**Acetone-butanol-ethanol production from Kraftpaper mill sludge by simultaneous
saccharification and fermentation**

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22 **Abstract**

23 Paper mill sludge (PS), a solid waste from pulp and paper industry, was investigated as a
24 feedstock for acetone-butanol-ethanol (ABE) production by simultaneous
25 saccharification and fermentation (SSF). *ABE fermentation of paper sludge by*
26 *Clostridium acetobutylicum* required partial removal of ash in PS to enhance its
27 *enzymatic digestibility*. Enzymatic hydrolysis was found to be a rate-limiting step in the
28 SSF. A total of 16.4-18.0 g/L of ABE solvents were produced in the SSF of de-ashed PS
29 with solid loading of 6.3-7.4% and enzyme loading of 10-15 FPU/g-glucan, *and the final*
30 *solvent yield reached 0.27 g/g sugars*. No pretreatment and pH control were needed in
31 ABE fermentation of paper sludge, which makes it an attractive feedstock for butanol
32 production. *The results suggested utilization of paper sludge should not only consider the*
33 *benefits of buffering effect of CaCO₃ in fermentation, but also take into account its*
34 *inhibitory effect on enzymatic hydrolysis.*

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36 **Keywords:**papermill sludge, *Clostridium acetobutylicum*,acetone-butanol-ethanol (ABE),
37 simultaneous saccharification and fermentation (SSF)

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43 1. **Introduction**

44 Butanol is an advanced biofuel and a versatile platform chemical usable for synthesis
45 of various industrial chemicals (Durre, 2008; Tashiro et al., 2013). Fermentative
46 production of butanol has failed to compete with the chemical synthesis route from
47 propylene since 1960s (Durre, 2008; Jones & Woods, 1986). Recently, bio-based butanol
48 has been proposed as the next generation biofuel due to its properties superior to ethanol
49 (higher energy density, less corrosiveness, and low vapor pressure) (Anbarasan et al.,
50 2012; Durre, 2008; Tashiro et al., 2013).

51 The bioprocess for butanol production, however, is challenged by low solvent titer,
52 high cost of feedstock and high energy consumption for solvent recovery. Strong end-
53 product inhibition is known to be the major cause for the low solvent titer and low
54 productivity, which in turn raises the cost of product recovery (Anbarasan et al., 2012;
55 Tashiro et al., 2013). High feedstock cost has been cited as the primary reason for
56 cessation of commercial bioprocess in 1960s (Jones & Woods, 1986). Conventional
57 feedstocks for ABE fermentation are food-based sugars, starch and molasses. Although
58 those substrates have good fermentability, their cost is too high to make it a viable
59 bioprocess for butanol production via fermentation. Lignocellulosic biomass has
60 therefore drawn attention as a feedstock for ABE production (Ezeji & Blaschek,
61 2008) (Ezeji & Blaschek, 2008; Lu et al., 2012; Qureshi et al., 2010a; Qureshi et al.,
62 2010b).

Paper mill sludge, a waste material generated from pulp and paper plants, is one of such with a great potential as it has a number of attractive features as a raw material. Being a waste material, it carries zero or, in some cases, negative cost (elimination of disposal cost). Sludge from common pulping process (Kraft pulping) has low lignin content. Therefore it does not require pretreatment prior to enzymatic hydrolysis. Pretreatment along with the detoxification of the post-pretreatment effluent is one of the major cost factors in bioconversion of lignocellulosic materials, especially in the case of ABE production from lignocellulose (Ezeji et al., 2007; Palmqvist & Hahn-Hagerdal, 2000; Qureshi et al., 2010a; Sun & Liu, 2012). Furthermore, the short fibers found in most Kraft mill sludges are readily hydrolyzed by enzymes into fermentable sugars (Lark et al., 1997; Marques et al., 2008). A major challenge for sludge as a feedstock is coping with high ash content originated from filler materials (clay, TiO_2 , and CaCO_3), which severely impede the enzymatic hydrolysis (Lynd et al., 2001; Nikolov et al., 2000). Ash removal is therefore necessary for efficient bioconversion.

