Appl Biochem Biotechnol (2010) 161:53–66
DOI 10.1007/s12010-009-8893-4

Bioconversion of Kraft Paper Mill Sludges to Ethanol by SSF and SSCF

Li Kang · Wei Wang · Yoon Y. Lee

Received: 19 May 2009 / Accepted: 16 December 2009 /
Published online: 23 January 2010
© Springer Science+Business Media, LLC 2010

Abstract Paper mill sludge is a solid waste material composed of pulp residues and ash generated from pulping and paper making processes. The carbohydrate portion of the sludge has chemical and physical characteristics similar to pulp. Because of its high carbohydrate content and well-dispersed structure, the sludges can be biologically converted to value-added products without pretreatment. In this study, two different types of paper mill sludges, primary sludge and recycle sludge, were evaluated as a feedstock for bioconversion to ethanol. The sludges were first subjected to enzymatic conversion to sugars by commercial cellulase enzymes. The enzymatic conversion was inefficient because of interference by ash in the sludges with the enzymatic reaction. The main cause was that the pH level is dictated by $CaCO_3$ in ash, which is two units higher than the pH optimum of cellulase. To alleviate this problem, simultaneous saccharification and cofermentation (SSCF) using cellulase (Spezyme CP) and recombinant *Escherichia coli* (ATCC-55124), and simultaneous saccharification and fermentation (SSF) using cellulase and *Saccharomyces cerevisiae* (ATCC-200062) were applied to the sludges without any pretreatment. Ethanol yields of 75–81% of the theoretical maximum were obtained from the SSCF on the basis of total carbohydrates. The yield from the SSF was also found to be in the range of 74–80% on the basis of glucan. The SSCF and SSF proceeded under stable condition with the pH staying near 5.0, close to the optimum for cellulase. Decrease of pH occurred due to carbonic acid and other organic acids formed during fermentation. The ash was partially neutralized by the acids produced from the SSCF and SSF and acted as a buffer to stabilize the pH during fermentation. When the SSF and SSCF were operated in fed-batch mode, the ethanol concentration in the broth increased from 25.5 and 32.6 g/L (single feed) to 45 and 42 g/L, respectively. The ethanol concentration was limited by the tolerance of the microorganism in the case of SSCF. The ethanol yield in fed-batch operation decreased to 68% for SSCF and 70% for SSF. The high-solids condition in the bioreactor appears to create adverse effects on the cellulase reaction.

L. Kang · W. Wang · Y. Y. Lee (✉)
Department of Chemical Engineering, Auburn University, Auburn, AL 36849, USA
e-mail: yylee@eng.auburn.edu

Keywords Paper mill sludges · Ethanol · SSF · SSCF · Bioconversion

Introduction

The pulp and paper industry generates 4–5 million tons of sludges per year in the US [1]. The sludges disposed by incineration or landfill cause substantial financial burden and a source of various environmental problems. Incineration is a significant cost factor because of low energy efficiency caused by high moisture content [1–3]. The fibers from the paper mill sludge have characteristics suitable for conversion to value-added products. Sludges from kraft paper mill have high carbohydrate and low lignin contents in comparison to natural lignocellulosic substrates. The composition of the organic portion of the sludge is very similar to that of commercial grade pulp. It also has well-dispersed structure and high surface area. It has been proven to be highly efficient for bioconversion to ethanol [4]. Although it is projected that a majority of ethanol is to be produced from natural lignocellulosic feedstock, paper sludge is a promising feedstock that needs to be considered for bioconversion.

As a feedstock for bioethanol, paper mill sludges offer two significant economic benefits. First, the cost of sludge is essentially zero and may even be a negative number considering that the cost of disposal is about $20 per wet ton (Randy Abston, Boise Paper, Jackson, AL, USA, personal communication, 2008). Second, elimination of pretreatment can significantly reduce the production cost of ethanol. Pretreatment opens up the biomass structure and makes it more amenable for enzymatic hydrolysis. It is a major processing unit in bioethanol plant accounting for about 30% of the total production cost of ethanol, second only to the raw material [5]. In alkaline delignification of biomass such as kraft pulping, lignin is predominantly degraded by various cleavage reactions including aryl ether linkages [6]. The kraft pulping process also removes about 50% hemicellulose and brings about changes in the structure of fiber [6]. The fibers in the sludge are short because they are screen rejects of paper machine and the digester. Because of small and well-dispersed structure, they are easily digested by cellulase enzymes [1, 2, 7–9].

In this study, sludge generated from a paper mill was investigated as a feedstock for ethanol production. The main objectives of the investigation were to characterize the sludges in terms of composition and physical properties and to test technical feasibility of converting the feedstock to ethanol without pretreatment. Bioconversion of pulp mill sludge has been previously investigated, wherein the pH of the sludge was pre-adjusted by hydrochloric acid [10] or by sulfuric acid [11]. This strategy may encounter a problem when the sludge has a large amount of ash, mainly CaCO3, such as the one used in this work since the amount of acid to neutralize the ash is excessive. Simultaneous saccharification and fermentation (SSF) of paper mill sludge was investigated by Fan et al. [7], in which pH in the medium was adjusted by sulfuric acid. However, the effect of ash on the pH during the course of SSF was not addressed. The high ash content in paper mill sludges, especially $CaCO_3$, causes several problems in the bioconversion process as it interferes with pH control. The high ash content also limits solid loading capacity in the bioreactor and reduces the efficiency of enzyme in a bioprocess. Additional technical issues arising from the contaminated substrate were included within the scope of this investigation.

