toxicity from inhibitors, SSL has been subjected to pretreatments such as steam stripping to raise the pH, treating in succession with anion and cation exchange resins and also by over-liming (Amartey and Jeffries, 1996; Van Zyl et al., 1988; Yu et al., 1987). These procedures have led to some improvements in SSL fermentability. However, extensive pretreatment is impractical when dealing with large volumes of SSL. Other than SSL pretreatments, some researchers have tested adapted yeast strains which have been transferred repeatedly in SSL (Nigam, 2001b). However, SSL-adapted pentose-fermenting yeasts generally gave lower ethanol yields from SSL compared to SSL-adapted *S. cerevisiae*, even though *S. cerevisiae* WT cannot utilize pentoses (Helle et al., 2004; Nigam, 2001b). As a result, companies such as Tembec continue to use *S. cerevisiae* for SSL fermentation in its alcohol plant, leaving the pentoses unfermented.

In this research, we used UV mutagenesis followed by screening to isolate mutants of *P. stipitis* having higher tolerance towards HW SSL while retaining the ability to ferment the main sugars in HW SSL to ethanol. Selected mutants were characterized and compared with the *P. stipitis* WT for their fermentative ability in a chemically defined medium and in HW SSL.

Materials and Methods

Yeast Strain and Chemicals

Pichia stipitis NRRL Y-7124 (NRC 2548) was obtained from the National Research Council Canada Culture Collection (Ottawa, Canada). All chemicals were purchased from Sigma–Aldrich. Hardwood spent sulfite liquor (HW SSL) was kindly provided by Tembec (Témiscaming, Québec, Canada) and stored at 4°C. The HW SSL contained, in (w/v): 0.076% arabinose, 2.2% xylose, 0.25% galactose, 0.33% glucose, 0.55% mannose, 1% acetic acid, 0.18% furfural, and 0.11% 5-hydroxymethyl furfural (HMF). The pH of HW SSL (initially at 2.5) was raised to 5.5 with 10 M NaOH and the HW SSL was boiled for 5 min in a microwave and then cooled to room temperature before use.

Culture Maintenance and Inoculum Preparation

P. stipitis WT and mutants were maintained on Potato Dextrose Agar (PDA) plates at 4°C and subcultured at 2-month intervals. The strains were lyophilized for long-term storage. For inoculum preparation, a loopful of cells from an isolated colony on PDA agar plate was aseptically transferred to 20 mL of broth containing 0.67% (w/v) yeast nitrogen base (YNB) without amino acids and 2% (w/v) xylose in a 125-mL Erlenmeyer flask. The culture was grown with shaking (180 rpm) at room temperature (23 ± 1°C) for 48 h.

UV Mutagenesis

One milliliter of the WT inoculum was transferred to an empty Petri dish and exposed to UV light at a distance of 40 cm for 20 s to achieve 50% survival rate. During UV exposure, the Petri dish was held stationary. The UV-irradiated culture was transferred to a sterile 15-mL centrifuge tube and incubated in the dark at 23 ± 1°C with shaking in a tube rotator in which the tube was placed at 60° from horizontal and rotated at 90 cycles per minute about its vertical axis (Barbosa et al., 1990). After 24 h, 100 μL of the UV-exposed culture were spread on HW SSL (pH 5.5) gradient plates and incubated at 28°C for 5–10 days. The square glass gradient plates (120 mm × 120 mm × 17 mm) were prepared according to Syzbalski and Bryson (1952).

Colonies growing at higher HW SSL concentrations, that is, showing better SSL tolerance, than the WT were isolated and inoculated on YNB plates supplemented with 2% (w/v) of either xylose or glucose to verify they continued to utilize each of these sugars. Putative mutant colonies were maintained on PDA plates and designated as first generation mutants. In the second round of mutagenesis, each of the first generation mutants was individually exposed to UV light. In the third round of mutagenesis, all the second generation mutants were pooled together and mutagenized. The procedures for the second and third rounds of UV mutagenesis and mutant selection were the same as described for the first round.

Growth Assessment in Diluted HW SSL

Mutants showing better tolerance than the WT on SSL gradient plates were further assessed for growth in liquid SSL. The *P. stipitis* WT was unable to grow in undiluted liquid HW SSL. Thus, HW SSL was diluted to 65%, 70%, and 75% (v/v) with sterile distilled water for these growth tests. One milliliter of the inoculum was transferred to 50 mL of the diluted HW SSL in 250-mL Erlenmeyer flask. The flasks were incubated with shaking (180 rpm) at 23 ± 1°C. Growth was monitored periodically by withdrawing samples and spreading serially diluted samples on PDA plates and enumerating colony forming units (CFU) after 48 h.

