Co-production of bio-ethanol, xylonic acid and slow-release nitrogen fertilizer from low-cost straw pulping solid residue

Chen Huang\textsuperscript{a,b,c}, Arthur J. Ragauskas\textsuperscript{c,d,e}, Xinxing Wu\textsuperscript{a,b}, Yang Huang\textsuperscript{a,b}, Xuelian Zhou\textsuperscript{a,b}, Juan He\textsuperscript{a,b}, Caoxing Huang\textsuperscript{a,b}, Chenhuan Lai\textsuperscript{a,b}, Xin Li\textsuperscript{a,b}, Qiang Yong\textsuperscript{a,b,}\textsuperscript{*}

\textsuperscript{a} College of Chemical Engineering, Nanjing Forestry University, Nanjing 210037, China
\textsuperscript{b} Co-Innovation Center for Efficient Processing and Utilization of Forest Resources, Nanjing Forestry University, Nanjing 210037, China
\textsuperscript{c} Department of Chemical & Biomolecular Engineering, University of Tennessee Knoxville, Knoxville, TN 37996, USA
\textsuperscript{d} Department of Forestry, Wildlife and Fisheries, Center for Renewable Carbon, The University of Tennessee, Institute of Agriculture, Knoxville, TN 37996, USA
\textsuperscript{e} Chemical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA

\begin{abstract}
A novel bio-refinery sequence yielding varieties of co-products was developed using straw pulping solid residue. This process utilizes neutral sulfite pretreatment which under optimal conditions (160 °C and 3% (w/v) sulfite charge) provides 64.3% delignification while retaining 90% of cellulose and 67.3% of xylan. The pretreated solids exhibited excellent enzymatic digestibility, with saccharification yields of 86.9% and 81.1% for cellulose and xylan, respectively. After pretreatment, the process of semi-simultaneous saccharification and fermentation (S-SSF) and bio-catalysis was investigated. The results revealed that decreased ethanol yields were achieved when solid loading increased from 5% to 30%. An acceptable ethanol yield of 76.8% was obtained at 20% solid loading. After fermentation, bio-catalysis of xylose remaining in fermentation broth resulted in near 100% xylonic acid (XA) yield at varied solid loadings. To complete the co-product portfolio, oxidation ammoniation of the dissolved lignin successfully transformed it into biodegradable slow-release nitrogen fertilizer with excellent agricultural properties.
\end{abstract}

1. Introduction

Facing continued growth of the human population and accompanying increases in GDP, the demand for energy has continued to grow over the past few decades. These energy demands are currently satisfied primarily by non-renewable fossil fuels, such as petroleum, coal and natural gas. The inevitable depletion of these fossil fuels and their unstable markets have spurred researchers to search for sustainable and renewable resources. Bio-ethanol, a product obtained from lignocellulosic biomass conversion, is an ideal substitute for gasoline. Lignocellulosic biomass is composed of cellulose, hemicellulose and lignin, and the three components are tightly associated and covalently linked in the plant cell wall (Talebnia et al., 2010). The complex structure of the lignocellulosic matrix requires significant degradation through the bio-refinery process to utilize lignocellulosic components for value added production of fuels and chemicals. Bio-ethanol generation based on the biological manufacturing processes utilizes at least five unit operations: chipping, pretreatment, enzymatic hydrolysis, fermentation and distillation (Narron et al., 2016). Pretreatment aims to overcome the recalcitrance of biomass and improve the yield of liberated sugars in the following enzymatic hydrolysis step. Over the years, various pretreatment methods have been investigated and applied successfully, such as dilute acid, dilute alkaline, steam explosion, and many more (Lin et al., 2017; Mosier et al., 2005; Yu et al., 2014). For a pretreatment method to prove to be viable, it should meet the following requirements: (1) improvement of enzymatic digestibility of the pretreated solids; (2) minimal loss of cellulose; (3) low-cost and preferably recyclable chemical reagents and (4) low-environmental impact.

After saccharification of the pretreated biomass, a microbial fermentation of the cellulolytically-liberated monosaccharides is performed to relinquish the final product, ethanol. The two most commonly implemented fermentation methods, separate hydrolysis and fermentation (SHF), and simultaneous saccharification and fermentation (SSF), both have drawbacks (Shen and Agblevor, 2010). Generally speaking, SSF is superior to SHF because the glucose produced from enzymatic hydrolysis is near-instantaneously converted by microorganism, lowering the threat of product inhibition (Akhtar et al., 2014).
In addition, SSF proves superior to SHF along the lines of processing time, contamination risk, energy consumption, ethanol yield and capital costs (Nikolić et al., 2010). In contrast to SSF, SHF can be more advantageous due to the optimized temperature it operates at (50 °C), resulting in enhancement of enzymatic activity. Thus, by applying an enzymatic pre-hydrolysis step prior to SSF, it is plausible that this process will have both the advantages of SHF and SSF. The nature of this process leads it to be labeled as semi-simultaneous saccharification and fermentation (S-SSF), born from manipulation of the advantages of SHF and SSF (Shen and Agblevor, 2010). The one-pot S-SSF process first conducts an enzymatic hydrolysis at an optimal temperature of 50 °C, and then SSF is conducted at 34 °C. One key requirement for S-SSF is high substrate loading for fermentation. This is due to the high ethanol titers required to curb distillation costs. However, high solid loadings are not without a drawback, as the increased system viscosity and inhibitor concentrations penalize ethanol yields (Jørgensen et al., 2007). One approach to overcome these challenges is the adoption of fed batch addition of substrate in combination with the S-SSF.

