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Hydrolysis of Birch Wood by Simultaneous Ball Milling, Dilute Citric Acid, and Fungus *Penicillium simplicissimum* Treatment at Room Temperature

Li Yuan,[1] Zhenhua Chen,[1] Yonghua Zhu,[2] Xuanming Liu,[2] Hongdong Liao,[2] Ding Chen[1]
[1]College of Materials Science and Engineering, Hunan University, Changsha 410082, People's Republic of China
[2]College of Biology, Hunan University, Changsha 410082, People's Republic of China
Correspondence to: D. Chen (E-mail: ma97chen@hotmail.com)

ABSTRACT: In this study, birch wood chips were treated in one-step ball milling (BM) hydrolysis with dilute citric acid and fungus *Penicillium simplicissimum* at room temperature and atmospheric pressure. An efficient conversion process for the production of fermentable sugars from woody biomasses using wet BM system was developed, in which wood lignocellulose was hydrolyzed into reducing sugars with the total yield of 245.3 mg/g wood. The concentrations of several major substances in the hydrolyzate were discussed in detail. The yields of the monomeric sugars were notably increased in the presence of fungus *P. simplicissimum*. Corresponding structure transformations before and after milling were analyzed by X-ray diffraction, UV spectroscopy, transmission Fourier transform infrared spectroscopy, and environmental scanning electron microscopy clearly indicated that this combined treatment could be attributed to the crystalline and chemical structure changes of wood lignocellulose during BM. When compared with traditional method of BM, this work showed a more simple, novel, and environmental friendly way in mechanochemical treatment of lignocellulosic biomass, especially woody biomass. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: biodegradable; biopolymers and renewable polymers; cellulose and other wood products; degradation; morphology

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INTRODUCTION

In light of the gradual depletion of fossil resources, many recent works have focused on converting renewable biomass to fuels, such as ethanol.[1,2] Lignocellulose biomass, including a wide range of materials, such as wood, crops, agricultural residues, food waste, and industrial waste,[3] is a highly desired reproducible and abundant resource that does not compete with food and feed.[4,5]

As one of the most abundant biomaterials on earth, woods, including birch,[6,7] spruce,[7,8] pine,[9] poplar,[10,11] aspen,[12] eucalyptus,[13] willow,[14] are widely investigated for ethanol production via a continuous technology of pretreatment—hydrolysis—fermentation. Ball milling (BM) as well as the combination with other treatment processes have been recently shown to be effective for lignocellulosic substrates,[15,16] such as eucalyptus,[17] rice straw,[18] and rice hull.[16] The objective of the milling pretreatment is a reduction of particle size and crystallinity of lignocellulose in order to increase the available surface and reduce the degree of polymerization.

In biological treatment processes, microorganisms such as brown-, white-, and soft-rot fungi are used to degrade cellulose, hemicellulose, and lignin in lignocellulose materials.[19] The advantages of biological treatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological treatment processes is very low.[20] So, biological treatments are sometimes used in combination with chemical treatments.[21,22] *Penicillium simplicissimum* strain H5 is one kind of filamentous fungi obtained from Centraalbureau voor Schimmelcultures (CBS), the Netherlands (CBS number 328.59). It is applicable in degrading natural lignocellulose substrate with high production of cellulase, hemicellulose enzyme, and some production of ligninolytic enzyme.[23,24]

Diluted acid treatment appears as more favorable method for industrial applications and have been studied for pretreating wide range of lignocellulosic biomass,[25] including certain hardwood species.[26] It can be performed at high temperature (e.g., 170–210°C) during a short period of time[27–29] or at lower temperature (e.g., 100–120°C) for longer retention time (30–90 min).[30,31] Nevertheless, while depending on the process temperature, some sugar degradation compounds and aromatic lignin degradation compounds are detected, and affect the microorganism metabolism in the fermentation step.[28,32]

Birch is one of the largest and most prominent hardwoods in Northeast China, and is extensively employed in the North China paper industry. However, birch wood chips, obtained from pruning, lack of alternative uses. Typically, birch wood chips is composed of 44.7% cellulose, 25.1% hemicellulose, 20.6% lignin, and 9.6% ash. The cellulose and hemicellulose, which typically comprise two-thirds of the dry mass, are polysaccharides that can be hydrolyzed to sugars and eventually be fermented to ethanol.[26] The dilute acid hydrolysis of birch wood at high temperature in an ethanol production scheme has been previously reported,[6,7] but there was no report about treatment at atmospheric pressure and room temperature reactor.

In our previous research, the utilization of immobilized cellulase combining with wet BM in degrading microcrystalline cellulose was investigated and the yield of glucose was 1.89 mg/mL.[33] Furthermore, in order to promote the saccharification process of agricultural biomass, the combined treatment of BM, mild acid and fungus hydrolysis was used to enable the conversion of the wheat straw (217 mg/g wheat straw, 200 small stainless steel beads, rotation speed 500 rpm, in citrate solvent of pH 4, BM for 48 h).[34] And the yielding of reducing sugar was much higher than the normal BM treatment without the combination of fungus hydrolysis.[16] Woody biomass is physically larger and structurally stronger and denser than agricultural biomass. Chemically, woody biomass has higher lignin content than agricultural biomass. Thus woody biomass could be used as substrate in the simultaneous BM, dilute citric acid and fungus *P. simplicissimum* hydrolysis treatment.