Simultaneous saccharification and fermentation (SSF) has been suggested to be more efficient bioconversion strategy than separate hydrolysis and fermentation (SHF) in ethanol production (Alkasrawi et al., 2003; Kadar et al., 2004). The advantages of the SSF over SHF include low equipment cost and alleviation of product inhibition of glucose and cellobiose on cellulase enzymes. Reduction of equipment and operation is achieved by performing enzymatic hydrolysis and fermentation concurrently in a single process. More importantly, SSF achieves high product yield and productivity keeping glucose level, consequently end-product inhibition on enzymatic hydrolysis low, since the sugar is simultaneously consumed by the microbes (Alkasrawi et al., 2003; Linde et al.,

2008).SSF has been investigated to produce butanol from corn stover by *Clostridium*
beijerinckii P260, however overliming detoxification of dilute acid pretreated substrates
and hydrolysates was required (Qureshi et al., 2014). Shah et al. (1991) identified the
effect pretreatment on converting hardwood substrates to butanol in a SSF process, they
revealed that both glucose and xylose could be utilized simultaneously by *C.*
acetobutylicum ATCC 824 (Shah et al., 1991). Calcium carbonate (CaCO_3) has been
found to increase butanol yield significantly in a SSF process by enhancing the buffer
capacities and the activities of NAD(P)H-dependent butyraldehyde and butanol
dehydrogenases (Li et al., 2015). Since paper mill sludge in this study is a pretreated
material from Kraft pulping, which contains mainly glucan, xylan and ash (CaCO_3), we
hypothesize that both glucan and xylan in paper mill sludge can be directly converted into
butanol in a SSF process without pH control.

Paper mill sludge has been evaluated as a feedstock for ethanol and lactic acid
production, and results have been positive (Budhavaram & Fan, 2009; Kang et al., 2011;
Lark et al., 1997; Marques et al., 2008). It is, however, yet to be studied for butanol
production. The objective of this study is to investigate the technical feasibility of
bioconversion of paper mill sludge into acetone-butanol-ethanol (ABE) through
simultaneous saccharification and fermentation (SSF). The main technical issues of our
interest are to assess the effects of enzyme loading and solid loading on the SSF and to
see how the results from the sludge compared with those of other biomass feedstocks.

2. Materials and method

2.1 Feedstock, enzyme and microorganism

Recycled Kraft paper mill sludge, collected from Boise Cascade (Jackson, AL), was used as feedstock for ABE production. Cellulase Cellic[®] C-Tec 2 (Batch No. VCNI0001) was a kind gift from Novozymes, North America (Franklinton, NC). The protein content and specific activity for C-Tec2 were 255 mg protein/mL and 119 FPU/mL. *Clostridium acetobutylicum* ATCC-824[™] (Lot NO. 58727357) was purchased from American Type Culture Collection (ATCC). Avicel PH-101 was purchased from Sigma-Aldrich (St. Louis, Mo.). Switchgrass was provided by Ceres Inc. (Thousand Oaks, CA). Switchgrass was pretreated by soaking in 2 % (w/w) sodium hydroxide solution at 60 °C for 24 h. The liquid-to-solid ratio in switchgrass pretreatment was 9:1. After pretreatment, the solids were washed with water until the pH reached 6.0 before subjecting to further analysis. The chemical compositions of paper mill sludge and switchgrass were determined according to the NREL standard procedure (NREL/TP-510-42618). The solid composition of NaOH pretreated switchgrass was analyzed to contain 54.1% of glucan, 23.8% of xylan, 10.4% of lignin and 6.2% of ash.

2.2 Ash removal of paper mill sludge

The ash in paper mill sludge was partially removed as previously described (Kang et al., 2011). Briefly, the sludge was first suspended in DI water at 3% (w/w) consistency and blended with bench-top stirrer (IKA[®] RW16, Germany) for 30 min. It was then dewatered by filtering through a 100-mesh screen. The dewatered sludge was dried at 45 °C until the moisture content reached below 10% for further processing and sample analysis. Multiple de-ashing cycles were applied to reduce the ash content of sludge to a level (6.1% ash) that can achieve acceptable saccharification rate. PS2 indicates the paper sludge de-ashed by two cycles of washing. PS3, PS4, etc. are defined accordingly.

