ENHANCED ETHANOL PRODUCTION FROM DE-ASHED PAPER SLUDGE BY SIMULTANEOUS SACCHARIFICATION AND FERMENTATION AND SIMULTANEOUS SACCHARIFICATION AND CO-FERMENTATION

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A previous study demonstrated that paper sludges with high ash contents can be converted to ethanol by simultaneous saccharification and fermentation (SSF) or simultaneous saccharification and co-fermentation (SSCF). High ash content in the sludge, however, limited solid loading in the bioreactor, causing low product concentration. To overcome this problem, sludges were de-ashed before SSF and SSCF. Low ash content in sludges also increased the ethanol yield to the extent that the enzyme dosage required to achieve 70% yield in the fermentation process was reduced by 30%. High solid loading in SSF and SSCF decreased the ethanol yield. High agitation and de-ashing of the sludges were able to restore part of the yield loss caused by high solid loading. Substitution of the laboratory fermentation medium (peptone and yeast extract) with corn steep liquor did not bring about any adverse effects in the fermentation. Fed-batch operation of the SSCF and SSF using low-ash content sludges was effective in raising the ethanol concentration, achieving 47.8 g/L and 60.0 g/L, respectively.

Keywords: Paper sludges; Ethanol; High solid loading; Simultaneous Saccharification and Fermentation; Simultaneous Saccharification and Co-Fermentation

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INTRODUCTION

Feedstock and the enzymes have been identified as two primary cost items in biomass conversion processes (Nguyen and Saddler 1991; Aden and Foust 2009). Our previous study demonstrated that SSF based on Spezyme CP and *Saccharomyces cerevisiae* (NREL-D5A), and SSCF based on the same enzyme and the recombinant *Escherichia coli* KO11 performed reasonably well in the bioconversion of untreated kraft paper sludges to ethanol in spite of the high ash content (Kang et al. 2010). SSCF performed with 13.5% solid loading of primary paper mill sludge resulted in an ethanol concentration of 3.25 wt.% and SSF with the same feed resulted in an ethanol concentration of 2.65 wt.%. Higher ethanol concentration is achieved by high solid loading; however, operation of high solid with high ash content creates extremely high viscosity, causing difficulty in mixing and pumping. High viscosity and non-Newtonian behavior of the broth require strong agitation in order to provide adequate mixing during fermentation. The ash content in the kraft paper mill sludge comes primarily from paper

filler materials such as clay (kaolin) and $CaCO_3$, etc. Nikolov et al. (2000) reported that fillers and other additives in the paper form an adhesive "envelope" around the cellulose fibers to obstruct the access of the enzymes to the cellulose substrate. In the previous studies, the enzymatic hydrolysis tests of sludge were carried out without pH control because of the high ash content, which neutralizes acid input. The pH of sludge digestion remained near neutral. For hardwood pulp, enzymatic hydrolysis was carried out under three different conditions: pH 4.8 (optimum for cellulase), pH 7.0, and with addition of $CaCO_3$. The enzymatic digestion of sludges was dismal, giving sugar yields of only 8 to 32% of theoretical maximum. The enzymatic digestibility of hardwood pulp which was tested at pH of 7.0 with buffer, although much lower than that of pH of 4.8, was significantly higher than that tested with addition of $CaCO_3$ (Kang et al. 2010). These data collectively indicate that the inefficient digestion of sludges is mainly due to the presence of ash in the sludges. Clay minerals are also known to form aggregates with organic molecules, and the formation of enzyme-clay complexes could alter the level of enzyme activity (Cabezas et al. 1991; Haska 1981; Tietjen and Wetzel 2003). Additional evidence also revealed that clay had an inhibitory effect on cellulase activity (Hamzehi and Pflug 1981; Pflug 1982; Tothill et al. 1993). The high ash content in paper mill sludges also limits the cellulose loading capacity in the bioreactor, since it lowers the cellulosic portion of the feedstock.

Much of these problems can be alleviated by de-ashing the sludges. For bioconversion purposes, the de-ashing operation needs to be optimized in order to recover as much fiber as possible while keeping the ash content low enough to be accepted as a fermentation feedstock. Various chemical treatments have been used to remove ash, and ethanol yields from 30.0 to 100.0% have been obtained (Lynd et al. 2001; Marques et al. 2008); however, they are not deemed appropriate for application in bioconversion feedstock because of high processing costs and safety issues related to the chemical treatments. Moreover, the effects of ash on the pH during the course of fermentation were not addressed.

The fermentation nutrient is a significant factor in the bioconversion of lignocellulosic biomass to ethanol. Expensive complex nutrients, such as yeast extract and peptone, are utilized in most laboratory studies, the cost of which is prohibitively high for commercial production. Corn steep liquor (CSL) is a byproduct of corn wet-milling and has been used as a fermentation nutrient in several different commercial fermentation processes (Kadam and Newman 1997; Lawford and Rousseau 1997; Amartey and Jeffries 1994; Tang et al. 2006).

This investigation was undertaken to improve the process of converting the kraft paper mill sludges to ethanol from the level that was previously developed in the laboratory. The focus of the work was on three main issues. The first was to develop a method to de-ash kraft paper mill primary sludge. The second was to ascertain the effects of de-ashing on the efficiency of the bioconversion to ethanol. The third was to improve the process economics deploying low-cost fermentation nutrients. As tools of bioconversion tests, SSCF and SSF were operated under batch and fed-batch modes. Special attention was paid on the product yield, concentration, and enzyme dosage in order to assess the overall process performance.