Growth Assessment in the Presence of Acetic Acid, Furfural, and HMF

Acetic acid, furfural, and HMF are among the most potent inhibitors generated from acid hydrolysis of lignocellulosic

substrates (Klinke et al., 2004). The effect of acetic acid was first assessed on solid medium. One hundred microliters of the WT or mutant inoculum cultures were spread on acetic acid gradient plates, prepared according to Syzbalski and Bryson (1952), and incubated at 28°C. Growth was assessed visually for 5–10 days. The effect of acetic acid was further assessed in liquid culture. Ten milliliters of pH unadjusted medium containing 0.67% (w/v) YNB w/o amino acids, 2% (w/v) xylose and varying concentrations (0.20–0.32%, w/v) of acetic acid were inoculated with 0.2 mL of the inoculum culture. A narrow range of acetic acid concentrations was chosen based on preliminary test of the acetic acid tolerance range of the WT strain. The cultures were grown in loosely capped glass test tubes shaken in the tube rotator as described above. Growth was monitored by direct measurement of the optical densities at 600 nm (OD600) of the cultures against the medium blank using a Spectronic 20 spectrophotometer.

For growth assessment in the presence of HMF and furfural, 0.2 mL of the WT or mutant inoculum cultures were separately inoculated in 10 mL of pH unadjusted medium containing 0.67% (w/v) YNB w/o amino acids, 2% (w/v) xylose and varying concentrations of HMF (0.4% and 0.5%, w/v) or furfural (0.1% and 0.2%, w/v). HMF was prepared as 8.8 M stock and was filter sterilized before use while furfural was directly used to amend the medium as described by Liu et al. (2004). The cultures were grown in loosely capped glass test tubes and growth monitored by direct OD600 measurements as described.

Growth Rate and Growth Yield of Mutants on Glucose or Xylose

An aliquot (0.2 mL) of the inoculum culture was added to 10 mL of YNB plus 4% (w/v) of either xylose or glucose held in loosely capped glass test tubes as described above. Growth was monitored by measuring OD600. Growth rate was determined during the early exponential phase. Growth yield (dry cell weight) was measured gravimetrically after 48 h. To do this, a sample of the cell culture was centrifuged; cells were washed twice with distilled water and dried at 90°C to constant weight.

Fermentation Assessment

Mutants were first assessed for their ability to ferment each of the lignocellulosic biomass sugars (glucose, mannose, galactose, xylose, and arabinose) individually or a mixture of glucose and xylose using high cell density inocula. Inocula were grown as described, except that 100 mL of the medium in 250-mL flask were used for each WT culture. Since the WT typically produced about twice the amount of biomass produced by the mutants, inoculum of each mutant was grown in two batches of 100 mL of medium. The flasks were incubated at 24°C and shaken at 180 rpm. After 48 h, the cells were harvested by centrifugation at 10,000*g* for 5 min at 4°C. Each pellet was washed twice with sterile distilled water and suspended to an OD600 of about 5.0–6.0 in 100 mL of 0.67% (w/v) YNB w/o amino acids supplemented with 4% (w/v) of one of xylose, glucose, mannose, galactose, and arabinose; or 4% (w/v) of xylose plus 4% (w/v) of glucose in the case of mixed sugar fermentation. An OD600 of 1.0 was equivalent to a cell dry wt of 0.22–0.27 mg/mL. Each culture was transferred to a 250-mL Erlenmeyer flask and incubated with shaking (180 rpm) at 23 ± 1°C. At periodic intervals, 1-mL samples were withdrawn for ethanol and sugar analysis.

The mutants were further tested for the ability to ferment the sugars in 50 and 60% (v/v) diluted HW SSL using high cell density inocula. The HW SSL was diluted with water and then supplemented with sugars so that the initial concentration of each sugar was the same as that in undiluted SSL. The inocula for the WT and mutants were grown in the same way as that described for the sugar fermentations above using high cell density inocula. After 48 h of growth, the cells were harvested and the pellet was suspended in 100 mL of diluted HW SSL held in a 250-mL Erlenmeyer flask. The flasks were incubated at 23 ± 1°C as described. Samples (2 mL) were withdrawn every 24 h for sugar and ethanol analysis.

Analytical Methods

Fermentation samples were centrifuged at 8,000*g* for 2 min in an Eppendorf centrifuge to obtain the supernatants before analysis. In chemically defined media, ethanol was measured enzymatically as described by Kägi and Vallee (1960). In cultures of defined media containing one sugar, the total reducing sugar was measured by the dinitrosalicylic acid method (Miller, 1959). In defined media containing mixed sugars, the residual sugars were measured by HPLC using an Aminex HPX-87H column (Barbosa et al. 1990). Ethanol and sugar concentrations in HW SSL were analyzed using an Equity-1 and Equity-1701 GC column (Supelco, Bellefont, PA), respectively, with an Agilent model 6890N GC-FID. Sugars were analyzed by GC as per-acetylated aldononitrile derivatives (McGinnis, 1982).

