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# Production of Ethanol from Sugar Cane Bagasse Hemicellulose Hydrolyzate by *Pichia stipitis*

## Scientific Note

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## ABSTRACT

The ability of *Pichia stipitis* to ferment D-xylose and D-glucose in the acid-hydrolyzed hemicellulose component of sugar cane bagasse depends on the alkali used to neutralize the hydrolyzate to pH 6.5. With $NH_4OH$ and NaOH no fermentation occurred, whereas neutralization with $Ca(OH)_2$ gave the best results ($Q_p max$ = 0.25 g/L-h; $Y_{p/s}$ = 0.38 g/g sugar). However, the volumetric productivity was still considerably less than observed in a semisynthetic medium with a sugar composition similar to the hydrolyzate. L-arabinose was not fermented but assimilated. Sequential neutralization methods failed to improve the fermentation. Acetic acid and lignin derivatives present in the hydrolyzate were major components that inhibited the fermentation.

**Index Entries:** Hemicellulose hydrolyzate; D-xylose fermentation; ethanol production; inhibitors; *Pichia stipitis*; neutralization techniques.

## INTRODUCTION

Plant biomass is considered the most abundant renewable resource available for the generation of liquid fuels and chemical feedstocks.

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Sugar cane bagasse is a particularly suitable waste for conversion to ethanol since it is produced in significant quantities ($3.0 \times 10^6$ t dry mass/annum) in South Africa (*1*). Sugar cane bagasse consists of 25–35% (dry wt) hemicellulose xylan, of which D-xylose is the major component (2).

Hemicellulose sugars are easily recovered from bagasse by mild acid hydrolysis, but various toxic compounds are formed that inhibit fermentation of the released sugars (D-xylose, D-glucose, and L-arabinose) to ethanol (*3,4*). Three sources of potential inhibitory compounds produced during hydrolysis are hemicellulose decomposition products (furfural, hydroxymethylfurfural, acetic acid) (*5*), soluble lignin, and heavy metal ions from equipment corrosion (iron, chromium, nickel, copper) (*6*).

Among the D-xylose-fermenting yeasts, *Pachysolen tannophilus* and *Candida* sp. are the most studied (*7*), but recently *Pichia stipitis* (the presumed teleomorph of *Candida shehatae*) has shown promise for industrial application, because it ferments D-xylose rapidly with a high ethanol yield and apparently produces no xylitol (*8*). Furthermore, *P. stipitis* has no absolute vitamin requirement for D-xylose fermentation (*9,10*) and is able to ferment a wider range of sugars (including cellobiose) than *C. shehatae* (*10*).

The objectives of this study were to develop suitable treatment procedures for bagasse hemicellulose hydrolysate to facilitate efficient fermentation of the sugars to ethanol by *Pichia stipitis*, and to identify the major inhibitory factors that retard the fermentation.

## METHODS

### Organism

*Pichia stipitis* CSIR-Y633 (CBS 7126) was obtained from J. P. van der Walt of the Council for Scientific and Industrial Research, Pretoria. The culture was routinely maintained on YM agar slants (*11*).

### Preparation and Composition of Bagasse Hemicellulose Hydrolyzate

Bagasse hemicellulose hydrolyzate was prepared in a polypropylene reactor with a metal heating coil by percolating bagasse with 1.8% $H_2SO_4$ at 95°C for 15 h with an initial liquid to solid ratio of 10:1. The liquid stream was withdrawn on a semicontinuous basis. This apparatus was shown previously (*4*) to result in a minimal release of metal ions by corrosion. The average composition of the hydrolyzate/L was, D-xylose, 46 g; D-glucose, 3 g; L-arabinose, 5 g; acetic acid, 10 g; furfural, 0.6 g; 5-hydroxymethylfurfural, 0.04 g; pH 1.0. In one instance, a hemicellulose hydrolyzate was prepared by replacing $H_2SO_4$ with $H_3PO_4$ at the same concentration. Otherwise, conditions were similar and the resultant hydrolyzate/L consisted of, D-xylose, 36.8 g; D-glucose, 7.2 g; L-arabinose, 4.7 g; acetic acid, 8.8 g; pH 1.6.

### *Hydrolyzate Neutralization and Pretreatment*

The hydrolyzate was treated by a number of procedures in order to reduce the effect of toxic components (*3*).