EXPERIMENTAL

Materials

Feedstocks

The primary paper sludge (PS) was collected from the primary wastewater clarifier unit of a Boise Paper Company kraft paper mill (Jackson, AL, USA). The consistency of the sludge slurry before the clarifier was between 0.5 and 3.0%. The consistency of sludge sample after the clarifier was around 20%. The PS was washed with tap water three times and further thickened to a 30% consistency using a vacuum filter, and 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 the 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 (NREL 2008). Analysis determined that the hardwood pulp contained 76.0% glucan, 21.1% xylan, and 2.9 wt.% unaccounted for components (lignin, acetyl group, ash, and protein). The PS contained 44.5% glucan, 9.9% xylan, 8.1% lignin, and 36.0 wt.% ash, which included 26.0% acid-soluble ash and 10.0% acid-insoluble ash. De-ashing was accomplished by floatation and screening. The screening procedure was that of Dorica and Simandl (1995) with a slight modification that included using CO_2 instead of air. One liter of resuspended paper sludge with a 3% consistency was placed in a 2 L beaker and mixed at 300 RPM using a laboratory stirrer (LR400A, Fisher Scientific) for 30 minutes with carbon dioxide bubbles flowing through two glass tubings, and put through 100 mesh screen. This procedure was repeated three times and thickened to about 45% consistency using a vacuum filter. The resulting solid then became referred to as the S-PS hereafter and was subjected to bioconversion tests. The PS after the two consecutive screenings was referred to as S-PS-1 and the PS after three consecutive screenings referred to as S-PS-2 (Fig. 1). S-PS-1 contained 64.8% glucan, 13.5% xylan, 5.6% lignin, and 14.0 wt. % ash, which included 8.0% acid-soluble ash and 6.0% acid-insoluble ash. S-PS-2 contained 71.2% glucan, 14.8% xylan, 6.2% lignin, and 6.1 wt. % ash, which included 4.0% acid-soluble ash and 2.1% acid-insoluble ash.

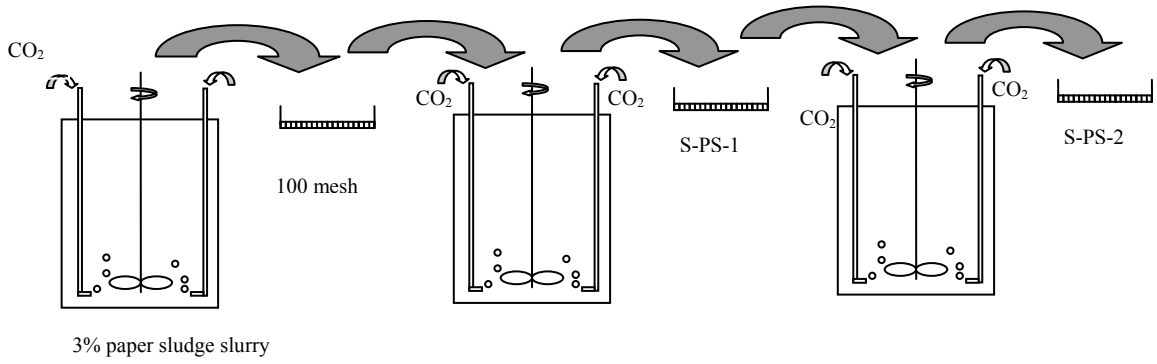

Fig. 1. De-ashing process of the primary sludge

Enzymes

Cellulase enzyme (Spezyme CP, Lot No. 301-00348-257) was a kind gift from Genencor-Danisco (Palo Alto, CA, USA). The activity of the Spezyme CP was 59 FPU/mL, as determined by the NREL standard procedure by Genencor-Danisco (NREL, 2008). Beta-glucosidase (Novozyme188, Cat. No. C-6150) was purchased from Sigma (St. Louis, MO, USA). Its activity was measured to be 750 CBU/mL by Novozymes, NC.

Microorganism

The microorganism used in the SSF was *S. cerevisiae* ATCC-200062 (NREL-D5A). This organism was grown on YPD agar plates containing a solid YPD medium, which contained 2.0% peptone (Sigma, P-6588), 1.0% yeast extract (Sigma, Y-0500), 1.5% agar (Sigma, A-1296), and 2.0% (w/v) glucose (Sigma, G-8270). The recombinant *E. coli* ATCC-55124 (KO11) was used for the SSCF. This organism was grown on LB agar plates containing a solid LB medium (Sigma, L-3152), which contained 1% tryptone, 0.5% yeast extract, and 1% NaCl, supplemented with 2% xylose (Sigma, X1500), 1.5% agar (Sigma, A-1296), and 40 or 600 mg/L of chloroamphenicol (Fluka, 23275) (Yomano et al. 1998).

Methods

Simultaneous Saccharification and Fermentation (SSF)/Co-fermentation (SSCF)

A 250 mL Erlenmeyer flask was used as a bioreactor with 100 mL of total working volume. It was operated in an incubator shaker (New Brunswick Scientific, Innova-4080) at 37 °C. Sludges and growth medium were added such that the glucan content became 3%, 6%, or 9% (w/v).

Hardwood pulp (HP-I) and hardwood pulp with 20% $CaCO_3$ based on dry basis of hardwood pulp (HP-II) were used as a control and put through the same bioconversion procedures. Analysis revealed that the ash content of HP-II was 16.7 wt. %, which was close to the ash content of S-PS-1.

The sludge samples were steam sterilized at 121 °C for 15 min. The growth media for SSF was a YP medium. The growth media for SSCF was a LB medium (Sigma, L-3152). For SSCF, 40 mg/L of chloroamphenicol was added after autoclaving to prevent bacterial contamination (Yomano et al. 1998).

The SSF and SSCF of hardwood pulp were carried out without further control of pH during fermentation. Three levels of cellulase were applied in the paper sludge: 5, 10, and 15 FPU /g-glucan. The cellulase enzyme was supplemented with β-glucosidase at a ratio of 2 CBU/FPU. The optical density (OD) was measured by a UV Spectrophotometer (BioTek Synergy HT Multidetection Microplate Readers) at 600 nm for yeast (NREL-D_5A) 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 yeast (NREL-D_5A)/L. In all of the microbial experiments, a sample from each flask was taken at the end of the run and streaked on an YPD plate to check for contamination. The presence of contaminants was also checked under an optical microscope. CFU tests were performed to check microorganism viability. The SSF and SSCF experiments requiring pH monitoring were carried out using a 1-L bioreactor (Multifors IHORS HT 2×1L) with 400 mL of working volume.

