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Development of Xylose-Fermenting Yeasts for Ethanol Production at High Acetic Acid Concentrations

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ABSTRACT

Mutants resistant to comparatively high levels of acetic acid were isolated from the xylose-fermenting yeasts *Candida shehatae* and *Pichia stipitis* by adapting these cultures to increasing concentrations of acetic acid grown in shake-flask cultures. These mutants were tested for their ability to ferment xylose in presence of high acetic acid concentrations, in acid hydrolysates of wood, and in hardwood spent sulfite liquor, and compared with their wild-type counterparts and between themselves. The *P. stipitis* mutant exhibited faster fermentation times, better tolerance to acid hydrolysates, and tolerance to lower pH.

Index Entries: Xylose fermentation; acetic acid tolerance; adaptation; acid hydrolysates; spent sulfite liquor.

INTRODUCTION

Lignocellulosic biomass presents a rich source of carbohydrates that could be fermented into fuel ethanol by suitable yeast strains. Of the carbohydrate content, the pentosan fraction can account for between 10 and 40% (*1*), and can be hydrolyzed by acid or enzymes. Agricultural and forestry plant residue is an abundant source of hemicellulosic material. A readily available lignocellulosic hydrolysate that can be used for ethanol fermentation is spent sulfite liquor (SSL), a byproduct of the sulfite pulp process. These hydrolysates typically contain both hexoses and pentoses,

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the latter being the dominant fraction. Hexose-fermenting yeast *Saccharomyces cerevisiae* has been used by several pulp and paper mills to convert the hexoses present to ethanol. The total ethanol yield could be increased by 25%, theoretically, through the use of an efficient pentose-fermenting yeast that can convert both hexoses and pentoses to ethanol (2). Since the discovery of *Pachysolen tannophilus* as a xylose-fermenting yeast (3), several such yeasts have been described, and *Pichia stipitis* and *Candida shehatae* strains have been used more extensively than the others in fermentation studies (4). In addition to the low pH, one of the drawbacks of pentosan substrates derived from agricultural waste by acid hydrolysis or from sulfite pulp process is the presence of sulfite and acetic acid, which are inhibitory to the yeasts. Ethanol production by *P. stipitis* has been shown to depend on a high initial medium pH when acetic acid was present in the medium (2).

Our purpose in the project reported here was to develop xylose-fermenting cultures that are tolerant to higher concentrations of acetic acid, a major inhibitory ingredient in lingocellulosic hydrolysates. Starting with known xylose-fermenting strains of *P. stipitis* and *C. shehatae*, we were able to produce stable mutants that are resistant to concentrations of acetic acid much higher than those found in hydrolysates. This study compares the fermentation performance of the acetic acid-resistant isolates with their wild types on acid hydrolysates and high acetic acid-containing synthetic media.

MATERIALS AND METHODS

Yeast Cultures, Media, and Culture Conditions

The primary cultures used for mutations were *P. stipitis* NRRL 7124 and *C. shehatae* ATCC 22984 from our collection. Cultures were maintained on YEPD (yeast extract, 5 g/L, peptone 10 g/L, glucose 20 g/L, and pH of 5.0) agar slants. The acetic acid-resistant mutants were maintained in PYN medium (Peptone, 3.5 g/L; yeast extract, 3.0 g/L; KH_2PO_4, 2.0 g/L; $MgSO_4 \cdot 7H_2O$, 1.0 g/L; $(NH_4)_2SO_4$, 1.0 g/L; sugar 20 g/L; and pH 5.0) containing xylose as the carbon source and acetic acid at desired concentrations. Purity of cultures was regularly checked by microscopic examination and colony morphology.

Screening of Spontaneous Mutants Resistant to High Concentrations of Acetic Acid

Semisolid Medium

Different densities of (1×10^6 to 2×10^8/plate) log-phase cells of *C. shehatae* and *P. stipitis* cultures were spread on the surface of YEPD agar incorporated with desired concentrations of acetic acid. The acid was separately added to the plate just before pouring the medium.