1. The pH was adjusted to 6.5 in one step by slowly adding sterile alkali ($NH_4OH$; NaOH; CaO; $CaCO_3$; $MgCO_3$ or $Ca(OH)_2$) with stirring. After neutralization to pH 6.5, the precipitate was removed by centrifugation (15300g for 20 min).
2. The hydrolyzate was neutralized sequentially (two step) by initially adjusting to pH 3 with sterile solid $Ca(OH)_2$. Subsequently, either NaOH or $NH_4OH$ was used to adjust the final pH to 6.5. The precipitate was removed by centrifugation.
3. The pH of the hydrolyzate was adjusted to 10 with $Ca(OH)_2$, the precipitate removed by centrifugation and the pH readjusted to 6.5, with $H_2SO_4$ followed by further centrifugation (*12*).
4. The pH of the hydrolyzate was adjusted to 10 with KOH, the precipitate removed by centrifugation, and the pH adjusted to 6.5 with HCl. Sodium sulfite (1 g/L $Na_2SO_3$) was slowly added with stirring at pH 6.5. The precipitate was removed and the pH readjusted to 6.5

### *Media*

The medium (INO) used for inoculum preparation/L contained, D-xylose (Merck), 32 g; D-glucose (Merck, anhydrous), 4.5 g; L-arabinose (Merck), 4.1 g; casamino acids (Difco), 5 g; citric acid, 0.5 g; $NH_4Cl$, 7.5 g; $KH_2PO_4$, 10 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; vitamin solution, 1 mL, and trace element solution, 1 mL (*13*).

The hydrolyzate medium (YCSP) consisted of neutralized nonsterile bagasse hydrolyzate and YC and SP concentrated media. YC medium/L (20-fold concentrated) consisted of, yeast nitrogen base (Difco), 134 g and casamino acids (Difco, vitamin free), 80 g, and was sterilized by ultrafiltration. SP medium/L (20-fold concentrated) consisted of, $KH_2PO_4$, 200 g; $MgSO_4 \cdot 7H_2O$, 10 g; $NH_4Cl$, 150 g, and casamino acids (Difco, vitamin free), 100 g. The pH was adjusted to 6.5 prior to sterilization at 121°C for 15 min. The sterilized nutrient concentrates, YC and SP media, were added aseptically to the neutralized bagasse hydrolyzate in order to achieve a 20-fold dilution. Trace element and vitamin solutions (*13*) were added at the same concentrations as in INO medium and the pH was adjusted to 6.5. This hydrolyzate medium (YCSP) was not sterilized further. No significant contamination of the media was observed during fermentation by *P. stipitis*. A slight precipitate was formed during nutrient addition and was not removed. Additions to the hydrolyzate (YCS) prepared with $H_3PO_4$ were as above, except that $KH_2PO_4$ was omitted from the SP medium.

In subsequent experiments the hydrolyzate medium was simplified/L to the following, $KH_2PO_4$, 10 g; casamino acids (Difco, vitamin

free), 5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; D-biotin, 0.1 mg; thiamine-HCl, 5 mg, and trace element solution, 1 mL (*13*), as indicated in the text as medium CP.

A semisynthetic medium (XAG) simulating the bagasse hemicellulose hydrolyzate contained/L D-xylose, 36.4 g; D-glucose, 1.3 g; L-arabinose, 10.4 g; YC-medium, 50 mL; SP-medium, 50 mL; trace element solution, 1 mL; vitamin solution, 1 mL; pH 6.5. Since the neutralization step with $Ca(OH)_2$ was not necessary with the semisynthetic medium, $CaCl_2 \cdot 2H_2O$ (0.05 g/L) was added. For the above XAG medium as well as the INO medium, the sugars were autoclaved separately from the other medium constituents.

The semisynthetic medium (XYL) used to examine the effect of acetic acid, cations and lignin derivatives on the ethanol fermentation by *P. stipitis* was similar to CP medium but in addition contained/L, D-xylose, 50 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; pH 6.5.

### Extraction and Effect of Lignin Derivatives on Ethanol Production

Bagasse hemicellulose hydrolyzate (1615 mL) was extracted with an equivalent volume of diethylether. The ether extract was evaporated to dryness *in vacuo*. The residue was dissolved in 20 mL water and 2–3 drops 40% NaOH were added, giving a solution containing 0.4 g/L paracoumaric acid, 0.6 g/L vanillic acid, vanillin, and ferulic acid, and 0.8 g/L glycosides of the above. Ninety percent of all the phenolic compounds in the above ether extract was further extracted with organic solvents (chloroform, hexane, and acetone) and separated on a Biogel P2 column at pH 10 with water as eluent at a flow rate of 0.5 mL/min. The components were identified by high performance liquid chromatography using a C-18 column (Waters Associates, Milford, MA) at room temperature with methanol–water (1:1) as solvent at a flowrate of 1 mL/min. Resuspended compounds from the dried ether extract were tested in a XYL medium at the same concentration as it would occur in bagasse hydrolyzate.

