ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

# Ethanol Production From Paper Sludge by Simultaneous Saccharification and Co-Fermentation Using Recombinant Xylose-Fermenting Microorganisms

**Jiayi Zhang,[1] Lee R. Lynd[1,2]**

[1]Chemical and Biochemical Engineering, Thayer School of Engineering, Dartmouth College, Hanover, New Hampshire 03755; telephone: 603-646-2231; fax: 603-646-2277; e-mail: lee.lynd@dartmouth.edu

[2]Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire

Received 14 January 2010; revision received 6 May 2010; accepted 10 May 2010

Published online 20 May 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.22811

**ABSTRACT:** Simultaneous saccharification and co-fermentation (SSCF) of waste paper sludge to ethanol was investigated using two recombinant xylose-fermenting microbes: *Zymomonas mobilis* 8b and *Saccharomyces cerevisiae* RWB222. *S. cerevisiae* RWB222 produced over 40 g/L ethanol with a yield of 0.39 g ethanol/g carbohydrate on paper sludge at 37°C, while similar titers and yields were achieved by *Z. mobilis* 8b at 30°C. Both *S. cerevisiae* RWB222 and *Z. mobilis* 8b exhibited decreasing cell viability at 37°C when producing over 40 g/L ethanol. A high ethanol concentration can account for *S. cerevisiae* RWB222 viability loss, but ethanol concentration was not the only factor influencing *Z. mobilis* 8b viability loss at 37°C. Over 3 g/L residual glucose was observed at the end of paper sludge SSCF by *Z. mobilis* 8b, and a statistical analysis revealed that a high calcium concentration originating from paper sludge, a high ethanol concentration, and a high temperature were the key interactive factors resulting in glucose accumulation. The highest ethanol yields were achieved by SSCF of paper sludge with *S. cerevisiae* RWB222 at 37°C and *Z. mobilis* 8b at 30°C. With good sugar consumption at 37°C, *S. cerevisiae* RWB222 was able to gain an improvement in the polysaccharide to sugar yield compared to that at 30°C, whereas *Z. mobilis* 8b at 30°C had a lower polysaccharide to sugar yield, but a higher sugar to ethanol yield than *S. cerevisiae*. Both organisms under optimal conditions achieved a 19% higher overall conversion of paper sludge to ethanol than the non-xylose utilizing *S. cerevisiae* D5A at its optimal process temperature of 37°C.

Biotechnol. Bioeng. 2010;107: 235–244.



**KEYWORDS:** saccharification and co-fermentation; paper sludge; ethanol; cellulose; *Saccharomyces cerevisiae*; *Zymomonas mobilis*

Correspondence to: Lee R. Lynd

## Introduction

Fuel ethanol produced from cellulosic feedstocks offers significant benefits if cost-effective processes can be developed (Farrell et al., 2006; Greene et al., 2004). Simultaneous saccharification and fermentation (SSF), featuring enzymatic hydrolysis of cellulose and fermentation of hexose sugars in one integrated step, significantly decreases inhibition to cellulase by cellulose hydrolysis products (Takagi et al., 1977; Van Dyke, 1972; Xiao et al., 2004; Zacchi and Axelsson, 1989). Simultaneous saccharification and co-fermentation of hexose and pentose sugars (SSCF) is a process similar to SSF except that the hexose and pentose fermentations occur in one step. SSCF offers potential for more streamlined processing and a lower capital cost (Chandrakant and Bisaria, 1998; Wyman, 1999) while reducing inhibition of hydrolysis by xylose (Kim and Lee, 2005). SSCF has been investigated for production of both ethanol (Kang et al., 2010; Kim and Lee, 2005; Linde et al., 2007; McMillan et al., 1999; Ohgren et al., 2006; Olofsson et al., 2008; Rudolf et al., 2007; Teixeira et al., 1999, 2000; Zhang et al., 2009a,b,c) and lactic acid (Patel et al., 2005; Zhu et al., 2007), with documentation of hydrolyzate inhibition a focus of most prior work.

*Saccharomyces cerevisiae* RWB222 and *Zymomonas mobilis* 8b are recombinant strains capable of fermenting both xylose and glucose to produce ethanol at high yields (Kuyper et al., 2005; Mohagheghi et al., 2004; Zhang et al., 2009a,b,c). *S. cerevisiae* RWB222 is an improved version of *S. cerevisiae* RWB218, with re-integration of the *ura*3 gene allowing greater stability in industrial media (Hans van Dijken, personal communication). *S. cerevisiae* RWB218 is an engineered version of the laboratory strain *S. cerevisiae* CEN.PK (Kuyper et al., 2005; van Dijken et al., 2000) containing the xylose isomerase from *Piromyces* sp. E2, along with over-

expression of native pentose phosphate pathway genes xylulokinase, ribose 5-phosphate isomerase, ribulose 5-phosphate epimerase, transketolase, and transaldolase (Kuyper et al., 2003, 2004, 2005). *Piromyces* sp. E2 isomerase, the first xylose isomerase found in a fungal microorganism (Harhangi et al., 2003), allows *S. cerevisiae* RWB218 to convert xylose to xylulose in one step. This allows for growth on xylose under strictly anaerobic conditions with very little xylitol production (Kuyper et al., 2003, 2004).

*Z. mobilis* 8b is a strain which can tolerate up to 16 g/L acetic acid derived from *Z. mobilis* ZM4, (Mohagheghi et al., 2004). Strain ZM4 (Joachimsthal et al., 1998, 1999) is an ethanol tolerant mutant of the xylose utilizing *Z. mobilis* CP4, which was first developed by integrating the genes encoding xylose isomerase, xylulokinase, transaldolase, and transketolase from *Escherichia coli* (Zhang et al., 1995).

A waste from paper making, paper sludge often contains over 50% carbohydrate on a dry basis, with glucan and xylan together comprising the major carbohydrate components (Lynd et al., 2001). As a substrate for biological conversion, paper sludge has a zero or negative feedstock cost, is fortuitously pretreated during the paper making process. Because of these features, paper sludge is a potential commercial feedstock for production of cellulosic ethanol as well as a model substrate for simultaneous saccharification and co-fermentation of glucan and xylan.

This study was undertaken to evaluate SSCF of paper sludge using two engineered xylose utilizing organisms. Initial paper sludge concentrations were chosen to yield final ethanol concentrations $\geq$40 g/L a point at which economical ethanol recovery becomes feasible (Zacchi and Axelsson, 1989). A commercial cellulase preparation (Spezyme CP) with supplemental β-glucosidase was used for enzymatic hydrolysis of cellulose and xylan.

## Materials and Methods

### Microorganisms

*Zymomonas mobilis* 8b was kindly provided by Dr. Zhang from NREL (Golden, CO). *Saccharomyces cerevisiae* RWB222 was kindly provided by Dr. van Dijken from Delft University of Technology (Delft, the Netherlands) via the Mascoma Corporation (Cambridge, MA). *Saccharomyces cerevisiae* D5A was provided by NREL (Zhang et al., 2009a).

### Substrates

Paper sludge was obtained from the cascade mill (Gorham, NH) operated by Pulp and Paper of America and subsequently sold to Fraser Papers. A single sample of fresh sludge was obtained from the mill, divided into aliquots in freezer bags, and stored at −20°C for long term use. Freezing at −20°C was shown to have no effect on the enzymatic hydrolysis of paper sludge (Shao, 2007). The paper sludge used in this study contains 70% moisture content and 30% solids. The dry paper sludge contains 47.7% glucan, 12.8% xylan, 1.5% mannose (% dry weight) and 32.6% minerals. Avicel PH-105 was kindly provided by FMC Biopolymer Corporation (Philadelphia, PA) as a gift.

