Green Chemistry

Natural deep eutectic solvent mediated extrusion for continuous high-solid pretreatment of lignocellulosic biomass

Journal:	*Green Chemistry*
Manuscript ID	GC-ART-05-2020-001560.R1
Article Type:	Paper
Date Submitted by the Author:	27-Jun-2020
Complete List of Authors:	Ai, Binling; University of Kentucky, Biosystems and Agricultural Engineering; Chinese Academy of Tropical Agricultural Sciences, Haikou Experimental Station Li, Wenqi; University of Kentucky, Biosystems and Agricultural Engineering Woomer, Joseph; University of Kentucky, Biosystems and Agricultural Engineering Li, Mi; University of Tennessee Pu, Yunqiao; Oak Ridge National Laboratory, Joint Institute of Biological Science, Biosciences Division Sheng, Zhanwu; Haikou Experimental Station, CATAS, Food research center Zheng, Lili; Haikou Experimental Station, CATAS, Food research center Adedeji, Akinbode; University of Kentucky, Biosystems and Agricultural Engineering Ragauskas, Arthur; University of Tennessee, Shi, Jian; University of Kentucky, Biosystems and Agricultural Engineering

SCHOLARONE™
Manuscripts

Natural deep eutectic solvent mediated extrusion for continuous high-solid pretreatment of lignocellulosic biomass

Binling Ai,[a, b] Wenqi Li,[b] Joseph Woomer,[b] Mi Li,[c] Yunqiao Pu,[d] Zhanwu Sheng,[a] Lili Zheng,[a] Akinbode Adedeji,[b] Arthur J. Ragauskas[c,d,e] and Jian Shi *[b]

[a]Haikou Experimental Station, Chinese Academy of Tropical Agricultural Sciences, Haikou, Hainan 571101, China

[b]Biosystems and Agricultural Engineering, University of Kentucky, Lexington, KY 40546, USA

[c]Department of Forestry, Wildlife, and Fisheries, Center for Renewable Carbon, University of Tennessee Institute of Agriculture, Knoxville, TN 37996, USA

[d]Joint Institute of Biological Science, Center for BioEnergy Innovation, Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA

[e]Department of Chemical and Biomolecular Engineering, University of Tennessee, Knoxville, TN 37996, USA

*Corresponding to: Dr. Jian Shi, University of Kentucky

Email: j.shi@uky.edu; Phone: (859) 218-4321; Fax: (859) 257-5671

Abstract

Several deep eutectic solvents (DESs) have been demonstrated to be highly effective for lignocellulosic biomass pretreatment, combining the advantages of simple synthesis, relatively low chemical cost and better biocompatibility. However, low biomass loading that is usually involved with DES pretreatment hinders its practical use. In this study, a twin-screw extruder was used for pretreating biomass sorghum bagasse at solid loadings up to 50%, mediated by a neutral-pH DES, choline chloride: glycerol (ChCl:Gly). This continuous extrusion process led to high glucose and xylose yields of >85% from enzymatic saccharification of the pretreated sorghum. A combination of microscopic, spectroscopic, and X-ray diffraction analyses demonstrate a high degree of defibration and disruption of the biomass cell wall structures; however, little or no change in chemical compositions. Further results from gel permeation chromatographic (GPC) and nuclear magnetic resonance (NMR) spectroscopic analyses indicate that ChCl:Gly-mediated extrusion preserved the basic lignin structural characteristics with no significant differences between extruded biomass at a solid loading of 30% and 50%. This study demonstrates the potential of DES-mediated extrusion as a highly effective continuous high-solid biomass pretreatment technology for industrially relevant applications.

Introduction

Processing and upgrading of bio-resources into biofuels and value-added chemicals have been viewed as part of a circular bioeconomy, promoting both economic growth and environmental sustainability. Biomass pretreatment, otherwise known as biomass deconstruction, is the first step in the refining of lignocellulose. Pretreatment is essential for enhancing cellulose accessibility by increasing surface area, disrupting the rigid cell wall structure and/or removing lignin and hemicelluloses.[1] Over the last two decades, a variety of pretreatment technologies have been investigated, which can be generally categorized into acidic (hot water and dilute acid, etc.), alkali (NaOH and ammonia, etc.), and solvent-based (organosolv, and ionic liquid) pretreatments.[2] Ionic liquid (IL) refers to the unique properties of a group of molten salts whose melting points are usually lower than 100 °C. The approximately infinite possible pairs of cations and anions to form an IL enable tailorable physicochemical property and functionality; therefore, ILs are often called "designer solvents".[3] Recently, Gschwend *et al*. have demonstrated that low-cost protic ionic liquids can be used to effectively fractionate Miscanthus grass and up to 77% of the glucose contained in the biomass could be released by enzymatic saccharification.[4] They also reported bioethanol production from highly contaminated waste wood using a low-cost ionic liquid, being able to efficiently recover metals and convert a low cost, toxic environmental hazardous feedstock into a valorized biorenewables.[5] Ionic liquid-based solvent has also been used in natural fiber welding process (Ioncell process) for produce cellulosic textile fibers operating on a commercial scale as an alternative to CS_2 based viscose and N-methylmorpholine N-oxide

(NMMO) monohydrate based Lyocell processes.[6, 7] However, the cost of ILs are still too high to be cost-effective for their large scale application in lignocellulosic biorefineries, especially in the pretreatment step.[8]

Recent advances in deep eutectic solvents (DESs) provide a relatively low-cost choice with comparable performance to ILs for biomass fractionation and lignin extraction application. DESs consist of two or more ionic components acting as either hydrogen-bond donors (HBD) or hydrogen-bond acceptors (HBA). The hydrogen-bonding interaction of HBD and HBA leads to the formation of the DES with a melting point (usually at room temperature) remarkedly lower than that of either individual component.[9] The HBD and/or HBA components of natural DESs are often based on natural metabolites, such as organic acids, amino acids, sugars, and choline salts (so-called natural DESs).[9, 10] Natural DESs, consisting of natural metabolites, are biocompatible,[11] generally non-toxic, and often biodegradable.[12] DESs were proven highly effective in pretreating a diverse range of biomass feedstocks, such as corn cob,[13] sorghum bagasse,[14] and even some feedstocks with high hardness such as endocarps.[15] Several studies have suggested that some DESs preferentially solubilize lignin but not cellulose.[16, 17]

Despite previous research on natural DESs based lignocellulosic biomass pretreatment, the chemical/material cost and energy consumption associated the recovery and separation processes still hinder their application as an industrial-relevant biorefinery technology at scale.[18] Humbird *et al*. conducted a cost analysis to assess the impact of solid loading (during enzymatic hydrolysis of dilute acid pretreated corn stover) on the capital and operation expenses.[19] As the solid loading

increases from 17% to 26%, the total installation cost will reduce about 7% due to the declined number and size of key equipment, such as enzymatic hydrolysis reactors, fermenters, and column diameter. The operation costs will also be significantly reduced because the steam demand for distillation decreases by 30%. Chen *et al.* reported a DESs-mediated pretreatment process at biomass loading of 27% using a batch glass reactor.[20] By acidifying the choline chloride: ethylene glycol (ChCl:EG) with 1% H_2SO_4, the glucose yield reached above 90%, as compared to 10% yield with unacidified ChCl:EG. However, the addition of mineral acid may cause corrosion of equipment. Further increasing the biomass loading in a batch reactor becomes very challenging due to mass transfer limitations. To overcome the aforementioned challenges, a continuous pretreatment process with greater than 50% biomass solid loading is highly desirable.

Extrusion has been frequently employed in the polymer and food industry. Several studies have examined high-solid biomass pretreatment processes based on extrusion because of the effective mixing and rapid heat transfer it provides.[21] Gu *et al*. employed a twin-screw extruder to enhance the enzymatic hydrolysis sugar yield (up to 60%) of wood residuals at solid loadings of 50% to 70% using just water.[22] Gatt *et al*. reported a so-called 'bioextrusion' pretreatment process for corn crop residues at high solid loadings of 14% to 40% supplementing with cellulase-xylanase enzyme cocktail.[23] This process increased the sugar conversion by 194% at 40% solid loading. Coimbra *et al*. reported an alkaline extrusion process pretreating wheat straw at a solid loading of 20% using a 10% NaOH solution, where glucose yield of 73.8% was obtained.[24] Da Silva *et al*. extruded sugarcane bagasse with IL, $[C_2C_1Im][Ac]$ at a high solid loading of 50% and 76.4% of

glucose yield was achieved.[25] The processes mentioned above demonstrate that extrusion enabled high-solid pretreatment of lignocellulosic biomass. Furthermore, extrusion can be operated continuously with controlled temperatures and residence times, which is critical for industrial applications.[21]

Despite the recent studies on DES pretreatment, most of the reported DES pretreatment processes employ batch processes at low biomass loadings ranging from 5% to 10%. This study, for the first time, investigated pretreatment of biomass sorghum bagasse with a neutral pH DES, choline chloride: glycerol (ChCl:Gly), *via* a twin-screw extruder to assess the overall pretreatment performance of continuous extruding process at high solid loadings. The effect of barrel temperature and residence time on pretreatment efficiency was investigated at biomass loadings of 30 wt% and 50 wt%. A combination of microscopic, spectroscopic, and X-ray diffraction analyses was used to evaluate the impact of the ChCl:Gly-mediated extrusion on the structural and chemical changes of biomass components. This study demonstrates the potential of DES-mediated extrusion as a promising high-solid loading continuous biomass pretreatment process for industrial-relevant applications.

