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Evaluation of recalcitrant features impacting enzymatic saccharification of diverse agricultural residues treated by steam explosion and dilute acid

Ruchi Gaur,[a] Ruchi Agrawal,[a] Rahul Kumar,[a] E. Ramu,[b] Veena Rani Bansal,[b] Ravi P. Gupta,[a] Ravindra Kumar,[a*] Deepak K. Tuli,[a] Biswapriya Das[c]

[a]DBT-IOC Centre for Advanced Bioenergy Research, Indian Oil Corporation Ltd., Research and Development Centre, Sector-13, Faridabad-121007, India.
[b]Analytical division, Indian Oil Corporation Ltd., Research and Development Centre, Sector-13, Faridabad-121007, India.
[c]Indian Oil Corporation Ltd., Research and Development Centre, Sector-13, Faridabad-121007, India.

Corresponding author:

Dr. Ravindra Kumar
Research Manager
DBT-IOC Centre for Advanced Bioenergy Research
R & D Centre, Indian Oil Corporation Ltd.
Sector-13, Faridabad-121007
INDIA

Abstract

Exploring the agricultural biomass for biofuel production necessitates pretreatment as a prerequisite step. However, due the variability in recalcitrance among biomasses, choosing an optimum pretreatment methodology suitable for multiple feedstocks is challenging. To assess which parameters of pretreated biomass may serve as useful indicators of potential subsequent enzymatic saccharification, an insight into the structural alteration during pretreatment and its impact on the downstream process is essential. In this study, two pretreatment methods, dilute acid (DA) and steam explosion (SE) have been employed on three different biomasses viz. rice straw (RS), cotton stalk (CS) and mustard stalk (MS). The alteration in recalcitrant features of the pretreated residues was measured by chemical analysis, XRD, BET and FT-IR. FT-IR proved useful to measure the cellulose related properties viz. lateral order index (LOI) and hydrogen bond index (HBI) besides lignin related features, i.e. cross-linked lignin (CLL), lignin/cellulose (L/C) and syringyl/guaiacyl (S/G) ratio. The results show that S/G ratio, specific surface area and HBI of the pretreated residues had a positive correlation with enzymatic saccharification across different biomasses and pretreatment methodologies employed. On the other hand, lignin content, CLL, L/C ratio and LOI showed a negative correlation. However, extent of xylan removal showed a positive correlation with the enzymatic saccharification only when a single pretreatment method was applied to different biomasses. The structure-activity correlation presented here would help to assess and predict the enzymatic saccharification while applying DA or SE pretreatment methods on different biomasses. This correlation could provide assistance in designing an optimum technology.

Keywords: Lignocellulosic biomass, pretreatment, physico-chemical parameters, enzymatic saccharification, cotton stalk, rice straw, mustard stalk

Introduction

Lignocellulosic biomasses (LCB) have been considered as a potent source for green fuels like bioethanol and biobutanol due to their richness in carbohydrates. However, owing to their complex cell wall structure formed by the intimate association among the three major structural polymers, viz. cellulose, hemicellulose and lignin, high resistance towards enzymatic and microbial deconstruction is prevalent.[1,2] Pretreatment methods have been employed to release fermentable sugars from biomass by altering the cell wall structural features responsible for recalcitrance.[3,4] Interestingly, plant cell wall structural construction and composition varies across biomass and so does the recalcitrance. This structural variability also accounts for the differences in the enzymatic digestibility/ saccharification among the biomass feedstocks after pretreatment.[5]

The factors that may contribute to biomass recalcitrance include: cellulose related features, viz. hydrogen bond index (HBI), lateral order index (LOI), crystallinity index (CrI) and degree of polymerization (DP); lignin related features, i.e. lignin content, syringyl to guaiacyl (S/G) ratio and cross-linked lignin (CLL) and biomass related features, i.e. specific surface area, cellulose sheathing by hemicelluloses, etc.[6,7] To reduce the biomass recalcitrance, pretreatment processes target the cell wall complexity by breaking some of the intercomponent linkages and therefore, make it amenable to enzymatic attack leading to the release of fermentable monosaccharides. Considering the future demand and commercial success of ethanol production, various kinds of agricultural residues like rice straw, cotton stalk, mustard stalk etc. are required to be processed. Due to the variability in structural features, the extent to which one biomass responds to one pretreatment method may not be applicable to others. Numerous pretreatment methods, including physical, chemical and biological have been investigated to reduce

recalcitrance and improve the sugar yields from LCB. All these technologies have their associated merits and demerits.[8-10]

Steam explosion (SE) and dilute acid (DA) pretreatment are the most promising technologies from both technical and economical points of view.[4,11] SE involves rapid expansion of biomass and vaporization of the saturated water within the cellulose fibrils, breaking down the molecular linkages to open up the plant cell wall matrix with partial removal/ disclocation of lignin.[1,12] DA pretreatment generally utilizes dilute sulphuric acid and effectively breaks down the lignocellulosic structure by solubilizing hemicellulose at moderate temperatures and pressure, thereby, removing linkages with cellulose and hence, increasing cellulose accessibility to enzymatic attack.[13] In a pursuit to find a suitable feature which can reflect the efficiency of pretreatment, while applying on different feedstocks, we have chosen rice straw (RS), cotton stalk (CS) and mustard stalk (MS). Ample availability and least alternative uses, except that for household burning, makes them attractive feedstocks for biofuel production [14, 15].

This study deals with the structural alteration of RS, CS and MS induced by DA and SE as pretreatment methodologies. The quantum of alteration in recalcitrant features of pretreated residues was measured by chemical analysis, crystallinity index, surface area and FT-IR spectroscopy. FT-IR was used for lateral order index (LOI), hydrogen bond index (HBI), cross-linked lignin (CLL), lignin/cellulose (L/C) ratio and syringyl/ guaiacyl (S/G) ratio. FT-IR has been reported as a tool in pulp and paper industry to measure the quality of cellulose after pulping.[16-18] However, in the present report a comprehensive analysis of various structural features of biomass and their impact on the performance of enzymatic saccharification has been

undertaken. This would help in selecting suitable pretreatment conditions giving lower recalcitrance for enhanced enzymatic saccharification for different biomasses.

2. Results and discussion

2.1 Compositional analysis vis-à-vis enzymatic saccharification

Both, DA and SE pretreatments solubilize hemicellulose and delocalize/ fragment lignin, thus increasing the accessibility of cellulose microfibrils to enzymatic attack.[19] Therefore, the quantity and the compositional features of these structural recalcitrants are likely to have a pronounced effect on enzymatic saccharification. The experimental design containing 10 different sets of pretreatment conditions based on our previous studies [11,12] along with the analysis of pretreatment hydrolyzate is given in the supplementary data (ST1). The solid residue was taken forward for enzymatic saccharification.

