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22Subcritical water and dilute acid pretreatments for bioethanol production from Melaleuca leucadendron shedding bark Ibrahim Nasser Ahmeda, Sylviana Sutantoa, Lien Huang Huynhb, Suryadi Ismadjic, Yi-Hsu

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Glucose Cellulase Fermentation Ethanol Subcritical water Melaleuca leucadendron abstract The feasibility of bioethanol production using the lignocellulose of the shedding bark of Melaleuca leuca- dendron (Paper bark tree) was investigated. The effects of pretreatment parameters (temperature, time and acid concentration) on the yields of sugars and inhibitors, and optimal pretreatment conditions were determined. At very low severity conditions (combined severity factor, CSF ≤ 0.335), 28% of xylan was recovered and this recovery increased with increasing CSF till it peaked to 64.4% (11.2 g xylose L⁻¹) at a CSF of 1.475. However, at CSF > 2.0, xylose yield declined due to degradation. Mild and progressive glu- cose yield was detected in prehydrolysate at CSF ≥ 1.514 , and subsequent enzymatic hydrolysis allowed complete glucan solubilization. Implementing environmentally friendly subcritical water pretreatment at CSF ≤ 0.335 on the shedding bark, about 85% of glucan solubilization was achieved after enzymatic hydrolysis. An industrial Saccharomyces cerevisiae strain readily fermented crude hydrolysate within 12 h, yielding 24.7 g L⁻¹ ethanol at an inoculum size of 2% (v/v), representing a glucose to ethanol conversion rate of 0.475 g g⁻¹ (91% ethanol yield). Based on our findings, the shedding bark is a potential feedstock for bio-ethanol production. © 2013 Elsevier B.V. All rights reserved. 1. Introduction Bioethanol has the potential as a supplement and/or replace- ment for gasoline. Currently most bioethanol is produced from starch and sugar producing crops. However, non-food plant sources like lignocellulosic biomass are far more abundant and cheaper. Among available lignocelluloses, wood and agricultural residues which have the advantage of being widely available are one of the better feedstock options, partly due to their higher cellulose content and compositional uniformity. Moreover, tree possesses a lignocellulosic

8energy conversion factor of 16 (compared to 1 and 8 for corn and sugarcane, respectively), and can be grown on marginal land, thereby minimizing encroachment on land for growing food

crops [1]. Thus the conversion of lignocellulosic biomass to fuel offers potential economical and environmental advantage. Lignocellulose consists of lignin (15–30%), and carbohydrates such as cellulose (41–53%) and hemicelluloses (14–35%), and minor components like proteins, ash, salt and minerals [2,3]. The chal- lenge in using trees bark or hardwoods as a feedstock is the difficulty associated with liberating cellulose from its lignin seal, *

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which

8is by far the most costly step of lignocellulose utilization, strongly affecting success and feasibility of prior and subsequent operations [4]. Although pretreatment

is costly, it costs more with- out pretreatment [5]. Depending on specific pretreatment, different effects may be observed on the substrate that can all contribute to improving hydrolysis. Some of these effects are: removal of some or all of the lignin which causes increased porosity in the substrate [6]; disruption of the lignin structure and its linkages with the rest of biomass; removal of hemicellulose that hampers access of cellulase to cellulose; disruption of the hemicellulose structure;

38reduction in the crystallinity of cellulose; reduction in the degree of polymer- ization of cellulose and reduction in the size of particles [7]. The

current choice of pretreatment method for lignocellulose material for ethanol production is dilute acid hydrolysis at moderate to high temperatures [8] and subcritical water (SCW) treatment [9,10].

24SCW is defined as hot water at temperatures ranging between 100 and 374 °C under high pressure to maintain water in liquid state.

It

9has been widely used for hydrolyzing organic compounds. Recently growing attention has led to extensive research activities using SCW for hydrolysis and conversion of bio-mass and carbohy- drates to useful compounds

[11–15]. On the other hand, dilute acid pretreatment predominantly solubilizes the hemicellulose fraction and disrupts the crystalline structure of cellulose fibrils, which favors fast enzymatic hydrolysis [16]. However, acid pretreatment can result in the formation of polysaccharide degradation products that are often inhibitory to downstream fermentation organisms and lower the overall sugar yields [17,18]. In addition the performance of different pretreatments may vary with feedstock types hence it is important to study the effect of pretreatment on poten- tial lignocellulose biomass. Melaleuca leucadendron (Paper-bark Tree, PBT) is a native Aus- tralian tree and now grown worldwide. The tree has grayish white, layered bark that is continually shed, exposing the whiter inner part. The bark feels soft like sponge. It has high adaptability and can grow well in poor soil, dry or submerged environments, and resists pests and pollution.