All liquid culture experiments were conducted at least three times using independently grown inocula. Since the sampling periods differed for each replicate experiment, figures presented show the general trends for one representative independent experiment.

Results

Isolation of HW SSL Tolerant Mutants

P. stipitis was subjected to three rounds of UV mutagenesis. After each round of mutagenesis, the cells were allowed to recover for 24 h before being spread on HW SSL gradient plates. After the first round of mutagenesis, six HW

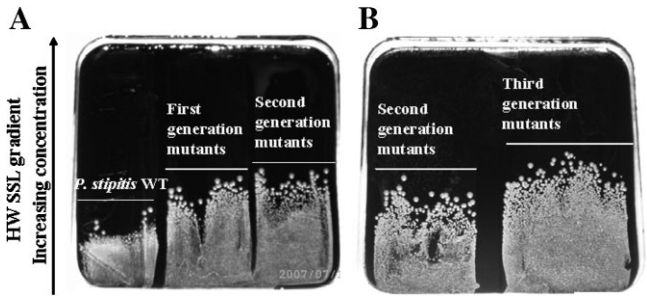

Figure 1. Growth of *Pichia stipitis* first, second (**A**) and third (**B**) generation mutants on HW SSL gradient plate.

SSL-tolerant mutants which appeared at higher SSL concentrations on the gradient plate relative to the WT were selected (Fig. 1A). These first generation mutants, designated as PS101, PS102, PS103, PS104, PS105, and PS106, were grown several times on HW SSL gradient plates to confirm their improved SSL tolerance, stability, and purity. Simultaneously, each mutant was also shown to grow on chemically defined media containing either xylose or glucose as the sole carbon source, like the WT. This was done to rule out the possibility of selecting for non-*P. stipitis* contaminants.

The WT did not grow in HW SSL unless the SSL was diluted to 65% (v/v) with distilled water. At this dilution, the WT did not grow until after day 6 (Fig. 2A). In contrast, all six first generation mutants grew in liquid HW SSL diluted to 65% (v/v) at a faster rate as compared to the WT (Fig. 2A). Further mutagenesis of the first generation mutants followed by screening resulted in the isolation of five second generation mutants, designated as PS201, PS203, PS204, PS205, and PS206, with further improved tolerance on the HW SSL gradient plate (Fig. 1). The second generation mutants could grow in 70% (v/v) HW SSL (Fig. 2B). At this concentration of HW SSL, the population of WT cells dropped by four orders of magnitude in 8 days (Fig. 2B). The second generation mutants were pooled and subjected to UV mutagenesis followed by screening. Six third generation mutants, designated as PS301, PS302, PS303, PS304, PS305, and PS306, showing further tolerance on HW SSL gradient plates were selected (Fig. 1B). The third generation mutants were capable of growing in liquid HW SSL diluted to 75% (v/v) with distilled water after an initial lag phase of 2–4 days (Fig. 2C). In particular, PS301 and PS302 were able to tolerate, and remain viable in, 80% (v/v) HW SSL although no increase in cell number was observed over a period of 10 days (Fig. 2D). All 6 third generation mutants were used for subsequent characterization studies. Most of them showed similar patterns of growth and fermentation. To avoid having too many overlapping lines, only the data for PS301 and PS302, the two strains most tolerant of HW SSL, are presented in most of the figures. All mutants were stable and retained their HW SSL tolerant

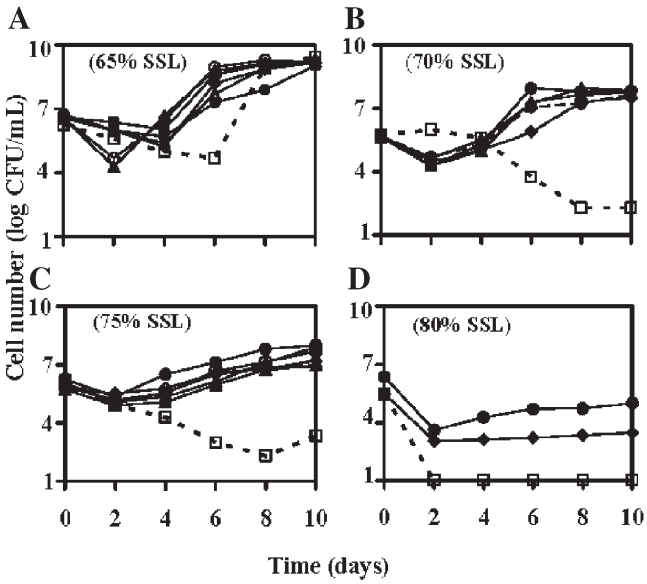

Figure 2. Growth of *P. stipitis* mutants in (**A**) 65%, (**B**) 70%, (**C**) 75%, and (**D**) 80% (v/v) HW SSL. A and B: Represent the first and second generation mutants, respectively. C and D: Represent the third generation mutants. Symbols in (A): WT (□), PS101 (■), PS102 (▲), PS103 (○), PS104 (◆), PS105 (Δ), and PS106 (●). Symbols in (B): WT (□), PS201 (▲), PS202 (◆), PS203 (●), PS204 (Δ), and PS205 (○). Symbols in (C): WT (□), PS301 (◆), PS302 (●), PS303 (▲), PS304 (○), PS305 (Δ), and PS306 (◇). Symbols in (D): WT (□), PS301 (◆), and PS302 (●).

phenotype even after ten cycles of growth on a non-selective medium.

