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**paper text:**

9 **Bioresource Technology 139 (2013) 410–414 Contents lists available at SciVerse ScienceDirect Bioresource Technology journal homepage: [www.elsevier.com/locate/biortech](http://www.elsevier.com/locate/biortech) Short Communication Impact of**

pretreatments on morphology and enzymatic saccharification of shedding bark of Melaleuca leucadendron  
Ibrahim Nasser Ahmed a, Shella Permatasari Santoso

3a, **Phuong Lan Tran-Nguyen a, Lien Huong Huynh b, Suryadi Ismadji c, Yi-Hsu Ju a,† a**

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**of Chemical Engineering, Can Tho University, 3-2 Street, Can Tho City, Viet Nam c Department of Chemical Engineering,**

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highlights SCW pretreatment primarily extracted amorphous parts of the PBT biomass. SCW pretreatment at 180 °C exposed crystalline cellulose nanofibers of PBT. Dilute acid pretreatment at 160 °C exhibited a large decrease in CrI. Dilute acid pretreated biomass had a disrupted intermolecular hydrogen bonds. Pretreatment of PBT with SCW and dilute acid resulted in high glucose release.

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**Keywords:**

Paper bark tree Subcritical water pretreatment Dilute acid pretreatment Crystallinity index XRD abstract The effects of subcritical water (SCW) and dilute acid pretreatments on

**10the shedding bark of Melaleuca leucadendron (paper bark tree, PBT)**

biomass morphology, crystallinity index (CrI) and enzymatic sacchar- ification were studied. The morphology of PBT bark was characterized

**4by X-ray diffraction, scanning elec- tron microscopy and Fourier transform infrared spectroscopy.**

SCW pretreatment mainly extracted amorphous parts of the biomass hence its CrI increased, partial decrystallization of cellulose and exposing of intact nanofibers of cellulose were observed for SCW pretreatment at 180 °C. On the other hand, dilute acid pretreatment at 160 °C exhibited a large decrease in CrI,

**6an increase in surface area, a decrease in lignin content and decrystallization of**

cellulose as well as the peel-off and degradation of some nanofiber bundles. Dilute acid and SCW pretreatments of PBT biomass resulted in about 4.5 fold enhancement in glucose release relative to the untreated one.

12 **© 2013 Elsevier Ltd. All rights reserved. 1. Introduction In**

biorefinery based on lignocellulosic materials with sugars as the intermediates, it is necessary to break down the structure of feedstock and obtain sugars from cellulose and hemicellulose. The main technological challenge in biological processing of lignocellulosic biomass into fuels and chemicals is to overcome the recalcitrance of cellulose to hydrolysis (Himmel et al., 2007). The main reasons are the presence of lignin (Grabber et al., 2008), the degree of crystallinity (Park et al., 2010), the degree of polymerization of polysaccharides (Merino and Cherry, 2007), available surface area and moisture content (Hendriks and Zeeman, 2009). ↑

2 **Corresponding author. Tel.: +886 2 27376612; fax: +886 2 27376644. E-mail address: yhju@mail.ntust.edu.tw (Y.-H. Ju). 0960-8524/\$ - see front matter © 2013 Elsevier Ltd. All rights reserved. http://dx.doi.**

org/10.1016/j.biortech.2013.04.095 The effects of pretreatment on the changes of chemical and physical features of lignocellulosic biomass have been investigated for the pretreatment of corn stover using aqueous ammonia (Kim et al., 2003), rice straw using organosolve (Sindhu et al., 2012), switch grass using dilute acid and ionic liquid (Li et al., 2010) and sugar cane bagasse using microwave assisted dilute acid (Chen et al., 2011). Recently,