9To the best of our knowledge, there is no report on bioethanol production from the pretreated shedding bark of PBT. The objective of

this study was to produce ethanol from pretreated PBT shedding barks. The study can be summarized into three parts: (1) to investigate the effects of pretreatments vari- ables (acid concentration, temperature and time) on sugar release, inhibitors generation and morphology of biomass. (2) To under- stand the effect of pretreatment on the enzymatic saccharification of the biomass; and (3) to evaluate the fermentation potential of sugar containing hydrolysates for bioethanol production. 2. Materials and methods 2.1. Materials Shedding bark of PBT was collected from experimental farm of National Taiwan University, Da'an District, Taipei, Taiwan. The location of the farm is N25°00'59.40"; E121°32'25.1". The air dried bark was ground by a blender and passed through a sieve with mesh no. 60. Cellulases and Novozyme 188 (from *Aspergillus niger*) with an activity of 0.3 U mg⁻¹ and 250 IU mL⁻¹, respectively were purchased from Sigma company. 2.2. Experimental design and Statistical analysis

4A 4 × 3 × 3 factorial design was implemented for evaluating the effects of three pretreatment parameters:

dilute sulfuric acid con- centration (0%, 0.5%, 1%, and 2%, v/v), pretreatment temperature (120, 140 and 160 °C) and pretreatment time (15, 30 and 60 min) on yield of sugars and inhibitors and to identify optimal pretreatment conditions. A sulfuric acid concentration of 0% refers to subcritical water (SCW) pretreatment. The four levels of acid concentration (X1) were represented by 0, 1, 2 and 3, and the three levels of temperature (X2) and time (X3) each were represented by 0, 1 and 2. A total of 36 runs were done and an overall experimental design is summarized in Table 1. Pretreatment values were chosen on the basis of previous works carried out on other lignocellulosic biomass [19,20] with slight modification. The statistical significance differ- ence among experimental result was checked by using a t-test with the level of

significance $P < 0.05$. For the purpose of pretreatment optimization, the concentration (g L^{-1}) of glucose, xylose, HMF and furfural of the prehydrolysate were the responses. Regression analysis was performed to estimate the effect of independent variables (acid concentration, temperature and time) on the responses by using statistical software of Minitab-16. Each response was tested for possible linear, quadratic and cubic models to find out the best fitting model. Cubic model was used to calculate regression coefficients for xylose, and full quadratic polynomial model was used for HMF, furfural and glucose. Backward elimination strategy was used to eliminate insignificant terms from the model. Significance of each model term was determined with

28 **analysis of variance (ANOVA). The fit of the models were evaluated by the**

value of R^2 . Contours were plotted based on the final equation in order to find out interaction and to determine optimum level of each variable for pretreatment. 2.3. Pretreatment Bark sample at a solid loading of $10\% (\text{g mL}^{-1})$ was soaked in sulfuric acid (0%, 0.5%, 1% or 2%, v/v).

6 **There are three main parts in the equipment for pretreatment: reactor, heater and control devices. The reactor is made of stainless steel with a total inner volume of about**

200 mL. It is 25 mm

3 **thick and can withstand an estimated maximum operation pressure of**

100 MPa. The reactor is equipped with

6a **thermocouple and a pressure gauge. The process was run under batch mode. For subcritical treatment, nitrogen gas (99.9% purity) purchased from Dong-Xing Company (Taiwan) was used to maintain constant pressure (10 bar) in the reactor.**

In SCW pretreatment, after a predetermined time (15, 30 or 60 min), subcritical condition was terminated immediately by venting vapor in the reactor. The reactor was rapidly cooled to room temperature and the slurry collected from the reactor was immediately filtered. The filtrate was stored at 4°C for further analysis of its monomeric sugar (glucose, xylose) and inhibitors (HMF and Furfural) contents. The collected solid was washed with deionized (DI) water and kept at 4°C . 2.4. Combined severity factor The

7 **combined severity parameter facilitates comparison of a broad range of data by coupling the reaction conditions of time,**

15 **temperature, and acid concentration into a single variable. The severity factor, R_o , is defined as [21]: $R_o = t \exp (TH - TR) 14.75$ where t is pretreatment time in min, TH is the pretreatment temperature in $^\circ\text{C}$, and TR is a reference temperature, most often**

7 **100°C . To include the effect of dilute acid,**

the

7 **combined severity factor (CSF) has been used**

[22]: $\log \text{CSF} = \log R_o - \text{pH}$ In this study, CSF of pretreatment varied from -1.515 to 2.885 (Table 1). 2.5.

35Scanning electron microscope (SEM) analysis Structural differences in the morphology of PBT bark before and after pretreatment were examined by

33using a JEOL JSM- 6390LV SEM [23]. The

31pretreated solid specimen were dehydrated using a freeze dryer (LABCONCO, 2.5 Free Zone, USA) since lyophilization preserves morphology

[24]. Prior to imaging, specimens

36were mounted on a conductive tape and coated with platinum using a JEOL JFC- 1300 auto fine coater

to make the fibers conductive, and to avoid buildup of charge on the specimen. Imaging was done using a voltage of 10 kV at 500× magnification. 2.6. Enzymatic saccharification Enzyme isodose saccharifications were done for all solid residues recovered from SCW and acid pretreatments after adjusted to pH 4.8 using Ca(OH)₂. A mixture of cellulase and β -glucosidase (Novozyme 188 from A. niger) were used

33at 50 °C for 72 h in a water bath shaker (200 rpm).