Table 1 shows that while applying DA as a pretreatment method for the same severity condition (160 ^{o}C), xylan removal was in the order of 160-RS-DA>160-CS-DA>160-MS-DA. An almost similar trend of xylan removal, when SE was applied was followed as well, i.e. 180-RS-SE>180-CS-SE>200-MS-SE. Interestingly, the three biomasses differ with respect to their texture and hardness and so is reflected in their response to DA or SE pretreatments with xylan removal and residual lignin content following a similar trend. It may be observed that, in all the sets of experiments, subsequent enzymatic saccharification of the pretreated residues gave an increased glucose release (Figure 1) due to higher removal of xylan although the the increase was obviously not proportional to the extent of xylan removal.

Figure 1 was plotted to find out the correlation between the extent of xylan removal and glucose release. For a given pretreatment method, e.g. DA, a positive correlation was obtained between xylan removal from RS, CS and MS biomass vs. glucose release (R^2=0.62 at 48 h).

Similarly, considering SE alone, xylan removal from the three biomasses showed a positive correlation with enzymatic saccharification (R^2 =0.74). Similar correlations were obtained at 2 and 24 h and shown in the supplementary data (SFigure 1a and 1b). However, xylan removal due to both DA and SE pretreatment when plotted together against respective glucose yields gave a poor correlation. This observation suggests that only when same pretreatment technology (DA or SE) is applied, can xylan removal serve as a structural feature to assess/ predict enzymatic saccharification across variable biomasses. The efficacy of this prediction feature is lost if different pretreatment methods are used (even on the same biomass).

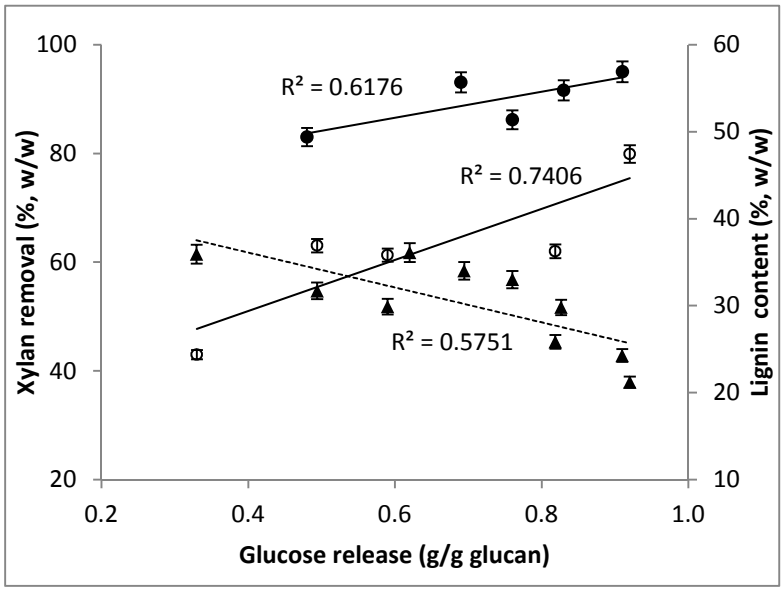


Figure 1. Correlation of xylan removal and residual lignin content with glucose release using 10 FPU of cellulase. Xylan removal and lignin content was determined in the cellulose rich residue of RS, CS and MS based on chemical composition and total solid obtained after pretreatment. DA pretreated xylan removal (-•-), SE pretreated xylan removal (-○-), residual lignin content (--▲--). Error bars show the percentage error (<3%) within individual sets of experiments.

2.2 *Enzymatic saccharification of pretreated biomass*

The pretreated solid from each experiment was subjected to enzymatic saccharification using 10 and 20 FPU of cellulase preparation per g substrate. 10% (w/w) solid loading was maintained in all the enzymatic saccharification experiments as beyond this, it was difficult to properly stir the slurry. The saccharification were monitored till 72 h and the glucose release profile is presented in Figure 2. It is very much evident from the figure that saccharification of RS was much more efficient under all pretreatment conditions than that for CS or MS; higher doses of enzyme somewhat improved the rate of saccharification and higher pretreatment temperature led to higher sugar recovery.

Figure 2 shows that in all sets of experiments, the most of the sugar release was achieved within 48 h. Therefore, 48 h saccharification efficiency were used for further studies. However, following the correlation between recalcitrance features and enzymatic saccharification to further support the inference, glucose release at 2 and 24 h was also considered (supplementary data). In case of CS, increase in DA pretreatment temperature from 150 to 160 ^{o}C (150-CS-DA and 160-CS-DA) resulted in an increase in glucose release from 0.48 to 0.69 (g/ g glucan) using 10 FPU of cellulase preparation. Similarly, an increase in SE temperature to 180 ^{o}C (160-CS-SE vs. 180-CS-SE), 1.5 fold higher saccharification efficiency was achieved in 48 h. While applying DA on RS, an increase in temperature from 150 to 160 ^{o}C led to an increase in glucose release from 0.83 to 0.91 (g/ g glucan) respectively in 48 h. Interestingly, increase in SE temperature from 160 to 180 ^{o}C for RS increased the glucose release from 0.81 to 0.90 (g/ g glucan) within 24 h. It is worth mentioning that although the glucose release in the case of RS using either SE or DA were the same, the enzymatic saccharification were much faster for SE pretreated RS.

The fact that 160-CS-DA gave a higher glucose release (0.69 g/ g glucan) than 180-CS-SE (0.49 g/ g glucan) makes DA pretreatment preferable over SE for CS. Whereas, considering the advantage of time saving, SE could be a suitable method for pretreatment of RS. Moreover, this observation is further supported by the formation of lesser inhibitors in SE as depicted by supplementary data (ST1). Increasing the enzyme dose from 10 to 20 FPU led to increased rate of glucan conversion for all the experiments. Therefore, in order to find out the impact of recalcitrants on enzymatic saccharification, 10 FPU was adopted for detailed studies.

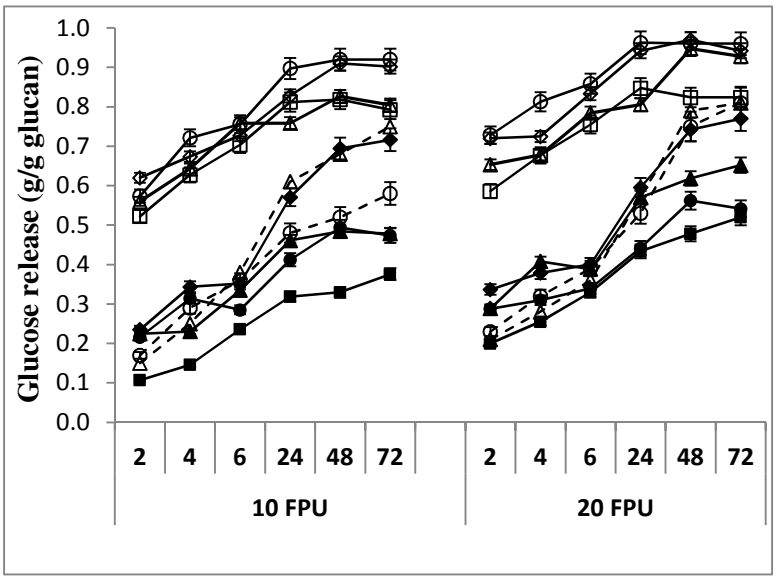


Figure 2. Time course of glucose release from DA and SE pretreated CS, RS and MS. The pretreated slurry obtained after each experiment was subjected to enzymatic saccharification using 10 and 20 FPU of cellulose. Samples withdrawn at various intervals and saccharification were monitored using HPLC by measuring glucose. 160-CS-SE (-■-); 180-CS-SE (-●-); 160-RS-SE (-□-); 180-RS-SE (-○-); 150-CS-DA (-▲-); 160-CS-DA (-♦-); 150-RS-DA (-Δ-); 160-RS-DA (-◊-); 160-MS-DA (- -Δ- -); 200-MS-SE (- -○- -). Error bars show the percentage error (< 3%) at each data point.

