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RESEARCH PAPER

Nitric-acid Hydrolysis of *Miscanthus giganteus* to Sugars Fermented to Bioethanol

Fuxin Yang, Waheed Afzal, Kun Cheng, Nian Liu, Markus Pauly, Alexis T. Bell, Zhigang Liu, and John M. Prausnitz

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Abstract *Miscanthus giganteus* (*M. giganteus*) is a promising feedstock for the production of bioethanol or biochemicals. Using only dilute nitric acid, this work describes a two-step process for hydrolyzing hemicellulose and cellulose to fermentable sugars. Primary variables were temperature and reaction time. The solid-to-liquid mass ratio was 1:8. No enzymes were used. In the first step, *M. giganteus* was contacted with 0.5 wt.% nitric acid at temperatures between 120 and 160°C for 5 to 40 min. The second step used 0.5 or 0.75 wt.% nitric acid at temperatures between 180 and 210°C for less than 6 min. Under selected conditions, almost all hemicellulose and 58% cellulose were transferred to the liquid phase. Small amounts of degradation products were observed. The xylose solution obtained from the nitric-acid hydrolysis was fermented for 96 h and the glucose solution for 48 h to yield 0.41 g ethanol/g xylose and 0.46 g ethanol/g glucose. To characterize residual solids and the liquor from both steps, nuclear-magnetic-resonance (NMR) spectroscopy was performed for each fraction. The analytical data indicate that the liquid phase from Steps 1 and 2 contain little lignin or lignin derivatives.

Fuxin Yang, Waheed Afzal, Kun Cheng, Nian Liu, Markus Pauly, Alexis T. Bell, John M. Prausnitz*
Energy Biosciences Institute, University of California, Berkeley, CA 94704, USA
Tel: +1-510-642-3592; Fax: +1-510-642-4778
E-mail: prausnit@cchem.berkeley.edu

Fuxin Yang, Waheed Afzal, Nian Liu, Alexis T. Bell, John M. Prausnitz
Department of Chemical & Biomolecular Engineering, University of California, Berkeley, CA 94720, USA

Fuxin Yang, Zhigang Liu
MOE Key Laboratory of Thermo-Fluid Science and Engineering, Xi'an Jiaotong University, Xi'an, Shaanxi 710049, China

Waheed Afzal
School of Engineering, University of Aberdeen, Aberdeen, United Kingdom

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1. Introduction

Stimulated by the depletion of fossil-fuel resources and by increasing CO_2 emissions, lignocellulosic biomass may provide an alternative feedstock for fuels and chemicals. The perennial grass *M. giganteus*, native to Southeast Asia, is a promising natural resource due to its high yield per acre and its ability to grow in marginal soil with little water [1,2]. *M. giganteus* is mainly composed of cellulose, hemicellulose and lignin, as indicated in Fig. 1. A typical biomass-to-ethanol process aims to hydrolyze polysaccharide components to sugars using chemical or biochemical methods; the sugars can then be fermented to alcohols [3]. However, hydrolysis is impeded by lignin that blocks access to cellulose fibers. Additional undesirable factors are cellulose crystallinity and low porosity. Thus, it is necessary to pretreat the biomass to make the polysaccharides more accessible to hydrolysis [3,4].

In the previous studies, alkali was used for pretreatment and enzymes to hydrolyze the pretreated solid [5-7]. Alkali pretreatment can be conducted at mild or even ambient conditions but, regrettably, some of the alkali is converted to irrecoverable salts or incorporated as salts into the biomass [8]. Moreover, pretreatment with alkali may take hours or days, followed by slow enzymatic hydrolysis to sugars.

However, acid can penetrate lignin to decompose cellulose and hemicellulose polymers in lignocellulosic biomass. Decomposition releases oligomers or monomeric sugars without using enzymes [9]. While hydrolysis with enzymes requires a few days, minutes are sufficient for acid hydrolysis. Acid processes were used industrially in the 1940s during World War II but they were not economically com-

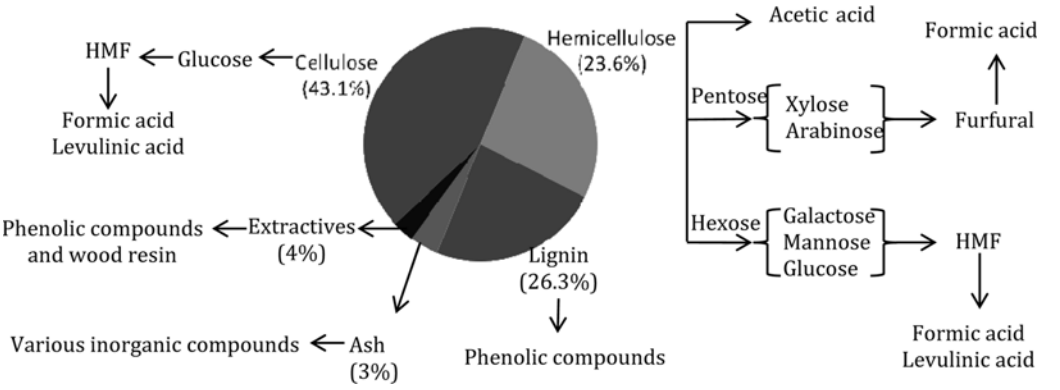

Fig. 1. Composition of *M. giganteus* and potential hydrolysis products.

petitive [10]. In recent years, acid hydrolysis has received extensive attention [11-17]. Hydrochloric acid, nitric acid, phosphoric acid and sulfuric acid have been investigated to obtain sugars from biomass [8,18,19]. Nguyen *et al.* reported a two-stage, dilute-sulfuric-acid pretreatment process for tree chips for large-scale applications [13].

Dilute nitric acid is a promising choice, because it is long-time compatible with stainless steel and can easily be neutralized with ammonia to produce harmless ammonium nitrate, a nutrient for microorganisms used in subsequent fermentation [20]. While some authors using nitric acid have reported results for a variety of feedstocks [18,21,22], no previous attention has been given to using nitric acid for conversion of *M. giganteus* without enzymes.

In an acid-hydrolysis process, severe conditions promote digestion of cellulose. However, such conditions cause significant degradation of sugars from hemicellulose. In this work, a two-step dilute-nitric-acid process was investigated. The primary purpose of the first step is to hydrolyze hemicellulose to xylose at mild conditions. In the second step, primarily hydrolyze cellulose to glucose, high temperature was used but only for a short time. The effect of temperature was investigated as well as acid concentration and reaction time to determine favorable conditions for hydrolysis with minimum degradation of sugars.

2. Materials and Methods

2.1. Materials

2.1.1. Lignocellulosic feedstock

M. giganteus was provided by the Energy Biosciences Institute (EBI), University of Illinois at Urbana-Champaign. A Retsch grinder and a 4-mm sieve produced 4-mm particles. The composition of moisture-free biomass was determined using the analytical procedure recommended by the National Renewable Energy Laboratory (NREL) [23]. By weight, it is 43.1% cellulose, 23.6% hemicellulose, 26.3% lignin, 3% ash and 4% extractables (pectins, tannins, and salts), as shown in Fig. 1, adapted from Taherzadeh and Karimi [10].

2.1.2. Reagents

69.4 wt.% nitric acid was purchased from Fisher Scientific (NJ, USA). For chemical analysis, sulfuric acid (95% aqueous solution) was obtained from Acros Organics (NJ, USA). Both were used without further purification. Nanopure water (18.2 MΩ) was used to prepare the solutions and for washing the recovered solid.