β -Glucosidase was used to supplement the insufficient β -glucosidase activity in cellulases. Sodium citrate buffer was used to maintain mixture pH at 4.8. The enzyme loadings were: Cellulases 33 FPU, β -glucosidase 66 CBU g⁻¹ pretreated dry biomass. Hydrolysis was performed at 50 °C on a rotating wheel at 200 rpm for 72 h. The hydrolysate samples were centrifuged (3500 × g, 5 min), filtered

4and stored at -20 °C. Table 1 Experimental design of

PBT shedding bark pretreatment with code values of the parameters, and associated combined severity factor, CSF. No. Pretreatment parameters H₂SO₄(%, v/v) Temp. (°C) Time (min) Code valuesa pH CSF

18X1 X2 X3 1 0 2 0 3 0 4 0 5 0 6 0 7 0 8 0 9 0 10 0.

5 11 0.5 12 0.5 13 0.5 14 0.5 15 0.5 16 0.5 17 0.5 18 0.5 19 1 20 1 21 1 22 1 23 1 24 1 25 1 26 1 27 1 28 2
29 2 30 2 31 2 32 2 33 2 34 2 35 2 36 2 120 15 120 30 120 60 140 15 140 30 140 60 160 15 160 30 160 60
120 15 120 30 120 60 140 15 140 30 140 60 160 15 160 30 160 60 120 15 120 30 120 60 140 15 140 30
140 60 160 15 160 30 160 60 120 15 120 30 120 60 140 15 140 30 140 60 160 15 160 30 160 60 0 0 0 0
0 0 1 0 1 0 1 0 2 0 2 0 2 1 0 1 0 1 0 1 1 1 1 1 1 2 1 2 1 2 2 0 2 0 2 0 2 1 2 1 2 1 2 2 2 2 2 3 0 3 0 3 0 3 1
3 1 3 1 3 2 3 2 3 2 0 3.28 1 3.26 2 3.22 0 3.2 1 3.15 2 3.17 0 3.25 1 3.26 2 3.25 0 0.98 1 1.05 2 1.02 0 1.03
1 1.17 2 1.08 0 1.02 1 1.19 2 1.17 0 0.87 1 0.9 2 0.89 0 0.84 1 0.86 2 0.93 0 0.82 1 0.81 2 0.95 0 0.7 1 0.69
2 0.66 0 0.77 1 0.72 2 0.67 0 0.72 1 0.7 2 0.66 -1.515 -1.194 -0.853 -0.816 -0.495 -0.244 -0.307 0.0004
0.335 0.78 1.016 1.347 1.324 1.475 1.8759 1.923 2.074 2.355 0.895 1.146 1.477 1.514 1.795 2.016 2.123
2.434 2.595 1.065 1.406 1.707 1.584 1.935 2.286 2.223 2.584 2.885 a Code represent H₂SO₄ is X1;
temperature is X2 and time is X3. Code values represent by 0 is low level, 3 is high level for X1. And 0 is low
level, 1 is central level, 2 is high level for X2 and X3. 2.7. Composition analysis Specific structural
carbohydrates and lignin composition of air dried native PBT shedding bark sample were determined using
the standard NREL method as described by Sluiter et al. [25]. Likewise the ash and moisture contents were
determined and a two-step extraction process was performed to quantify extractives using NREL procedure
[26]. The contents of glucose and xylose in the filtrate from pre- treatment and hydrolysate were determined
by using a high performance liquid chromatography, HPLC (Jasco, Japan) equipped with Jasco 830-R1
Intelligent RI detector and a Cosmosil sugar-D col- umn (4.6 mm I.D. × 250 mm).

21Acetonitrile: water (80:20, v/v) was used as the mobile phase at a flow rate of 1.0 mL min⁻¹. The concentrations of

5-hydroxymethylfurfural (HMF) and furfural were analyzed by HPLC (Jasco, Japan) equipped with a PU-2089 pump, a degasser, an UV-2077 detector and a Luna C-18 column (5 μ m particle size, 250 mm \times 4.6 mm, Phenomenex, USA). The sample was diluted appropriately with deionized water, filtered through a 0.22 μ m PVDF syringe filter (Testhigh) and then

16injected into the column under the conditions: 25 $^{\circ}$ C column temperature,

mobile phase

16acetonitrile: water: acetic acid (11:88:1, v/v/v) with a flow rate of 1 mL min⁻¹, 25 μ L injection volume and 276 nm absorption wavelength. Finally, the concentrations of

d-glucose, d-xylose, furfural and HMF was calculated by using calibration curves obtained from standard d-glucose, d-xylose, furfural and HMF solutions, respectively. Ethanol content was determined by gas chromatography (GC-14B,

3Shimadzu, Japan) with a flame ionization detector after the sample was

centrifuged at 13,000 \times g and the supernatant was filtered using 0.22 μ m PES membrane filters (Pall, USA). Other conditions of operation were: nitrogen as mobile phase (30 mL min⁻¹), column temperature 40 $^{\circ}$ C, injector temperature 200 $^{\circ}$ C and injection volume 1 μ L. The

29concentrations of ethanol were calculated based on elution time and peak areas of known concentration of ethanol. Separations were carried out

on a column stabilwax[®] – DA (fused silica, polar phase; crossbond[®] carbowax[®] polyethylene glycol). 2.8. Fermentation Ethanol Red[®] Saccharomyces cerevisiae was used in the fermentation studies and was routinely cultured at 30 $^{\circ}$ C on YPD agar plates

26(20 g L⁻¹ glucose, 20 g L⁻¹ peptone, 10 g L⁻¹ yeast extract and 16 g L⁻¹ agar).