In this work, saccharification of birch wood in a simultaneous BM combined microorganisms was studied. To evaluate the effects of this treatment, sugar yields and chemical composition change in the process were tested and the structure transformations, including crystalline structure, surface morphology, and molecular structure, were also investigated by X-ray diffraction (XRD), environmental scanning electron microscopy (ESEM), UV spectroscopy, and transmission fourier transform infrared spectroscopy (FTIR), respectively.

EXPERIMENTAL

Biomass and Chemicals

Birch chips (from Haerbin, Heilongjiang Province, $\sim$ 10 years old) was about 10 mm in length, 3 mm in width, and 0.2 mm in thickness on average. It was washed five times with distilled water, dried at 60°C for 24 h, and stored at room temperature.

3,5-dinitrosalicylic acid (DNS) and citrate solvent were prepared as described by Zhou et al.[16] All the chemical reagents used were of analytical reagent grade and purchased from Country Medicine, Shanghai, China.

P. simplicissimum strain H5 was obtained from CBS (CBS 328.59). Fungal cultures were maintained on melt extract agar (MEA) plates (malt extract 20 g L^{-1}, glucose 20 g L^{-1}, peptone1 g L^{-1}) at 4°C for 4 days. The spores on the agar surface were gently scraped and blended in the sterile physiological saline as spore suspension. Then the spore concentration was assessed by microscope with a blood cell counting chamber and adjusted to 1.0×10^8 spores mL^{-1}. H5 was cultured in the sterile melt extract (ME) liquid media, and the initial spore concentration in each flask was 3.0×10^4 spores mL^{-1}. The culture flasks were shaken at 180 rpm at 28°C for 9 days. The mycelium of *P. simplicissimum* H5 were obtained and centrifuged at 4000 rpm for 3 min, washed with distilled water to remove the redundant sugar.

Hydrolysis in the Ball Milling Reactor

Hydrolysis reaction was performed in a QM-ISP04 planetary BM reactor (Instrument Company of Nanjing University, China), using a 100 mL steel milling cup and 200 spheres with a diameter of 6 mm and a mass of 500 mg and rotation speed of 500 rpm as the optimal grinding elements. Wood chips was loaded at 1.7% (w/v) solid concentration in 5 mmol L^{-1} citrate solvent and distilled water with and without *P. simplicissimum* H5, respectively. After 12, 24, 36, 48, and 60 h, samples were removed and centrifuged at 13,000 rpm for 5 min. The supernatant was taken for sugar analysis and UV spectroscopy. The remnant was freeze-dried for 24 h and then used for structure and morphology observations.

Analysis Methods

Enzyme Assays. Cellulase activity (filter paper activity, FPase) were measured as described by Ghose.[35]

Sugar Analysis. Total reducing sugars were measured as glucose according to the DNS assay.[35] Sugar conversion was defined as the amount of reducing sugars released, expressed in milligram per gram of wood chips.

Monomeric sugars were analyzed using an Agilent1100 ChemStation system provided by Agilent Company equipped with a UV detector (G1314A, Agilent, USA). A special ZORBAX Eclipse XDB-C18 HPLC column (150 mm length, 4.6 mm I.D., and 5 μm particle size) (Agilent, USA), optimized for the separation of monosaccharide-PMP(1-phenyl-3-methyl-5-pyrazolone) derivatives (The method of derivatization of monosaccharide with PMP was described as mentioned by Daia et al.,[36] was used at ambient temperature of 30°C. The PMP derivatives elution was performed with a mixture of 0.1 *M* phosphate buffer (pH 6.8) and acetonitrile in a ratio of 83: 17 (v/v, %) at a flow rate of 1 mL/min, and UV absorbance of the effluent was monitored at 245 nm. The PMP derivatives were quantified by comparing their integration values of peak area to a calibrated standard curve.

Morphology Observation. XRD measurements were performed on a ZLB-2 X-ray diffractometer (Dandong Radiative Instrument, China). The X-ray diagrams were recorded at 2θ (Bragg angle) from 10° to 60° by a goniometer equipped with scintillation counter, at a scanning speed of 0.06° s^{-1}.

UV spectra were recorded on a SHIMAZU-UV2450 spectrophotometer using 1 cm cells.

FTIR was performed using a Nexus 870 FTIR/NIR spectrometer (Thermo Electron Corporation, America), taking 100 scans for each sample with a resolution of 4 cm^{-1}, ranging from 400 to 4000 cm^{-1}.

