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Pretreatment of *Miscanthus*×*giganteus* using aqueous ammonia with hydrogen peroxide to increase enzymatic hydrolysis to sugars

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Abstract

BACKGROUND: *Miscanthus*×*giganteus* (*M.*×*giganteus*) is a potential source for bioethanol or other useful products. Pretreatment of lignocellulosic biomass is an essential step prior to enzymatic hydrolysis to sugars and fermentation to bioethanol.

RESULTS: In this work, a one-step process uses aqueous ammonia with or without hydrogen peroxide; a proposed two-step process uses aqueous ammonia in the first step and hydrogen peroxide in the second step. In the two-step process, overall 89.5% lignin is removed. The pretreated biomass is followed by using cellulase and β-glucosidase to convert cellulose and hemicellulose from the recovered solid to fermentable sugars. The conversion of cellulose to glucose is 90.2% and to xylose is 73.4%. Characterization data are obtained for the recovered solid using scanning electron microscopy (SEM), attenuated total reflection-infrared spectroscopy (ATR-IR), and X-ray diffraction (XRD) for better understanding of the two-step process.

CONCLUSION: Results from the two-step process using aqueous ammonia and hydrogen peroxide separately are much better than those from the one-step process for removing lignin and for enhancing conversion to sugars by enzymatic hydrolysis.

Keywords: *Miscanthus*×*giganteus*; pretreatment; aqueous ammonia; hydrogen peroxide; enzymatic hydrolysis

INTRODUCTION

Miscanthus×*giganteus* (*M.*×*giganteus*) is a perennial C_4 grass that grows rapidly to over 3 m, as shown in Fig. 1. It has high yield (20–25 tons of dry matter per hectare), is resistant to pests/diseases and tolerant to cold and drought. Compared with corn and sugar cane, *M.*×*giganteus* is much cheaper and avoids concerns associated with using potential food resources for biofuels.[1–3]

Miscanthus×*giganteus* contains ∼40 wt% cellulose, ∼25 wt% hemicelluloses and ∼26 wt% lignin.[4–6] Lignin, a crosslinked hydrophobic polymer, forms a protective sheath around carbohydrates; it hinders enzymatic hydrolysis to sugars.[7–9] The purpose of pretreatment is to remove as much lignin as possible.

Previous studies reported pretreatment of *M.*×*giganteus* using sulfuric acid/ethanol/water,[10] formic acid/acetic acid/water,[11–13] formic acid/hydrogen peroxide/water,[14] aqueous NaOH,[15] ethylenediamine/DMSO,[6] ethylenediamine/1-butyl-3-methylimidazolium dimethylphosphate,[6] 1-ethyl-3-methylimidazolium acetate with ammonia and/or oxygen,[16] autohydrolysis in water with and without 2-naphthol,[17] ozone/ethanol[18] and electrolyzed water with or without alkaline peroxide.[19] For these processes, solvent recovery and/or waste-water disposal are often difficult, expensive and energy-consuming.

Aqueous ammonia is a promising candidate for pretreatment. Because ammonia is volatile, it is easily regenerated; it reacts strongly with lignin but only weakly with carbohydrates; it is an effective swelling agent. On a molar basis, the cost is about one-fourth that of sulfuric acid.[20] Hydrogen peroxide is a commercially available oxidizing agent friendly to the environment. Some studies indicated that the addition of small amounts of hydrogen peroxide can enhance lignin removal and modify cellulose structure toward favoring enzymatic hydrolysis.[21–24] Aqueous ammonia with or without hydrogen peroxide has been used to pretreat corn stover,[20,25–29] hybrid poplar,[24] switchgrass,[30–32] rice straw,[33–35] wastepaper,[36,37] rapeseed straw,[38] wheat straw,[39] sorghum fiber[40] and barley hull.[41]

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Figure 1. Miscanthus grows up to 10 ft in one season in Illinois, USA (photo from S. P. Long, University of Illinois).

This work concerns pretreatment of *M.×giganteus* using aqueous ammonia with or without hydrogen peroxide. We studied three pretreatment conditions: (1) using aqueous ammonia in one step; (2) using a mixture of aqueous ammonia and hydrogen peroxide in one step; (3) using aqueous ammonia in the first step followed by hydrogen peroxide in the second step. After each pretreatment, the solid residue was enzymatically hydrolyzed for 96 h using 65 μL cellulase and 65 μL β-glucosidase per gram of *M.×giganteus*. Experimental studies indicate the effect of aqueous ammonia and hydrogen peroxide concentrations, temperature, reaction time, and liquid:solid ratio. Finally, the effect of each pretreatment method is assessed qualitatively, based on characterization data for the recovered solid from scanning electron microscopy (SEM), attenuated total reflection-infrared spectroscopy (ATR-IR), and X-ray diffraction (XRD).

EXPERIMENTAL

Materials

Miscanthus×giganteus was obtained from the University of Illinois at Urbana-Champaign; 28–30 wt% aqueous ammonia from EMD Chemicals Inc., Germany; 50 wt% hydrogen peroxide from Acros; 0.5% (w/v) sodium azide aqueous solution from Ricca Chemical Company; cellulases (Celluclast 1.5L Product # C2730-50 mL), and β-glucosidase (Novo188 Product # C6105-50 mL) from Sigma-Aldrich (St. Louis, MO, USA). *Miscanthus×giganteus* was milled to 4 mm particles using a Retsch grinder.