Hydrolysate obtained from PBT bark at various CSF was combined and adjusted to pH 5.05 using Ca(OH)₂ and glucose concentration was adjusted to 52 g L⁻¹. Any un-hydrolyzed fiber and gypsum (CaSO₄) was removed by filtration (0.22 μ m) prior to use. Inoculums for yeast shake-flask studies were prepared by selecting a single colony from YPD culture plates and inoculating into 40 mL pre-seed medium. The media consisted of 20 mL YPD broth, 20 mL filter-sterilized hydrolysate

12containing 2 g L⁻¹ KH₂PO₄, 5 g L⁻¹ yeast extract, 10 g L⁻¹ peptone and 1 g L⁻¹ MgSO₄

at pH 5.05. After incubation at 30 $^{\circ}$ C for 24 h, the optical density (OD 660) reading of the seed culture reached between 0.8 and 1.0. Aliquots (1%, 2%, 5% and 10%, v/v) were used to inoculate the main fermentation medium independently. The yeast fermentation 12 10 Sugar composition (g/L) 8 6 4 2 0 (Glucose) (Xylose) -2 -1 0 1 2 3 CSF Fig. 1. Xylose and glucose composition in PBT shedding bark prehydrolysates

4(10%, w/v solid load) presented as a function of

CSF. media consisted of filter-sterilized hydrolysate

12containing 2 g L⁻¹ KH₂PO₄, 5 g L⁻¹ yeast extract, 10 g L⁻¹ peptone and 1 g L⁻¹ MgSO₄.

The pH was 5.05. Fermentations were conducted

23with a working volume of 100 mL and were incubated at 30 °C with slow agitation. Samples were taken at regular time intervals for

measurement of biomass as well as glucose and ethanol concentrations. 3. Results and discussion 3.1. Effect of pretreatment on composition of hydrolysate and biomass morphology 3.1.1. Effect of pretreatment on xylan dissolution The yields of sugars for both SCW and acid pretreated hydrolysates (prehydrolysates) are shown in Fig. 1. For SCW pre-treatment at very low CSF (−1.515 to −0.853), little xylose (<5% xylan) was recovered in the prehydrolysates, while as CSF was increased to 0.0004, the xylose yield reached 5.0 g L⁻¹, which is about 28% of available xylan (Table 2). This does not reflect poor solubilization of PBT xylan. Previous studies on using compressed hot water for pretreatment of lignocelluloses biomass from different sources indicated that the solubilized hemicelluloses appeared mainly in oligomeric form [27–31]. Our result yielded >20% xylan dissolution

4in the absence of an acid catalyst (SCW) at a

lower temperature (140–160 °C) in contrast to previous works using higher temperature (>180 °C) and compressed hot water [27,29]. Similarly when using dilute acid pretreatment at low CSF (0.78–1.347), little xylose was obtained (<5% xylan), which is less than the maximum recovery obtained from SCW pretreatment at a CSF of 0.0004 (Fig. 1). This unusual event indicates that the main factor among Table 2 Chemical compositions of the native PBT shedding bark. Component Compositiona Glucan Xylan Galactan Extractives Ash Moisture Acid insoluble lignin Acid soluble lignin 47.2 17.4 ND 9.2 1.14 5.37 17.6 1.53 ND: not detected. a Percent composition on dry-weight basis. pretreatment variables in CSF was temperature. Xylose recovery increased with increasing CSF and peaked at a CSF of 1.475, with a value of 11.2 g L⁻¹, which is 64.4% of xylan. However, at CSF > 2.0, xylose yield declined, presumably due to degradation. Hence dilute acid had better effect on xylan solubilization in comparison with the SCW for PBT bark. Carolina et al., [16] reported a comparison of pretreatment methods and the report indicate that dilute acid had better effect on solubilization of hemicelluloses than compressed hot water pretreatment. During SCW pretreatment, temperature had the greatest impact (p = 0.002) on xylose recovery among the pretreatment variables. An increase in temperature from 120 to 160 °C was accompanied with an increase in xylose yield from 0.21 g L⁻¹ to 5.0 g L⁻¹ in the prehydrolysate while less effect was observed for reaction time (p = 0.046). Similarly, at low H₂SO₄ concentration (0.5%), comparable effect of temperature was observed from 120 to 140 °C. Further rise to 160 °C reduced xylose recovery drastically. At 1% and 2% H₂SO₄, temperature was still the most determinant factor (p < 0.01) on xylose yield with optimum values at 140 and 120 °C, respectively. Using 1% H₂SO₄ at 140 °C, significantly higher (p < 0.01) xylose yield (8.3 g L⁻¹) was obtained after 30 min pretreatment compared to 1.92 g L⁻¹ at 15 min. Further increasing reaction time to 60 min resulted in xylose degradation (about 2.65 fold loss). At the most acidic condition (2% H₂SO₄), reaction time became a more important factor at both extreme ends of temperature (120 and 160 °C). At 120 °C, xylose yield sharply increased from 1.72 to 10.8 g L⁻¹ as reaction time progressed from 15 to 60 min. However, extending reaction time from 30 to 60 min at 160 °C significantly (p < 0.05) reduced the xylose yields. Indeed, pretreating PBT samples under extreme conditions (2% H₂SO₄, 160 °C, 60 min) showed complete degradation of xylose. High xylose yields were achieved when the pretreatment took place using 2% H₂SO₄ at 120

