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Towards comprehensive lignocellulosic biomass utilization for bioenergy production: Efficient biobutanol production from acetic acid pretreated switchgrass with *Clostridium saccharoperbutylacetonicum* N1-4

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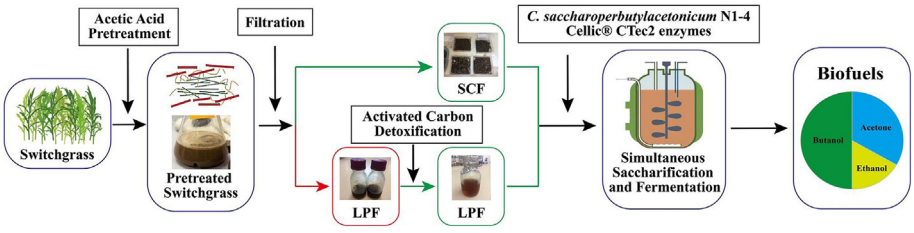
HIGHLIGHTS

- An innovative acetic-acid-based biomass pretreatment method was developed.
- Both the prehydrolysates and the solid cellulosic fraction were highly fermentable.
- 8.6 g/L butanol was obtained from simultaneous saccharification and fermentation.
- Comprehensive carbon utilization was realized with a tailored pretreatment strategy.

GRAPHICAL ABSTRACT



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ABSTRACT

For bioenergy production from lignocellulosic biomass, most biomass pretreatment processes need to use some chemical reagent as the catalyst to overcome the biomass recalcitrance barrier. Such reagents are usually severe inhibitors for the subsequent microbial fermentation process. Therefore, in many cases, the liquid prehydrolysates fraction (LPF) is discarded after the pretreatment, which is a tremendous waste of materials and leads to additional pollution. Biobutanol produced from the acetone, butanol and ethanol (ABE) fermentation process has been of great interests recently due to its high value as a bioenergy source or biochemical. During ABE fermentation, acetic acid (AA) is produced and then re-assimilated as a carbon source. Thus, AA is a substrate rather than an inhibitor for biobutanol production. In this study, we employed AA as the chemical catalyst for the pretreatment of switchgrass which then be used for ABE production through simultaneous saccharification and fermentation (SSF) with hyper-butanol producing *Clostridium saccharoperbutylacetonicum* N1-4. Through systematic investigation of the pretreatment conditions and fermentation, we concluded that the optimized condition for switchgrass pretreatment was with 3 g/L AA at 170 °C for 20 min. Both LPF and solid

Abbreviations: AA, acetic acid; ABE, acetone, butanol and ethanol; ATP, adenosine triphosphate; BA, butyric acid; F-C, Folin-Ciocalteu; FPU, filter paper unit; HMF, hydroxymethylfurfural; HPLC, high performance liquid chromatography; LPF, liquid prehydrolysates fraction; NREL, National Renewable Energy Laboratory; RID, refractive index detector; SCF, solid cellulosic fraction; SHF, separate hydrolysis and fermentation; SSF, simultaneous saccharification and fermentation; TGY, tryptone-glucose-yeast extract; TPC, total phenolic compounds

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cellulosic fraction (SCF) of the pretreated biomass were highly fermentable. In the fermentation with the SCF/LPF mixture as the substrate, 8.6 g/L butanol (corresponding to a yield of 0.16 g/g) was obtained. Overall, here we demonstrated an innovative and tailored biomass pretreatment strategy for comprehensive and efficient carbon source utilization for bioenergy production.

1. Introduction

It is globally recognized that the energy crisis due to the exhaustion of fossil fuels is a big program that human beings are facing in the near future. The production of bioenergy from renewable resources is considered as a promising solution to the energy issue as well as the associated environmental problems. For bioenergy production, lignocellulosic biomass is widely considered as a sustainable feedstock because it is inexpensive, highly abundant and broadly distributed [1–4]. Prior to converting lignocellulosic biomass into bioenergy through microbial fermentation, a pretreatment process is generally required to overcome the biomass recalcitrance barrier. Various approaches, including biological, physical, chemical, and physical-chemical ones have been employed for biomass pretreatment. For most of the known pretreatment processes (especially for the chemical and physical-chemical processes), chemical reagents (such as diluted acid, alkaline, organosolv or ionic liquid) are usually used as the catalyst for breaking down the recalcitrant structure of the biomass [1,5–7]. These reagents, even at low levels, are severe inhibitors (besides the phenolic inhibitors generated from the degradation of biomass during the pretreatment) for the subsequent microbial fermentation processes [5]. In addition, a significant level of acetic acid (AA) is usually produced by cleaving off the acetyl group from the hemicellulose backbone during the biomass pretreatment process [8], which is also a strong inhibitor, for example, for the microbial ethanol fermentation [9]. With such issues, the liquid prehydrolysates fraction (LPF) after a regular biomass pretreatment is usually discarded as waste or combusted for power energy generation after evaporating the water [10]. This is a tremendous waste of carbon sources, and meanwhile reduces the energy conversion efficiency and leads to additional pollution issues. For bio-based process, the feedstock cost accounts for a major fraction out of the overall cost [3,11]. The wasting of feedstock carbon sources would lead to inefficient conversion and recovery of the energy source for practical application. Therefore, it is highly desirable to develop a bioprocess capable of more comprehensive and efficient utilization of lignocellulosic biomass for the production of biofuel and bioenergy.

Recently, biobutanol produced from renewable biomass carbon sources through the clostridial acetone, butanol and ethanol (ABE) fermentation has been of great interests, because it not only can be used as a biofuel and energy source with various advantages over ethanol, but also has vast applications as a chemical feedstock in many industries [12–17]. As a fuel source, butanol has comparable energy content as gasoline, which is much higher than ethanol. Compared to ethanol (the well accepted biofuel as an additive to gasoline), butanol is less soluble in water, less evaporative, and less hygroscopic, making it easier to handle and more compatible with the existing pipeline infrastructure and regular vehicle engines. As a biochemical, butanol can be used in food, cosmetic, pharmaceutical, and plastic industries [16].

ABE fermentation is a unique bi-phasic process. In the first phase (acidogenesis), carbohydrate is degraded into acids (mostly AA and butyric acid, or BA); while in the second phase (solventogenesis), the acids generated from the first phase are re-assimilated and converted into solvents along with the uptake of additional carbohydrate [12]. In this sense, AA (as well as BA) is a substrate rather than an inhibitor for biobutanol production. Indeed, it has been reported by various researchers that the supplementation of exogenous AA can efficiently improve butanol production and stabilize ABE fermentation [18–20].

Therefore, if AA is employed as the biocatalyst in biomass pretreatment, this reagent along with the AA generated during the pretreatment can both be utilized for biobutanol production. In such an approach, no exogenous chemical reagent is introduced, and thus can save cost and meanwhile avoid the inhibition on the subsequent butanol fermentation. In addition, the AA (as a weak organic acid) pretreatment can potentially generate lower level phenolic inhibitors when compared to the regular pretreatment process (with strong chemical reagents involved) under similar conditions [21,22]. Thus, the LPF from the biomass pretreatment with AA could be possibly utilized as the carbon source (rather than being discarded) for ABE fermentation, ending up with more comprehensive and efficient utilization of the biomass carbon source and minimizing pollution.