10 **the shedding bark of Melaleuca leucadendron (Paper-bark Tree, PBT)**

as feedstock for bioethanol production was investigated (Ahmed et al., 2013a). The objective of this work was to study the morphology of untreated PBT biomass and PBT biomass treated with dilute acid and SCW, and compare the saccharification efficiencies of treated and untreated PBT biomasses into fermentable sugars. Biomass crystallinity and morphology were characterized by XRD, FTIR and FE-SEM. I.N. Ahmed et al. / Bioresource Technology 139 (2013) 410–414 411 2. Methods 2.1. Raw material PBT shedding bark was collected from the experimental farm of National Taiwan University, Taipei, Taiwan. The air dried bark was milled to pass 8 mm screen and stored in a desiccator. 2.2. Pretreatment Dry milled bark (10 g) was pretreated with SCW or dilute sulfuric acid (100 mL) in a reactor. Specification of the reactor was described by Ahmed et al. (2013b). The suspension was heated to the desired temperature (120–180 °C) for a predetermined time (1–3 h). After that the reactor was rapidly cooled to room temperature and the slurry collected from the reactor was filtered. The filtrate (prehydrolysate) was analyzed for its glucose, xylose, 5-hydroxymethylfurfural (HMF) and furfural contents. The collected solid was washed with deionized water and dried using a freeze dryer (LABCONCO, 2.5 Free Zone, USA). 2.3. Chemical composition of PBT shedding bark Contents of specific structural carbohydrates and lignin in untreated and pretreated barks were determined following the method described by Sluiter et al. (2011). The total amount of oligomeric sugars in the sample was calculated after the prehydrolysate was

15 **autoclaved with 4% sulfuric acid for 1 h at 121 °C to break down oligomeric sugars into monomeric**

ones as described by Ahmed et al. (2013a). The contents of HMF and furfural in the filtrate were determined by using an HPLC (Jasco, Japan) equipped with a PU-2089 pump, a degasser, a UV-2077 detector and a Luna C-18 column (5  $\mu$ m particle size, 250 mm  $\times$  4.6 mm, Phenomenex, USA) as described by Ahmed et al. (2013b). 2.4.

**16 Fourier transform-infrared (FT-IR) spectroscopy FT-IR spectra were recorded using a FT-IR spectrometer**

(FTS- 3500, Bio Rad). The sample and KBr pellet for analysis were mixed at a ratio of 1:100. Each sample was recorded from 4000 to 400  $\text{cm}^{-1}$  with 2  $\text{cm}^{-1}$  resolution in transmission mode. A set of 64 scans were collected for each sample (Moniruzzaman and Ono, 2013). 2.5. X-ray diffraction (XRD) XRD pattern of the sample was conducted according to the method described by Chang and Holtzapple (2000). Freeze dried and powdered sample was positioned on a quartz sample holder and scanned (speed 0.55° min<sup>-1</sup>, range 2 $\theta$  = 5–50°, step size 0.02°) at room temperature by using an X-ray diffractometer (Bruker D2 PHASER)

**14 in conjunction with a Cu K $\alpha$  radiation source ( $\lambda$  = 0.154 nm) operated at 30 kV.**

Biomass crystallinity (expressed as CrI) was determined as follows.  $I_{002} / (I_{002} + I_{am})$  where  $I_{002}$

**11 is the intensity for the crystalline portion at  $2\theta = 22.5^\circ$  and  $I_{am}$  is the peak for the amorphous portion at**

$2\theta = 18.7^\circ$ . 2.6. Scanning electron microscopy (SEM) Prior to acquiring images, the sample was

**7 mounted with double sided carbon tape on precut brass sample stub and sputter coated with approximately 30 Angstrom of**

platinum (Ahmed et al., 2013b) and characterized by FE-SEM (JEOL JSM-6500F). Images reported here

**7 were acquired with a 10 kV accelerating voltage**

at various magnifications (2500 –30000 ). Low magnification imaging (200 , 10 kV) of the biomass was examined by using a SEM (JEOL JSM-6390LV SEM). 2.7. Enzymatic saccharification Freeze dried solid (10 g) was suspended in sodium citrate buffer (100 mL, 50 mM, pH 4.8). A mixture of cellulase (33 FPU/g biomass) and  $\beta$ -glucosidase (66 CBU/g biomass) was used.  $\beta$ -Glucosidase (Novozyme 188 from *Aspergillus niger*) was used to supplement the insufficient  $\beta$ -glucosidase activity in cellulose. Hydrolysis