ESEM was used to observe the microstructure and the surface morphology of wood chips, using an FEI Quanta 200 FEG

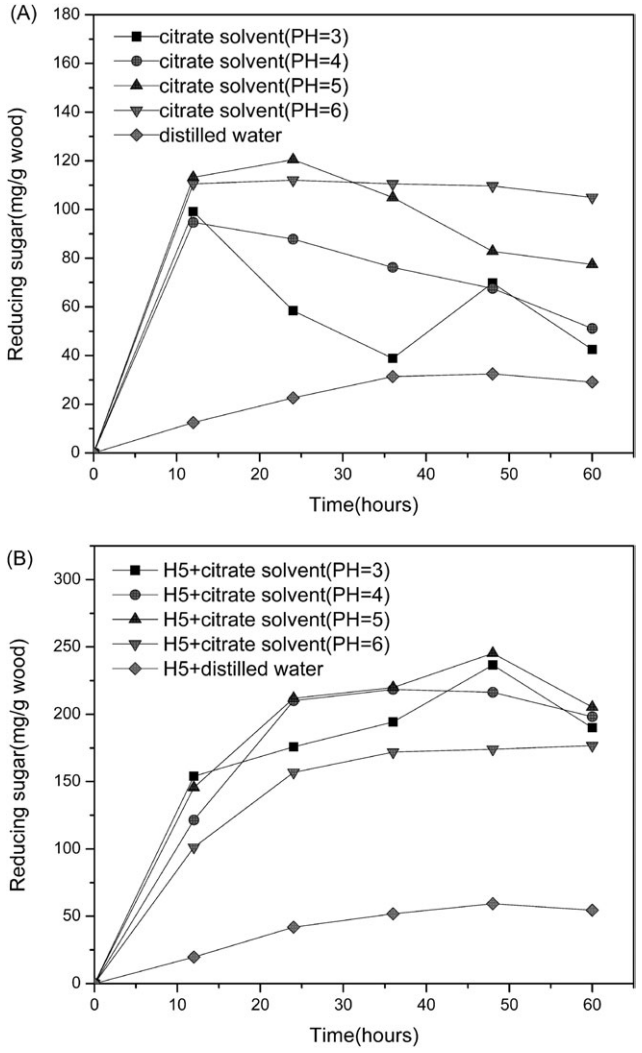

Figure 1. Effect of mild acid and ball milling time on wood chips hydrolysis with and without *P. simplicissimum* H5: (A) wood chips hydrolysis without H5 and (B) wood chips hydrolysis with H5.

ESEM with an acceleration voltage of 142 eV (American FEI Company).

RESULTS AND DISCUSSION

Sugar Yield and Chemical Composition Change

The total yield of reducing sugar resulting from wood chips by BM treatment with and without *P. simplicissimum* H5 in different medium [citrate solvent (pH = 3, 4, 5, 6) and distilled water] assayed are summarized in Figure 1. The wood chips was hardly hydrolyzed in water, the sugar production was only 32.5 mg/g wood until BM for 48 h. While in other medium, the best sugar production was almost achieved rapidly at the first 12 h. In citrate solvent (pH = 5) the sugar production was reached to 120.5 mg/g wood at 24 h. [Figure 1(A)].

With the addition of *P. simplicissimum* H5, the total yield of reducing sugar raised obviously, in which citrate solvent (pH = 5) combined with *P. simplicissimum* H5 and BM exhibited the best sugar production at 48 h (245.3 mg/g wood) [Figure 1(B)]. With extended milling time, sugar production at other pH levels gradually increased until 48 h.

The composition of monomeric sugars (glucose, mannose, galactose, xylose, and arabinose), resulting from BM treatment in different medium [citrate solvent (pH = 3, 4, 5, 6), and distilled water] with and without *P. simplicissimum* H5 referred to 1 g raw material, is summarized in Table I. When BM in citrate solvent (pH = 5), with extending BM time, the yield of glucose increased from 2.8 at 12 h to 4.0 mg/g wood at 48 h. With the addition of *P. simplicissimum* H5, the yield of glucose increased to 91.2 and 141.5 mg/g wood at 12 and 48 h, respectively. On the contrary, the value of xylose produced by BM was reduced from highest 34.2 at 12 h to 6.3 mg/g wood at 48 h. With the *P. simplicissimum* H5 addition the yield of xylose decreased to 5.8 and 2.6 mg/g wood at 12 and 48 h, respectively. That could be attributed to degradation of xylose under combined treatment. Sun et al., Larsson et al., and Cara et al. already clearly demonstrated that hemicellulose sugars could be easily solubilized by dilute acid hydrolysis pretreatment.[20,27,37] Cruz et al. also found that if the acid hydrolysis reaction time is longer than 1 h, xylose concentration decreases due to degradation.[38] Moreover, almost all the pentoses and hexoses (glucose, mannose, galactose, xylose, and arabinose) can be detected with the addition of *P. simplicissimum* H5 in BM treatment at 12 or 48 h, while galactose and arabinose were hardly detected without *P. simplicissimum* H5 in BM treatment at the same time. Thus, BM and *P. simplicissimum* H5 exerted significant different influence on the different hydrolysates. And the degradation of xylose may be the reason of sugar concentration decreases after BM for 48 h. Reaction time and fungi treating medium were crucial factor for hemicellulose degradation.