Besides the aforementioned technical limits, more attention should be paid to identifying a low-cost feedstock for bio-refineries to rely upon. Most traditional studies are based on heavily-investigated biomasses like wheat straw, corn stover, and wood, whose price varies from 30 to 90 US$ per ton (Lee et al., 2009). For a hypothetical bio-ethanol plant with annual output of 1 × 10^5 ton, feedstock costs would reach as high as 1.8 × 10^7 US$ (based on 6 tons of feedstock producing 1 ton of ethanol). Such a significant cost necessitates us to search for a more reliably obtainable and low-cost lignocellulosic starting material. Waste wheat straw (WWS), the solid residue from the straw pulping industry, can meet both the requirements of availability and low price. Prior to pulping, harvested crude wheat straw needs a screening and sorting process to remove the wheat leaves, ears and ash, which are undesirable materials for paper making. This separation step produces large amount of solid residue termed “WWS”, which is used in this study as bio-refinery feedstock. It is reported that the generation of WWS from a Chinese straw pulp mill can reach values of 2 × 10^5–4 × 10^5 ton per year (Huang et al., 2017a). However, most of these residues are currently burned for energy, causing serious air pollution. Considering the relatively high polysaccharides content of WWS, it is feasible to imagine that WWS could be targeted for large-scale bio-refinery applications. Furthermore, bio-refinery co-location within a functioning pulp mill could foster symbiotic economic and technical advantages.

Another obstacle towards the successful industrialization of the bio-refinery is a failure to realize a process which involves utilization of the three biopolymer constituents of lignocellulosic solids (i.e., cellulose, hemicellulose, and lignin). It is well known that cellulose can be easily hydrolyzed into fermentable glucose for conversion to ethanol by Saccharomyces cerevisiae with yields greater than 90% (Balat, 2011). However, a drawback of S. cerevisiae is its inability to ferment xylose (Chu et al., 2013). Other strains such as Pichia stipitis and Candida shehatae utilize both glucose and xylose as precursor for ethanol fermentation, but the low ethanol yields reported limit their application (Karakash et al., 2007). Recently, researchers have reported another mode of xylose utilization, involving xylose conversion to xylonic acid (XA) through oxidation (Zhang et al., 2017). XA is listed as one of the 30 high-value chemicals by US Department of Energy and is a valuable platform chemical with a wide range of applications. For example, it has been successfully applied as a concrete deflocculant to improve the dispersion (Zhang et al., 2017). The XA is also reported to be utilized in some pharmaceuticals as a skin-penetrating antiaging agent to prevent the harm from some toxic metal wastes and radioactive materials. Moreover, it is the precursor for the synthesis of 1,2,4-butanetriol, which is an important intermediate substance for cholesterol-lowering drugs and energetic material 1,2,4-butanetriol trinitrate (Hummel et al., 2010). In this study, we used ammonium sulfite (AS) as pretreatment to conserve most of the cellulose and xylan for downstream utilization. After AS pretreatment, the aforementioned S-SSF protocol was implemented to produce ethanol from cellulose. Following glucose fermentation, an additional step of bio-catalysis was performed upon the remaining xylose to produce XA. Finally, the lignin dissolved during the pretreatment was then successfully converted into slow-release nitrogen fertilizer through oxidation ammoniation (ammoxidation). This work has been performed in effort to validate a novel bio-refinery sequence that can be optimized across a variety of co-products, thereby demonstrating the potential of co-production of ethanol and other value-added products using a low-cost starting feedstock.

2. Material and methods

2.1. Starting materials

Straw pulping solid residue (waste wheat straw, WWS) was kindly provided by Quanlin Industry of Paper (Shandong Province, China). WWS was air dried at room temperature until constant mass was reached, and then subjected to pretreatment.

Cellulase (Cellic Ctec2) was kindly provided by Novozymes (Franklinton, NC, USA). The xylanase (X-2753) and all other chemical reagents in this study were purchased from Sigma-Aldrich (Shanghai, China).

The glucose fermenting yeast S. cerevisiae and xylose bio-catalysis strain G. oxydans NL71 were obtained from the Biochemical Engineering Research Institute of Nanjing Forestry University and ATCC (American Type Culture Collection), respectively.