Results and discussion

Continuous extrusion pretreatment and enzymatic hydrolysis

Batch pretreatments were performed in high-pressure glass reactors at 180 ºC to assess the effect of biomass loading and pretreatment time on pretreatment efficiency. With increasing biomass

loading, starting from 10% to 20%, the biomass recovery increased from 77.0% to 81.2% (Table 1). When the biomass loading continued to increase to 30%, and the pretreatment time was extended from 2 h to 4 h, the biomass recovery reverted to 76.9%, showing the negative correlation between solid loading and biomass dissolution. Very little glucan (1.9-2.3%), xylan (1.7-3.2%), and lignin (4.0-5.4%) were removed during the batch pretreatments. The decrease in efficiency of enzymatic hydrolysis was also correlated with biomass loading, though by extending pretreatment time hydrolysis efficiency was retained (Fig. 1). At 10% solid loading, both glucose, and xylose yields increased to greater than 63% during the enzymatic hydrolysis of the pretreated solids, as compared with the glucose and xylose yields of 28% and 24%, respectively for raw biomass. Further increase of solid loading to 20-30%, both glucose and xylose yields fell to approximately 40%.

Compared to batch pretreatment, DES-mediated extrusion can tolerate much higher solid loading of up to 50%, which is not possible with batch pretreatment. Extrusion pretreatment led to little changes in biomass composition, but a great improvement on enzymatic hydrolysis sugar yields. At same pretreatment temperature of 180 ºC, the sugar yields (65.0% and 60.6% for glucose and xylose, respectively) from enzymatic hydrolysis of extrusion pretreated biomass at 30% solid loading for 16 min residence time were comparable to that of batch pretreatment at 10% solid loading for 2 h. Increasing extrusion time from 16 min to 40 min (4 to 10 passes), glucose and xylose yields increased to 85.8% and 84.4%, respectively. When solid loading was increased to 50%, similar glucose and xylose yields (87.0% and 86.5%, respectively) were obtained, and the

same trend was observed with the increasing of extrusion time. Lowering the extrusion temperature to 150 ºC, sugar yields declined markedly even if the residence time was extended to 60 min. Results from this work are comparable to a previous study, where sugarcane bagasse was pretreated with IL, $[C_2C_1Im][Ac]$ using a twin-screw extruder.[25] Approximately 90% of glucose yield was obtained from enzymatic saccharification of extruded bagasse at a solid loading of 25%, while the glucose yield fell to 76.4% as the solid loading increased to 50%.

Fig. 2 shows the mass flow of glucan, xylan, and lignin in sorghum bagasse during pretreatment and enzymatic saccharification for two representative batch and extrusion pretreatment conditions. In batch-type pretreatment (with 30% of biomass loading at 180 ºC for 240 min), about 3.9 g lignin and 3.3 g xylan were removed during pretreatment as compared to 2.4 g lignin and 2.1 g xylan removal in continuous extrusion (with 50% of biomass loading at 180 ºC for 40 min). Results indicate that ChCl:Gly only solubilized a small portion of lignin and xylan from sorghum bagasse. It is generally believed that a mild alkaline pretreatment is needed to solubilize reactive lignin *via* proton extraction, while much stronger alkaline conditions are needed to solubilize xylan.[26, 27] The limited effectiveness of ChCl:Gly was hypothesized to be due to limited solvation effects with lignin and xylan. High biomass loading, on the other hand, means low DES to biomass ratio, further limiting lignin solubilization at high biomass loading. Upon extrusion pretreatment, 29.7 g of glucan and 17.6 g of xylan were recovered, and 27.9 g of glucose and 14.5 g of xylose were obtained from enzymatic hydrolysis. The overall yield of glucose and xylose from were 75.6% and 65.1% of the theoretical yield with extrusion, compared with 42.5%

and 47.7% with batch pretreatment. Despite the lower lignin and xylan removal, biomass digestibility has been significantly improved by extrusion pretreatment. It is speculated that the improvement of enzymatic saccharification is not only achieved by lignin removal but also the structural changes of biomass components.[28]

Adding acids to DES pretreatment usually leads more effective solubilization of hemicellulose sugars.[29] DES pretreatment under alkali conditions was reported to remove a high fraction of lignin in biomass.[2, 30] The better availability and reactivity of acidic protons is critical for acid-catalyzed cleavage of lignin-polysaccharide interlinkages in a DES environment.[20] Chen *et al*. reported that acidified ChCl:Gly with 1% H_2SO_4 in batch DES pretreatment was effective in fractionating switchgrass and subsequently improving biomass digestibility.[31] Xia *et al*. reported a three-constituent DES, acidified ChCl:Gly with $AlCl_3$ (as a Lewis acid), where lignin fractionation efficiency was drastically increased from 3.6% to 95.5%.[32] Acidic DESs, such as ChCl:Oxalic acid, ChCl:Formic acid, and ChCl:Acetic acid, also showed impressive fractionation efficiency.[33-35] Another acidic DES, ChCl:Lactic acid, was used in a previous study to fractionate lignin from high-lignin content endocarps. As high as 64.3% (for walnut endocarp) and 70.2% (for peach endocarp) of lignins were removed during pretreatment at 10% biomass loading, demonstrating the superior lignin solubility of ChCl:Lactic acid DES.[15]

Extrusion was used as an effective pretreatment for many types of lignocellulosic biomass,[21] and is usually performed in the presence of additives, such as alkali,[36] ethylene glycol,[37] glycerol,[38] and ILs.[25] DES (ChCl:Gly) could be a promising additive for extrusion partially due to its neutral

pH, which causes less corrosion to extruders and elimination of neutralization step before enzymatic hydrolysis. It has reported that extrusion in the absence of additives causes accumulation, burning, and die blockage owing to the poor flow of characteristics lignocellulosic feedstocks.[25] Flow instability (i.e., flow pattern and rate), burning, and carbonization were observed in the trials without ChCl:Gly in this study (data not shown). The instability is likely caused by varying rheological behavior of the biomass feedstock at high solid loading at extrusion conditions (high temperature and pressure in different extrusion zones). In contrast, the addition of DES greatly improved the flow stability and pretreatment performance, possibly due to less abrasion of the extrusion barrel, decreased operational torque, and less local overheating. Moreover, DES with better cellulose affinity and wettability could lead to more effective fibrillation of the crystalline cellulose structure into submicron- or nano-scale fibers, resulting in improved enzymatic accessibility.[37]

Characterization of raw and pretreated biomass

The ChCl:Gly-mediated high-solid extrusion, as stated above, resulted in minor removal of lignin and hemicelluloses. Despite the minimal compositional changes, it was believed that the pretreated biomass underwent physicochemical structural changes. To understand the structural and chemical changes of biomass after the DES-mediated extrusion, complementary spectral and chemical approaches, such as scanning electron microscopy (SEM), X-ray diffraction (XRD), were carried out in addition to the compositional analysis to better understand what causes the great improvement of enzymatic digestibility of the pretreated biomass.

Mechanical pulverization and fibrillation of the biomass occurred during extrusion. In the case of extrusion with biomass loading of 30%, extruded sorghum bagasse was of black color and in the form of pellets or blocky fragments. Extruded at biomass loading of 50%, sorghum bagasse become granular or powdery and looked dark brown. After washing, the extruded residue with biomass loading of either 30% or 50% was a brown powder. The SEM images presented in Fig. 3 demonstrate the changes in the surface morphology of the materials after extrusion. Clearly, DES-mediated extrusion had resulted in the collapse and disruption of the surface structures of biomass in addition to size reduction. The extruded biomass exhibited highly disordered structures and irregular fibers. No visible differences can be regarded as significant between the extruded samples at biomass loading of 30% and 50%, suggesting that DES-mediated extrusion can tolerate different biomass loadings.