Stationary Liquid Medium

PYN broth supplemented with desired quantities of acetic acid in test tubes were inoculated with log-phase cells of *P. stipitis* and *C. shehatae*, and incubated at 30°C. Cultures growing in low acetic acid concentrations were subsequently transferred to tubes containing higher concentrations of acetic acid.

Shake-Flask Cultures

Log-phase cells of cultures were inoculated in 50 mL of PYN media containing the desired level of acetic acid in 250 mL conical flasks and incubated in a Psychotherm incubator shaker (New Brunswick Scientific Co.) maintained at 30°C and rotating at 150 rpm. Growing cultures were inoculated into media containing the next higher concentration of acetic acid.

Preparation of Acid Hydrolysate of Steam-Exploded Aspen

Acid hydrolysates were prepared by a modification of the method described by Prior et al. (*4*). A 30% (w/v) suspension of steam-exploded aspen was adjusted to pH 1.5 with hydrochloric acid and autoclaved for 30 min. This hydrolysate was filtered through Whatman No. 1 filter paper, producing a dark yellow filtrate. The pH was readjusted to 5.0 with sodium hydroxide, and the solution was then stored at 4°C after autoclaving.

Pretreatment of Hardwood SSL

Hardwood SSL that had an initial pH of about 2.5 was neutralized to pH 5.0 with sodium hydroxide. The neutralized liquor was filtered through Whatman No. 1 filter paper and stored at 4°C before addition to PYN medium.

Fermentation Experiments

All fermentation trials were done in 50 mL of media in 250 mL conical flasks. Generally, the inoculum was at a concentration of 1% (v/v) from a log-phase culture of the test organism. When more than one type of organism was used for comparison purposes, the density was adjusted to give equal optical densities in the medium after inoculation. Cell growth, utilization of substrate, and production of ethanol and xylitol were normally monitored in all fermentation experiments. Growth in the defined PYN medium was monitored at 620 nm in a Pye Unicam UV spectrophotometer after the sample was diluted 1:10 in distilled water. When media-containing complex substrates, such as aspen hydrolysates or SSL, were monitored for growth, the medium was diluted 1:5 with 0.1*M* HCl, incubated at room temperature for 10 min, harvested by centrifugation, resuspended in distilled water, and absorbance at 620 nm was measured.

Analytical Methods

Sugars, acetic acid, ethanol and xylitol were measured with a Shodex SE-51 refractive index detector using a Hewlett Packard 1084B HPLC. A Shodex SH1011 sugar column maintained at 50°C was used for the separation, and an HP 3396 series II integrator was used for collecting and analyzing the data. The samples were frozen and thawed twice, spun for 10 min in a microcentrifuge, and made up to 0.01*N* final concentration of sulfuric acid before injecting into the column. Calculations were made against standard values obtained for pure compounds.

Materials

Steam-exploded aspen was a gift from Stake Technology Ltd, Norval, Ontario, Canada. Hardwood SSL was obtained from Temeco Enterprises Temiscaming, Quebec. Acetic acid, xylose, and glucose for media preparation, and all standards for HPLC were obtained from Sigma Chemicals (St. Louis, MO). Other media ingredients from Accumedia manufacturers (Baltimore, MD) and all other chemicals were of research or analytical grade.

RESULTS

Studies on acetic acid tolerance of the xylose-fermenters *C. shehatae* and *P. stipitis* revealed very low resistance to acetic acid. On agar plates, the cultures did not show any growth when 0.2% (v/v) acetic acid was incorporated in the medium. In test tube broth cultures, growth could be seen only up to 0.2% (v/v) acetic acid, whereas the cells grew well at 0.40% (v/v) acetic acid in shake flasks after a week's incubation. Where no growth could be seen, deformed or degraded cells could be observed on microscopic examination.