2.2. Ammonium sulfite (AS) pretreatment

In this study, the pretreatment conditions of temperature and AS charge were optimized. First, the effect of varied AS charges (0–5% (w/v)) on the pretreatment was investigated at the constant temperature of 160 °C. And then, the pretreatment temperatures (130–170 °C) were further optimized. AS pretreatment was conducted in 1 L reactors heated through submersion in a hot oil bath. WWS (50 g, dry weight) was loaded into reactors with 0–5% (w/v) of AS solution (accounting for biomass moisture content) to force the final solid/liquid ratio of 1:10. Each reactor was heated to target temperatures at the rate of 1 °C/min, with target temperatures being maintained for 40 min. After pretreatment, the reactors were cooled via submersion into an ice water bath. Pretreated WWS was then separated by a cloth bag and washed with excessive deionized water until the effluents were clear and colorless. After washing, pretreated WWS was refrigerated at 4 °C until further experimentation. The liquid phase was also bottled and stored at the same temperature.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis was performed at 5% (w/v) substrate loading with a working volume of 50 mL in 150 mL flasks at 50 °C, pH 4.8 and 150 rpm for 48 h. The enzyme cocktail utilized was comprised of cellulase at 25 FPU/g-cellulose and xylanase at 150 U/g-xylan. At the conclusion of enzymatic hydrolysis, aliquots were taken from the system and the sugar concentrations were measured by HPLC (high-performance liquid chromatography). All assays were performed in duplicate, and the results represented the average value between two experimental replications.

2.4. Microorganisms and cultivation

First, the yeast of S. cerevisiae was inoculated in a medium containing 20 g/L glucose, 3 g/L yeast extract and 5 g/L peptone at 30 °C and 150 rpm for 24 h, and then transferred to another fresh medium for further cultivation. After three rounds of sufficient cultivation, the cells...
were harvested by centrifuging at 5000 rpm for 10 min, and then washed with sterile water three times to remove residual medium contents. Next, 50 ml of deionized water was used to re-suspend the cells, and the optical density (OD) of the cells was determined spectrophotometrically at 600 nm. Finally, the cells were refrigerated at 4 °C prior to fermentation experimentation.

The cultivation of G. oxydans was conducted in a medium containing sorbitol (100 g/L) and yeast extract (10 g/L), and the inocula was grown in the medium at 30 °C and 220 rpm for 24 h, followed by transferring to another fresh medium for cultivation. After three rounds of sufficient cultivation, the cells were collected by centrifugation (5000 rpm for 10 min), water-washed three times, and then measured for OD (as described in the previous paragraph).

2.5. Semi-simultaneous saccharification and fermentation (S-SSF) and biocatalysis

S-SSF was conducted in 250 mL flasks with a working volume of 50 mL. First, the enzymatic hydrolysis stage (pre-hydrolysis) was performed at 50 °C and 150 rpm mixing with solid loadings of 5%, 10%, 20% and 30%. For the experiments where the solid loading was more than 10%, a fed-batch process was adopted. In the fed-batch processing, the remaining substrate was dropped into the system at a loading of 5% every 6 h interval until achieving target substrate loading. Importantly, the entire enzyme dosage (cellulase: 25 FPU/g-cellulose, and xylanase: 150 U/g-xylan) were added at the beginning of the pre-hydrolysis, unlike the substrate. After 48 h of enzymatic pre-hydrolysis, yeast (with an initial fermentation OD of 5.0) and nutrients (concentration of 0.08 g/L MgSO4, 0.08 g/L ZnCl2, 0.20 g/L CaCl2 and 0.24 g/L urea) were added to the mixture to perform the following SSSF at 34 °C and 150 rpm mixing for another 120 h. Aliquots were withdrawn during the S-SSF process for the determination of sugar and ethanol concentrations.

After the designated fermentation time, the system was supplanted into a hot water bath to distill the ethanol, and then sterilized at 121 °C for 15 min in an autoclave. To compensate for the water loss during the aforementioned processes, the flasks after sterilization were supplemented with an addition volume of sterile water to re-reach the initial weight of the system. Next, bio-catalysis of xylose was conducted at 30 °C and 220 rpm for 72 h. The initial OD of G. oxydans was set to 5.0. The medium for bio-catalysis includes 5.0 g/L yeast extract, 0.5 g/L MgSO4, 1.0 g/L KH2PO4, 2.0 g/L KHPO4 and 5.0 g/L (NH4)2SO4, which was added to the system along with the strain. Calcium carbonate powder was used to maintain pH ~ 4 during the catalysis process.

2.6. Ammoniation of dissolved lignin for the fixation of nitrogen

Ammoniation was performed in a 1 L stainless steel reactor with mechanical agitation. Spent liquor from AS pretreatment (300 mL) was loaded into the reactor, followed by addition of 10 mL of H2O2 solution (30 wt% H2O2). The reactor was heated to 90 °C and maintained for 90 min with the agitation speed of 300 rpm. After completion of this reaction, the reactor was cooled down at RT. The ammoniated liquid was then centrifuged and filtered to remove suspended solids, and finally freeze-dried to obtain a solid and dry powder.