The X-ray diffraction profiles for raw and extruded sorghum bagasse are presented in Fig.4. The extrusion at biomass loading of 30% led to a decrease of the crystallinity index (CrI) from 55.8 to 35.8%. The bagasse extruded at biomass loading of 50% showed less change/decrease in CrI (49.8%) as compared with untreated bagasse (55.8%). Cellulose crystallinity is reported among the factors affecting enzymatic digestibility of cellulose, however, in the present study no significant difference of enzymatic digestibility between the samples extruded at biomass loading of 30% and 50% was observed despite their different CrI. Laureano-Perez *et al*. found that the cellulose crystallinity mostly influenced the initial hydrolysis rate.[39] This is in support of our results that crystallinity effect was not reflected in the enzymatic digestibility after 72 h of

enzymatic hydrolysis. Moreover, cellulose hydrolysis is also governed by other factors, such as lignin/hemicellulose contents and distribution, porosity, and particle size,[40] polymerization degree as well.[41] Crystallinity effects alone do not explain the cellulose hydrolysis trends.[41] To further determine the effect of the ChCl:Gly and extrusion on the lignin structure, GPC, and HSQC NMR analysis were performed.

Characterization of lignin streams

The weight-average molecular weight (M_w), number-average molecular weight (M_n) and polydispersity index (PDI) of lignins extracted from raw and extruded sorghum bagasse are presented in Table 2. The molecular weights of the lignins from extruded biomass shifted to lower values than that from the untreated. The average M_w of lignin from raw sorghum bagasse was 6109 Da, while the average M_w of lignin from extruded bagasse declined to 3479 and 3252 Da for 30% and 50% solid loading, respectively. This decline indicated the depolymerization of lignin caused by the DES-mediated extrusion. The PDI values reveal the width of the size distribution of lignin samples. The increase of PDI from 1.4 to 1.7-1.9, showing a wider span of M_w, suggested that extrusion of biomass lead to a wider molecular size distribution of lignin in comparison to the non-treated biomass lignin. No significant difference in either average molecular weight or DPI was observed between the lignin from samples extruded at 30% and 50% biomass loading.

To examine the structural changes of lignin during the extrusion, 2D ^{1}H-^{13}C HSQC NMR was applied to characterize the extracted lignins from raw and extruded biomass. The aromatic region between 6.0-8.0 (^{1}H) and 90-150 (^{13}C) ppm examining key lignin monolignol subunits is shown

in Fig. 5 (top). The spectra of the aromatic region of CEL (the lignin from raw sorghum bagasse) revealed that natural sorghum lignin is SGH type lignin with hydroxycinnamates such as ferulate (FA) and p-coumarate (pCA). The S:G:H ratio was 0.33:0.63:0.04, based on the total SGH amount. Also, the natural sorghum lignin had 50.9% of pCA and 6.3% of FA. After DES-mediated extrusion, the lignin samples maintained the basic aromatic lignin structure characteristics but also underwent structural changes. The abundance of S:G:H turned to 0.52:0.47:0.01 when the biomass was extruded at the loading of 30%, and 0.46:0.53:0.01 at a loading of 50%. It suggested that some condensation reactions occurred during the extrusion process. The results also showed the decrease of H units in the lignin after extrusion. In addition, the signals of tricin in the lignin was completely depleted after the DES-mediated extrusion. During the CEL extraction, complete removal of dioxane as the solvent was challenging and trace amount of dioxane typically remained in the samples as the impurity from the procedure of dioxane extraction, rotary evaporation, and freeze drying. This leads to the dioxane signal in the NMR HSQC spectra of the isolated CELs.

Another important parameter, the relative content of linkages (such as β-*O*-4′, β-5′, and β-β′), were also calculated to determine the changes of lignins. The aliphatic region between 2.5-6.0 (^{1}H) and 50-90 (^{13}C) ppm examining lignin inter-units and side chains is shown in Fig. 5 (bottom). The results illustrated that β-*O*-4′ linkage was the major inter-unit linkages with the presence of a small amount of β-5′ and a limited amount of β-β′. The relative content of β-*O*-4′ linkage in raw lignin was 42.7%, while it was reduced to 30.1% and 23.5% after extrusion at biomass loading of 30% and 50%. This result suggested that a small quantity of this linkage was cleaved during the

extrusion. The relative contents β-5′ were increased to 5.2% and 5.4% after the extrusion. Lignin is available as a by-product of lignocellulosic biorefineries, but it also can serve as a renewable resource for the production of bio-based materials and platform chemicals and fuels.[42] Further valorization of lignins requires detailed insight into their structure and composition, as the final molecular structure determines which lignin is best suited for what valorization strategy.[43] The above structural results are presented as useful background information when devising lignin valorization strategies for this DES-mediated extrusion process.

Effect of residual DES on enzymatic hydrolysis

In order to evaluate the effect of residual DES on enzymatic hydrolysis, different concentrations of ChCl:Gly was added into the washed pretreated sorghum bagasse (extruded with 50% of biomass loading at 180 ºC for 40 min) to assess compatibility with cellulolytic enzymes. The solid loading in this enzymatic hydrolysis is 20%. Fig. 6 shows the effect of ChCl:Gly addition on enzymatic hydrolysis. With ChCl:Gly addition up to 5%, no reduction in glucan hydrolysis was observed, but some reduction in xylan hydrolysis. This data suggests that that ChCl:Gly is probably more inhibitory to hemicellulases than cellulases. With 10% ChCl:Gly addition, glucose yield decreased from slightly from 79.7% to 71.7%; while xylose yield decreased from 65.8% to 53.7%. Further increasing ChCl:Gly concentration to 25% led to 20-30% inhibition in both glucan and xylan hydrolysis sugar yields. Results from this study are complementary to a previous report from Xu *et al*. who evaluated the growth of *Saccharomyces cerevisiae*, a well-studied and established strain for the industrial production of ethanol, in the presence of 5% of DESs prepared

by mixing ChCl with urea, ethylene glycol, xylitol, isosorbide, and glycerol in different molar ratios.[44] DES made of ChCl:Gly (1:2 molar ratio) showed excellent biocompatibility; yeast growth was not compromised even with the presence of 5% ChCl:Gly DES. The biocompatible DESs enabled one-pot production of cellulosic ethanol that integrated biomass pretreatment, enzymatic saccharification, and ethanol fermentation in a single process.[44]

To explore the possibility of washing-free or less washing processes, the effect of residual ChCl:Gly (2-10%) on enzymatic hydrolysis was investigated. The sugar yields with residual ChCl:Gly were slightly lower than the sugar yields obtained by adding the same percentage of pristine ChCl:Gly (Fig. 6). With 2% and 5% residual ChCl:Gly, glucose yield decreased about 5% and 10%, respectively, compared with almost no reduction by adding pristine ChCl:Gly to the same percentage. In the present study, 64.8% glucose and 47.9% xylose were liberated with commercial enzyme cocktail (Novozymes Cellic® CTec2 and HTec2) in the presence of 10% ChCl:Gly. Xylan hydrolysis appeared more vulnerable than glucan hydrolysis to the residual DES. With 10% residual ChCl:Gly, the glucose yield decreased about 20% as compared to 35% reduction in xylose yield on the basis of no-DES control; glucose and xylose yields decreased about 7% and 6%, respectively, compared with adding the same percentage of pristine ChCl:Gly. Inhibitors such as hydroxymethyl furfural and furfural formed from sugar degradation were reported to be inhibitory to fermentation but not inhibitory to cellulases at concentration of < 4g/L.[45-47] Since we didn't detect hydroxymethylfurfural (HMF) and furfural (in noticeable peaks using HPLC analysis, data not shown) in the pretreatment liquid, it is possible that the phenolic

compounds derived from lignin degradation could have played a major role hindering enzymatic hydrolysis.[44, 48]

Because DES-mediated extrusion pretreatment only removed a small portion of xylan, hemicellulase to cellulase ratio in the enzyme mixture might need to be adjusted to improve the xylose yield further. Previous reports showed that increasing hemicellulase loading or adding a surfactant to ammonia fiber expansion (AFEX) or hot water pretreated corn stover, containing high xylan content, can greatly improve xylose yield.[49-51] Combining the results from the biocompatibility study, as compared to a conventional DESs pretreatment process, where large amounts of water were used to wash pretreated solid thoroughly, it is possible to develop a wash-free or less washing process to bring down the residual ChCl:Gly to less than 10%. Engineering DES-tolerant enzymes could be another strategy to promote sugar yield.[52]

The cost of DESs is still a key determinant of the technological and economic viability of DESs-based pretreatment processes. The price of pretreatment medium is the most important parameter in determining the economic viability of pretreatment technology.[53] Many efforts have been made to control the cost using DESs as an alternative pretreatment medium, including developing low-priced DESs, recycling and reusing DESs. ChCl:Gly is a relative low-cost DES and can be mass-produced. The waste glycerol can be used to prepare ChCl:Gly with similar properties to that using pure glycerol.[54] Approximately 300,000 m^3 of waste glycerol was produced in 2015 from the biodiesel industry and will reach 400,000 m^3 in 2022.[55] Chen *et al*. have demonstrated recycle and reuse of ChCl:Gly for at least five more pretreatment cycles (120°C, 60

min) while maintaining its pretreatment capability.[31] Klein-Marcuschamer *et al*. showed that reducing solvents loading is more important than increasing the rate of solvents recycling, as it brings several simultaneous advantages for biorefineries when recycling rate is higher than 95%, including lower capital cost, lower electricity use, and lower working capital.[53] In addition, value-added products, such as lignin-derived products, will impact the feasibility of the process.[53, 56] This DES-mediated high-solid extrusion at a short time without compromising the benefits of high sugar yields obtained at high DESs loading could improve the overall economics of DESs-based pretreatment processes.