### Enzymes

Spezyme CP, an enzyme mixture derived from *T. reesei*, was kindly provided by Genencor International, Inc. (Rochester, NY). β-Glucosidase (Novozyme 188) was purchased from Sigma–Aldrich (St. Louis, MO). 1 mg protein Spezyme CP was found to correspond to 0.46 FPU cellulase activity, and 1 mg Novozyme 188 corresponded to 19 IU β-glucosidase activity. The protein content was measured by a modified Lowry Peterson's method total protein kit (Sigma–Aldrich). Cellulase and β-glucosidase activities were measured using standard methods (Ghose, 1987).

### Media

Rich medium (RM) consisting of 10 g/L yeast extract and 2 g/L $KH_2PO_4$ supplemented with different concentrations of sugars was used for inoculum preparation of *Z. mobilis* 8b (Mohagheghi et al., 2004). Corn steep liquor supernatant (Sigma–Aldrich) was added at 1% v/v for *Z. mobilis* 8b fermentation media.

For *S. cerevisiae*, shaking synthetic medium was used for inoculum preparation and synthetic medium was used for fermentation studies as described (Kuyper et al., 2005). The synthetic medium (Kuyper et al., 2005) used in fermentation includes 5 g/L $(NH_4)_2SO_4$, 3 g/L $KH_2PO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 15 mg/L EDTA, 4.5 mg/L $ZnSO_4 \cdot 7H_2O$, 0.3 mg/L $CoCl_2 \cdot 6H_2O$, 0.84 mg/L $MnCl_2 \cdot 2H_2O$, 0.3 mg/L $CuSO_4 \cdot 5H_2O$, 4.5 mg/L $CaCl_2 \cdot 2H_2O$, 3.0 mg/L $FeSO_4 \cdot 7H_2O$, 0.4 mg/L $Na_2MO_4 \cdot 2H_2O$, 1.0 mg/L $H_3BO_3$, 0.1 mg/L KI, 0.05 mg/L biotin, 1.0 mg/L calcium pantothenate, 5.0 mg/L nicotinic acid, 25.0 mg/L myo-inositol, 1.0 mg/L thiamin HCl, 1.0 mg/L pyridoxin HCl, and 0.2 mg/L para-amino-benzoic acid. The shaking flask synthetic medium used for inoculum preparation is similar to the synthetic medium as described except with 6.59 g/L $K_2SO_4$ and 1.2 g/L urea $((NH_2)_2CO)$ instead of 5 g/L $(NH_4)_2SO_4$. Anaerobic growth factors ergosterol (0.01 g/L) and Tween-80 (0.42 g/L) were added in the paper sludge fermentation and sugar fermentation.

### Inoculum Preparation

Two milliliters of a *Z. mobilis 8b* stock culture stored at −80°C was incubated in 10 mL RM medium with 20 g/L glucose and 10 g/L xylose for 8 h at 30°C. This culture was then transferred to a 500 mL screw-cap bottle containing 200 mL RM medium with 20 g/L glucose and 20 g/L xylose,

and incubated for 16–20 h at 30°C or 37°C as indicated with a final $OD_{600}$ between 1.2 and 2.0. For quantification, dilutions were made by adding fresh medium to achieve an optical density between 0.1 and 0.6 blanked with fresh medium. This method was used for all the OD measurements reported in this article. The inoculum culture was centrifuged at 5,000*g* for 15 min, and the pelleted cells were collected and re-suspended in sterilized water as the inoculum for the fermentation.

Three milliliters of a *S. cerevisiae* RWB222 stock culture stored at −80°C was inoculated in a 500 mL orbital shaking flask containing 150 mL shaking synthetic media supplemented with 40 g/L xylose at 30°C or 37°C as indicated for 24–36 h with the final $OD_{600}$ of about 3.0. The inoculum was collected by centrifugation at 5,000*g* for 15 min and re-suspended in sterilized water as the inoculum for the fermentation. A similar procedure was used for *S. cerevisiae* D5A except the inoculum was prepared in 40 g/L glucose.

### Batch Paper Sludge SSCF and Avicel SSF

Experiments for paper sludge SSCF were carried out at 30 or 37°C in a 2 L reactor (Applikon, Schiedam, the Netherlands) with a modified impellor to reduce the effects of mass transfer limitation (Zhang et al., 2009c). The working volume was 900 ± 25 mL. A filter sterilized enzyme mixture and *Z. mobilis* 8b were added into the reactor to make final concentrations of concentration of 10 FPU cellulase/g cellulose, 60 IU β-glucosidase/g cellulose, 0.09 g/L *Z. mobilis* 8b, 1% v/v clarified corn steep liquor supernant (cCSL) and 82 g/L cellulose from paper sludge. For *Z. mobilis*, a dry cell weight correlation of 0.35 g/L per unit OD600 was used to prepare initial cell mass during inoculation. Nitrogen was purged 2 h before inoculation. Similar operation conditions were carried out for Avicel SSF, except that pH was controlled by a combination electrode pH probe (EASYFERM PLUS K8 200, Hamlton, Reno, NV) at 5.5 ± 0.1 with 1 M KOH and the stirring speed was controlled at 300 rpm from the beginning of the experiment. Fermentations using paper sludge as substrate were started at pH 5.8, and pH dropped slowly to pH 5.5. A histogram of pH trend in the fermentation can be found in Zhang (2008). Similar pH trends during fermentations were also reported with lower concentrations of paper sludge (Kang et al., 2010).

Paper sludge SSCF or Avicel SSF by *S. cerevisiae* RWB222 and by *S. cerevisiae* D5A were carried out as described for *Z. mobilis* 8b except for the inoculum amount and medium composition. The inocula for *S. cerevisiae* RWB222 and *S. cerevisiae* D5A were about 1.1 g/L, and the SSCF and SSF experiments used synthetic medium. For *S. cerevisiae*, a dry cell weight correlation of 0.56 g/L/U OD600 was used to prepare initial cell mass during inoculation. Each experiment reported was carried out at least twice.

### Batch Low Concentration Paper Sludge SSCF

Low concentration paper sludge SSCF reactions were carried out in 250 mL serum bottles with a working volume of about 100 mL. Paper sludge and inoculum were added in the bottles at one third the concentration of the high concentration batch paper sludge SSCF in the reactor as described in Batch Paper Sludge SSCF and Avicel SSF Section. Enzymes were loaded at the same level as high concentration paper sludge SSCF, namely 10 FPU cellulase/g cellulose and 60 IU β-glucosidase/g cellulose. The serum bottles were purged with nitrogen before autoclaving. The pH trend of lower concentration paper sludge fermentations was found to be the same as higher concentration paper sludge fermentations, as indicated in Batch Paper Sludge SSCF and Avicel SSF Section.

### Calcium Inhibition Experiments With *Z. mobilis* 8b

Investigation of inhibition of *Z. mobilis* 8b by calcium was carried out in 250 mL serum bottles containing 100 mL 1% v/v nitrogen-purged corn steep liquor supernatant supplemented with 10 g/L glucose, and different concentrations of calcium and ethanol. Serum bottles were prepared according to a factorial design with levels of ethanol (0, 10, 20, and 40 g/L), temperature (30 and 37°C) and calcium (0, 1 g/L) in duplicates to test the individual and interaction effects on the consumption of glucose by *Z. mobilis* 8b. The inoculum was 0.05 g/L *Z. mobilis* 8b with an initial pH at 5.75. Glucose concentrations after 24 h were measured and statistic analysis was carried out using Minitab, Inc. (State College, PA).