2.7. Analytical methods

Nitrogen content of the ammonized lignin powders was determined according to the Kjeldahl method, providing values for each sample's total nitrogen (t-N) and organic nitrogen (o-N) contents (Bradstreet, 1965). The carbon content of the powders was quantified using a carbon-nitrogen analyzer (FLASH 2000, Thermo Scientific, Waltham, MA, USA). In addition, quantification of carboxyl group content was analyzed using a conductometric method with modification to previous report (Fras et al., 2004). Finally, FT-IR spectra were obtained using a Bruker VERTEX 80v FT-IR spectrometer (Bruker, Madison, WI, USA).

The chemical compositions of each solid sample were determined according to the two-step acid hydrolysis procedure provided by National Renewable Energy Laboratory (Sluiter et al., 2011). The sugars found in spent liquor were quantified as monosaccharides after 4% sulfuric acid hydrolysis at 121 °C for 1 h to convert oligosaccharides into monosaccharides. Each monosaccharide, as well as ethanol, were analyzed in solution by a HPLC system (high-performance liquid chromatography) equipped with a refractive index detector. A Bio-Rad Aminex HPX-87H column was used for separation with 0.05 M H2SO4 solution as mobile phase flowing at a rate of 0.6 mL/min. The column temperature was set at 55 °C.

For determination of xylose and xylonic acid concentrations during the bio-catalysis process, high-performance anion-exchange liquid chromatography (HPAEC) coupled with a pulsed amperometric detector was used. The HPAEC (Dionex ICS 3000, Thermo Scientific, Waltham, MA, USA) was equipped with a CarboPac™ PA10 column with 0.2 M NaOH and 0.5 M NaOAc containing 0.05 M NaOH as eluent at an elution gradient of 0.3 mL/min. The working temperature of the column was set at 30 °C. Finally, enzymatic hydrolysis yield, ethanol yield, and bio-catalytic XA yield were calculated as follows:

\[
\text{Enzymatic hydrolysis yield(%) = } \frac{\text{glucose or xylose in enzymatic hydrolyzate(g)}}{\text{initial glucose or xylose in substrate(g)}} \times 100\%
\]

\[
\text{Ethanol yield(%) = } \frac{\text{ethanol in fermentation liquid(g)}}{\text{initial glucose in substrate=g.01(g)}} \times 100\%
\]

\[
\text{XA yield(%) = } \frac{\text{xyloic acid in liquid=0.934(g)}}{\text{initial xylene in liquid(g)}} \times 100\%
\]

3. Results and discussion

3.1. Ammonium sulfate (AS) pretreatment

3.1.1. Chemical components of AS pretreated WWS

Generally, WWS is made up of wheat leaves, ears and straw rejects which cannot be utilized for paper making. The structural constituents of the starting WWS used in this study were determined to be 26.5% cellulose, 17.6% xylan and 20.4% lignin. Uniquely, the WWS utilized in this work has an ash content as high as 29.5%, a value that is much higher than most conventional lignocellulosic biomass’s. The ash in WWS is derived primarily from the soil and dust present in the farmland, and is introduced to the material during the feedstock collection process (Huang et al., 2017a). Prior studies have demonstrated that WWS ash is detrimental to hot water pretreatment due to its buffering capacity (Huang et al., 2016). Whereas a neutral ammonium sulfate pretreatment could minimize the negative effect of the ash, because it is expected that the system pH will remain constant (around 7) during the pretreatment. Furthermore, it was reported that the ash weights before and after the pretreatments were almost changeless (Huang et al., 2017b). In this study, the solids after pretreatments were washed with water to remove the free ash, dissolved lignin and other chemicals. To explore the effects of AS pretreatment on the WWS composition, both pretreatment temperature and chemical charge were investigated.

The effect of AS concentration upon chemical composition of pretreated solids at identical temperatures (160 °C) is displayed in Fig. 1A. It can be seen that the extent of delignification significantly increased with increasing AS concentrations. Specifically, hot water pretreatment alone (AS concentration of 0%) only removed 28.5% of lignin. With the addition of 1% (w/v) of AS, lignin removal reached 54.6%. And finally, lignin removal was at its highest value (70.1%) when pretreatment was conducted with a chemical charge of 5%. The increasing delignification was mainly caused by a greater extent of lignin sulfonation, forming the...
soluble lignin derivative of lignosulfonate (Zhu et al., 2009). The AS pretreatment also resulted in partially dissolution of cellulose and xylan. As expected, the removal of xylan was more pronounced than cellulose removal. In particular, cellulose recovery was nearly constant (around 90%) in spite of increases to sulfite loading. In particular, cellulose recovery was nearly constant (around 90%) in spite of increases to sulfite charge. However, xylan recovery reduced from 83.7% (hot water pretreatment) to 64.9% at maximum sulfite loading (5%). In conflict with our finding, Zhang et al. (2017) reported an enhanced xylan recovery at increasing sulfite charge. However, more lignin was removed with increasing sulfite charge, resulting in extended cleavage of various linkages between lignin and hemicellulose. Loss of these linkages can lead to further degradation of hemicellulose, providing a feasible explanation as to why a decrease in xylan recovery was observed during our AS pretreatment operations (Yang et al., 2013).