Tolerance of Third Generation Mutants Towards Acetic Acid, HMF, and Furfural

Mutants PS301 and PS302 grew in the presence of 0.32% (w/v) acetic acid, as demonstrated by increases in cell densities of both mutants by more than one log unit over a period of 4 days (Fig. 3F). Other third generation mutants tolerated 0.28–0.30% (w/v) acetic acid. In contrast, the *P. stipitis* WT could grow only up to 0.25% (w/v) acetic acid after a lag period of 4 days (Fig. 3C).

The concentrations chosen for growth assessment in the presence of furfural and HMF were similar to or higher than those in the HW SSL. In general, no difference was seen between the WT and mutants in their growth responses in the absence of the inhibitors (Fig. 4D) or at the lower HMF (0.4%, Fig. 4A) or furfural (0.1%, Fig. 4C) concentrations. Difference was only seen at the higher inhibitor concentrations. The mutants tolerated HMF better than the WT. For example, at 0.5% (w/v) HMF, mutants PS301 and PS302 grew faster and with a shorter lag phase as compared to the WT (Fig. 4B). However, the WT tolerated furfural better than the mutants. In the presence of 0.2% (w/v) furfural, the WT had a lag phase of 2 days while PS301 and PS302 had a prolonged lag phase of 5–6 days (Fig. 4E). The reason for higher growth tolerance of the WT to furfural is not known.

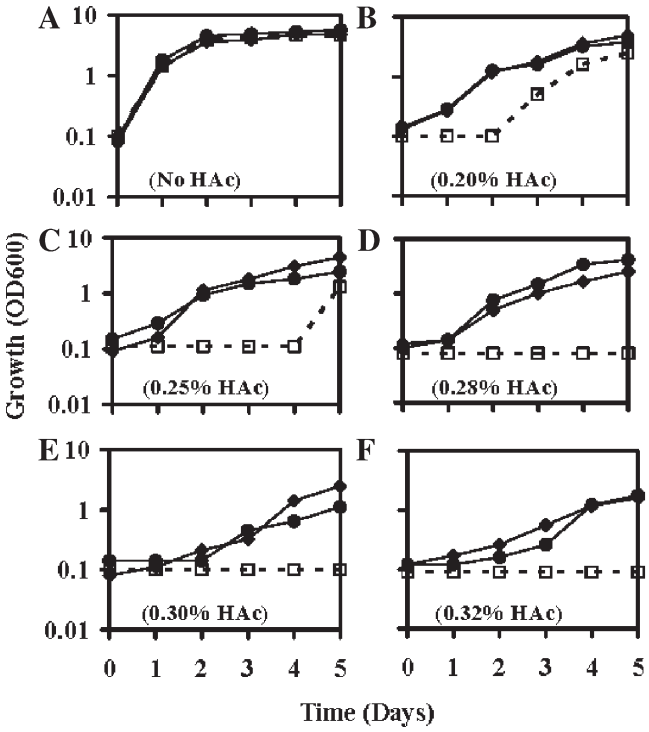

Figure 3. Growth of *P. stipitis* WT and mutants in the absence (**A**) and in the presence of 0.20% (**B**), 0.25% (**C**), 0.28% (**D**), 0.30% (**E**), and 0.32% (**F**) (w/v) acetic acid (HAc). Symbols: WT (□), PS301 (◆), and PS302 (●).