### Inoculum Preparation

*P. stipitis* was transferred to a fresh YM-slant containing D-xylose (7.5 g/L) and D-glucose (2.5 g/L) as carbon sources at pH 5.0 and incubated at 30°C for 24–72 h. The yeast was transferred from this slant to 100 mL INO medium in a 1-L Erlenmeyer flask that was incubated at 30°C on a rotary shaker (160 rpm; 27.5 mm throw) for 30 h. Twenty mL were transferred to each of five 1-L Erlenmeyer flasks containing 180 mL INO medium. The cultures were grown aerobically for 18 h at 30°C, as described above. The cells were harvested aseptically by centrifugation (2460g for 5 min), washed twice in sterile water and then resuspended in approximately 60 mL sterile water to give a cell concentration of ca. 1 g/L in the bagasse medium after inoculation.

### *Shake Flask Experiments*

Erlenmeyer flasks (250 mL) containing 150 mL medium and stoppered with cotton wool plugs were shaken (130 rpm; 20 mm throw) at 30°C. This condition was designated semianoxic cultivation.

### *Fermentor Cultivation*

Batch cultivations were conducted in a Multigen F-2000 bioreactor (New Brunswick Scientific Co.) equipped with a 2-L glass vessel containing 1.5 L medium. A reflux cooler was fitted to the gas exhaust port to minimize evaporation. An agitation speed of 300 rpm and an aeration rate of 100 mL/min were maintained. The fermentation temperature was controlled at 30°C, or as stated otherwise in the text. The initial pH was 6.5, but was not controlled during fermentation.

### *Analytical Methods*

The ethanol concentration was determined in cell-free media with a gas chromatograph (Hewlett-Packard 5710 A) equipped with a flame ionization detector and a stainless steel column (1.5 mm ID × 1.5 m) packed with Porapak N 80-100 mesh (Waters Associates). The column temperature was 175°C and the nitrogen flow rate was 60 mL/min.

D-xylose, D-glucose, L-arabinose, and xylitol in bagasse hemicellulose hydrolyzate were determined with a high performance liquid chromatograph equipped with a refractive index detector (Waters Associates). The components were separated on a Bio-Rad Aminex HPX-87C column maintained at 85°C using double distilled deionized and degassed water as eluent.

Acetic acid in the supernatant fluid acidified with formic acid was determined with a gas chromatograph (Hewlett-Packard 5790 A) equipped with a flame ionization detector and a glass column (1.5 mm ID × 2.0 m) packed with Porapak Q 80-100 mesh (Waters Associates). The column temperature was 175°C and the nitrogen flow rate was 50 mL/min.

## RESULTS

### *Effect of Bagasse Hemicellulose Hydrolyzate Preparation Procedures*

#### *Effect of One Step Neutralization on Fermentation*

The chemical used to raise the pH of the bagasse hemicellulose hydrolyzate to pH 6.5 had a marked effect upon the ability of *P. stipitis* to ferment the sugars present in the hydrolyzate (Table 1). No fermentation occurred under semianoxic conditions when the hydrolyzate was neu-

Table 1
Effect of Neutralizing Sugar Cane Bagasse Hydrolyzate with Different Alkalis on Ethanol Fermentation by *Pichia stipitis*

| Substrate | Neutralized to pH 6.5 with | Medium used | Method of cultivation | Fermentation time, h | $Q_p$max, g/L-h | $Y_{p/s}$ | Max. ethanol concn, g/L |
|---|---|---|---|---|---|---|---|
| Xylose, arabinose and glucose | NaOH | XAG | Flask | 44 | .58 | .35 | 17.0 |
| $H_2SO_4$-hydrolyzed bagasse[a] | $NH_4OH$ | YCSP | Flask | 120 | 0 | 0 | 0 |
| | NaOH | YCSP | Flask | 120 | 0 | 0 | 0 |
| | CaO | YCSP | Flask | 96 | .13 | .26 | 9.4 |
| | $CaCO_3$ | YCSP | Flask | 120 | .14 | .23 | 9.7 |
| | $MgCO_3$ | YCSP | Flask | 120 | .07 | .20 | 5.8 |
| | $Ca(OH)_2$ | YCSP | Flask | 96 | .15 | .27 | 11.1 |
| | $Ca(OH)_2$ | CP | Fermentor | 79 | .25 | .38 | 15.0 |
| $H_3PO_4$-hydrolyzed bagasse[b] | $Ca(OH)_2$ | YCS | Flask | 72 | .18 | .32 | 10.5 |

[a]Hydrolyzed at 95°C with 1.8% $H_2SO_4$ for 15 h. After neutralization and additions the average composition of the hydrolyzate/L was, 40.9 g D-xylose; 4.5 g L-arabinose; 3.1 g D-glucose and 8.3 g acetic acid.