2.2. Hydrolysis of biomass using dilute nitric acid

Dried *M. giganteus* and solutions were weighed using analytical balances (Mettler Toledo, Model AB204-S and XS6002S), and then placed into a pressure reactor (18.10 mL) with stirring. The solid-to-liquid ratio was 1:8. The reactor was submerged into a silicone-oil bath at a pre-set temperature. The pre-set temperature was above the desired temperature to allow the reactor and oil bath to reach thermal equilibrium at the desired operating temperature in a short time (3 ~ 7 min). After a predetermined reaction time, the reactor was taken from the oil bath and cooled to 70°C using an ice-water bath. For the high-temperature step, a microwave reactor (Milestone, ETHOS EZ) was used to minimize the time for reacting conditions to reach a temperature above 180°C. After cooling, the pulp was filtered to separate the solid from the liquid. The recovered liquid was then analyzed to determine the yields of sugars and degradation products; subsequently, sugars were fermented. The recovered solid was washed several times with Nanopure water until the pH was adjusted to between 6 and 7. A small sample was dried in a 105°C oven overnight to determine its dry weight and composition. The rest of the recovered solid was air-dried prior to the second step where the experimental procedures were similar to those used in the first step. Fig. 2 shows the proposed two-step process for hydrolysis of *M. giganteus*.

2.3. Composition analysis for the recovered solid

The composition of the recovered *M. giganteus* was determined using the analytical procedure proposed by the NREL [23]. First, the polysaccharides were hydrolyzed by 72 wt.% sulfuric acid and second, by 4 wt.% sulfuric acid. Each sample was analyzed in triplicates.

50 mg of dried, ball-milled biomass were put into a glass vial; 0.5 mL of 72 wt.% sulfuric acid was added. The samples were stirred every 15 min for one hour to ensure that the biomass was impregnated with acid. 14 mL Nanopure water was added to dilute the sulfuric acid to 4 wt.%. Then, the glass vial was capped and placed into an autoclave reactor (Steris, Amsco Lab 250) for 60 min at 121°C. Thereafter, the samples were cooled to normal temperature and then stored in a refrigerator overnight. The liquid was separated from the solid using a glass-microfiber filter (Millipore). The contents of lignin and ash were determined by weighing the recovered solids before and after drying in a 105°C oven as well as ash in a furnace at 575°C. To determine the monosaccharide concentration, a Shimadzu HPLC was used at 50°C equipped with an Aminex HPX 87H column (300 × 7.8 mm) and a refractive-index detector. The flow rate of 0.01 N sulfuric acid eluent was 0.6 mL/min. Galactose and mannose could not be separated from xylose and arabinose due to the characteristics of the column. However, because the concentrations of galactose and mannose were only 1 ~ 2%, and because their response factors were similar, the error for calculating hemicellulose content was insignificant.

Eqs. (1) and (2) quantify cellulose and hemicellulose contents. When calculating the concentration of the polymeric sugars from the concentration of the corresponding monomeric sugars, a correction factor 0.90 (162/180) was used for C6 sugars (glucose, galactose and mannose) and a correction factor 0.88 (132/150) for C5 sugars (xylose and arabinose) as suggested by Sluiter *et al.* [23].

$$\textit{Cellulose to glucose conversion}\ (\%) = \frac{\textit{Equiv. glucose conc.}\left(\frac{\text{mg}}{\text{mL}}\right) \times \textit{hydrolysis volume}\ (\text{mL}) \times 0.90 \times 100}{\textit{Cellulose}\ (\text{mg})\ \textit{in the pretreated miscanthus}} \quad (1)$$

$$\textit{Hemicellulose to xylose conversion}\ (\%) = \frac{\textit{Equiv. xylose conc.}\left(\frac{\text{mg}}{\text{mL}}\right) \times \textit{hydrolysis volume}\ (\text{mL}) \times 0.88 \times 100}{\textit{Hemicellulose}\ (\text{mg})\ \textit{in the pretreated miscanthus}} \quad (2)$$

2.4. Composition analysis for the recovered liquid

The concentrations of degradation products in the liquid were determined by HPLC together with those for the monomeric sugars. In addition, a Dionex HPLC system (ICS 3000, equipped with CarboPac PA200 Carbohydrate Column) was used to obtain concentrations of oligomeric sugars.

2.5. Characterization by NMR

Two-dimensional solution-state nuclear magnetic resonance (2D-NMR) analysis of the solid residue after each step was performed according to a method reported previously [24]. In brief, solid plant residue (300 mg) was ball-milled for 7 h with an interval of 5 min grinding and 5 min standing using a Retsch PM 100 mill (Retsch, Germany). Milled material (25 mg) was dissolved in DMSO-d_6/EmimOAc-d_{14} (0.75 mL/10 μL). To analyze the liquid phase after each step, the liquor (1 mL) was rota-vaporated and the remaining solid was dissolved in 0.75 mL DMSO-d_6. The HSQC 2D-NMR spectra were acquired using a Bruker standard pulse sequence 'hsqcetgpsisp.2' on a Bruker AVANCE 600 MHz NMR spectrometer equipped with an inverse gradient 5-mm TXI $^{1}H/^{13}C/^{15}N$ cryoprobe using parameters previously reported [24]. All spectra were calibrated using the central DMSO-d_6 solvent peak (δ_C 39.9 ppm, δ_H 2.49 ppm). The peak integrals were normalized by the signal of an internal standard (0.8 μM 1,3,5-trimethoxybenzene, Sigma-Aldrich, 98%). The NMR data processing and analysis were performed using Bruker's Topspin 3.1 software.

2.6. Fermentation

The fermentation at three conditions was conducted to study the effects of inhibitors on ethanol yield: control without inhibitors, control with inhibitors, and hydrolysate. The first control samples only contain sugars; the second controls with inhibitors, contain sugars, some weak acids and furan derivatives, but no phenolic compounds (not detected in this work). Hydrolysate is collected from the dilute-nitric-acid process.

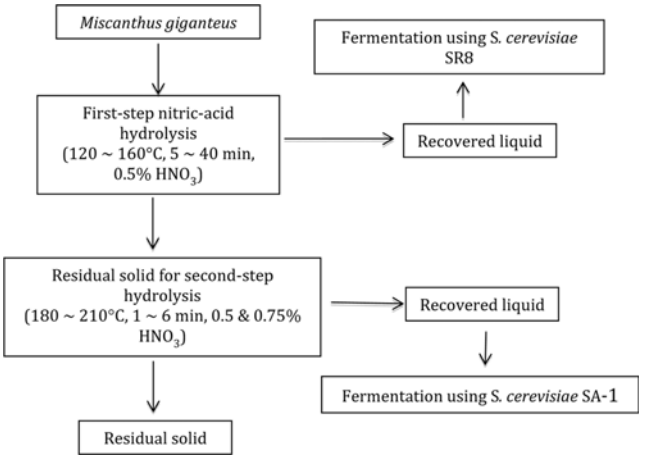

Fig. 2. The two-step process for hydrolysis of *M. giganteus* and subsequent fermentation.

For fermentation of the first-step hydrolysate (mainly containing xylose), the yeast strain *Saccharomyces cerevisiae* (*S. cerevisiae*) SR8 was used, kindly supplied by Professor Cate (EBI at Berkeley) [25]. For fermentation of the second-step hydrolysate (mainly containing glucose), *S. cerevisiae* SA-1 (APA2156) was used, kindly supplied Professor Arkin (EBI at Berkeley) [26]. The stock cultures were grown on a petri dish with YPAD solid media at 30°C in an incubator for three days (YPAD solid media: 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 100 mg/L adenine hemisulfate and 20 g/L agar). The biologic colonies were placed in conical tubes filled with 10 mL YPAD liquid media (YPAD solid media without agar) for growing at 30°C and 220 rpm in an incubator shaker (Innova 44) overnight; the cells were then harvested by centrifugation.