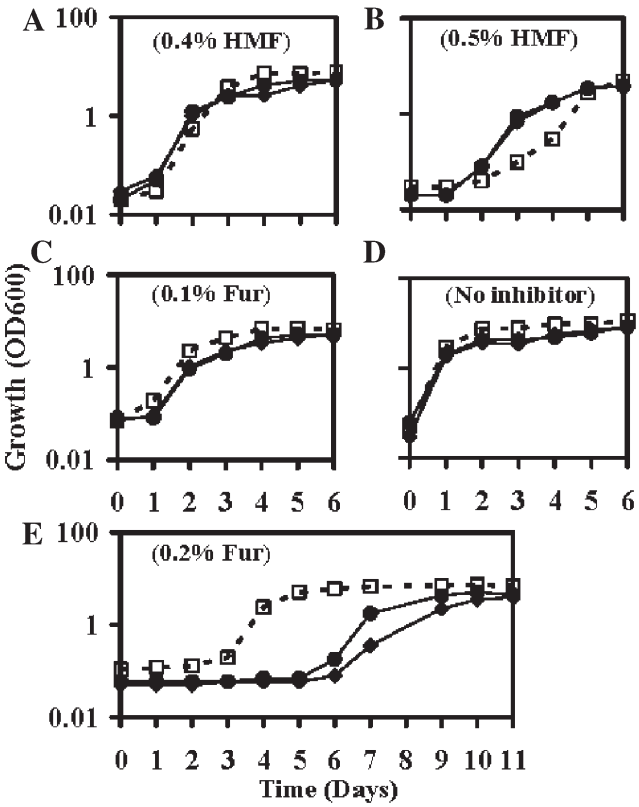

Figure 4. Growth of *P. stipitis* WT and mutants in the presence of 0.4% w/v HMF (**A**), 0.5% w/v HMF (**B**), 0.1% w/v furfural (Fur) (**C**), 0.2% w/v furfural (**E**) and in the absence of inhibitors (**D**). Symbols: WT (□), PS301 (◆), and PS302 (●).

Growth and Fermentation Characteristics of Third Generation Mutants on Biomass Sugars

The growth rates of *P. stipitis* WT strain on 4% (w/v) xylose and glucose were 0.20 ± 0.01 and $0.23 \pm 0.005\ h^{-1}$, respectively (Table I). The growth rates of mutants on 4% (w/v) xylose ranged from 0.23 ± 0.01 to $0.33 \pm 0.02\ h^{-1}$ while on 4% (w/v) glucose, they ranged from 0.29 ± 0.02 to $0.37 \pm 0.02\ h^{-1}$. The growth yield for the WT on 4% (w/v) xylose (1.87 ± 0.01 g/L) was more than twice of that for the mutants (range of 0.63 ± 0.1–0.83 ± 0.1 g/L). The growth yields of the mutants on 4% (w/v) glucose ranged from 1.33 ± 0.05–1.8 ± 0.145 g/L, which were also considerably lower than that of the WT strain (2.60 ± 0.06 g/L).

The ability of the mutants to grow and ferment each of the five biomass sugars was assessed in chemically defined media using high initial cell density inocula. Glucose was readily consumed by all the mutants within 24 h and fermented to ethanol (Fig. 5A). The maximum ethanol concentrations achieved by the mutants, about 1.76% (w/v), were slightly higher than that of the WT at 1.68% (w/v). Xylose was also utilized and fermented to ethanol by the *P. stipitis* mutants, although at a slower rate than glucose. All the mutants consumed xylose to a greater extent than the WT and this led to slightly higher peak ethanol concentrations at 48 h. Among the mutants, PS301, PS303, and PS306 were most efficient with about 87.5% of the initial 4% (w/v) xylose being consumed after 48 h. Mutants PS302, PS304, and PS305 consumed about 75%, while the WT consumed about 70% of the 4% xylose after 48 h (Fig. 5B). The maximum ethanol concentration produced by the six mutants ranged from 1.28% to 1.42% (w/v) while that for the WT was slightly lower (1.21%, w/v).

Mannose was almost completely utilized after 36 h of incubation and the peak ethanol concentration for the mutants and WT ranged from 1.46% to 1.53% (w/v)

Table I. Growth rate (h^{-1}) and growth yield (g/L) of *P. stipitis* WT and mutants on 4% (w/v) xylose or 4% (w/v) glucose.

	Xylose		Glucose	
Strain	Growth rate	Growth yield	Growth rate	Growth yield
PS (WT)	0.20 ± 0.01	1.87 ± 0.01	0.23 ± 0.005	2.60 ± 0.06
PS301	0.23 ± 0.01	0.64 ± 0.07	0.34 ± 0.03	1.77 ± 0.23
PS302	0.26 ± 0.01	0.83 ± 0.11	0.32 ± 0.02	1.8 ± 0.01
PS303	0.28 ± 0.005	0.68 ± 0.04	0.37 ± 0.02	1.63 ± 0.10
PS304	0.29 ± 0.005	0.64 ± 0.14	0.30 ± 0.005	1.8 ± 0.145
PS305	0.27 ± 0.01	0.76 ± 0.1	0.29 ± 0.02	1.33 ± 0.05
PS306	0.33 ± 0.02	0.63 ± 0.1	0.35 ± 0.02	1.65 ± 0.11

Values are the means ± SD.