### Carbon Mass Balance Calculation

The carbon recovery rate was calculated based on the total moles of carbon at the final time, divided by the moles of carbon at the beginning of the fermentation. The carbon equation includes the carbon present as part of glucan, xylan, and mannan in the paper sludge, as well as glucose and xylose in the enzyme mixtures and that arising from enzymatic hydrolysis and fermentation products. Carbon dioxide production was estimated assuming one mole of $CO_2$ formed per mole of acetic acid or ethanol produced, and one mole of $CO_2$ consumed per mole of succinic acid produced. For the purpose of mass balance calculations, the carbon content of cells was assumed to be 44% on a dry basis (Graaf et al., 1999; Lange and Heijnen, 2001).

### Analysis

Concentrations of cellobiose, glucose, xylose, xylitol, succinic acid, lactic acid, glycerol, acetic acid and ethanol in fermentation broths were analyzed by HPLC with an Aminex-87H column (Bio-Rad Laboratories, Hercules, CA) maintained at 60°C. The carbohydrate content of dried cellulose-containing solid paper sludge was determined via

quantitative saccharification based on a 2 h incubation in 72 wt% $H_2SO_4$ at 30°C followed by a second 1 h incubation in 4 wt% $H_2SO_4$ at 121°C (NREL analytical procedures, 1995). The cell mass was determined by counting colony forming units on agar plates, as described in Zhang et al. (2009c).

## Results

### Paper Sludge SSCF at 37°C

Paper sludge SSCF was carried out with *S. cerevisiae* RWB222 and *Z. mobilis* 8b at 37°C with an initial concentration of 170 g/L dry paper sludge (82 g/L glucan, 22 g/L xylan) and an inoculum corresponding to about 10% of the final cell mass produced in a soluble sugar fermentation with a similar total potential carbohydrate concentration (data not shown). As may be seen from Figure 1A, *S. cerevisiae* produced about 45 g/L ethanol with no glucose and about 1 g/L xylose left in the medium, and about 5.5 g/L glucan and 1.2 g/L xylan left in the solid residual after 120 h (Table I). *Z. mobilis* 8b performed somewhat less well at 37°C (Fig. 1B), with about 3.3 g/L glucose and 5.5 g/L xylose present in the medium, and about 12.2 g/L glucan and 4.7 g/L xylan left in the solid residual at the end of fermentation. The viable cell number per mL for the *S. cerevisiae* RWB222 fermentation dropped dramatically beginning at 65 h, at which point the ethanol concentration was around 36 g/L ethanol, while the viable cell number per mL of *Z. mobilis* 8b started decreasing at 41 h at which point the ethanol concentration was about 20 g/L ethanol.

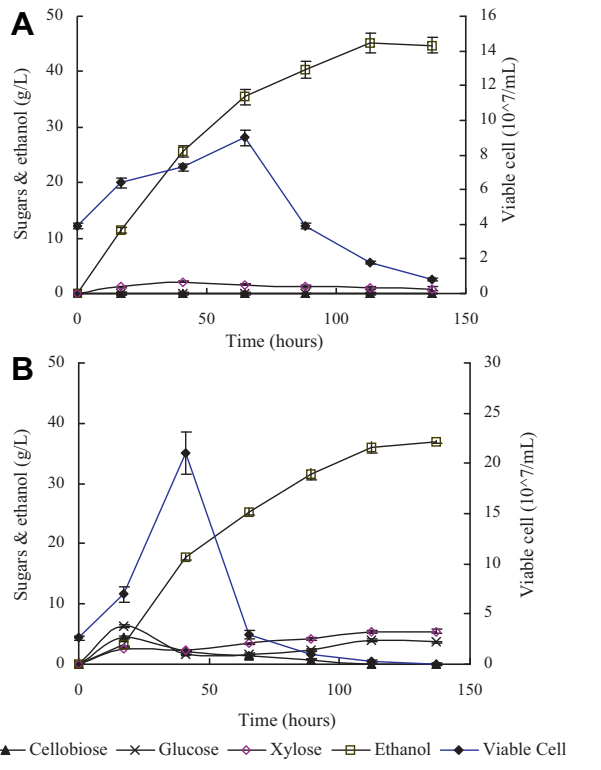


**Figure 1.** 82 g/L cellulose paper sludge SSCF at 37°C. **A**: *S. cerevisiae* RWB222; (**B**) *Z. mobilis* 8b. (—▲—) Cellobiose, (—×—) glucose, (—◇—) xylose, (—□—) ethanol, and (—◆—) viable cell. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

### Paper Sludge SSCF at 30°C

SSCF of paper sludge at 30°C was performed to test the effect of temperature on cell viability and sugar utilization. Paper sludge SSCF using *S. cerevisiae* RWB222 at 30°C had a similar fermentation profile as at 37°C, except that the viable cell numbers remained relatively constant after reaching 36 g/L ethanol, as seen from Figure 2A. *Z. mobilis* 8b maintained good cell viability at 30°C, and while some cellobiose and glucose accumulated during the first 50 h, no residual cellobiose and glucose were detected at the end of fermentation (Fig. 2B). Residual glucan after 120 h is around 10 g/L and residual xylan is around 2.6 g/L in the solid residue of SSCF by both *Z. mobilis* and *S. cerevisiae* at 30°C, a concentration higher than by *S. cerevisiae* RWB222 at 37°C as shown in Table I.

### Low Concentration Paper Sludge SSCF

A set of experiments was designed to assess the contribution of ethanol inhibition to the loss of cell viability observed in paper sludge SSCF at 37°C. If ethanol inhibition were a major factor responsible for loss of viability, then good cell viability would be expected in SSCF experiments at lower initial cellulose concentrations. As seen from Figure 3A and C, *S. cerevisiae* RWB222 established high viability at both 30 and 37°C under conditions where the final ethanol reached 15 g/L, suggesting that ethanol concentration is the major factor for declining viability at high substrate concentrations for SSCF with this organism. However, even at a reduced final ethanol concentration, cell viability in SSCF with *Z. mobilis* 8b still decreases dramatically at the end of fermentation at 37°C (Fig. 3B). For high concentration paper sludge SSCF with *Z. mobilis*, good cell viability is again observed at 30°C (Fig. 3D). These results indicated that one or more factors other than ethanol inhibition contributed substantially to loss viability in SSCF using *Z. mobilis* 8b at 37°C.