2.3 Culture maintenance and inoculum preparation

The stock of *C. acetobutylicum* was maintained as spores at -20 °C in Elliker broth (BDDifco™) supplemented with 20 % (v/v) of glycerol. The spores were revived by inoculating one loop of the spore suspension in the Reinforced Clostridial HiVeg™ Broth (HiMedia Laboratories, India) and anaerobically incubated for 24 h at 36 °C. The inoculum was prepared by transferring 1 mL of actively growing culture into 50 mL of P2 medium supplemented with 10 g/L of glucose in a 100 mL screw-capped Pyrex bottle and anaerobically incubated for 24 h. The P2 medium formula was as follows (g/L): KH₂PO₄, 0.5; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.2; FeSO₄·7H₂O, 0.01; MnSO₄·H₂O, 0.01; NaCl, 0.01; ammonium acetate, 2.2; yeast extract, 1.0.

2.4 Enzymatic hydrolysis of paper mill sludge

Enzymatic hydrolysis was performed to estimate the digestibility of de-ashed paper mill sludge. The experiment was carried out in batch mode using 125 mL Erlenmeyer flasks for 120 h under 50 °C and 150 rpm. Sodium citrate (0.05 M, pH 4.8) was used as buffer and 0.01% (w/v) of sodium azide was used as biocide to prevent microbial contamination. To assess the effect of ash content on enzymatic hydrolysis, the de-ashed paper mill sludge (4% glucan) was hydrolyzed with cellulases under an enzyme loading of 10 FPU/g glucan.

2.5 ABE fermentation with mixed glucose and xylose as substrates

ABE fermentation with mixed glucose (44.8 g/L) and xylose (16.1 g/L) as substrates was carried out in 125 mL serum bottle with a working volume of 50 mL. P2 medium was used as inorganic mineral fermentation nutrients. The initial pH of the fermentation broth was adjusted to 6.7 with calcium carbonate (5.0 g/L). The fermentation bottle was

flushed with nitrogen gas for 5 min and crimp-sealed with rubber stopper. The bottles were loaded with sugars and fermentation medium, autoclaved at 121 °C for 15 min, inoculated with the actively growing seed-culture at 5 % (v/v). Fermentation was carried out at 36 °C, 150 rpm, and under strict anaerobic condition. Aliquots of samples were taken with 12-h interval.

2.6 Simultaneous saccharification and fermentation (SSF) of de-ashed paper mill sludge, Avicel and alkali-pretreated switchgrass

SSF of paper mill sludge was performed at 36 °C and 150 rpm in a 125 mL serum bottle containing 50 mL of P2 medium with 3.8-7.4% of PS7. Anaerobic condition was developed by sparging nitrogen gas into the headspace of the bottle. The sealed bottles were autoclaved at 121 °C for 15 min. Cellulase enzyme (C-Tec2) was added after sterilization. Different solid loading of PS7 (3.8, 5.0, 6.3 and 7.4% w/v) was compared in SSF processes, which are equivalent to 36.6, 48.6, 60.6, 72.7 g/L sugars (glucose and xylose) given the carbohydrates were completely hydrolyzed. For each solid loading, three enzyme loadings were applied: 5, 10 and 15 FPU/g-glucan. For the solid loadings of 3.8% and 5.0%, *C. acetobutylicum* culture was inoculated at the same time as enzyme addition. For the solid loadings of 6.3% and 7.4%, there was insufficient fluidity to perform fermentation due to limited level of free water available in the beginning, the culture was inoculated after 12 h enzymatic hydrolysis, at which point the substrate was partially liquefied. Aliquots of samples were taken at 24, 48, 72, 96, and 120 h. SSF of Avicel and alkali-pretreated switchgrass were conducted under the same conditions.

2.7 HPLC analysis

The enzymatic digestibility of de-ashed paper mill sludge was calculated from the

released glucose content, as a percentage of the theoretical sugars available in the substrates. The released glucose and xylose content during enzymatic hydrolysis were quantitated by HPLC with Aminex HPX-87P column (Bio-Rad Laboratories, Hercules, CA). The fermentation product in this study was analyzed by HPLC equipped with refractive index detector (Shodex, Japan) and Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA). The solvent yield was calculated on weight basis as the amount of ABE formation divided by the total sugars in the feed.