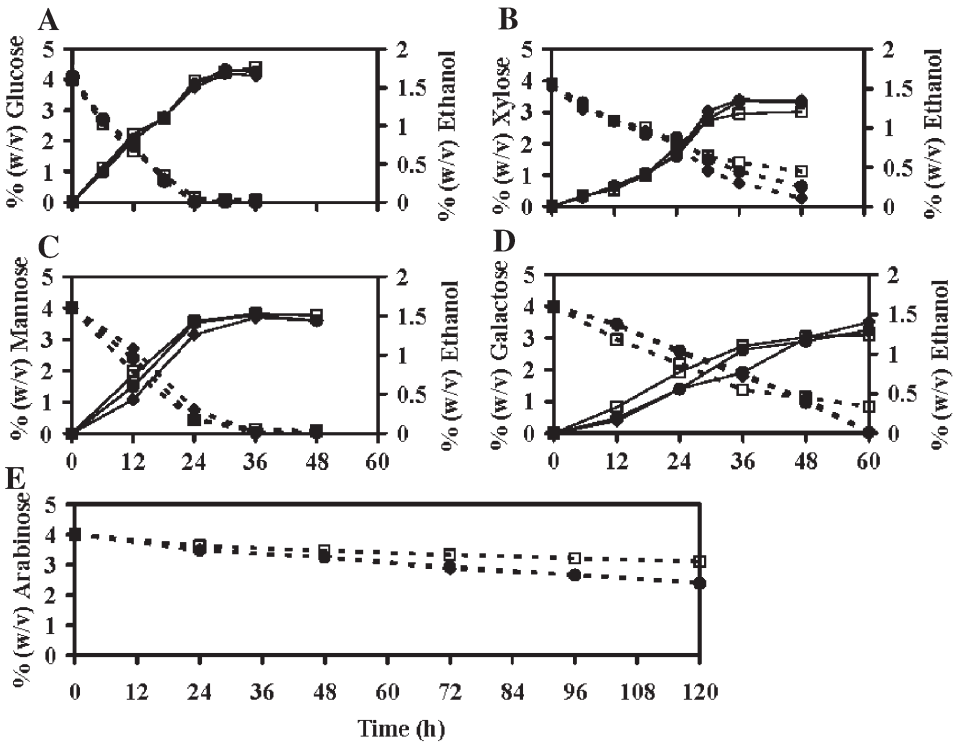

Figure 5. Ethanol production from 4% (w/v) of glucose (**A**), xylose (**B**), mannose (**C**), galactose (**D**), and arabinose (**E**) by *P. stipitis* WT (□), PS301 (◆), and PS302 (●) using high initial cell density inocula. Dotted lines represent sugar utilization and solid lines represent ethanol production.

(Fig. 5C). Galactose was mostly consumed by PS301, PS302, and PS305 after 60 h of fermentation while during the same time interval, the WT utilized about 80%. For the other mutant strains, the percentage of galactose consumed ranged from 88.4% to 91.6%. The maximum ethanol concentration produced from galactose by the mutants and WT ranged from 1.24% to 1.4% (w/v) (Fig. 5D). Arabinose was assimilated at a slower rate by all the strains. After 96 h, the mutants consumed 31–34% of the initial 4% (w/v) arabinose while the WT consumed only 20% (Fig. 4E). None of the strains produced any ethanol from arabinose.

The ability of the third generation mutants to utilize and ferment a mixture of 4% (w/v) each of glucose and xylose was also assessed. As expected, glucose was utilized preferentially over xylose by all the mutants. Most of the glucose was consumed after 36–42 h (Fig. 6A). For the WT, xylose utilization was slow and incomplete and there was a lag period after glucose was completely consumed before xylose consumption began (Fig. 6B). As a result, very little additional ethanol was produced from xylose. All third generation mutants started utilizing xylose while about 1% (w/v) of glucose remained in the medium. The rate of xylose utilization was slower as compared to glucose consumption. Interestingly, all the mutants almost completely utilized the 4% (w/v) xylose after 102 h while the WT consumed only about 26% of the initial xylose during the same period. In all the cultures, the presence of xylose did not affect glucose utilization. However, the utilization of xylose was adversely affected by glucose and the effect was more pronounced for the WT. During mixed glucose–xylose fermentation, the peak ethanol accumulated by the third generation mutants ranged from 2.43% to 2.85% (w/v). These values are approximately 50% higher than those on 4% (w/v) glucose alone as seen in Figure 5A. The WT accumulated 1.71% (w/v) ethanol from a 4% (w/v) glucose–xylose mixture, and this was the same as that accumulated on 4% (w/v) glucose alone. The highest concentration of ethanol (2.85%, w/v) from 4% (w/v) glucose + 4% (w/v) xylose mixture was produced by strain PS301 (Fig. 6C).