Conclusions

To obtain a high biomass loading in the pretreatment process, a twin-screw extruder was employed for pretreatment of sorghum bagasse, mediated by a neutral pH DES choline chloride:glycerol (ChCl:Gly). This continuous extrusion process showed a high pretreatment performance at a biomass loading as high as 50%, both glucose and xylose yields reaching up to 85%. These sugar yields were much higher than those from batch-type pretreatment in high-pressure glass reactors at biomass loading of 10%. Characterization of the pretreated biomass showed that the DES-mediated extrusion effectively pulverized and fibrillated the biomass and decreased the crystallinity. Also, the characterization of lignin streams demonstrated that lignin maintained the basic lignin structure characteristics during the extrusion. Results demonstrate the DES-mediated extrusion process as a promising technology that enables continuous pretreatment of lignocellulosic biomass at high solid loading.

Experimental section

Material

Sorghum bagasse (*Sorghum bicolor*, forage variety ES5200) was provided by the Bioenergy Feedstock Library, Idaho National Laboratory (Idaho Falls, ID). The air-dried bagasse was ground by a Wiley Mill to pass a 2 mm sieve. The ground sample was dried in a convection oven at 40 °C overnight and stored in Ziploc bags at room temperature for subsequent use. All chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Waltham, MA, USA). Cellulase (Cellic® CTec2) and hemicellulase (Cellic® HTec2) were provided by Novozymes North America (Franklinton, NC, USA). The DES, ChCl:Gly was prepared by mixing choline chloride (ChCl), and glycerol (Gly) at a molar ratio of 1:2, followed by heating at 60 °C with continuous stirring until a homogeneous and clear solvent was obtained.[57] The DES was allowed to cool in a desiccator to ambient temperature and stored for use in pretreatment. The pH of the synthesized DES is measured to be 7.1.

Continuous pretreatment using a twin-screw extruder

A laboratory-scale twin-screw extruder (EuroLab 16, Thermo Scientific, Germany) was used for the pretreatment. The extruder consisted of a barrel and rotatable twin screws that convey material continuously from the input to the output. The barrel and screws are composed of EN40B wear-resistant steel. The reaction zone was divided into a conveying zone, kneading block zones, and reaction and compression zones by screws of different pitch lengths and with a reverse conveying

screw element (Fig. 7).

Sorghum bagasse was mixed with ChCl:Gly at biomass loadings of 30 wt% and 50 wt%, respectively, and allowed to impregnate overnight. The set temperature of each extrusion zone was kept constant at either 150 ºC or 180 ºC depending on the pretreatment conditions. The extrusion screw speed was set at 35 rpm. Bagasse-DES mixtures were run through the extruder in passes until the desired number of passes was achieved. The average residence time of one pass was 4 min, with slight variations due to the feeding rate. Extruded biomass was rinsed with 20 ml of ethanol and centrifuged at 4000 $\times g$ for 10 min to separate the pretreated solid and liquid fraction. The solid fraction was washed five additional times with 20 ml of ethanol each time to remove residual DES, and then freeze-dried for future use.[15] The liquid fraction was stored at 4 ºC for sugar determination using HPLC.

Batch pretreatment in high-pressure glass reactors

As a control for the extrusion experiment, batch-type pretreatment was performed in ACE glass pressure vessel reactors. ChCl:Gly was added into 38-mL-capacity glass pressure tubes containing sorghum bagasse to biomass loadings equivalent to 10 wt%, 20 wt%, and 30 wt%, respectively. The mixture (10 g mass in total) was impregnated overnight and then pretreated at 180 °C in an oil bath for 2 or 4 h with constant stirring using a magnetic stir bar at 200 rpm. After pretreatment, the slurry was rinsed and centrifuged as described above.

Enzymatic saccharification

Enzymatic saccharification of raw and pretreated sorghum bagasse samples was conducted according to the NREL laboratory analytical procedure.[58] In the enzymatic hydrolysis experiments to evaluate the pretreatment efficiency, the glucan loading was 1% with 50 mM sodium citrate buffer (pH 4.8) and 0.02% sodium azide as a microbial inhibitor. The cellulase (Cellic® CTec2, protein content 188 mg/ml) was used at an enzyme loading of 20 mg protein/g glucan supplemented with hemicellulase (Cellic® HTec2, protein content 27 mg/ml) loading of 0.26 mg/g glucan.[15] In the enzymatic hydrolysis experiments to evaluate the inhibitory effect of ChCl:Gly, biomass with different residual ChCl:Gly concentrations (2%, 5%, and 10%) was obtained by mixing the washed and unwashed pretreated biomass in different proportions, and the total solid loading was 20%. After 72 h of incubation at 50°C with rotational shaking, suspension liquid was sampled and soluble sugars were measured by HPLC, as described in the next step.

Characterization of raw and pretreated biomass

The composition of raw and pretreated biomass was determined with two-stage acid hydrolysis according to the NREL laboratory analytical procedure.[59] The concentration of sugars were determined using an HPLC (Ultimate 3000, Dionex, USA) equipped with a refractive index detector (RID) and a Biorad Aminex HPX-87H column.

A scanning electron microscope (S-4800, Hitachi, Japan) was used to take images of the raw and pretreated samples at 10 kV acceleration voltage after gold coating. X-ray diffraction analysis was done by an X-ray powder diffractometer (D8-Advance, Bruker, Germany) to determine the

crystallinity of samples. The samples were scanned over a 2θ range of 10-50° at a scanning rate of 1 degree per minute. The crystallinity index (CrI) was calculated as the following equation

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100\%$$

where I_{002} is the overall intensity of the peak at 2θ about 22° and I_{am} is the intensity of the baseline at 2θ about 18°.[40]

Characterization of lignin

Lignin isolation with a cellulolytic enzyme. The raw and extruded bagasse samples were subjected to enzymatic hydrolysis with a mixture (1:1 by volume) of Cellic® CTec2 and HTec2 in 5 mM citrate buffer (pH 4.8, 50°C) under continuous agitation at 200 rpm for 48 h. The residue was isolated by centrifugation and was hydrolyzed once more with freshly added enzymes mixture. The residue obtained was rich in lignin and was washed with deionized water, centrifuged, and freeze-dried. The lignin-enriched residue was extracted with dioxane-water (96% *v/v*, 10.0 mL/g biomass) for 24 h. The extracted mixture was centrifuged, and the supernatant was collected. Dioxane extraction was repeated once by adding fresh dioxane-water. The extracts were combined, roto-evaporated to reduce the volume at less than 45°C and freeze-dried. The dioxane extracted CEL yields from the raw sorghum bagasse, the extruded 30% biomass, and the extruded 50% biomass are 3.5%, 3.7%, and 3.8%, respectively, based on the dry weight of biomass, which have been included in the revised manuscript. The purity of the CELs, determined as Klason lignin, extracted from the raw sorghum bagasse, the extruded 30% biomass, and the extruded 50%

biomass are 88%, 90%, and 87%, respectively. The results are comparable to the CEL purity of 84-89% measured from literature.[60] To preserve structural features of the isolated CEL, no further purification was performed in this study, therefore carbohydrates signals were observed in NMR spectra. The obtained lignin samples were used for further analysis.

Gel permeation chromatographic (GPC) analysis. The weight-average molecular weight (M_w) and number-average molecular weight (M_n) of lignin were measured by GPC after acetylation, as previously described.[14] Briefly, lignin derivatization was conducted based on ~3 mg lignin in 1 mL of pyridine/acetic anhydride (1:1 *v/v*) in the dark at room temperature for 24 h at 200 rpm. The solvent/reagents were removed by co-evaporation at 45°C with ethanol several times, using a rotatory evaporator until dry. The resultant acetylated lignin was dissolved in tetrahydrofuran (THF), and the solution was filtered through a 0.45 μm membrane filter before GPC analysis. Size-exclusion separation was performed on an Agilent 1200 HPLC system (Agilent Technologies, Inc, Santa Clara, CA, US) equipped with Waters Styragel columns (HR0.5, HR3, and HR5E; Waters Corporation, Milford, MA, US). A UV detector (270nm) was used for detection. THF was used as the mobile phase at a flow rate of 0.3 mL/min. Polystyrene narrow standards were used for establishing the calibration curve.