Pretreatment of *M.×giganteus*

Figure 2 shows a schematic diagram illustrating pretreatment, enzymatic hydrolysis, and composition analysis. In a typical experiment, *M.×giganteus* and aqueous ammonia at a fixed mass ratio, with or without hydrogen peroxide, are placed into a pressure reactor (Moline Parr Instruments). The reactor is sealed and submerged into an oil bath. After a fixed reaction time at a fixed temperature, the reactor is transferred into an ice bath. Then, the recovered solid is separated by filtration. The solid is washed several times with water until pH 7. The final solid residue is subjected to enzymatic hydrolysis. In the two-step process, the residue from the first step is dried for 24 h at 105°C and then pretreated with hydrogen peroxide in the second step.

Enzymatic hydrolysis

Enzymatic hydrolysis for the wet solid is performed following the standard NREL protocol.[42] Prior to hydrolysis, the solid is not dried to avoid hornification of cellulose.[43,44] For 1 g dry *M.×giganteus*, 50 g 0.05 mol L^{-1} sodium citrate buffer (pH 4.8), 65 μL of cellulases, 65 μL of β-glucosidase, and 400 μL 0.5% (w/v) sodium azide are added into the liquid. Sodium azide is used to inhibit microbial growth during enzymatic hydrolysis. The liquid is put into a bottle and incubated at 50°C and 150 rpm in a shaker for 96 h. Samples are regularly withdrawn for analysis using HPLC to determine the content of glucose and xylose.

Composition analyses of *M.×giganteus* prior to enzymatic hydrolysis and of the liquid after enzymatic hydrolysis

Using the standard NREL Analytical Procedure[45] we obtained the content of cellulose, hemicellulose, and lignin in *M.×giganteus* and the content of glucose and xylose in the liquid after enzymatic hydrolysis. For this analysis we used an autoclave

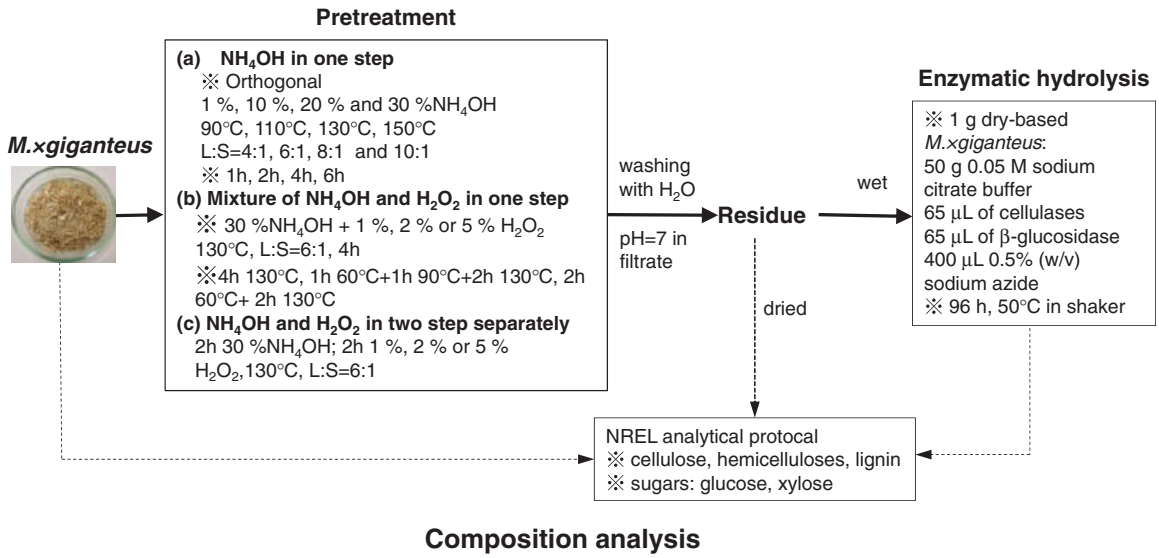

Figure 2. Schematic diagram for pretreatment of *M.×giganteus*; NH_4OH is aqueous ammonia and H_2O_2 is hydrogen peroxide.

Table 1. Pretreatment of *M.×giganteus* using aqueous ammonia in one step*

	Pretreatment conditions			Pretreatment results		
Trial No.	Aqueous ammonia concentration (wt%)	Temperature (°C)	Liquid:solid mass ratio	Cellulose recovered (%)	Hemicellulose recovered (%)	Lignin removal (%)
1	1	90	4:1	97.6	69.7	25.6
2	1	110	6:1	97.3	69.4	29.2
3	1	130	8:1	96.9	66.5	30.1
4	1	150	10:1	94.0	66.8	32.5
5	10	90	6:1	96.5	68.3	32.6
6	10	110	4:1	96.0	67.8	33.3
7	10	130	10:1	96.4	64.1	46.7
8	10	150	8:1	93.7	60.9	55.1
9	20	90	8:1	95.8	68.7	37.7
10	20	110	10:1	95.9	68.5	44.8
11	20	130	4:1	95.6	63.8	59.2
12	20	150	6:1	93.2	60.0	61.1
13	30	90	10:1	93.8	64.0	44.3
14	30	110	8:1	93.5	62.6	52.2
15	30	130	6:1	92.0	63.7	62.2
16	30	150	4:1	91.3	50.3	71.6
A(1,j)§	29.35	35.05	47.43			
A(2,j)§	41.93	39.88	46.58			
A(3,j)§	50.70	49.85	43.78			
A(4,j)§	57.88	55.08	42.08			
Rj§	28.53	20.03	5.35			

* Pretreatment time: 1 h.
§ The objective is lignin removal; A(n,j) is the average amount of objective results, n refers to the level, and j refers to the factor; Rj=max{A(n,j)} − min{A(n,j)}.