28°C for 60 min or 2% H₂SO₄ at 140 °C for 15 min. For the

lowest H₂SO₄ concentration (0.5%), moderate temperature (140 °C) and 30 min hydrolysis time resulted higher xylose release with minor degradation. 3.1.2. Effect of pretreatment on glucan dissolution Glucose yield increased with increasing CSF, which is an indication of cellulose disruption. These trends were observed in several previous lignocellulosic biomass pretreatment studies [32–34]. Glucan depolymerization apparently resulted in the release of monomeric glucose. Subcritical water prehydrolysate (CSF ≤ 0.335) was free from monomeric glucose hence complete glucan recovery in pretreated solid residue was possible; however an increase in CSF was accompanied by an increase in glucose release. At a CSF of 1.475, where xylose yield peaked, significant glucan depolymerization was witnessed; with a glucose recovery of 3.36 g

L-1, which is about 7.15% of the available glucan. Glucose release kept increasing with increasing CSF. At a CSF of ~2.123, no xylose was detected while glucose yield reached 9.25 g L⁻¹. Similar to xylan solubilization, temperature had the greatest impact ($p = 0.001$) on glucan depolymerisation followed by reaction time and acid strength. For instance, at 0.5% H₂SO₄ (pH 1.1) and 30 min, the glucose released increased by 5.15-fold to 24.8% of the total glucan when temperature was raised from 140 °C (CSF = 1.475) to 160 °C (CSF = 2.074). A notable difference between solubilisation of the xylan and glucan fractions was the fact that the latter did not reach its potential maximum under the study conditions (Fig. 1). This glucan depolymerisation during pretreatment of PBT shedding bark requires further investigation. Söderström et al. [35] reported that up to 40% cellulose hydrolysis was possible for softwoods at high CSF (3.1–3.2), albeit at the expense of pentose sugars and accumulation of degradation products (furfural, formic acid). Moreover they reported that at CSF > 3.2, glucose yield dramatically declined through degradation to HMF and levulinic acid. From results in 0.06 Inhibitors (g/L) 0.04 0.02 0.00 (HMF) (Furfural) -2 -1 0 1 2 3 CSF Fig. 2. Furfural and HMF composition in PBT shedding bark prehydrolysates

4(10%, w/v solid load) presented as a function of

CSF. glucose release, it was observed that temperature had the most pronounced effect ($p < 0.01$) on glucose yield when implementing SCW or the most dilute acid pretreatment. However, reaction time had dominant effect ($p < 0.01$) at 2% H₂SO₄. The degrading effect of temperature on glucose was not significant at the most acidic condition used in this study (2% H₂SO₄), unlike in the case of xylose. 3.1.3. Effect of pretreatment on inhibitors accumulation During acid pretreatments, various inhibitors may be formed, such as phenolics, furfural and HMF [36,37]. These inhibitory compounds

4originate from the release and subsequent degradation of carbohydrate and

lignin.