[b]Hydrolyzed at 95°C with 1.8% $H_3PO_4$ for 15 h. After neutralization and additions the hydrolyzate/L contained, 31.8 g D-xylose; 4.4 g L-arabinose; 0.4 g D-glucose and 8.3 g acetic acid.

tralized with NaOH or $NH_4OH$, whereas with $MgCO_3$, fermentation occurred at a slow rate and the ethanol concentration was low. Neutralization with calcium compounds allowed fermentation to proceed more effectively. The highest yield, ethanol concentration and volumetric rate of ethanol production were achieved in hydrolyzate neutralized with $Ca(OH)_2$ (Table 1). $CaCO_3$ and CaO were less effective than $Ca(OH)_2$ as neutralizing agents. Considerably more $CaCO_3$ (147.9 g/L) than $Ca(OH)_2$ (21.0 g/L), was required to neutralize the hydrolyzate. The fermentation of hydrolyzate neutralized with $Ca^{2+}$ ions was probably more effective than other cations because of the precipitation of toxic factors that are removed by subsequent centrifugation (*14*). When the fermentation of $Ca(OH)_2$-neutralized hydrolyzate and the XAG medium were compared, the fermentation rate, yield, and ethanol concentration were much lower in the hydrolyzate, which suggests that inhibitors were present which retarded ethanol production (Table 1).

The semianoxic fermentation of the XAG medium by *P. stipitis* resulted in an initial utilization of D-glucose followed by D-xylose (Fig. 1A). The concentration of L-arabinose decreased slightly at the end of the fermentation but it was assimilated rather than fermented, since no increase in the ethanol concentration occurred during its utilization. During the fermentation the pH dropped from 6.5 to 4.6. When $Ca(OH)_2$-neutralized hydrolyzate (YCSP) was fermented, the rates of sugar utilization and ethanol production were much slower than in the XAG medium

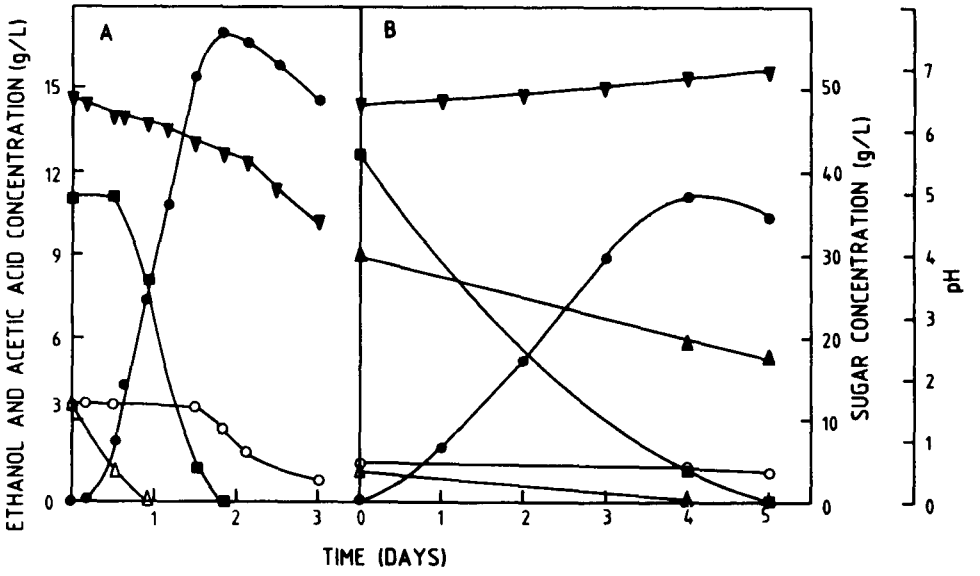


Fig. 1. Production of ethanol and sugar utilization by *Pichia stipitis* at 30°C and pH 6.5 in (A) a D-xylose-L-arabinose-D-glucose semisynthetic (XAG) medium, and (B) $Ca(OH)_2$-neutralized hydrolyzate (YCSP) under semianoxic conditions. Symbols: Ethanol (●), D-xylose (■), L-arabinose (○), D-glucose (△), pH (▼), acetic acid (▲).

with a parallel uptake of D-glucose and D-xylose (Fig. 1B). L-arabinose was not fermented. The pH of the hydrolyzate increased from 6.5 to 7.0 during fermentation and can be ascribed to the simultaneous utilization of acetic acid and D-xylose (Fig. 1B). The ethanol yield coefficient of 0.35 found in the XAG medium was slightly less than the value of 0.37 reported by Du Preez et al. (*15*) at pH 6.5 from a similar D-xylose concentration. The pH of the hydrolyzate was raised to 6.5 in order to minimize the toxicity of acetic acid during fermentation (*3*). However, this pH is not optimal for fermentation since Du Preez et al. (*15*) reported the highest ethanol yields between pH 4 and 5.5.