The pH value of dilute acid strongly impacts the hydrolysis of wood chips. The yield of the total monosaccharide were higher when BM for 48 h in citrate solvent pH = 5 and pH = 6 than BM in citrate solvent pH = 3 and pH = 4. With the *P. simplicissimum* H5 addition, the yield of the total monosaccharide were higher when BM for 48 h in citrate solvent pH = 4 and pH = 5 than BM in citrate solvent pH = 3 and pH = 6 (Table I). *P. simplicissimum* H5 was reported to produce cellulase and liginnase.[23,24] Correspondingly, our research found that the cellulase activity (filter paper activity, FPase) of *P. simplicissimum* H5 was 3.141 FPU/mL after cultured 9 days before BM. and the optimum pH value of cellulase was pH 4.5, that is may be the reason that *P. simplicissimum* H5 could promote the hydrolysis of wood chips, and the BM treatment in citrate solvent pH = 4 and citrate solvent pH = 5 combined with *P. simplicissimum* H5 present a dramatic increase in glucose yield as compared with BM treatment in other medium.

Many researchers adapted two-stage acid hydrolysis of wood chips or wood mill to produce fermentable monomeric sugars at high temperatures.[27,29,30,39,40] In addition to the pentose and hexose present in the hydrolysates, which can be converted into ethanol by specific microorganisms,[41] the sugar degradation products had strongly negative effect in the fermentation step.[42] The generation of by-products is increased with increasing temperature and residence time.[2] Therefore, the dilute acid hydrolysis at atmospheric pressure and room temperature could more

Table I. Composition of the Monomeric Sugars (mg/g Wood) Resulting from Ball milling in Different Medium with and Without *P. simplicissimum* H5

	Mannose	Glucose	Galactose	Xylose	Arabinose	Total
$BMW_{Ph5,12h}$	3.5	2.8	nd	34.2	nd	40.5
$BMW_{PH5,48h}$	1.6	4.0	nd	6.3	nd	11.9
$BMW_{PH3,48h}$	0.5	0.9	nd	0.3	nd	1.7
$BMW_{PH4,48h}$	2.3	1.8	nd	1.8	nd	5.9
$BMW_{PH6,48h}$	3.5	6.7	2.9	11.5	nd	24.6
$BMWwater_{,48h}$	0.6	1.8	2.1	nd	2.9	7.4
$BMPW_{Ph5,12h}$	17.0	91.2	5.3	5.8	5.7	125
$BMPW_{Ph5,48h}$	13.4	141.5	10.3	2.6	4.5	172.3
$BMPW_{Ph3,48h}$	13.6	115.2	4.9	3.7	11.4	148.8
$BMPW_{Ph4,48h}$	20.7	161.1	10.0	7.6	2.9	202.3
$BMPW_{Ph6,48h}$	26.4	35.3	4.9	7.2	3.0	76.8
$BMPWwater_{,48h}$	10.8	42.4	2.0	Nd	2.8	58

nd, not detected; BMW, ball milling wood chips in different medium without *P. simplicissimum* H5; BMPW, ball milling wood chips in different medium with *P. simplicissimum* H5; Total, total yield of mannose, glucose, galactose, xylose, and rabinose.

optimal, safer, and economically feasible way of wood hydrolysis.[43] As compared to traditional methods, our approach that combined with mild acid, *P. simplicissimum* H5 and BM at atmospheric pressure and room temperature could provide a simple, novel, and environmental friendly treatment for saccharification of lignocellulose.

Effects of Wet Ball Milling on Wood Crystalline Structure

The effect of wet BM and *P. simplicissimum* H5 on the crystal structure of wood cellulose measured by X-ray powder diffraction is shown in Figure 2. Curve *f* collected for untreated wood chips exhibits the well resolved spectrum of cellulose I with the three characteristic reflections [1 0 1], [101(−)], and [0 0 2].[44] The first two reflections, of medium-strong intensity, are obtained with 2θ ranging between 13° and 18° and the third, almost totally resolved, very sharp and with a very strong intensity, is obtained for a 2θ value of 22.5°. Moreover, another peak of weak intensity, characteristic of the [040] reflection, can be observed at a 2θ value of 35.2°. The reflection [002] became less intensive and wider as the wet BM proceeded [Figure 2(A)]. After more than 48 h treatment in citrate solvent, almost no peaks corresponding to [002] could be found [Figure 2(B)], indicating crystalline ordered scattering units was reduced because of a breakage of original crystallites and a considerable distortion of the three-dimensional crystalline order after BM.