Attempts to isolate spontaneous mutants resistant to higher concentrations of acetic acid were done in shake-flask cultures since that was the only condition in which these cultures could grow above 0.2% (v/v) acetic acid in the medium. Acetic acid tolerance of *C. shehatae* on different media and adaptation of these cultures to higher acetic acid concentrations in shake-flask culture is shown in Fig. 1. Essentially, the cells were challenged with increasing concentrations of acetic acid in the medium and monitored for growth. Adaptation took about 2 wk at the highest concentration tested. The highest concentrations of acetic acid against which the cells were adapted to were 1.3% (v/v) for *C. shehatae* and 0.8% (v/v) for *P. stipitis*. At these concentrations, growth was found to be inconsistent, and hence the mutant of *C. shehatae* was grown and maintained at 1.0% (v/v) acetic acid containing medium and that of *P. stipitis* at 0.6% (v/v) acetic acid. These mutants were maintained in acetic acid containing PYN media,

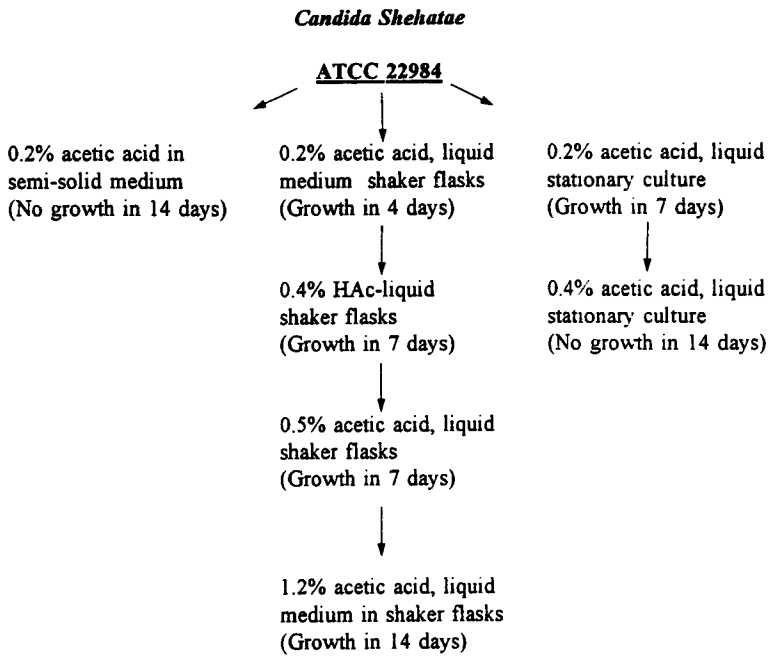

Fig. 1. Adaption of *C. shehatae* in medium containing xylose and high concentration of acetic acid.

since they did not grow on solid media incorporating similar concentrations of acetic acid. The mutants were found to be stable for acetic acid tolerance when tested after storage in acid-free agar for 4 mo at 4°C, or after six passages through normal PYN broth.

Preliminary experiments were done to look at the production of ethanol by these mutants in PYN medium in the presence of high acetic acid concentrations. The concentrations of acetic acid used were 0.9% (v/v) in the case of *C. shehatae* mutant and 0.52% (v/v) for the *P. stipitis* mutant. Fermentation experiments were as described in Materials and Methods. The results are shown in Fig. 2. As can be seen in Fig. 2A, at the acetic acid concentration of 0.9% (v/v), the *C. shehatae* mutant exhibited a growth lag for the first 2 d. It consumed only about 40% of the xylose and acetic acid in the medium at the end of 6 d. A small amount of ethanol was detectable after 4 d, suggesting that ethanol can be produced in the presnce of 0.9% (v/v) acetic acid by this organism, under the conditions used. Figure 2B shows the results of the fermentation using the *P. stipitis* mutant in PYN medium containing 0.52% (v/v) acetic acid. The organism did not show any growth lag in this case, and all the xylose in the medium was utilized in 6 d, supporting a steady growth. In the same period, more than 50% of acetic acid in the medium was consumed. The most important result is that ethanol production started at 60 h, and increased steadily to 6 d, and reached a maximum concentration of 10 mg/mL (Fig. 2B).