The third generation mutants were further assessed for the ability to ferment the sugars in 50% and 60% (v/v) HW SSL using high initial cell density. In 50% (v/v) HW SSL, glucose, xylose, mannose, and galactose were utilized and fermented to ethanol by the mutants at a slightly faster rate as compared to the WT (data not shown). Arabinose was only slightly utilized (<10%) by the WT and the mutants. In 60% (v/v) HW SSL, the sugars were poorly utilized and no ethanol was produced by the WT (Fig. 7). In contrast, glucose, mannose, and xylose were completely utilized by the mutants (Fig. 7A–C). Glucose was completely consumed within the first 24 h of incubation. There was an initial lag of 3–5 days before the utilization of mannose, galactose, and xylose started. Mannose was completely utilized in 7–9 days

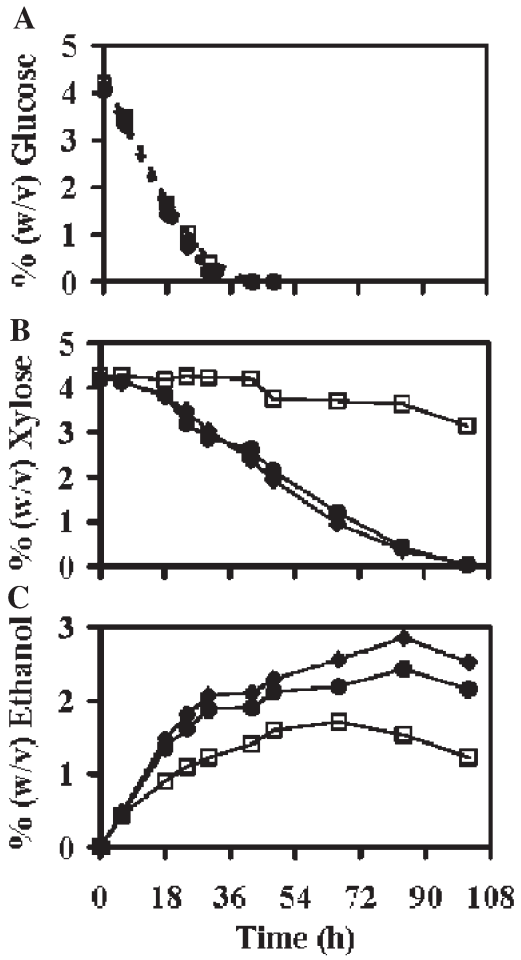

Figure 6. Mixed sugar [4% w/v xylose (**A**) and 4% w/v glucose (**B**)] utilization and ethanol production (**C**) by *P. stipitis* WT (□), PS301 (◆), and PS302 (●) using high initial cell density inocula.

while xylose was completely consumed in 8–9 days by the mutants. Galactose and arabinose were incompletely utilized by the mutant and WT strains, although galactose was consumed to a much greater extent by the mutants compared to the WT. The mutants fermented the SSL sugars to ethanol, with maximum concentrations (ranging from 0.33% to 0.64%, w/v) peaking around 7 days. The maximum concentration of ethanol (0.64% w/v) was produced by PS301.

Discussion

Inhibitor-tolerant yeast strains capable of fermenting all the sugars in non-detoxified lignocellulosic hydrolysates are absolutely required for the development of an efficient biomass conversion process. A number of improved *P. stipitis* mutants have been isolated by different selection schemes. Sreenath and Jeffries (1997) obtained a mutant of *Pichia stipitis* FPL-061 by selecting for growth on L-xylose in the presence of respiratory inhibitors. This nitrosoguanidine-induced mutant strain produced higher ethanol yield as compared to the parental strain. From *Pichia stipitis* FPL-061, Sreenath and Jeffries (1999) further isolated glucose derepressed mutants of *P. stipitis* through chemical (ethyl methane sulfonate) mutagenesis followed by selection in the presence of 2-deoxyglucose. Shi et al. (1999) isolated a mutant of *P. stipitis* CBS 6054 by disrupting the cytochrome *c* gene. The ethanol yield from 8% (w/v) xylose by the mutant strain (0.46 g/g sugar) was 21% higher than the parental strain (0.38 g/g sugar). Some researchers have obtained mutants of *P. stipitis* tolerant to wood hydrolysates by adaptation. For example, Nigam (2001b) reported twofold improved ethanol productivity by *P. stipitis* in wood hydrolysates through continuous adaptation for 6 weeks. However, adaptation can be time-consuming and labor-intensive. In contrast, random mutagenesis, as used in the present study, can rapidly produce improved micro-organisms. In this study, we used random UV mutagenesis followed by selection to obtain mutants of *P. stipitis* with improved tolerance to inhibitors in HW SSL. A relatively low UV dose resulting in 50% cell survival was used to minimize the occurrence of multiple mutations during mutagenesis. With each round of mutagenesis, the tolerance to HW SSL was further improved. The third generation mutants also exhibited improved growth tolerance to acetic acid and HMF, two of the primary inhibitors in HW SSL.