2.8 SEM image analysis

The surface morphology of de-ashed sludge sample was examined with scanning electron microscopy (Carl Zesis, Model EVO-50, Thornwood, NY). For SEM examination, the samples were sputter-coated with gold using EMS 550X Sputter Coating Device (Electron Microscopy Sciences, Hatfield, PA). The SEM images were taken with 1.0 and 3.0 kX magnifications under 20.0 kV beam.

3. Results and discussion

3.1 Chemical composition and enzymatic digestibility of de-ashed paper mill sludge

The chemical composition and enzymatic digestibility of unwashed and de-ashed paper mill sludges were determined (Table 1). Glucan (51.8%) and xylan (12.6%) were the two major organic components in the unwashed paper mill sludge. The ash content was as high as 32.7%. Ash in paper mill sludge appeared to inhibit enzymatic hydrolysis significantly (Table 1). After each cycle of water washing, the ash content in paper mill sludge was reduced gradually to 25.1%, 20.5%, 12.3% and 6.1% in PS2, PS4, PS6 and PS7, respectively. Correspondingly, the glucan digestibility increased significantly from 0 in unwashed PS to 0.2%, 6.4%, 65.4% and 74.1% in PS2, PS4, PS6 and PS7, respectively.

There was a strong inverse association ($r^2=0.85$) between ash content and glucan and xylan digestibility in paper mill sludge. SEM images (not shown) revealed clean and smooth surface of the washed sludge, contrasting with the rough surface with small particles attached on the surface of unwashed sludge. However, the de-ashing also led to considerable loss of short fibers. For example, about 24-25% of glucan was washed away in PS6 and PS7. The results indicated that de-ashing by water washing is critically needed for improving the glucan digestibility of paper mill sludge, but minimizing short fiber loss should be considered as well.

Ash in the sludge is composed of inorganic salts, calcium carbonate being the predominant component. The presence of calcium carbonate can dramatically increase the pH of paper mill sludge suspension in water or buffer. The buffer pH changed from 4.8 with unwashed PS to 7.4, 7.4, 6.4, 5.5 and 5.3 with PS2, PS4, PS6 and PS7, respectively. The change of pH in solution has a significant impact on cellulase activity. Wang et al. (2005) previously showed the specific activity of endoglucanase III decreased more than 90% at pH 7.0 (Wang et al., 2005). This negative effect of pH increase on enzyme was amplified by the high temperature in the enzymatic hydrolysis. Kang et al. (2010) observed enzymatic hydrolysis at higher temperature (50 °C) resulted in much lower glucan digestibility of primary paper mill sludge as comparing to the hydrolysis at 37 °C (Kang et al., 2010).

3.2 Simultaneous saccharification and fermentation of Avicel, alkali-pretreated switchgrass and de-ashed paper mill sludge

SSF of Avicel, alkali-pretreated switchgrass and de-ashed paper mill sludge were compared and fermentation of mixed sugars (glucose and xylose) was used as a control. Mixed sugars fermentation showed both glucose and xylose were consumed and the final solvent concentration reached 17.3 g/L (Fig. 1) and butanol was 10.4 g/L. At the end of fermentation (120), small amount of glucose (3.3 g/L) and xylose (2.1 g/L) were not utilized. ABE fermentation of mixed sugars gave a total solvent yield of 0.31(g/g sugars).

SSF of Avicel was performed with a solid loading of 5.8% (w/v) and enzyme loading of 20 FPU/g-glucan (Fig.2a).During the initial 24 h, glucose was quickly released to 12.0 g/L, and acetic and butyric acids were accumulated to 2.9 and 3.2 g/L, respectively. After that, glucose concentration decreased and more acids were re-assimilated to butanol, acetone and ethanol. Acetic and butyric acids were stabilized at 1.9 and 1.2 g/L after 72 h. At the end of SSF (120 h), ABE total solvents reached 16.8 g/L with 9.5 g/L of butanol. The ABE solvent yield was 0.28 (g/g glucose) and the productivity was 0.14 g/L/h.