Materials and Methods

Materials

Feedstock

The paper mill sludges were collected from a kraft paper mill, Boise Paper Company (Jackson, AL, USA). There were two different types of sludges. One was primary sludge (PS), 90a discharge from the paper machine and the digester, which was collected from the primary wastewater clarifier unit. The other one was recycle sludge, which came from a reprocessing unit of recycled paper. Both were stored at 4 °C. A sample quantity of wet hardwood pulp was also obtained from Boise Paper Company. It was used as a reference substrate in bioconversion studies. The paper sludge and hardwood pulp were analyzed for carbohydrates, moisture content, and ash content according to the National Renewable Energy Laboratory (NREL) standard procedure [12]. Hardwood pulp was analyzed to contain 76.0% glucan, 21.1% xylan, and 2.9 wt.% unaccounted for (lignin, acetyl group, ash, and protein).

Enzymes

Cellulase enzyme (Spezyme CP, Lot No. 301-00348-257) was a kind gift from Danisco Genencor (Palo Alto, CA, USA). The activity of Spezyme CP was 59 FPU/mL, as determined by NREL standard procedure [12]. β-glucosidase (Novozyme188, Cat. No. C-6150) was purchased from Sigma (St. Louis, MO, USA) and its activity was measured to be 750 CBU/mL.

Microorganism

The microorganism used in the SSF was *Saccharomyces cerevisiae* ATCC-200062 (NREL-D5A). The growth media was YP medium, which contained 1% yeast extract (Sigma, Y-0500) and 2% peptone (Sigma, P-6588). Recombinant *Escherichia coli* ATCC-55124 (KO11) was for the simultaneous saccharification and cofermentation (SSCF) experiments. This organism was grown in LB medium (Sigma, L-3152), which contained 1% tryptone, 0.5% yeast extract, 1% NaCl, and 40 mg/L chloroamphenicol.

Contaminant Microorganism Test

A YPD agar (Sigma, Y-1500) or LB agar (Sigma, L-3027) plate was placed on a turntable, and 10 μL of sludge solution was dispersed evenly on the plate. Tetracycline and cycloheximide were added to a separate plate for comparison purpose. The YPD or LB agar plate was then incubated at 30 °C for 48 h. Samples from each flask were streaked on YPD or LB agar plate to check the microbial contamination. The gram stain was used to differentiate gram-positive and gram-negative bacteria. Colony forming unit (CFU) tests were performed to determine the concentration of microorganism cells. YPD or LB agar plates were prepared for this purpose by diluting the culture with sterile saline (0.89% NaCl solution) to obtain a spread plate cell count of 30–300 cells per plate.

Digestibility Test

The enzymatic digestibility of the sludge was determined according to the NREL Chemical Analysis and Testing Standard Procedures [12]. Screw-capped 250 mL Erlenmeyer flasks were used as the hydrolysis reactor. The sludge samples were suspended in DI water and put into the flasks to reach a total working volume of 100 mL. The sludge was loaded into the bioreactor that the initial glucan content in the reactor becomes 1% (*w*/*v*). Sludges were steam sterilized by autoclaving at 121 °C for 15 min. The enzymatic digestibility tests were carried out at 50 °C in an incubator shaker (New Brunswick Scientific, Innova-4080) agitated at 150 RPM. For hardwood pulp, enzymatic hydrolysis was carried out under three different conditions: pH4.8 (0.05 M sodium citrate buffer), pH7.0 (0.02 M sodium phosphate buffer), and with addition of $CaCO_3$ to the level of 0.5 g/g-glucan matching that of sludge without pH control. In all digestibility tests, enzyme loading of 15 FPU of Spezyme CP and 30 CBU of Novozyme-188 was applied per g-glucan in the feedstock. The enzymes were filter-sterilized. Hydrolyzate samples were taken at 6, 12, 24, 48, and 72 h and analyzed for glucose, xylose, and cellobiose. Total released glucose and cellobiose after 72 h of hydrolysis were used to calculate the enzymatic digestibility:

$$\text{Digestibility}(\%) = \frac{\text{Glucose_released(g)} + 1.053 \times \text{Cellobiose_released(g)}}{1.111 \times \text{Glucan_added(g)}} \times 100$$

Hardwood pulp was put through the same procedure as a control. The digestibilities for xylan content were determined in a similar manner. For xylan digestibility, hydration factor of 1.136 was used in the equation. Digestibility tests were done in duplicates.

Simultaneous Saccharification and Fermentation/Cofermentation

A 250-mL Erlenmeyer flask was used as the bioreactor. It was operated in an incubator shaker (New Brunswick Scientific, Innova-4080) at 37 °C with 150 RPM with100 mL working volume. Sludges and growth medium were added such that the glucan content becomes 3% *w*/*v* or 6% *w*/*v*. Hardwood pulp was used as a reference substrate. The sludge samples were steam sterilized at 121 °C for 15 min. The growth media for SSF was YP medium. The growth media for SSCF was LB medium (Sigma, L-3152). For SSCF, 40 mg/L chloroamphenicol was added after autoclaving to maintain sterility. KO-11 is resistant to these antibiotics.

SSF and SSCF of the sludges were carried out at 37 °C without pH control. SSF and SSCF of the hardwood pulp were carried out at 37 °C with pH4.8 (0.05 M sodium citrate buffer) and pH6.0 (0.05 M sodium phosphate buffer), respectively. Cellulase and β-glucosidase were also added to achieve enzyme loading at 15 FPU and 30 CBU/g-glucan, respectively. Optical density (OD) was measured by UV Spectrophotometer (BioTek Synergy HT Multidetection Microplate Readers) at 600 nm for yeast (NREL-D5A) and 550 nm for recombinant *E. coli* (KO11). The initial OD after inoculation was 0.05, equivalent to 16 mg dry cell weight of KO11/L and 50 mg of NREL-D5A/L. At the conclusion of all of the microbial experiments, a sample from each flask was streaked on a YPD or LB agar plate and checked under an optical microscope for presence of microbial contamination. CFU tests were performed using YPD or LB agar plates to check microorganism viability. The SSF and SSCF experiments requiring pH monitoring were carried out using a1-L bioreactor (Multifors IHORS HT 2×1 L) with 400 mL working volume and 3% *w*/*v* loading.