When the hemicellulose fraction of bagasse was hydrolyzed with phosphoric instead of sulfuric acid and fermented with *P. stipitis* after $Ca(OH)_2$-neutralization (to pH 6.5), the fermentation rate and yield were greater compared to $H_2SO_4$-hydrolyzed $Ca(OH)_2$-neutralized bagasse (YCSP) but the maximum ethanol concentration was less (Table 1). Hydrolysis of bagasse with $H_3PO_4$ formed slightly less acetic acid, and $H_3PO_4$ is also less corrosive than $H_2SO_4$. This slight advantage is probably not justified in terms of the higher cost of $H_3PO_4$.

### *Other Treatment Procedures*

Sequential neutralization of hydrolyzate initially with $Ca(OH)_2$ (to pH 3) followed by NaOH or $NH_4OH$ (to pH 6.5) was attempted to reduce the costs of neutralization. Furthermore, the precipitation of $CaSO_4$ should be almost complete at pH 3 based on dissociation constants of $H_2SO_4$ (*16*). This treatment method resulted in lower rates of ethanol production, ethanol yields, and ethanol concentrations (Table 2) when compared to fermentation of hydrolyzate neutralized with $Ca(OH)_2$ alone (Table 1).

Fermentation of hydrolyzate initially adjusted to pH 10 with $Ca(OH)_2$ (Table 2), and subsequently neutralized with $H_2SO_4$ to pH 6.5,

Table 2
Effect of Other Treatment Procedures on Ethanol Fermentation of $H_2SO_4$-hydrolyzed Sugar Cane Bagasse by *Pichia stipitis* in Shake Flasks[a]

| First neutralization step | Sequential step to pH 6.5 | Medium used and additions | Fermentation time, h | $Q_p$max, g/L-h | $Y_{p/s}$ | Max. ethanol concn, g/L |
|---|---|---|---|---|---|---|
| $Ca(OH)_2$ to pH 3 | $NH_4OH$ | YCSP | 120 | .07 | .20 | 4.6 |
| | NaOH | YCSP | 120 | .12 | .22 | 9.3 |
| | NaOH | YCSP + 2 g/L PEG 20000 | 136 | .13 | .21 | 10.2 |
| $Ca(OH)_2$ to pH 10 | $H_2SO_4$ | YCSP | 72 | .20 | .25 | 12.2 |
| KOH to pH 10 | HCl | YCSP + 1 g/L $Na_2SO_3$ | 118 | .05 | .14 | 5.2 |

[a]Sugar cane bagasse hydrolyzed at 95°C with 1.8% $H_2SO_4$ for 15 h. After neutralization and additions the average composition of the hydrolyzate/L was, 42.0 g D-xylose; 4.5 g L-arabinose; 3.2 g D-glucose; and 8.5 g acetic acid.

yielded results slightly higher than with the fermentation of $Ca(OH)_2$-neutralized hydrolyzate (Table 1). However, this procedure would be less economic since 13% more $Ca(OH)_2$ was required than when neutralizing to only pH 6.5. Increasing the pH to 10 with KOH followed by HCL to pH 6.5 plus 1 g/L $Na_2SO_3$ was the most effective treatment for hemicellulosic hardwood hydrolyzates and fermentation by *P. tannophilus* (*12*). However, this method was not an effective treatment of bagasse hemicellulose hydrolyzate prior to fermentation by *P. stipitis* (Table 2).

The addition of polyethylene glycol (PEG) 20 000 (2 g/L) to hemicellulose hydrolyzate failed to improve the fermentation of a hydrolyzate neutralized with $Ca(OH)_2$ and NaOH (Table 2).

### *Optimization of Fermentation*

When the supplemental nutrient additions to the $Ca(OH)_2$-neutralized hydrolyzate were reduced (CP medium), based on the optimization experiments of Du Preez et al. (*10*), and the fermentation was conducted in a fermentor, an improvement in the ethanol yield coefficient, volumetric rate of ethanol production, and ethanol concentration was observed compared to previous experiments conducted in shake flasks (Table 1).