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.

The fed-batch experiments were started with 100 mL of initial working volume, and squeezed sludge cakes were added to the bioreactor at the desired time to achieve a total glucan content of 6% (w/v). Addition of sludge was done after 24 hours for SSCF and after 12, 24, and 48 hours for SSF. The cellulase and β-glucosidase were also added to maintain the overall enzyme loading at 10 FPU and 20 CBU/g-glucan. Samples were analyzed for glucose, xylose, organic acid, and ethanol by HPLC. Aseptic conditions were maintained in all of the microbial experiments. The fed-batch fermentation runs were made in triplicates.

The fermentation runs for the low-cost medium study were operated in an incubator shaker (New Brunswick Scientific, Innova-4080) at 37 °C with 150 RPM. The cellulase and β-glucosidase were also added to maintain the enzyme loading at 10 FPU and 20 CBU/g-glucan. Two growth media were used for the SSF test of screen de-ashed paper sludges: (I) "YP medium" containing 10 g/L of yeast extract (Difco, Detroit, MI) and 20 g/L of peptone (Difco, Detroit, MI), and (II) "low-cost medium" containing 0.45% by volume of CSL (Sigma C4648, 50% solid) and 5 mM $MgSO_4$ as described by Newman et al. (Kadam and Newman 1997).

For the SSCF test of screening de-ashed paper sludges, seven growth media were used: (A) a "LB medium" containing 5 g/L of yeast extract and 10 g/L of peptone; (B) 1.0% CSL by volume (Sigma C4648, 50% solid) without mineral supplement; (C) 1.0% CSL by volume with mineral supplements as described by Martinez et al. (1999); (D) 3.0% CSL by volume without mineral supplements; (E) 3.0% CSL by volume with mineral supplements; (F) 5.0 % CSL by volume without mineral supplements; and (G) 5.0 % CSL by volume with mineral supplements. Mineral supplements per liter for CSL + M medium were: 1 g of KH_2PO_4, 0.5 g of K_2HPO_4, 3.1 g of $(NH_4)_2SO_4$, 0.4 g of $MgCl_2 \bullet 6H_2O$, and 20 mg of $FeCl_3 \bullet 6H_2O$.

Colony Forming Unit Test

The colony forming unit (CFU) tests were performed to determine the viable cell population. The YPD or LB agar plate was 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/plate.

Analytical Methods

The solid samples were analyzed for carbohydrates and Klason lignin following the NREL CAT standard procedures (NREL 2008). The moisture content was measured by an infrared moisture balance (Denver Instrument, IR-30). Sugars were determined by HPLC using an HPX-87P column. For the SSF or SSCF tests, a BioRad-HPX-87H column was used for the measurement of sugar, organic acid, and ethanol. A refractive index detector was used with the HPLC. The acid-insoluble ash was determined

following the TAPPI test method (T 244 cm-99). Liquid sample analysis and ash determination were done in triplicates. Where applicable, statistical data including the mean value and standard deviation were computed using Microsoft Office Excel 2003.

RESULTS AND DISCUSSION

De-ashing of Sludges by Screen-Washing

The PS was further analyzed to determine ash contents of 26% acid soluble and 10% acid-insoluble based on oven-dry untreated sludge. In reference to the Boise Paper papermaking process and the data analysis results, it was concluded that the acid-soluble ash was primarily from $CaCO_3$ and the acid-insoluble ash was primarily from clay. Part of the insoluble ash content was physically associated with the fibers, in agreement with the findings of Middleton and Scallan (1991). The suspended fine particles not associated with fibers are presumed to pass through the screen with the filtrate. Table 1 shows the glucan/xylan content and losses in the screening process. With S-PS-1, the glucan/xylan loss was 11.2%/17.2% when the ash removal was 76.4%. As indicated by the data on screening of S-PS-2, ash removal increased the glucan/xylan loss; glucan/xylan loss corresponding to 90.6% ash removal was 25.6%/34.3%. The CO_2 bubbles enhanced the extent of separation of the ash from the fibers. The gas bubbles rose to the surface, carrying the particles of low density, such as fine fibers, to the surface of the liquid phase. Carbon dioxide was used in place of air since a large amount of it is produced during the fermentation process, and it is also a by-product of the manufacture of lime from calcium carbonate during the kraft chemical recovery process (Biermann 1996).

Table 1. Composition of De-ashed Sludges

(%)	PS	S-PS-1[d]	S-PS-1[e]	S-PS-2 [d]	S-PS-2 [e]
Sample Weight(g)	100.0	63.0	61.0	52.0	48.0
Glucan	44.5	38.4	39.5	32.4	33.1
Xylan	9.9	7.8	8.2	5.9	6.5
Ash	36.0	10.7	8.5	4.5	3.4
Glucan loss	-	13.7	11.2	27.2	25.6
Xylan loss	-	21.2	17.2	40.4	34.3
Ash removal	-	70.3	76.4	87.5	90.6

a. The data of glucan, xylan and ash in table are based on oven-dry untreated primary sludge.

b. The data of glucan loss, xylan loss and ash removal in table are based on the initial glucan, xylan and ash of oven-dry untreated primary sludge.

c. All data are the mean value of duplicate (n=2; standard deviation<1.0).

d. With air, no carbon dioxide.

e. With carbon dioxide.

The use of CO_2 has an additional benefit; it increases the dissolution of $CaCO_3$. The solubility of CO_2 in water is very high (88 mL CO_2/100 mL of water at 20 °C), and its aqueous solution is carbonic acid, a weak unstable acid. The water insoluble calcium carbonate in the presence of water and carbon dioxide dissolves as water-soluble calcium bicarbonate, which is a compound existing only in solution.