It was observed that the major crystalline peak, which appeared around $2\theta = 22.5°$ by cellulose crystalline plane 002, was higher as *P. simplicissimum* H5 was added in the degradation treatment (Figure 3). That is, the crystallinity of cellulose increased when *P. simplicissimum* H5 participated in wood degradation. When biodegradation progresses, disordered noncrystalline region in the internal cellulose fiber would be the first target for microorganisms, thus increasing the crystallinity. After the amorphous region has been decomposed, crystalline part would be attacked by acid-BM treatment. That can explain why the crystallinity increased relatively as compared to non*P. simplicissimum* treatment, and decreased as degradation progressed after a certain period of BM time. Similar phenomenon was also fund by Park et al.[45]

Mazeau and Heux used molecular dynamics simulation to calculate the cohesive energy density of cellulose. The cohesive energy density of Iα cellulose is 46 kcal/mol larger than that of amorphous cellulose, which suggests that crystalline cellulose is much more stable than amorphous cellulose.[46] Mikushina et al. demonstrated that long-range crystalline order was decreased by mechanical milling and more amorphous characteristic might be obtained and it can be more active in chemical modifying.[47] In this work, BM disrupted the long-range crystalline structure of cellulose. It made wood more accessible to citrate solvent and *P. simplicissimum* H5, which enabling conversion of carbohydrate polymers into sugar.

UV Spectroscopy

UV spectroscopy was used to semiquantitatively determine the purity of lignin in respect of the concentration.[48] In this study, UV spectras of the water-insoluble lignin fractions were performed, which verify the purity of lignin at $\lambda = 200$–400 nm. Figure 4 shows that the UV absorption spectra of the citrate solvent (pH = 3, 4, 5, 6 BM 48 h)-soluble lignin fractions, water (BM 48 h)-soluble lignin fractions and water (BM 12 h)-soluble lignin fractions, respectively. Evidently, lignin fractions (water-BM 48 h) and (water-BM 12 h) exhibit the basic UV spectra typical of lignin, which have a maximum at ∼ 280 nm, originating from nonconjugated phenolic groups in lignin.[49,50] Interestingly, the maximum wavelength was 272 nm (water-BM 48 h) and 269 nm (water-BM 12 h), implied a relatively higher content of syringyl units in water (BM 48 h) and water (BM 12 h)-fractions since syringyl units exhibit the bands at somewhat shorter wavelengths, specifically in the 268–276 nm.

Moreover, a higher absorption of water (BM 48 h)-fractions (2.5 L.0.1/g.cm) than water (BM 12 h)-fractions (1.7 L.0.1/g.cm) indicated a higher purity of the lignin with the extending BM time in water. On the other hand, citrate solvent (pH = 3,4,5,6 BM 48 h)-soluble lignin fractions did not exhibit the

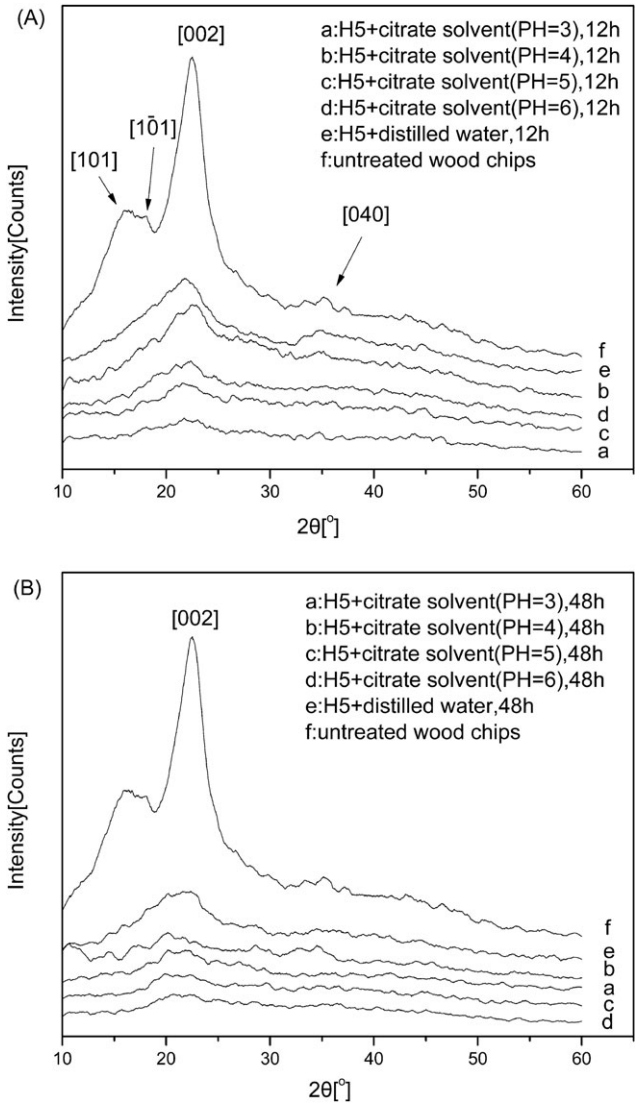

Figure 2. XRD patterns of wood chips before and after ball milling in different treating medium: (A) milling time = 12 h and (B) milling time = 48 h.

basic UV spectra typical of lignin, was undoubtedly due to more nonlignin materials such as polysaccharide degradation products, ash, salt, etc. Clearly the wood lignin was hardly hydrolyzed in water-BM, but the BM treatment in citrate solvent combined with *P. simplicissimum* H5 present a dramatic increase in lignin hydrolysis.