4Formation of these compounds is directly proportional to pretreatment severity

[38]. In Fig. 2, the formation of furfural and HMF, the two major degradation products of pentose and hexose sugars, is presented as a function of CSF. SCW prehydrolysate was free of detectable inhibitors or negligible accumulation of furfural at its highest CSF, while in dilute acid prehydrolysate the accumulation of furfural and HMF increased with increasing CSF. Treatment conditions which increased low level accumulation of furfural strongly correlated to conditions associated with the progression of xylose loss (CSF > 1.75). In contrast, the appearance of low level of HMF does not appear to correlate to any glucose loss during pretreatment but presumably results from the degradation of minor hexose sugars associated with the hemicelluloses fraction. These results support similar observations in the pretreatment of both hard and softwoods [32,35,39]. 3.1.4. Modeling fit The data of sugars and inhibitors were successfully modeled using cubic and quadratic polynomial multiple regression equations, which define predicted responses in terms of the independent variables: Xylose = $0.03 + 33.47X_1 - 1.173X_2 + 0.019X_3 - 21.16X_2^2 - 0.14X_1X_2 + 7.21X_1X_3 - 5.6X_2X_3$ Glucose = $5.85 + 2.23X_1 + 3.27X_2 + 0.8X_3 - 3.11X_1^2 + 0.097X_2^2 - 0.5X_3^2 + 1.29X_1X_2 + 0.74X_1X_3 - 0.09X_2X_3$ HMF = $0.017 + 0.0093X_1 + 0.0192X_2 + 0.006X_3 - 0.0115X_1^2 + 0.011X_2^2 - 0.003X_3^2 + 0.008X_1X_2 + 0.0042X_1X_3 + 0.0053X_2X_3$ Table 3 ANOVA table of the adjusted models from SCW and dilute sulfuric acid pretreated PBT shedding bark. Source Sum of squares DF F value P-value Xylose Model 561.095 Residual 47.6 R² 0.9593 Glucose Model 416.65 Residual 34.8 R² 0.9882 HMF Model 128.44 Residual 31.93 R² 0.9721 Furfural Model 539.5 Residual 31.1 R² 0.9910 8 11.52 28 --- 9 14.01 26 --- 9 11.62 26 --- 9 11.89 26 --- 0.0000 --- 0.0001 --- 0.0001 --- 0.0001 --- Furfural = $0.0212 + 0.0064X_1 + 0.0125X_2 + 0.004X_3 - 0.0097X_1^2 + 0.0023X_2^2 - 0.005X_3^2 + 0.004X_1X_2 + 0.0026X_1X_3 + 0.0022X_2X_3$ where, X₁ is acid concentration, X₂ is temperature, X₃ is time. The regression equations obtained from ANOVA ensured a satisfactory

32adjustment of the theoretical values to the experimental data

(Table

343). The proportion of total variation attributed to each fit can be evaluated by the value of

R² and Chauhan and Gupta [40] reported a value of R² > 0.75 which indicates the aptness of the model. The relationship between responses (sugars and inhibitors yield) and

32 variables is visualized by the contour plot (Supplementary Material). The

plots present the polynomial models which showed the response of the factors varied within their experimental range and holding the third factor at fixed center level. The contour plot of xylose and furfural (Fig. S1) shows that, at 1% H₂SO₄ concentration (fixed at center level) xylan conversion to xylose is most favored

7 at a pretreatment temperature of 137 °C and a reaction time of 37 min,

but under harsher conditions (>150 °C, and >50 min) xylose conversion to furfural began to dominate. Similarly the xylose yield plot of pretreatment temperature and acid concentration when the time was kept at central value clearly shows that, the interaction of these two variables had a significant effect on response of xylose. In general the optimum condition for monomeric xylose is at moderate temperature (135–140 °C) and acidity (0.5–1% H₂SO₄). If implementing higher acidity (>1.5% H₂SO₄) it is important to use lower temperature (<130 °C) for higher xylose yield. However applying of simultaneously higher temperature (>150 °C) and higher acidity (>1.5% H₂SO₄) resulted in an increase of furfural accumulation. On the other hand, the response contour plots of glucose (Fig. S2) show that its solubility in prehydrolysis liquor depends on pretreatment severity, and did not reach its potential maximum in the model as also observed in the experiment. Although glucose and HMF yields share contour region, however more severity favored HMF accumulation. In general the optimum pretreatment condition of PBT shedding bark, which is characterized by high dissolution of xylan and low loss of glucan, was found to be 135–140 °C, 30–40 min and 1% acid concentration.

3.1.5. Effect of pretreatment on morphology of biomass SEM micrographs of native and pretreated PBT shedding bark sample are shown in Fig. 3. The native sample displayed a well-separated macrofibrils with smooth surface and length and diameter of 150–300 μm and 5–10 μm, respectively, indicating a Fig. 3. SEM images comparing the morphology of PBT shedding bark (A: native, B: pretreated at CSF -0.495, C: pretreated at CSF 1.795 and D: pretreated at CSF 1.935) samples at 500× and 10 kV. highly ordered surface structure (Fig. 3A). The pretreated sample showed morphological changes on macrofibers (Fig. 3B–D). Higher pretreatment severity was accompanied with the reduction in macrofibrils size, agglomeration and exposed more internal areas in the biomass than the native one. After pretreatment at low CSF (-0.495), the macrofibrils are still well separated and their diameters are almost the same, but the lengths of macrofibrils dropped and lost the smooth surface (Fig. 3B). Under moderate severe condition (CSF = 1.795), some agglomerate of macrofibrils appeared and their lengths are reduced and shrunken in size (Fig. 3C) which indicates depolymerization of the reactive cellulose region. Samir et al. [41] found out that under controlled conditions, hydrolysis may remove the amorphous region of cellulose fiber before crystalline region. At high severe condition (CSF = 1.935), agglomeration of macrofibrils increased greatly (Fig. 3D). Zhao et al. [42] proposed that when macrofibrils lose amorphous cellulose, the remaining microfibrils bundles have large surface potential, which could drive the agglomeration to lower the system energy. Alternatively, acid catalyzed intermolecular surface dehydration could also result in agglomeration. In general SEM shows that the long macrofibrils of PBT bark

10 dissociated by the pretreatment were fine, and had smaller average size and more roughness and surface area than the

untreated bark.

10 Moreover, the smooth and contiguous surface of the original PBT bark was perforated by pretreatment. The

porosity

10 in pretreated fibrils greatly increased the enzyme-accessible surface area.