The ethanol yield was calculated as follows:

$$\text{Ethanol yield}[\%\ \text{of theoretical maximum}] = \frac{\text{Ethanol produced(g)in reactor}}{\text{Initial sugar(g)in reactor} \times 0.511} \times 100$$

Sugar is interpreted as glucose in the SSF and glucose plus xylose in the SSCF.

Fed-batch experiments were started with 100 mL initial working volume and 6% *w/v* glucan loading. Squeezed sludge cakes equivalent to 6 g-glucan were added to the bioreactors at desired times. The cellulase and β-glucosidase were also added at beginning of fermentation process to achieve overall enzyme loading at 15 FPU and 30 CBU/g-glucan based on total glucan. Addition of sludge was done at 24 h point for SSCF and at 24 and 48 h points for SSF. Samples were analyzed for glucose, xylose, organic acid, and ethanol by high performance liquid chromatography (HPLC). Aseptic conditions were maintained in all of the microbial experiments. The fed-batch fermentation runs were made in triplicate.

Analytical Methods

The solid samples were analyzed for carbohydrates and Klason lignin following the NREL standard procedures [12]. The moisture content was measured by an infrared moisture balance (Denver Instrument, IR-30). Sugars were determined by HPLC using a HPX-87P column. For the SSF or SSCF tests, BioRad-HPX-87H column was used for measurement of organic acid and ethanol. The acid-insoluble ash was determined following the Tappi test method [13]. Liquid sample analysis and ash determination were done in triplicate.

The alkalinity of the sludge was determined by titration with 1 N H_2SO_4 to pH5.0 and expressed in terms of milliliters of 1 NH_2SO_4/10 g dry sludge. The buffering capacity of the paper sludges was determined by titration with 20 g/L acetic acid. The experiments requiring pH monitoring were carried out using 1-L bioreactor (Multifors IHORS HT 2× 1 L) with 400 mL working volume at 37°C and 150 RPM. Where applicable, statistical computation including mean value and standard deviation was performed using Microsoft Office Excel 2003.

Results and Discussion

Composition

The compositions of primary sludge and recycle sludge are shown in Table 1. All data are based on oven-dry original feedstocks. The composition of corn stover, determined by the National Bioenergy, Center, NREL, Golden, CO, USA, is also included as a reference. Paper mill sludges are high in glucan and low in lignin in comparison to corn stover. They contain high amount of ash, about one third of the total mass. The majority of ash content in sludge is from filler material added during the paper making process: clay, TiO_2, and $CaCO_3$. It is added as fine particles of 0.1~10 μm [14]. The ash was further analyzed to determine acid-soluble fraction and acid-insoluble fraction. Based on the composition data of ash provided by Boise Paper, Jackson, AL, USA (Randy Abston, Boise Paper, Jackson, AL, USA, personal communication, 2008), we conclude that the acid-soluble ash is mainly $CaCO_3$, and acid-insoluble ash is composed of clay and TiO_2.

Table 1 Composition of kraft paper mill and corn stover.

	Primary sludge	Recycle sludge	Corn stover
Glucan (%)	44.5	49.6	36.1
Xylan (%)	9.9	13.7	21.4
Lignin (%)	8.1	4.6	17.2
Total ash (%)	36.0	30.0	7.1
Acid-soluble ash (%)	26%	22%	-
Acid-insoluble Ash (%)	10%	8%	-
Others (%)	1.5	2.1	18.2
Alkalinity (milliliters of 1 NH_2SO_4/10 g dry sludge)	60	50	-

Data of sludges is the mean value of triplicate (n=3; SD<0.5%)

Enzymatic Digestibility

The enzymatic digestibility data of sludges and a reference substrate are summarized in Table 2. Hardwood pulp was taken as the reference substrate because the composition is similar to the organic portion of the sludges. Digestibility tests were made at 37°C and 50°C. Although the optimum temperature for enzymatic hydrolysis was 50°C, tests at 37°C were also made in consideration of SSF/SSCF investigated in this work. Digestibilities were determined for glucan and xylan separately. For sludges, tests were carried out without pH control because of the high ash content which carries enormous buffering capacity. The pH of sludge digestion remained near neutral. For hardwood pulp, enzymatic hydrolysis was carried out under three different conditions: pH4.8 (optimum for cellulase), pH7.0, and

Table 2 Enzymatic digestibility of paper mill sludges.

	Temperature (°C)	Sugar released at 72-h enzymatic hydrolysis (g/L)			72-h digestibility (%)	
		Glucose	Cellobiose	Xylose	Glucan	Xylan
Primary sludge[a]	50	0.6	0.3	0.2	8.3	7.9
	37	2.5	1	0.8	32.0	31.7
Recycle sludge[a]	50	0.7	0.3	0.3	9.2	9.6
	37	2.4	0.9	0.9	30.2	28.7
Hardwood pulp with 0.5 gCaCO3/g-glucan[a]	50	1.4	0.7	0.7	19.3	22.1
	37	2.6	1	0.8	32.9	25.2
HP with 0.05 M citrate buffer (pH=4.8)[a]	50	10.5	0	2.1	94.6	66.3
	37	9.1	0	1.8	82.0	56.8
HP with 0.05 M phosphate buffer (pH=7)[a]	50	3.6	0.6	0.8	38.1	25.2
	37	4.8	0.5	0.9	48.0	28.4
Primary sludge[b]	37	0.7	0.3	0.1	9.2	4.0
Recycle sludge[b]	37	0.6	0.3	0.1	8.3	3.2

Data of sludges and hardwood pulp is the mean value of duplicate (n=2; SD<1.0 for digestibility)