The positive effect of de-ashing is its improvement in enzyme digestibility, fermentation efficiency, and titer of ethanol. Although there is a loss of carbohydrates, a net gain in the overall process economics is expected from the de-ashing process.

SSF and SSCF of Screen De-ashed Sludges

Data on S-PS-1,S-PS-2 and PS in Table 2 clearly indicate that ash removal increased the ethanol fermentation yield. The ethanol fermentation yields of the S-PS-2 with the lowest ash content at very low enzyme loading of 5 FPU cellulase /mL were higher than those of the original sludge at medium enzyme loading of 10 FPU cellulase/mL. The ethanol fermentation yields of the S-PS-2 at 10 FPU cellulase /mL were close to those of the original sludge at 15 FPU cellulase/mL.

Table 2. Ethanol Yield from SSF and SSCF of Treated Paper Sludges

Enzyme loading	15 FPU of Cellulase + 30 CBU of β-glucosidase /g-glucan			10 FPU of Cellulase + 20 CBU of β-glucosidase/g-glucan			5 FPU of Cellulase + 10 CBU of β-glucosidase/g-glucan		
	P.S.	S-PS-1	S-PS-2	P.S.	S-PS-1	S-PS-2	P.S.	S-PS-1	S-PS-2
120h SSF Ethanol Yield(%) [a]	74.5	77.6	81.2	67.1	72.8	75.7	60.5	64.5	70.2
120h SSF Ethanol Conc.(g/L)	25.3	26.4	27.2	22.8	24.7	25.7	20.5	21.9	23.8
120h SSCF Ethanol Yield(%) [b]	78.0	80.4	82.8	68.2	73.6	76.0	59.6	65.1	71.8
120h SSCF Ethanol Yield(%) [c]	95.8	97.5	100.4	83.7	89.3	92.2	73.2	78.9	87.1
120h SSCF Ethanol Conc.(g/L)	32.5	33.1	34.1	29.1	30.3	31.3	24.9	26.8	29.6

a. The ethanol yield of SSF based on glucan only.
b. The ethanol yield of SSCF based on glucan and xylan.
c. The ethanol yield of SSCF based on glucan only.
d. SSF and SSCF based on 6% (w/v) glucan loading/ 100 mL working volume.
e. Data of sludges are the mean value of duplicate (n=2; standard deviation<0.1).

The adverse effect of ash in the sludge is primarily on the enzymatic reaction part of the SSF/SSCF rather than the microbial reaction. A number of researchers found that various components in the ash interfere with the cellulase reaction (Kang et al., 2010; Nikolov et al., 2000; Tietjen and Wetzel 2003; Cabezas et al. 1991; Haska, 1981; Hamzehi and Pflug 1981; Pflug 1982; Tothill et al. 1993). Improvement of yield by de-ashing is therefore due to improvement in the enzymatic hydrolysis. It is quite obvious that de-ashing removed the chemicals (clay minerals and other additives in paper) that inhibit enzymatic hydrolysis.

The fact that SSF/SSCF proceeds under glucose-limited conditions proves that the hydrolysis is the rate-limiting step of the SSF/SSCF (Kang et al. 2010; Kim and Lee 2005). On the other hand, ash removal caused loss of fine fibers and therefore loss of the glucan/xylan. As shown in the case of S-PS-2, 90% removal of ash was accompanied by a loss of glucan/xylan as high as 25.6%/34.3% during the screen de-ashing. Considering the large glucan/xylan loss during the screening of S-PS-2, and that there was only a 6% difference in ethanol yield between the two enzyme loadings of 15 and 10 FPU cellulase /g-glucan, S-PS-1 was used in the subsequent bioconversion tests in which medium enzyme loading of 10 FPU cellulase/g-glucan was applied.

Ash removal also decreases the total bulk solid loading for a given glucan content in the feedstock. As an example, for 6% w/v glucan loading, the total solid loading of the original primary sludge, S-PS-1, and S-PS-2 were 13.5 wt.%, 9.3 wt.%, and 8.4 wt.%, respectively. Fermentation of high viscosity broth requires strong agitation to provide adequate mixing and mass transfer. Agitation intensity as it relates to solid loading thus became of interest. When sludge and enzyme loadings increased, the viscosity of the reaction mixture increased accordingly.

Fermentation results indicated that dense sludges (high solid loading) required longer time for liquefaction (breakdown of viscosity). The ethanol yields from fermentation were also lower at higher solid loadings (Table 3). With high solid loading (21.6% w/v for PS), the enzymatic hydrolysis was insufficient to liquefy the sludge, resulting in a very low ethanol yield (data not shown here). In recent work, results showed that the binding capacity of cellulase decreased when the substrate concentration increased (Wang et al. 2011). This has a negative effect on the productivity because the enzymatic hydrolysis is the rate-limiting step in the SSF.

There are a number of reports that suggest that agitation enhances the hydrolysis yield of cellulosic substrates. The literature information along these lines collectively indicates that agitation enhances the adsorption of cellulase to the substrate, therefore increasing the activities of the endoglucanases and the cellobiohydrolases (Cavaco-Paulo and Almeida 1994; Cavaco-Paulo et al. 1996; Sakata et al. 1985). Most of the data in Table 4, except for the ethanol yield from SSCF of PS at relatively high agitation (250 RPM), clearly show that agitation has a positive effect on ethanol production in the SSF and SSCF, which agree with the previous findings. Enhanced interaction between the enzyme and the solid substrates of sludge due to high mixing is a plausible reason for the improvement of ethanol production. In addition, agitation may also have improved the mass transfer of sugar into microorganisms.