(Amsco 3043 vacamatic), a HPLC (Shimadzu, Aminex Column HPX-87H 300×7.8 mm (BioRad), refractive-index detector), and an Agilent 8453 UV-vis spectrophotometer with quartz cuvettes. Details are given in previous publications.[4–6,16] On a water-free basis, raw *M.×giganteus* contains 42.0% cellulose, 25.0% hemicellulose, 26.0% lignin, 4.0% ash, and 3.0% others (pectin, lipid, salts).

Characterization of *M.×giganteus*

SEM studies used a Hitachi S-5000 electron microscope at 15 kV accelerating voltage. ATR-IR at room temperature used a Nicole 6700 FT-IR spectrometer (Thermo scientific). XRD was with a Bruker diffractometer at 40 kV and 20 mA with Cu Kα radiation (λ=0.154 nm); the scanning rate was 0.02° s^{-1} in the 2θ range from 3 to 60°. The cellulose crystallinity index (CrI) is calculated using[46]

$$CrI = (I_{002} - I_{am})/I_{002} \times 100$$

where I_{002} and I_{am} are the intensity of diffraction at $2\theta = 22°$ and at $2\theta = 18°$, respectively.

RESULTS AND DISCUSSION

Pretreatment using aqueous ammonia

An orthogonal experiment is useful for obtaining the effects of aqueous ammonia concentration, temperature, and liquid:solid mass ratio (aqueous ammonia:*M.×giganteus*). There were four levels for each factor, i.e. 1, 10, 20, and 30 wt% for aqueous ammonia concentration; 90, 110, 130, and 150°C for temperature; 4:1, 6:1, 8:1, and 10:1 for liquid:solid mass ratio.

Table 1 shows results. As indicated by analysis of range and variance, shown in Tables 1 and 2, an orthogonal design software was used to design and analyze the experiments.[47,48] It is seen that aqueous ammonia concentration and temperature were the most significant factors affecting lignin removal while the liquid:solid ratio was less important. Lignin removal increased with aqueous ammonia concentration and temperature, while some cellulose and hemicelluose were dissolved in the liquid phase. Lignin removal of 62.2% was achieved at 30 wt% aqueous ammonia, 130°C, and liquid:solid ratio 6:1; the recovery ratios for the recovered solid were 92.0% and 63.7% for cellulose and hemicellulose, respectively. Figure 3 shows that the contents of lignin, cellulose, and hemicelluloses were nearly unchanged when the contact time was increased to 2 h.

Pretreatment using a mixture of aqueous ammonia and hydrogen peroxide

Table 3 shows that addition of hydrogen peroxide increased lignin removal remarkably while more cellulose and hemicellulose were dissolved. Comparing test No.15 in Table 1 and test No.1 in Table 3 (30 wt% aqueous ammonia), the addition of 5 wt% hydrogen peroxide raised lignin removal from 62.2 to 79.3% but reduced cellulose recovery from the recovered solid from 92.0 to 85.8% and hemicellulose recovery from 63.7 to 50.2%. Lignin removal was not strongly sensitive to the concentration of

Table 2. Analysis of variance for the orthogonal experimental results in Table 1*

Factors	Sum of square of deviations	Degrees of freedom	F	F_c (probability =0.01)
aqueous ammonia concentration	1810.5	3	4.278	9.780
temperature	1001.2	3	2.365	9.780
liquid:solid ratio	73.6	3	0.174	9.780
error	846.5	6		

*F, explained variance/unexplained variance; F_c, critical value of F.

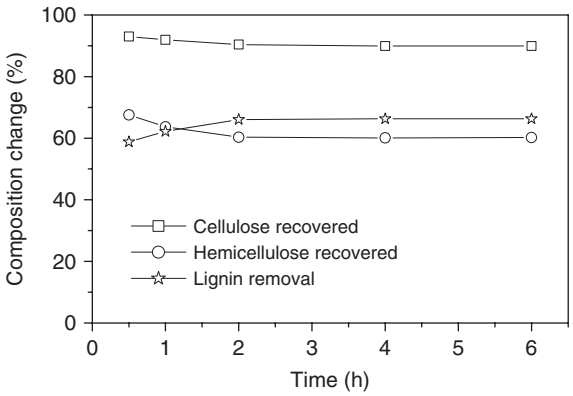

Figure 3. Pretreatment of *M.×giganteus* by 30 wt% aqueous ammonia as a function of time at130°C and liquid:solid ratio 6:1.

hydrogen peroxide: addition of 1 wt%, 2 wt%, or 5 wt% hydrogen peroxide removed 72.7, 73.1, and 79.3% lignin, respectively.