Fermentation was carried out anaerobically in 100 mL serum bottles. The density of the culture was determined by measuring its optical density at 600 nm (OD600) using a SpectraMax M2. Before the fermentation, the hydrolysate was adjusted to pH 6.0 with a small amount of aqueous ammonium hydroxide. 40 mL hydrolysate were then taken into the serum bottle in addition to synthetic anaerobic fermentation media and harvested yeast cells with initial OD600 3.0 (for first-step hydrolysate fermentation) and 0.3 (for second-step hydrolysate fermentation). Synthetic anaerobic fermentation media without carbon sources contains 1.7 g/L YNB (yeast nitrogen base without amino acids and without ammonium sulfate), 5.0 g/L ammonium sulfate, 21.3 g/L MES buffer (2-(N-morpholino)ethanesulfonic acid), 1.54 g/L CSM, 1 mL/L ergosteral solution and a small amount of KOH to adjust to pH 6.0. 1 mL ergosteral solution was made by 10 mg ergosterol, 393 μL tween-80 and 607 μL ethanol. Control experiments with and without inhibitors were performed under the same conditions.

During the fermentation, 1.0 mL of fermentation broth was taken for measuring OD600; 200 μL were centrifuged, filtered and analyzed for ethanol using a Shimadzu HPLC at 55°C equipped with a Rezex RFQ-Fast Acid H column (100 × 7.8 nm) and a refractive-index detector. The flow rate of 0.01 N sulfuric acid eluent was 1.0 mL/min.

The fermentation was studied in triplicates. All experimental materials were sterile.

3. Results and Discussion

3.1. First Step

The goal of this work was to establish a two-step dilute-nitric-acid process. In the first step, hemicellulose hydrolysis was maximized using 0.5 wt.% nitric acid at a temperature between 120 and 160°C. The reaction time varied from 5 to 40 min.

3.1.1. Effects of temperature and reaction time on hydrolysis

Table 1 presents results for the first step at several operating conditions, and gives the percent removal of cellulose, hemicellulose and lignin based on the original contents. There are two independent variables: temperature and time. As shown in Fig. 3, only one variable (temperature or time) is changed for a given run, while maintaining the other constant. In Fig. 3D, at a fixed temperature (120, 140 or 160°C), increasing reaction time gives only a small gain in the hydrolysis of cellulose. In Fig. 3E, experimental data

Table 1. Analysis of the recovered solid after the first step with liquid-to-solid ratio is 8:1; nitric acid concentration is 0.5 wt.%

Process condition[a]		Solids recovery (wt.%)	Removal (wt.%)[b]			pH before[c]	log(R_o)[d]	CSF[e]
T	t		Cel	Hem	Lig			
120	10	88.3	5.6	17.8	17.8	1.15	1.59	0.44
	20	79.9	8.0	46.0	18.3	1.15	1.89	0.74
	30	76.1	9.2	58.4	18.7	1.15	2.07	0.92
	40	72.0	12.3	72.3	19.4	1.15	2.19	1.04
140	10	76.9	8.3	68.9	14.9	1.15	2.18	1.03
	20	70.7	9.3	87.5	17.6	1.15	2.48	1.33
	30	68.5	10.0	91.3	19.1	1.15	2.65	1.50
	40	67.0	13.1	93.8	19.8	1.15	2.78	1.63
160	5	70.9	10.1	86.6	17.8	1.15	2.47	1.32
	10	66.3	11.9	92.4	18.4	1.15	2.77	1.62
	20	63.4	15.4	96.7	19.2	1.15	3.07	1.92
	30	58.9	23.1	98.1	20.2	1.15	3.24	2.09

[a]T: temperature (°C); t: time (minutes).
[b]Removal represents the percentage removal of cellulose (Cel), hemicellulose (Hem), and lignin (Lig), based on contents in the untreated biomass.
[c]pH of the reaction solution prior to the first step.
[d,e]Calculated from Eq. (3), CSF: combined severity factor.

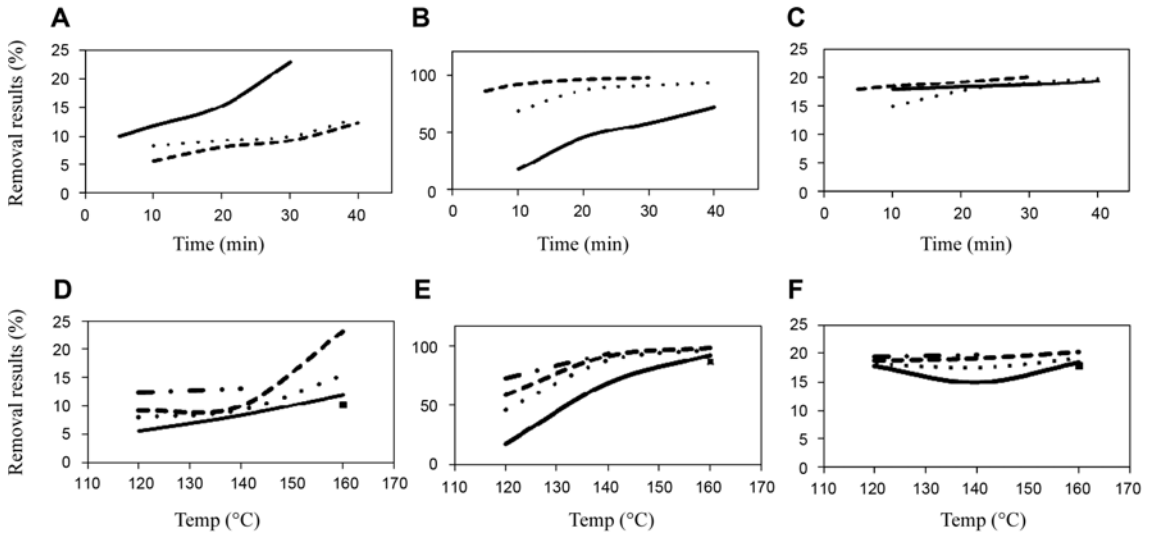

Fig. 3. Effect of process conditions on removal of various components based on recovered-solid analysis after the first step. (A, B, C) percent removal as a function of time, — 120°C, ⋯ 140°C, – – 160°C; (D, E, F) percent removal as a function of temperature, ■ 5 min, — 10 min, ⋯ 20 min, – – 30 min, –•– 40 min. A, D: cellulose; B, E: hemicellulose; C, F: lignin.

show that a high hydrolysis conversion of hemicellulose is accomplished at 140 or 160°C. Fig. 3B shows that, when the reaction time exceeds 20 minutes, the hydrolysis of hemicellulose does not improve significantly at temperatures above 140°C. Thus, for maximizing hemicellulose hydrolysis, a temperature between 140 and 160°C is preferred. At these temperatures, the effect of reaction time is not significant.

In the first step only little lignin, about 20 wt.% based on its original content is removed as demonstrated in Figs. 3C and 3F. A higher temperature (160°C) and a longer reaction time (30 minutes) do not appear to enhance lignin removal.

In summary, for the first step, the yield of sugars derived from hemicellulose is favorable at 140°C for 20 ~ 40 min or at 160°C for 5 to 30 min. Within these ranges, the hydrolysis of hemicellulose is not sensitive to reaction time.

Table 2 and Fig. 4 show small concentrations of byproducts in the liquid, obtained by HPLC. Furfural originates from xylose and other five-carbon sugars, while acetic acid comes from hydrolysis of the acetyl groups in hemicellulose, as shown in Fig. 1.