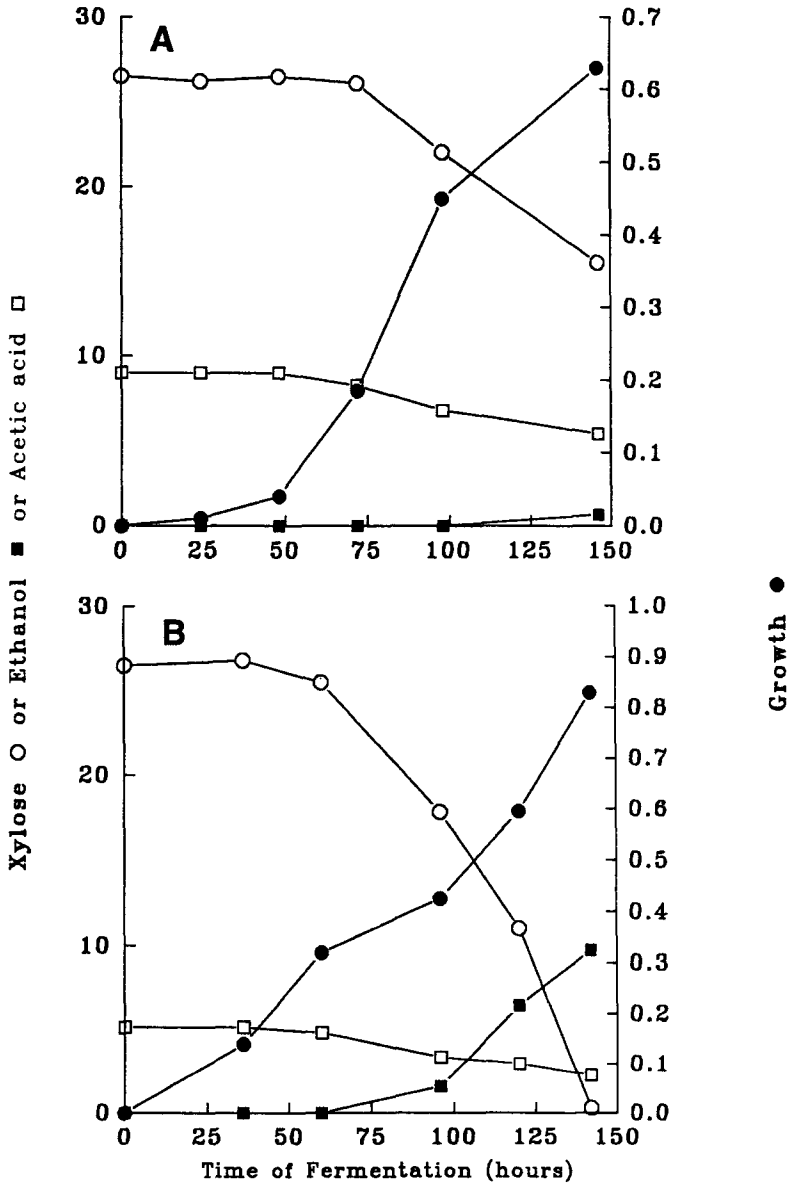

Fig. 2. Fermentation of xylose in PYN medium in the presence of added acetic acid by mutant cultures. (**A**) *C. shehatae* mutant; acetic acid concentration—0.9%. (**B**) *P. stipitis* mutant; acetic acid concentration—0.52%. ●, growth (A620/10); ○, xylose (mg/mL); ■, ethanol (mg/mL).

Fermentation experiments were performed in substrates based on acid hydrolysates of plant biomass and SSL from the paper industry. Media made from acid hydrolysates of steam-exploded aspen (Materials and Methods) were supplemented with 20 g/L xylose and inoculated with wild-type and mutant cultures of *P. stipitis*. Samples were analyzed at