Hildebrand solubility parameter can be used to numerically estimate the interaction between different materials; materials with similar values of Hildebrand solubility parameter are likely more soluble with one in the other [23]. For example, solvents which display good lignin solubility have Hildebrand solubility parameter close to 11. AA has a Hildebrand solubility parameter value of 10.1, and therefore is conferred to be a very effective reagent for lignin solubilization and biomass pretreatment. It has been reported in a recent study that AA-based pretreatment on lignocellulosic biomass could increase the irregularity of the pore structure, which would facilitate the later processing and bioconversion of the biomass [24]. Xu et al. reported that the pretreatment with 10 g AA/kg on raw corn stover at 195 °C for 15 min results in xylose recovery up to 81.82% in the prehydrolysates [22]. In another report, the same research group reported that pretreatment of corn stover with combined AA and lactic acid yielded a higher glucan recovery and the simultaneous saccharification and fermentation (SSF) leads to a high ethanol yield (88.7% of the theoretical yield based on pure glucose) [21]. However, to our best knowledge, there was no research so far in which biomass was pretreated with AA and then used for the downstream biobutanol production by taking advantage of the unique acid re-assimilation feature of ABE fermentation.

Therefore, in this study, the objective was to explore AA as an innovative and efficient reagent for biomass pretreatment, and meanwhile utilize the pretreated biomass for biobutanol production through ABE fermentation. Such an integrated approach would increase the efficiency for the biomass conversion for biobutanol production, and thus improve the overall techno-economic feasibility of the bioprocess for bioenergy generation. In detail, we systematically optimized the conditions for the biomass pretreatment. Both the solid cellulosic fraction (SCF) and LPF were successfully fermented for efficient butanol production. In addition, with the mixture of SCF/LPF, high titer and yield for the solvent production were achieved after the fermentation. This study provides valuable references for developing an efficient, economical and sustainable bioprocess for bioenergy production from low-value lignocellulosic biomass.

2. Materials and methods

2.1. Feedstock, enzymes, microorganism and reagents

The Alamo-I switchgrass (*Panicum virgatumis*) was provided by Ceres, Inc. (Thousand Oaks, CA). Before the pretreatment, the biomass was milled to pass through a 0.25-in. screen and then stored at room temperature. The content of glucan, xylan, and lignin in the untreated switchgrass (in % based on dry weight) was 35.6, 19.2, and 20.0, respectively (Table 1). Comparing with the wooden biomass, the herbaceous switchgrass biomass is less recalcitrant and thus can be processed more easily under relatively mild conditions [25]. The

Table 1
The chemical composition of the raw switchgrass and the solid cellulosic fraction (SCF) after pretreatment.[a]

Pretreatment conditions		Composition (%)			
	Acetic acid used (g/L)	Glucan	Xylan	Total Carbohydrate	Lignin
Raw switchgrass		35.6 ± 0.6	19.2 ± 0.8	65.5 ± 1.2	22.6 ± 0.3
SCF (150 °C)	0	34.9 ± 2.3	20.9 ± 0.2	64.5 ± 2.9	28.0 ± 0.6
	3	35.3 ± 3.1	19.6 ± 1.0	61.9 ± 3.2	28.3 ± 0.9
	7	37.1 ± 0.7	19.3 ± 0.7	63.8 ± 0.9	29.2 ± 0.7
	11	35.7 ± 2.4	17.7 ± 0.6	60.1 ± 2.8	29.5 ± 0.3
SCF (170 °C)	0	44.5 ± 0.8	11.8 ± 1.1	62.6 ± 3	31.1 ± 1.1
	3	45.1 ± 1.9	10.7 ± 1.1	61.9 ± 1.4	32.4 ± 1.3
	7	48.2 ± 2.6	7.5 ± 0.7	61.7 ± 2.2	35.0 ± 1.7
	11	48.8 ± 0.3	6.7 ± 0.9	61.3 ± 1.3	35.8 ± 0.6
SCF (190 °C)	0	45.3 ± 1.6	15.5 ± 0.3	62.2 ± 1.1	29.1 ± 0.5
	3	44.6 ± 2.0	15.1 ± 0.1	61.4 ± 2.3	30.5 ± 0.4
	7	45.7 ± 2.1	17.2 ± 0.3	64.8 ± 1.9	29.6 ± 0.4
	11	44.5 ± 1.7	15.6 ± 1.3	61.7 ± 1.4	30.3 ± 0.3

[a] All results shown are the average value ± standard deviation from replicated experiments.

commercial enzyme cocktail Cellic® CTec2 was obtained from Novozymes (Franklinton, NC) for the hydrolysis purpose. The enzyme activity was determined as 119 FPU/ml using Whatman #1 filter paper as the substrate, and the β-glucosidase activity as 343 IU/ml using p-nitrophenyl-β-D-glucoside (PNPG) as the substrate. *Clostridium saccharoperbutylacetonicum* N1-4 (HMT) (DSM 14923, = ATCC 27021) was obtained from DSMZ (Braunschweig, Germany) and used for ABE fermentation in this study. All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

2.2. Pretreatment

For the pretreatment, 200 ml stainless steel batch reactors (1.375″ID × 6″L) were used to hold the biomass slurry, which were heated up in the oven of a gas chromatography (GC; an old GC has been modified and repurposed for precisely heating up the reactor) [26]. Switchgrass (10 g) was loaded into the tubular reactor at a liquid/solid weight ratio of 10:1. Various amounts of AA (0 g, 30 g, 70 g, or 110 g per kg of dry biomass, thus equivalent to 0, 3, 7 and 11 g/L) were applied for the biomass pretreatment at three different temperatures (150, 170 or 190 °C) for 20 min [22]. After the pretreatment, the slurry was immediately fractionated into the SCF and the LPF through vacuum filtration using a filter paper (Whatman®, Grade 802 Fluted, size 32.0 cm). The SCF was washed with tap water (300 ml for each run) for five times and dried at room temperature. Then it was stored in a climate cabinet at 25 °C and 65% relative humidity. The LPF was collected and stored at 4 °C until later use. All the experiments were performed in duplicate.

2.3. Detoxification

Detoxification of the LPF was performed with activated carbon to improve its ability to be hydrolyzed and fermented in the following steps. Granular activated carbon with particle size of 20–40 mesh was used for this purpose. Before use, the activated carbon was rinsed with DI water on a filter paper to remove the impurities and then dried at 45 °C in an oven for an overnight. The adsorbent was then loaded into the LPF at a ratio of 5% (w/v). The mixture was then incubated in a shaker at 150 rpm of agitation and 60 °C for 6 h to reach the adsorption equilibrium. The detoxified LPF was recovered through centrifugation. Then, the pH was adjusted to around 6.5 with 5 N of NaOH. Microfiltration (with 0.45-μm filter) was then applied to remove the suspended particles inside. The chemical composition of the detoxified LPF was then analyzed.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis of the LPF was carried out by mixing the LPF with concentrated sodium citrate buffer (pH 4.8) to obtain a final total volume of 100 ml (and the final sodium citrate buffer concentration of 50 mM). The cellulase at a loading of 15 FPU/g glucan was added, and the reaction mixture was incubated in a shaker at 150 rpm of agitation and 50 °C. Samples were taken at various time intervals (0, 3, 6, 12, 24, 36 and 72 h) and centrifuged to remove the insoluble materials (solid phase). The glucose or xylose yield (%) at the specific time was calculated based on the amount of glucose or xylose in the liquid phase, as a percentage of the theoretical total sugar equivalent available in the original feedstock. Each enzymatic hydrolysis was carried out in duplicate.