SSF of alkali-pretreated switchgrass was performed with a solid loading of 5% and enzyme loading of 15 FPU/g-glucan (Fig.2b). In the first 24h, glucose (10 g/L) and xylose (1.0 g/L) was released, small amount of acetic and butyric acids (2 g/L) was produced. No solvents were produced until 48 h, but the solvents production quickly stopped at 72h. The final total solvents was very low (3 g/L). More than 9 g/L of glucose was present in the solution from 24 to 120 h. This indicated microbes could not actively consume sugars in the SSF of Alkali-pretreated switchgrass, and enzyme hydrolysis was not a rate-limiting step in SSF. It was most likely that residual lignin (10.4%) and other degradation compounds in alkali-pretreated switchgrass inhibited the microbial fermentation step.Similar results have been reported on converting dilute acid pretreated

corn stover to butanol in a SSF process(Qureshi et al., 2014), they found the pretreated corn stover still contained toxic chemicals that inhibited cell growth and fermentation activity. A further detoxification of pretreated solid was required to improve the ABE yield.

In contrast to alkali-pretreated switchgrass, SSF of de-ashed paper mill sludge (7.4% solid loading and 10 FPU) showed very good ABE solvents yield (Fig. 2c). Specifically in the first 24h, glucose and xylose reached 10 and 6 g/L, respectively. Both acetic and butyric acids reached 4.0 g/L, respectively. After 24 h, the glucose concentration continued to increase and reached at 16.1 g/L at 48h, then decreased gradually to 5.0 g/L at 120 h as SSF progressed. The xylose concentration was kept at 6 g/L from 24 to 120 h. Butanol was steadily produced from 24 to 120 h and reached 9.7 g/L at the end of fermentation. The total ABE solvent concentration was 17.1 g/L, the solvent yield was 0.24 (g/g sugars) and the productivity was 0.20 g/L/h. As comparing to the SSF of Avicel, the total acids concentration (5.7 g/l) was significantly higher than that in SSF of de-ashed sludge. Most likely, it is due to the buffering effect of ash in paper mill sludge. The culture tends to produce more acids to maintain the medium pH near its optimum for solvent production. Most of the solvent-producing cultures used for ABE production has shown the tendency to produce high level of acids when it comes to the fermentation of the hydrolysates of lignocellulosic substrate(Qureshi et al., 2010b; Sun & Liu, 2012).

3.3 Effect of enzyme loading on SSF of de-ashed paper mill sludge

To examine the effect of enzyme loading on ABE production, SSF of PS7 was performed (3.8% solid loading) with different enzyme loading (5, 10, and 15 FPU/g-glucan) (Fig. 3a-c). Under 5 FPU, the glucose and xylose were released in a small amount

(1 g/L) in the solution from 24 to 96 h (Fig. 3a). At 120 h, the glucose surged to 5.2 g/L. However, the low glucose concentration did not appear to inhibit the butanol production. Butanol increased from 0.3 g/L at 24 h to 5.3 g/L at 120 h. At the end of fermentation, the total solvent reached 7.4 g/L, the solvent yield was 0.20 g/g sugars and the productivity was 0.062 g/L/h.

When the enzyme dosage was increased to 10 FPU, about 2.3 and 0.40 g/L of glucose and xylose were released in the solution at 24 h. After that, both glucose and xylose concentration were kept less than 2 g/L until 120 h. The butyric acid first increased to 4.7 g/L at 24 h, and decreased gradually to 2.2 g/L at 120 h. The acetic acid increased to 4.5 g/L and was kept at the same level until 120h. The butanol concentration increased linearly and reached 6.8 g/L at 120 h. The total solvent concentration reached 10.6 g/L, the solvent yield was 0.29 g/g sugars and the productivity was 0.088 g/L/h. As comparing to the SSF process at 5 FPU, both solvent yield and the productivity increased by 45%. This indicated enzymatic hydrolysis was a rate-limiting step in SSF process. It should be noted that xylose was also consumed in the SSF process, this agreed well with previous reports on *C. acetobutylicum* being able to ferment xylose to butanol as well (Ezeji & Blaschek, 2008; Gao et al., 2014; Ounine et al., 1985).