### Avicel SSF

SSF was carried out with Avicel, a model substrate that is free of inhibitors and far less viscous than paper sludge at the start of a high solids fermentation. Figure 4 shows the fermentation profile of Avicel SSF at 37°C by *S. cerevisiae* RWB222 and *Z. mobilis* 8b, in which viable cell number

**Table I.** Carbon balance calculation for high solid paper sludge SSCF by *S. cerevisiae* RWB222 and *Z. mobilis* 8b at 30 and 37°C.

| Unit (g/L) | RWB222 at 37°C | RWB222 at 30°C | 8b at 37°C | 8b at 30°C |
|---|---|---|---|---|
| Carbon in | | | | |
| Glucan | 80.72 ± 2.24 | 79.54 ± 1.99 | 78.57 ± 1.96 | 81.88 ± 2.46 |
| Xylan | 21.59 ± 0.60 | 21.28 ± 0.53 | 21.01 ± 0.53 | 21.90 ± 0.66 |
| Mannan | 2.59 ± 0.07 | 2.54 ± 0.06 | 2.52 ± 0.06 | 3.09 ± 0.09 |
| Glucose from enzyme | 0.90 ± 0.02 | 0.82 ± 0.02 | 0.89 ± 0.02 | 0.94 ± 0.03 |
| Xylose from enzyme | 0.82 ± 0.02 | 0.75 ± 0.02 | 0.81 ± 0.02 | 0.86 ± 0.03 |
| Total carbon (mol/L) | 3.96 ± 0.09 | 3.90 ± 0.10 | 3.86 ± 0.10 | 4.04 ± 0.12 |
| Carbon out | | | | |
| Glucan | 5.51 ± 1.09 | 10.50 ± 1.56 | 12.18 ± 1.54 | 9.59 ± 1.68 |
| Xylan | 1.17 ± 0.37 | 2.60 ± 0.35 | 4.66 ± 0.34 | 2.56 ± 0.29 |
| Mannan | 0.83 ± 0.08 | 0.79 ± 0.08 | 0.66 ± 0.08 | 0.99 ± 0.11 |
| Xylotriose | 0.00 ± 0.00 | 0.31 ± 0.00 | 0.69 ± 0.05 | 0.53 ± 0.00 |
| Xylobiose | 0.53 ± 0.02 | 0.73 ± 0.00 | 1.38 ± 0.16 | 0.94 ± 0.00 |
| Cellobiose | 0.00 ± 0.00 | 0.00 ± 0.00 | 1.46 ± 0.54 | 0.00 ± 0.00 |
| Glucose | 0.00 ± 0.00 | 0.10 ± 0.00 | 3.77 ± 1.14 | 0.00 ± 0.00 |
| Xylose | 0.95 ± 0.15 | 0.48 ± 0.00 | 5.47 ± 0.83 | 1.37 ± 0.70 |
| EXP | 5.33 ± 0.32 | 5.28 ± 0.37 | 3.59 ± 0.25 | 5.66 ± 0.34 |
| Lactic acid | 0.56 ± 0.04 | 0.57 ± 0.01 | 1.12 ± 0.22 | 0.00 ± 0.00 |
| Glycerol | 4.92 ± 0.11 | 3.54 ± 0.02 | 0.79 ± 0.13 | 0.41 ± 0.07 |
| Acetic acid | 1.27 ± 0.06 | 0.35 ± 0.01 | 0.91 ± 0.18 | 0.38 ± 0.53 |
| Ethanol | 45.23 ± 0.65 | 40.14 ± 0.96 | 36.58 ± 0.80 | 46.32 ± 0.99 |
| Cell mass | 1.76 ± 0.18 | 3.89 ± 0.54 | 0.53 ± 0.00 | 0.56 ± 0.00 |
| Total carbon (mol/L) | 3.80 ± 0.06 | 3.71 ± 0.09 | 3.78 ± 0.10 | 3.88 ± 0.10 |
| Carbon recovery | 0.96 | 0.95 | 0.98 | 0.96 |
| EXP/xylose consumed | 0.27 | 0.33 | 0.36 | 0.34 |

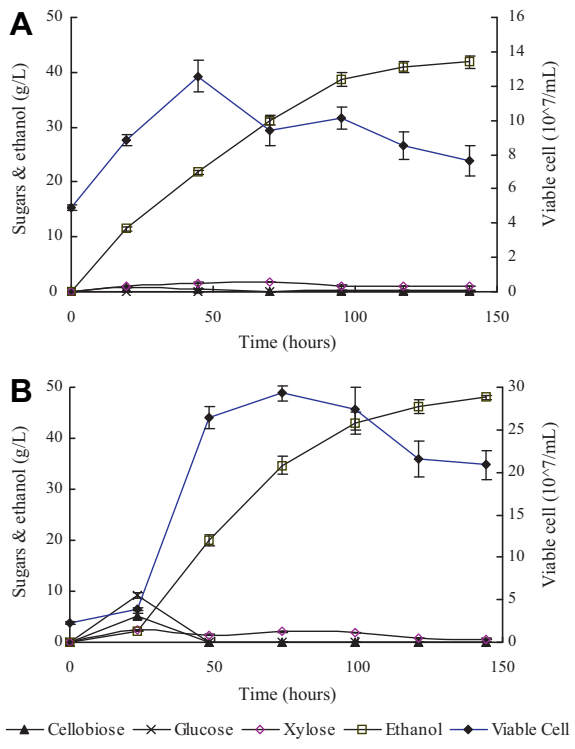


**Figure 2.** 82 g/L cellulose paper sludge SSCF at 30°C. **A**: *S. cerevisiae* RWB222; (**B**) *Z. mobilis* 8b. (—▲—) Cellobiose, (—×—) glucose, (—◇—) xylose, (—□—) ethanol, and (—◆—) viable cell. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

starts decreasing at 44 h (around 29 g/L ethanol) for *S. cerevisiae* RWB222, and at 25 h (around 23 g/L ethanol) for *Z. mobilis* 8b. The viable cell growth curves of both *S. cerevisiae* RWB222 and *Z. mobilis* 8b shown in Figure 4 were similar to those in the paper sludge SSCF's shown in Figure 1. This indicates that mass transfer limitations associated with an initially viscous feedstock and any inhibitors present in paper sludge are not responsible for the observed loss of cell viability.

No soluble glucose or cellobiose was detected in Avicel SSF by *Z. mobilis* 8b at 37°C after 24 h as shown in Figure 4B, while about 3.5 g/L glucose accumulated at the end of fermentation in paper sludge SSCF by *Z. mobilis* 8b under similar conditions as shown in Figure 1B. This difference suggests that although *Z. mobilis* cell viability is not affected by paper sludge, some component of paper sludge inhibits the ability of *Z. mobilis* to consume glucose.

### Effect of Calcium on Glucose Utilization by *Z. mobilis* 8b

An experimental design with three factors—temperature (30 and 37°C), ethanol concentration (0, 10, 20, 40 g/L), and calcium ion concentration (0, 1 g/L)—was designed to examine the effect on glucose utilization by *Z. mobilis* 8b. Utilization of 10 g/L glucose after 24 h incubation was measured. Glucose (10 g/L) was chosen because this concentration is close to the maximum glucose concentration detected in paper sludge SSCF in the first 24 h. Calcium (1 g/L) was selected because it is the maximum concentration detected in the paper sludge fermentation broth. Figure 5