Table 1. Chemical analysis of pretreated Rice straw (RS), cotton stalk (CS) and mustard stalk (MS) using DA and SE

Components of pretreated solid (%)	Native CS[a]	150CSDA	160CSDA	160CSSE	180CSSE	Native RS[a]	150RSDA	160RSDA	160RSSE	180RSSE	Native MS[a]	160MSDA	200MSSE
Glucan	37.4±0.20	56.2±1.80	56.5±1.25	49.0±2.0	48.8±1.25	39.2±0.81	53.0±1.0	52.0±2.0	55.7±2.20	55±2.0	39.5±0.5	59.6±0.2	53.7±0.3
Xylan	22.7±0.56	6.0±0.60	2.2±0.10	16.0±1.20	10.7±0.45	20.5±1.40	2.3±0.10	3.8±0.10	6.8±0.32	4.8±0.24	18.7±0.3	4.2±0.6	10.5±0.2
Lignin	20.8±1.16	34.2±0.68	39.0±0.40	28.8±0.57	35.8±1.95	21.1±0.95	32.6±0.85	32.3±1.10	26.2±0.20	29.8±1.10	22.5±0.4	33.0±0.5	29.9±0.4
Ash	6.7±0.5	2.1±0.10	0.8±0.02	4.5±0.30	4.5±0.30	12.6±0.50	11.2±0.40	10.8±0.60	14.5±0.50	11.3±0.75	4.3±0.2	1.8±0.2	2.7±0.10
Acetic acid	3.4±.25	0.8±0.01	0.5±0.01	2.5±0.20	2.1±0.10	2.5±0.10	0.5±0.02	0.5±0.02	0.7±0.02	0.4±0.01	2.6±0.3	0.7±0.2	1.20±0.3
Solid Content	NA	72.9	72.3	79.2	78.1	NA	77.2	78.1	81.0	80.2	NA	62.0	69.0
Xylan removal	NA	78.5	93.1	44.4	63	NA	91.6	94.7	76.4	79.9	NA	86.2	61.3

[a]The composition has been reported following the NREL protocol and extractives have not been included

Naming of pretreated solids: Temperature - Biomass (CS/RS/ MS) - Pretreatment method (DA or SE)

All the values are shown with standard deviation.

The residual lignin content and its structural type gets altered as a result of pretreatment. Table 1 shows that the content of residual lignin was in the range of 26.2 to 39.1% (w/w) and followed the order: 160-CS-DA>180-CS-SE>150-CS-DA>160-MS-DA>150-RS-DA>160-RS-DA>200-MS-SE>180-RS-SE>160-CS-SE>160-RS-SE. Primarily, it is observed from Table 1 that following pretreatment, CS residue retained more lignin than RS residue irrespective of the pretreatment method used. Secondly, the amount of lignin retained in residues of the SE pretreated biomass is lower than the ones pretreated by DA. This may be attributed to the fact that SE had lower acid charge, as a result had lower lignin condensation than DA. Also, SE in addition to partially removing xylan also partly removes the water-soluble lignin.

It may be worth noting that the amount of lignin retained in pretreated biomass could have a significant impact on the subsequent enzymatic saccharification. Figure 1 shows the correlation between lignin content and glucose release. Increased lignin content in the cellulose rich residue (as given in Table 1) had a negative correlation with the enzymatic saccharification (R^2 =0.58 at 48 h) across the pretreatment method and the biomass. Moreover, 2 and 24 h saccharification data also supported this observation (SFigure 1a and 1b). The inverse correlation of glucose release with lignin removal is due to the fact high lignin removal was achieved at reduced xylan removal at acidic pretreatments such as DA and SE here. This observation is consistent with the earlier reports on lignin content being a hindrance for enzymatic saccharification [20,21] and suggests that lignin content is an important structural parameter to assess/ predict enzymatic saccharification across variable pretreatment technologies and diverse biomasses.

2.3 *Structural features of lignin vis-à-vis enzymatic saccharification*

To get an insight into the participation of lignin related features in affecting the enzymatic saccharification, FT-IR studies were conducted for native and pretreated CS, RS and MS.[22] Figure 3 shows the FT-IR spectra of native and pretreated residues of CS and RS. The values for FT-IR determined parameters are presented in Table 2.