[a] Sterilization method applied was sterilization by autoclaving

[b] Sterilization method applied was sterilization by antibiotics

with addition of $CaCO_3$ to the level of 0.5 g/g-glucan matching that of sludge. The enzymatic digestion of sludges was dismal giving sugar yields of only 8–32% of theoretical maximum. The highest digestibility observed was that of hardwood conducted at pH4.8 and 50°C which stood at 94.6% and 66.3% for glucan and xylan, respectively. The data clearly indicate that the digestibilities of sludges are far below to those of hardwood pulp tested at pH 4.8. Hardwood pulp tested at pH7.0, although much lower than that tested at pH4.8, was significantly higher than that tested with addition of $CaCO_3$. These data collectively indicate that the inefficient digestion of sludges is mainly due to the presence of ash in the sludges. The high level of ash in sludge made it difficult to control pH because of the buffering action of $CaCO_3$. Also to be noted here is that the sugar yield obtained at 37°C was higher than that obtained at 50°C. This result is unexpected since 50°C is close to the optimum temperature for the cellulase enzymes. We have no clear explanation to offer at this time. It may be that the cellulose-enzyme reaction is hampered by certain ionic components released from the sludges, and more of such components are released at high temperature.

The cellulase enzyme used in the test was supplemented with β-glucosidase at the level of 2 CBU/FPU, to suppress inhibition by cellobiose. Cellobiose was nonexistent in the enzymatic hydrolysis of hardwood pulp at pH4.8 with this enzyme formulation, but its accumulation was significant in all of the neutral pH runs. Cellobiose was higher also at 37°C versus 50°C where two conditions were not favorable for fungal cellulases and β-glucosidase. Their activities peak at 50±5°C and pH of 4.0–5.0 [15]. This perhaps is an indication that β-glucosidase is more sensitive to pH and temperature than the other cellulose components. Marques et al. found that the optimum condition of enzymatic hydrolysis of recycle sludge is 35°C and pH5.5. The recycle sludge used in their study was neutralized with hydrochloric acid before enzymatic hydrolysis [10].

Microbial Contamination of Sludges

Enzymatic hydrolysis of sludges conducted with addition of antibiotics without sterilization has shown very poor results giving digestibility of less than 10% (Table 2). To test bacterial contamination, sludge solution was sampled on YPD agar plate. After the incubation, the gram-negative bacteria and molds were identified from the gram stain test and the morphology of the microorganism. It appears that tetracycline and cycloheximide provide only a partial protection as we observed that some microorganisms still grew during the enzymatic hydrolysis. Steam sterilization of sludges was therefore necessary to eliminate the contamination problem.

Simultaneous Saccharification and Fermentation

Recognizing the difficulty of enzymatic hydrolysis of sludges, other means of improving the process were sought. The first was to remove the ash content of the sludges before subjecting them to bioconversion. This work is currently in progress in our laboratory. The second approach was to apply a different and more advanced bioconversion scheme for the sludges, namely, the simultaneous saccharification and fermentation. The SSF is now considered as the norm for the bioconversion of lignocellulosic feedstocks for a number of reasons. Simultaneous use of cellulase and microorganism makes the SSF proceed under sugar-limited conditions so that the sugar concentration in the bioreactor is kept below the inhibition threshold. According to Varga et al. [16], product inhibition starts to occur at 15 g/L of glucose. The sugar yield obtained from the enzymatic digestion of sludges was very low since the pH of sludge digestion remained near neutral because of high ash

content in the sludge. Some ethanologen microorganisms, especially yeast, have a broad range of pH optimum, 3.0–6.0. The pH optimum for *E. coli* is near 7.0.

With this understanding, a number SSF runs were attempted using the paper mill sludges without removing the ash to assess the adverse effects arising from pH mismatch between the enzymatic and the microbial reactions. The enzyme mix and loading were identical to those of the digestibility test. *S. cerevisiae* ATCC-200062 (NREL-D5A) was used for the conversion of glucose to ethanol. The SSF of sludges was run at 37 °C without pH control. Two different substrate loadings were applied: 3% and 6% glucan (*w*/*v*). The time-course results of the SSF run are shown in Fig. 1, and the yield data are shown in Table 3. The data summarized in Table 3 shows that approximately 80% of the maximum theoretical yield is achieved with 3% glucan loading and 75% yield with 6% glucan loading, corresponding to ethanol concentration of 13.6 and 25.5 g/L, respectively. The yield of SSF is calculated on the basis of glucan in the sludge because *S. cerevisiae* can only convert glucan, xylan being left unconverted. The 1:1 mixture of the two sludges gave about the same ethanol yield.

The results show that conversion of sludges through SSF is far superior to enzymatic digestion. The 75–80% yield of ethanol from the SSF of the sludges is a drastic improvement over the 8–32% sugar yield obtained from the enzymatic digestion. It is surprising because the front part of the SSF is the enzymatic digestion. The profiles of glucose, xylose, and ethanol follow the pattern of the normal SSF (Fig. 1). The glucose