13 was performed at 50 °C for 72 h. The hydrolysate samples were

centrifuged (3500g, 5 min), filtered and stored at 20 °C. 2.8. Statistical analysis Each experiment was done at least in triplicate and the statistical significance difference of the results was checked by a t-test with the level of significance  $p < 0.05$  using Microsoft Excel. 3. Results and discussion 3.1. Composition of PBT bark and mass balance The compositions of PBT bark prehydrolysate and solid residue are listed in Table 1. A maximum of 47% of available xylan was solubilized into monomeric xylose at a SCW pretreatment temperature of 180 °C. Under the same pretreatment condition a significant amount of glucose was detected in the prehydrolysate which may result from the dissolution of minor hexose sugar associated with hemicelluloses and amorphous cellulose. Similarly in dilute acid pretreatment monomeric xylose recovery increased with temperature until 140 °C and a maximum of 65.7% solubilized xylan was obtained. However, further increase of temperature to 160 °C resulted in a drastic decline in xylose yield, presumably due to degradation. Thus weight loss of the residue solid after SCW pretreatment was mainly attributed to xylan and acid soluble lignin dissolution into prehydrolysate as monomeric/oligomeric xylose, furfural, solubilized lignin and others which were not analyzed in this work. In general, the analysis of carbohydrate and non carbohydrate compositions of bark showed good mass balance (Table 1), which agrees with previous report on mass balance of bark during SCW pretreatment (Ahmed et al., 2013a). The calculation incorporated the including total extractives into prehydrolysate and the balance of ash. 3.2. Biomass crystallinity and XRD analysis The XRD patterns of PBT biomass show cellulose I or native cellulose with high crystalline characteristics.

6 CrI measures the relative amount of crystalline cellulose in the total solid.

The CrI of

PBT bark increased ( $p < 0.05$ ) with increasing SCW pretreatment temperature; reaching maximum at 160 °C (Table 2). However further increase of the temperature to 180 °C caused a significant ( $p < 0.05$ ) reduction in CrI, and a partial transition of cellulose I into cellulose II. This indicates that SCW pretreatment at lower temperature mainly extracted xylan and lignin, which are amorphous, and SCW pretreatment above 180 °C was severe enough to disrupt the crystalline structure of cellulose I of PBT biomass. In the same way pretreatment time played an important role in the crystallinity of PBT. For instance an increase in SCW pretreatment time caused a 412 I.N. Ahmed et al. / Bioresource Technology 139 (2013) 410–414 Table 1 Compositions of SCW prehydrolysate and solid residue from PBT shedding bark (0.1 g mL<sup>-1</sup> air dried solid load). Composition of solid (wt.%) Untreated SCW (120 °C) SCW (140 °C) SCW (180 °C) 1% H<sub>2</sub>SO<sub>4</sub> (120 °C) 1% H<sub>2</sub>SO<sub>4</sub> (140 °C) 1% H<sub>2</sub>SO<sub>4</sub> (160 °C) Xylan Glucan Lignina Ash Extractives 18.4 ± 0.7 49.7 ± 1.12 19.8 ± 0.64 1.62 ± 0.05 9.70 ± 1.10 Composition of prehydrolysate (wt.%) Xylose Glucose Olig-xylose Olig-glucose Furfural HMF Extractives Over all mass balance 99.7 16.1 ± 0.53 49.2 ± 1.52 19.7 ± 0.51 1.62 ± 0.05 9.70 ± 0.81 47.6 ± 1.03 18.1 ± 0.77 1.62 ± 0.05 5.80 ± 0.68 42.2 ± 1.75 16.2 ± 0.94 1.62 ± 0.05 14.7 ± 0.52 47.8 ± 1.10 19.3 ± 0.20 1.62 ± 0.05 6.10 ± 0.52 43.9 ± 1.44 16.6 ± 0.47 1.62 ± 0.05 4.08 ± 0.78 38.3 ± 1.75 15.4 ± 1.08 1.62 ± 0.05 0.24 ± 0.01 0.00 ± 0.00 0.92 ± 0.01 0.00 ± 0.00 0.000 ± 0.00 0.00 ± 0.00 9.70 ± 1.10 97.9 3.37 ± 0.01 0.00 ± 0.00 4.50 ± 0.12 0.42 ± 0.10 0.001 ± 0.00 0.00 ± 0.00 9.70 ± 1.10 95.5 8.60 ± 0.31 4.50 ± 0.10 4.41 ± 0.20 1.10 ± 0.01 0.006 ± 0.00 0.001 ± 0.00 9.70 ± 1.10 94.6 2.30 ± 0.06 0.24 ± 0.01 1.02 ± 0.02 1.09 ± 0.01 0.003 ± 0.00 0.00 ± 0.00 9.70 ± 1.10 98.3 11.2 ± 0.13 3.36 ± 0.22 0.89 ± 0.03 4.50 ± 0.20 0.02 ± 0.00 0.03 ± 0.00 9.70 ± 1.10 98.3 1.50 ± 0.08 11.1 ± 0.33 0.23 ± 0.01 1.30 ± 0.02 0.04 ± 0.00