NMR spectroscopic analysis. Nuclear magnetic resonance (NMR) spectra of lignin samples products were acquired in a Bruker Avance III HD 500-MHz spectrometer, and spectral processing was carried out using a Bruker Topspin 3.5 (Mac) software. The lignin samples (~20 mg) were dissolved in 100 mg DMSO-d_6 in a micro-NMR tube independently. Heteronuclear single quantum

coherence (HSQC) experiments were carried out with a Bruker pulse sequence (hsqcetgpspsi 2.2) on an N_2 cryoprobe (BBO 1H&19F-5mm) with the following acquisition parameters: spectra width 12 ppm in F2 (^{1}H) dimension with 1024 data points (acquisition time 85.2 ms), 200 ppm in F1 (^{13}C) dimension with 256 increments (acquisition time 5.1 ms), a 1.0-s delay, a $^1J_{C–H}$ of 145 Hz, and 128 scans. The central DMSO-d_6 solvent peak (δ_C/δ_H at 39.5/2.49) was used for chemical shifts calibration. Assignment and the relative abundance of lignin compositional subunits and inter-unit linkage were estimated using volume integration of contours in HSQC spectra according to published literature.[14] For volume integration of monolignol compositions of syringyl (S), guaiacyl (G), *p*-hydroxyphenyl (H), *p*-coumarate (*p*CA), and ferulate (FA), the cross-peaks of $S_{2/6}$, G_2, $H_{2/6}$, $pCA_{2/6}$, and FA_2 contours were used with G_2, and FA_2 integrals doubled were integrated. The C_α signals were used for volume integration for inter-unit linkages estimation. The abundances of aromatics and side-chain linkages were presented as percentage of total SGH units and total side-chain linkages, respectively.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors acknowledge the National Institute of Food and Agriculture, U.S. Department of Agriculture, Sustainability Challenge Area grant under accession number 1015068 and Hatch-Multistate project under accession number 1018315. This work was also supported by the National

Natural Science Foundation of China (No. 51708548), China Scholarship Council (No. 201803260012), and Central Public-interest Scientific Institution Basal Research Fund for Chinese Academy of Tropical Agricultural Sciences (No. 17CXTD-05). Oak Ridge National Laboratory is managed by UT-Battelle, LLC, under Contract DE-AC05-00OR22725 with the U.S. Department of Energy (DOE). Y.P. and A.J.R acknowledge the support from the Center for Bioenergy Innovation (CBI), a U.S. DOE Bioenergy Research Centers supported by the Office of Biological and Environmental Research in the DOE Office of Science. The views and opinions of the authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, expressed or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed or represents that its use would not infringe privately owned rights. We thank Novozymes for providing enzyme samples and the Idaho National Laboratory for providing biomass materials.

List of figures and tables

Table 1 Effect of pretreatment processes and conditions on the chemical composition of sorghum bagasse.

	Biomass loading (%)	Pretreatment temperature and time	Biomass recovery (%)	Biomass composition (%)		
				Glucan	Xylan	Lignin
Untreated	-	-	-	33.2±0.4	19.7±1.0	16.8±1.2
Batch processing	10	180 ºC, 120 min	77.0±0.4	40.1±1.3	21.8±0.6	14.8±0.9
	20	180 ºC, 120 min	81.2±1.4	38.5±0.5	22.2±0.8	14.2±0.9
	30	180 ºC, 240 min	76.9±0.7	40.7±1.5	21.4±0.7	16.7±1.1
Continuous extrusion	30	180 ºC, 16 min	84.8±0.7	35.4±1.7	20.5±1.3	17.1±0.5
	30	180 ºC, 28 min	86.3±0.8	35.2±0.6	20.8±0.2	17.9±0.9
	30	180 ºC, 40 min	85.5±1.1	34.4±1.7	21.0±0.6	18.1±0.8
	50	180 ºC, 16 min	85.6±1.5	33.2±3.0	17.2±2.2	18.0±1.0
	50	180 ºC, 28 min	85.5±1.0	34.4±1.0	20.2±1.1	18.1±0.1
	50	180 ºC, 40 min	85.4±0.8	33.9±1.6	20.2±1.4	18.0±0.2
	50	150 ºC, 28 min	85.2±1.1	33.7±1.4	20.0±0.6	18.0±0.7
	50	150 ºC, 40 min	85.6±0.6	34.1±2.1	20.4±1.1	18.2±0.2
	50	150 ºC, 60 min	85.9±0.3	32.7±2.3	19.2±1.4	17.7±0.4

Table 2 The weight-average (M_w) and number-average (M_n) molecular weights, and polydispersity index (PDI) of lignins from raw and extruded sorghum bagasse

Lignin source	M_w (Da)	M_n (Da)	PDI
Raw sorghum bagasse [14]	6109	4216	1.4
Extruded bagasse at loading of 30%	3479±62	1855±24	1.9±0.0
Extruded bagasse at loading of 50%	3252±132	1864±38	1.7±0.0

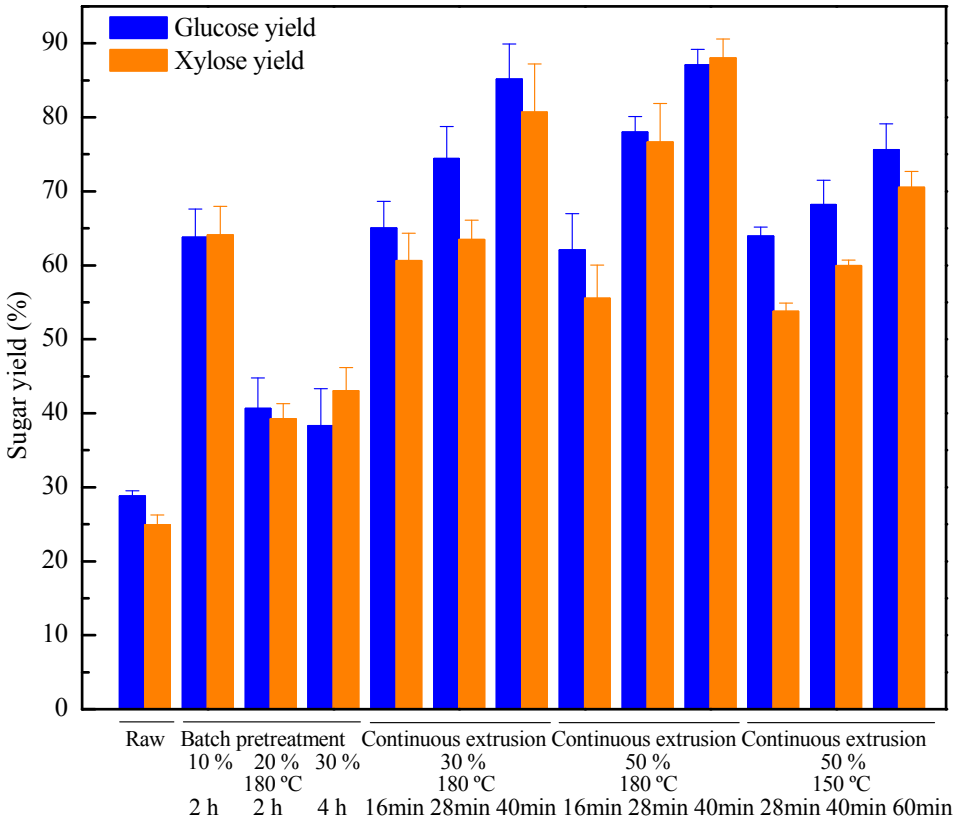

Fig. 1 Effect of pretreatment conditions on the enzymatic hydrolysis sugar yield of raw and pretreated sorghum bagasse.