Concerning pretreatment temperature (as shown in Fig. 1B), increasing temperature (at the AS charge of 3%) promoted the removal of lignin, indicating that a high temperature was beneficial to the degree sulfonation induced upon lignin. In addition, elevated temperatures also enhanced the degradation of xylan. However, only a slight increase in cellulose solubilization was detected with increasing temperatures. Specifically, the recovery of cellulose and xylan decreased from 96.5% and 84.3% to 86.1% and 54.5%, respectively, when the temperature was raised from 130 °C to 170 °C.

It should be noted that xylan recovery in this study was more than 50% under all the tested pretreatment conditions, with a highmark of 84.3% xylan recovery obtained at 130 °C and 3% (w/v) AS. To the best of our knowledge, almost all the sulfite pretreatments reported were conducted in acidic conditions, which resulted in a high cellulose selectivity due to its high delignification and near-complete removal of xylan (Rajan and Carrier, 2014; Zhu et al., 2009). The dissolved xylan in the acidic pretreatment liquor is problematically mixed with lignosulfonate, sulfite, and pretreatment byproducts, creating several issues for downstream utilization of xylan. The main advantage of utilizing neutral sulfite pretreatment is the retention of most of the xylan and nearly all of the cellulose, making the downstream conversion of the two carbohydrates less onerous in addition to reducing the clutter of soluble components in AS pretreatment hydrolysate.

3.1.2. Effect of pretreatment on the enzymatic digestibility of the pretreated solid
It is agreed upon that the presence of hemicellulose and lignin limit both the rate and extent of cellulosic saccharification (Kristensen et al., 2008). The neutral AS pretreatment utilized in this study removes most of the lignin, however, the majority of xylan remains in solid phase after pretreatment. Because of this, an enzyme cocktail containing both cellulase and xylanase was used. Conversion results from enzymatic hydrolysis are shown in Fig. 2. It can be observed that the introduction of AS to the pretreatment system greatly benefitted enzymatic digestibility of the pretreated solids (pretreatment conducted at the same temperature of 160 °C). The condition of hot water pretreatment (0% AS) showed a low cellulose hydrolysis yield of 48.1%. Increasing AS charge enhanced cellulose hydrolysis yields up to 86.9% at sulfite concentration of 3%. However, further increasing the sulfite charge to 4% and 5% resulted in constant cellulose hydrolysis yield. In addition, a similar trend was observed in xylan hydrolysis yield, which increased from 47.3% up to 81.1% with increasing AS charges from 0 to 3%. Enzymatic hydrolysis yield results were consistent with the removal of lignin (Fig. 1), verifying lignin’s role as a barrier to efficient cellulose hydrolysis by cellulase.

Pretreatment temperature was another key parameter affecting
enzymatic digestibility. As shown in Fig. 2B, the temperatures of 130 °C and 140 °C only garnered enzymatic hydrolysis yields of ~50% for cellulose and ~40% for xylan. However, when the temperature was over 140 °C, a significant increase in saccharification of both cellulose and xylan was observed. After pretreatment at 160 °C, optimal cellulose and xylan hydrolysis yields of 86.9% and 81.1% were achieved, respectively. Interestingly, pretreatment conducted at temperature above 160 °C resulted in decreased extents of enzymatic saccharification (82.0% cellulose hydrolysis yield and 81.5% xylan hydrolysis yield at 170 °C). The same findings have also been reported by other researchers, indicating that the excessive delignification incited a negative effect that hampered enzymatic digestibility (Yang et al., 2013). The removal of lignin and hemicellulose can facilitate enzymatic access to cellulose, however, excessive delignification and xylan removal may cause the recrystallization of cellulose, thus depressing the enzymatic hydrolysis (Wei and Cheng, 1985). Based on the above enzymatic hydrolysis results, the optimized pretreatment conditions of 160 °C and 3% AS charge were therefore chosen for the following experiments.

3.1.3. Spent pretreatment liquor analysis

The quantities of components identified in the spent liquor (pretreatment hydrolyzate) from AS pretreatment are presented in Table 1. From these results it can be seen that the pH of all hydrolyzates were in the range of 7–8, except for that obtained from hot water pretreatment (0% AS, pH 6.3). Specifically, the pH increased from 6.3 to 7.9 with increasing sulfite concentrations, explained by the weak basicity of the AS. Moreover, the pH of the AS hydrolyzates decreased at increasing pretreatment temperatures (from 7.9 to 7.1 at 130 °C to 170 °C). This can be explained as increasing pretreatment temperatures facilitating additional degradation of hemicellulose, which leads to production of different organic acids in solution (Garrote and Parajó, 2002). Analysis of dissolved sugar concentrations revealed that only a limited amount of glucose and xylose were produced during the AS pretreatment. At the optimized conditions of 160 °C and 3% sulfite concentration, only 2.5 g/L glucose and 3.2 g/L xylose was detected. These quantities are in agreement with the high carbohydrate recovery from pretreated solids. Concerning fermentation inhibitors, acetic acid derived from hemicellulose degradation was almost the only inhibitor quantifiable. Specifically, acetic acid concentrations were less than 1 g/L at all the pretreatment conditions. This quantity is mostly negligible when compared to inhibitor production from other pretreatment technologies, such as dilute acid pretreatment and bisulfite pretreatment (Zhu et al., 2009). The findings from the AS pretreatment hydrolyzate characterization indicates that AS pretreatment is a mild pretreatment technology capable of extensive lignin solubilization coupled with a minor extent of carbohydrate depolymerization.