In Fig. 4A the xylose concentration in the liquid increases with rising reaction time and temperature, achieving a maximum at 140°C and 40 min. At 160°C, however, the concentration of xylose is largest at 5 min and then gradually declines as reaction time increases. The concentration

Table 2. Analysis of the recovered liquid after the first step

Process condition		Composition of liquid phase (mg/mL)[a]										Theoretical recovery (%)[b]									
T	t	glu	xyl	ara	for	ace	lev	HMF	fur	X2	X3	glu	xyl	ara	for	ace	lev	HMF	fur	X2	X3
120	10	0.7	1.7	0.8	0.0	0.4	0.0	0.0	0.0	0.6	0.7	1.1	5.1	2.5	0.0	1.0	0.0	0.0	0.0	1.9	2.1
	20	1.2	7.2	1.1	0.0	1.3	0.0	0.0	0.0	2.5	1.6	1.9	21.4	3.4	0.0	3.2	0.0	0.0	0.0	7.6	4.9
	30	1.6	11.1	1.3	0.0	2.1	0.0	0.0	0.0	2.1	1.0	2.7	33.1	3.8	0.0	5.1	0.0	0.0	0.0	6.5	3.1
	40	2.0	16.5	1.4	0.0	3.2	0.0	0.0	0.1	0.7	0.2	3.3	49.2	4.1	0.0	7.7	0.0	0.1	0.3	2.3	0.6
140	10	0.9	15.2	1.9	0.0	2.5	0.0	0.1	0.0	2.0	0.9	1.5	45.5	5.7	0.0	5.9	0.0	0.2	0.1	6.1	2.7
	20	1.5	20.9	2.0	0.0	3.5	0.0	0.2	0.5	0.7	0.1	2.6	62.4	5.9	0.0	8.6	0.0	0.4	2.1	2.2	0.4
	30	2.1	21.1	2.2	0.0	3.9	0.0	0.2	0.7	0.2	0.0	3.6	63.1	6.5	0.0	9.5	0.1	0.4	3.5	0.5	0.1
	40	2.1	21.2	2.2	0.0	3.8	0.1	0.2	1.1	0.1	0.0	3.6	63.4	6.4	0.0	9.2	0.4	0.4	5.1	0.2	0.0
160	5	1.8	20.1	2.7	0.0	3.3	0.0	0.2	0.5	2.4	0.7	3.0	60.0	8.0	0.0	8.0	0.0	0.4	2.2	4.0	1.2
	10	2.8	20.0	3.5	0.0	3.8	0.1	0.2	1.4	0.3	0.0	4.6	59.7	10.6	0.3	9.2	0.2	0.5	6.3	0.5	0.0
	20	6.0	18.2	3.4	0.1	4.0	0.3	0.2	3.4	0.3	0.0	10.0	54.4	10.2	0.7	9.6	0.6	0.6	15.8	0.5	0.0
	30	7.4	17.0	2.9	0.2	3.9	0.4	0.3	4.3	0.2	0.0	12.3	50.9	8.8	1.1	9.4	0.9	0.7	20.2	0.3	0.0

[a]Glu: glucose; xyl: xylose; ara: arabinose; for: formic acid; ace: acetic acid; lev: levulinic acid; fur: furfural; X2: xylobiose; X3: xylotriose.
[b]Calculated from Eq. (4) in Supplementary Material.

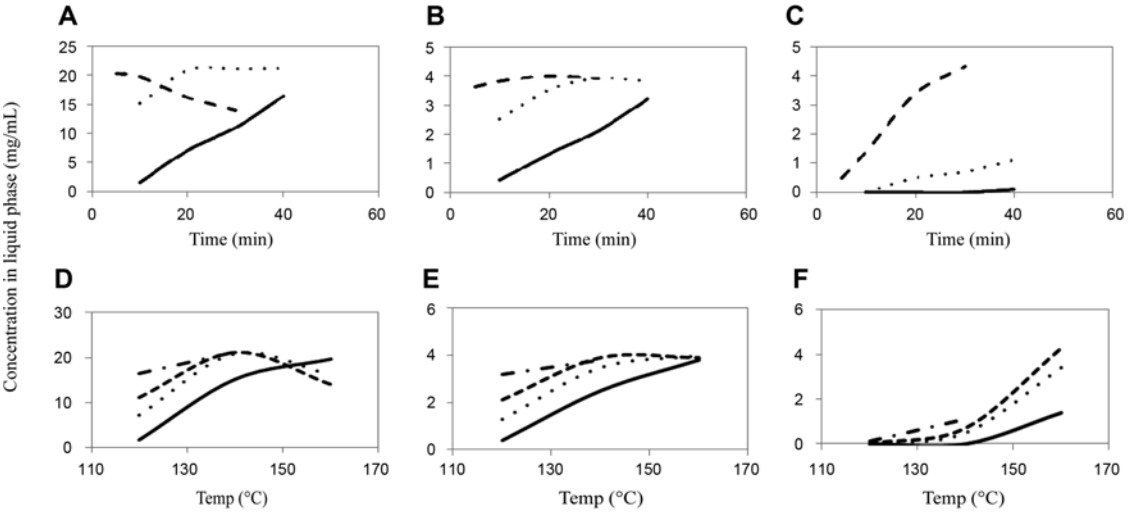

Fig. 4. Effect of process conditions on the concentrations in recovered liquid phase after the first step: (A, B, C) concentration as a function of time, — 120°C, ⋯ 140°C, – – 160°C; (D, E, F) concentration as a function of temperature, ■ 5 min, — 10 min, ⋯ 20 min, – – 30 min, –•– 40 min. A, D: xylose; B, E: acetic acid; C, F: furfural.

of furfural in the liquid increases with reaction time at 160°C as shown in Fig. 4C, indicating that at high temperature, undesired conversion of xylose into furfural and other products is significant only at long reaction times.

3.1.2. Preliminary optimization of the first step

Tables 1 and 2 and Figs. 3 and 4 show analyses for recovered solid and liquid from the first step. Compositions of the recovered solid and the liquid show significant differences as process conditions change. To obtain best conditions for the first step, the combined severity factor (CSF) is used to determine the effects of temperature, acid concentration and reaction time [27]. CSF gives a rough estimate of the relative severity of reaction conditions. CSF is defined by:

$$\mathrm{CSF} = \log R_0 - pH \tag{3}$$

where

$$R_0 = t \cdot exp[(T_r - T_0)/14.75]$$

where t is the reaction time in minutes; T_r is the reaction temperature in degrees Celsius; T_o is the reference temperature, 100°C; the pH of the solution is measured by a pH meter (Mettler-Toledo, SevenCompact™ pH/Ion S220) before the reaction begins. Table 1 shows CSF and pH used here.

For dilute-nitric-acid hydrolysis in the first step, CSF ranges from 0.44 to 2.09. Fig. 5A shows the trend of cellulose, hemicellulose and lignin removal based on solid-phase analysis. Removal of hemicellulose increases only slightly when CSF rises to above 1.5. Hemicellulose can almost be completely removed and solubilized in the liquid under severe reaction condition (CSF above 2.09), whereas,

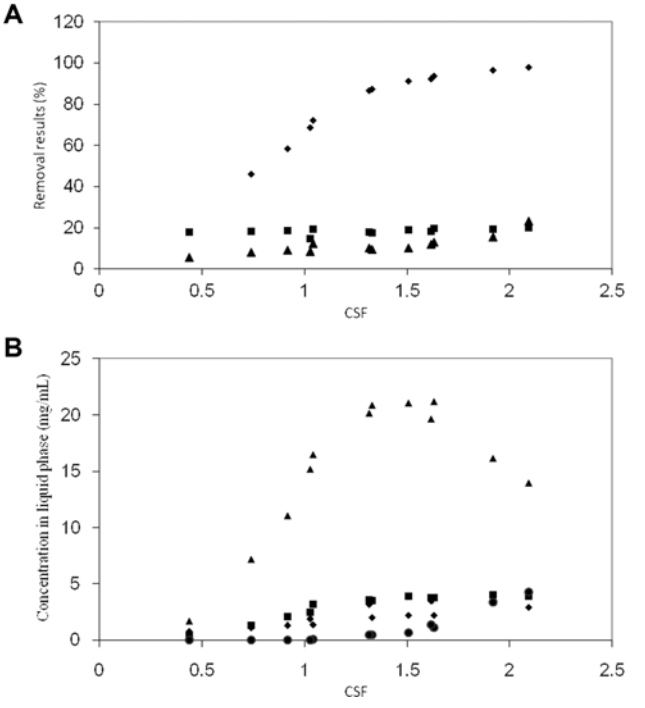

Fig. 5. Composition of the recovered solid and liquid phases after the first step: (A) percent removal based on the recovered-solid analysis; ▲ cellulose; ◆ hemicellulose; ■ lignin; (B) concentration based on the recovered-liquid analysis; ▲ xylose, ◆ arabinose, ■ acetic acid; ● furfural. CSF (combined severity factor) is defined in Eq. (3).

at best, only 23% cellulose is dissolved in the liquid.