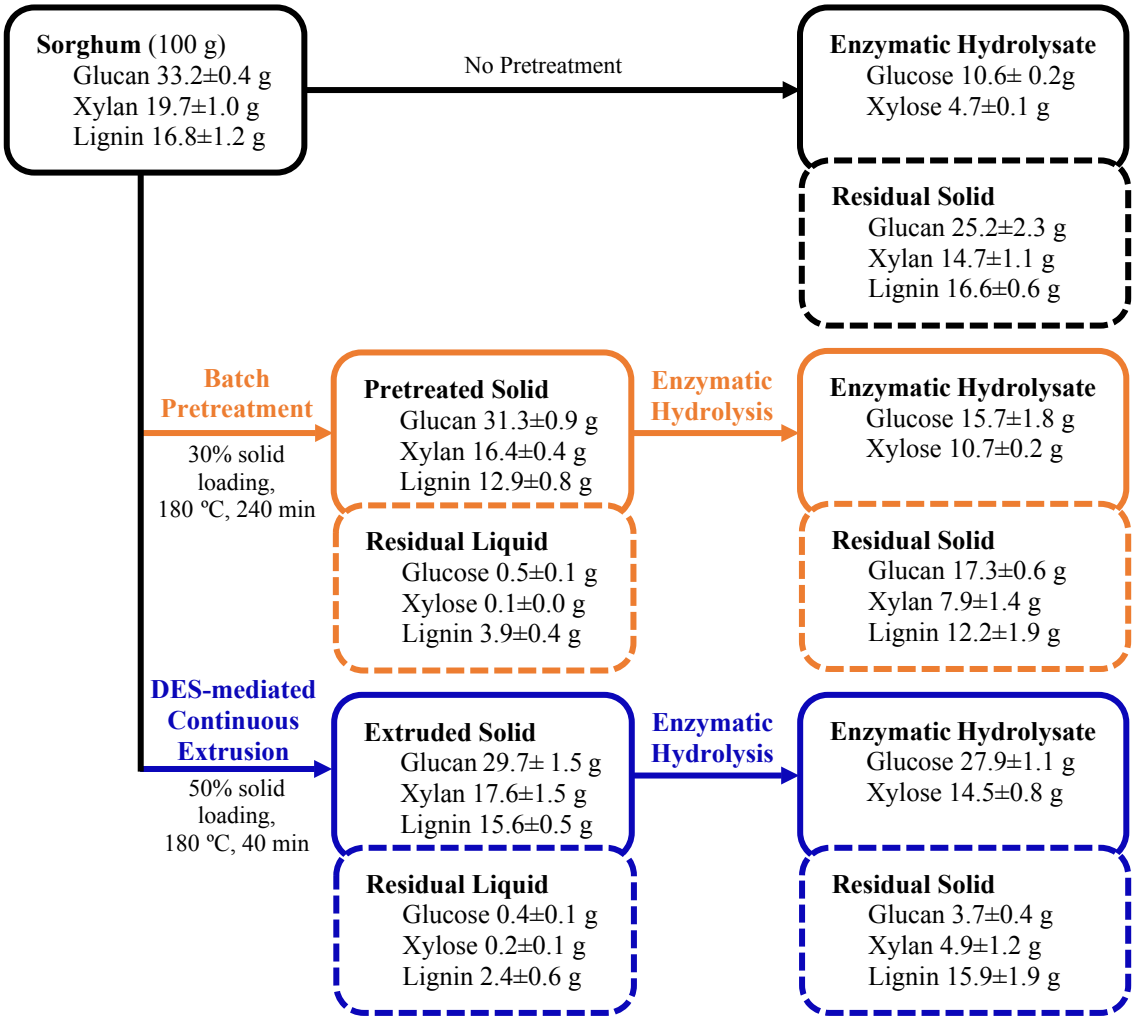

Fig. 2 Mass flow of glucan, xylan and lignin during the pretreatment and enzymatic saccharification of sorghum bagasse.

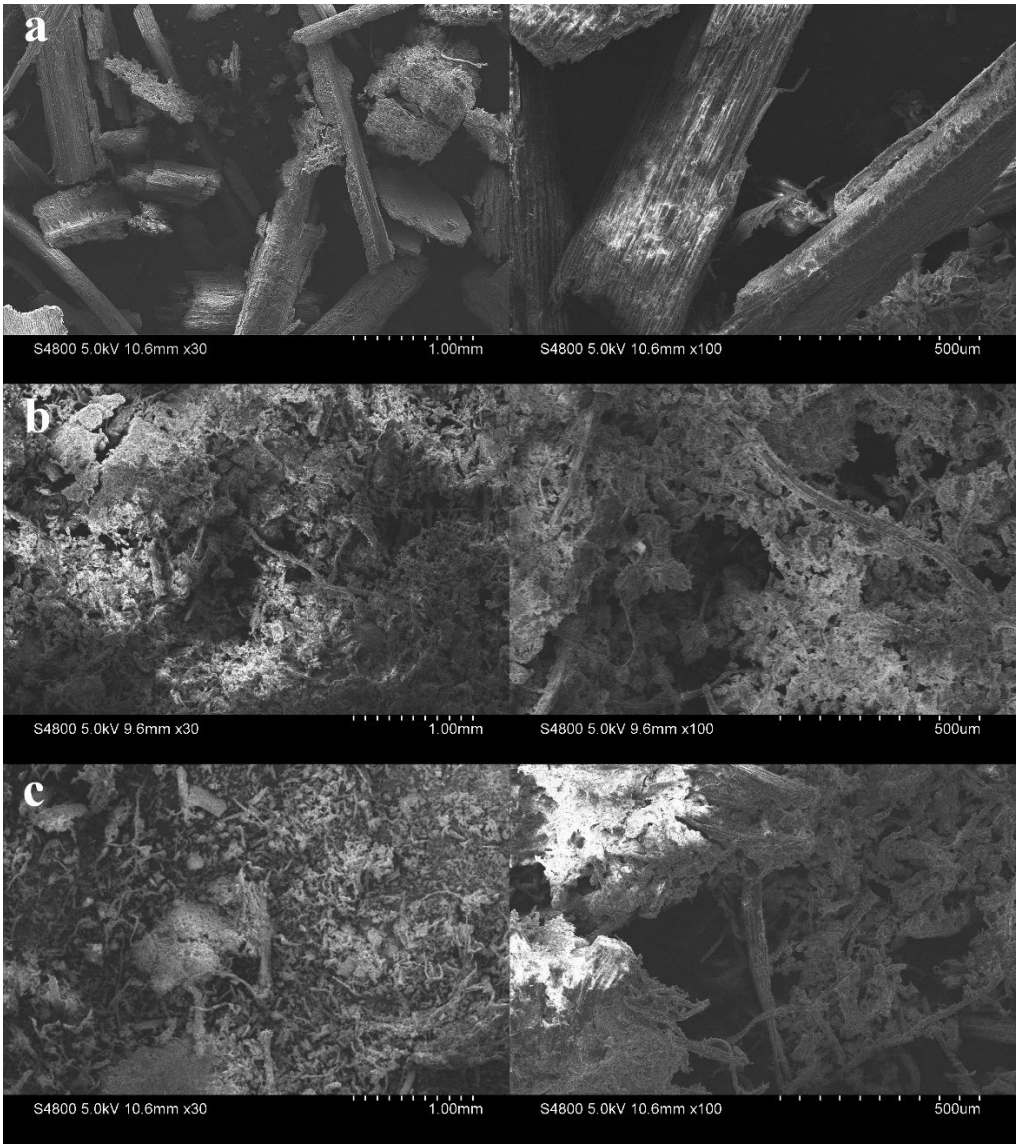

Fig. 3 Scanning electron microscopic images of **(a)** raw and extruded sorghum bagasse at **(b)** 30% and **(c)** 50% solid loadings at two magnifications of 30× (left) and 100× (right).