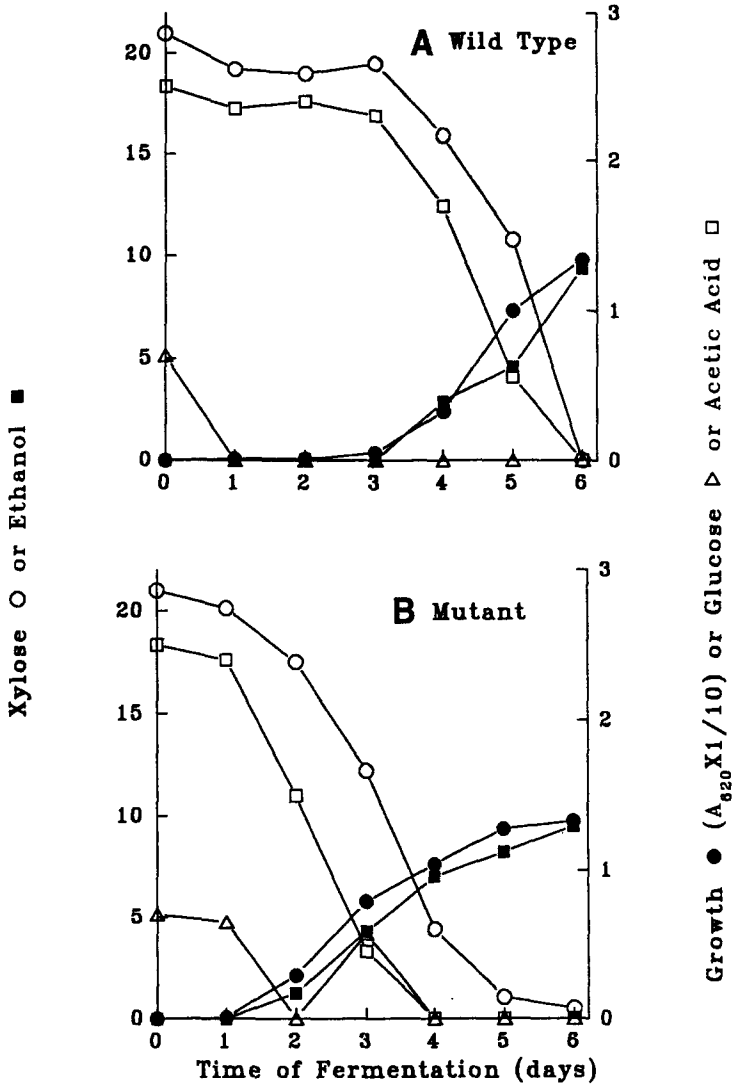

Fig. 3. Fermentation of aspen hydrolysate medium supplemented with xylose by *P. stipitis* cultures. (**A**) Wild-type *P. stipitis*. (**B**) Mutant *P. stipitis*. ○, xylose (mg/mL); △, glucose (mg/mL); □, acetic acid (mg/mL); ●, growth (A620/10); ■, ethanol (mg/mL).

regular intervals for fermentation parameters, and the results are shown in Fig. 3. The medium contained only 0.26% (v/v) acetic acid, permitting the wild-type cells to grow in this medium after 3 d (Fig. 3A). Both xylose and acetic acid were consumed simultaneously. Ethanol production was also observed from the 4th d. Results of experiments done using the *P. stipitis* mutant in the same medium are given in Fig. 3B. The mutant cells were able to grow well in this medium and started growing after 24 h, 48 h earlier than the wild-type culture. Utilization of xylose, acetic acid, and

Table 1
Carbohydrate and Acetic Acid Contents in SSL

Type of SSL	Glucose mg/mL	Xylose mg/mL	Acetic acid mg/mL
Hardwood	3.5	25.4	5.95
Softwood	6.3	30.7	2.7