FTIR Analysis

FTIR analysis is a very useful technique to monitor structural changes undergone by lignin during chemical and mechanical treatments.[44] The most conspicuous changes are at wavenumbers 1034, 1115, 1240, 1329, 1371, 1425, 1462, 1593, 1738 cm^{-1} and in the range from 3200 to 3500 cm^{-1} (Figure 5). Band assignments according to the literature and band shifts are listed in Table II. The most strongly affected regions in the spectra of wood include those bands that are mainly assigned to lignin (between 1800 and 3200 cm^{-1}). Hon concluded that the

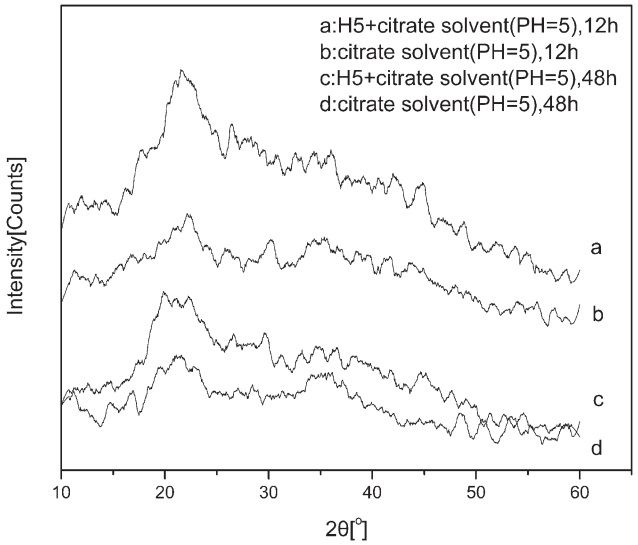

Figure 3. XRD patterns of wood chips at ball milling in citrate solvent (pH = 5) with and without *P. simplicissimum* H5.

mechanical degradation of cellulose is much less severe than that of lignin.[53] Spectra of untreated wood chips and H5-water BM wood lignin show absorbance centered at 1738 cm^{-1}, assigned to C=O in unconjugated ketones, carbonyls, and ester groups.[51] This band does not appear in other acid BM lignin, suggesting the hydrolysis of these structures.

Moreover, untreated wood chips and H5-water BM wood were showing absorbance near 1329 cm^{-1} (syringyl), which was typical for hardwood lignin,[50] and were not found in other Spectra of H5-acid BM wood (similar results also detected in UV spectroscopy). But the intensity of absorbance at 1329 cm^{-1} was lower in case of H5-water BM wood, which suggested of lower syringyl unit than untreated wood chips lignin. The bands at 1034 and 1157 cm^{-1} was associated with guaiacyl units in lignin molecules, which indicated the presence of guaiacyl unit in the

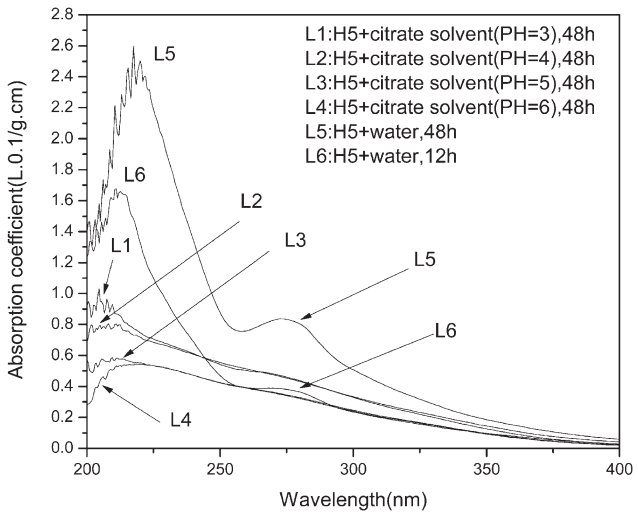

Figure 4. Ultraviolet (UV) spectra of water-insoluble lignin fractions extracted with different medium combined *P. simplicissimum H5* from wood chips.