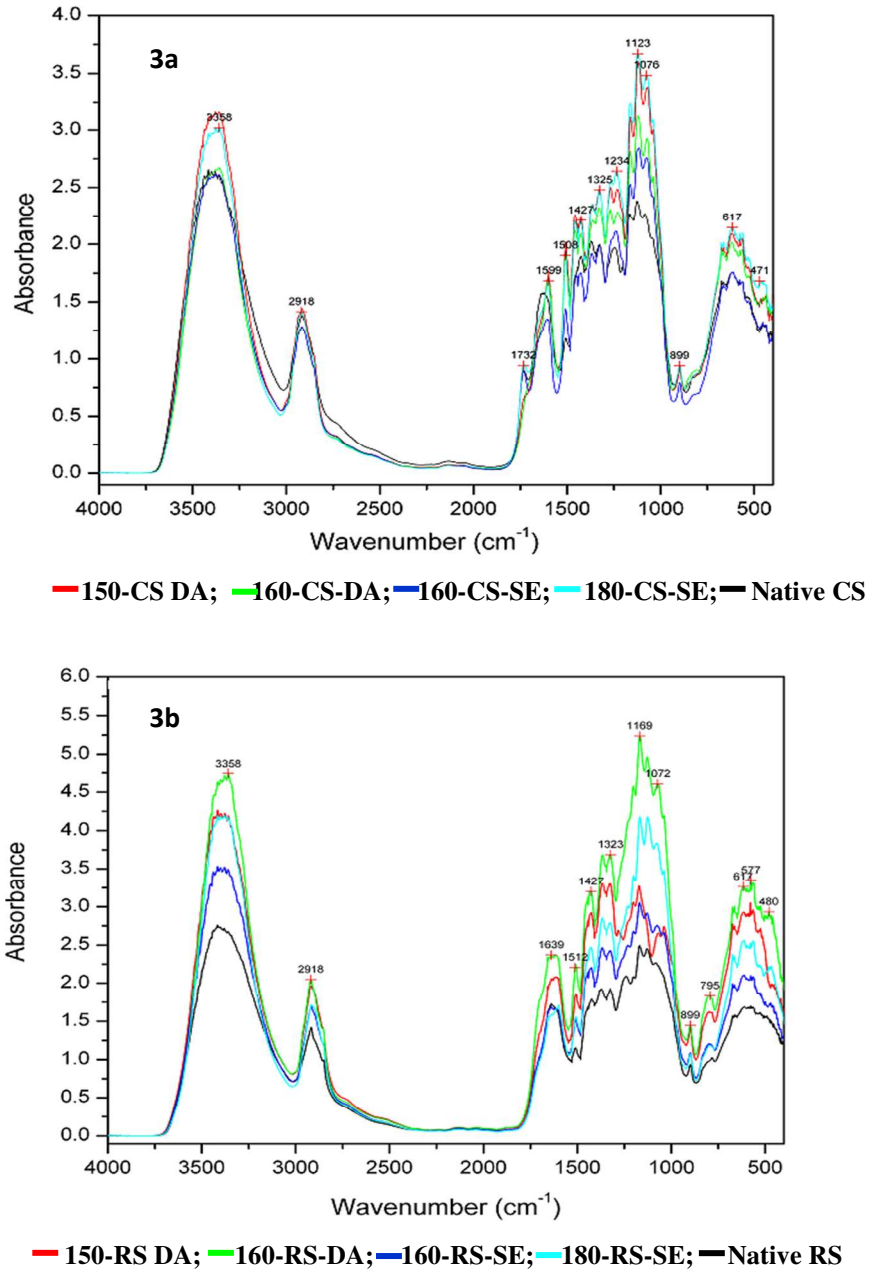
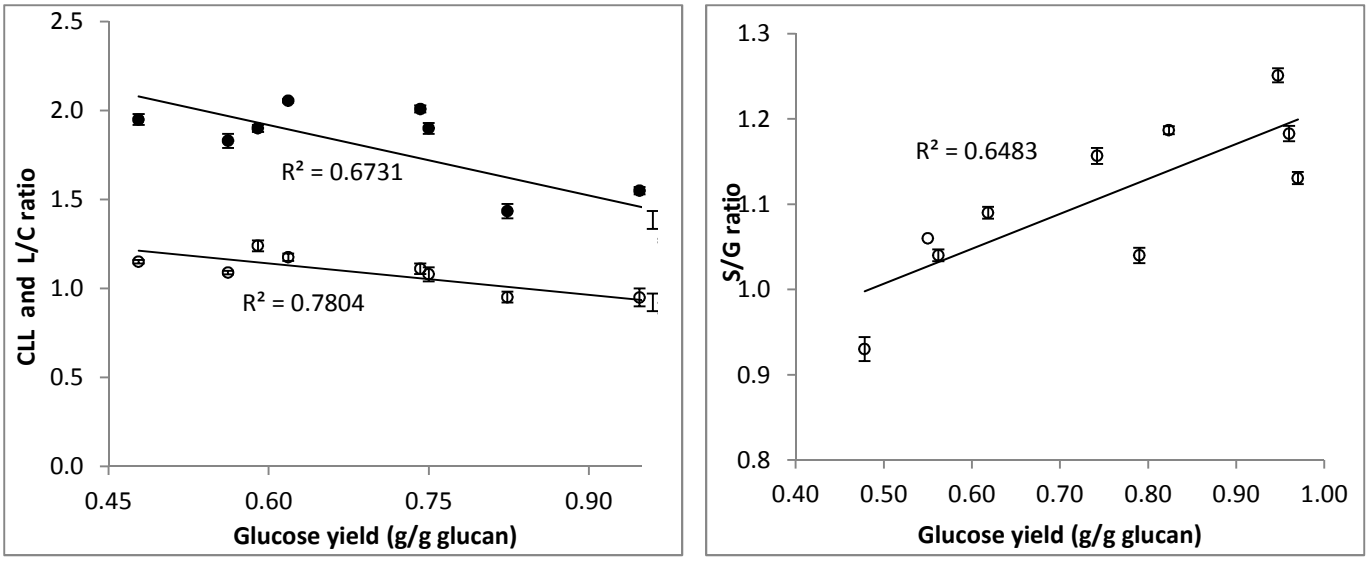


Figure 3. FTIR spectra for native and pretreated (a) CS and (b) RS

The characterictic peaks have been marked in the spectra. The peaks at 1500 and 1600 cm^{-1} originate from the aromatic skeletal vibrations of lignin. Peak at 1600 cm^{-1} also signifies C=O stretching and indicative of lignin in condensed or cross-linked form. Cross-linked lignin (CLL) is a characteristic structural feature of lignin arising due to the presence of more guaiacyl (G) units. Thus, lignin with condensed and cross-linked structures are measured using the ratio of intensities at 1500 and 1600 cm^{-1}. Higher this value, higher is the condensed and cross-linked structures present in the pretreated biomass. The FT-IR data show that CLL in pretreated CS upon pretreatment ranged between 1.18 and 1.09, however, for RS, the range was relatively narrower, i.e. 0.95 to 0.89. In the pretreated CS, decrease in CLL from 1.18 to 1.09 reflected an increase in the glucose release from 0.33 to 0.69 (g/ g glucan) upon enzymatic saccharification.



4a **4b**

Figure 4. Correlation of lignin related features with enzymatic saccharification. RS, CS and MS were pretreated by DA and SE. FTIR determined (a) CLL (-○-) and L/C ratio (-●-) and (b) S/G ratio of resulting pretreated residues were plotted against the glucose release determined by their enzymatic saccharification. Error bars indicate standard deviation within the sets of experiments ($p<0.05$).

A similar observation was obtained for RS. It is worth mentioning that, in all set of experiments, the glucose release increased after pretreatment and was evidenced by the decreasing values of CLL. Hence, to draw a correlation between CLL and glucose release, Figure 4a was plotted. It shows a strong negative correlation between CLL of DA and SE pretreated CS RS and MS (R^2 = 0.82). Likewise, at 2 and 24 h of saccharification, a negative correlation was persistent as shown in the supplementary data (SFigure 2a and 2b). It shows that CLL may act as an important structural feature of lignin for assessing/predicting the enzymatic saccharification across different pretreatment methods applied to diverse biomasses.