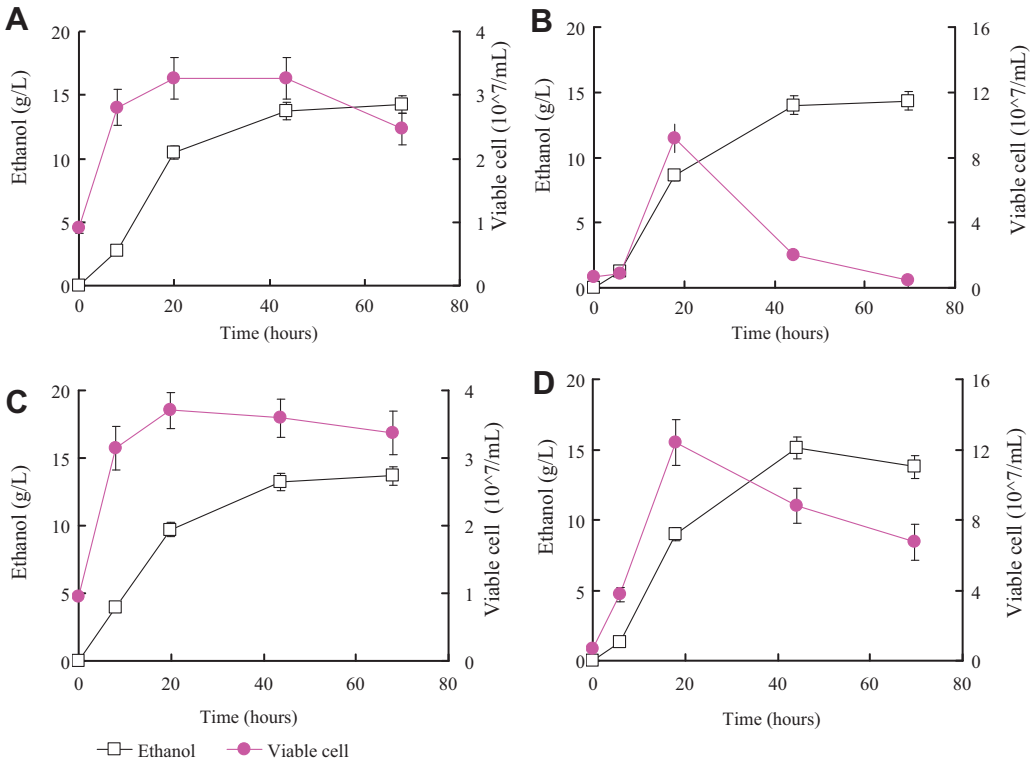


**Figure 3.** 25 g/L cellulose paper sludge SSCF. **A**: *S. cerevisiae* RWB222 at 37°C; (**B**) *Z. mobilis* 8b at 37°C; (**C**) *S. cerevisiae* RWB222 at 30°C; (**D**) *Z. mobilis* 8b at 30°C. (—□—) Ethanol, (—●—) viable cell. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

shows the interactive effect of ethanol, calcium, and temperature on residual glucose. The residual glucose was calculated as the mean value under certain conditions, for example, the data point at 20 g/L ethanol and 37°C in Figure 5 was the mean value of residual glucose for bottles at 37°C with 20 g/L ethanol and 0 g/L calcium and 1 g/L calcium. As shown in Figure 5, residual glucose increased when ethanol increased from 20 to 40 g/L at 37°C, and no residual glucose was detected at 30°C. No residual glucose was detected when there was no calcium, and residual glucose increased with increasing ethanol concentration when 1 g/L calcium was present. Figure 6 shows the single factor effect on residual glucose, with the data indicating that residual glucose was only present with 1 g/L calcium, 37°C and ≥20 g/L ethanol. We conclude that the interaction of calcium, ethanol, and high temperature is responsible for the accumulation of glucose in high concentration paper sludge SSCF, which slows down cellulose enzymatic hydrolysis and lowers final ethanol titer.

### Mass Balance of *Z. mobilis* 8b and *S. cerevisiae* RWB222 in Paper Sludge SSCF at 30 and 37°C

Carbon balances are presented in Table I based on the initial carbohydrate present, carbohydrates left in the residual solids, residual soluble sugars and sugar oligmers, and fermentation products. Trace amounts of mannose, xylitol, and succinic acid, which were masked by ethyl β-xylopyranoside (EXP) (Zhang et al., 2009a), were not quantified. Cell carbon is based on an assumed carbon content of 44% of cell weight for both *S. cerevisiae* RWB222 and *Z. mobilis*, with cell carbon attributed to cell mass equal to the maximum cell mass detected minus the inoculation amount. Values for the carbon recovery calculated in this manner were between 95% and 98%. Of note, the compound ethyl β-xylopyranoside, a significant xylan enzymatic hydrolysis byproduct in the presence of ethanol, was present and accounted for about ~30% of the xylan yield loss (Zhang et al., 2009a).

### Comparative Performance of *Z. mobilis* 8b and *S. cerevisiae* RWB222 in Paper Sludge SSCF at 30 and 37°C

Data from high concentration paper sludge SSCF using *Z. mobilis* 8b and *S. cerevisiae* RWB222 after 5 days are tabulated and compared in Table II. In order to understand the metabolic performance of the organism under different culture conditions, the ethanol yield based on sugar consumption was calculated. Ethanol yield based on the sugar consumed by *Z. mobilis* 8b was higher than for *S. cerevisiae* RWB222. Specifically, the average metabolic

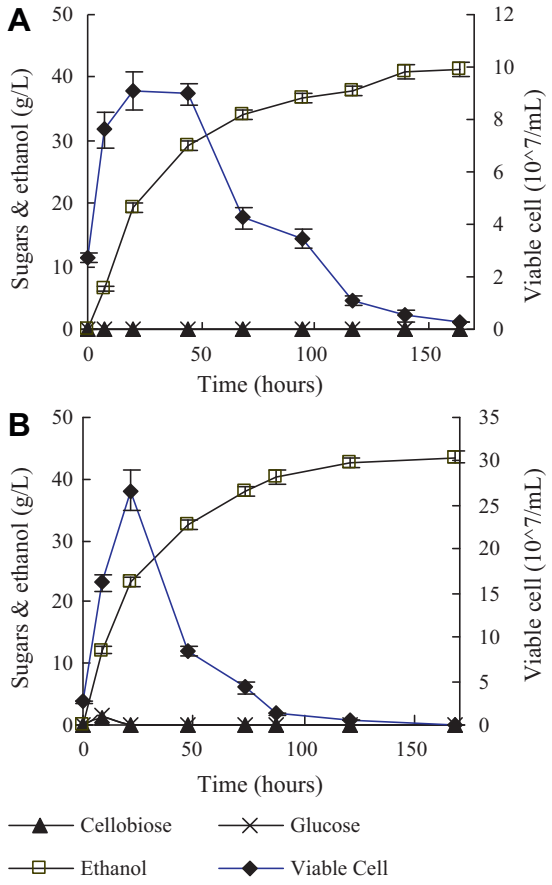


**Figure 4.** 100 g/L Avicel SSF at 37°C. **A**: *S. cerevisiae* RWB222; (**B**) *Z. mobilis* 8b. (—▲—) Cellobiose, (—×—) glucose, (—□—) ethanol, and (—◆—) viable cell. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

ethanol yield for *Z. mobilis* 8b was 0.46 g ethanol/g sugar consumed at 30°C and 0.47 g ethanol/g sugar at 37°C, which was higher than the 0.40 g ethanol/g sugar at 30°C and 0.42 g ethanol/g sugar 37°C for *S. cerevisiae* RWB222.

To understand the enzyme hydrolysis performance under different conditions, the percentage of glucan and xylan solublized (glucan and xylan conversion) was calculated. For *S. cerevisiae* RWB222, the average glucan conversion to monomer sugar or ethanol was 93%, and the average xylan conversion to monomer sugar or ethanol was 95% at 37°C. For *Z. mobilis* 8b, the average glucan and xylan conversion to monomer sugar or ethanol was much lower at 37°C, presumably due to cellulase inhibition by the high concentration of residual glucose and xylose remaining (Fig. 1B). Conversions of glucan and xylan at 30°C were similar for both *Z. mobilis* 8b and *S. cerevisiae* RWB222, at almost 90%. SSCF is a hydrolysis dominated process if the residual glucose and xylose are at very low concentrations in the broth, consistent with the similar enzymatic hydrolysis yield of cellulose and hemicellulose achieved by both organisms at 30°C as shown in Table I.

