0273-2289/92/3435—0359$02.00

# Performance of Microorganisms in Spent Sulfite Liquor and Enzymatic Hydrolysate of Steam-Pretreated *Salix*

## Scientific Note

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**Index Entries:** Xylose fermentation; spent sulfite liquor; enzymatic hydrolysate; genetic engineering.

## INTRODUCTION

The earth's oil reserves are limited, and the availability and price are subject to constant variations. In this perspective, ethanol produced from renewable resources, such as wood, represents an alternative to fossil fuels. Ethanol is "cleaner" than hydrocarbons in terms of toxicity and emission. Furthermore, there will be no net addition to the atmospheric carbon dioxide from ethanolic fuels.

A process for ethanol from lignocellulose has to be cost effective enough to compete with the price of petrol. It has been shown that the price of the raw material represents a major fraction of the final price of lignocellulose-derived ethanol (*1*). Therefore, the highest possible conversion yield is a prerequisite for a future ethanol production from lignocellulose, which means that all the sugars in a wood hydrolysate have to be fermented. In addition, the pretreatment and hydrolysis of a ligno-

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cellulosic substrate may give rise to inhibitors that reduce the rate of fermentation and prevent a complete conversion of the fermentable sugars to ethanol (2).

Lignocellulose consists of 10–40% xylose (3), which can be fermented to ethanol by bacteria, yeasts, or fungi. None of these species fulfill the demands of efficiently fermenting a lignocellulosic substrate to ethanol. Xylose-fermenting fungi are usually slow and low yielding (4). Xylose-fermenting bacteria generally produce acidic byproducts that reduce the yield and make product purification laborious. In general, bacteria also prefer a higher pH than the yeasts and the fungi, which increases the contamination risk and thereby the yield in a nonsterile industrial process.

Xylose-fermenting yeasts, such as *Pichia stipitis* (5), *Candida shehatae* (5), and *Pachysolen tannophilus* (6) require a detoxified, e.g., streamstripped (7), substrate for efficient ethanol production, which adds costs to the process. Detoxification was not necessary in the fermentation of spent sulfite liquor (SSL) with baker's yeast *Saccharomyces cerevisiae* (2). However, this yeast cannot ferment xylose to ethanol, only its isomer xylulose. Therefore, the addition of the bacterial enzyme xylose isomerase, which catalyzes the conversion of xylose to xylulose, is needed for the efficient fermentation of a xylose-containing substrate (8). Using this approach, the enzyme cost has to be balanced against detoxification costs. However, a cost-efficient process for the production of fuel ethanol may not be able to carry the cost of either enzyme or detoxification. It is therefore desirable either to adapt or construct an organism that efficiently ferments all sugars in a nondetoxified lignocellulose hydrolysate.

Adaptation has been described (9) for *P. stipitis* and *C. shehatae*. This resulted in high yields of ethanol. However, the fermentations were performed with lignocellulosic substrates that had undergone steam-stripping. Cocultures of one hexose and one pentose fermenting yeast have also been described (2).

The construction of xylose-fermenting microorganisms by genetic engineering has been done (10–12) and is under current investigation. Efforts to transform *S. cerevisiae* with the bacterial gene for xylose isomerase has not been fruitful with respect to ethanol production (10). More promising is the transformation of *S. cerevisiae* with the genes for xylose reductase and xylitol dehydrogenase from *P. stipitis* (11,12). With bacteria, there are two major strategies, either to clone pyruvate decarboxylase and alcohol dehydrogenase from the bacterium *Zymomonas mobilis* into xylose-fermenting bacteria, e.g., *Lactobacillus*, *Lactococcus*, and *Escherichia*, which has been successfully carried out with *E. coli* (13,14), or to supply *Z. mobilis* with a xylose-metabolizing-pathway (15).

In this article, we have studied the performance of some prospective candidates for genetic transformation in two different technical substrates, spent sulfite liquor and steam-pretreated enzymatically hydrolyzed *Salix*.

## METHODS

### Microorganisms

The yeasts *Saccharomyces cidri* NCYC 775, *S. cerevisiae* NCYC 1047, and ATCC 4132 were studied. The bacteria *Escherichia coli* ATCC 11303, *Zymomonas mobilis* ATCC 31821, *Lactobacillus brevis* DSM 20054, and *Lactococcus lactis* ATCC 19435 were also investigated. The yeasts were kept on agar slants at 4°C, and the bacteria at −80°C in freezing medium, except *L. brevis*, which was kept in liquid medium at 4°C.