Fig. 5B shows that the concentration of xylose in the liquid increases slightly when CSF rises from 0.44 to 1.63, but falls when CSF rises from 1.63 to 2.09. In the first step, the maximum concentration of xylose is 21.2 mg/mL when

Table 3. Analysis of the recovered solid after the second step with liquid-to-solid ratio is 8:1

No.[a]	Process condition[b]			Solids recovery (wt. %)	Removal (wt.%)			Overall removal (wt.%)[c]			pH before[d]	log(R_o)[e]	CSF[f]
	T	t	NC		Cel	Hemi	Lig	Cel	Hemi	Lig			
1	180	3	0.5	87.9	16.1	69.5	13.2	23.9	96.2	28.4	1.15	2.83	1.68
2		6	0.5	83.5	17.8	71.8	22.5	25.4	96.5	36.1	1.15	3.13	1.98
3		3	0.75	87.2	13.9	66.4	11.5	21.9	95.8	27.0	0.98	2.83	1.85
4		6	0.75	78.1	20.7	97.3	12.0	28.1	99.7	27.5	0.98	3.13	2.15
5	195	3	0.5	55.5	53.4	99.9	17.9	57.7	100.0	32.3	1.15	3.27	2.12
6		3	0.75	65.7	34.2	100.0	32.1	40.3	100.0	44.0	0.98	3.27	2.29
7	210	1	0.5	64.2	39.7	100.0	16.4	45.3	100.0	31.1	1.15	3.24	2.09
8		1	0.75	70.9	22.6	100.0	30.6	29.8	100.0	42.8	0.98	3.24	2.26
9	180	3	0.5	84.9	15.4	61.5	17.1	23.9	94.8	31.9	1.15	2.83	1.68
10		6	0.5	79.2	15.5	94.9	26.3	24.1	99.3	39.4	1.15	3.13	1.98
11		3	0.75	82.7	14.7	69.3	16.8	23.3	95.9	31.6	0.98	2.83	1.85
12		6	0.75	80.2	19.0	96.3	17.3	27.2	99.5	32.0	0.98	3.13	2.15
13	195	3	0.5	60.8	42.4	100.0	21.7	48.3	100.0	35.6	1.15	3.27	2.12
14		3	0.75	64.7	33.5	100.0	25.8	40.2	100.0	39.0	0.98	3.27	2.29
15	210	1	0.5	64.6	37.0	100.0	22.6	43.4	100.0	36.4	1.15	3.24	2.09
16		1	0.75	67.6	32.9	100.0	20.1	39.7	100.0	34.3	0.98	3.24	2.26

[a]For 1-8, the samples are treated with 0.5 wt.% nitric acid at 140°C for 20 minutes in the first step; For 9-16, the samples are treated with 0.5 wt.% nitric acid at 160°C for 5 minutes in the first step.
[b]T: temperature (°C); t: time (minutes); NC: nitric acid concentration (wt.%).
[c]Overall removal is combined results of the first step and the second step.
[d]pH of the reaction solution prior to the second step.
[e,f]Calculated from Eq. (3).

the operating temperature is 140°C and the reaction time is 40 min (CSF 1.63). For sugar-cane bagasse, Rodriguez-Chong *et al.* obtained an optimal 18.6 mg/mL at 122°C after 9.3 min using 6% nitric acid when the liquid-to-solid ratio is 10 [18]. Degradation to acetic acid and furfural rises as process conditions become more severe. The acetic acid concentration is 2.5 mg/mL at 140°C after 10 min and then rises to 4.0 mg/mL at 160°C after 20 min, while the concentration of furfural rises from 0 to 4.3 mg/mL. Degradation to furfural increases almost linearly as CSF rises from 1.5 to 2.1 because at these conditions, almost all hemicellulose is removed into the liquid phase in a short time.

Optimization of biomass hydrolysis requires a compromise: maximize hydrolysis of hemicellulose, but minimize production of degradation products. The data suggest that for the first step, 140°C for 20 min or 160°C for 5 min are optimal. Samples treated under these conditions were selected for further treatment in a second step.

A material balance for first step is given in the Supplementary Material (SM-1).

3.2. Second step

In the second step, the biomass originating from the first step was treated with 0.5 or 0.75 wt.% nitric acid at more severe conditions to hydrolyze cellulose to glucose. Sixteen

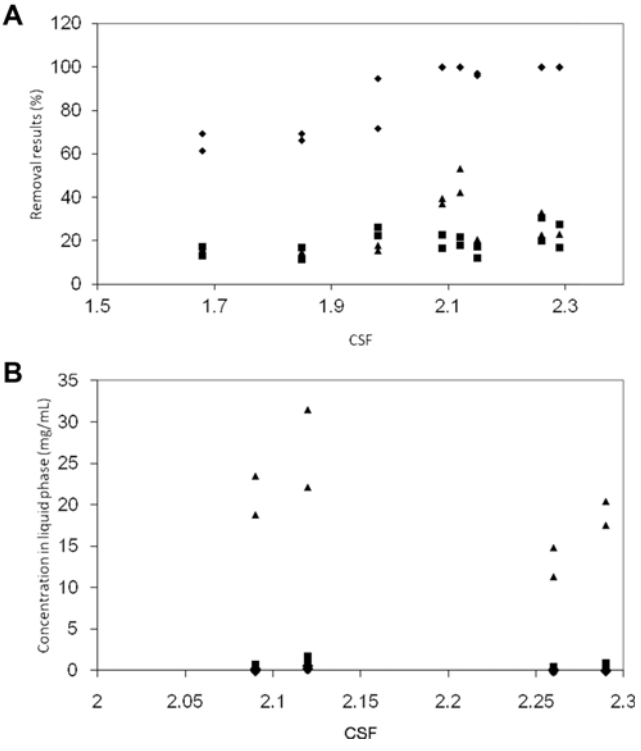

Fig. 6. Composition of the recovered solid and liquid phases after the second step: (A) percent removal based on the recovered-solid analysis; ▲ cellulose; ◆ hemicellulose; ■ lignin; (B) concentration based on the recovered-liquid analysis; ▲ glucose; ◆ formic acid; ■ HMF. CSF (combined severity factor) is defined in Eq. (3).

Table 4. Analysis of the recovered liquid after the second step

No.[a]	Process condition[b]			Composition of liquid phase (mg/mL)[c]									Theoretical recovery (%)[d]								
	T	t	NC	glu	xyl	ara	for	ace	lev	HMF	fur	G2	G3	glu	xyl	ara	for	ace	lev	HMF	fur	G2	G3
1	195	3	0.5	31.5	1.4	0.7	0.5	1.0	1.5	1.6	1.3	0.1	0.1	41.0	24.7	12.7	2.6	14.5	2.5	3.1	36.3	0.1	0.1
2		3	0.75	20.4	2.3	0.4	0.0	1.0	0.1	0.9	0.9	0.2	0.0	26.6	42.2	7.6	0.2	14.2	0.2	1.6	25.5	0.1	0.0
3	210	1	0.5	23.5	2.6	0.3	0.2	0.7	0.5	0.7	0.8	0.1	0.1	30.6	47.2	6.2	1.1	9.8	0.9	1.3	22.5	0.3	0.1
4		1	0.75	11.3	2.3	0.4	0.1	0.9	0.0	0.3	0.7	0.3	0.0	14.7	42.7	7.4	0.5	13.2	0.0	0.5	19.7	0.4	0.0
5	195	3	0.5	22.1	1.8	0.4	0.2	0.6	0.6	0.9	0.7	0.1	0.1	33.5	39.8	8.8	1.4	10.9	1.4	1.9	24.5	0.2	0.2
6		3	0.75	17.5	2.0	0.3	0.0	0.5	0.1	0.5	0.4	0.2	0.0	26.6	44.8	7.2	0.1	8.3	0.1	1.1	14.5	0.3	0.0
7	210	1	0.5	18.8	2.1	0.4	0.0	0.5	0.1	0.6	0.5	0.2	0.1	28.5	47.8	8.0	0.2	9.9	0.2	1.3	18.8	0.3	0.2
8		1	0.75	14.8	2.3	0.3	0.0	0.5	0.1	0.4	0.4	0.3	0.0	22.4	52.4	6.1	0.2	9.2	0.2	0.9	13.3	0.5	0.0