The kinetic parameters for fermentation of $Ca(OH)_2$-neutralized hydrolyzate at pH 6.5 (CP medium) were affected by temperature (Fig. 2). The maximum ethanol yield (0.42 g/g sugar) and ethanol concentration (15.2 g/L) were observed at 27°C, whereas the maximum volumetric rate of ethanol production (0.20–0.25 g/L-h) occurred in the range of 24–30°C. Above 30°C all parameters dropped sharply. These values show a considerable improvement over the data obtained in shake flasks. Xylitol production was also temperature dependent, since it increased from zero at 18°C to 2.2 g/L at 33°C (Fig. 2). Du Preez et al. (*15*) reported 2.95 g/L xylitol at 36°C in a semisynthetic medium but no detectable amounts at lower temperatures, and also noted a decrease in fermentation rates at temperatures above 30°C.

### *Identification of Major Inhibitors of Ethanol Production*

The addition of acetic acid to XYL medium at a concentration similar to that found in the hydrolyzate resulted in a reduction in the rate of ethanol production, yield, and maximum ethanol produced (Table 3) and these values were similar to those observed in $Ca(OH)_2$-neutralized hydrolyzate (Table 1).

The addition of an ether extract of lignin derivatives to XYL medium reduced the rate of ethanol production (Table 3). Although the ethanol yields with and without the ether extract were similar, in the presence of the ether extract D-xylose was incompletely utilized, with a residual value of 15.5 g/L upon attainment of maximum ethanol concentration.

Cations added as the sulfate salt to XYL medium in the concentration that occurred in neutralized hydrolyzate inhibited the fermentation