When the enzyme loading was increased 15 FPU, there was no further improvement on the total solvent concentration. In the first 24 h, 2.8 and 1.8 g/L of glucose and xylose were released in the solution. The glucose concentration continued to increase and reached 6.2 g/L at 48 h. After that, glucose decreased gradually and reached 0.1 g/L at 120 h. Xylose decreased quickly to less than 0.5 g/L and was kept at the same level until 120 h. The total solvent concentration reached 10.6 g/L, the solvent yield was 0.29 g/g

sugars and the productivity was 0.088 g/L/h, which were similar to those in 10 FPU. This indicated that 10 FPU was sufficient in converting of paper mill sludge to butanol at 3.8% of solid loading.

3.4 Effect of solid loading on the SSF of de-ashed paper mill sludge

To assess the effect of solid loading on ABE production, SSF of PS7 was performed under 15 FPU and different solid loading (5.0, 6.3 and 7.4%). At 6.3% solid loading and above, the slurry exhibited extremely high viscosity, which limited the nutrients transportation. Hence, a pre-hydrolysis was performed to liquefy the slurry before culture inoculation.

When the 5.0 % solid loading was used in SSF, glucose (8.2 g/L) and xylose (3.7 g/L) were quickly released in the solution at 24 h. After that, glucose further increased to 9.5 g/L at 48 h, and then decreased gradually to 0.4 g/L at 120 h. Xylose concentration were kept around 3.5 g/L until 120 h. The butyric acid first increased to 3.3 g/L at 24 h, and decreased gradually to 1.6 g/L at 120 h. The acetic acid increased to 3.7 g/L at 24 h and was kept at 3.0-3.7 until 120h. The butanol production was initiated early and reached 2.2 g/L at 24 h. At the end of fermentation, the total solvent concentration reached 14.5 g/L, the solvent yield was 0.30 g/g sugars and the productivity was 0.12 g/L/h. When the solid loading increased to 6.3%, about 12.7 g/L of glucose and 5.3 g/L of xylose were released in the solution at 24 h. Glucose continued to increase and reached 15.4 g/l at 48 h. After that, glucose decreased quickly to 1.5 g/L from 48 to 120 h. Xylose decreased gradually to 2.3 g/L at 120 h. Butyric acid reached 3.7 g/L at 24 h, then decreased slowly to 1.8 at 120 h. Acetic acid reached 3.7 g/L at 24 h and was not changed until 120 h. At the end of fermentation, the total solvent concentration reached 16.3 g/L, the solvent yield was

0.27 g/g sugars and the solvent productivity was 0.14 g/L/h.

When the solid loading increased to 7.4, about 14.5 g/L of glucose and 6.7 g/L of xylose were released in the solution at 24 h. Glucose continued to increase and reached 18.0 g/L at 48 h. After that, glucose decreased quickly from 17.0 to 1.5 g/L during the period of 72-120 h. Xylose decreased slowly from 6.7 to 4.3 g/L at 120 h. Butyric acid reached 4.1 g/L at 24 h, then decreased to 1.8 at 120 h. Acetic acid reached 4.1 g/L at 24 h and was not changed until 120 h. At the end of fermentation, the total solvent concentration reached 18.0 g/L, the solvent yield was 0.275g/g sugars and the solvent productivity was 0.15 g/L/h. Comparing the solvent yield at different solid loading, higher solid loading resulted in lower solvent yield. It was most likely due to the decreasing hydrolysis yield at higher solid loading, which has been documented as the “solids effect” (Jorgensen et al., 2007; Kristensen et al., 2009).

3.5 Discussion

Paper mill sludge was utilized directly as feedstock for butanol production via simultaneous saccharification and fermentation. Effects of solid loading and enzyme loading on SSF of PS7 were compared (Table 2). Under a solid loading of 6.3-7.4% and enzyme loading of 10-15 FPU/g-glucan, a total of 16.4-18.0 g/L of ABE solvent was produced, which is comparable to that of the control test (pure cellulose). In comparing ABE production from different substrates in SSF processes, the final ABE solvent concentrations from paper sludge were 5-34% higher than that from dilute acid pretreated wheat straw (Table 3). The ABE yields from paper sludge were similar to that from dilute acid pretreated wheat straw. In comparing ABE production in SSF and SHF processes, the ABE yields in SHF process appeared relatively higher than those in SSF process, but