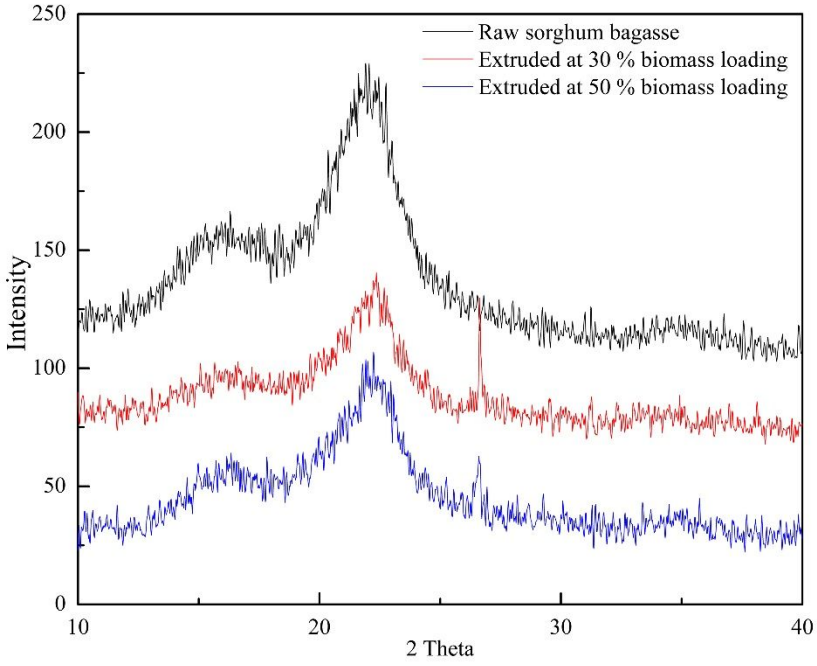

Fig. 4 X-ray diffraction profiles of raw and extruded sorghum bagasse

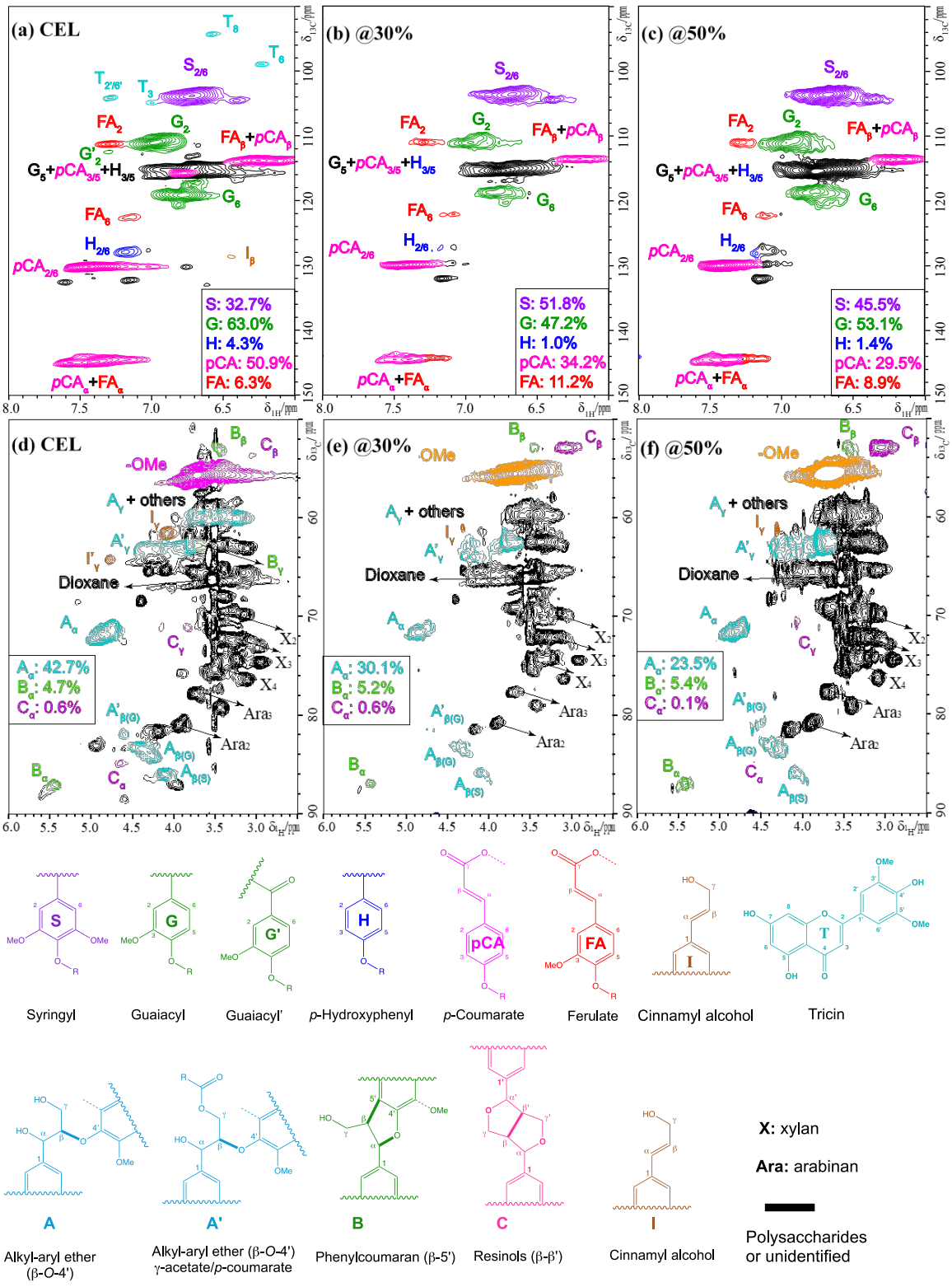

Fig. 5 ^{13}C–^{1}H (HSQC) spectra of aromatic regions (top: a-c) and aliphatic region (bottom: d-f) of the lignin from raw sorghum bagasse (CEL),[14] and extruded bagasse at 30% biomass loading

(@30%), and extruded bagasse at 50% biomass loading (@50%). The structures of lignin compositional units and side-chain linkages were coded with colors corresponding to the cross peaks in the spectra.

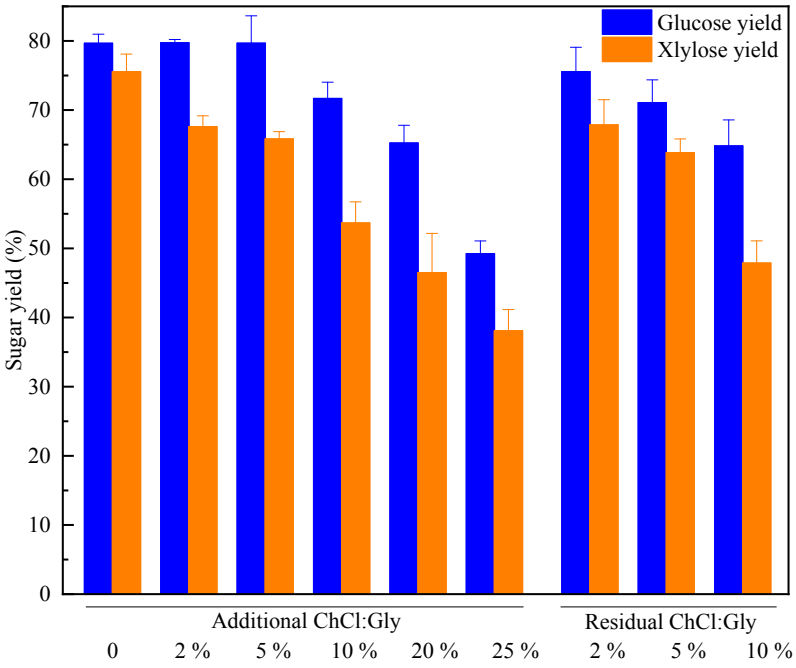

Fig. 6 Inhibitory effect of additional ChCl:Gly and residual ChCl:Gly on enzymatic hydrolysis of extruded sorghum bagasse.

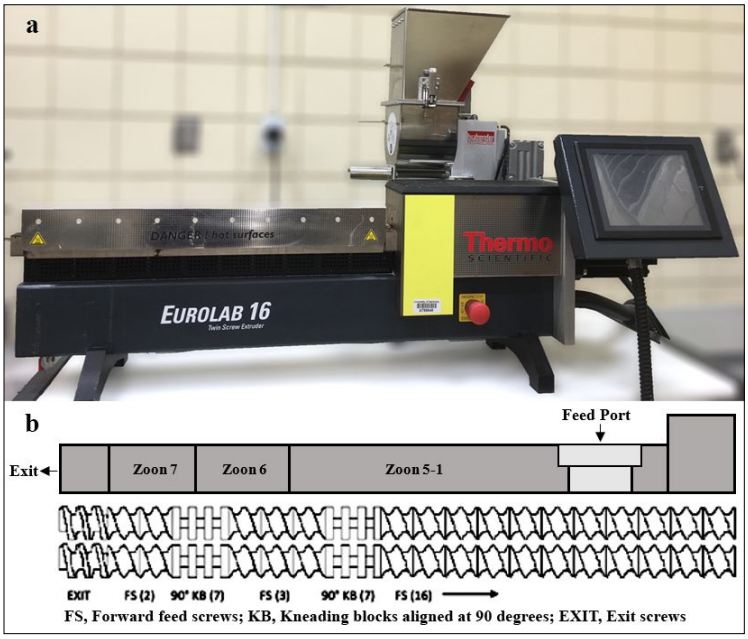

Fig. 7 Image of the extruder system **(a)** and a diagram of the Eurolab 16 co-rotating twin-screw extruder configuration **(b)**.

Notes and references

1. A. K. Chandel, V. K. Garlapati, A. K. Singh, F. A. F. Antunes and S. S. da Silva, *Bioresource Technol*, 2018, **264**, 370-381.

2. X. Meng and A. J. Ragauskas, *Curr Opin Biotech*, 2014, **27**, 150-158.

3. N. V. Plechkova and K. R. Seddon, *Methods Reagents Green Chem*, 2007, 105-130.

4. A. Brandt-Talbot, F. J. Gschwend, P. S. Fennell, T. M. Lammens, B. Tan, J. Weale and J. P. Hallett, *Green Chem*, 2017, **19**, 3078-3102.

5. F. J. V. Gschwend, L. M. Hennequin, A. Brandt, F. E. Bedoya-Lora, G. H. Kelsall, K. Polizzi, P. Fennell and J. Hallett, *Green Chem*, 2020, DOI: 10.1039/D0GC01241F.

6. A. M. Stepan, A. Michud, S. Hellstén, M. Hummel and H. Sixta, *Ind Eng Chem Res*, 2016, **55**, 8225-8233.

7. L. K. Hauru, M. Hummel, A. Michud and H. Sixta, *Cellulose*, 2014, **21**, 4471-4481.

8. A. George, A. Brandt, K. Tran, S. M. S. N. S. Zahari, D. Klein-Marcuschamer, N. Sun, N. Sathitsuksanoh, J. Shi, V. Stavila, R. Parthasarathi, S. Singh, B. M. Holmes, T. Welton, B. A. Simmons and J. P. Hallett, *Green Chem*, 2015, **17**, 1728-1734.

9. A. Paiva, R. Craveiro, I. Aroso, M. Martins, R. L. Reis and A. R. C. Duarte, *ACS Sustainable Chem Eng*, 2014, **2**, 1063-1071.