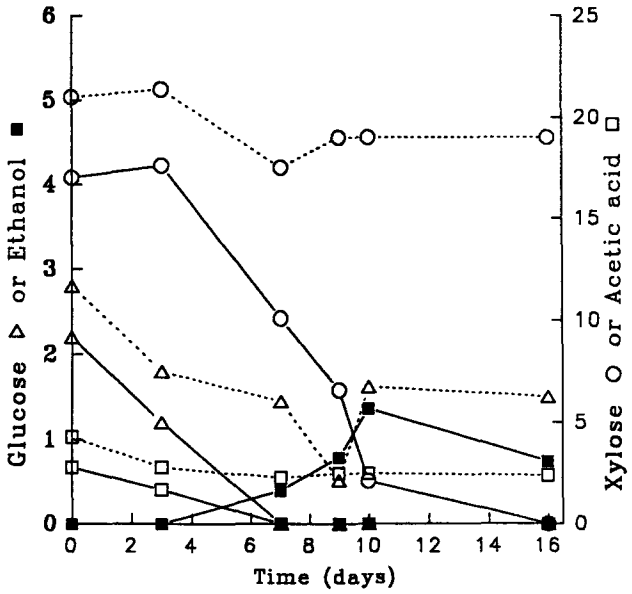

Fig. 4. Fermentation of xylose by *P. stipitis* mutant in media containing 60 (solid lines) or 80% (dashed lines) HSSL. ○, xylose (mg/mL); △, glucose (mg/mL); □, acetic acid (mg/mL); ■, ethanol (mg/mL).

the small amount of glucose also started before 24 h in the case of the mutants. Ethanol production likewise also started 48 h earlier with the mutants than in the case of the wild-type culture (Fig. 3A and B). These results suggest that the *P. stipitis* mutant cells can utilize xylose in the presence of acid hydrolysates of steam-exploded aspen more efficiently. Another important waste substrate is SSL from pulp and paper industry. The carbohydrate and acetic acid contents of hardwood and softwood SSL samples are given in Table 1. Since hardwood SSL contained a higher acetic acid concentration, we used it in all of the experiments reported here. Preliminary studies using 10–50% of hardwood SSL in PYN medium demonstrated the inability of the wild-type cultures of *P. stipitis* to grow in concentrations of HSSL of more than 20% (v/v), whereas the *P. stipitis* mutant could grow and ferment xylose in that medium (data not shown). Attempts were done to find out the highest concentration of HSSL at which the *P. stipitis* mutant could grow and produce ethanol. Sixty percent and 80% HSSL in PYN media were inoculated with *P. stipitis* mutant, and the results obtained were shown in Fig. 4. At 60% HSSL concentration,

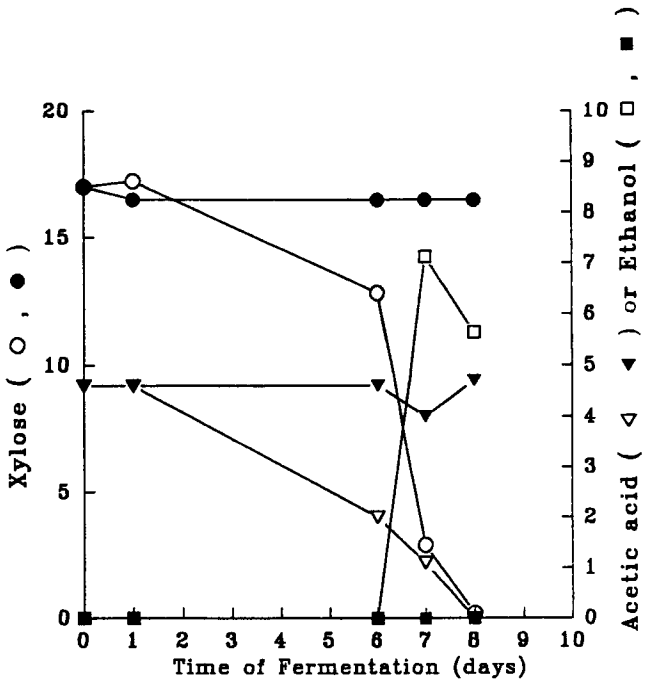

Fig. 5. Xylose fermentation by mutant (open symbols) and wild-type (filled symbols) *P. stipitis* in media containing 0.5% acetic acid and an initial pH of 5.0. ○, ●, xylose (mg/mL); △, ▲ acetic acid (mg/mL); □, ■, ethanol (mg/mL).

xylose, glucose, and acetic acid were utilized almost completely by 10 d, with ethanol production measurable after the 3rd d. Conversely, at 80% HSSL concentration, the substrates were not utilized to any appreciable extent (Fig. 4). This was corroborated by the growth profiles. There was no growth at all in the 80% HSSL medium (data not shown). These data suggest that the maximum concentration of HSSL at which the *P. stipitis* mutant can grow and produce ethanol is between 60 and 80% (v/v).