Lignin to cellulose (L/C) ratio is another parameter obtained by the ratio of absorption intensities at 1510 and 898 cm^{-1}. Upon increasing the severity of DA pretreatment from 150 to 160 ^{o}C, conversion of crystalline to amorphous cellulose (increase in absorption at 898 cm^{-1}) is anticipated which is reflected in the decreased values of L/C. Again, with an increase in SE pretreatment temperature from 160 to 180 ^{o}C for CS and RS, a decrease in L/C values was observed. L/C values for pretreated CS ranged between 1.83 and 2.06 while the glucose release obtained was between 0.33 and 0.69 (g/ g glucan). The L/C values for pretreated RS were in the range of 1.27 and 1.55 with a glucose release between 0.82 and 0.92 (g/ g glucan). Figure 4a shows a negative correlation between L/C ratio of pretreated RS, CS and MS on enzymatic saccharification (R^2=0.69) showing the L/C may serve as an important property to assess/ predict enzymatic saccharification across different pretreatment methods and biomasses. Thus, by running an FT-IR spectra of a pretreated biomass sample and by calculating L/C value, it is possible to predict as to how a pretreatment method will respond to enzymatic saccharification.

Another lignin related important parameter is the ratio of syringyl (S) and guaiacyl (G) units measured by the ratio of peak intensities at 1329 and 1268 cm^{-1}. The S/G ratio of pretreated

CS was in the range 0.94 to 1.14 while the corresponding glucose release ranged between 0.33 and 0.69 (g/g glucan). For RS, range of S/G ratio was between 1.14 and 1.26 yielding glucose in the range 0.82 and 0.92 (g/g glucan). It was worth analyzing the relation between pretreatment induced alteration of S/G ratio and subsequent glucose obtained by saccharification of pretreated biomasses. Figure 4b shows that the S/G ratio of pretreated biomasses (RS, CS and MS) and enzymatic saccharification shared a linear correlation (R^2=0.65) across different pretreatment technology and diverse biomasses. Enzymatic saccharification at 2 and 24 h also showed the similar correlation (SFigure 4). Thus, the S/G ratio of lignin may serve as a crucial feature to assess the pretreatment efficiency. Looking at the structure of syringyl (S) and guaiacyl (G) units in lignin, "S" offers two sites for cross-linking while, "G" has three such sites. Thus, the presence of a large number of G units relative to the S is believed to lead to a higher degree of lignin cross-linking or polymerization. In the paper-pulp industry, biomass with higher S/G ratio is preferable due to the ease of delignification.[23] In contrast, a slight decrease in S/G ratio as determined by mass-spectrometry gave better saccharification in case of DA pretreated poplar.[24] However, in the present study, increase in S/G ratio resulted in better saccharification possibly due to the presence of less cross-linked form of residual lignin. This observation is supported by the inverse relationship found between cross-linked lignin and enzymatic saccharification as depicted in Figure 4a. The reduced cross-linking within the lignin can be attributed to the removal of G units because of pretreatment, thereby increasing the enzymatic accessibility.

2.4 Structural features of cellulose vis-à-vis enzymatic saccharification

Using FT-IR, properties of cellulose viz. hydrogen bond index (HBI) and lateral order index (LOI) were calculated for native and pretreated biomass. [16] The hydrogen bond index (HBI) is a property specific to cellulose, considering the chain mobility and bond distance; the HBI of

cellulose is closely related to the crystal system and the degree of intermolecular regularity, i.e., crystallinity.[25] The ratio of the absorbance bands at 3400 cm^{-1} (H-bonded absorption) and 1320 cm^{-1} (CH_2 rocking vibration) was used to measure HBI of the cellulose samples. [22, 26] The range of HBI for pretreated CS, RS and MS was found to be 1.16-1.45 leading to a glucose release between 0.33 and 0.92 g/g glucan (data not shown). Although, increase in DA or SE pretreatment severity for RS and CS resulted in decreased HBI values (Table 2) no significant correlation with saccharification efficiency was observed when variable biomasses were studied. In case of SE pretreated *Eucalyptus grandis,* increased values of HBI have been reported.[22]

Another feature of cellulose rich residues worth analyzing was LOI which is correlated to the overall degree of order in cellulose. The band at 1430 cm^{-1} assigned to CH_2 scissoring motion in cellulose associated with the amount of crystalline structure, while the band at 898 cm^{-1} corresponding to C-H deformation is assigned to the amorphous region in cellulose. The ratio between the band intensities at 1430 cm^{-1} and 898 cm^{-1} is used as an LOI.[27] Lower values of LOI indicate less ordered structure of cellulose and hence, lower crystallinity.[26]

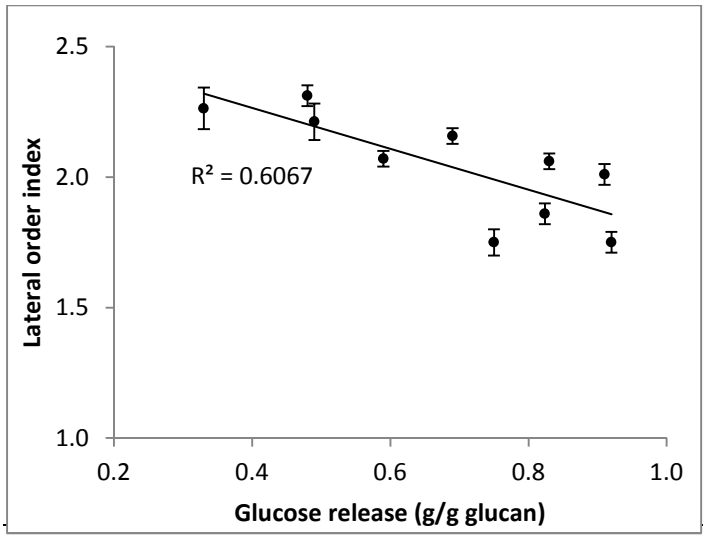


Figure 6. Correlation between lateral order index (LOI) with enzymatic saccharification. RS, CS and MS were pretreated by DA and SE. FT-IR determined LOI (-●-) of the resulting pretreated biomasses were plotted against the glucose release determined by their subsequent enzymatic saccharification. Error bars indicate standard deviation within the sets of experiments ($p<0.05$).

As shown in Figure 6a, pretreatment seems to have disturbed the cellulose structure to different extent leading to varying reorganization taking place (variable LOI value). As the LOI values decreased upon pretreatment, susceptibility to enzymatic attack increased. Thus, a negative correlation (R^2=0.61) was observed making LOI an indicator to assess/ predict efficiency of enzymatic saccharification across biomasses and pretreatment technology. An increase in LOI values upon steam explosion pretreatment of *E. grandis* has been reported by Xiao et al. (2014),[22] whereas, Isroi et al. (2012)[28] has observed an improved digestibility with a decrease in LOI values upon phosphoric acid pretreatment.