### Cultivation Media and Conditions

Chemicals used for cultivation were from Merck, Germany of pro analysis quality, if not otherwise stated. The growth medium for cell mass production for the yeasts was 10 g/L xylose (Fluka, Switzerland, purum), 10 g/L glucose (BDH, England), 10 g/L galactose (Merck, Germany, puris), and 3 g/L yeast extract (Difco, USA), 3 g/L malt extract broth (Difco, USA), 5 g/L Bacto-peptone (Difco, USA), 19 g/L $KH_2PO_4$, 3 g/L $(NH_4)_2HPO_4$, and 1.1 g/L $MgSO_4 \cdot 7H_2O$. The growth medium was inoculated directly from agar slants.

*E. coli* was grown in a medium containing 10 g/L Bactotryptone (Difco, USA), 5 g/L yeast extract (Difco, USA), 5 g/L NaCl, 10 g/L glucose (BDH, England), 10 g/L xylose (Fluka, Switzerland, purum), and 10 g/L galactose (Merck, Germany, puris), with pH adjusted to 7.5. The medium was inoculated with a 2.0% (v/v) inocula of an overnight culture.

The growth medium for cell mass production of *Z. mobilis* was 10 g/L yeast extract (Difco, USA), 2 g/L $KH_2PO_4$, 150 g/L glucose (BDH, England), 10 g/L xylose (Fluka, Switzerland, purum), and 10 g/L galactose (Merck, Germany, puris). Inocula (2.0% [v/v]) of an overnight culture was used.

The growth medium for *L. brevis* was 10 g/L sodium acetate, 10 g/L Bacto-peptone (Difco, USA), 2 g/L yeast extract (Difco, USA), 0.5 g/L $MnSO_4 \cdot 4H_2O$, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CoCl_2 \cdot 6H_2O$, 10 g/L xylose (Fluka, Switzerland, purum), and 1 g/L glucose (BDH, England). Ten milliliters of the stored organism were inoculated to 100 mL of growth medium and were grown at 30°C overnight. This culture (3.3% [v/v]) was inoculated to the main culture.

*L. lactis* was grown in a medium consisting of 5 g/L Bactotryptone (Difco, USA), 5 g/L yeast extract (Difco, USA), 1 g/L casoamino acids (Difco, USA), 2.5 g/L $K_2HPO_4$, 2.5 g/L $KH_2PO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 3.3 g/L glucose (BDH, England), 3.3 g/L xylose (Fluka, Switzerland, purum), and 3.3 g/L galactose (Merck, Germany, puris). The growth medium was inoculated with a 2.0% (v/v) inocula of an overnight culture.

The yeasts and *E. coli* were grown in 1 L baffled Erlenmeyer flasks with 200 mL growth medium in rotary shaking water bath at 30°C (yeasts)

or 37°C (*E. coli*). *Z. mobilis*, *L. brevis*, and *L. lactis* were grown in standing cultures at 30°C.

All cultures were harvested in late log phase by centrifugation (at 18,000*g* [yeast] or 11,000*g* [bacteria]) for 15 min at 4°C, (Beckman model J2-21). The dry wt was determined, and the cells were used for fermentations.

### Fermentation Substrate

SSL, sodium based, was supplied by MoDo AB, Örnsköldsvik, Sweden, and the enzymatic hydrolysate of steam-pretreated *Salix* (EH) was supplied by the Department of Chemical Engineering I, University of Lund (*16*). The substrates were supplemented with 0.25% (w/v) yeast extract, 0.025% (w/v) $(NH_4)_2HPO_4$, and 0.0025% (w/v) $MgSO_4 \cdot 7H_2O$. The substrates were buffered with solid 0.1*M* sodium phosphate and pH was adjusted to 5.5.

### Fermentation Conditions

A 25-mL filled beaker, sealed with a rubber stopper supplied with a cannula for carbon dioxide removal, was inoculated with 2 g cells (wet wt). The beaker was slowly stirred and thermostated at 30°C. Each organism fermented both substrates at pH 5.5. All fermentations were run for at least 1 wk and sampled regularly.

### Analysis

All samples were diluted 50 times and then centrifuged in a Wifug table centrifuge at 5700 rpm for 1 min. The samples were then filtered through a 0.45-μm membrane filter (Schleicher & Schuell). The samples were analyzed on an HPLC equipped with an RI-detector. Ethanol, xylitol, glycerol, lactic acid, and acetic acid were analyzed on a Bio-Rad aminex HPX-87H column at 65°C. As a mobile phase, 0.005*M* $H_2SO_4$ was used at a flow rate of 0.6 mL/min. Glucose, xylose, galactose, arabinose, and mannose were analyzed on a Bio-Rad aminex HPX-87P column at 85°C, with water as a mobile phase and a flow rate of 0.6 mL/min. The samples for the lead column were deionized with a mixed-bed ion-exchange resin.