0.06 ± 0.00 9.70 ± 1.10 83.3 a Acid soluble lignin plus acid insoluble lignin. Table 2 Effect of SCW and dilute acid pretreatments on crystallinity index (Crl). Untreated SCW H<sub>2</sub>SO<sub>4</sub> 120 °C 140 °C 160 °C 180 °C 0.5% (140 °C) 1% (140 °C) 2% (140 °C) 0.5% (160 °C) 1% (160 °C) 2% (160 °C) 53 ± 1.1 54 ± 1.3 67 ± 2.1 98 ± 1.8 47 ± 1.3 98 ± 2 84 ± 1.7 66 ± 2.1 70 ± 1.2 6 ± 0.3 0.01 ± 0.00 significant (p < 0.05) increase in Crl at 120 °C (data not shown) and a significant (p < 0.05) reduction in Crl at 180 °C. Dilute acid pretreatment of PBT biomass at 140 °C resulted in dissolution of amorphous layers and decrystallization of cellulose I. For instance, PBT biomass pretreated with 0.5% H<sub>2</sub>SO<sub>4</sub> at 140 °C had lesser deformation of crystalline cellulose hence an increases of I002 peak and Crl were significantly (p < 0.05) higher relative to the untreated biomass (Table 2). However, raising the temperature to 160 °C resulted in a lower Crl (Table 2) indicating the crystalline morphology of PBT biomass is strongly affected by the pretreatment temperature. Increasing of acid concentration to 1% and 2% significantly decreased the Crl. Hence depolymerization of cellulose chains may be the major event during dissolution in dilute acid pretreatment of PBT bark at high temperature. 3.3. FT-IR analysis The intensity of FT-IR spectra of PBT bark peaks at 1043, 1110, 1155 and 1234 cm<sup>-1</sup> decreased significantly after SCW pretreatment. Liu et al. (2007) reported that the

### 13 reduction of the degree of polymerization of cellulose

was indicated by a change in the intensity and position of the CAO stretch band at 1045 cm<sup>-1</sup>. The peak at 1155 cm<sup>-1</sup> arose from CAOAC stretching at the β-1,4-glucosidic linkages in cellulose and hemicelluloses.

### 4 The peak at 1234 cm<sup>-1</sup> is generally associated with syringyl lignin and the CAO stretch in lignin and

xylan (Popescu et al., 2007); hence the decrease in this peak intensity was associated with the extraction of lignin during pretreatment. In the same way dilute acid pretreated bark showed great differences in band intensities at 1043 (CAO stretching), 1110 (anti-symmetric in-phase ring stretching), 1155 (CAOAC anti-symmetric stretching), 1234 (syringyl lignin and the CAO stretch out lignin and xylan), 1281 (CAH bending), 1323 (OAH rocking), 1372 (CAH bending), 1420 (CH<sub>2</sub> symmetric bending), and 1507 cm<sup>-1</sup> (aromatic ring of lignin). Moreover the broad peak near 3450 cm<sup>-1</sup> representing OAH stretching showed large reduction in intensity which indicates that highly ordered hydrogen bonds were disrupted through cellulose dissolution and regeneration. 3.4. Scanning electron microscopy SEM micrographs of the untreated bark displayed well-separated macrofibrils with length and diameter of 150–300 μm and 5–10 μm, respectively (Supplementary material, Fig. S1A). The SEM of SCW pretreated PBT shedding bark at 120 and 180 °C for 1 h (Figs. S1B and S1C) showed morphological changes such as reduction in sizes and agglomeration of fibers. FE-SEM images of untreated biomass macrofibril's surface (Fig. S2A) shows hairy like structures which might be amorphous carbohydrates and lignin. These structures completely covered up the inner ordered and intact crystalline fibers. The micrographs of biomass after 3 h SCW pretreatment at 120 °C show obvious surface changes (Fig. S2C). After 1 h SCW pretreatment at 180 °C, most hairy like amorphous structures on the surface were lost and some of them agglomerated. In addition cracks and holes on the macrofibrils structures appeared (Fig. S3A). Prolonged pretreatment time (3 h) at 180 °C resulted in an increase in the agglomeration of fibers. Moreover inner nanofibers are clearly revealed (Fig. S3B). Zhao et al. (2006) proposed that when macrofibrils lose amorphous cellulose, the remaining microfibril bundles have large surface potential, which could induce agglomeration to lower system energy. FE-SEM images of the dilute acid pretreated biomass (Fig. S4) show that after pretreatment pores are visible and the inside of the