Comparison between tests No.3 and No.4 in Table 3 indicates that the concentration of aqueous ammonia was important for removing lignin. These results are consistent with previous observations that addition of hydrogen peroxide helps to remove lignin in lignocellulosic materials.[31,36,37] Three heating strategies were studied; the results in Table 4 indicate that cellulose and hemicellulose recovery were only mildly sensitive to heating-strategy.

Pretreatment using aqueous ammonia and hydrogen peroxide separately in two steps

Table 5 shows results for the two-step process. Comparison between test No.1 in Table 3 and test No. 1 in Table 5 indicates that two-step pretreatment significantly enhanced removal of lignin; lignin removal increased from 79.3 to 89.5% using 30 wt% aqueous ammonia and 5 wt% hydrogen peroxide, while cellulose and hemicellulose recovery were reduced from 85.8 to 80.9% and from 50.2 to 44.5%, respectively. Hydrogen peroxide reacts with chromophoric and reactive groups in lignin to break aryl ether bonds and other linkages between lignin and carbohydrates, similar to the role of hydrogen peroxide as a bleaching reagent in the pulp-and-paper industry.[21–23] As shown in Table 5, lignin removal increased with hydrogen peroxide concentration; lignin removal increased from 81.1 to 89.5% as the concentration of hydrogen peroxide was increased from 1 to 5 wt%. Cellulose recovery and hemicellulose recovery were less sensitive to the concentration of hydrogen peroxide. Comparing tests No.1, No.4 and No. 5 in Table 5, lower temperature in the second step using hydrogen peroxide reduced delignification; lignin removal decreased from 89.5 to 72.0% when the temperature was reduced from 130 to 90°C. Table 6 indicates that lignin removal obtained in this work is comparable with that reported in the literature.[6,10–12]

Enzymatic hydrolysis of the pretreated recovered *M.×giganteus*

Figure 4 shows results from enzymatic hydrolysis of *M.×giganteus* pretreated with aqueous ammonia in the one-step process. The results are not satisfactory; the highest conversion of cellulose to glucose and the highest conversion of hemicellulose to xylose were near 40% and 50%, respectively. For raw *M.×giganteus* conversions were only 4.9% and 2.3%, respectively. Conversion of carbohydrates to sugars increased with aqueous ammonia concentration; when aqueous ammonia concentration was increased from 1 to 30 wt%, conversion of cellulose to glucose increased from 18.0 to 39% and conversion of hemicellulose to xylose increased from 24.3 to 48.1%. Higher pretreatment temperature did not significantly improve conversion of carbohydrates.

Figure 5 shows results of enzymatic hydrolysis following one-step pretreatment using a mixture of aqueous ammonia and hydrogen peroxide. Comparison between Figs 4 and 5 indicates that addition of hydrogen peroxide into aqueous ammonia did not significantly affect conversion of carbohydrates to sugars, although it did enhance removal of lignin, as shown in Tables 1 and 3. This enhancement suggests that there are other factors affecting hydrolysis besides lignin content. The major factors affecting hydrolysis include lignin and hemicellulose content, cellulose fiber crystallinity, accessible surface area, and chemical bonds such as C–C and C–O bonds, etc. Figure 5 also shows that following pretreatment using a mixture of aqueous ammonia and hydrogen peroxide, the concentration of hydrogen peroxide has almost no effect on enzymatic hydrolysis.

Table 3. Pretreatment of *M.×giganteus* by a mixture of aqueous ammonia and hydrogen peroxide in one step*

Trial No.	Pretreatment solvents	Pretreatment results		
		Cellulose recovered (%)	Hemicellulose recovered (%)	Lignin removal (%)
1	30 wt% aqueous ammonia + 5 wt% hydrogen peroxide	85.8	50.2	79.3
2	30 wt% aqueous ammonia + 2 wt% hydrogen peroxide	87.0	54.3	73.1
3	30 wt% aqueous ammonia + 1 wt% hydrogen peroxide	88.3	54.9	72.7
4	10 wt% aqueous ammonia + 1 wt% hydrogen peroxide	91.6	63.1	59.7

*Pretreated at 130°C, liquid:solid ratio 6:1, and time 4 h.

Table 4. Pretreatment of *M.×giganteus* by a mixture of 30 wt% aqueous ammonia and 5 wt% hydrogen peroxide in one step using different heating strategies*

Trial No.	Heating-strategies	Pretreatment results		
		Cellulose recovered (%)	Hemicellulose recovered (%)	Lignin removal (%)
1	4 h 130°C	85.8	50.2	79.3
2	1 h 60°C+1 h 90°C+2 h 130°C	86.0	55.1	73.5
3	2 h 60°C+2 h 130°C	86.2	56.4	73.8

*Pretreated with liquid:solid mass ratio 6:1.