2.5. Batch fermentation

The *C. saccharoperbutylacetonicum* culture was maintained in the glycerol stock at −80 °C. To prepare the seed culture, 1 ml of the glycerol stock was anaerobically inoculated into 100 ml tryptone-glucose-yeast extract (TGY) medium containing 30 g/L of tryptone, 20 g/L of glucose, 10 g/L of yeast extract, and 1 g/L of L-cysteine. The TGY culture was incubated in an anaerobic chamber under a N_2-CO_2-H_2 (volume ratio of 85:10:5) atmosphere at 35 °C for 12–14 h till the OD_{600} reaching ~0.8 [27], which would then be used as the inoculum for the fermentation.

When SCF and/or LPF was used as the substrate, SSF was performed. Fermentations were carried out in 500 ml bioreactors (GS-MFC, Shanghai Gu Xin biological technology Co., Shanghai, China) with a 250 ml working volume. The modified P2 (MP2: by eliminating the ammonium acetate within the P2 medium) medium contains the following (in g/L): KH_2PO_4, 0.5; K_2HPO_4, 0.5; $(NH_4)_2SO_4$, 2; $MgSO_4{\cdot}7H_2O$, 0.2; $MnSO_4{\cdot}H_2O$, 0.01; $FeSO_4{\cdot}7H_2O$, 0.01; NaCl, 0.01; p-aminobenzoic acid, 0.001; thiamine-HCl, 0.001; biotin, 0.00001. For the fermentation with SCF, pretreated SCF (based on a total sugar equivalent of 20 g/L as glucose and xylose) along with yeast extract (2 g/L), tryptone (6 g/L), and MP2 medium were mixed together. For the fermentation with LPF, the MP2 medium along with yeast extract (2 g/L) and tryptone (6 g/L) was directly supplemented into the LPF, making a final volume of 100 ml. The mixture was then filter-sterilized by passing through a bottle top filtration system (0.2 μm; VWR, Radnor, PA) and then decanted into a sterile serum bottle. For the fermentation with the mixture of SCF and LPF, similar as the fermentation with LPF, the MP2 medium along with yeast extract (2 g/L) and tryptone (6 g/L) was firstly supplemented into the LPF, making a final volume of 100 ml. Then, the SCF (based on the ratio of SCF and LPF after pretreatment from certain amount of biomass) was added.

For all the fermentations, the initial pH was adjusted to 6.5 with 2 N NaOH. To generate an anaerobic condition, oxygen-free nitrogen was sparged through the fermentation broth starting several hours before the inoculation until the cell culture initiated its own gas production during the fermentation. The Cellic CTec2 enzyme of 15 FPU/g glucan and active growing preculture (5% v/v) were added at the same time to initiate the fermentation. The fermentation was performed at 30 °C and 150 rpm of agitation for 96 h with the pH controlled > 5.0. All fermentations were performed in triplicates.

2.6. Analytical procedure

The lignin and carbohydrate composition of SCF was analyzed following the National Renewable Energy Laboratory protocol (NREL/TP-

510-42618). The chemical composition of LPF was analyzed via secondary hydrolysis as described in the protocol (NREL/TP-510-42623). The LPF was characterized for the carbohydrate content (oligosaccharides and monomeric sugars) and degradation products (AA, furfural, hydroxymethylfurfural (HMF) and total phenolic compounds (TPC)). The amount of oligosaccharides in LPF was calculated by subtracting the monomeric sugar content in the LPF from the total monomeric sugar content after secondary hydrolysis. The TPC was determined using the Folin-Ciocalteu (F-C) assay [28]. In brief, 100 μL of LPF, standard (gallic acid) or 95% (vol/vol) methanol blank was added into 2 ml microtubes and mixed with 200 μL of F-C reagent by vortex. The total volume was made to 1.1 ml by adding 800 μL sodium carbonate into each tube and incubate at room temperature for 2 h. Transfer 200 μL sample, standard or blank from the assay tube to a clear 96-wells microplate and read the absorbance of each well at 765 nm using the spectrophotometer equipped in Tecan infinite M100 pro (Tecan Trading AG, Switzerland). The calibration curve was obtained in a similar manner as described above for quantifying the samples by using gallic acid solutions as standards. Results were expressed as mg per gram of dry material (mg g^{-1} DW).

The sugar analysis was performed with an Agilent 1260 Infinity HPLC system (Agilent Technologies, CA) equipped with a refractive index detector (RID) and a 300 mm × 7.8 mm (i.d.), 9 μm Aminex HPX-87P column and a 30 mm × 4.6 mm (i.d.) guard column (Bio-Rad, Hercules, CA). Nano-pure water was used as the mobile phase at an isocratic flow rate of 0.6 ml/min and the temperature was maintained at 85 °C during the 35-min elution. The fermentation products were quantified with the same Agilent 1260 Infinity HPLC system equipped with a Varian MetaCarb 87H Column (300 × 7.8 mm) along with a 50 × 4.6 mm MetaCarb 87H guard column (Agilent Technologies, CA). 0.005 N H_2SO_4 was used as the mobile phase at an isocratic flow rate of 0.6 ml/min, and the temperature of the column was maintained at 25 °C during the elution.

2.7. Mass balance calculation

The recovery rate and solvent yield from the pretreatment were calculated according to the literature [22]. In details, the recovery rate (of either glucan or xylan) was obtained by dividing the mass of glucan (or xylan) in both the SCF and LPF after pretreatment with the mass of glucan (or xylan) in the original biomass used for the pretreatment.

$$\text{Recovery Rate}_{Glucan} = \frac{\text{Mass}_{Glucan\ in\ SCF} + \text{Mass}_{Glucan\ in\ LPF}}{\text{Mass}_{Glucan\ in\ raw\ biomass}} \times 100\%$$

$$\text{Recovery Rate}_{Xylan} = \frac{\text{Mass}_{Xylan\ in\ SCF} + \text{Mass}_{Xylan\ in\ LPF}}{\text{Mass}_{Xylan\ in\ raw\ biomass}} \times 100\%$$

Total biomass recovery rate was obtained by dividing the total dry weight of the biomass after pretreatment (combined SCF and LPF) with the total dry weight of the raw biomass (before the pretreatment).

Total carbohydrate presented in the following context refers to the total content of gluan, xylan, galactan, arabinan, and mannan.

The solvent yield was calculated by dividing the amount of solvent generated in the fermentation with the total carbohydrate that has been consumed during the fermentation:

$$\text{Solvent yield \%} = \frac{\text{Solvent generated in fermentation}_{g/L}}{\text{Total carbohydrate consumed during fermentation}_{g/L}} \times 100\%$$

2.8. Statistical analyses

All the statistical analyses were conducted using SAS software (SAS Institute Inc, Cary, NC). Results with *p*-values below the conventional 5% threshold were regarded as significant.