the ABE final concentrations were similar. It should be noted that different enzyme loading and different *Clostridium* strains will affect the final ABE concentration and ABE yields was well. Two major advantages of paper mill sludge as feedstock for butanol production are that no pretreatment is needed and no pH control is required as ABE fermentation from sugars. Yang et al. previously found that supplementation of calcium carbonate in ABE fermentation from glucose significantly improved the solvent yield by stabilizing the pH of fermentation broth(Yang et al., 2013). Remarkably, significant amount of CaCO_3 already exists in paper mill sludge and can be used as a buffering reagent in fermentation process. Previously, Budhavaram et al. investigated lactic acid production from paper sludge with *Bacillus coagulan* in a SSF process and found high lactic acid yield (>80%) were achieved without pH control because of the buffering effect of CaCO_3 (Budhavaram & Fan, 2009). However, excessive CaCO_3 inhibited enzymatic hydrolysis. Margues et al. showed paper sludge needs to be neutralized before enzymatic hydrolysis (2008). It be noted that calcium carbonate also showed inhibition on glucose utilization in ethanol production from paper sludge by *zymomonasmobilis*(Zhang & Lynd, 2010), they found calcium carbonate and high temperature were responsible for the accumulation of glucose in high solid loading of paper sludge SSF process. This indicated utilization of paper sludge should not only consider the benefits of buffering effect of CaCO_3 in fermentation, but also take into account its inhibitory effect on enzymatic hydrolysis and glucose utilization. In addition, attempts to convert alkali-pretreated switchgrass to butanol in a SSF process have not been successful, residual lignin or other toxic compounds on the pretreated substrates potentially inhibit the cell growth and fermentation activity.

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361 4. Conclusion

362 Kraft paper mill sludge has great potential as a feedstock for ABE production. It does
363 not require chemical pretreatment or detoxification, but only needs to be partially de-
364 ashed to improve the enzymatic hydrolyzability. Remarkably, the buffering effect of
365 CaCO_3 in paper mill sludge can be effectively used in the SSF process to produce butanol,
366 because pH control is a critical issue in butanol fermentation by *C. acetobutylicum*. The
367 results showed butanol production from de-ashed paper mill sludge could be carried out
368 under the solid loading of 6.3-7.4 wt% and enzyme loading of 10-15 FPU.

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Table 1

Chemical composition and enzymatic digestibility of paper mill sludge.

Component (%)	Unwashed	PS2 ^a	PS4 ^a	PS6 ^a	PS7 ^a
Glucan	51.8	55.5	59.4	66.4	71.8
Xylan	12.6	13.2	13.6	14.8	15.2
Mannan	1.5	1.3	1.5	1.1	1.7
Ash	32.7	25.1	20.5	12.3	6.1
Mass Closure	98.6	95.1	94.9	94.5	94.8
Solid Recovery (%)	-	85.3	68.1	59.1	54.0
Glucan Loss (%)	-	8.5	22.0	24.3	25.0
Xylan Loss (%)	-	11.1	27.0	31.0	34.9
Ash Removal (%)	-	34.6	57.5	77.7	90.0
Solid Loading ^b (%)	8.1	7.5	7.0	6.3	5.7
pH	7.4	7.4	6.4	5.5	5.3
Glucan Digestibility ^c (%)	0	0.2	6.4	65.4	74.1
Xylan Digestibility ^c (%)	0	0	9.2	71.7	94.3

a. In each de-ashing cycle, the paper mill sludge slurry was blended for 30 min at 3% (w/w) consistency and de-ashed sludge was collected by filtrating the slurry with a 100 mesh sieve. For the reference, PS4 was the de-ashed sample after 4-times of washing.

b. The enzymatic hydrolysis was evaluated for a given glucan loading of 4 % (w/v) and an enzyme loading of 10 FPU/g-glucan.

c. Enzymatic digestibility was based on 120 h.

Table 2

Effects of solid loading and enzyme loading on SSF of paper mill sludge.