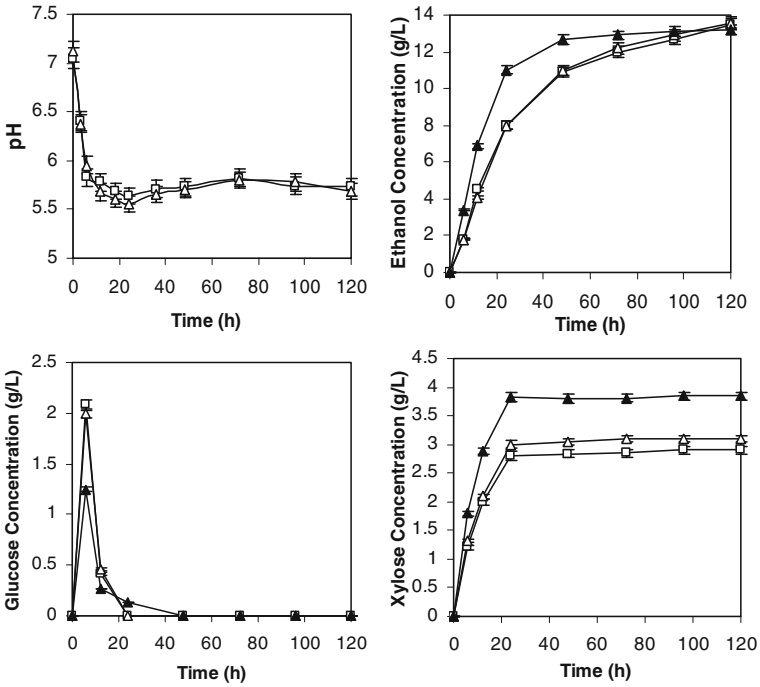

Fig. 1 Simultaneous saccharification and fermentation (SSF) of sludges by *Saccharomyces cerevisiae* (ATCC-200062). *Squares with connecting lines* represent primary sludge. *Triangles with connecting lines* represent recycle sludge. *Filled triangles with connecting lines* represent hardwood pulp. The *data points* represent average of triplicate runs. The pH was controlled at 4.8 for runs with hardwood pulp. Other conditions of the SSF were: 3% (*w*/*v*) glucan loading, 15 FPU Spezyme CP+30 CBU of Novozyme-188/g-glucan

Table 3 Ethanol yield from SSF and SSCF of paper mill sludges.

	Primary sludge (PS)		Recycle sludge (RS)		Mixture of PS and RS (1:1)	
	6% glucan loading	3% glucan loading	6% glucan loading	3% glucan loading	6% glucan loading	3% glucan loading
SSF ethanol yield (%) [a]	74.5	79.5	75.0	80.0	74.6	80.0
SSCF ethanol yield (%) [b]	78.0	80.1	75.1	77.1	77.0	80.5
SSCF ethanol yield (%) [c]	95.8	98.3	96.3	98.9	94.5	98.8

Data of sludges is the mean value of triplicate (n=3; SD<3.0)

[a] The ethanol yields of SSF were based on glucan only

[b] The ethanol yields of SSCF were based on total carbohydrate

[c] The ethanol yields of SSCF were based on glucan only

concentrations remained below 0.1 g/L after 24 h signifying that the yeast was rapidly consuming available glucose. The xylose concentrations remained unchanged after 24 h since the yeast NREL-D5A cannot convert xylose to ethanol. The two different sludges showed identical conversion pattern. Although the terminal ethanol yields were the same, hardwood pulp was converted slightly faster than the sludges.

The main reason for the improved performance of SSF appears to be the low pH level observed in the SSF. The SSF run started at a pH of 7.05 for primary sludge and 7.12 for recycle sludge and decreased to pH of 5.73 and 5.69, respectively, a level close to the optimum for cellulase and for *S. cerevisiae*. After the initial drop, the pH stayed relatively constant throughout the SSF. Since pH is a critical factor in bioconversion of sludges, we investigated further on it to verify the reason for the pH drop. We have measured the level of acetic acid and lactic acid in our SSF experiments. The maximum acetic acid level was 1.8 g/L, and lactic acid was 2.8 g/L. We speculated that these acids may be the reason for the pH drop. These organic acids produced in quantities are not enough to dissolve all acid-soluble ash. But they may interact with $CaCO_3$ in the sludge forming buffers, Ca-acetate or Ca-lactate, eventually lowering the pH.

To test this possibility, we have carried out titrations adding acetic acid into deionized water and water-sludge mixtures, simulating the SSF condition. As shown in Fig. 2, pH drop in water was rather drastic changing from 7.0 to 3.5 with addition of 45 mL of 20 g/L of acetic acid to 500 mL deionized water (equivalent to 4.5 g acetic acid per liter matching the total acid produced in the SSF). In the same test with water-sludge mixtures, the pH drop was much lower, decreasing from 7.0 to 6.6 with addition of same amount of acetic acid. The results indicate that acid production by yeast, although contributing to pH drop, cannot explain the entire pH drop in the SSF.

Another reason one can deduce is carbon dioxide produced by yeast. It is produced in large quantities; about the same amount as ethanol, although most of it escapes from the broth during the SSF due to its low solubility in water. Carbon dioxide in the broth may exist in a supersaturated condition before it is released from the broth. One example of supersaturated carbon dioxide is carbonated soft drinks, which show pH in the range of 2.5–4.2. The extent of supersaturation during fermentation can be quite high even under atmospheric pressure because it has to overcome the surface tension before it is released. We note that the surface tension in a bubble is inversely proportional to the diameter. If so, contribution of carbon dioxide to pH change is further increased to the level that it can

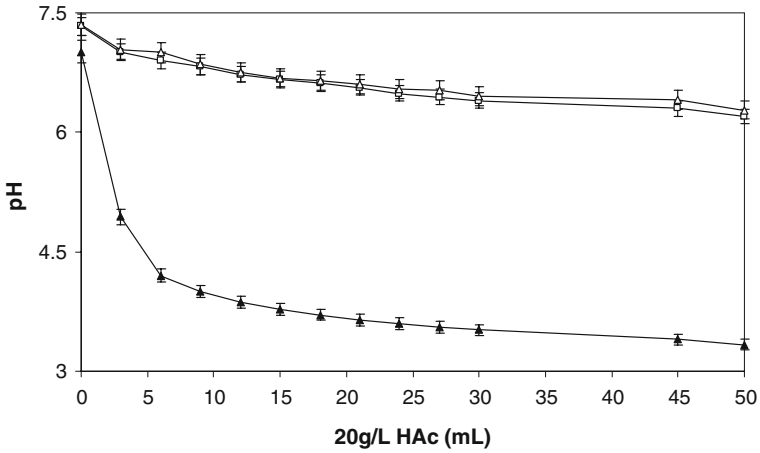

Fig. 2 Buffer capacity titration curves with acetic acid for paper sludge slurries and deionized water. *Squares with connecting lines* represent primary sludge. *Triangles with connecting lines* represent recycle sludge. *Filled triangles with connecting lines* represent deionized water. *Data point* represents the average of duplicate runs

explain the observed pH drop. Kraemer et al. found that CO_2 was indeed supersaturated during fermentative hydrogen production regardless of N_2 sparging [17]. To what extent carbonic acid contributes to the pH drop of the SSF is unknown at this time. The organic acid and carbonic acid may interact to give additive effects to account for the observed pH drop. Whatever the reason may be, we emphasize that a normal operation of SSF of untreated paper mill sludge proceeds well without pH control to give satisfactory performance as a process converting the feedstock to ethanol.