3.2. Enzymatic hydrolysis of pretreated bark Pretreatment typically leads to degradation of hemicelluloses into sugars (mostly xylose) and solid residues with modified surface morphology, which is more accessible to

enzymatic hydrolysis. Although high conversion yield

11 **can be realized by applying high enzyme loading following biomass pretreatment, enzyme dose need to be significantly reduced to make a conversion process commercially attractive** [43]. Thus **pretreatment conditions and subsequent enzymatic hydrolysis must be optimized for maximum sugar release with minimum amount of enzyme.**

In this study, cellulase to β -glucosidase (Novozym 188) at a Filter Paper Unit (FPU): Cellobiose Unit (CBU) of 1:2 combinations ratio was implemented [44]. β -Glucosidase was used to supplement the insufficient β -glucosidase activity in cellulases. The two enzymes cooperate in a synergistic fashion to degrade the substrate. The success of enzymatic hydrolysis generally depends in part on the pretreatment's capacity to remove cellulase-specific barriers [45]. To evaluate the effectiveness of pretreatment on cellulose to glucose conversion, pretreated slurries were subsequently used in trials with fixed enzyme activity dose. Excess enzyme combination loading (33 FPU and 66 CBU per g of pretreated dry biomass) and prolonged reaction time (72 h) was implemented to exclude the effect of enzyme and time limitation on sugar production. 50 (Prehydrolysate) (Hydrolysate) (Total) Glucose release (g/L) 40 30 20 10 0 -2 -1 0 1 2 3 CSF Fig. 4. Glucose composition (g L⁻¹) in hydrolysates obtained after PBT shedding bark

14 **pretreatment (10%, w/v solid load) and enzyme saccharification (50 °C; pH 5.2; 48 h) presented as a function of CSF.**

Table 4 Typical examples of the pretreatment conditions and the results obtained in this work and in related studies. Biomass Pretreatment Prehydrolysate composition Cellulase Hydrolysate glucosed Reference Xylosea Glucosea HMFb Furfuralb Salix, wood chips Spruce, wood chips Poplar Eucalyptus Paper bark tree Paper bark tree Steam explosion at 200 °C, 0.25% H₂SO₄ Steam explosion at 200 °C, 2.5% SO₂ Organosolv at 180 °C, 1.25% H₂SO₄, 50% ethanol Dilute acid 0.75% H₂SO₄ at 160 °C Dilute acid 0.5% H₂SO₄ at 140 °C (CSF = 1.475) Subcritical water at 160 °C (CSF = 0.0004) 36 4 – 17 50 1.2 77.3 8.3 64.4 7.17 28 0.1 0.4 1.8 0.55 0.31 0.45 0.1 0.13 1.37 <0.01 0.015 ND < 0.01 15 15 20 20 33 33 81 50 82 76 92 85 [47] [48] [49] [50] This work This work ND: not detected. a Sugars dissolved (monomers only)

2 **in liquor during pretreatment, reported in weight percent (wt.%) of**

native biomass composition. b Inhibitors concentration (g L⁻¹) in liquor during pretreatment. c

2 **FPU (filter paper unit) per g**

substrate. d

2 **Enzymatic hydrolysis glucose yield; reported in weight percent (wt.%) of original glucose in wood.**

Enzyme hydrolysis of SCW pretreated material at its least severity conditions (CSF from -1.515 to -0.853) yielded moderate conversion of available glucan, with maximum reaching about 52% (24.4 g glucose L⁻¹). These are better than the un-pretreated samples which yielded only 31% conversion. Lower CSF (-0.495 to -0.244) of SCW pretreated hydrolysate resulted in $\geq 85\%$ recovery of available glucan into glucose (Fig. 4). Similarly,

37 **dilute acid pre-treatment enhanced enzymatic hydrolysis of cellulose and release of**

monomeric glucose. A close look at data from saccharification only shows that proximate results were obtained using SCW (CSF > -0.816) and dilute acid pretreatments. Implementing dilute acid pretreatment at CSF ≥ 1.75 the total recovered glucose reach a plateau of 43–48 g L⁻¹, which indicates complete solubilization of available glucan in the biomass. The small difference among samples

37 is attributed to the accumulation of inhibitors during pre-treatment

and unavailability of glucan. For PBT shedding bark pretreated by dilute acid or SCW, although significant difference was observed in the removal of hemicelluloses, equivalent amount of glucose was obtained after enzymatic hydrolysis. Therefore, SCW can be considered as a better alternative for pretreatment PBT shedding bark since it has advantages in environmentally friendly, free from inhibitors accumulation, and no need for detoxification of hydrolysate. More-over SCW pretreatment sounds economically feasible and simple to implement when compared to other biomass pretreatment methods. For instance the typical organosolv pretreatment of woody biomass requires high temperature (160–190 °C) and high ethanol concentration (40–60%) [46]. In organosolv pretreatment, despite good

2 cellulose conversion, hemicelluloses recovery was low because of sugar decomposition at high temperatures in the presence of acid;

hence it requires

2 extensive detoxification due to the high concentration of inhibitors such as furfural and

HMF (Table 4).