Table 3. Ethanol Yield from SSF and SSCF of Paper Sludges at Different Solid Loadings

Sludge	PS			S-PS-1		
Solid loading (%w/v)	6.7	13.5	20.2	4.6	9.3	13.9
Glucan loading (%w/v)	3.0	6.0	9.0	3.0	6.0	9.0
120h SSF Ethanol Yield(%) a	68.8	66.1	-	74.3	72.8	71
120h SSF Ethanol Conc.(g/L)	23.4	22.5	-	25.2	24.7	24.1
120h SSCF Ethanol Yield(%) b	72.4	69.8	-	75.1	73.6	70.8
120h SSCF Ethanol Yield(%) c	87.8	85.7	-	91.1	89.3	85.9
120h SSCF Ethanol Conc.(g/L)	29.8	29.1	-	30.9	30.3	29.2
a. Simultaneous Saccharification and Fermentation (SSF) based on glucan only.						
b. Simultaneous Saccharification and co-Fermentation (SSCF) based on glucan and xylan.						
c. Simultaneous Saccharification and co-Fermentation (SSCF) based on glucan.						
d. Data of sludges are the mean value of duplicate (n=2; standard deviation<0.1).						
e. Enzyme loading, 10 FPU of Cellulase + 20 CBU of β-glucosidase /g-glucan.						

The ethanol yield from SSCF of PS at relatively high agitation (250 RPM) was lower than the medium agitation (150 RPM) (Table 4). It was probably due to the fact that *E.coli* is less viable under high agitation as measured by CFU tests. It is well known that growth robustness of *E.coli* is low compared to that of yeast (Dien et al. 2003). The yeast used in this work is very robust and can withstand high agitation under anaerobic conditions. It was reaffirmed by the CFU tests that showed no observable damage after the SSF. Mechanical damage of the *E.coli* by shear stress could result in a slower growth under high agitation.

Table 4. Ethanol Yield from SSF and SSCF of Sludges at Different Agitation Intensities

Sludge	PS			S-PS-1		
Agitation intensity (rpm)	250	150	100	250	150	100
120h SSF Ethanol Yield(%) a	67.7	66.1	58.4	74.4	72.8	69.0
120h SSF Ethanol Conc.(g/L)	23.0	22.5	19.8	25.3	24.7	23.4
120h SSCF Ethanol Yield(%) b	64.4	69.8	61.1	74.9	73.6	70.1
120h SSCF Ethanol Yield(%) c	79.1	85.7	75.0	90.9	89.3	85.0
120h SSCF Ethanol Conc.(g/L)	26.9	29.1	25.5	30.9	30.3	28.9
a. Simultaneous Saccharification and Fermentation (SSF) based on glucan only.						
b. Simultaneous Saccharification and co-Fermentation (SSCF) based on glucan and xylan.						
c. Simultaneous Saccharification and co-Fermentation (SSCF) based on glucan.						
d. Data of sludges are the mean value of duplicate (n=2; standard deviation<0.1).						
e. Enzyme loading, 10 FPU of Cellulase + 20 CBU of β-glucosidase /g-glucan.						
f. 13.5% (w/v) PS loading/100mL working volume.						
g. 9.3% (w/v) S-PS-1 loading/100mL working volume.						

Evaluation of Low-cost Fermentation Medium for Fermentation

The yeast extract and peptone are high quality nitrogen sources containing vitamin B complexes and amino acids, yet the cost is prohibitively high to be used as a nutrient for industrial fermentation. Replacement of the laboratory medium with practical nutrients is one of the major technical hurdles to be cleared for the development of industrial fermentation from laboratory work. The fermentation route producing ethanol from biomass is no exception. Substitution of the laboratory medium with an alternative inexpensive nitrogen source medium, however, has often resulted in slow rates and low yield in ethanol production. CSL, which is low in carbohydrates and rich in proteins, is considered as a suitable replacement for yeast extract in fermentation media (Thomsen 2006). It is currently used widely in industrial fermentation processes.

A number of SSF runs were made using screen de-ashed paper mill sludges without pH control. The ethanol yield of S-PS-1 from the SSF with lean medium (II) was 71.1% of theoretical maximum with 6% glucan loading at 10 FPU/g-glucan and 20 CBU/g-glucan. It is very close to the ethanol yield achieved with the rich medium (I) - 72.8% under the same conditions. This is in agreement with the previous findings that CSL is a good substitute for yeast extract and peptone (Kadam and Newman 1997; Amartey and Jeffries 1994; Tang et al. 2006).

In the previous study (Kang et al. 2010), the ash in the sludge, especially calcium carbonate, was partially neutralized by the acids and carbonic acid (CO_2 dissolved in water) produced from the SSCF and SSF and acted as a buffer to stabilize the pH during fermentation. After screening, S-PS-1 still contained 14.0 wt. % ash, which is primarily $CaCO_3$. The time-course results of the SSF run are shown in Fig. 2.