2.5 Surface area and enzymatic saccharification

Table 2 shows an increase in the specific surface area after pretreatment leading to an increase in glucan conversion. For example, an increase in surface area of native RS, CS and MS from 1.66, 1.88 and 1.00 to 6.66, 4.13 and 3.21 m^2/g (after DA pretreatment at 160 ^oC) resulted in an

increase in glucose release from 0.32, 0.16 and 0.15 (g/g glucan) to 0.91, 0.69 and 0.81 (g/g glucan) respectively. The spurt in surface area was much more prominent in RS than that for CS or MS. Its impact is also reflected in the enzymatic saccharification of their respective pretreated residues. Figure 7 shows a positive correlation between DA and SE pretreated biomasses and their respective enzymatic saccharification (R^2=0.82) at 48 h, intuitively due to increased accessibility to cellulase. Almost similar correlation was observed at 2 and 24 h of saccharification (SFigure 5a and 5b). Yang et al. (2011) [29] have also discussed about the specific surface area and increase in enzymatic saccharification. From the above data, it may be proposed that the surface area of pretreated biomass may serve as a structural characteristic to assess/ predict the enzymatic saccharification across different biomass and different pretreatment technologies.

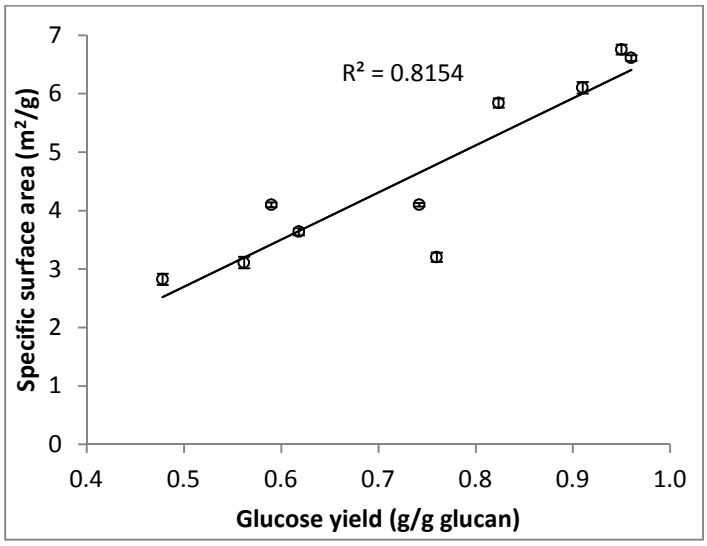


Figure 7. Correlation of specific surface area and glucose release. RS, CS and MS were pretreated by DA and SE. Specific surface area of the pretreated biomasses was plotted against the glucose release determined by enzymatic saccharification. Error bars indicate standard deviation within the sets of experiments ($p<0.05$)

Table 2. Cellulose and lignin related properties of native and pretreated biomass and surface area thereof

Sample ID	HBI (3400/1323)	CLL (1510/1600)	L/C (1512/898)	LOI (1423/898)	S/G (1329/1268)	Specific surface area (m^2/g)
CS-Native	1.36±0.02	0.79±0.01	1.33±0.01	1.97±0.01	1.03±0.007	1.88±0.01
150-CS-DA	1.19±0.006	1.18±0.01	2.06±0.02	2.31±0.01	1.06±0.03	3.67±0.03
160-CS-DA	1.16±0.01	1.12±0.01	2.01±0.03	2.16±0.01	1.14±0.01	4.13±0.03
160-CS-SE	1.17±0.02	1.15±0.01	1.95±0.01	2.26±0.01	0.94±0.01	2.82±0.09
180-CS-SE	1.28±0.03^{A}	1.09±0.01	1.83±0.01	2.21±0.01^{B}	1.02±0.01	3.11±0.07
RS-Native	1.44±0.02	0.69±0.01	1.31±0.02	2.21±0.02	1.09±0.01	1.66±0.01
150-RS-DA	1.39±0.02	0.95±0.01	1.55±0.04	2.06±0.02	1.26±0.01	6.10±0.03
160-RS-DA	1.36±0.02	0.89±0.01	1.27±0.05	2.01±0.02	1.14±0.01	6.75±0.03
160-RS-SE	1.32±0.04^{A}	0.95±0.04	1.43±0.01	1.86±0.01	1.20±0.01	5.84±0.08
180-RS-SE	1.45±0.03	0.92±0.04	1.39±0.01	2.20±0.02^{B}	1.18±0.005	6.61±0.08
MS-Native	1.51±0.02	0.74+0.02	1.39+0.01	2.17±0.02	1.03±0.006	1.00±0.01
160-MS-DA	1.29±0.01	1.08±0.02	1.90±0.01	1.75±0.01	1.04±0.008	3.21±0.02
200-MS-SE	1.22±0.01	1.24±0.01	1.91±0.01	2.07±0.02	1.06±0.01	4.11±0.03

DA: Dilute acid; **SE**: Steam explosion; **CS**: Cotton stalk; **RS**: Rice straw, **MS**: Mustard stalk; All the values are shown with standard deviation having $p<0.05$ within the same column. $p>0.05$ within the same column is depicted by superscripts ^{A} and ^{B}.

For a better presentation, a summary of various cellulose and lignin related properties of native and pretreated biomass and their correlation with enzymatic saccharification is given in Table 3.

Table 3. Correlation between biomass recalcitrance features and enzymatic saccharification

Properties/ parameters	Correlation with saccharification efficiency	R^2
a. Composition related		
1. Xylan removal (%)	Positive	0.62 (DA); 0.74 (SE)
2. Lignin content (%)	Negative	0.58
b. Cellulose related		
1. LOI	Negative	0.65
2. Surface area (BET)	Positive	0.82
c. Lignin related		
1. CLL	Negative	0.82
2. S/G ratio	Positive	0.65
3. L/C ratio	Negative	0.69

HBI: hydrogen bond index; LOI: lateral order index; CLL: Cross-linked lignin; S/G: syringyl to guaiacyl ratio; L/C: lignin to cellulose ratio; R^2 values are correlation coefficients across SE or DA pretreatment methods and variable biomasses

3. Experimental

3. 1 Biomass

Rice (*Oryza sativa*) straw was collected from Mathura District (27.49 ºN, 77.67 ºE) in Uttar Pradesh (India) in August 2013. Cotton (*Gossypium hirsutum*) stalk was collected from Sirsa district, west of Haryana (29.14-30.0 North latitude and 74.29-75.18 East longitudes), India in the month of March 2013. The biomass was air dried and shredded to the particle size ~5 mm by knife mill and stored in plastic bags at room temperature (25 ^{o}C) until further use. All the experiments were conducted using a single lot of each biomass.

Cellobiose, glucose, xylose, arabinose, acetic acid, 5-hydroxymethyl furfural (HMF), furfural, $CaCO_3$ and sulfuric acid were obtained from Merck (India) and were of analytical

grade. The cellulase enzyme preparation was a kind gift from Novozymes (India). All the chemicals were used without any further purification.