3. Results and discussion

3.1. Biomass pretreatment and composition analysis

Alamo switchgrass was selected as the lignocellulosic feedstock in this study. In comparison with the wooden biomass, it is less recalcitrant and thus can be processed easily with relatively mild pretreatment conditions [25]. Generally, the biomass pretreatment through the hydrothermal approach is carried out at temperatures between 160 and 230 °C for a residence time of 10–60 min. One of the primary objectives for hydrothermal pretreatment is to remove as much hemicellulose as possible from the biomass [29]. Using AA as the chemical catalyst in the biomass pretreatment, the recalcitrance of the biomass can be overcome at relative milder conditions, and thus lead to improved yield of sugars and biofuel production.

In this study, for the pretreatment, 10 g of dried switchgrass (for each reaction) was mixed with 100 ml water, and then pretreated under four different AA concentrations (0, 3, 7, 11 g/L) at three different temperatures (150 °C, 170 °C, 190 °C; thus totally 12 different pretreatment conditions) for 20 min. The composition of the SCF of the pretreated biomass (as compared to the untreated raw biomass) was illustrated in Table 1. In the raw switchgrass, the total carbohydrate (cellulose and hemicellulose) represented approximately 65.5% of the total weight (wt/wt; the same below unless otherwise indicated) and the total lignin accounted for 22.6%. After pretreatment, especially when the pretreatment temperature was above 170 °C, the glucan content in the SCF increased while the xylan content decreased significantly. The total biomass recovery rate decreased significantly from above 70% to lower than 40% when the pretreatment temperature was increased from 150 °C to 190 °C (Fig. 1). At 170 °C, when the AA concentration increased from 0 to 11 g/L, the xylan content in the SCF decreased remarkably from 11.8% to 6.7%, while the other components (glucan and lignin) increased slightly (Table 1). At 150 °C (the mildest condition employed in this study), the residual cellulose, hemicellulose, lignin and total sugars were almost unchanged compared to the raw biomass. Furthermore, the SCF composition was almost not influenced by the increase of AA concentration under this pretreatment condition. This indicated that 150 °C was not effective for the biomass pretreatment. While at 190 °C, most of the components were carbonized due to

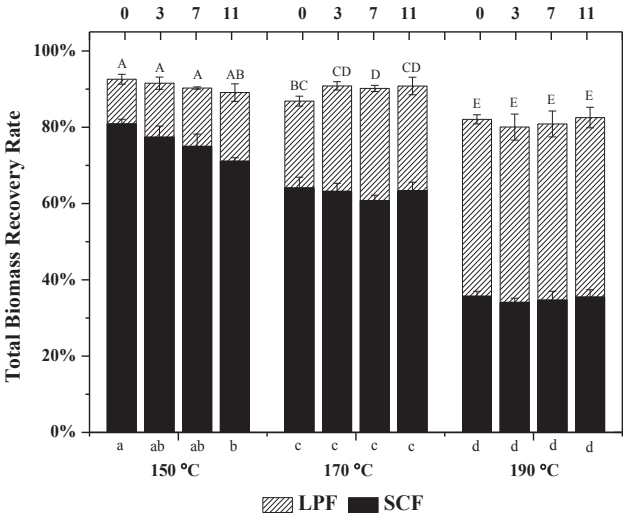

Fig. 1. The total biomass recovery rate in the SCF and LPF under different pretreatment conditions. The number on the top indicates the acetic acid concentration (0, 3, 7, an 11 g/L) used for the pretreatment. The reported value represents the mean of replicated samples, and the error bar represents the standard deviation. The bars with the same letter (uppercase for LPF on the top and lowercase for SCF on the bottom) across all pretreatment conditions are not significantly different at the 0.05 level based on Tukey's HSD test.

the harsh pretreatment condition, as illustrated by the low biomass recovery rate in Fig. 1, demonstrating that this high temperature was unfavorable for the biomass pretreatment. Therefore, based on the SCF composition under various pretreatment temperature conditions, 170 °C was likely the optimal temperature for the switchgrass pretreatment in this study.

The composition of the LPF was presented in Table 2. At 170 °C when no AA was added, a total of 15.5 g/L sugars with xylose (7.7 g/L) as the dominant component was detected in the LPF. With the increase of AA concentration for the pretreatment, more sugars were released with 21.0 g/L (11.1 g/L xylose) was detected when 7 g/L AA was used. However, when 11 g/L AA was employed, the total sugars decreased slightly possibly due to the degradation. It should be noticed that, with 7 g/L AA was used for the pretreatment, the majority of xylose was as the oligomer (9.1 g/L) and with only a small portion in the form of the monomer (2.1 g/L). It has been reported that the hydrothermal pretreatment (also called self-hydrolysis) works by cleaving off the acetyl group from the hemicellulose backbone, and simultaneously releasing the polysaccharides and AA into the LPF [8]. This was confirmed by the increase of acetate concentration in the LPF and decrease of hemicellulose (xylan) content in SCF after the pretreatment as illustrated in Tables 1 & 2. At 150 °C, with the increase of added AA concentration from 0 to 11 g/L, the total sugar in LPF increased from 7.5 to 12.7 g/L. For xylose, however, even when 11 g/L AA was used, only 3.6 g/L was detected most of which was oligomer. Therefore, again, these results suggested that 150 °C was not adequate for the biomass pretreatment. On the other hand, at 190 °C, the concentration of all the sugars in LPF (generated under conditions with various AA concentrations) was very low, because most of released sugars were further degraded into other products such as furfural and HMF (Fig. 1 and Table 2). The concentration of these side products (HMF, furfural and TPC) increased with the pretreatment temperature and reached the highest at 190 °C. Taken together, 170 °C was concluded as the optimal temperature for the switchgrass pretreatment, based on the analysis of the composition for both SCF and LPF. Therefore, for the following steps, the biomass pretreated at 170 °C (with various concentrations of AA employed for the pretreatment) was subjected to further processing and fermentation.