[a]For 1-4, the samples are treated with 0.5 wt.% nitric acid at 140°C for 20 min in the first step; For 5-8, the samples are treated with 0.5 wt.% nitric acid at 160°C for 5 min in the first step.
[b]T: temperature (°C); t: time (minutes); NC: nitric acid concentration (wt.%).
[c]Glu: glucose; xyl: xylose; ara: arabinose; for: formic acid; ace: acetic acid; lev: levulinic acid; fur: furfural; G2: cellobiose; G3: cellotriose.
[d]Calculated from Eq. (4) in Supplementary Material.

operating conditions were studied. Temperatures varied from 180 to 210°C and reaction times from 1 to 6 min.

Results are summarized in Table 3. Similar to step 1, it was observed that how the composition of the recovered solid varies with CSF, as shown in Fig. 6. The overall removal of cellulose increases slightly to a maximum when CSF is 2.12 and then declines when CSF increases further. Hydrolysis of cellulose above 195°C is promising. For further analysis, the composition of the liquid phase was shown in Table 4 and Fig. 6B. In the second step, 53% of the cellulose is removed into the liquid, resulting in an overall removal of 58% in the two steps combined. For the second step, the theoretical recovery of cellulose is 41%; the experimental recovery is 40%, as shown in Supplementary Material (SM-2). For comparison, for softwoods, Nguyen *et al.* obtained a theoretical yield of 38% for glucose using 2.5% sulfuric acid at 210°C for 2 min (CSF = 3.03) [13].

3.3. Preliminary optimization of the two-step dilute-nitric-acid process

Fig. 2 shows the proposed two-step process for hydrolysis of *M. giganteus.* Using 0.5 wt.% nitric acid for the second step following a first step at 140°C, results are better than those following a first step at 160°C. This result is not surprising because when the first step is at 160°C, the solid may become more recalcitrant, requiring a more severe condition for the second step.

Maximum glucose is obtained at 195°C after 3 min using 0.5 wt.% nitric acid in the second step. The analysis suggests that 140°C for 20 min using 0.5 wt.% nitric acid is the best operating condition for the first step.

Hydrolysis of lignocellulosic biomass at a more severe condition enhances yields but increases production of degradation products.

3.4. Characterization of the recovered solids and liquids by NMR

Under the conditions studied here, the apparent optimum process uses 0.5 wt.% nitric acid to treat biomass at 140°C for 20 min or at 160°C for 5 min in the first step followed by a treatment of the recovered solid residue at 195°C for 3 min with the same concentration nitric acid in the second step. The resulting recovered solid residues and the aqueous liquor after each step were characterized using solution-state 2D-NMR spectroscopy toward understanding the structural compositional changes of the material. Comparison of the 2D-HSQC NMR spectra between non-treated *M. giganteus* with those for recovered solid after each step is shown in Supplementary Material (SM-3 and SM-4). The peak assignments were based on previous data [24,28].

For the recovered solids, the contour integrals of the α peaks of lignin side-chains show that the β-O-4' linked aryl ether linkage (A) is reduced by 39% and 5-5'/4-O-β' linked dibenzodioxocin (D) is reduced by 90% after the first step compared to non-pretreated *M. giganteus*. However, β-5' linked phenylcoumaran (B) and β-β' linked resinol (C) are enriched. As Fig. 7A shows, the cleavages of lignin-side chains become more apparent when a higher temperature (160°C) is used in the first step. After the second step at 195°C, nearly all aryl ether bonds are cleaved in the solid residue. However, the contents of phenylcoumaran and resinol remain similar to those in the original biomass, suggesting that the hydrolysis may also cause lignin condensation. In addition, the 2D-NMR spectra show that

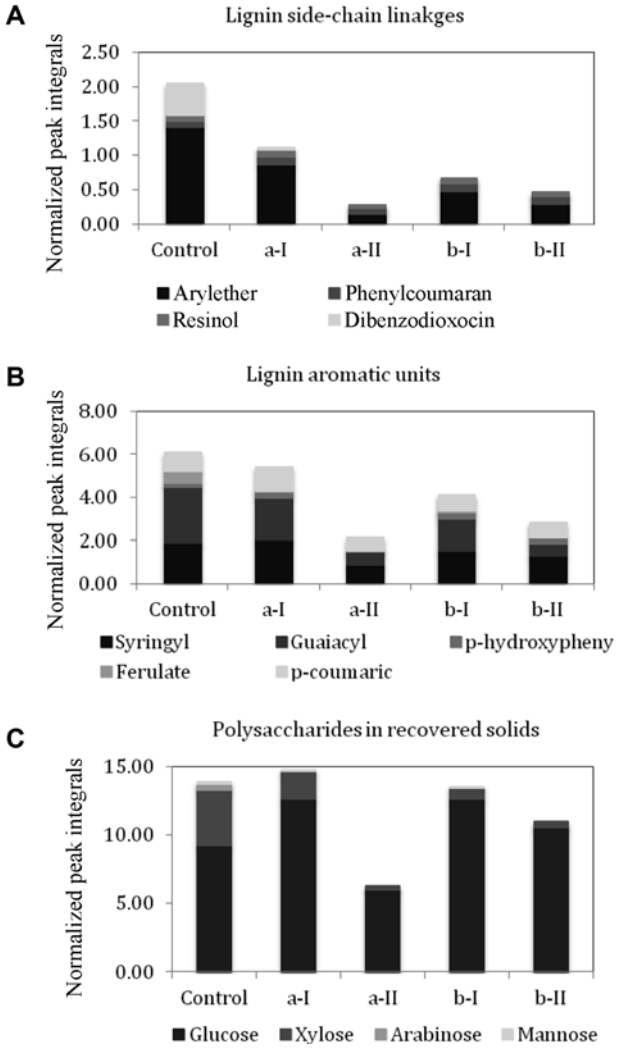

Fig. 7. Lignocellulosic compositions of non-treated *M. giganteus* and recovered solid after each step as determined by quantitative 2D-NMR. Control: untreated material; a-I: residue after first step with 0.5 wt.% HNO_3 at 140°C for 20 min; a-II: residue after second step with 0.5 wt.% HNO_3 at 195°C for 3 min and the first step at 140°C for 20 min; b-I: residue after first step with 0.5 wt.% HNO3 at 160°C for 5 min; b-II: residue after second step with 0.5 wt.% HNO_3 at 195°C and the first step at 160°C for 5 min.