biomass had clearly been opened. Individual nanofiber with widths of 10–30 nm can be seen. Moreover some nanofiber bundles peeled off from the crystalline stacks and degraded into smaller size, hence the lignocellulosic structure was destroyed significantly which resulted in generating a lot of debris (Figs. S4B and S4C). The event is more significant when implementing 1% and 2% H<sub>2</sub>SO<sub>4</sub> at 160 °C which resulted in biomass with nanofiber structure. Apertures of various sizes, fibers with reduced length and loose and disordered fibers at some points were observed. The morphological changes are consistent with the I.N. Ahmed

12et al. / **Bioresource Technology** 139 (2013) 410–414 413 **Fig. 1.** Comparison of

enzymatic saccharification of PBT biomass (10% w/v) at different pretreatment conditions. Pretreatment time = 72 h. observation that higher glucose release and faster hydrolysis rate were obtained during enzymatic saccharification. 3.5. Enzymatic saccharification Enzymatic hydrolysis of the pretreated bark showed significantly higher cellulose digestibility than the untreated one (Fig. 1). Enzyme hydrolysis of untreated biomass for 72 h yielded only 10.0 g L<sup>-1</sup> glucose. However, for shedding bark pretreated by SCW at 120, 140, 160 and 180 °C the glucose obtained is 16.4, 28.0, 34.4 and 40.7 g L<sup>-1</sup>, respectively. Hence significant ( $p < 0.05$ ) increase in cellulose digestibility with increasing SCW pretreatment temperature was observed. Similarly, dilute acid pretreated shedding bark exhibited significantly higher glucan saccharification than that of the SCW pretreated shedding bark over the time course of 72 h. This is in agreement with the results of XRD and FT-IR analyses which showed that SCW pretreatment of the bark mainly extracted amorphous xylan and lignin, leaving crystalline cellulose for enzymatic hydrolysis. The FE-SEM micrographs showed that maximum impact of SCW pretreatment was observed at 180 °C in which nanofibers of cellulose were visible but the biomass still maintained its original structure. In the case of dilute acid pretreated bark, cellulose nanofibers were degraded and deformation of crystalline cellulose I structure was observed which enhanced the enzymatic saccharification.

8**The loss of intra and inter molecular hydrogen bonding**

in cellulose provided enhanced surface

8**area leading to better enzyme accessibility and increased binding sites in recovered cellulose fibers**

from dilute acid pretreated biomass. In general the difference in the amount of glucose released from barks pretreated by SCW and dilute acid can be attributed to the difference between the decrystallized cellulose in the dilute acid pretreated bark and the largely crystalline cellulose in the SCW pretreated bark. Moreover, the hemicellulose fraction of lignocellulose is amorphous. Lignin is closely associated with cellulose fiber and acts as a binder. Both are in the non-crystalline zone of biomass and form a physical barrier to cellulase reaching cellulose (Hendriks and Zeeman, 2009). Their removal increases the surface area and porosity within the biomass, thus providing easier enzyme access to cellulose. The success of enzymatic hydrolysis generally depends partly on the pretreatment's capacity to remove cellulase-specific barriers (Jeoh et al., 2007). 4. Conclusion Study on the impacts of pretreatments on PBT bark indicates that SCW pretreatment