The recovery rate of glucan, xylan and the total carbohydrate from the pretreatment at 170 °C was calculated and illustrated in Fig. 2. For the glucan, only a small fraction was released into the LPF, varying from 13.8% without AA added to 17.2% when 7 g/L AA was added. However, more than 75% of the glucan was conserved in the SCF. The total glucan recovery rate was more than 90% for all the pretreatment conditions. In the opposite, most of the xylan was released into the LPF,

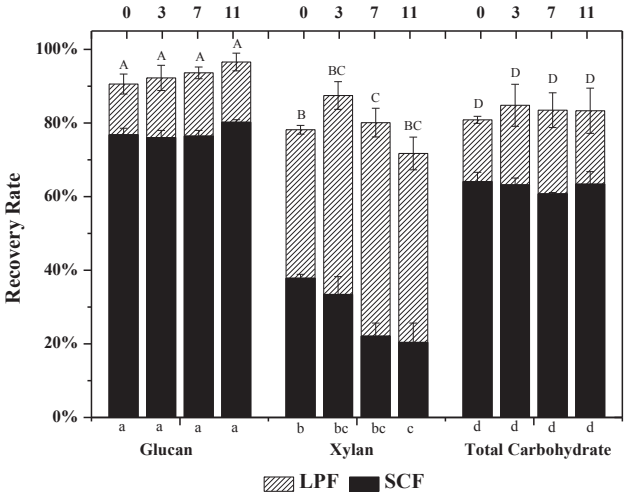

Fig. 2. The carbohydrate recovery rate in the SCF and LPF from the pretreatment at 170 °C with various concentrations of acetic acid used for the pretreatment (with the number on the top indicating the corresponding acetic acid concentration used for the pretreatment). The value represents the mean of replicated samples, and the error bar represents the standard deviation. The bars with the same letter (uppercase for LPF on the top and lowercase for SCF on the bottom) within the same component fraction (glucan, xylan, or carbohydrate) are not significantly different at the 0.05 level based on Tukey's HSD test.

ranging from 40.3% (when no AA was added) to 58% (when 7 g/L AA was used). The recovery rate of xylan in the SCF decreased with the increase of the AA concentration, from 37.8% (0 g/L AA) to 20.4% (11 g/L AA). These results indicated that the xylan recovery in LPF was much more subjected to the influence of AA concentration. However, when the recovery rate of the total carbohydrate was considered, it was not significantly influenced by the AA concentration that has been employed for the pretreatment, with a recovery rate of 60.8–61.1% was observed within SCF. The recovery rate in LPF was only 16.8% when no AA was added, and slightly increased when various concentrations of AA was employed for the pretreatment (Fig. 2). Overall, these results indicated that the concentration of AA employed for the pretreatment at 170 °C did not significantly influence the total carbohydrate recovery rate.

Table 2
Chemical composition of the liquid prehydrolysates fraction (LPF).[a]

Pretreatment conditions		Carbohydrate (g/L)							Degradation products (g/L)			
Acetic acid used (g/L)		Glucose			Xylose			Total Carbohydrate	Acetic acid	HMF	Furfural	Total phenolic compounds
		Monomer	Oligomer	Total	Monomer	Oligomer	Total					
LPF (150 °C)	0	2.1 ± 0.1	1.8 ± 0.2	3.8 ± 0.4	0.1 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	7.5 ± 0.1	2.2 ± 0.3	0.2 ± 0.3	0.2 ± 0.1	3.8 ± 0.1
	3	2.2 ± 0.2	2.1 ± 0.3	4.3 ± 0.2	0.2 ± 0.3	1.7 ± 0.2	1.9 ± 0.3	9.8 ± 0.3	3.9 ± 0.2	0.2 ± 0.3	0.2 ± 0.1	4.0 ± 0.1
	7	2.0 ± 0.2	2.3 ± 0.6	4.4 ± 0.4	0.2 ± 0.2	2.4 ± 0.5	2.6 ± 0.2	10.6 ± 0.4	6.2 ± 0.4	0.2 ± 0.1	0.3 ± 0.1	4.2 ± 0.2
	11	2.4 ± 0.1	2.7 ± 0.4	5.1 ± 0.4	0.3 ± 0.2	3.4 ± 0.1	3.6 ± 0.3	12.7 ± 0.1	9.7 ± 0.8	0.3 ± 0.2	0.6 ± 0.1	4.4 ± 0.2
LPF (170 °C)	0	1.0 ± 0.1	3.9 ± 0.2	4.9 ± 0.4	0.6 ± 0.1	7.1 ± 0.4	7.7 ± 0.4	15.5 ± 0.1	2.4 ± 0.4	0.4 ± 0.4	0.7 ± 0.2	6.2 ± 0.2
	3	1.6 ± 0.1	4.2 ± 0.3	5.8 ± 0.5	0.8 ± 0.1	9.5 ± 0.5	10.4 ± 0.5	20 ± 0.1	4.9 ± 0.6	0.5 ± 0.5	0.8 ± 0.1	6.3 ± 0.2
	7	1.6 ± 0.3	4.5 ± 0.5	6.1 ± 0.3	2.1 ± 0.1	9.1 ± 0.4	11.1 ± 0.5	21 ± 0.5	7.8 ± 0.6	0.7 ± 0.3	1.3 ± 0.2	6.4 ± 0.2
	11	1.8 ± 0.3	4.0 ± 0.6	5.8 ± 0.5	2.7 ± 0.2	7.2 ± 0.8	9.8 ± 0.7	18.4 ± 0.5	11.7 ± 0.8	0.8 ± 0.6	1.7 ± 0.3	6.5 ± 0.1
LPF (190 °C)	0	1.3 ± 0.2	1.0 ± 0.5	2.3 ± 0.6	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	3.1 ± 0.5	3.5 ± 0.4	1.3 ± 0.1	3.2 ± 0.2	11.8 ± 0.2
	3	1.1 ± 0.1	1.0 ± 0.3	2.1 ± 0.5	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	2.8 ± 0.2	5.3 ± 0.3	1.4 ± 0.6	2.9 ± 0.3	11.9 ± 0.2
	7	0.6 ± 0.0	1.2 ± 0.3	1.8 ± 0.2	0.13 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	3.0 ± 0.1	6.6 ± 0.0	1.3 ± 0.5	2.6 ± 0.2	12.3 ± 0.2
	11	0.3 ± 0.1	2.1 ± 0.1	2.4 ± 0.1	0.12 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	2.2 ± 0.1	10.8 ± 0.1	1.8 ± 0.5	3.7 ± 0.3	12.3 ± 0.3

[a] All results shown are the average value ± standard deviation from replicated experiments.

3.2. Detoxification of LPF

During the pretreatment, cellulose and hemicellulose can be degraded to HMF and furfural. Additionally, lignin degradation compounds partially precipitate as high-molecular-weight insoluble particles with the other parts dissolved as soluble TPC in the LPF [10]. As shown in Table 2, generally, the concentration of HMF, furfural and TPC increased with the temperature and AA concentration that was employed for the pretreatment. Comparatively, the temperature played a more significant role than AA concentration for the generation of these degradation products. Under the same pretreatment condition, the TPC concentration was much higher than that of furfural and HMF.

These degradation products (furfural, HMF, and TPC) are all common inhibitors for the downstream fermentation process [10]. As a preliminary test, we tried to carry out SSF with *C. saccharoperbutylacetonicum* for ABE production using the LPF as the substrate. The results showed no cell growth or solvent production (data not shown). Based on our previous study [30], furfural or HMF at the level of < 3 g/L in the fermentation medium does not have significant inhibition on *C. saccharoperbutylacetonicum* for ABE fermentation, while the TPC (*p*-coumaric, ferulic acid, vanillic acid, 4-hydroxybenzoic acid, etc.) derived from lignin, are much more toxic. The TPC inhibit the cell growth even at a very low concentration (< 1 g/L). The inhibitory mechanism of these phenolic compounds has been proposed as disrupting the function of cell membrane via hydrophobic interactions [31].