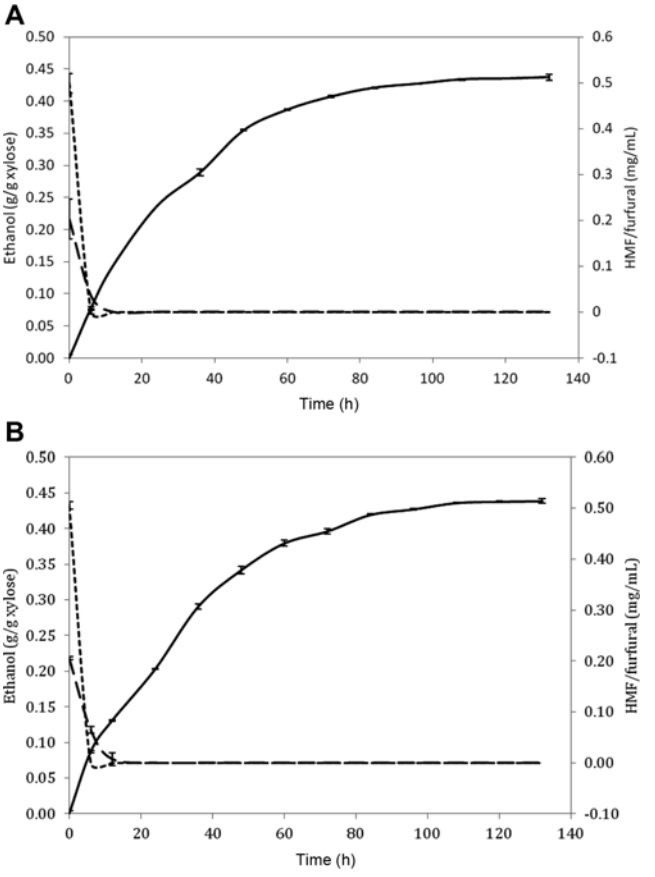

Fig. 8. Anaerobic fermentation of first-step hydrolysate using *S. cerevisiae* SR8. (A) Control with inhibitors, — ethanol, ⋯ furfural, – – HMF; (B) hydrolysate obtained from first step using 0.5% nitric acid at 140°C for 20 min, — ethanol, ⋯ furfural, – – HMF.

ferulate (FA) units and arabinosyl side-chain of hemicellulose are simultaneously removed in the first step, while *p*-coumaric acid (*p*CA) lignin units seem resistant to this step as well as to the second step. Syringyl (S) lignin units are more resistant to the first step of 140°C and 20 min than guaiacyl (G) units (Fig. 7B). When 160°C is used in the first step, the content of S and G in the recovered solid decrease significantly. Fig. 7B shows that these lignin units are reduced further by the second step at higher temperature. The total amount of lignin can be reduced by 65% by two-step 0.5 wt.% nitric acid hydrolysis (first step: 140°C for 20 min and second step: 195°C for 3 min). Lignin quantified here by 2D-NMR here only takes those structures into account that can be found in native lignin. Additionally, co-densed lignin or other hitherto unknown lignin derivatives during hydrolysis were not taken into account with the 2D-NMR analysis, but might be obtained by Klason lignin measurements.

The first step at 140°C for 20 min removes more than 50% of the hemicellulose, mainly arabinoxylan. Increasing the temperature of the first step raises removal of hemicellulose (Fig. 7C). The second step at 195°C for 3 min further removes hemicellulose as well as cellulose. 140°C for 20 min for the first step and 195°C for 3 min for the second step with 0.5 wt.% nitric acid, hydrolyzes nearly 50% of the cellulose into glucose after the second step. However, degradation products of glucose such as HMF seem to increase at 140°C rather than at 160°C according to the 2D-NMR spectra of the liquid phase (SM-5 and SM-6). However, less cellulose can be hydrolyzed using 160°C in the first step (Fig. 7C), probably because the higher temperature in the first step changes cellulose morphology and hence makes it more resistant to deconstruction. The liquor from each step appears to lack any lignin compounds. Very few

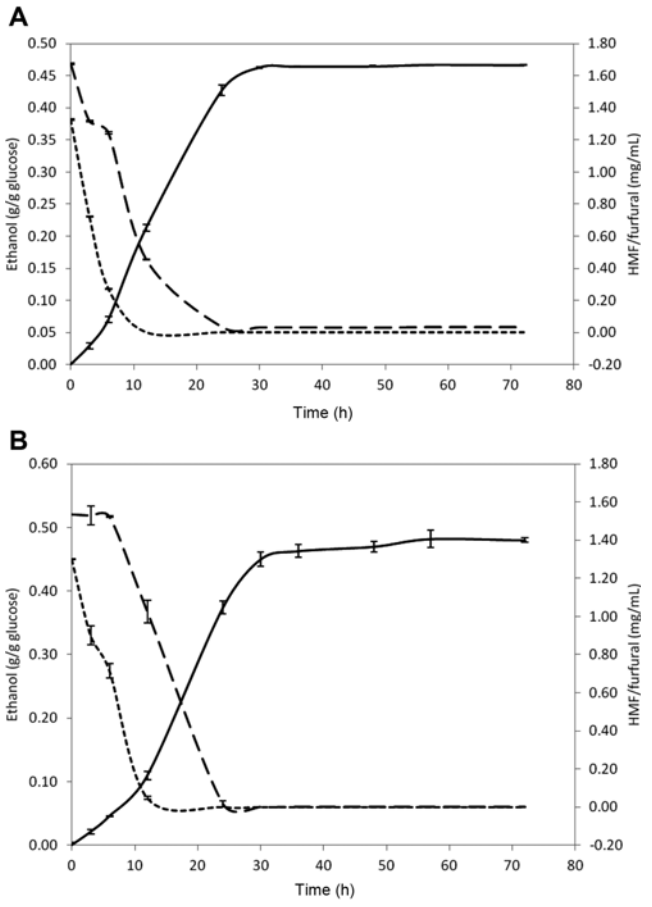

Fig. 9. Anaerobic fermentation of second-step hydrolysate using *S. cerevisiae* SA-1. (A) Control with inhibitors, — ethanol, ⋯ furfural, – – HMF; (B) hydrolysate obtained from first step using 0.5% nitric acid at 195°C for 3 min, — ethanol, ⋯ furfural, – – HMF.

oligosaccharides of xylose or glucose were observed. Following pH adjustment, the liquor can be fermented.

3.5. Fermentation

For fermentation of first-step hydrolysate, the sample collected after the first step using 0.5% nitric acid at 140°C for 20 min was neutralized with aqueous ammonia hydroxide and fermented by the yeast strain *S. cerevisiae* SR8 with initial OD600 3.0, as shown in Figs. 8 and SM-7. Also fermented were controls with xylose and the same amount inhibitors detected in the hydrolysate: 20.9 mg/mL xylose, 3.5 mg/mL acetic acid, 0.2 mg/mL HMF and 0.5 mg/mL furfural. In the control samples without inhibitors, 96 hours were required to ferment xylose; the ethanol yield was 0.45 g/g xylose (theoretical ethanol yield is 0.46 g/g xylose) [29]. Because the hydrolysate contains some glucose, it is assumed that all of the glucose is converted to ethanol in theoretical yield. In the hydrolysate and the control with inhibitors, 120 hours were required for fermentation; the ethanol yields are 0.41 g/g xylose and 0.44 g/g xylose, respectively. Furfural and HMF were reduced to furfuryl alcohol and 2,5-furandimethanol during the first 12 h [30].

For fermentation of second-step hydrolysate, sample was collected after the second step using 0.5% nitric acid at 195°C for 3 min. Fermentation was performed with the yeast strain *S. cerevisiae* SA-1 with initial OD600 of 0.3. Controls with glucose and inhibitors contained 31.5 mg/mL glucose, 0.5 mg/mL formic acid, 1.0 mg/mL acetic acid, 1.5 mg/mL levulnic acid, 1.6 mg/mL HMF and 1.3 mg/mL furfural. As shown in Figs. 9 and SM-8, 24 h were required to reduce furfural and 31 h to reduce HMF. Furfural was reduced more rapidly than HMF. Because the hydrolysate contains some xylose, it is assumed that all of the xylose is converted to ethanol in theoretical yield. Ethanol yields for the control without inhibitors, for control with inhibitors, and for hydrolysate samples were 0.49 g/g glucose, 0.47 g/g glucose and 0.46 g/g glucose (theoretical ethanol yield is 0.51 g/g glucose), respectively.

Results from control samples with inhibitors are similar to those from hydrolysate, indicating that any potential phenolic byproducts produced in the nitric-acid hydrolysis process (not detected in this work) do not affect the fermentation. The ethanol yields for control samples without inhibitors are almost the same as those for hydrolysate, suggesting that weak acids and furan derivatives do not influence the ethanol yield. However, these byproducts reduce the growth rates of the yeasts and the initial rate of ethanol production.