The typical pH range at which *P. stipitis* ferments high acetic acid-containing substrates is between pH 5.0 and 6.0. We evaluated the possible advantage of the *P. stipitis* mutant for fermenting xylose in the presence of high acetic acid concentrations at lower initial pH values. Mutant and wild-type cultures were inoculated into PYN media containing 0.5% (v/v) acetic acid and initial pH values from 3.0–6.0. At pH 6.0, both mutant and wild-type cells grew comparably and fermented xylose (data not shown). Only the *P. stipitis* mutant grew at pH 5.0, at this acetic acid concentration, where it exhibited a 4 d lag in growth. Other fermentation parameters of mutant and wild-type cells growing in media containing 0.5% (v/v) acetic acid and initial media pH of 5.0 are shown in Fig. 5. The wild-type cells did not utilize xylose or acetic acid, or produce any ethanol during the 8 d of fermentation. On the other hand, the mutant cells started consuming both xylose and acetic acid from the 2nd d of incubation, and continued until these substrates were exhausted, at the end of 8 d. Ethanol was

also produced (about 7 mg/mL from 17 mg/mL of xylose present) during this period (Fig. 5). The results suggest that the initial pH of a fermentation medium with a high acetic acid content can be reduced below that required for the wild-type culture, when the *P. stipitis* mutant is used for the fermentation.

DISCUSSION

The isolation of acetic acid-tolerant mutants of *P. stipitis* and *C. shehatae* was done with the aim of obtaining strains of yeast that can ferment lignocellulosic acid hydrolysates more efficiently. We did not find any growth of *P. stipitis* or *C. shehatae* cultures on agar plates containing acetic acid concentrations above 0.1% (v/v), nor could we adapt these cells to higher acetic acid tolerance on semisolid media. Our attempts to produce mutants using UV irradiation following standard prodedures (*5*) were not successful (data not shown). Even mutants resistant to high acetic acid concentrations obtained through adaptations in shake-flask cultures did not grow on agar plates incorporating more than 0.1% (v/v) acetic acid. These organisms seem to have an inherent inability to grow in the presence of acetic acid under these conditions. This is the likely reason for our failure to pick up any acetic acid-tolerant mutants in our mutation experiments, where selection trials were done on semisolid media incorporating 1% (v/v) acetic acid. From the results of trials to isolate spontaneous mutants in stationary broth media and shake-flask cultures, it is clear that aeration is a requirement for these cells to grow in the presence of acetic acid. Once the mutants were isolated, they were routinely grown in stationary tube cultures containing the desired acetic acid concentrations. The mutant cells of both the *Candida* and *Pichia* cells had clearly different morphology compared to their wild-type counterparts. They occurred as circular single cells compared to the mostly irregularly shaped wild-type cells often occurring in small and branched chains (data not shown).

In experiments done to determine the capability of acetic acid-resistant mutants to produce ethanol in the presence of high acetic acid concentrations (Fig. 2), it was found that the *P. stipitis* mutant was more suitable, since it could ferment xylose in a shorter period (about 40 h less) and gave a 10-fold better yield. It should be noted that there is no direct comparison between these cultures in this experiment, since each was exposed to different acetic acid concentrations, below their respective tolerance levels (0.6% [v/v] for *P. stipitis* mutant and 1.0% [v/v] for *C. shehatae* mutant). Our reasons for choosing *P. stipitis* mutants in most of the work presented here are as follows: The *Candida* mutant showed consistently slower growth in the presence of acetic acid, low pH, and in media containing acid hydrolysates of wood or SSL. Ethanol production in the above media was very low compared to that of the *P. stipitis* mutant in presence of acetic acid.

Finally, in fermentation trials in media containing 5% or more xylose, *C. shehatae* produced considerably higher quantities of xylitol compared to *P. stipitis* (data not shown).