Table 5. Pretreatment of *M.×giganteus* by aqueous ammonia and hydrogen peroxide separately in two steps*

No.	Pretreatment method Step 1; step 2	Pretreatment results for the recovered solid		
		Cellulose recovered (%)	Hemicellulose recovered (%)	Lignin removal (%)
1	30 wt% aqueous ammonia, 130°C, 2 h; 5 wt% hydrogen peroxide, 130°C, 2 h	80.9	44.5	89.5
2	30 wt% aqueous ammonia, 130°C, 2 h; 2 wt% hydrogen peroxide, 130°C, 2 h	81.6	48.9	82.0
3	30 wt% aqueous ammonia, 130°C, 2 h; 1 wt% hydrogen peroxide, 130°C, 2 h	82.0	53.6	81.1
4	30 wt% aqueous ammonia, 130°C, 2 h; 5 wt% hydrogen peroxide, 110°C, 2 h	84.0	47.0	80.0
5	30 wt% aqueous ammonia, 130°C, 2 h; 5 wt% hydrogen peroxide, 90°C, 2 h	88.0	56.0	72.0

*The mass ratio of liquid:solid is 6:1 for both step 1 and step 2.

Figure 6 shows results of enzymatic hydrolysis following pretreatment using aqueous ammonia and hydrogen peroxide separately in two steps. Pretreatment using hydrogen peroxide in the second step significantly improved conversions by enzymatic hydrolysis. Conversion of cellulose to glucose was high, up to 90.2% after 96 h; conversion of hemicellulose to xylose was 73.4%. Conversion increased with rising hydrogen peroxide concentration. Conversion of cellulose to glucose was 4.9, 43.9, 59.2, and 90.2% for unpretreated, 1 wt%, 2 wt%, and 5 wt% hydrogen peroxide, respectively; corresponding conversion of hemicellulose to xylose was 2.3, 42.7, 50.4, and 73.4%, respectively. Table 5 and Fig. 6 show that two-step pretreatment using aqueous ammonia and hydrogen peroxide in sequence favors both removal of lignin and subsequent conversion by enzymatic hydrolysis.

Table 7 presents results for enzymatic hydrolysis when pretreated using different methods. The conversion by enzymatic hydrolysis in this work is comparable with that reported in the literature.[10,11,19]

Characterization of the recovered solid

SEM, ATR-IR, and XRD data provide characterization for four selected samples of the solid, i.e. untreated; pretreated with 30 wt% aqueous ammonia; pretreated in one step with a mixture of 30 wt% aqueous ammonia and 5 wt% hydrogen peroxide; pretreated with 30 wt% aqueous ammonia and 5 wt% hydrogen peroxide separately in two steps. Figures 7, 8, and 9 show results.

The SEM micrograph in Fig. 7 shows that the morphology of the solid significantly changed after pretreatment. The untreated, raw *M.×giganteus* exhibited rigid and ordered fibrils, while the pretreated solid had reduced structure with larger external surface

Table 6. Lignin removal after pretreatment*

Method	Description	Lignin Removal (%)	Reference
A	130°C and liquid:solid of 6:1 with 30 wt% aqueous ammonia 2 h in the first step and 5 wt% hydrogen peroxide 2 h in the second step	89.5	this work
B	formic acid/acetic acid/water mixture (30/50/20 v% ratio), liquid:solid of 25:1, 107°C, 3 h	86.6	11,12
C	ethylenediamine/DMSO (50/50 wt% ratio), liquid:solid of 10:1, 70°C, 6–10 h	77.8	6
D	ethylenediamine/1-butyl-3-methylimidazolium dimethylphosphate (50/50 wt% ratio), liquid:solid of 10:1, 70°C, 6–10 h	68.8	6
E	25 wt% aqueous ammonia, liquid:solid ratio of 12:1, 60°C, 6 h	36.3	12
F	in the first step: sulfuric acid solution (0.15 mol·L^{-1}), liquid:solid of 10:1, heated to reflux for 17 h; in the second step: aqueous ethanol with H_2SO_4 (0.5 wt% H_2SO_4 based on dry *M.×giganteus*, ethanol/water=0.8), liquid:solid of 8:1, 170°C, 1 h	70.5	10

*The highest lignin removals reported in the respective literature.

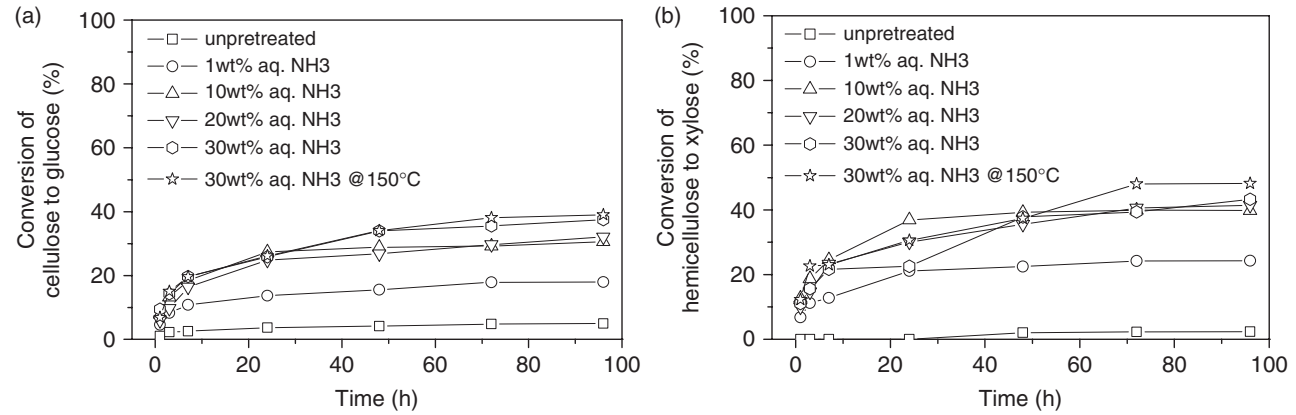

Figure 4. Enzymatic hydrolysis of *M.×giganteus* pretreated with aqueous ammonia: (a) conversion of cellulose to glucose and (b) conversion of hemicellulose to xylose. Pretreatment conditions: 130°C; liquid:solid ratio 6:1, time 4 h.