In order to compare strain performance in a process context, ethanol yields were calculated based on grams ethanol produced per gram potential sugars from the paper sludge initially present, and also compared to the same amount of paper sludge fermentation by the non-xylose fermenting strain *S. cerevisiae* D5A at 37°C. The results (Table II) showed that the highest yields were achieved by SSCF of paper sludge with *S. cerevisiae* RWB222 at 37°C and *Z. mobilis* 8b at 30°C, with both organisms achieving a 19% higher ethanol yield than *S. cerevisiae* D5A at 37°C.

## Discussion

Our results show that SSCF at 30°C using both *S. cerevisiae* RWB222 and *Z. mobilis* 8b results in good cell viability and high conversion of both glucan and xylan to ethanol in 5 days with low residual sugars present. At 37°C, paper sludge SSCF with *S. cerevisiae* RWB222 results in high conversion of glucan and xylan to ethanol although cell viability decreases at ethanol concentrations around 36 g/L. During Avicel SSF with *S. cerevisiae* RWB222, a similar pattern of cell growth was observed, although the viable cell count started declining around 29 g/L ethanol instead of 36 g/L, as seen during paper sludge SSCF. The lower ethanol concentration observed in Avicel SSF might due to the sampling frequency or a lower rate of sugar release at the later stage of the Avicel fermentation. Under the conditions tested, a similar cell viability curve was observed in paper sludge SSCF and Avicel SSF; however, soluble glucose accumulated only in paper sludge SSCF at 37°C using *Z. mobilis* 8b. Further investigation showed that the interactive effect of $\geq$20 g/L ethanol, 1 g/L calcium, and 37°C cause the accumulation of glucose in paper sludge SSCF. Cell viability loss was observed in the later stage of low and high concentration paper sludge SSCF by *Z. mobilis* 8b at 37°C, which indicates that ethanol concentration is not the only factor effecting the loss of cell viability.

Glucan and xylan in paper sludge used in this paper were converted to monomer sugars at almost the same rate by Spezyme CP and Novozyme 188 (Zhang et al., 2009c). However, we observed higher residual xylose accumulation than residual glucose during paper sludge SSCF by both *S. cerevisiae* RWB222 and *Z. mobilis* 8b. The slower consumption rate of xylose than glucose in SSCF was consistent with soluble sugar fermentations of glucose and xylose by both engineered xylose utilizing *S. cerevisiae* (Kuyper et al., 2005) and *Z. moblis* (Kim and Barrow, 2000).

Commercial cellulase prepared from *Trichoderma reesei* has activity optimums at a temperature at 50°C and pH 4.8 (Mandels and Weber, 1969). Researchers have tried to develop a process or find a thermal tolerant organism to match the optimum conditions of enzymatic hydrolysis for purposes such as SSF fermentations. However, increasing the fermentation temperature of traditional ethanologens has been shown to decrease ethanol production and increase cell death rate (Ballesteros et al., 1991; Banat et al., 1992,

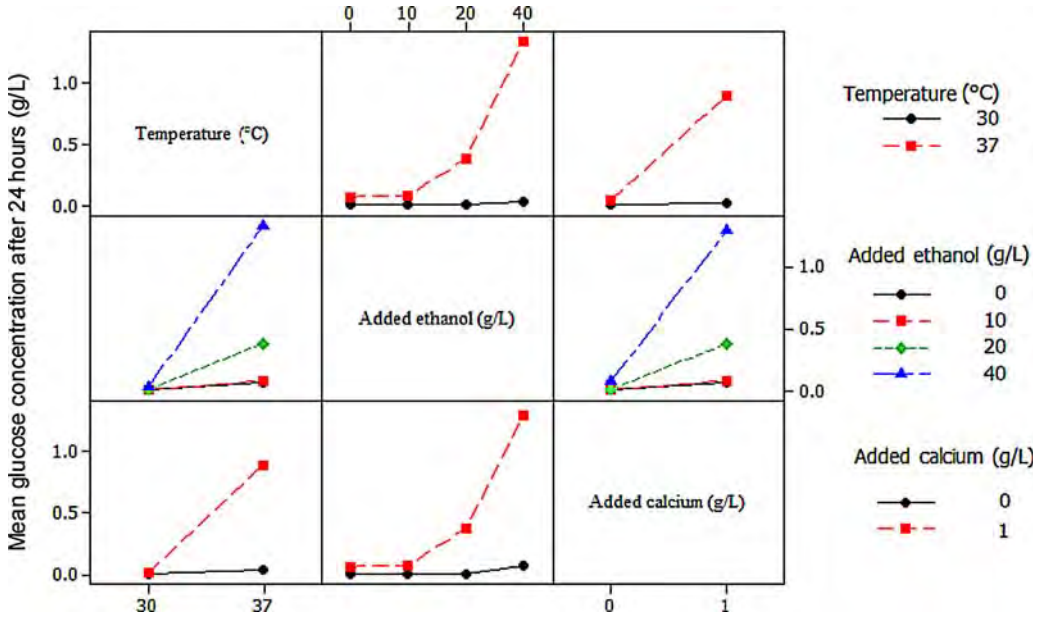


**Figure 5.** Interaction effects of ethanol, calcium, and temperature on 10 g/L glucose utilization by *Z. mobilis* 8b in factorial designed experiments. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

1996; D'Amore et al., 1989; Spindler et al., 1988; Szczodrak et al., 1988; van Uden, 1984). Alternatively, use of engineered thermophilic bacteria shows the potential to match optimal microbial growth to the optimal enzymatic hydrolysis temperature (Shaw et al., 2008), but its application in industry has yet to be demonstrated. Here we compared paper sludge SSCF by two engineered microorganisms at the optimum cell growth temperatures of 30 and 37°C, the highest temperature we know that over 40 g/L ethanol was produced with a good cell viability in soluble sugar fermentation. Paper sludge SSCF by *Z. mobilis* 8b has much higher ethanol yields at 30°C than 37°C due to better consumption of residual sugar, and higher final glucan and xylan conversion. Paper sludge SSCF by

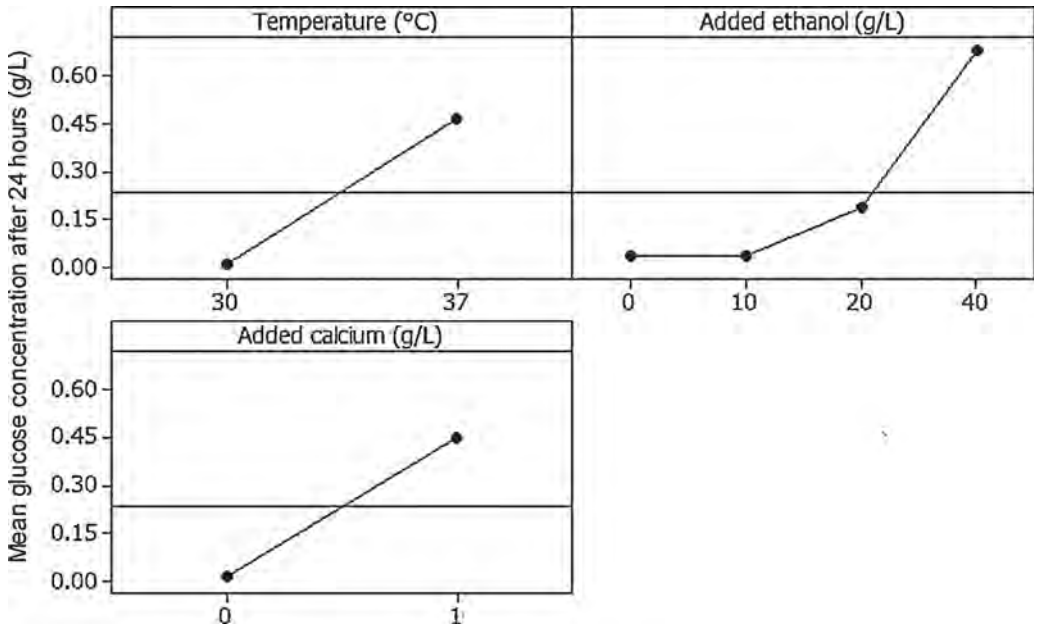


**Figure 6.** Effect of ethanol, calcium, and temperature on 10 g/L glucose utilization by *Z. mobilis* 8b in factorial designed experiments.