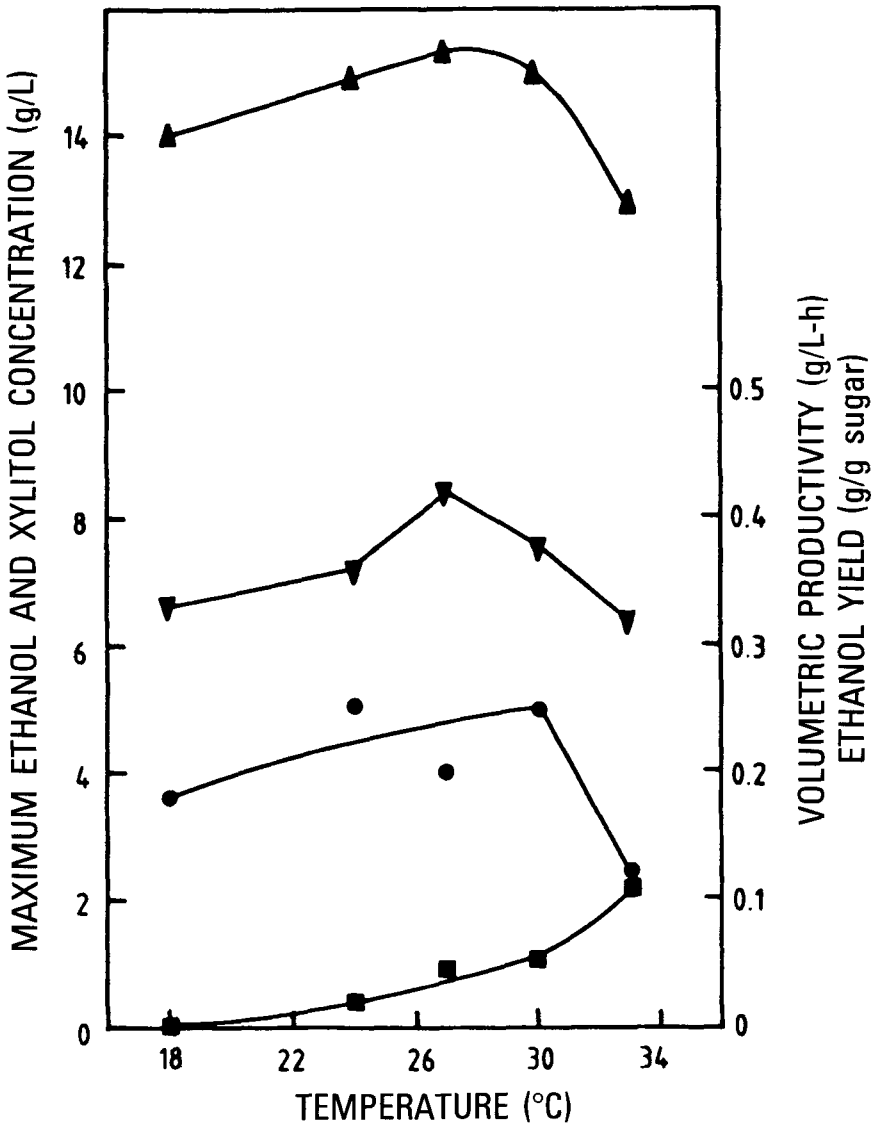


Fig. 2. Effect of temperature on the kinetic parameters for fermentation by *Pichia stipitis* of $Ca(OH)_2$-neutralized hydrolyzate at pH 6.5 (CP medium) in a fermentor. Symbols: Maximum ethanol concentration (▲), ethanol yield (▼), maximum volumetric productivity (●), maximum xylitol concentration (■).

to differing degrees (Table 4). The monovalent cations $NH_4^+$, $Na^+$, and $K^+$, but not the divalent cation $Ca^{2+}$ retarded the volumetric productivity compared to the control, but none of the cations affected the yield or maximum ethanol concentration. The failure of calcium ions to retard the fermentation rate could be related to the poor solubility of calcium salts formed during neutralization and the possible removal of unknown toxic factors during precipitation.

Table 3
Effect of Acetic Acid and Lignin Derivatives on Ethanol Fermentation by *Pichia stipitis* in XYL Medium

| Toxic factor | Fermentation time, h | $Q_p$ max, g/L-h | $Y_{p/s}$ | Max. ethanol concn, g/L |
|---|---|---|---|---|
| None | 44 | .50 | .42 | 17.5 |
| Acetic acid (9.2 g/L) | 105 | .22 | .28 | 13.0 |
| Lignin derivatives[a] | 67 | .26 | .42 | 11.5 |

[a]Resuspended compounds from a dried ether extract were added to XYL medium at the same concentration as found in bagasse hydrolyzate.

Table 4
Effect of Cations on Ethanol Fermentation by *Pichia stipitis* in XYL Medium

| Cation | Concn of sulfate salt[a], g/L | Solubility of salt at 30°C[b], g/L | $Q_p$max, g/L-h | $Y_{p/s}$ | Ethanol concn[c], g/L |
|---|---|---|---|---|---|
| None | 0 | — | .57 | .33 | 15.8 |
| $NH_4^+$ | 41.3 | 438 | .23 | .32 | 15.8 |
| $Na^+$ | 45.1 | 180 | .23 | .32 | 16.1 |
| $K^+$ | 71.7 | 115 | .22 | .31 | 15.6 |
| $Ca^{2+}$ | 46.5 | 0.64 | .57 | .35 | 16.6 |

[a]Concentration of salt found in bagasse hemicellulose hydrolyzate adjusted to pH 6.5 with the corresponding hydroxide.
[b]Vogel (*17*).
[c]Ethanol concentration after 66 h when experiment was terminated.

## DISCUSSION

The results obtained here show that the fermentation of hemicellulose sugars in acid-hydrolyzed bagasse was most effective if the hydrolyzate was neutralized to pH 6.5 with $Ca(OH)_2$. This could be ascribed to a number of factors. First, compared to the sulfates of $NH_4^+$, $Na^+$, and $K^+$, the $CaSO_4$ formed during neutralization is poorly soluble and the $Ca^{2+}$ concentration in the hydrolyzate was not inhibitory. The monovalent cations, on the other hand, were more soluble and the concentrations found in the hydrolyzate inhibited the fermentation rate (Table 4). Second, the $Ca^{2+}$ ions may bind and precipitate toxic factors present in the hydrolyzate and thus improve the fermentation of the hydrolyzate (*18*). However, a complete lack of fermentation in hydrolyzate neutralized with NaOH or $NH_4OH$ suggests that these cations failed to remove the inhibitors.

Acetic acid was a major inhibitory compound present in the hydrolyzate but was only removed slightly (ca. 17%) by $Ca(OH)_2$ neutral-

ization. Acetic acid toxicity was minimized by fermentation at pH 6.5 (*3*). However, this pH was not optimal for D-xylose fermentation by *P. stipitis* (*15*) and therefore other means should be attempted to remove acetic acid from the hydrolyzate.

The inhibition of the rate of fermentation and the final ethanol concentration by the lignin derivatives (Table 3) agrees with the results of Tran and Chambers (*19,20*). They found that derivatives such as vanillin and vanillic acid present in red oak hydrolyzate reduced the final ethanol concentration produced. These compounds were also present in bagasse hydrolyzate. However, they evaluated the individual compounds present in the extract, whereas we tested the extract itself.