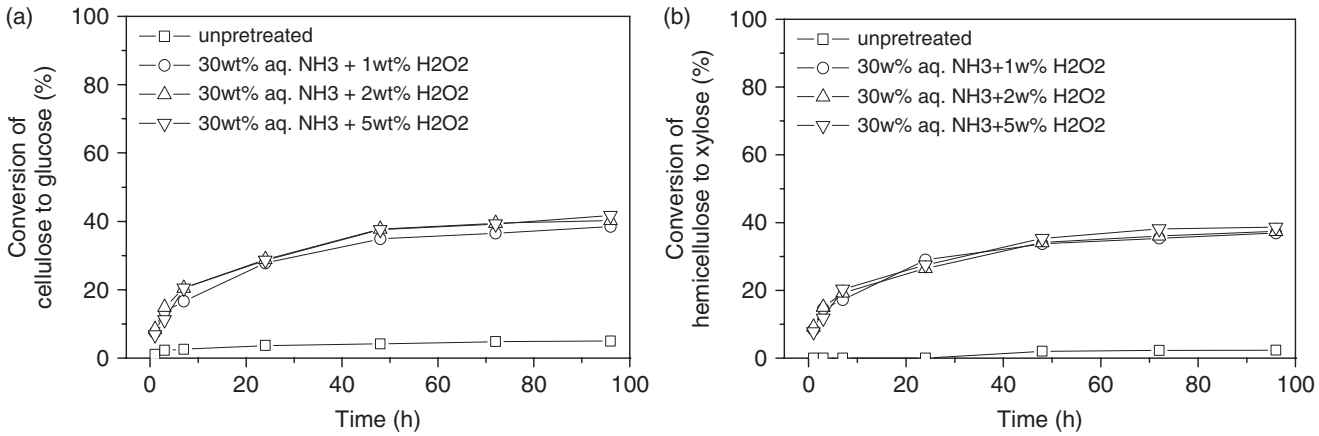

Figure 5. Enzymatic hydrolyses of *M.×giganteus* pretreated by a mixture of aqueous ammonia and hydrogen peroxide: (a) conversion of cellulose to glucose and (b) conversion of hemicellulose to xylose for the recovered solid. Pretreatment conditions: 130°C, liquid:solid ratio 6:1, time 4 h.

area and porosity. The surface structure (d) of the solid pretreated with 30 wt% aqueous ammonia and 5 wt% hydrogen peroxide separately in two steps seems to be more distorted and broken than those in (b) and (c), consistent with data showing that two-step pretreatment gives highest lignin removal and best efficiency for enzymatic hydrolysis.

ATR-IR spectra in Fig. 8 show that, compared with raw *M.×giganteus*, the pretreated solids have reductions in band intensities at positions characteristic of lignin peaks (1200–1300 cm^{-1}, C–O of guaiacyl and syringyl rings; 1500–1700 cm^{-1}, aromatic ring vibration),[20,49] consistent with the observation that preatreatment substantially removes lignin. Further comparison