**Table II.** Comparison of paper sludge SSCF by *S. cerevisiae* RWB222 and *Z. mobilis* 8b at 30 and 37°C, and with *S. cerevisiae* D5A at 37°C.

| | RWB222 at 37°C | RWB222 at 30°C | 8b at 37°C | 8b at 30°C | D5A at 37°C |
|---|---|---|---|---|---|
| g ethanol/g sugar consumed | 0.42 ± 0.01 | 0.40 ± 0.01 | 0.46 ± 0.01 | 0.47 ± 0.01 | 0.44 ± 0.01 |
| g ethanol/g sugars fed in paper sludge | 0.39 ± 0.01 | 0.35 ± 0.01 | 0.31 ± 0.01 | 0.39 ± 0.01 | 0.33 ± 0.01 |
| Final xylan conversion | 0.95 ± 0.02 | 0.89 ± 0.02 | 0.78 ± 0.02 | 0.90 ± 0.01 | 0.93 ± 0.02 |
| Final glucan conversion | 0.93 ± 0.01 | 0.88 ± 0.02 | 0.74 ± 0.02 | 0.88 ± 0.02 | 0.94 ± 0.01 |
| Ethanol production % compare to *S. cerevisiae* D5A | 119% | 105% | 93% | 119% | 100% |

*S. cerevisiae* RWB222 has a higher ethanol yield at 37°C than 30°C, presumably due to higher cellulase and xylanase enzymatic activity at the higher temperature. The final glucan and xylan conversion at 37°C is about 5% higher than that at 30°C. *Z. mobilis* 8b has a higher maximum ethanol yield (0.47 g ethanol/g sugar consumed) than *S. cerevisiae* RWB222 (0.42 g ethanol/g sugar consumed). The ethanol yield based on sugar consumed is consistent for both strains at both temperatures. The higher ethanol yield of *Z. mobilis* 8b and lower glucan and xylan conversion explains why *Z. mobilis* 8b at 30°C has a similar ethanol titer from the same amount of paper sludge as *S. cerevisiae* RWB222 at 37°C. We can imagine the best ethanol productivity will be achieved at a temperature between 30 and 37°C, at which *Zymomonas mobilis* 8b has good growth and good glucose utilization ability in the presence of 1 g/L calcium; however, the improvement in ethanol production will not be higher than 5%. Use of microbes capable of fermenting xylose conferred a significant advantage compared to microbes that ferment glucose only during paper sludge SSCF. In particular, about 19% more ethanol was produced from the same amount paper sludge in 5 days by the xylose utilizing organisms compared to results for the non-xylose utilizing organism *S. cerevisiae* D5A.

Our experience suggests that a good organism for soluble glucose and xylose co-fermentation is not necessarily a good candidate for SSCF. In contrast to soluble sugar fermentation, hydrolysis is usually the rate-limiting step during SSCF, which means that the ability to rapidly use soluble substrates is less important for SSCF. There are no high concentrations of glucose and xylose in the medium, which means the ability to use trace residual glucose and xylose, and tolerance to starvation under high concentrations of ethanol are more important than tolerance of high concentrations of sugars. Good ethanol yields from both glucose and xylose are important for SSCF whereas only yields from glucose are important for SSF. It is also desirable to use an organism which can grow under strictly anaerobic conditions, since it can be difficult to effectively distribute trace oxygen in a large industrial reactor with high solids (Wyman, 1999). Based on the above discussion, we consider *S. cerevisiae* RWB222 and *Z. mobilis* 8b to be two promising candidates for paper sludge SSCF.

This research was supported by the grants from Mascoma Corporation, the National Institute of Standards and Technology, and the BioEnergy Science Center (BESC), Oak Ridge National Laboratory, a U.S. Department of Energy (DOE) Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science.

## References

Ballesteros I, Ballesteros M, Cabanas A, Carrasco J, Martin C, Negro M, Saez F, Saez R. 1991. Selection of thermotolerant yeasts for simultaneous saccharification and fermentation (SSF) of cellulose to ethanol. Appl Biochem Biotechnol 28–29:307–316.

Banat IM, Nigam P, Marchant R. 1992. Isolation of thermotolerant, fermentative yeasts growing at 52°C and producing ethanol at 45°C and 50°C. World J Microbiol Biotechnol 8(3):259–263.

Banat IM, Singh D, Marchant R. 1996. The use of a thermotolerant fermentative *Kluyveromyces marxianus* IMB3 yeast strain for ethanol production. Acta Biotechnol 16(2–3):215–223.

Chandrakant P, Bisaria VS. 1998. Simultaneous bioconversion of cellulose and hemicellulose to ethanol. Crit Rev Biotechnol 18:295–331.

D'Amore T, Celotto G, Russell I, Stewart G. 1989. Selection and optimization of yeast suitable for ethanol production at 40 degree C. Enzyme Microb Technol 11:411–416.

Farrell AE, Plevin RJ, Turner BT, Jones AD, O'Hare M, Kammen DM. 2006. Ethanol can contribute to energy and environmental goals. Science 311(5760):506–508.

Ghose TK. 1987. Measurement of cellulase activities. Pure Appl Chem 59(2):257–268.

Graaf AAD, Striegel K, Wittig RM, Laufer B, Schmitz G, Wiechert W, Sprenger GA, Sahm H. 1999. Metabolic state of *Zymomonas mobilis* in glucose-, fructose-, and xylose-fed continuous cultures as analysed by 13C- and 31P-NMR spectroscopy. Arch Microbiol 171(6):371–385.

Greene N, Celik FE, Dale B, Jackson M, Jayawardhana K, Jin H, Larson ED, Laser M, Lynd L, MacKenzie D. 2004. Growing energy: How biofuels can help end America's oil dependence. Natural Resources Defense Council.

Harhangi HR, Akhmanova AS, Emmens R, van der Drift C, de Laat WTAM, van Dijken JP, Jetten MSM, Pronk JT, Op den Camp HJM. 2003. Xylose metabolism in the anaerobic fungus *Piromyces* sp. strain E2 follows the bacterial pathway. Arch Microbiol 134–141.

Joachimsthal E, Haggett KD, Jang JH, Rogers PL. 1998. A mutant of *Zymomonas mobilis* ZM4 capable of ethanol production from glucose in the presence of high acetate concentrations. Biotechnol lett 20:137–142.

Joachimsthal E, Haggett KD, Rogers PL. 1999. Evaluation of recombinant strains of *Zymomonas mobilis* for ethanol production from glucose/xylose media. Appl Biochem Biotechnol 77–79:147–157.

Kang L, Wang W, Lee Y. 2010. Bioconversion of kraft paper mill sludges to ethanol by SSF and SSCF. Appl Biochem Biotechnol 161(1–8):53–66.