## RESULTS AND DISCUSSION

### Fermentation Substrate

When choosing the substrate to be used in this study, two were selected that could be considered as extremes (Table 1). The composition of the substrates depends both on the raw material and on the pretreatment.

In EH, the main sugars were glucose and xylose, whereas in SSL the sugar content was more evenly distributed: mannose (43%), xylose (27%), galactose (14%), and glucose (11%). This is because EH was made from

Table 1
Content of SSL and EH

| | SSL, g/L | EH, g/L |
|---|---|---|
| Glucose | 4 | 61 |
| Xylose | 10 | 8 |
| Galactose | 5 | <1 |
| Arabinose | 2 | <1 |
| Mannose | 16 | <1 |
| Acetic acid | 5 | 10 |
| Ionic strength | High ≈ 2*M* | Probably low |
| Microbial inhibitors | High level | Probably low |

hardwood and SSL was made from softwood (*17*), as well as because of the different pretreatments (steam pretreatment combined with enzymic hydrolysis and sulfite pulping, respectively). The low xylose content of EH is the result of incomplete hydrolysis of the xylan fraction. The sugar composition of the substrate will be one parameter that determines the choice of fermenting organism.

A second parameter is the acetic acid content. SSL contains 5 g/L acetic acid and EH 10 g/L. The inhibition of yeasts by acetic acid is well known (*18–20*). One way to circumvent the inhibiting effect of acetic acid is steam-stripping, which has been shown to improve the fermentation efficiency with *P. stipitis* (*21*).

A third parameter is inhibiting compounds, such as Browning reaction products, formed during the pretreatment. It was assumed that a substrate pretreated by steam in combination with enzymatic hydrolysis would contain less inhibitors than a substrate pretreated by sulfite pulping, as it is known that pretreatment by acid hydrolysis produces more inhibitors than enzymatic hydrolysis (*22*). In addition, SSL was produced in a recycling system, whereas EH was not. This may result in an accumulation of inhibiting compounds. An earlier study has indicated that an enzymatically hydrolyzed substrate may be less inhibitory for *P. stipitis* (*23*).

Finally, the ionic strength may influence the choice of fermenting organism, and it was assumed that it differed between the two substrates. In SSL, high amounts of sodium sulfite have been added in the pulping process, and the ionic strength is estimated to be 2*M*. In EH, it should be considerably lower since only neutralizing compounds have been added.

## Fermentation Results

The products after 48 h of fermentation in SSL and EH are shown in Tables 2 and 3, respectively. Only one *S. cerevisiae* is represented, since both strains gave similar results. The yeasts produced almost the same

Table 2
Fermentation Products in SSL after 48 H

| Organism | Consumed sugar, g/L | Ethanol, g/L | Lactic acid, g/L |
|---|---|---|---|
| *S. cidri* | 25 | 10 | 0 |
| *S. cerevisiae* ATCC 4132 | 23 | 9 | 0 |
| *L. brevis* | 17 | <1 | 8 |
| *L. lactis* | 8 | 0 | 8 |
| *E. coli* | 4 | 0 | 3 |
| *Z. mobilis* | 4 | 2 | 0 |

Table 3
Fermentation Products in EH after 48 H

| Organism | Consumed sugar, g/L | Ethanol, g/L | Lactic acid, g/L |
|---|---|---|---|
| *S. cidri* | 60 | 29 | 0 |
| *S. cerevisiae* ATCC 4132 | 56 | 30 | 0 |
| *L. brevis* | 46 | 9 | 25 |
| *L. lactis* | 28 | 0 | 25 |
| *E. coli* | 23 | 1 | 19 |
| *Z. mobilis* | 61 | 32 | 0 |

amount of ethanol, giving a yield of 0.27–0.30 g/g in SSL (expressed as g/L EtOH divided with g/L initial sugars) and a yield of 0.41–0.47 g/g in EH. The byproducts from the yeast fermentations were mainly xylitol, glycerol, and acetic acid, with some variations in concentrations between the different strains. *L. brevis* showed typical heterofermentative product formation, and *L. lactis* showed homofermentative product formation. *E. coli* mainly produced lactic acid and utilized mainly glucose. *Z. mobilis* produced ethanol very fast and efficiently, but could utilize only glucose.