Solid loading (w/v)%	Enzyme Loading (FPU/g-glucan)	Residual sugars (g/L)		Acids (g/L)		Solvents(g/L)				Solvent Yield ^a (g/g)
		Glucose	Xylose	Acetic	Butyric	Acetone	Butanol	Ethanol	Total	
3.8	5	5.2	1.4	4.1	2.1	1.9	5.3	0.2	7.4	0.20
	10	0.3	0.4	4.9	2.2	3.5	6.8	0.4	10.6	0.29
	15	0.1	0.3	4.1	1.9	3.4	6.9	0.4	10.6	0.29
5	5	8	3	4.5	2.7	3.5	5.8	0.4	9.7	0.20
	10	6	3.2	4.2	2.2	4.1	7.7	0.7	12.6	0.26
	15	0.5	3.1	3.5	1.6	5.2	8.5	0.8	14.5	0.30
6.3	5	7.2	3.8	4.2	1.9	4.6	7.7	1.0	13.3	0.22
	10	3.2	3.3	4.0	1.9	5.6	9.1	1.1	15.7	0.26
	15	1.5	2.3	3.5	1.8	5.4	9.6	1.4	16.3	0.27
7.4	5	8.9	4.1	4.1	1.4	5.8	8.7	1.2	15.8	0.22
	10	4.9	5.3	4.1	1.6	5.7	9.7	1.7	17.1	0.24
	15	4.1	4.3	4.1	1.8	6.2	10.2	1.6	18.0	0.25

^a the solvent yield was calculated from the total solvents over the theoretical amounts of sugars (glucose and xylose) in the PS7.

Table 3

ABE production from different feedstocks in SSF and SHF processes

Feedstock	Pretreatment	Detoxification	Fermentation	Culture	ABE (g/L)	ABE Yield (g/g sugars)	References
Paper sludge	None	NA	SSF	<i>C. acetobutylicum</i> ATCC 824	12.6-18.0	0.24-0.30	This study
Wheat straw	Dilute acid	NA	SSF SSF+GS ^d	<i>C. beijerinckii</i> P260	11.9 21.4	0.27 0.31	(Qureshi et al., 2008)
DDGS ^a	Dilute acid Liquid hot water AFEX	Overliming NA NA	SHF	<i>C. acetobutylicum</i> ATCC 824	12.1 11.4 9.0	0.31 0.31 0.32	(Ezeji & Blaschek, 2008)
Switchgrass	Dilute acid	NA Dilution ^c	SHF	<i>C. beijerinckii</i> P260	1.5 14.6	NA 0.39	(Qureshi et al., 2010b)
Maple ^b	Liquid hot-water	Overliming	SHF	<i>C. acetobutylicum</i> ATCC 824	11.0	0.28	(Sun & Liu, 2012)
Switchgrass	NaOH	NA	SHF	<i>C. saccharobutylicum</i> DSM 13864	22.7	0.40	(Gao et al., 2014)

a. DDGS: Dried distillers' grains and solubles; b. Maple: maple hemicellulose hydrolysate; c. Dilution: the hydrolysate was diluted 2 times with DI water and then was supplemented with extra pure sugar; SSF+GS: SSF was performed together with gas stripping.

Figure Legends

Fig.1. ABE fermentation of mixed glucose and xylose.

Fig. 2. Simultaneous saccharification and fermentation (SSF) of different feedstocks (a: Avicel under the solid loading of 5.8% and enzyme loading of 20 FPU/g-glucan, b: alkali-pretreated Switchgrass under the solid loading of 5.0% and enzyme loading of 15 FPU/g-glucan, c: PS7 under the solid loading of 7.4% and enzyme loading of 10 FPU/g-glucan).

Fig. 3. Effect of enzyme loading on simultaneous saccharification and fermentation (SSF) of PS7 under 3.8% of solid loading (a: 5 FPU/g-glucan, b: 10 FPU/g-glucan, c: 15 FPU/g-glucan).

Fig. 4. Effect of solid loading on simultaneous saccharification and fermentation (SSF) of PS7 under different solid loadings applying enzyme loading of 15 FPU/g-glucan (a: 5.0% solid loading, b: 6.3% solid loading, c: 7.4% of solid loading).

Fig. 1

Fig. 2a.

Fig. 2b.

Fig. 2c.

Fig. 3a.

Fig. 3b.

Fig. 3c.

Fig. 4a.

Fig. 4b.

Fig. 4c.