To decrease the toxicity of LPF, a detoxification procedure is necessary prior to the hydrolysis and fermentation. Various methods for the detoxification of biomass hydrolysates have been previously reported including neutralization, over-liming, evaporation, ion exchange resin adsorption and activated carbon adsorption [10]. Activated carbon has been used as an adsorbent for hundreds of years in wastewater, drinking water, refinery waste, and chemical clarification applications. Comparing to other detoxification methods, activated carbon adsorption is less costly, easier to operate, and the activated carbon is easy to be regenerated [10,32,33]. Thus, activated carbon adsorption was employed in this study for the detoxification of LPF generated at 170 °C with various levels of AA used for the pretreatment.

As shown in Fig. 3, after the activated carbon adsorption, HMF and furfural decreased by 50–60% in the LPF from all pretreatment conditions. Similarly, the TPC concentration decreased by $> 50\%$. The effectiveness of activated carbon adsorption on the removal of these inhibitors has also been reported previously [31,34,35]. It is known that activated carbon detoxifies the pre-hydrolysate by physical adsorption and such efficient removal of the inhibitors is probably due to the high affinity of these compounds to the activated carbon [35]. A common yet unfavorable feature is that detoxification will also cause the adsorption of carbohydrates. Results in Fig. 3 showed that the decrease of glucose, xylose and total carbohydrate was only around 8%, 9% and 11%, which was much lower than that for the removal of inhibitors. Additionally, the adsorption of inhibitors and carbohydrates did not change remarkably for the LPF obtained from the pretreatment with different AA concentrations applied. The adsorption has also removed 6–21% unbound AA (with higher percentage of AA been removed when lower AA was employed for the pretreatment). However, most of the AA was still left in the solution, which could be used as the carbon source for the following ABE fermentation. All these results indicated that activated carbon adsorption was an ideal detoxification approach for selectively removing the inhibitors while keeping most sugars and acetate in LPF.

3.3. The effect of detoxification on enzymatic hydrolysis

Enzymatic hydrolysis was carried out with an enzyme loading of 15 FPU/g glucan for both the detoxified LPF and undetoxified LPF, to evaluate the effects of detoxification on the enzymatic hydrolysis. As shown in Fig. 4, without detoxification, the glucan-to-glucose yield reached a maximum of 73.9% in the LPF when no AA was added for the pretreatment, and a minimum of 56.4% in the LPF when 11 g/L AA was used for the pretreatment. With detoxification, the maximum of glucose yield increased slightly to 77.7% in the LPF when no AA added for the pretreatment, while a minimum of 58.5% (also slightly increased from the case without detoxification) was achieved in the LPF when 11 g/L AA was used for the pretreatment. For the xylan-to-xylose hydrolysis yield, neither the detoxification nor the amount of AA used for the pretreatment made a significant difference. For LPF from all pretreatment conditions, the final hydrolysis yield of xylose was around 78%. Furthermore, the hydrolysis kinetics was similar to each other as well for the detoxified and undetoxified LPF from the same pretreatment conditions. These results indicated that detoxification did not significantly influence the enzymatic hydrolysis in term of the hydrolysis kinetics or the final sugar yield.

3.4. Simultaneous saccharide and fermentation (SSF) of SCF and LPF

For the ABE production from lignocellulosic feedstocks, SSF has been proven to be a preferable approach with various advantages when compared to the separate hydrolysis and fermentation (SHF) [1,36]. In SSF, the feedback inhibition of sugars on cellulases is mitigated because the sugars are consumed by the fermenting organism as soon as it is formed. Moreover, the saccharification (enzymatic hydrolysis) and fermentation are carried out in the same reactor, which simplifies the operation and decreases the cost [36–38]. Generally, for the regular biomass pretreatment, the LPF which contains high levels of inhibitors (including the chemical reagents for pretreatment and the degradation products from the biomass) is discarded and not used for the fermentation. However, in this study, AA was used as the chemical reagent which could be used as a carbon source for ABE fermentation, and also the biomass was pretreated in a relatively mild condition. Besides, the acid-based pretreatment could help release large fraction of the hemicellulose into the LPF. Therefore, with the detoxification process, we expect that the LPF (and thus the mixture of SCF/LPF) in this study could be used for efficient fermentation for ABE production. Thus, we carried out SSF using LPF, SCF and SCF/LPF mixture respectively for ABE production. An enzyme loading of 15 FPU/g glucan was employed for each fermentation. A control fermentation (for the fermentation with SCF) was meanwhile performed using Avicel (20 g/L) as the

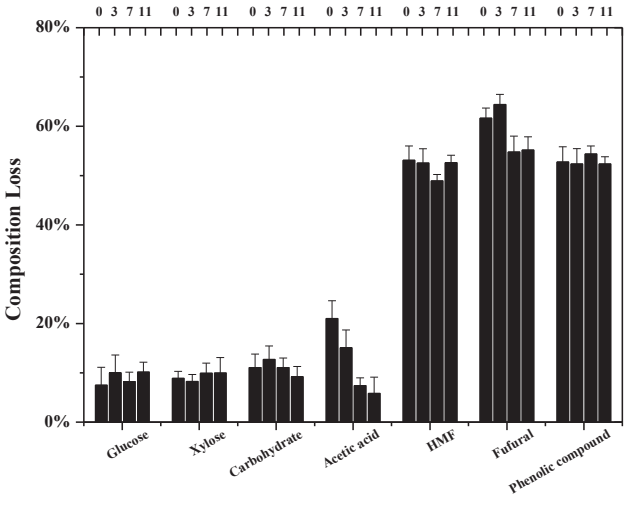

Fig. 3. Effects of activated carbon detoxification on the variation of the composition of the LPF obtained from the pretreatment at 170 °C. The composition loss refers to the decrease in percentage for each compositional content in the LPF after the detoxification comparing to that before the detoxification. The number on the top indicates the acetic acid concentration (0, 3, 7, an 11 g/L) used for the pretreatment. The reported value represents the mean of replicated samples, and the error bar represents the standard deviation.