Figures 1 and 2 show two typical fermentation time-courses. *S. cerevisiae* ATCC 4132 (Fig. 1) consumed glucose and mannose very fast, whereafter galactose was more slowly consumed. Xylose was consumed very slowly at a rate that corresponded to the xylitol production rate, and glycerol reached a steady-state concentration. *L. brevis* (Fig. 2) consumed glucose as fast as xylose, but utilized galactose much slower. This organism produced lactic acid as well as acetic acid and ethanol. The decreased glucose consumption after 10 h of fermentation might be owing to inhibition by the acids and the ethanol.

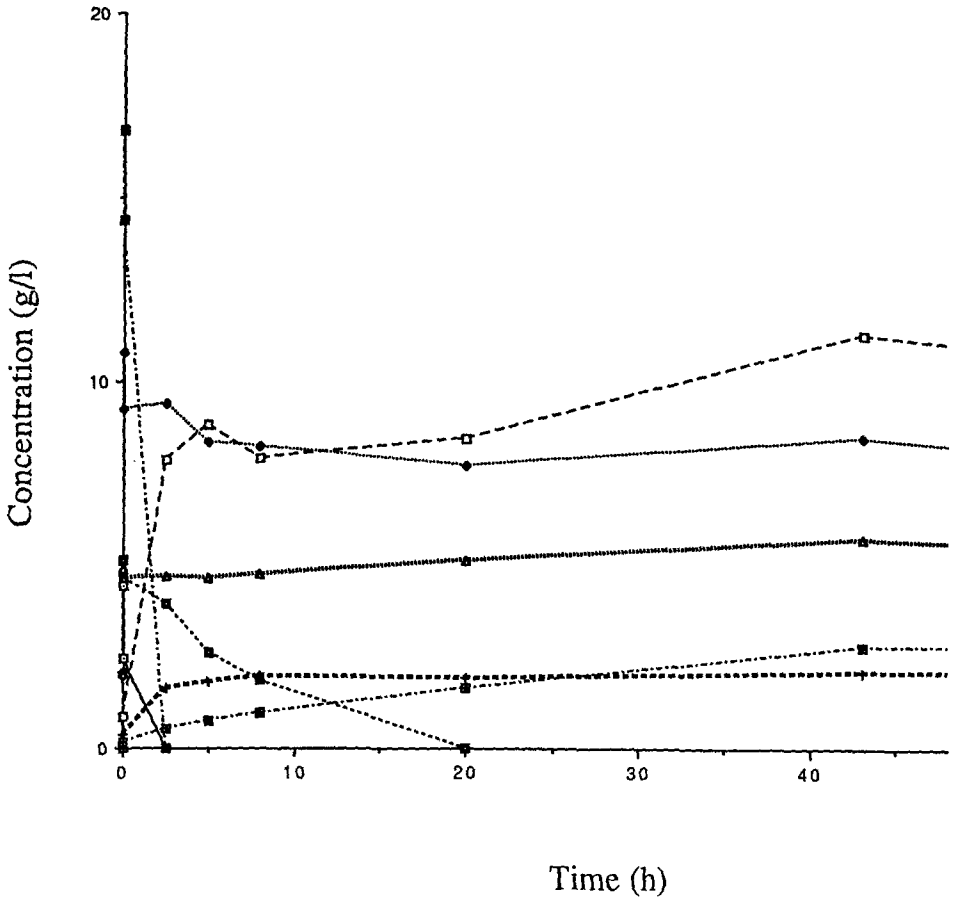


Fig. 1. Sugar consumption and product formation during a 48-h fermentation in SSL by *S. cerevisiae* ATCC 4132. —⊡— Glucose; ···◆··· xylose; ---◘---galactose; ---◇---arabinose; -·-·■-·- mannose; - - □ - - ethanol; —△— acetic acid; --- + --- glycerol; -·-·⊞-·- xylitol.

## Suitability of the Organisms for Fermentation

This study was carried out to evaluate the organisms for future genetic engineering approaches, with the goal to construct an efficient ethanol-producing organism to be used in nondetoxified lignocellulosic hydrolysates. It is also desirable that the ideal organism can ferment efficiently at a low pH with a high sugar consumption rate to minimize the contamination, which is important to consider in fuel ethanol production, where it is not realistic to work under sterile conditions.