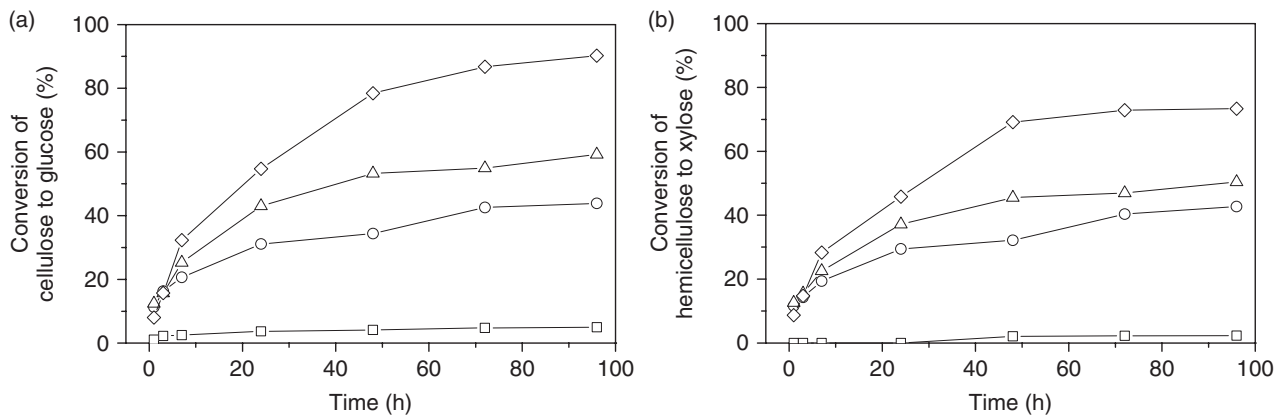

Figure 6. Enzymatic hydrolysis of *M.×giganteus* pretreated by aqueous ammonia in the first step and hydrogen peroxide in the second step: (a) conversion of cellulose to glucose and (b) conversion of hemicellulose to xylose for the recovered solid. Pretreatment conditions: 130°C, liquid:solid ratio 6:1. □, untreated; ◯, 2 h 30 wt% aqueous ammonia + 2 h 1 wt% hydrogen peroxide; △, 2 h 30 wt% aqueous ammonia + 2 h 2 wt% hydrogen peroxide; ◇, 2 h 30 wt% aqueous ammonia + 2 h 5 wt% hydrogen peroxide.

Table 7. Conversion of cellulose and hemicellulose to sugars for the recovered solid after enzymatic hydrolysis of pretreated *M.×giganteus**

Method§	Carbohydrate	Time (h) : Conv. (%)	Loading of Celluclast 1.5 L and Novozym 188	Reference
A	cellulose	72 : 86.7; 96 : 90.2	20 FPU and 20 CBU g^{-1} cellulose	this work
A	hemicelluloses	72 : 72.9; 96 : 73.4	20 FPU and 20 CBU g^{-1} cellulose	this work
B	cellulose	72 : 77.7	7.5 FPU and 37 CBU g^{-1} cellulose	11
F	cellulose	72 : 98.2	20 FPU and 40 CBU g^{-1} cellulose	10
M1£	cellulose	72 : 63.5; 168 : 71	15 FPU and 15 CBU g^{-1} cellulose	19
M2£	cellulose	72 : 65.1; 168 : 70	15 FPU and 15 CBU g^{-1} cellulose	19
M3£	cellulose	72 : 80.4; 168 : 81	15 FPU and 15 CBU g^{-1} cellulose	19
M4£	cellulose	72 : 92.8; 168 : 94.7	15 FPU and 15 CBU g^{-1} cellulose	19
M5£	cellulose	72 : 94.7; 168 : 95.1	15 FPU and 15 CBU g^{-1} cellulose	19

*The highest carbohydrate conversions from the literature are listed.
§See Table 6 for pretreatment method.
£M1 pretreatment method: acidic electrolyzed water (AEW) (0.2 wt% NaCl, pH=2.6, oxidation reduction potential >1100 mV), liquid:solid 8:1, 200°C, 24 min; M2 pretreatment method: alkline electrolyzed water (ALEW) (0.2 wt% NaCl, pH 11.7, oxidation reduction potential < −795 mV) , liquid:solid 8:1, 200°C, 24 min; M3 pretreatment method: 1 wt% H_2SO_4, liquid:solid 8:1, 200°C, 8 min; M4 pretreatment method: alkaline peroxide solution (pH 11.5, 4.0% hydrogen peroxide), liquid:solid 20:1, 50°C, 24 h in the first step and AEW, liquid:solid 16:1, 121°C, 50 min; M5 pretreatment method is similar to M4, using ALEW instead of AEW.