The third generation *P. stipitis* mutants isolated in this study exhibited slightly faster growth rates but lower growth yields on xylose and glucose compared to the WT. They also retained the ability to utilize and ferment four of the major sugars found in HW SSL. In 60% (v/v) HW SSL, the WT strain could not ferment any of the SSL sugars while the mutant strains completely utilized glucose, mannose and xylose and produced ethanol. Moreover, the mutants utilized xylose (as sole carbon source or in mixed sugars) more efficiently than the WT strain.

None of the mutants produced ethanol from arabinose, the other pentose sugar. Similar results were reported for *P. stipitis* and other pentose-fermenting yeasts, *C. shehatae* and *P. tannophilus* (Du Preez et al., 1986; Neirinck et al., 1982). However, in the present study, the mutants appeared to utilize slightly more arabinose than the WT in both defined media and diluted HW SSL.

One of the problems associated with fermentation of mixed sugars in lignocellulosic hydrolyzates is glucose repression of xylose utilization, resulting in sequential glucose–xylose utilization (Bicho et al., 1988; Lee, 1992). While the *P. stipitis* mutants isolated in this study continued to exhibit a sequential pattern of glucose-xylose utilization (Fig. 6), they showed considerable improvement in ethanol production from a glucose-xylose mixture as a result of more efficient xylose utilization. In a 4% xylose and 4% glucose mixture, only 26.2% of xylose was utilized by the WT while 100% xylose was utilized by the mutants in 4 days. Glucose caused a noticeable lag in xylose utilization by the

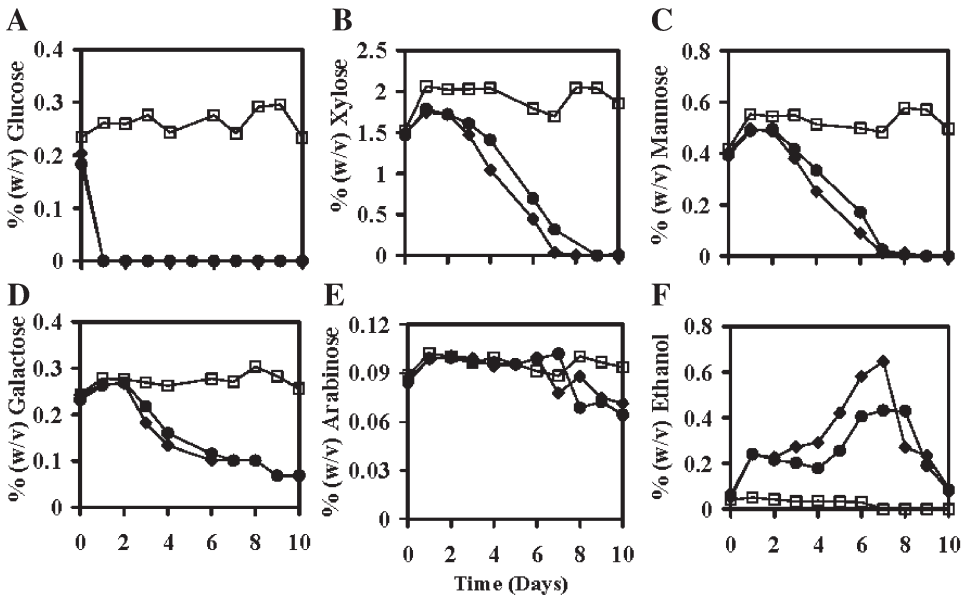

Figure 7. Utilization of HW SSL (60% v/v) sugars (**A–E**); and ethanol production (**F**) by *P. stipitis* WT (□), PS301 (◆), and PS302 (●) using high initial cell density inocula.

WT strain. However, xylose utilization by all the mutants commenced while about 1% (w/v) glucose was still present in the medium. As a result, the peak ethanol concentrations accumulated by the mutants were about 1.6-fold higher than the WT. The reason(s) for more efficient utilization of xylose by the mutants is not known.

In summary, we have successfully isolated several mutants of *P. stipitis* with higher tolerance to HW SSL. These mutants also exhibited better xylose-fermenting ability in a glucose–xylose mixture. Furthermore, they were able to utilize and ferment the main sugars glucose, mannose, galactose, and xylose in 60% (v/v) HW SSL. In contrast, the WT was unable to ferment the sugars in 60% (v/v) HW SSL. HW SSL contains many of the same inhibitors commonly found in other lignocellulosic hydrolysates. Thus, the mutants isolated in this study may also be tolerant of, and it would be of interest to test their performance in, other lignocellulosic hydrolysates. This research demonstrates the utility of random mutagenesis to generate industrially advantageous mutations in *P. stipitis*. Efforts are in progress to further improve the SSL tolerance of these mutants. In addition, separate lines of mutagenesis are envisioned to obtain mutants that are tolerant of ethanol and not subject to glucose repression. It is likely that these randomly generated mutations are additive in combination, and the genetic mating system of *P. stipitis* (Melake et al., 1996) provides a method of testing this possibility.

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