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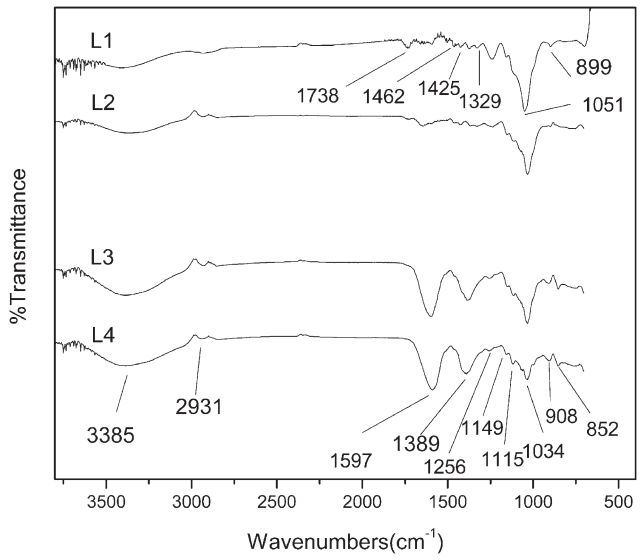

Figure 5. Infrared spectra of wood chip. L1: untreated wood chips; L2:H5 + water, 48 h; L3: citrate solvent (pH = 5), 48 h; L4:H5 + citrate solvent (pH = 5), 48 h.

lignin molecule,[52] the intensity were lower at H5-acid and H5-water wood than untreated wood chips, due to the lignin degradation in BM treatment.

As can be seen, the two spectral profiles of the citrate solvent (pH = 5, 48 h) with and without *P. simplicissimum* H5 BM wood (L4 and L3) were rather similar, only slightly differences in band intensities, indicating a similar structure of the two lignocellulose. However, differences between the spectra become visible in the regions of Aromatic skeletal vibrations plus C=O stretch at 1597 cm^{-1} and of the CH deformation vibration at 1389 cm^{-1}. Clearly, the spectrum of acid-H5 BM wood fraction L3 and L4 gives more intense bands both at 1597 and 1389 cm^{-1}, whereas only two shoulders occurred in the spectrum of untreated wood chips fraction L1 and water-H5 BM wood fraction L2. Accordingly, it can safely be concluded that acid-H5 BM wood fraction has a higher amount of Aromatic skeletal vibrations plus C=O stretch and the CH deformation vibration bonds. These can be caused by carbonyl groups of some residual aliphatic esters from hydroxycinnamic acids.[53] The intensity were higher at H5-acid wood and acid wood than untreated wood chips and water-H5 BM wood, due to the lignin degradation in BM treatment.

Morphological Observation

ESEM imagings of untreated and ball milled wood chips immediately revealed an extraordinary effect of the BM process on the shape of the wood lignocelluloses. It can be seen that

Table II. Assignment of FTIR Spectra of Wood Chips Before and After Ball milling in Different Medium with and Without *P. simplicissimum* H5

Peak location range (cm^{-1})	Assignment[48,51,52]	L1	L2	L3	L4
3400-3200	Valence vibration of hydrogen bonded OH-groups	3383	3383	3385	3385
3000-2842	C—H stretch in methyl and methylene groups	2933	2933	2939	2931
1738-1709	C=O stretch in unconjugated ketones, carbonyls and in ester groups (frequently of carbohydrate origin); conjugated aldehydes and carboxylic acids absorb around and below 1700 cm^{-1}	1738	1738	-	-
1605-1593	Aromatic skeletal vibrations plus C=O stretch; S > G; $G_{condensed}$ > $G_{etherified}$	1593	1597	1591	1597
1460-1470	C—H deformations (asym in $—CH_3$ and $—CH_2—$)	1462	1462	-	-
1422-1430	Aromatic skeleton vibrations combined with C—H in plane deformations	1425	1421	-	-
1375-1374	CH deformation vibration	1371	1367	1389	1389
1330-1325	Phenolic OH; Condensed S and G ring (G ring bound via position 5)	1329	1329	-	-
1235-1225	OH plane deformation, also COOH	1240	1238	1258	1256
1162-1125	C—O—C asymmetric valence vibration	1157	1151	1157	1149
1120-1115	Asymmetric in-phase ring stretching, C—C and C—O stretching	-	1113	1118	1115
1060-1015	C—O valence vibration mainly from C3—O3H	1051	-	-	-
1030-1035	Aromatic C—H in-plane deformation (G > S) plus C—O deform. in primary alcohols plus C—H stretching (unconjugated)	1051	1032	1034	1034
895-892	Anomere C-groups, C1—H deformation, ring valence vibration	899	901	904	908
858-853	C—H out-of-plane in position 2, 5, and 6 of G units	-	852	852	852

L1, untreated wood chips; L2, H5+water, 48 h; L3, citrate solvent (pH = 5), 48 h; L4, H5+citrate solvent (pH = 5), 48 h.