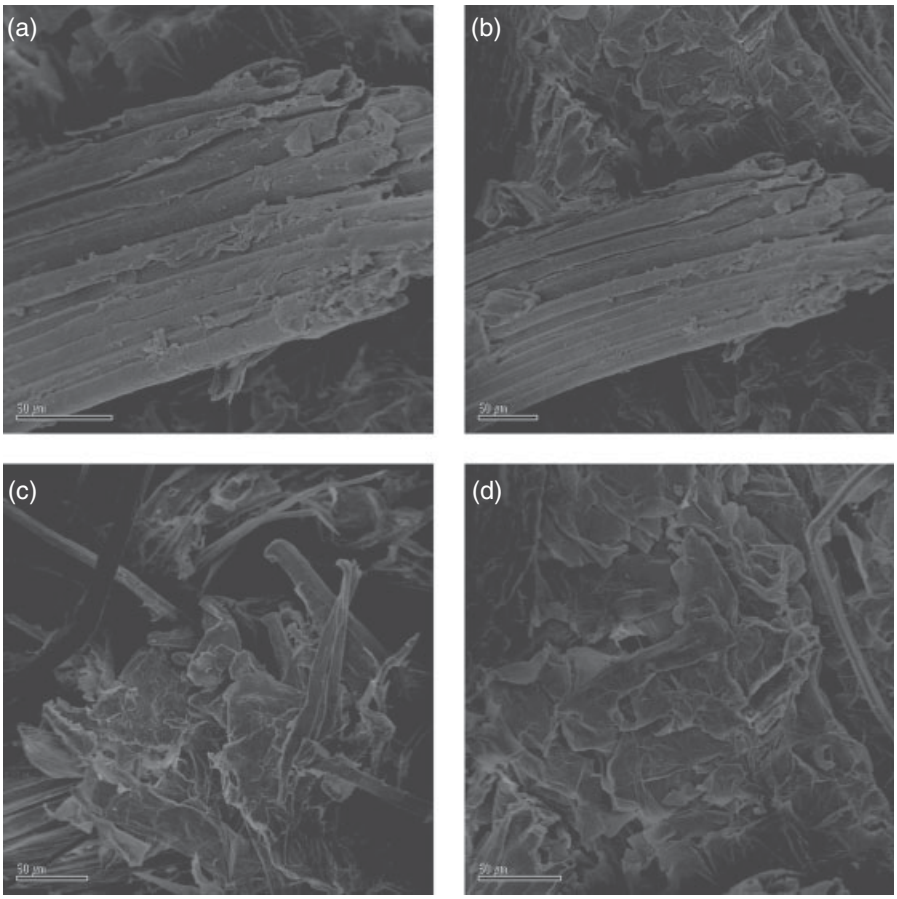

Figure 7. SEM of the *M.×giganteus* (a) unpretreated and (b) pretreated by 30 wt% aqueous ammonia, (c) by the mixture of 30 wt% aqueous ammonia and 5 wt% hydrogen peroxide, and (d) by 30 wt% aqueous ammonia and 5 wt% hydrogen peroxide separately in two steps.

among Fig. 8(b), (c), and (d) shows that the band intensity at the characteristic lignin position for (d) is weaker than that for (b) and (c), in agreement with data showing that two-step pretreatment gives the highest lignin removal and best carbohydrate conversion.

The results of XRD shown in Fig. 9 indicate that pretreatment changes the apparent crystallinity of *M.×giganteus*. Crystallinity index CrI increases after pretreatment; this increase has been ascribed to higher concentration of cellulose following removal of amorphous lignin and hemicellulose by pretreatment.[15,20,28] The solid pretreated with 30 wt% aqueous ammonia and 5 wt% hydrogen peroxide separately in two steps, i.e. (d) has the highest index, consistent with data showing that (d) gives the most removal of lignin and hemicellulose.

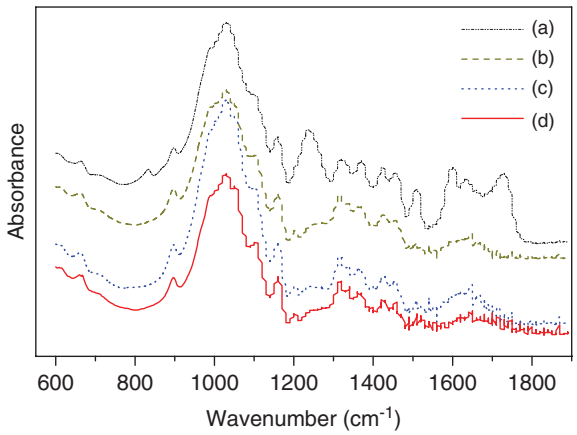

Figure 8. ATR-IR of the *M.*×*giganteus* (a) unpretreated and (b) pretreated by 30 wt% aqueous ammonia, (c) by the mixture of 30 wt% aqueous ammonia and 5 wt% hydrogen peroxide, and (d) by 30 wt% aqueous ammonia and 5 wt% hydrogen peroxide separately in two steps.

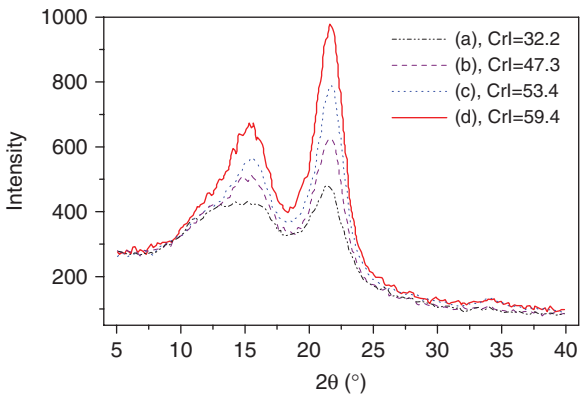

Figure 9. XRD of the *M.*×*giganteus* (a) unpretreated and (b) pretreated by 30 wt% aqueous ammonia, (c) by the mixture of 30 wt% aqueous ammonia and 5 wt% hydrogen peroxide, and (d) by 30 wt% aqueous ammonia and 5 wt% hydrogen peroxide separately in two steps.

CONCLUSION

Three methods are studied to pretreat *M.*×*giganteus*, (a) aqueous ammonia in one step, (b) a mixture of aqueous ammonia and hydrogen peroxide in one step, (c) aqueous ammonia in the first step and hydrogen peroxide in the second step. The two-step pretreatment at 130°C gives the greatest lignin removal and the highest conversion to sugar by enzymatic hydrolysis; 89.5% lignin removal is achieved after pretreatment using 30 wt% aqueous ammonia for 2 h in the first step and 5 wt% hydrogen peroxide for 2 h in the second step. The ratio of liquid:solid is 6:1. After 96 h, conversion of cellulose to glucose is 90.2% and conversion of hemicellulose to xylose is 73.4%. Although not studied in this work, cellulose and hemicellulose dissolved in the liquid phase can also be converted to fermentable sugars. Removal of lignin changes the physical structure of the recovered solid, as indicated by SEM, ATR-IR and XRD data. Aqueous ammonia concentration and temperature are more important for delignification than liquid:solid mass ratio. The two-step pretreatment process using aqueous ammonia and hydrogen peroxide separately is effective for removing lignin and for enhancing conversion to sugars by enzymatic hydrolysis.

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