Simultaneous Saccharification and Cofermentation

In order to utilize the xylan content in the sludge, another process scheme was investigated, namely, simultaneous saccharification and cofermentation. This process uses recombinant *E. coli* ATCC® 55124 (KO11) in place of the yeast in SSF. This organism is known to convert xylose as well as glucose to ethanol with high efficiency [18]. The overall performance data and the time-course data of SSCF are presented in Table 3 and Fig. 3. The SSCF of sludges was also run at 37 °C without pH control. Two different substrate loadings were applied: 3% and 6% glucan (*w*/*v*). The ethanol yields obtained from SSCF are similar to those of SSF ranging from 75% to 80%. These yields were calculated on the basis of total carbohydrates (glucan and xylan) for obvious reason. The yields of SSCF were also calculated on the basis of glucan only for direct comparison with the SSF. The 3% glucan-based yield of ethanol was 98.3% for primary sludge and 98.9% for recycle sludge. Glucan-based yield (6%) of ethanol was 95.8% for primary sludge and 96.3% for recycle sludge, corresponding to ethanol concentration of 32.5 and 32.7 g/L, respectively. In theory, this yield can surpass 100%. On the average, SSCF can produce 25% more ethanol from the same feedstock primarily because of the ability of KO11 to convert xylose to ethanol. The yields were slightly higher with 3% glucan loading than with 6% glucan loading. Elshafei et al. reported that substrate loading between 0.5–5% total solids in SSF was not affected by substrate or ethanol inhibition [19]. In this work, total solid loading was much higher, ranging from 6% to14% (*w*/*v*). Here again the presence of high ash content in the sludges was not a problem in operation of the SSCF.

The time-course profiles of various observable parameters are shown in Fig. 3. There were discernable differences in sugar and pH profiles from those of SSF. The pH again dropped sharply in the early phase of the SSCF and remained relatively constant afterwards. The SSCF was started at pH7.0, decreased to pH of 5.93 for primary sludge and 5.89 for recycle sludge, which is close to the optimum for KO11 (pH of 6.0) but slightly higher than those of SSF. The same argument made for the pH drop of SSF also applies here. During the SSCF, acetic acid and lactic acid were detected at the level of 3.0 and 1.0 g/L, respectively. The acid-soluble ash can dissolve under weak acidic condition and acts as a buffer. The ethanol conversion rate of hardwood pulp was faster than the sludges, but the terminal ethanol yields were essentially the same. The xylose concentration of both sludges decreased after 12 h. This is a positive proof that the xylan fraction is utilized in the SSCF by KO11.

The profiles of glucose in Figs. 1 and 3 showed that both SSF and SSCF proceed under a glucose-limited condition, which means the enzymatic reaction is the rate-limiting step. In the SSCF, xylose consumption occurred shortly after depletion of glucose. This is a classic example of diauxic consumption of substrates wherein glucose is preferred to xylose. The SSCF applies only to a feedstock that contains both glucan and xylan. It also requires cellulase enzyme that has built-in xylanase activity. Most of the commercial "cellulases" including Spezyme CP have xylanase activity. The reacted residues after SSF and SSCF were analyzed for glucan and xylan contents (Table 4). For both sludges, the SSF residues have higher unreacted glucan and xylan than SSCF residues. The fact that two different microorganism are used has no bearing in this observation because the microbial actions are

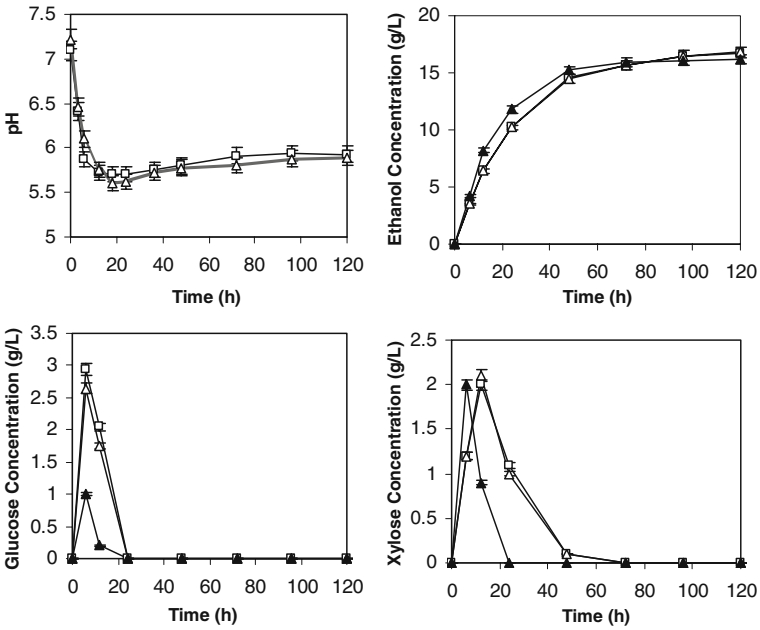

Fig. 3 Simultaneous saccharification and cofermentation (SSCF) of sludge by recombinant *E. coli* (KO11). *Squares with connecting lines* represent primary sludge. *Triangles with connecting lines* represent recycle sludge. *Filled triangles with connecting lines* represent hardwood pulp. The *data points* represent average of triplicate runs. The pH was controlled at 6.0 for runs with hardwood pulp. Other conditions of the SSCF were: 3% (*w*/*v*) glucan loading, 15 FPU Spezyme CP+30 CBU of Novozyme-188/g-glucan

Table 4 Composition Analysis of fermentation residues (based on initial dry solids).