3.2 Pretreatment of biomass

3.2.a Steam explosion

Impregnation of biomass (RS, CS and MS) before SE was carried out as described previously by us. [11,12] Batches of 15 kg biomass were placed in a stainless steel (SS) wire mesh bin and dipped into a plastic tank having 1% (w/w) sulfuric acid. The liquid was recirculated using a pneumatic pump for 30 min at 30 ºC. The soaked biomass was dewatered using a hydraulic press at 100 bars. The pressed biomass was properly mixed to determine moisture content using an IR based moisture analyzer (MA 150 Sartorius, Germany) following NREL protocol.[30]

The SE experiments were carried out in an in-house designed pilot plant comprising of high pressure reactor of 10 L (SS) equipped with feeding device, cyclone separator, quick opening pneumatic butterfly valve, electric heater and a noise absorber. Before starting the experiment, steam explosion digester was flushed 3-4 times with steam at 10 bar to quickly attain the desired operating temperature. 400 g (dry basis) of acid soaked and pressed RS, CS and MS (containing ~60% moisture) was introduced into the digester and temperature was increased by injecting high pressure steam (15 bar) rapidly. The plant was run at two operating temperatures: 160 and 180 ºC and retention time was kept as 10 min. For MS, the operating temperature was 200^{o}C. After the completion of the reaction, the ball valve was opened that caused the rapid, explosive decompression and the disintegration of biomass material. The total solid recovered was estimated by NREL protocol.[31] The treated biomass received from the cyclone separator was filtered to obtain two fractions: xylose rich hydrolyzate and cellulose rich residue.

3.2.b Dilute acid pretreatment

DA pretreatment was carried out in a specially designed 2L high pressure reactor (HPR) system. The system had an additional feature to add acid to the biomass at the desired temperature by pressure equalizing mechanism and could be cooled rapidly after the completion of the reaction by circulating chilled water in reactor internal tubes. The reactor containing 100 g cotton stalk, rice straw or mustard stalk (dry basis) and 800 ml of water was heated to the desired temperature (150 and 160 ^{o}C) and the catalyst (1.23%, w/w H_2SO_4) prepared in 90 ml water was added to the reactor under pressure. The reaction was allowed to proceed for 30 min, after which cooling commenced. The total solid content was estimated and the slurry was filtered to obtain xylose rich hydrolyzate and cellulose rich residue.

3.2.c Analysis of pretreatment hydrolysate

The hydrolysates (20 ml) obtained from SE and DA pretreatments were neutralized using lime to pH 5, clarified through 0.22 µm filter and subjected to sugar analysis using HPLC (Waters, Switzerland) fitted with Biorad Aminex HPX-87H column at 50 ^{o}C equipped with a guard column. Sulphuric acid (0.008N) was used as mobile phase at 0.6 ml/min. Sugars, acetic acid and formic acid were analyzed by Refractive Index detector and other degradation products, HMF and furfural by UV detector.[32]

3.2.d Compositional analysis of pretreated biomass

The compositional analysis of native and pretreated cotton stalk was carried out by two stage acid saccharification following the standard protocol of NREL.[33,34] Total cellulose, hemicellulose and lignin were determined by NREL protocol.[35] Sugar and inhibitors released were measured as described above.

3.3 FT-IR and BET analysis

Infrared analysis was carried out using FT-IR spectrometer (IRPrestige-21, Shimadzu, Japan) and DRS8000 diffuse reflectance measurement accessory (Shimadzu) with 4 mm sampling cup. Spectra acquisition parameters were: wave number range 4000-400 cm^{-1}, resolution 4 cm^{-1} and number of scans 200. Background correction was performed using reference mirror prior to sample spectrum acquisition. Spectrum was processed using IR Solution software for base line (Zero), smooth (10), KubelkaMunk function, and second order derivative with 13 points. Peak maxima of required negative peak and height were measured with reference to 1900 cm^{-1}.

Different FT-IR based features viz. lateral order index (LOI), hydrogen bond index (HBI), cross-linked lignin (CLL) and syringyl/ guiacyl (S/G) ratio were calculated as the ratio of intensities at particular wave numbers (cm^{-1}) as: 1429/898; 3400/1323; 1510/1600 ; 1372/2920 and 1327/1267 respectively.[17]

The specific surface area of the biomass samples were calculated based on the BET theory using nitrogen adsorption isotherms at -196 °C in a surface-area analyzer (Quantachrome autosorb® IQ) using the method reported by Hsu et al. (2010).[36]

X-ray diffraction (XRD) used to determine the crystallinity index (CrI) of biomass was analyzed by Rigaku XRD in Panalytical (Netherlands), X-pert pro diffractometer set at 40 kW, 30 mA; radiation was Cu Kα (λ=1.54A) and grade range between 300 to 600 with a step size of 0.0030. CrI was calculated according the empirical method proposed by Segal et al. (1959).[37]

3.4. Biomass saccharification

Two gram pretreated biomass (on dry basis) was suspended in 20 ml of 0.05M sodium citrate buffer (10% loading) containing 0.02% sodium azide. The mixture was pre-incubated at 50 ^{o}C

for 10 min and varying FPUs of cellulose preparation were added. The mixture was incubated in a orbital shaker at 50 ^{o}C upto 72 h. Samples were withdrawn at varying intervals and centrifuged at 8,000 x g for 5 min. The supernatant was analyzed for sugars through HPLC as described above.

3.5 Statistical analysis

The compositional analysis and FTIR data has been reported as the average of three replicates. Standard deviation at each data point has been calculated by one way ANOVA using the Post Hoc Tuckey test available at Statistica.mooo.com. $p<0.05$ was set as the level of statistical significance.

Conclusions

In summary, biomass recalcitrance to deconstruction for making it amenable to enzymatic saccharification is a key controlling factor, which in turn can be influenced by its physico-chemical characteristics. Correlation curves prepared in this study between different biomass recalcitrance parameters and enzymatic saccharification would help in assessing/ predicting the saccharification efficiency of an unknown biomass by extrapolation. Chemical analysis of the pretreated biomass for residual xylan and lignin content showed that removal of hemicellulose facilitated the enzymatic saccharification while, the resultant increase in lignin hampered it. BET determined specific surface area of the pretreated biomass might serve as an indicator of efficient enzymatic saccharification. FT-IR, a simple tool, provided detailed insight into the changes occurring after pretreatment. Important physico-chemical parameters of cellulose and lignin, i.e. lateral order index (LOI), hydrogen bond index (HBI), cross-linked lignin (CLL), lignin/ cellulose (L/C) and syringyl/ guaiacyl (S/G) ratio were measured. Among these parameters, CLL, L/C ratio and LOI had a negative correlation with enzymatic saccharification while, HBI,

S/G ratio along with specific surface area had a positive correlation. These were found to be potential recalcitrant features in predicting enzymatic hydrolyzability across diverse biomasses and pretreatment methods used. Xylan removal (%) however, showed a positive correlation with the enzymatic saccharification only when single pretreatment method (DA or SE) was applied to different biomasses. LOI which represents directional orderness or crystallinity of biomass was considered an indicator of efficient pretreatment and hence, enzymatic saccharification.