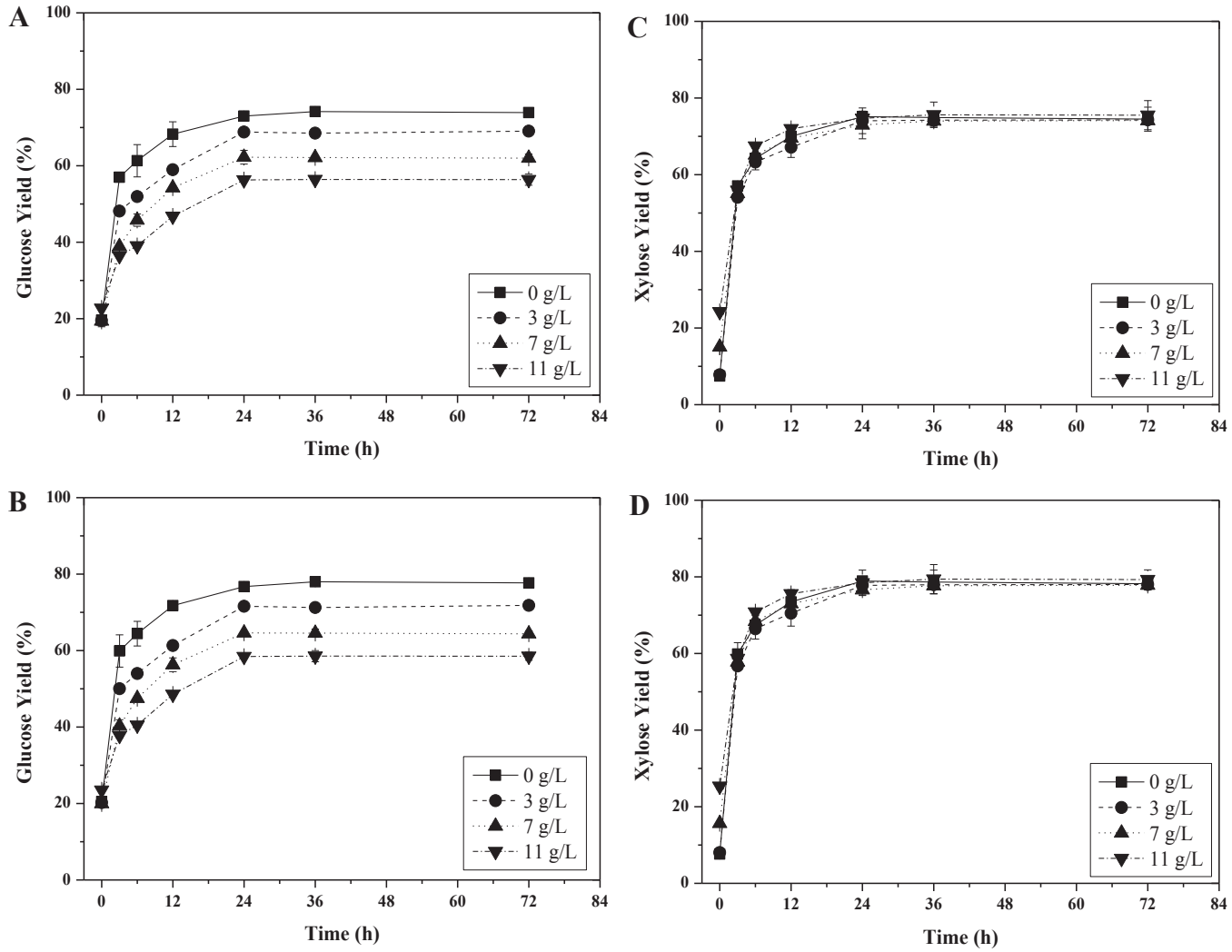

Fig. 4. Effects of detoxification on the enzymatic hydrolysis of glucan (A: before detoxification vs. B: after detoxification) and xylan (C: before detoxification vs. D: after detoxification) in the LPF obtained from the pretreatment at 170 °C with various amounts of acetic acid used for the pretreatment. The reported value represents the mean of replicated samples, and the error bar represents the standard deviation.

feedstock [39]. The fermentation was run for 96 h with the results illustrated in Fig. 5 and Table 3.

For all the fermentations, the reducing sugars (glucose and xylose) were completely consumed at the end (data not shown). This demonstrated that the activated carbon detoxification was very effective for removing the inhibitory compounds in the LPF and enabling the successful fermentation (especially for the LPF and the mixture). In the SCF, 1.5 to 2.4 g/L acetate was detected at the end of the fermentation (Table 3). The production of butyrate was generally low, with 0.7 g/L (when no AA was used for the pretreatment) and 0.3 g/L (when 3 g/L AA was used for the pretreatment) produced. While there was no BA was detected in the fermentation with SCF generated through the pretreatment with 7 or 11 g/L AA was employed. The butanol production increased from 3.0 g/l to 4.3 g/L with the increase of AA concentration from 0 to 11 g/l for the pretreatment, along with the increase of butanol yield from 0.15 to 0.21 g/g correspondingly. The total ABE, like butanol, also increased with the increase of AA concentration that was used for the pretreatment (Fig. 5).

For the fermentation with Avicel, 1.3 g/L acetate and 0.8 g/L butyrate were produced. While this butyrate level was comparable, the acetate level was only about half of that from the fermentation with SCF (when 0 g/L AA was employed for the pretreatment). In the clostridial metabolic pathways, other than glycolysis, the main route for energy (ATP) generation is through the acetate and butyrate production pathways. In the fermentation with pretreated biomass as substrate, the recalcitrant structure and toxic substances after pretreatment will inhibit the cell growth to some extent. To compensate such inhibition and sustain the cell growth, the cell will direct more carbon flow for the acetate and butyrate formation to generate energy, leading to the increased production of acids (especially acetate, because the acetate production pathway is more efficient for energy generation than the butyrate production pathway) [40]. There was 4.8 g/L butanol and 8.0 g/L total ABE produced in the fermentation with Avicel (Fig. 5).

For the fermentation with LPF, the final acetate concentration was kept at approximately the same level as in the original LPF (Table 3). This indicated that the produced acetate during the fermentation has been mostly re-assimilated; however, not all the acetate in the medium could be re-assimilated. The butyrate production was at similar levels (from 1.2 to 1.8 g/L) in all the fermentations with the LPF generated in pretreatment with various AA concentrations. However, these values were much higher than that from the fermentation with SCF. There was high level acetate in the LPF (but not in the SCF); the re-assimilation of acetate led to increased butyrate production (Table 3). For the butanol production, the final concentration ranged from 1.2 to 4.3 g/L depending on different levels of AA used for the pretreatment, with corresponding yields varying from 0.07 to 0.25 g/g. The lowest butanol

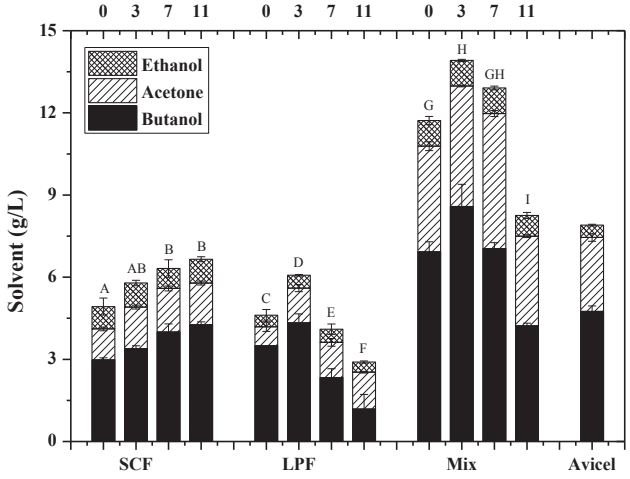

Fig. 5. Solvent production in the simultaneous saccharification and fermentation with *C. saccharoperbutylacetonicum* N1-4 using the pretreated biomass as substrate. The samples were characterized after 96 h of fermentation at 30 °C. The reported value represents the mean of replicated samples, and the error bar represents the standard deviation. The bars with the same letter for the fermentations with the same type of substrate (SCF, LPF, Mix, or Avicel) are not significantly different at the 0.05 level based on Tukey's HSD test.