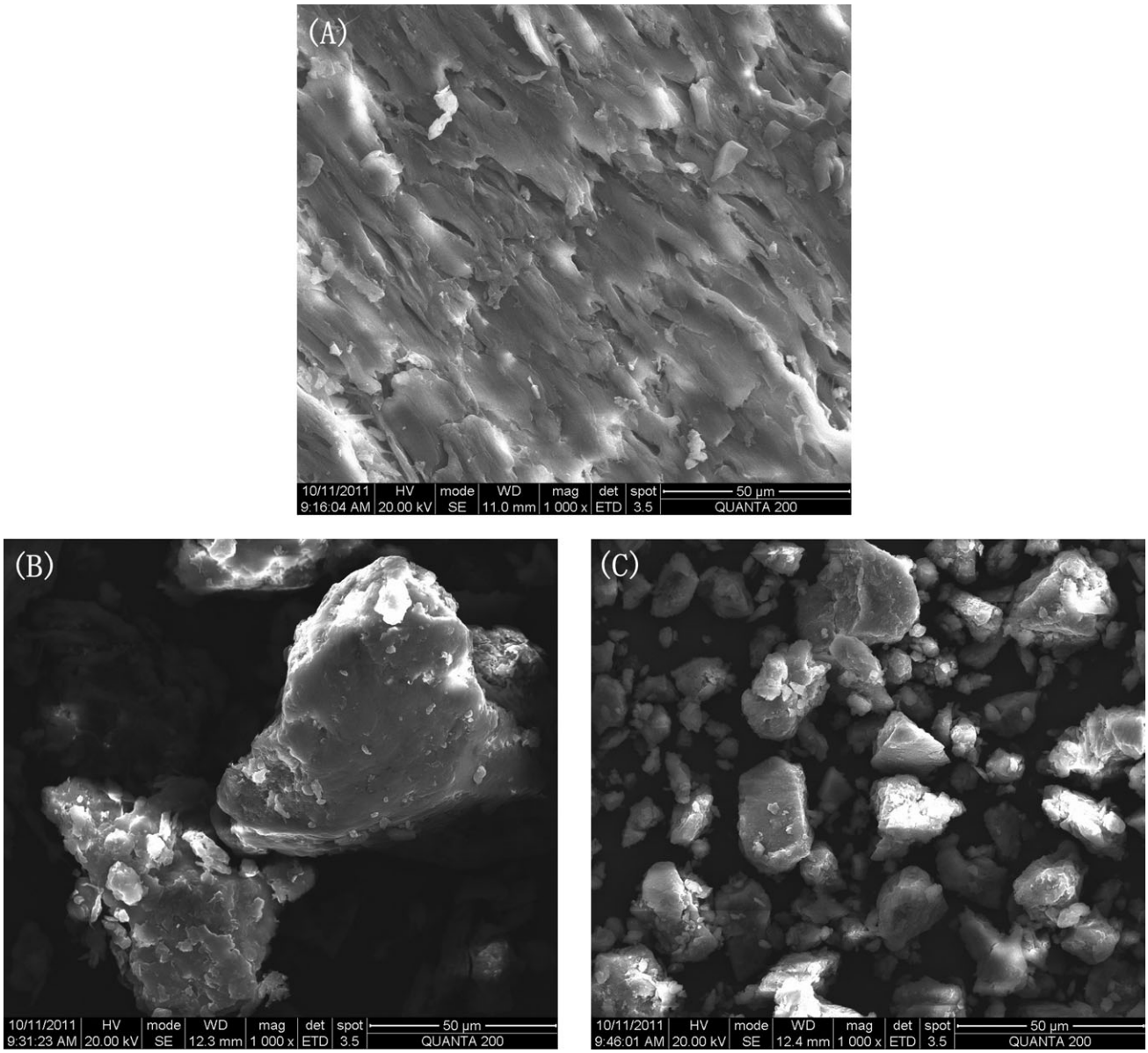

Figure 6. ESEM photos of wood chips before and after milling: (A) untreated wood chips; (B) H5 + citrate solvent (pH = 5), 12 h; and (C) H5 + citrate solvent (pH = 5), 48 h.

untreated wood chips has a typical wood fibrous morphology [Figure 6 (A)], while a progressive reduction of the fiber size was observed during the milling time, after 12 h of BM wood chips began to lost the original fibrous shape and it was reduced to small particles with dimensions ranging in the micrometers [Figure 6 (B)]. From the images an intuitionistic view of the particles size reduction under citrate solvent with increased milling time can be obtained [Figure 6 (B, C)]. After 48 h milling, the wood fibers were destroyed and converted to tiny powders with frayed surface, which had volume mean diameter about 20 μm, thus a greater surface area was introduced [Figure 6(C)]. So, BM loosed the crystalline surface and made amorphous structure, which was more accessible for chemical and biological treatment, these reactions proceeded repeatedly and synergistically until the wood chips was degraded efficiently.

CONCLUSIONS

Sugar yields and chemical composition were obtained from a simultaneous process employing mechanical BM, dilute acid hydrolysis and *P. simplicissimum* H5 function. When 200 small stainless steel beads, rotation speed of 500 rpm, citrate solvent pH 5 and *P. simplicissimum* H5 were used, produced sugar was 245.3 mg/g wood. XRD, ESEM, UV spectroscopy, and infrared spectra of wood chips milled indicated that BM broken the crystalline structure and made it more amorphous, which increased the accessibility for mild acid and fungi hydrolysis.

This technique can be recommended as a simple and environmental friendly pretreatment for wood hydrolysis.

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