This structure-activity correlation helps to assess the enzymatic saccharification while applying different pretreatment methods on different biomasses resulting to help in designing the process technology. Besides chemical analysis, surface area and crystallinity index; LOI, CLL S/G and L/C ratio may serve as important recalcitrance features for quick analysis of pretreated biomass and may prove to be a tool for performance assessment during pretreatment scale-up processes. Nevertheless, inclusion of other biomasses would further support the argument presented in the current study and validate the extrapolation of the results.

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References

1. Y. Pu, F. Hu, F. Huang, B.H. Davison, A.J. Ragauskas, *Biotechnol. Biofuels* **2013**, 6, 15
2. M. Adsul, B. Sharma, R.R. Singhania, J.K. Saini, A. Sharma, A. Mathur, R.P. Gupta, D.K. Tuli, *RSC Adv.* **2014,** 4, 44726
3. R. Samuel, S. Cao, B.K. Das, F. Hu, Y. Pub, A.J. Ragauskas, *RSC Adv*. DOI: 10.1039/c3ra40578h
4. B. Yang, C.E. Wyman, *Biofuels Bioprod. Biorefin*. **2008,** 2, 26
5. M. Li, S. Pattathil, M.G. Hahn, D.B. Hodge, *RSC Adv.* **2014,** 4, 17282

6. W. Zhang, Z. Yi, J. Huang, F. Li, B. Hao, M. Li, S. Hong, Y. Lv, W. Sun, A. Ragauskas, F. Huf, J. Peng, L. Peng, *Bioresour. Technol.* **2013,** 130**,** 30
7. N. Mosier, C.E. Wyman, B.E. Dale, R. Elander, Y.Y. Lee, M.T. Holtzapple, *Bioresour. Technol.* **2005,** 96, 673
8. N.K. Christos, A.M. Konstantinos, S.T. Kostas. *ChemSusChem.* **2013**, 6, 110
9. F. Hu, A. Ragauskas, *RSC Adv.,* **2014**, 4, 4317
10. G. Bali, X. Meng, J. Deneff, Q. Sun, A.J. Ragauskas. *ChemSusChem.* **2015**, 8, 275
11. M. Kapoor, T. Raj, M. Vijayaraj, A. Chopra, R.P. Gupta, D.K. Tuli, R. Kumar, *Carbohydr. Polym.* **2015,** 124**,** 265
12. S. Sharma, R. Kumar, R. Gaur, R. Agrawal, R.P. Gupta, D.K. Tuli, B. Das, *Bioresour. Technol.* **2015,** 175, 350
13. J.N. Saddler, L.P. Ramos, C. Breuil, in: *Bioconversion of forest and agricultural plant residues,* ed. J.N. Saddler, Oxford, UK: CAB International, **1993**, ch. 3, pp. 73
14. P. Binod, R. Sindhu, R.R. Singhania, S. Vikram, L. Devi, , S. Nagalakshmi, N. Kurien, R.K. Sukumaran, A. Pandey, *Bioresour. Technol.* **2010,** 101, 4767
15. A.J. Shaikh, R.M. Gurjar, P.G. Patil, K.M. Paralikar, P.V. Varadarajan, R.H. Balasubramanya, *Particle Boards from Cotton Stalk*, Technical Report CIRCOT, Mumbai, India
16. S.Y. Oh, D.I. Yoo, Y. Shin, G. Seo, *Carbohydr. Res.* **2005a,** 340, 417.
17. M. Polettoa, V. Pistorc, R. M. C. Santanaa, A.J. Zatterab, *Mater. Res*. **2012,** 15, 421
18. X. Duan, C. Zhang, X. Ju, Q. Li, S. Chen, J. Wang, Z. Liu, *Bioresour. Technol*. **2013,** 140, 363
19. S.H. Ghaffar, M. Fan, *Biomass Bioenerg*. 2013, DOI:10.1016
20. S. Y Leu, J. Y. Zhu. *Bioenerg. Res.* **2013,** 6, 405
21. W. Zhu, C.J. Houtman, J.Y. Zhu, R. Gleisner, K.F. Chen. *Process Biochem*. **2012,** 47, 785
22. L.P. Xiao, Z. Lin, W.X. Peng, T.Q. Yuan, F. Xu, N.C. Li, Q.S. Tao, H. Xiang, R.C. Sun, *Sustain. Chem. Process*. **2014,** 2, 9
23. R.B. Santos, E.A. Capanema, M.Y. Balakshin, H.M. Chang, H. Jameel, *BioRes.* **2011,** 6, 3623
24. B.N. Davison, S.R. Drescher, G.A. Tuskan, M.F. Davis, N.P. Nghiem, *Appl. Biochem. Biotechnol*. **2006,** 129–132, 427
25. S.Y. Oh, D.I. Yoo, Y. Shin, H.C. Kim, H.Y. Kim, Y.S. Chung, W.H. Park, J.H. Youk, , *Carbohydr. Res*. **2005b,** 340**,** 2376
26. A. Kljun, H.M. El-Dessouky, T.A.S. Benians, F. Goubet, F. Meulewaeter, J.P. Knox, R.S. Blackburn, *Eur. Polym. J.* **2014,** 51, 57
27. F. Carrilo, X. Colom, J.J. Suñol, J. Saurina, *Eur. Polym. J.* **2004,** 40, 2229
28. Isroi, M.M. Ishola, R. Millati, S. Syamsiah, M.N. Cahyanto, C. Niklasson, M.J. Taherzadeh, *Molecules* **2012,** 17, 14995
29. B. Yang, Z. Dai, S.Y. Ding, C.E. Wyman, *Biofuels* **2011,** 2, 421
30. A.D. Sluiter, B. Hames, D. Hyman, C. Payne, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, J. Wolfe, Laboratory Analytical Procedure (LAP). Technical Report NREL/TP-510-42621. Colorado, **2008a.**
31. A. Sluiter, D. Hyman, C. Payne, J. Wolfe, Laboratory Analytical Procedure (LAP). Technical Report NREL/TP-510-42627, Colorado, **2008b**
32. A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, Laboratory Analytical Procedure (LAP). Technical Report NREL/TP-510-42623, Colorado, **2006**
33. B. Hames, R. Ruiz, C. Scarlata, A. Sluiter, J. Sluiter, D. Templeton, Laboratory Analytical Procedure (LAP). Technical Report NREL/TP-510-42620, Colorado, **2008**

34. A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, Laboratory Analytical Procedure (LAP). Technical Report NREL/TP-510-42622, Colorado, **2005**
35. A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D. Crocker, Laboratory Analytical Procedure (LAP). Technical Report NREL/TP-510-42618, Colorado, **2008c**
36. T.C. Hsu, G.L. Guo, W.H. Chen, W.S. Hwang, *Bioresour. Technol.* **2010,** 101, 4907
37. L. Segal, A.E. Martin Jr., C.M. Conrad. *Text Res. J.* **1959**, 29, 786

Graphical Abstract