Component (%)	Primary sludge	Primary sludge residue		Recycle sludge	Recycle sludge residue	
		SSF	SSCF		SSF	SSCF
Glucan	44.5	1.6	0.5	49.6	1.9	0.5
Xylan	9.9	2.4	0.8	13.7	4.1	0.9

Data of sludges is the mean value of triplicate (n=3; SD<0.5 for glucan and xylan). SSF and SSCF were based on 3% (w/v) glucan loading

not the rate-controlling step. Therefore, the main difference between the two processes is that SSF has a buildup of xylose. It is well known that xylose has an inhibitory effect on xylanase [20–22]. Low xylanase activity results in less xylan removal. Unreacted xylan in solid influences the glucan hydrolysis because xylan physically surrounds cellulose fibrils in the structure of lignocelluloses and becomes a barrier impeding glucanase action. It has indeed been proven that xylan removal improves the access of cellulase enzyme to cellulose and consequently the activity of cellulase [23]. It has also been reported that xylose has a direct inhibitory effect on cellulase [24–26].

Fed-Batch Operation

The concentration of ethanol in the bioreactor significantly affects the cost of the downstream separation process. The threshold level of ethanol concentration for economically feasible distillation is reported to be about 4 wt.% [27, 28]. We have attempted the fed-batch operation (intermittent input of feed) of SSF and SSCF in an effort to increase the terminal ethanol concentration in the bioreactor. The bioreaction was started with a sludge loading of 60 g-glucan/L. Additional feedstock of the same level was put into the reactor at 24 h point (one additional input) for SSCF and at 24 and 48 h points for SSF (two additional inputs). At these input points, the reaction has proceeded far enough to liquefy the dense solid slurry and retain fluidity high enough to accept additional solid feed. The ethanol production from the fed-batch runs are shown in Fig. 4. In the case of SSCF with one additional feeding, ethanol concentration of 42 g/L was obtained at the end of the run. The yields were identical for both sludges. This is equivalent to ethanol yield of 68% on the basis of total sugar, which is substantially lower than the yield of single batch with 6% glucan loading. *E. coli* KO11 is known to utilize several pentoses and hexoses for producing ethanol with high metabolic yield [18]. However, its growth robustness and ethanol tolerance are relatively low compared to yeast [29]. We believe it is the main reason for the low ethanol yield observed from the fed-batch SSCF. Additional buildup of ash from the sludge may also have played a role in lowering the ethanol yield. Xylose utilization in the fed-batch SSCF was less efficient compare to single-feed runs, as evidenced by relatively high xylose concentration in the reactor which remained at 2 g/L even after 72 h. On the other hand, glucose had a short initial buildup and then remained below 0.1 g/L after 24 h. In the fed-batch SSF runs with two additional feedings, the final ethanol concentration of 45 g/L was achieved. The overall ethanol yield in the fed-batch SSF was approximately 70% based on glucan, which was also lower than the yield of single batch with only 6% glucan loading. In fed-batch SSF using yeast (ATCC-200062), ethanol concentration higher than SSCF was obtained, yet the overall yield was lower. Viability of the cells at the end of fed-batch SSF was tested by measurement of cell count on agar plate. The measured cell

concentration of 5×10^7 CFU/mL indicated that the cells remained highly viable at the end of the operation. It appears that the cellulase enzyme somehow becomes less effective under high solid loading. The interference of high ash with the enzyme, unproductive binding with increased lignin content after successive feeding, product inhibition by ethanol and/or acids, and substrate inhibition by unreacted xylose are the potential reasons for the low yield of the fed-batch SSF. Mass transfer resistance at the solid–liquid interface and within the solids may further increase the local concentration of some of the inhibitory components. The exact reasons and the mechanisms associated with these phenomena are yet to be determined.

Conclusion

Kraft paper mill sludges have features desirable for bioconversion to value-added products. The organic component of the sludges is essentially the same as commercial grade pulp, high in carbohydrates and low in lignin. Its small size and high surface area make it amenable for biological conversion without pretreatment. However, enzymatic hydrolysis of the sludges is inefficient because of the high ash content. The buffering action of the calcium carbonate in the ash keeps the pH near neutral and two units higher than the optimum pH of cellulase.

SSF based on Spezyme CP and *S. cerevisiae* (D_5A), SSCF based on the same enzyme and the *E. coli*-KO11 performed well despite the high ash content. The main reason for the improved performance is the pH drop to 5.5 and 5.8 that occurred in the SSF and SSCF, respectively. The pH drop was caused by carbonic acid and organic acids producing during fermentation. The glucan-based ethanol yield for the primary sludge and recycle sludge in the SSF were 79.5% and 80.0%, respectively. Xylose was effectively utilized in the SSCF. Ethanol yields based on glucan and xylan was 80.1% and 77.1%, respectively, for the primary sludge and recycle sludge. Fed-batch SSF and SSCF were attempted to increase the ethanol concentration. Ethanol concentrations in the broth was increased to 45 and 42 g/L for three-feed SSF and two-feed SSCF, respectively. The yields of the fed-batch runs were lower at 68% for SSF and 70% for SSCF. Further increase of ethanol concentration in the