at 180 °C can be a promising alternative to dilute acid pretreatment in minimizing biomass recalcitrance and enhancing enzymatic saccharification. At mild pretreatment temperature (160 °C) the recovered PBT biomass from dilute acid pretreatment exhibited a disturbed intermolecular hydrogen bonds with the order of cellulose I structure decreased significantly which resulted in high surface area. Enzymatic saccharification of pretreated biomass resulted in significantly high glucose release than that of the untreated biomass by a factor of 4.5. Acknowledgements Financial supports from the National Science Council of Taiwan (NSC100-2623-E-011-001-ET) and National Taiwan University of Science and Technology (100H451403) are acknowledged. References Ahmed, I.N., Nguyen, P.L.T., Huynh, L.H., Ismadji, S., Ju, Y.H., 2013a. Bioethanol production from pretreated *Melaleuca leucadendron* shedding bark- simultaneous saccharification and fermentation at high solid loading. *Bioresour. Technol.* 136, 213–221. Ahmed, I.N., Sutanto, S., Huynh, L.H., Ismadji, S., Ju, Y.H., 2013b. Subcritical water and dilute acid pretreatments for bioethanol production from *Melaleuca leucadendron* shedding bark. *Biochem. Eng. J.* in press. <http://dx.doi.org/10.1016/j.bej.2013.03.008>. Chang, V.S., Holtzaple, M.T., 2000. Fundamental factors affecting biomass enzymatic reactivity. *Appl. Biochem. Biotechnol.* 84–86, 5–37. Chen, W.H., Tu, Y.J., Sheen, H.K., 2011. Disruption of sugarcane bagasse lignocellulosic structure by means of dilute sulfuric acid pretreatment with microwave-assisted heating. *Appl. Energy* 88, 2726–2734. Grabber, J.H., Hatfield, R.D., Lu, F., Ralph, J., 2008. Coniferyl ferulate incorporation into lignin enhances the alkaline delignification and enzymatic degradation of cell walls. *Biomacromolecules* 9, 2510–2516. Hendriks, A.T.W.M., Zeeman, G., 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour. Technol.* 100, 10–18. Himmel, M.E., Ding, S.Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D., 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* 315, 804–807. Jeoh, T., Ishizawa, C.I., Davis, M.F., Himmel, M.E., Adney, W.S., Johnson, D.K., 2007. Cellulase digestibility of pretreated biomass is limited by cellulose accessibility. *Biotechnol. Bioeng.* 98, 112–122. Kim, T.H., Kim, J.S., Sunwoo, C., Lee, Y.Y., 2003. Pretreatment of corn stover by aqueous ammonia. *Bioresour. Technol.* 90, 39–47. Li, C., Knierim, B., Manisseri, C., Arora, R., Scheller, H.V., Auer, M., Vogel, K.P., Simmons, B.A., Singh, S., 2010. Comparison of dilute acid and ionic liquid 414 I.N. Ahmed et al. / *Bioresource Technology* 139 (2013) 410–414 pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. *Bioresour. Technol.* 101, 4900–4906. Liu, C.F., Sun, R.C., Zhang, A.P., Ren, J.L., 2007. Preparation of sugar cane bagasse cellulosic phthalate using an ionic liquid as reaction medium. *Carbohydr. Polym.* 68, 17–25. Merino, S.T., Cherry, J., 2007. Progress and challenges in enzyme development for biomass utilization. *Adv. Biochem. Eng. Biotechnol.* 108, 95–120. Moniruzzaman, M., Ono, T., 2013. Separation and characterization of cellulose fibers from cypress wood treated with ionic liquid prior to laccase treatment. *Bioresour. Technol.* 127, 132–137. Park, S., Baker, J.O., Himmel, M.E., Parilla, P.A., Johnson, D.K., 2010. Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance. *Biotechnol. Biofuels* 3, 1–10. Popescu, C., Popescu, M., Singurel, G., Vasile, C., Argyropoulos, D.S., Willfor, S., 2007. Spectral characterization of Eucalyptus wood. *Appl. Spectrosc.* 6, 1117–1168. Sindhu, R., Binod, P., Janu, K.U., Sukumaran, R.K., Pandey, A., 2012. Organosolvent pretreatment and enzymatic hydrolysis of rice straw for the production of bioethanol. *World J. Microbiol. Biotechnol.* 28, 473–483. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2011. Determination of structural carbohydrates and lignin in biomass, NREL. Technical, Report NREL/TP-510-42618. Zhao, H., Kwak, J.H., Wang, Y., Franz, J.A., White, J.M., Holladay, J.H., 2006. Effects of crystallinity on dilute acid hydrolysis of cellulose by cellulose ball-milling study. *Energy Fuel* 20, 807–811.