2 Furthermore, complete solvent (ethanol) recovery is a critical issue in process economy. Similarly, the

most commonly used

2 steam pretreatment (acid catalyzed steam explosion)

method also has drawbacks such as inhibitor accumulation, relatively A 60 50 40 (Biomass) Glucose (g/ L) (Ethanol) 30 20 10 0 -2 0 2 4 6 8 10 12 14 16 18 20 22 24 26 Time (h) C 60 50 40 (Biomass) Glucose (g/ L) (Ethanol) 30 20 10 0 -2 0 2 4 6 8 10 12 14 16 18 20 22 24 26 Time (h) 25 B 60 25 50 20 20 Ethanol (g/ L) 40 (Biomass) 15 Glucose (g/ L) (Ethanol) 30 15 10 20 10 Ethanol (g/ L) 5 10 5 0 0 0 -2 0 2 4 6 8 10 12 14 16 18 20 22 24 26 Time (h) 25 D 60 50 20 20 15 10 Ethanol (g/ L) 5 0 Glucose (g/ L) (Biomass) 40 (Ethanol) 15 30 10 20 Ethanol (g/ L) 10 5 0 0 -2 0 2 4 6 8 10 12 14 16 18 20 22 24 26 Time (h) Fig. 5. Time courses of glucose consumption, ethanol and yeast biomass production at inoculums size of 1% (A), 2% (B), 5% (C) and 10% (D) (v/v) *Saccharomyces cerevisiae*. low sugar recovery especially when applied to softwood and energy-intensive (operation temperature above 200 °C). In general, SCW pretreatment is advantageous in that very low levels of fermentation inhibitors than those generated by organosolv, steam explosion and dilute acid pretreatments. Moreover the milder temperature (120–160 °C) used requires lower energy consumption and prevents glucan and xylan degradation, resulting in better monomeric sugar recovery (>85%) in saccharification. In addition, since no chemicals is required making SCW treatment an environmentally benign and economical approach. 3.3. Hydrolysate fermentation Identifying optimum inoculums size is important in reducing the production cost of cellulosic ethanol. In this study the fermentation of hydrolysate with various *S. cerevisiae* seed cultures was performed.

13 The time courses of ethanol production, residual glucose and

dry cell biomass during fermentation are shown in Fig. 5. At low inoculums sizes (1–5%, v/v),

13 ethanol production rate in the early phase of culture was slow but rapidly increased after 5 h.

This phenomenon is more pronounced at 1% inoculum. At high inoculum size (10%, v/v), fast glucose consumption and ethanol production were observed. In the first 4 h, both biomass and ethanol

concentrations changed little since cells were adapting to the new environment. After that, biomass increased rapidly due to fast utilization of glucose and nitrogen. Between 6 and 10 h sharp increases in biomass and ethanol were observed while glucose decreased rapidly. After 10 h a gradual decrease in xylose concentration was observed (data not shown) with negligible change on ethanol concentration, which indicated that the yeast may consume xylose under glucose stress condition. Biomass concentration remained fairly constant

13after 12 h and fermentation was almost completed after 12 h. The highest ethanol concentration was 24.7 g L⁻¹

1, corresponding to a conversion of 0.475 g g⁻¹ glucose (91% ethanol yield) when using 2–5% (v/v) yeast inoculum size. At 1% inoculum, the yield was a bit lower (87%). In the case of the highest inoculum size (10%, v/v), glucose consuming and fermentation time was shorter (8 h), while conversion reduced to 0.41 g g⁻¹ glucose (80.4% ethanol yield). Turhan et al. [51] reported that 3%

19(v/v) inoculum size was optimum for ethanol production from

carob. Similarly, Sharma et al. [52]

19showed an inoculum level of 3% v/v

as

19optimum for maximum ethanol production from sunflower hulls hydrolysate.

A report by Fadel [53] showed that increasing inoculum size up to 4% increased production of ethanol from starchy industrial waste, which is also in agreement with this study. 4. Conclusion M. leucadendron shedding barks have higher glucan and xylan components. Although optimal conditions for glucan and xylan solubilization

14were found to be different, significant glucose recovery was detected under moderate severity conditions (CSF

≥ 1.514). The optimum pretreatment conditions of PBT shedding bark, which is characterized by high dissolution of hemicellulose and low loss of cellulose, is at 1% (v/v) H₂SO₄ acid concentration, 135–140 °C pretreatment temperature and 30–40 min pretreatment time. Implementing environmentally friendly subcritical water pretreatment at very low severity condition (CSF ≤ 0.335) on PBT biomass, 85% of glucan solubilization can be achieved after enzymatic hydrolysis. Fermentation trials confirmed the feasibility to convert the hydrolysate into ethanol with high yield (91%) at lower inoculums, which implies paper bark tree shedding is a promising feedstock for bioethanol production. Acknowledgments The authors would like to acknowledge the National Science Council of Taiwan (NSC100-2623-E-011-001-ET) and National Taiwan University of Science and Technology (100H451403) for financing

17this work. Appendix A. Supplementary data Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bej.2013.03>.

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