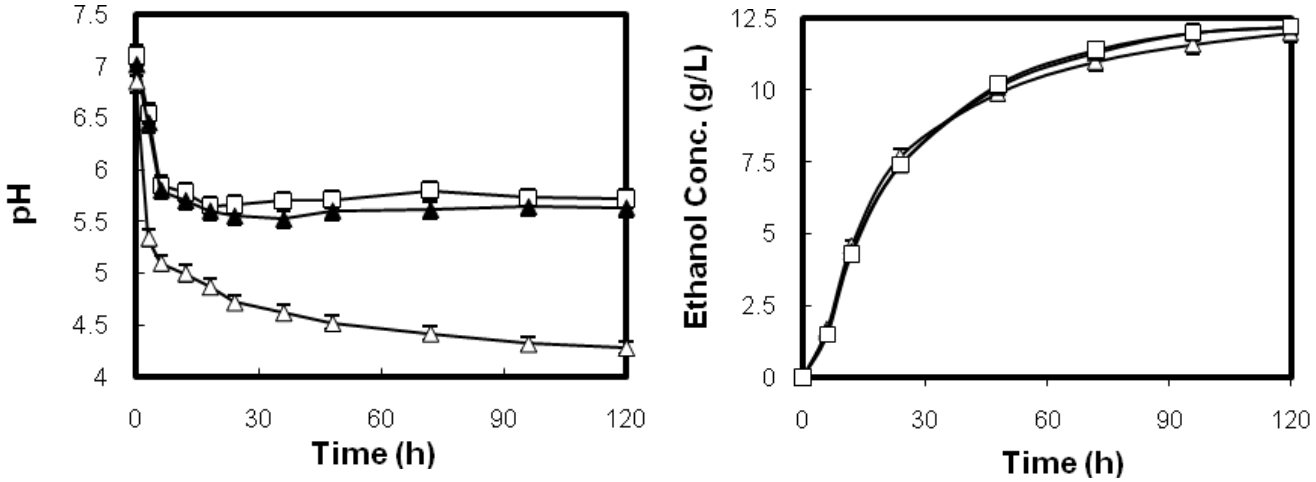

Fig. 2: Simultaneous Saccharification and Fermentation of de-ashed Sludges by *Saccharomyces cerevisiae* (ATCC-200062)
Squares represent de-ashed primary sludge (S-PS-1). Triangles represent hardwood pulp (HP-I). Filled triangles represent hardwood pulp with 20% $CaCO_3$ on dry basis of hardwood pulp (HP-II). The data points represent the average of triplicate runs. The pH was not controlled. Other conditions of the SSF were: 3% (w/v) glucan loading, 37°C, 10 FPU Spezyme CP + 20 CBU of Novozyme-188/g-glucan.

During the fermentation of S-PS-1, HP-I, and HP-II, the pH quickly dropped and reached final values (at 120 h) of 5.7, 4.3, and 5.6, respectively. The decrease in pH occurred due to carbonic acid (CO_2 dissolved in water) and other organic acids that formed during fermentation. The maximum acetic acid (AA) level was 2.0 g/L, and lactic acid (LA) was 1.8 g/L. This pH drop by CO_2 and organic acid is counteracted by the buffering action of ash in the S-PS-1, calcium carbonate in the HP-II, and CSL in the lean medium to reach the respective final pH values in the bioreactor. Since CSL contains proteins, peptides, and amino acids, the addition of CSL may strengthen the buffering capacity of the medium (Stanburg and Whitaker 1984).

In general, the activity of *S.cerevisiae* is stable under neutral or slightly acidic and anaerobic conditions. Under the anaerobic conditions, the intracellular pH of *S.cerevisiae* is usually maintained between 5.5 and 5.75 when the external pH is 3.0 (Imai and Ohno 1995a) or between 5.9 and 6.75 when the external pH is between 6.0 and 10.0 (Imai and Ohno 1995b). The various enzymes involved in yeast metabolism of sugars are located within the yeast cell. The enzyme activity is unaffected if the internal pH of the yeast cell is stable. The ethanol yields of S-PS-1, HP-I, and HP-II with 3% glucan loading were essentially the same: 73.7%, 73.7%, and 72.4%, respectively. This yield level was also very close to that from the fermentation of hardwood pulp under a pH of 4.8 (73.0%). The previous study (Kang et al. 2010) showed that although the terminal ethanol yields were the same, hardwood pulp was converted faster than the untreated sludges. After screen de-ashing, S-PS-1 attained an almost identical ethanol production rate as that of HP.

In order to utilize the xylan content in the sludge, the SSCF was also performed. In this process, recombinant *E.coli* KO11 was used in place of the yeast. This organism is known to convert xylose as well as glucose to ethanol with high efficiency (Ohta et al. 1991). This strain has performed well with high metabolic yields in rich media; however, the yields of both strains declined in a minimal media (Martinez et al. 1999; York and Ingram 1996). The poor performance in a minimal media may be attributed to NADH-mediated inhibition of citrate synthase, limiting the availability of glutamate, a protective osmolyte (Underwood et al. 2002a and 2002b). In this work, different media were tested for their performance in ethanol fermentation by *E.coli* KO11 from the screen de-ashed paper sludges. The ethanol yield data are shown in Table 5. The yields were calculated on the basis of total carbohydrates (glucan and xylan). The yields of SSCF were also calculated on the basis of glucan only for direct comparison with the SSF. On average, the SSCF produced 15% more ethanol than the SSF from the same feedstock because KO11 can convert both glucose and xylose to ethanol.

This study's initial approach was to use CSL as a source of protein and vitamin, and NH_4Cl as an inorganic nitrogen source with other minerals (Table 5). For 1% CSL supplementation, addition of minerals increased the ethanol yield from 53.1% to 57.4%. At CSL levels above 1%, supplementation minerals showed no significant improvement in ethanol production. With 1% CSL and a very low amount of yeast extract (0.05%) (Medium H), the ethanol yield reached 73.5%, which was almost identical to that of the rich medium.

Table 5. Ethanol Yield from SSCF of De-ashed Paper Sludges on Different Mediums

Medium	Yeast Extract (%w/v)	Peptone (%w/v)	CSL (%v/v)	Mineral Supplement	SSCF Ethanol Yield(%) [a]	SSCF Ethanol Yield(%) [b]	SSCF Ethanol Conc. (g/L)
A	0.5	1	0	0	73.6	89.3	30.3
B	0	0	1	0	53.1	64.4	21.9
C	0	0	1	Yes	57.4	69.6	23.6
D	0	0	2	0	64.4	78.1	26.5
E	0	0	2	Yes	65.6	79.6	27.0
F	0	0	3	0	72.1	87.5	29.7
G	0	0	3	Yes	71.2	86.4	29.3
H	0.05	0	1	0	73.5	89.2	30.3

a. The ethanol yield of SSCF based on glucan and xylan.
b. The ethanol yield of SSCF based on glucan only.
c. SSCF based on 6% (w/v) glucan loading.
d. All Data are the mean value of duplicate (n=2; standard deviation<1.0).
e. The Enzyme loading: 10 FPU of Cellulase + 20 CBU of β-glucosidase /g-glucan.