Table 3
Summary of results from the simultaneous saccharification and fermentation with pretreated switchgrass that has been pretreated at 170 °C.[a]

Fractions	Acetic acid used (g/L)	Starting sugars (g/L)	Acid (g/L)		Solvent Yield (g/g)	
			Acetate	Butyrate	Butanol	ABE
SCF	0	20	2.4 ± 0.2	0.7 ± 0.2	0.15^{a}	0.25^{a}
	3	20	1.7 ± 0.0	0.3 ± 0.1	0.17^{b}	0.29^{b}
	7	20	1.6 ± 0.3	0.0 ± 0.0	0.20^{c}	0.32^{c}
	11	20	1.5 ± 0.1	0.0 ± 0.0	0.21^{c}	0.33^{c}
LPF	0	12.8	3.1 ± 0.2	1.6 ± 0.1	0.25^{d}	0.33^{d}
	3	16.4	4.5 ± 0.1	1.8 ± 0.3	0.25^{d}	0.35^{d}
	7	16.4	6.9 ± 0.2	1.6 ± 0.3	0.12^{e}	0.22^{e}
	11	14.9	11.1 ± 0.9	1.2 ± 0.4	0.07^{f}	0.17^{f}
Mix	0	50.0	1.7 ± 0.4	1.6 ± 0.2	0.14^{gh}	0.23^{g}
	3	53.6	3.8 ± 0.4	1.7 ± 0.1	0.16^{g}	0.26^{h}
	7	53.5	5.8 ± 0.3	1.6 ± 0.2	0.13^{h}	0.24^{gh}
	11	52.1	10.5 ± 0.0	1.1 ± 0.2	0.08^{i}	0.16^{i}
Avicel	1.6	20	1.3 ± 0.1	0.8 ± 0.1	0.24	0.39

[a] The data presented are the average of the results from three independent experiments, and error bars represent standard deviations. Butanol or ABE production values indicated with the same superscript letter within the same faction section (SCF, LPF, Mix, or Avicel) are not significantly different at the 0.05 level based on Tukey's HSD test.

production (1.2 g/L) was observed when 11 g/L AA was used for the pretreatment, while the highest butanol production (4.3 g/L; corresponding to the highest yield of 0.25 g/g) was obtained when 3 g/L AA was employed for the pretreatment. Similar as butanol production, the total ABE reached the highest concentration of 6.1 g/L (corresponding to a yield of 0.35 g/g) when 3 g/L AA was used for the pretreatment, while decreased to 2.9 g/L (a yield of 0.17 g/g) when 11 g/L AA was used for the pretreatment (Table 3). Based on the results from the fermentation with LPF, 3 g/L AA was an appropriate concentration for the switchgrass pretreatment, which could lead to the highest solvent production in the following fermentation. This was different from the results for the SCF fermentation (in which the solvent production increased with the increase of AA level used for the pretreatment). This is because, the AA used for the pretreatment (along with those generated in the pretreatment) all ended up in the LPF (but not SCF). For the SCF, the higher AA used, the harsher condition for the pretreatment (and thus the better accessibility for the enzymatic hydrolysis and microbial fermentation). While in LPF, when AA was too high (> 7 g/L), it will inhibit the cell growth and fermentation (although it benefits at a lower concentration).

Finally, the SCF and LPF was mixed together and used as the carbon source for the SSF. At the end of the fermentation, there was still tremendous acetate left depending on the different conditions (that is the AA concentration used for the pretreatment). However, compared to the fermentation with LPF, clearly under each condition, more acetate was been re-assimilated (Table 3). This was probably because in the SCF/LPF mixture, more carbon source (sugars) was available, and thus led to more efficient acetate re-assimilation, as well as high levels of solvent production. However, the butyrate production was at the similar levels as compared to the fermentation with LPF. The butanol and total ABE production ranged from 4.2 to 8.6 g/L and 8.3–13.9 g/L, respectively, corresponding to the yield of 0.08–0.16 g/g and 0.16–0.26 g/g, respectively. The lowest solvent production was observed when 11 g/L AA was employed for the pretreatment, while the highest solvent production was obtained when 3 g/L AA was used for the pretreatment. When the AA concentration used for the pretreatment was at 0–3 g/L, the solvent yield with the SCF/LPF mixture was lower than that with LPF but higher than that with SCF. However, when the AA concentration for the pretreatment was high (7–11 g/L), on the contrary, the solvent yield with the mixture was higher than that with LPF but lower than that with SCF (Fig. 5 and Table 3). Taken together, these results demonstrated that when no AA (0 g/L AA) was employed for the pretreatment, the biomass recalcitrance barrier could not be effectively overcome, thus leading to lower enzyme digestibility and fermentability of the SCF. While when high concentration (7–11 g/L) of AA was used, the degradation products as well as the high concentration AA in the LPF will inhibit the fermentation. Therefore, 3 g/L AA was determined as the optimal concentration for the switchgrass pretreatment for the ABE production purpose with *C. saccharoperbutylacetonicum*.

The final butanol production of 8.6 g/L in the fermentation with the SCF/LPF mixture was much lower than that from a regular batch fermentation with high concentrations of glucose as carbon source [27]. However, since the total amount of carbon source (as pretreated biomass rather than pure glucose) in the SCF/LPF mixture was low (only around 50 g/L, comparing to, for example, 80 g/L glucose for a regular fermentation), the final butanol production (8.6 g/L) in our SSF process is deemed decent and significant. On the other hand, a large fraction of acetate within the LPF has not been re-assimilated for the biosolvent production. Therefore, metabolic engineering efforts are desired to enable the strain for enhanced acid re-assimilation for the fermentation of the AA-pretreated lignocellulosic biomass. This is currently underway in our lab.

Overall, the novelty of this work is to use AA pretreated biomass for ABE fermentation. This has taken on the consideration that, for an efficient overall bioprocess design, biomass pretreatment needs to be put in the context with subsequent processing steps which can be strongly affected by the kind of biomass pretreatment. AA, which is usually regarded as a strong inhibitor for the microbial ethanol fermentation, is not inhibitory for ABE fermentation but rather acts as a substrate stimulating the fermentation. Therefore, such an approach ends up with more comprehensive and efficient utilization of the lignocellulosic biomass carbon source and minimized pollution. The results from this work have provided important references for real applications for biofuel production from lignocellulosic biomass.

4. Conclusions

Acetic acid (AA) was explored as a catalyst for efficient hydrothermal pretreatment of switchgrass, and the pretreated biomass was further utilized for biobutanol production through acetone, butanol,

and ethanol (ABE) fermentation taking advantage of the acid re-assimilation capability of the *Clostridium* strain. Our results demonstrated that the optimal condition for switchgrass pretreatment was with 3 g/L AA at 170 °C for 20 min, under which most of the xylan was released into the liquid prehydrolysates fraction (LPF) while most of the glucan was reserved in the solid cellulosic fraction (SCF), thus leading to efficient biobutanol production. After detoxification with activated carbon, both the LPF and SCF could be fermented for butanol production through SSF. In the fermentation with the SCF/LPF mixture, 8.6 g/L butanol and 13.9 g/L ABE was obtained, corresponding to high yields of 0.16 g/g and 0.26 g/g, respectively. The results from this study presented an innovative and efficient strategy for comprehensive conversion of lignocellulosic biomass into high value biofuel.

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