Kim IS, Barrow KD, Rogers PL. 2000. Kinetic and nuclear magnetic resonance studies of xylose metabolism by recombinant *Zymomonas mobilis* ZM4(pZB5). Appl Environ Microbiol 66(1):186–193.

Kim TH, Lee YY. 2005. Pretreatment of corn stover by soaking in aqueous ammonia. Appl Biochem Biotechnol 121:1119–1131.

Kuyper M, Harhangi HR, Stave AK, Winkler AA, Jetten MSM, de Laat WTAM, den Ridder JJ, op den Camp HJM, van Dijken KP, Pronk JT. 2003. High-level functional expression of a fungal xylose isomerase:

The key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? FEMS Yeast Res 4(3):69–78.

Kuyper M, Winkler AA, van Dijken JP, Pronk JT. 2004. Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: A proof of principle. FEMS Yeast Res 4:655–664.

Kuyper M, Toirkens MJ, Diderich JA, Winkler AA, van Dijken JP, Pronk JT. 2005. Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. FEMS Yeast Res 5(10):925–934.

Lange HC, Heijnen JJ. 2001. Statistical reconciliation of the elemental and molecular biomass composition of *Saccharomyces cerevisiae*. Biotechnol Bioeng 75(3):334–344.

Linde M, Galbea M, Zacchia G. 2007. Simultaneous saccharification and fermentation of steam-pretreated barley straw at low enzyme loadings and low yeast concentration. Enzyme Microb Technol 40(5):1100–1107.

Lynd LR, Lyford K, South CR, van Walsum PG, Levenson K. 2001. Evaluation of paper sludges for amenability to enzymatic hydrolysis and conversion to ethanol. Tappi J 84(2):50.

Mandels M, Weber J. 1969. The production of cellulases. Cellulases and their Application, 391–413.

McMillan JD, Newman MM, Templeton DW, Mohagheghi A. 1999. Simultaneous saccharification and cofermentation of dilute-acid pretreated yellow poplar hardwood to ethanol using xylose-fermenting *Zymomonas mobilis*. Appl Biochem Biotechnol 77–79:649–665.

Mohagheghi A, Dowe N, Schell D, Chou Y, Eddy C, Zhang M. 2004. Performance of a newly developed integrant of *Zymomonas mobilis* for ethanol production on corn stover hydrolysate. Biotechnol Lett 26:321–325.

Ohgren K, Bengtsson O, Gorwa-Grauslund MF, Galbe M, Hahn-Hagerdal B, Zacchi G. 2006. Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with *Saccharomyces cerevisiae* TMB3400. J Biotechnol 126(4):488–498.

Olofsson K, Rudolfa A, Lidéna G. 2008. Designing simultaneous saccharification and fermentation for improved xylose conversion by a recombinant strain of *Saccharomyces cerevisiae*. J Biotechnol 134(1–2):112–120.

Patel M, Ou MS, Ingram LO, Shanmugam KT. 2005. Simultaneous saccharification and co-fermentation of crystalline cellulose and sugar cane bagasse hemicellulose hydrolysate to lactate by a thermotolerant acidophilic *Bacillus* sp. Biotechnol Prog 21(5):1453–1460.

Rudolf A, Baudel H, Zacchi G, Hahn-Hägerdal B, Lidén G. 2007. Simultaneous saccharification and fermentation of steam-pretreated bagasse using *Saccharomyces cerevisiae* TMB3400 and Pichia stipitis CBS6054. Biotechnol Bioeng 99(4):783–790.

Shao XJ. 2007. Cellulosic Biomass to Ethanol: Kinetic Modeling, Scale up, and Reactor Design. Ph.D. thesis, Dartmouth Colledge, USA.

Shaw AJ, Podkaminer KK, Desai SG, Bardsley JS, Rogers SR, Thorne PG, Hogsett DA, Lynd LR. 2008. Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. Proc Natl Acad Sci 105(37):13769–13774.

Spindler DD, Wyman CE, Mohagheghi A, Grohmann K. 1988. Thermotolerant yeast for simultaneous saccharification and fermentation of cellulose to ethanol. Appl Biochem Biotechnol 17(1–3):279–294.

Szczodrak J, Targo Z, Nacute SKI. 1988. Selection of thermotolerant yeast strains for simultaneous saccharification and fermentation of cellulose. Biotechnol Bioeng 31(4):300–303.

Takagi M, Abe S, Suzuki S, Emert GH, Yata N. 1977. A method for production of alcohol directly from cellulose using cellulase and yeast. Proc Bioconversion Symp, IIT, Delhi, 551–571.

Teixeira LC, Linden JC, Schroeder HA. 1999. Optimizing peracetic acid pretreatment conditions for improved simultaneous saccharification and co-fermentation (SSCF) of sugar cane bagasse to ethanol fuel. Proceedings of the 1998 world renewable energy congress V. Part 2 16(1–4): 1070–1073.

Teixeira LC, Linden JC, Schroeder HA. 2000. Simultaneous saccharification and cofermentation of peracetic acid–pretreated biomass. Appl Biochem Biotechnol 84–86:111–128.

van Dijken JP, Bauer J, Brambilla L, Duboc P, Francois JM, Gancedo C, Giuseppin MLF, Heijnen JJ, Hoare M, Lange HC, et al. 2000. An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. Enzyme Microb Technol 26(9):706–714.

Van Dyke BH. 1972. Enzymatic hydrolysis of cellulose: A kinetic study. Ph.D. thesis, Massachusetts Institute of Technology, USA.

van Uden N. 1984. Temperature profiles of yeasts. Adv Microb Physiol 25:195–251.

Wyman CE. 1999. Biomass ethanol: Technical progress, opportunities, and commercial challenges. Annu Rev Energy Environ 24:189–226.

Xiao Z, Zhang X, Gregg DJ, Saddler JN. 2004. Effects of sugar inhibition on cellulases and β-glucosidase during enzymatic hydrolysis of softwood substrates. Appl Biochem Biotechnol 115(1–3):1115–1126.

Zacchi G, Axelsson A. 1989. Economic evaluation of preconcentration in production of ethanol from dilute sugar solutions. Biotechnol Bioeng 34(2):223–233.

Zhang J. 2008. Simultaneous saccharification and co-fermentation of paper sluge. Ph.D thesis, Dartmouth college, USA: 275.

Zhang M, Eddy C, Deanda K, Finkelstein M, Picataggio S. 1995. Metabolic engineering of a pentose metabolism pathway in ethanologenic zymomonas mobilis. Science 267(5195):240–243.

Zhang J, Heiss C, Thorne PG, Bal C, Azadi P, Lynd LR. 2009a. Formation of ethyl-xylopyranoside during simultaneous saccharification and co-fermentation of paper sludge. Enzyme Microb Technol 44:196–202.

Zhang J, Shao X, Lynd LR. 2009b. Simultaneous saccharification and co-fermentation of paper sludge to ethanol by *Saccharomyces cerevisiae* RWB222. Part II: Investigation of discrepancies between predicted and observed performance at high solids concentration. Biotechnol Bioeng 104(5):932–938.

Zhang J, Shao X, Townsend OV, Lynd LR. 2009c. Simultaneous saccharification and co-fermentation of paper sludge to ethanol by *Saccharomyces cerevisiae* RWB222- part I: Kinetic modeling and parameters. Biotechnol Bioeng 104(5):920–931.

Zhu Y, Lee YY, Elander RT. 2007. Conversion of aqueous ammonia-treated corn stover to lactic acid by simultaneous saccharification and cofermentation. Appl Biochem Biotechnol 137–140(1):721–738.