### Table 1

<table>
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<tr>
<th>Pretreatment conditions</th>
<th>pH</th>
<th>Fermentation inhibitors (g/L)</th>
<th>Sugar concentration (g/L)</th>
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<td>Temperature (°C)</td>
<td>Formic acid</td>
<td>Acetic acid</td>
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3.2. Semi-simultaneous saccharification and fermentation (S-SSF) and biocatalysis

#### 3.2.1. Fed batch S-SSF for bio-ethanol production

High solid loading with respect to enzymatic hydrolysis and fermentation is known to exert several advantages versus low solid loading, like lowered thermal energy input, decreased hydraulic loads, and decreased cost of ethanol distillation (Modenbach and Nokes, 2013). It has been reported that ethanol concentrations should be greater than 4% (v/v) prior to distillation to avoid needless energy consumption during distillation. This demand translates to a solid loading above 15% being prescribed (Koppram et al., 2014). To achieve this benchmark, the S-SSF process utilizing a fed batch strategy was adopted for this study, with the solid pretreated at the optimized conditions as substrate. Results from the S-SSF unit operation are shown in Fig. 3. First, it can be seen that after 48 h of enzymatic pre-hydrolysis, glucose concentrations reached 22.9, 44.7, 78.7 and 103.3 g/L with substrate loadings of 5%, 10%, 20% and 30%, respectively (Fig. 3A). Decreased cellulose hydrolysis yield (from 85.1% to 64.0%) was observable during enzymatic hydrolysis, indicated a degree of product inhibition, in spite of our adoption of the fed batch strategy. After being transferred to the following SSSF process, glucose was consumed completely after 12 h for the 5%, 10% and 20% solid runs, demonstrating the efficiency with which S. cerevisiae utilized glucose. Unique to the 30% solid loading experiment, glucose concentration remained increasing in the system during the first 12 h, suggesting no ethanol fermentation during the initial half day of fermentation. This phenomenon may be attributed to the pronounced osmotic stress at extremely high glucose concentrations, which suppresses glucose consumption of microorganisms (Otterstedt et al., 2004). After 12 h of yeast adaptation to the system, glucose concentrations finally began to decrease. Further after 72 h of fermentation, no glucose was quantifiable in the broth for all the experiments.

Concurrently with the uptake of glucose, ethanol concentrations began to increase (Fig. 3C). After 48 h of fermentation, ethanol concentrations in 5%, 10%, and 20% solid loadings became constant. However, ethanol concentrations continued increasing until the end during the 30% solid loading run, indicating simultaneous occurrence of both enzymatic hydrolysis and fermentation. After S-SSF, the highest concentrations of ethanol were 11.5, 22.4, 43.1 and 57.2 g/L as the solid loading increased from 5% to 30%. These ethanol concentrations correspond to decreasing ethanol yields of 80.4%, 78.6%, 76.8% and 66.1%, respectively. The reason for the decrease in ethanol yield is likely due to high substrate loading, resulting in poor mass transfer (Wirawan et al., 2012). It should be noted that when increasing solid loading from 5% to 20%, a mere 3.6% decrement in ethanol yield was observed. However, further increasing solid loading to 30% resulted in a 10.7% decrement, indicating the rise of significant mass transfer issues. After the S-SSF experiment, increased ethanol productivity was...
observed (from 0.1 to 0.2, 0.4 and 0.5 g/(L·h)) at solid loadings ranging from 5% to 30%. It can be found that the yeast employed tends to use glucose at a greater rate under the high solid loadings, leading to the higher ethanol productivity.

In addition to glucose and ethanol, a large amount of xylose was also quantified in the fermentation broth, as shown in Fig. 3B. To our surprise, a slight decrease in xylose concentrations was observable across fermentation experiments. Take the 10% solid loading run for example: xylose concentration decreased from 21.6 g/L (initial concentration) to 18.8 g/L at the end of the S-SSF. This phenomenon may ascribe to an interaction between xylose and ethanol that results in the formation of ethylxyloside, thus decreasing the concentration of xylose (Li et al., 2013). At the conclusion of S-SSF, xylose concentrations of 8.8, 18.8, 33.4 and 44.8 g/L were obtained in the fermentation broth at varied solid loadings. These high xylose concentrations support the argument that additional co-product value from xylose will benefit bio-refinery process viability.