Although the terminal ethanol yields were almost identical, S-PS-1 in the rich medium was converted faster than that in medium H. The 3% CSL alone (medium G) appears to provide sufficient nutrients to achieve an ethanol yield of 71.2 %, which is comparable to that from rich medium A (73.6%). There was no apparent improvement in ethanol yields with CSL levels above 3%. These results support the findings of previous studies that the recombinant strain of *E. coli* KO11 does not produce ethanol efficiently without the addition of large amounts of complex nutrients (Asghari et al. 1996; York and Ingram 1996).

In this study, CSL was tested as a nutritional supplement for *E.Coli*-KO11. The 3% CSL was shown to meet the nutrient requirement, enough to attain cell growth to the level obtainable from rich medium A. The economic feasibility of using medium G and H in this process is yet to be investigated.

There are some technical issues concerning the use of CSL as a fermentation medium: 1) need for sterilization to remove bacterial contaminants, 2) difficulty of product separation caused by impurities in the broth that are originated from CSL; 3) increase of waste treatment burden due to high BOD in the spent fermentation liquor, and 4) inconsistent composition of CSL that varies with the source (Lee 2005; Lawford and Rousseau 1997). Handling of CSL is not easy because it is highly viscous and tends to gelate turning into a sticky liquid. In view of the aforementioned problems associated with CSL, the subsequent tests were performed using medium H containing a low level of CSL (1%) and a low amount of yeast extract (0.05%). The time-course profiles of various observable parameters of SSCF based on medium H are shown in Fig. 3.

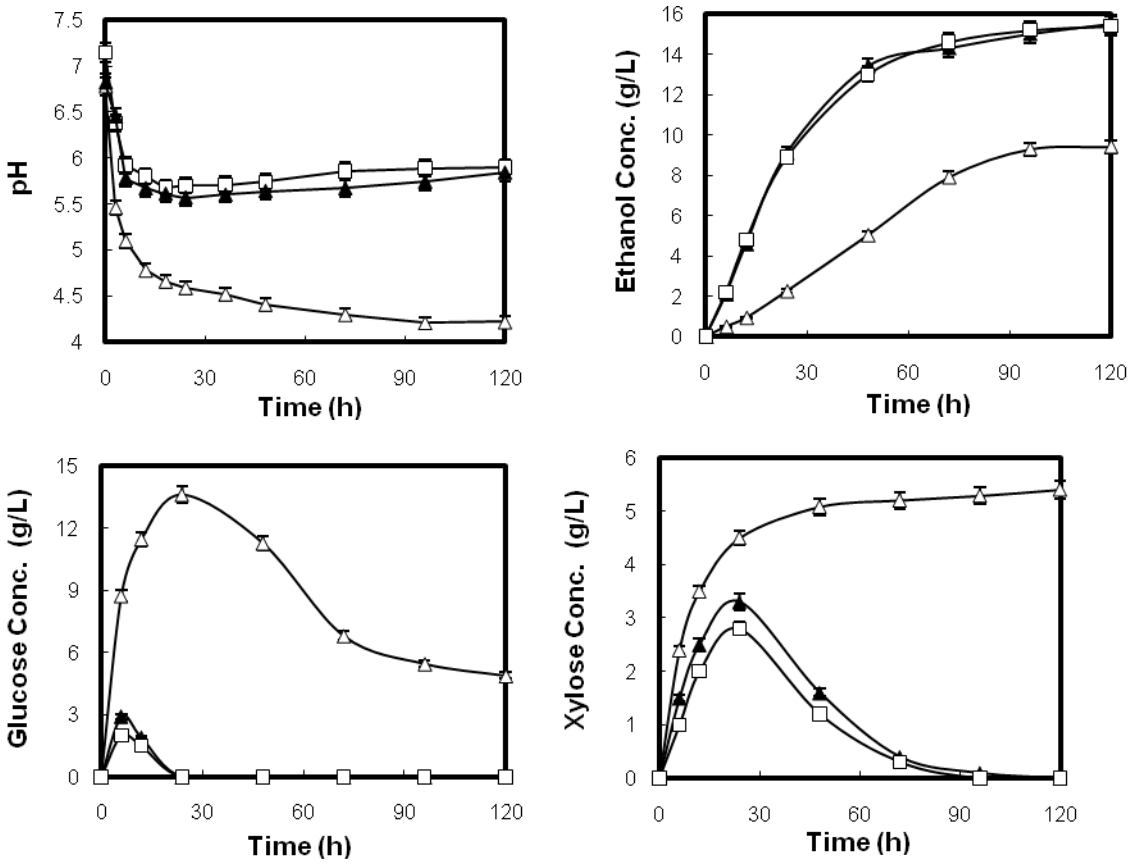

Fig. 3. Simultaneous saccharification and co-fermentation of de-ashed sludges by *Escherichia coli* KO11 (ATCC-55124)
Square tagged curves represent de-ashed primary sludge (S-PS-1). Triangles represent hardwood pulp (HP-I). Filled triangles represent hardwood pulp with 20% $CaCO_3$ on the basis of dry hardwood pulp (HP-II). The data points represent the average of triplicate runs. The pH was not controlled. Other conditions of the SSF were: 3% (w/v) glucan loading, 37°C, 10 FPU Spezyme CP + 20 CBU of Novozyme-188/g-glucan.