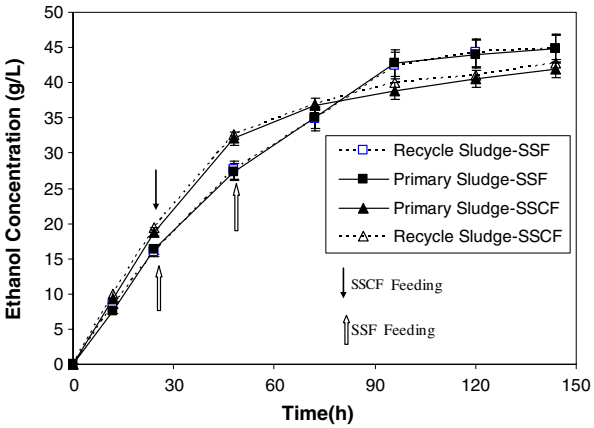

Fig. 4 Fed-batch SSF and SSCF of sludges. The *data points* represent average of triplicate runs. The enzyme loading in the SSF/SSCF were: 15 FPU Spezyme CP+30 CBU of Novozyme-188/g-glucan

SSCF was difficult because of the low ethanol tolerance of *E. coli* KO11. Yields are lower for multiple-feed fed-batch runs than the single-feed runs for both SSF and SSCF. The low conversion yield is related to the cellulase enzyme reaction. High-solid conditions in the bioreactor and the inhibition due to product and substrate are believed to be the major factors suppressing the cellulase enzyme reaction.

Acknowledgment The authors wish to acknowledge the financial support provided by Masada, LLC, Alabama Center for Renewable Resources Engineering, and Laboratory of Biofuels and Biomaterials, Auburn University, AL, USA. They are grateful to Boise Paper, Jackson, AL, USA, for providing paper mill sludges and technical information and to Danisco Genencor, Palo Alto, CA, USA for providing cellulase enzymes.

References

1. Fan, Z., & Lynd, L. R. (2006). *Bioprocess and Biosystems Engineering, 30*(1), 35–45.
2. Scott, G. M., Abubakr, S., & Smith, A. (1995). *Proceedings of the 1995 international environmental conference May 7–10* (pp. 269–279). Atlanta, GA: Tappi Press.
3. Sjöde, A., Alriksson, B., Jönsson, L. J., & Nilvebrant, N. (2007). *Applied Biochemistry and Biotechnology, 136–140*, 327–337.
4. Lark, N., Xia, Y., Qin, C.-G., Gong, C. S., & Tsao, G. T. (1997). *Biomass Bioenergy, 12*, 135.
5. Galbe, M., & Zacchi, G. (2002). *Applied Microbiology and Biotechnology, 59*, 618–628.
6. Sjöström, E. (1993). *Wood chemistry: fundamentals and applications* (2nd ed.). San Diego: Academic Press.
7. Fan, Z., & Lynd, L. R. (2006). *Bioprocess and Biosystems Engineering, 30*(1), 27–34.
8. Jeffries, T. W., & Schartman, R. (1999). *Applied Biochemistry and Biotechnology, 78*(1–3), 435–444.
9. Fan, Z., South, C., Lyford, K., Munsie, J., Walsum, P., & Lynd, L. R. (2003). *Bioprocess and Biosystems Engineering, 26*(2), 93–101.
10. Marques, S., Alves, L., Roseiro, J. C., & Gírio, F. M. (2008). *Biomass Bioenergy, 32*, 400–406.
11. Lynd, L. R., Lyford, K., South, C. R., van Walsum, P., & Levenson, K. (2001) *TAPPI* **84**, 50; full text at http://www.tappi.org.
12. NREL (2008) Laboratory Analytical Procedure (LAP), National Renewable Energy Laboratory,Golden, CO. Available from: http://www.nrel.gov/biomass/analytical_procedures.html.
13. Acid-Insoluble Ash in Wood, Pulp, Paper, and Paperboard, Test Method T 244 cm-99.
14. Biermann, C. J. (1996). *Handbook of pulping and papermaking* (2nd ed.). San Diego: Academic Press.
15. Saddler, J. N., & Gregg, D. J. (1998). In Bruce, A., & Palfreyman, J. W. (Eds.), *Forest products biotechnology.* Taylor & Francis, London, pp. 183–207.
16. Varga, E., Szengyel, Z., & Reczey, K. (2002). *Applied Biochemistry and Biotechnology, 98/100*, 73–87.
17. Kraemer, J. T., & Bagley, D. M. (2006). *Biotechnological Letters, 28*, 1485–1491.
18. Ohta, K., Beall, D. S., Mejia, J. P., Shanmugam, K. T., & Ingram, L. O. (1991). *Applied Environmental Microbiology, 57*(4), 893–900.
19. Elshafei, A. M., Vega, L. L., Kalsson, K. T., Clausen, E. C., & Gaddy, J. L. (1991). *Bioresource Technology, 35*, 73–80.
20. Paul, J., & Varma, A. K. (1990). *Biotechnological Letters, 12*(1), 61–64.
21. Copa-Patiño, J. L., Kim, Y. G., & Broda, P. (1993). *Applied Microbiology and Biotechnology, 40*(1), 69–76.
22. Rapp, P., & Wagner, F. (1986). *Applied and Environmental Microbiology, 51*(4), 746–752.
23. Öhgren, K., Bura, R., Saddler, J. N., & Zacchi, G. (2007). *Bioresource Technology, 98*, 2503–2510.
24. Xiao, Z., Zhang, X., Gregg, D. J., & Saddler, J. N. (2004). *Applied Biochemistry and Biotechnology, 113/116*, 1115–1126.
25. Nigam, P., & Prabhu, K. A. (1991). *Journal of Basic Microbiology, 31*(4), 279–283.
26. Kim, T. H., & Lee, Y. Y. (2005). *Applied Biochemistry and Biotechnology, 121/124*, 1119–1131.
27. Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M. F., Lidén, G., & Zacchi, G. (2006). *Trends Biotechnology, 24*(12), 549–556.
28. Wingren, A., Galbe, M., & Zacchi, G. (2003). *Biotechnology Progress, 19*, 1109–1117.
29. Dien, B. S., Cotta, M. A., & Jeffries, T. W. (2003). Bacteria engineered for fuel ethanol production: Current status. *Applied Microbiology and Biotechnology, 63*(3), 258–266.