10. Y. Liu, J. B. Friesen, J. B. McAlpine, D. C. Lankin, S.-N. Chen and G. F. Pauli, *J Nat Prod*, 2018, **81**, 679-690.

11. B.-Y. Zhao, P. Xu, F.-X. Yang, H. Wu, M.-H. Zong and W.-Y. Lou, *ACS Sustainable Chem Eng*, 2015, **3**, 2746-2755.

12. K. Radošević, M. Cvjetko Bubalo, V. Gaurina Srček, D. Grgas, T. Landeka Dragičević and I. Radojčić Redovniković, *Ecotox Environ Safe*, 2015, **112**, 46-53.

13. C.-W. Zhang, S.-Q. Xia and P.-S. Ma, *Bioresource Technol*, 2016, **219**, 1-5.

14. L. Das, M. Li, J. Stevens, W. Li, Y. Pu, A. J. Ragauskas and J. Shi, *ACS Sustainable Chem Eng*, 2018, **6**, 10408-10420.

15. W. Li, K. Amos, M. Li, Y. Pu, S. Debolt, A. J. Ragauskas and J. Shi, *Biotechnol Biofuels*, 2018, **11**, 304.

16. J. G. Lynam, N. Kumar and M. J. Wong, *Bioresource Technol*, 2017, **238**, 684-689.

17. M. Francisco, A. van den Bruinhorst and M. C. Kroon, *Green Chem*, 2012, **14**, 2153-2157.

18. K. D. O. Vigier, G. Chatel and F. Jerome, *ChemCatChem*, 2015, **7**, 1250-1260.

19. D. Humbird, A. Mohagheghi, N. Dowe and D. J. Schell, *Biotechnol Progr*, 2010, **26**, 1245-1251.

20. Z. Chen, X. Bai and C. Wan, *ACS Sustainable Chem Eng*, 2018, **6**, 12205-12216.

21. A. Duque, P. Manzanares and M. Ballesteros, *Renew Energ*, 2017, **114**, 1427-1441.

22. B.-J. Gu, M. P. Wolcott and G. M. Ganjyal, *Bioresource Technol*, 2018, **269**, 262-268.

23. E. Gatt, V. Khatri, J. Bley, S. Barnabé, V. Vandenbossche and M. Beauregard, *Bioresource Technol*, 2019, **282**, 398-406.

24. M. C. Coimbra, A. Duque, F. Saéz, P. Manzanares, C. H. Garcia-Cruz and M. Ballesteros, *Renew Energ*, 2016, **86**, 1060-1068.

25. A. S. A. Da Silva, R. S. S. Teixeira, T. Endo, E. P. Bon and S.-H. Lee, *Green Chem*, 2013, **15**, 1991-2001.

26. M. Nagy, Georgia Institute of Technology, 2009.

27. M. Ragnar, C. T. Lindgren and N.-O. Nilvebrant, *Journal of Wood Chemistry and Technology*, 2000, **20**, 277-305.

28. Q. Zheng, T. Zhou, Y. Wang, X. Cao, S. Wu, M. Zhao, H. Wang, M. Xu, B. Zheng, J. Zheng and X. Guan, *Sci Rep*, 2018, **8**, 1321.

29. R. Kumar, G. Mago, V. Balan and C. E. Wyman, *Bioresource Technol*, 2009, **100**, 3948-3962.

30. W. Li, Y. Zhang, L. Das, Y. Wang, M. Li, N. Wanninayake, Y. Pu, D. Y. Kim, Y.-T. Cheng and A. J. Ragauskas, *RSC Adv*, 2018, **8**, 38721-38732.

31. Z. Chen, W. D. Reznicek and C. Wan, *Bioresource Technol*, 2018, **263**, 40-48.

32. Q. Xia, Y. Liu, J. Meng, W. Cheng, W. Chen, S. Liu, Y. Liu, J. Li and H. Yu, *Green Chem*, 2018, **20**, 2711-2721.

33. X.-J. Shen, J.-L. Wen, Q.-Q. Mei, X. Chen, D. Sun, T.-Q. Yuan and R.-C. Sun, *Green Chem*, 2019, **21**, 275-283.

34. W. Xing, G. Xu, J. Dong, R. Han and Y. Ni, *Chem Eng J*, 2018, **333**, 712-720.

35. X.-D. Hou, K.-P. Lin, A.-L. Li, L.-M. Yang and M.-H. Fu, *Ind Crop Prod*, 2018, **120**, 322-329.

36. A. Duque, P. Manzanares, I. Ballesteros, M. J. Negro, J. M. Oliva, F. Saez and M. Ballesteros, *Process Biochem*, 2013, **48**, 775-781.

37. S.-H. Lee, Y. Teramoto and T. Endo, *Bioresource Technol*, 2009, **100**, 275-279.

38. M. K. Moro, R. S. S. Teixeira, A. S. A. da Silva, M. D. Fujimoto, P. A. Melo, A. R. Secchi and E. P. da Silva Bon, *Ind Crop Prod*, 2017, **97**, 509-517.

39. L. Laureano-Perez, F. Teymouri, H. Alizadeh and B. E. Dale, *Appl Biochem Biotech*, 2005, **124**, 1081-1099.

40. S. Park, J. O. Baker, M. E. Himmel, P. A. Parilla and D. K. Johnson, *Biotechnol Biofuels*, 2010, **3**, 10.

41. V. P. Puri, *Biotechnol Bioeng*, 1984, **26**, 1219-1222.

42. Y. Cao, S. S. Chen, S. Zhang, Y. S. Ok, B. M. Matsagar, K. C. Wu and D. C. Tsang, *Bioresource Technol*, 2019, 121878.

43. S. Constant, H. L. Wienk, A. E. Frissen, P. de Peinder, R. Boelens, D. S. Van Es, R. J. Grisel, B. M. Weckhuysen, W. J. Huijgen and R. J. Gosselink, *Green Chem*, 2016, **18**, 2651-2665.

44. F. Xu, J. Sun, M. Wehrs, K. H. Kim, S. S. Rau, A. M. Chan, B. A. Simmons, A. Mukhopadhyay and S. Singh, *ACS Sustainable Chem Eng*, 2018, **6**, 8914-8919.

45. L. J. Jönsson and C. Martín, *Bioresource Technol*, 2016, **199**, 103-112.

46. D. Kim, *Molecules*, 2018, **23**, 309.

47. E. Palmqvist, J. S. Almeida and B. Hahn-Hägerdal, *Biotechnol Bioeng*, 1999, **62**, 447-454.

48. Y. Kim, E. Ximenes, N. S. Mosier and M. R. Ladisch, *Enzyme Microb Tech*, 2011, **48**, 408-415.

49. J. Shi, M. A. Ebrik, B. Yang, R. J. Garlock, V. Balan, B. E. Dale, V. R. Pallapolu, Y. Lee, Y. Kim and N. S. Mosier, *Bioresource Technol*, 2011, **102**, 11080-11088.

50. Q. Qing, B. Yang and C. E. Wyman, *Bioresource Technol*, 2010, **101**, 9624-9630.

51. Q. Qing and C. E. Wyman, *Biotechnol Biofuels*, 2011, **4**, 18.

52. J. Shi, J. M. Gladden, N. Sathitsuksanoh, P. Kambam, L. Sandoval, D. Mitra, S. Zhang, A. George, S. W. Singer and B. A. Simmons, *Green Chem*, 2013, **15**, 2579-2589.

53. D. Klein-Marcuschamer, B. A. Simmons and H. W. Blanch, *Biofuels, Bioprod Biorefin*, 2011, **5**, 562-569.

54. B. R. Bewley, A. Berkaliev, H. Henriksen, D. B. Ball and L. S. Ott, *Fuel Process Technol*, 2015, **138**, 419-423.

55. M. R. Monteiro, C. L. Kugelmeier, R. S. Pinheiro, M. O. Batalha and A. da Silva César, *Renewable and Sustainable Energy Reviews*, 2018, **88**, 109-122.

56. A. K. Kumar, S. Sharma, G. Dixit, E. Shah, A. Patel and G. Boczkaj, *Biofuels, Bioprod Biorefin*, 2020.

57. A. Satlewal, R. Agrawal, P. Das, S. Bhagia, Y. Pu, S. K. Puri, S. Ramakumar and A. J. Ragauskas, *ACS Sustainable Chem Eng*, 2018, **7**, 1095-1104.

58. M. Selig, N. Weiss and Y. Ji, *Enzymatic saccharification of lignocellulosic biomass: laboratory analytical procedure (LAP)*, National Renewable Energy Laboratory, 2008.

59. A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton and D. Crocker, *Determination of structural carbohydrates and lignin in biomass: laboratory analytical procedure (LAP)*, National Renewable Energy Laboratory, 2008.

60. A. Zhang, F. Lu, R.-C. Sun and J. Ralph, *J. Agric. Food. Chem.*, 2010, **58**, 3446-3450.