3.2.2. Bio-catalysis for XA production

High efficiency xylose conversion is a key factor in the economic performance of lignocellulosic bio-refinery. Conventional xylose-derived products such as ethanol and xylitol are inefficiently produced with respect to time and/or microorganism selection (Nigam, 2001; Rocha et al., 2014). In this study, the product XA was produced from the unconsumed xylose remaining in fermentation broth. To remove the effect of ethanol upon xylose bio-transformation, ethanol was evaporated prior to introduction of the bio-catalysis (as described in Section 2.5).

After ethanol distillation, the bio-conversion process was conducted and the results are shown in Fig. 4. It can be seen that xylose was completely exhausted after 24 h of catalysis at varied solid loadings (Fig. 4A). At the same time, XA accumulated and reached its highest concentrations of 9.9, 19.4, 35.0 and 46.5 g/L after 24 h of bio-treatment. These concentrations correspond to XA yields of 100%, 97.6%, 99.8% and 96.5%, respectively (Fig. 4B). As for XA productivity, it increased from 0.4 to 0.8, 1.5 and 1.9 g/(L·h) with the solid loading increasing from 5% to 30% after 24 h of bio-catalysis. The same with the S. cerevisiae, G. oxydans seems able to convert xylose faster under high substrate loading, resulting in increased XA productivity. The results from our experiments indicate a fast conversion process with high efficiency, achieving near 100% XA yield at varying solid loadings.

Fig. 3. Glucose, xylose and ethanol concentrations in the S-SSF system at different solid loadings.

Fig. 4. Bio-catalysis of xylose for XA production.
3.3. Ammoxidation of dissolved lignin to produce slow-release nitrogen fertilizer

During AS pretreatment, more than 60% of WWS lignin was dissolved into the liquid phase. Traditionally, these lignin enriched liquors are burned after concentration as a source of energy. Unfortunately for some less-developed countries, black liquors are fully discharged into rivers, causing significant environmental pollution. Therefore, exploration of an easily performed and economically-feasible application for black liquors needs to be developed. It has been reported that plants have the potential to use this lignin-contained black liquor as their micro- and macronutrients, especially lignin coupled with nitrogen (Xie et al., 1991). The dissolved lignin (i.e., lignosulfonate) produced from AS pretreatment contains negatively charged sulfonate groups, hydroxyl, phenolic and carboxyl groups which have the potential to participate in a variety of biological and chemical reactions in soil (Meier et al., 1993). Moreover, after AS pretreatment, both free and lignin-linked ammonium can serve as nitrogen source, reducing the required dosage of inorganic nitrogen-providing fertilizers. Importantly, free nitrogen in the form of the ammonium cation is subject to loss by several mechanisms such as volatilization, denitrification, and ground water leaching (Meier et al., 1993). These mechanisms lead to decreased retention of nitrogen within soil. Slow-release nitrogen fertilizers, whose nitrogen can only be released through soil-level microbiological degradation, present an alternative approach to maximizing soil’s nitrogen retention (Ramirez et al., 1997). Oxidation ammoniation (ammoxidation) is a conventional technology for producing slow-release fertilizers from the lignin. During the ammoxidation reaction of the dissolved lignin, ammonium is chemically incorporated into the lignin molecules in the form of amides. Table 2 shows the main parameters of the dissolved lignin before and after the ammoxidation reaction. It can be seen that the abundance of carboxyl groups increased from 3.2 to 8.5 mmol/g after ammoxidation, corresponding to a pH decrease from 7.8 to 4.2 (determined in liquid phase). During the reaction, the aromatic ring is degraded by H2O2, resulting in formation of new carboxylic acid structures similar to muconic acid (Xiang and Lee, 2000). In addition, a slight decrease of total nitrogen (t-N) was observed, which may be caused by the evaporation of ammonia at elevated temperatures. Significantly, 4.9% N was fixed into the lignin as organic nitrogen (o-N), an increment of 3.7% compared to the lignin without ammoxidation. This increase indicates the successful addition of nitrogen to lignin’s chemical structure, forming the slow-release nitrogen fertilizer.

C/o-N ratio is another significant parameter for soil-based microbial degradation of the ammonized lignin fertilizer. According to Meier et al. (1994), biological degradation of fertilizers is hindered at C/o-N ratio > 25. On the other hand, significant organic nitrogen release is caused at C/o-N ratio < 20, therefore values lower than 20 are the industrial benchmark. It was measured that the lignin before nitrogen fixation had a C/o-N ratio of 23.3, which decreased to the acceptable value of 5.2 after ammoxidation (Table 2). These results indicated that the ammoxidation process not only introduces more organic N into the lignin structure, but also increased the biological degradability of the fertilizer product created.