Treatment with sulfite or PEG failed to improve ethanol production, whereas treatment with excess $Ca(OH)_2$ resulted in a slight improvement. Strickland and Beck (*12*) found that neutralization to pH 10 and then to pH 5.5 plus sulfite treatment improved the fermentation of red oak hydrolyzate and ascribed the improvement to the removal of furfural or conversion to furfuryl alcohol (*21*) or reactions between furfural and other hydrolyzate components such as phenolics or the removal of cations. While furfural and cations inhibit yeast fermentation (*4,19,20*), the concentrations of these compounds in this hydrolyzate were too low to inhibit the fermentation and therefore neutralization first to pH 10 would be unlikely to improve the fermentation of bagasse hydrolyzate. How sulfite treatment improves fermentation has not been fully established, but changes in redox potential after adding reducing compounds were correlated with improved D-glucose conversion in wood hydrolyzates by *Saccharomyces cerevisiae* (*5,12*).

Although an effective treatment to alleviate the effect of inhibitory compounds in bagasse hydrolyzate was found, the rate of ethanol production remained low. Complete removal of toxic factors should be addressed so that optimal fermentation conditions can be applied.

## NOMENCLATURE

$Q_p max$ Maximum volumetric rate of ethanol production, g/L-h, calculated from the slope of ethanol vs time curve.

$Y_{p/s}$ Ethanol yield coefficient, g ethanol/g fermentable sugars utilized.

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## REFERENCES

1. Dekker, R. F. H., and Lindner, W. A. (1979), *S. Afr. J. Sci.* **75,** 65.
2. Schneider, H., Wang, P. Y., Chan, Y. K., and Maleszka, R. (1981), *Biotechnol. Lett.* **3,** 89.
3. Lee, Y. Y., and McCaskey, T. A. (1983), *Tappi J.* **66,** 102.
4. Watson, N. E., Prior, B. A., Lategan, P. M., and Lussi, M. (1984), *Enzyme Microb. Technol.* **6,** 451.
5. Hajny, G. J. (1981), *Biological utilization of wood for production of chemicals and foodstuffs.* US Dept. Agric. For. Serv. For. Prod. Lab., Madison, WI, Research Paper FPL 385.
6. Magee, R. J., and Kosaric, N. (1985), *Adv. Biochem. Eng./Biotechnol.* **32,** 61.
7. Jeffries, T. W. (1985), *Trends Biotechnol.* **3,** 208.
8. Du Preez, J. C., and Prior, B. A. (1985), *Biotechnol. Lett.* **7,** 241.
9. Dellweg, H., Rizzi, M., Methner, H., and Debus, D. (1984), *Biotechnol. Lett.* **6,** 395.
10. Du Preez, J. C., Bosch, M., and Prior, B. A. (1986), *Appl. Microbiol. Biotechnol.* **23,** 228.
11. Wickerham, L. J. (1951), *Taxonomy of yeasts.* US Dept. Agric., Washington, DC, Tech. Bull. No. 1029.
12. Strickland, R. C., and Beck, M. J. (1984), Effective pretreatments and neutralization methods for ethanol production from acid-catalyzed hardwood hydrolyzates using *Pachysolen tannophilus. Proc. 6th Int. Symp. Alcohol Fuels Technol.*, Ottawa, Canada, vol. 2, pp. 220–226.
13. Du Preez, J. C., and Van der Walt, J. P. (1983), *Biotechnol. Lett.* **5,** 357.
14. Gong, C-S., and Tsao, G. T. (1982), Production of ethanol from biomass hemicellulose hydrolyzate by yeasts. *Proc. Natl. Meet. Am. Inst. Chem. Eng.*, Orlando, Florida, Paper No. 43, pp. 1–17.
15. Du Preez, J. C., Bosch, M., and Prior, B. A. (1986), *Enzyme Microb. Technol.* **8,** 360.
16. Weast, R. C., Selby, S. M., and Hodgeman, C. D., eds. (1965), *Handbook of Chemistry and Physics,* 46th ed., Chemical Rubber Co., Cleveland, OH, p. D-79.
17. Vogel, A. I. (1961), *A Textbook of Quantitative Inorganic Analysis,* 3rd ed., Longmans, Green & Co. Ltd., London, pp. 1144–1145.
18. Šestáková, M. (1979), *Folia Microbiol.* **24,** 318.
19. Tran, A. V., and Chambers, R. P. (1985), *Biotechnol. Lett.* **7,** 841.
20. Tran, A. V., and Chambers, R. P. (1986), *Enzyme Microb. Technol.* **8,** 439.
21. Noller, C. R. (1958), *Textbook of Organic Chemistry,* Saunders, Philadelphia, PA, pp. 457–460.