One of this study's main interests here was to observe how the pH profiles vary with different nutrient media and substrates. The pH dropped sharply in the early phase of the SSCF due to production of carbonic acid (CO_2 dissolved in water) and other organic acids, and remained relatively constant afterwards. The maximum acetic acid (AA) level was 2.0 g/L, and lactic acid (LA) was 1.8 g/L. The SSCF was started with a pH of 7, but decreased to 5.9 for S-PS-1. In the HP-I run, however, the pH quickly dropped from 6.8 to 4.2, similar to the pH profile of the SSF process. Much like CSL, the yeast extract and peptone also had a certain level of buffering capacity (Terzaghi and Sandine 1975; Hugo and Lund 1968). However, at low levels, their buffering capacity was not high enough to significantly affect the pH behavior of the SSCF of the HP-I. The presence of $CaCO_3$ enhances the buffering capacity of the medium. The pH of SSCF of HP-II with $CaCO_3$ dropped from 6.8 (0 h) to 5.8 (120 h), which is similar to the pH profile of the SSCF of S-PS-1. Ethanologenic derivatives of *E. coli* B function efficiently between a pH of 5.8 and a pH of 7.5 (Beall et al. 1991). Often a pH of 6.0 is used as a

practical optimum because it lowers the solubilization of CO_2 (Moniruzzaman et al. 1998). The pH in the fermentation HP-I remained below 5 after 6 hours because of low enough buffer capacity. Insufficient use of xylose, even glucose, and low ethanol yield indicates that *E. coli* KO11 was inhibited under low pH conditions. Moniruzzaman et al. also found that only 2 hours of exposure at a pH of 3 could cause long delays in cell growth and low ethanol yield (Moniruzzaman et al. 1998).

Fed-batch Operation

The concentration of ethanol in the bioreactor is a factor that significantly affects the cost of the downstream separation process. Alcohol concentration of 40 g/L has been mentioned as a target value in biomass fermentations (Hohmann and Rendleman 1993). This study 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 the initial sludge loading of 60 g-glucan/L. Additional feedstock of the same level was put into the reactor after 24 h for SSCF and after 12, 24, and 48 h for SSF. At these input points the reaction had 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, an ethanol concentration of 47.8 g/L was obtained at the end of the run. This is equivalent to an ethanol yield of 70% on the basis of total sugar with 10% w/v total glucan loading, which was slightly lower than the yield of the single-batch with 6% w/v glucan loading; however, it was still higher than the ethanol yield of 68% from untreated PS even with a lower enzyme loading and a low-cost medium. This is a significant benefit in the SSF gained specifically by de-ashing the sludge. The reason for the low ethanol yield observed from the fed-batch SSCF is that the ethanol tolerance of *E. coli* KO11 is relatively low compared to yeast (Dien et al. 2003; Kang et al. 2010).

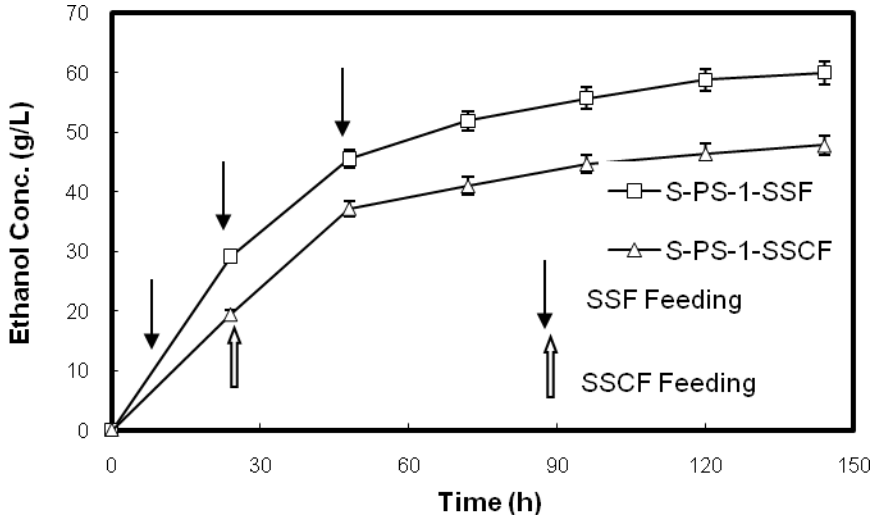

Fig. 4. Fed-Batch SSF and SSCF of S-PS-1: The data points represent the average of triplicate runs. Other conditions of the SSF were: 6g initial and additional feeding of glucan loading (9.3 g dry basis S-PS-1), 37°C, and 10 FPU Spezyme CP + 20 CBU of Novozyme-188/g-glucan. Total glucan loading for SSCF was 10% w/v and for SSF was 15% w/v.

In the fed-batch SSF runs with three additional feedings, a final ethanol concentration of 60 g/L was achieved, which was much higher than that of the fed-batch SSF of untreated primary sludge, 45 g/L, with two additional feedings. The overall ethanol yield in the fed-batch SSF was approximately 70% based on total glucan loading-15%w/v (23.1%w/v total solid loading), which was also lower than the yield of the single batch with 6% w/v glucan loading, yet comparable to that of the fed-batch SSF of untreated PS with a lower enzyme loading and a low-cost medium. The yield and product concentration data collectively indicate that the removal of ash and other extraneous components had positive effects on the bioconversion process.

CONCLUSIONS

1. Kraft paper mill sludges have features desirable for their bioconversion to value-added products. Without any cleaning, the sludges can be converted to ethanol by SSF or SSCF with reasonable efficiency (Kang et al. 2010). The bioconversion processes, however, can be significantly improved by de-ashing of the sludges.
2. The ethanol yields in the SSF or SSCF are improved significantly after de-ashing of the sludges. The ethanol yields for the de-ashed primary sludge (S-PS-1) reached 72.8% and 73.6% of the theoretical maximum for SSF and SSCF, respectively. These yields were achieved with a relatively low enzyme loading of 10 FPU/g-glucan and 20 CBU/g-glucan.
3. Often-used laboratory fermentation media, such as yeast extract and peptone, can be replaced by CSL or at least supplemented at a reduced level, without adversely affecting the ethanol yield. With fed-batch operation of SSF and SSCF, the ethanol concentrations in the broth were increased to the respective levels of 47.8 g/L and 60 g/L.

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