In *E. coli*, the genes for pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis* already have been successfully incorporated (*13, 14,24*). *L. brevis* and *L. lactis* may be genetically engineered in the same way. The results from the present study showed that *L. brevis* has the ad-

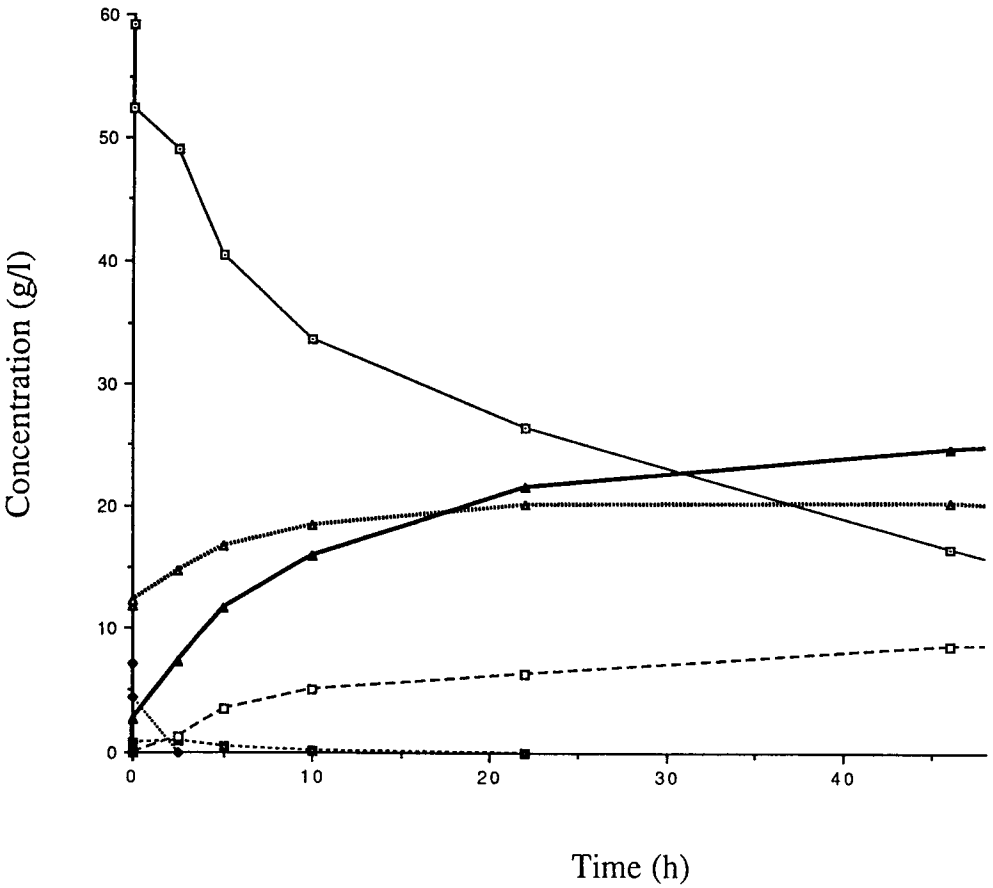


Fig. 2. Sugar consumption and product formation during a 48-h fermentation in EH by *L. brevis*. —⊡— Glucose; ···◆··· xylose; ---◘---galactose; - - □ - - ethanol; —▲— lactic acid; —△— acetic acid.

vantage of utilizing xylose as fast as glucose. *L. lactis* has the advantage of being homofermentive, and it might thus be easier to close unwanted metabolic pathways genetically. The drawbacks for *E. coli*, *L. brevis*, and *L. lactis* were their considerably lower sugar consumption rates compared with *S. cerevisiae* and *Z. mobilis*, and their inability to readily utilize all hexoses and pentoses in the two lignocellulosic substrates.

*Z. mobilis* fermented glucose very well in the investigated technical substrates. Xylose-utilizing enzymes would be needed to make *Z. mobilis* xylose fermenting. Hydrolysates from softwood will contain mannose and some galactose. Therefore, in order to transform *Z. mobilis* into an efficient organism for these substrates, additional genes would be necessary.

*S. cerevisiae* consumed sugar and formed ethanol most efficiently in the two nondetoxified lignocellulosic substrates investigated. It fermented all hexoses well at low pH, which means that only xylose-metabolizing enzymes would have to be incorporated. Either the gene for xylose

isomerase from *L. brevis*, or the genes for xylose reductasae and xylitol dehydrogenase from *P. stipitis* have to be transformed into *S. cerevisiae*. Xylose isomerase is a bacterial enzyme, and the gene has so far not been successfully transformed into *S. cerevisiae* (*10*). Incorporation of xylose reductase (*12*) and xylitol dehydrogenase (*11*) might be a possible way, and genes from *P. stipitis* would be appropriate. *S. cidri* was included in this study because, according to the literature, it should be able to utilize xylose (*25*). Unfortunately, the present results showed that it utilized only minor quantities of xylose and formed xylitol.

## ACKNOWLEDGMENT

This study was supported by the National Swedish Energy Administration.

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