The results illustrated in Fig. 3 demonstrate the advantage of the *P. stipitis* mutant over its wild-type counterpart in fermenting xylose in a medium based on acid hydrolysates made from steam-exploded aspen. The medium was supplemented with xylose to obtain quantities of ethanol sufficient for a reasonable comparison. The acetic acid concentration in the extracts was only 0.26% (v/v), and the wild-type cells showed considerable lag in growth, whereas the mutants showed detectable growth at least 36 h earlier than the wild type. Even though the final ethanol yield obtained with either culture was essentially the same, ethanol production occurred about 48 h earlier in the case of the mutants. Acetic acid concentration in some acid hydrolysates can be as high as 0.48% (v/v) (*6*), and the wild-type cultures will be unsuitable for fermentation in these media. The acetic acid-tolerant mutant of *P. stipitis* can ferment xylose at these concentrations of acetic acid.

SSL has been used for ethanolic fermentation since as early as 1878 (*8*). Because of the presence of acetic acid and other inhibitory substances, the amount of HSSL that can be added to the fermentation medium is currently limited to about 20% (v/v) (Temeco Enterprises, personal communication). Our preliminary experiments on the effect of HSSL on xylose fermentation using wild-type *P. stipitis* suggested that cell growth was totally inhibited above 20% (v/v) HSSL. The *P. stipitis* mutant could ferment xylose from HSSL at a high concentration of 50% (v/v) HSSL. The maximum concentration of HSSL that could be used for fermentation was found to be between 60 and 80% (v/v) (Fig. 4). The growth curves that are not presented here clearly showed inhibition of growth of the mutants at 80% HSSL (v/v). The fermentation profiles (Fig. 4) conform to the results of the growth curves and suggest that the *P. stipitis* mutant was capable of producing ethanol in media containing 60% (v/v) HSSL.

Wild-type *P. stipitis* cultures have a high pH optimum for xylose fermentation, compared to other commonly used xylose fermenters (*7*). In acid hydrolysates and SSL, they ferment xylose at a pH of around 5.5 (*2,6*). Since mutants tolerant to high concentrations of acetic acid might show tolerance of lower pH, we compared wild-type and mutants of *P. stipitis* on their fermentation capabilities at pH 3.0–6.0 and at 0.5% acetic acid concentration. At pH 5.0, we found that the mutant cells grew well and fermented xylose where the wild-type *P. stipitis* was inhibited (Fig. 5). The minimum pH at which these mutant cells could grow, at 0.5% acetic acid concentration, was 4.7 (data not shown). Bjorling and Lindman (*2*) had reported complete inhibition of ethanol production by *P. stipitis* in media containing 0.39% acetic acid at pH 4.0. We tried fermentation at 0.4% acetic acid concentration and a pH range of 3–6. We were able to detect ethanol production at pH 4.0 in these experiments (data not shown).

The pH-dependent inhibition of growth in the presence of acetate and metabolism for glucose adapted yeast cultures has been described (*9,10*). Briefly, when the medium pH is acidic, a portion of the acetate has a neutral charge and can freely diffuse across the cell membrane. The result is a steep, pH dependent decline in the cellular ATP pool. ATP depletion inhibits the ability of the glucose-adapted cells to synthesize the necessary apparatus of respiratory metabolism and culture recovery can require several days, if, indeed, recovery is possible. At lower concentrations of acetate, the cells can adapt to the presence of acetate (*9*), and this phenomenon may be operating in our experiments described here.

In this article, we describe the development of a *P. stipitis* isolate tolerant to 0.6% acetic acid in growth media. The mutant is capable of fermenting xylose from media containing acid hydrolysates of aspen and HSSL, where its wild-type parent was either slower or inhibited. It is also capable of fermentation at lower initial pH values in the presence of high acetic acid concentrations. Specific or critical comparisons of kinetics of fermentation or yields of ethanol obtained are deliberately avoided in the discussion, since optimal conditions for fermentation using these mutants are currently being worked out using a laboratory fermenter.

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