4. Conclusion

A two-step, dilute-nitric-acid process was investigated to hydrolyze hemicellulose and cellulose in *M. giganteus* to sugars that are subsequently fermented to bioethanol. In the two-step process, no enzymes were used. In the first step at 140°C for 20 min, 88% hemicellulose is removed into the liquid and 61% of original hemicellulose is converted to xylose. In the second step at 195°C for 3 min, 53% cellulose is removed into the liquid and 40% of original cellulose is converted to glucose. Overall for the two steps combined, 58% cellulose and nearly all hemicellulose are removed; 47% of original total hemicellulose and cellulose are converted to sugars. Xylose and glucose are fermented separately using different yeasts. Fermentation data with controls show that byproducts do not lower the ethanol yield, but reduce the initial rate of fermentation. Based on dissolved cellulose and hemicellulose, overall ethanol yield is close to the theoretical yield.

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References

1. Carroll, A. and C. Somerville (2009) Cellulosic biofuels. *Annu. Rev. Plant Biol.* 60: 165-182.
2. Heaton, E. A., F. G. Dohleman, A. F. Miguez, J. A. Juvik, V. Lozovaya, J. Widholm, O. A. Zabotina, G. F. McIsaac, M. B. David, T. B. Voigt, N. N. Boersma, and S. P. Long (2010) Miscanthus: A promising biomass crop. *Adv. Bot. Res.* 56: 75-137.
3. McMillan, J.. D. (1994) Pretreatment of Lignocellulosic Biomass. pp. 292-324. *Enzymatic Conversion of Biomass for Fuels Production*. American Chemical Society, Wahington DC, USA.
4. Shill, K., S. Padmanabhan, Q. Xin, J. M. Prausnitz, D. S. Clark, and H. W. Blanch (2011) Ionic liquid pretreatment of cellulosic biomass: Enzymatic hydrolysis and ionic liquid recycle. *Biotechnol. Bioeng.* 108: 511-520.
5. Liu, Z., S. Padmanabhan, K. Cheng, P. Schwyter, M. Pauly, A. T. Bell, and J. M. Prausnitz (2013) Aqueous-ammonia delignification of miscanthus followed by enzymatic hydrolysis to sugars. *Bioresour. Technol.* 135: 23-29.
6. Liu, Z., S. Padmanabhan, K. Cheng, H. Xie, A. Gokhale, W. Afzal, H. Na, M. Pauly, A. T. Bell, and J. M. Prausnitz (2014) Two-step delignification of miscanthus to enhance enzymatic hydrolysis: Aqueous ammonia followed by sodium hydroxide and oxidants. *Energy Fuels*. 28: 542-548.
7. Yu, G., W. Afzal, F. Yang, S. Padmanabhan, Z. Liu, H. Xie, M. A. Shafy, A. T. Bell, and J. M. Prausnitz (2014) Pretreatment of miscanthus×giganteus using aqueous ammonia with hydrogen peroxide to increase enzymatic hydrolysis to sugars. *J. Chem. Technol. Biotechnol.* 89: 698-706.
8. Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Lee, M. Holtzapple, and M. Ladisch (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol*. 96: 673-686.
9. Wyman, C. E. (1994) Ethanol from lignocellulosic biomass: Technology, economics, and opportunities. *Bioresour. Technol.* 50: 3-15.
10. Taherzadeh, M. J. and K. Karimi (2007) Acid-based hydrolysis processes for ethanol from lignocellulosic materials: A review. *BioResources*. 2: 472-499.
11. Lloyd, T. A. and C. E. Wyman (2005) Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. *Bioresour. Technol.* 96: 1967-1977.
12. Moe, S. T., K. K. Janga, T. Hertzberg, M.-B. Hägg, K. Øyaas, and N. Dyrset (2012) Saccharification of lignocellulosic biomass for biofuel and biorefinery applications – A renaissance for the concentrated acid hydrolysis? *Energy Procedia*. 20: 50-58.
13. Nguyen, Q., M. Tucker, F. Keller, and C. Eddy (2000) Two-stage dilute-acid pretreatment of softwoods. *Appl. Biochem. Biotechnol*. 84-86: 561-576.
14. Sannigrahi, P., A. Ragauskas, and S. Miller (2008) Effects of two-stage dilute acid pretreatment on the structure and composition of lignin and cellulose in loblolly pine. *BioEnergy Res.* 1: 205-214.
15. Söderström, J., L. Pilcher, M. Galbe, and G. Zacchi (2003) Two-step steam pretreatment of softwood by dilute H2SO4 impregnation for ethanol production. *Biomass Bioenergy*. 24: 475-486.
16. Monavari, S., M. Galbe, and G. Zacchi (2009) The influence of solid/liquid separation techniques on the sugar yield in two-step dilute acid hydrolysis of softwood followed by enzymatic hydrolysis. *Biotechnol. Biofuels*. 2: 6.
17. Nguyen, Q. A., M. P. Tucker, B. L. Boynton, F. A. Keller, and D. J. Schell (1998) Dilute acid pretreatment of softwoods - Scientific note. *Appl. Biochem. Biotechnol.* 70-2: 77-87.
18. Rodríguez-Chong, A., J. Alberto Ramírez, G. Garrote, and M. Vázquez (2004) Hydrolysis of sugar cane bagasse using nitric acid: A kinetic assessment. *J. Food Eng.* 61: 143-152.
19. Zhang, R., X. Lu, Y. Sun, X. Wang, and S. Zhang (2011) Modeling and optimization of dilute nitric acid hydrolysis on corn stover. *J. Chem. Technol. Biotechnol.* 86: 306-314.
20. Brink, D. L. (1996) Hydrolyzing lignocellulose. *US Patent* 5,536,325.
21. Brink, D. L. (1993) Two stage hydrolysis or depolymerization of polysaccharide material as cellulose, hemicellulose and lignocellulose to monosaccharides using nitric acid. *US Patent* 5,221,357.
22. Brink, D. L. (1994) Method of treating biomass material. *US Patent* 5,366,558.
23. Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, and D. Crocker (2008) Determination of Structural Carbohydrates and Lignin in Biomass. *National Renewable Energy Laboratory*, Golden, CO, USA.
24. Cheng, K., H. Sorek, H. Zimmermann, D. E. Wemmer, and M. Pauly (2013) Solution-state 2D NMR spectroscopy of plant cell walls enabled by a Dimethylsulfoxide-d_6/1-Ethyl-3-methylimidazolium acetate solvent. *Anal. Chem.* 85: 3213-3221.
25. Kim, S. R., J. M. Skerker, W. Kang, A. Lesmana, N. Wei, A. P. Arkin, and Y. S. Jin (2013) Rational and evolutionary engineering approaches uncover a small set of genetic changes efficient for rapid xylose Fermentation in *Saccharomyces cerevisiae*. *Plos One* 8: 13.
26. Greer, D. R., T. P. Basso, A. B. Ibanez, S. Bauer, J. M. Skerker, A. E. Ozcam, D. Leon, C. Shin, A. P. Arkin, and N. P. Balsara (2014) Fermentation of hydrolysate detoxified by pervaporation through block copolymer membranes. *Green Chem.* 16: 4206-4213.
27. Chum, H., D. Johnson, S. Black, and R. Overend (1990) Pretreatment-Catalyst effects and the combined severity parameter. *Appl. Biochem. Biotechnol.* 24-25: 1-14.
28. Kim, H. and J. Ralph (2010) Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d_6/pyridine-d_5. *Org. Biomol. Chem.* 8: 576-591.
29. Jin, Y. S. and T. W. Jeffries (2004) Stoichiometric network constraints on xylose metabolism by recombinant *Saccharomyces cerevisiae*. *Metab. Eng.* 6: 229-238.
30. Lewis Liu, Z., J. Moon, B. Andersh, P. Slininger, and S. Weber (2008) Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxymethylfurfural by Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol.* 81: 743-753.