FT-IR analysis was used in this study to investigate the functional changes of the lignin during the ammoxidation reaction. The signals observed at 928 cm⁻¹ and 1103 cm⁻¹ represent glycosidic linkages between glucose units, and C–O and C–C stretching of cellulose and hemicelluloses, respectively (Nikonenko et al., 2005; Shen et al., 2016). However, these two bands disappeared in the ammonized lignin, revealing that the dissolved cellulose and hemicellulose may also participate in the ammoxidation reactions. The signals observed in control sample at 1509 cm⁻¹ (aromatic skeletal vibrations), and 1224 cm⁻¹ (C–O stretching of syringyl and guaiacyl units) became inconspicuous (trace amounts of signals) in the ammonized lignin (Lapierre et al., 1994; Shen et al., 2016; Thangavelu et al., 2014). These results demonstrate that lignin ring opening chemical reactions have occurred during the ammoxidation process. The signal at 1411 cm⁻¹ (N–H stretching of ammonium ions) weakened after the reaction, and this corresponded to the emergence of the C=O stretching vibration in various amides at 1634 cm⁻¹ (Koslick et al., 1997). Another feature of the spectra data is the disappearance of band at 1037 cm⁻¹ (C–O stretching in lignin), which is probably caused by a decrease in methoxy groups in lignin (Movasaghi et al., 2008; Shen et al., 2008). After ammoxidation, the sample no longer shows the functional characteristics of the control lignin, exhibiting strong signals of carbonyl and amide groups.

3.4. Mass balance

When considering large-scale implementation of lignocellulosic bio-refineries, many factors must be simultaneously considered. The cost of feedstock and enzymes, process energy demand, and even the price of the ethanol are significantly decisive. Recently, the price of enzymes has decreased due to rapid development of genetic engineering technology (van Bellen and Li, 2002). However, the cost in feedstock, including harvesting, transportation and storage, remains significant and increasing (Lee et al., 2009). WWS, the residue of straw pulping residue, is a potentially viable bio-refinery residue due to a near-zero cost when co-located in a functioning straw pulp mill. A flowsheet of our entire bio-refinery process utilizing WWS to produce three co-products is shown in Fig. 5.

As can be seen, WWS only contains 264.8 g cellulose, 176.2 g xylan and 204.2 g lignin from 1000 g raw WWS, which can be best explained by the large amount of ash that comes with the bioresource (295.0 g). After pretreatment, 564.0 g solid was recovered, including 239.8 g cellulose, 118.6 g xylan and 73.0 g lignin. Next, we modeled a 20% substrate loading S-SSF and bio-catalysis, resulting in theoretical production of 121.6 g ethanol and 98.6 g xylocidic acid. Our lab-scale results reveal the investigated process to be a high-efficiency conversion route for polysaccharides in WWS, with about 8.2 tons raw WWS consumed for 1 ton bio-ethanol production, accompanied by 810.6 kg XA. In addition, the spent liquor containing dissolved lignin (lignosulfonate) and the lignin residue from S-SSF can both be used as feedstock for ammoxidation to produce slow-release nitrogen fertilizer. Through our work, diverse co-production by a bio-refinery process has been demonstrated as possible. Co-production by lignocellulosic bio-refineries will secure better financial positioning due to diversification of share of revenue streams, relinquishing its dependence upon a single product.

Table 2

Comparison of ammonization product and the control lignin (based on the dry products).

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>t-N (%)</th>
<th>α-N (%)</th>
<th>o-N (%)</th>
<th>C (%)</th>
<th>Carboxyl groups (mmol/g)</th>
<th>C/o-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.8 ± 0.1</td>
<td>14.5 ± 0.9</td>
<td>13.3 ± 0.7</td>
<td>1.2 ± 0.3</td>
<td>27.9 ± 1.1</td>
<td>3.1 ± 0.8</td>
<td>23.3 ± 2.1</td>
</tr>
<tr>
<td>Ammonized</td>
<td>4.2 ± 0.3</td>
<td>14.1 ± 1.1</td>
<td>9.2 ± 0.1</td>
<td>4.9 ± 0.2</td>
<td>25.3 ± 0.7</td>
<td>8.5 ± 0.3</td>
<td>5.2 ± 1.5</td>
</tr>
</tbody>
</table>

* Total nitrogen.
* Ammonium nitrogen.
* Organic nitrogen.
4. Conclusion

AS pretreatment at 160 °C and 30% AS charge retained most of the polysaccharides with high enzymatic digestibility. S-SSF results showed decreased ethanol yields when increasing the substrate loadings, with an acceptable yield of 76.8% at 20% solid loading. Next, bio-catalysis of xylan remaining in the fermentation broth showed nearly 100% XA yields despite varying solid loadings. Finally, ammoxidation of dissolved lignin successfully generated a slow-release nitrogen fertilizer with excellent agriculture properties. About 8.2 tons raw WWS will be consumed for 1 ton of bio-ethanol, in addition to 810.6 kg XA produced for 1 ton of bio-ethanol, in addition to 810.6 kg XA production and large amount of slow-release nitrogen fertilizer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.11.060.

References


