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Pretreatment of Rice Straw by a Hot-Compressed Water Process for Enzymatic Hydrolysis

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Abstract Hot-compressed water (HCW) is among several cost-effective pretreatment processes of lignocellulosic biomass for enzymatic hydrolysis. The present work investigated the characteristics of HCW pretreatment of rice straw including sugar production and inhibitor formation in the liquid fraction and enzymatic hydrolysis of pretreated material. Pretreatment was carried out at a temperature ranging from 140 to 240 °C for 10 or 30 min. Soluble oligosaccharides were found to constitute almost all the components of total sugars in the liquid fraction. The maximal production of total glucose at 180 °C and below accounted for 4.4–4.9% of glucan in raw material. Total xylose production peaked at 180 °C, accounting for 43.3% of xylan in raw material for 10-min pretreatment and 29.8% for 30-min pretreatment. The production of acetic acid increased at higher temperatures and longer treatment time, indicating more significant disruption of lignocellulosic structure, and furfural production achieved the maximum (2.8 mg/ml) at 200 °C for both 10-min and 30-min processes. The glucose yield by enzymatic hydrolysis of pretreated rice straw was no less than 85% at 180 °C and above for 30-min pretreatment and at 200 °C and above for 10-min pretreatment. Considering sugar recovery, inhibitor formation, and process severity, it is recommended that a temperature of 180 °C for a time of 30 min can be the most efficient process for HCW pretreatment of rice straw.

Keywords Rice straw · Pretreatment · Hot-compressed water · Sugar production · Inhibitor formation · Enzymatic hydrolysis · Fermentation

Introduction

Lignocellulosic biomass is one potential resource for the production of fuels such as ethanol, and the bioconversion of lignocellulosic biomass to ethanol is a multi-step process

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consisting of pretreatment, enzymatic hydrolysis, and ethanol fermentation. Among these steps, pretreatment is particularly crucial in view of the recalcitrance of lignocellulosic structure to enzymatic hydrolysis, i.e., the lignin seal and the hemicellulose sheath of cellulose and the crystallinity of cellulose itself, and often dominates the cost of the whole conversion process [1, 2].

Hot-compressed water (HCW) pretreatment, in which biomass is exposed to pressured hot water, is one of several most promising pretreatment methods [1–4]. Water under pressure penetrates the cell structure of biomass, hydrates cellulose, and dissolves hemicellulose and lignin, and the acidity of water at high temperature (around 200 °C) and organic acids released from hemicellulose facilitate the disruption of lignocellulosic structure during pretreatment. HCW pretreatment does not require the addition of any chemical, can generate reactive cellulose fiber and recover most of the pentosans, and produce only an amount of degradation products with little inhibition to subsequent hydrolysis and fermentation [1, 2, 5]. So far, some research work has been conducted on HCW pretreatment of lignocellulosic biomass for sugar production, outlining fundamental characteristics of the process [5–23]. However, specific features of the process are still to be sufficiently clarified for improvement of the promising technology such as details incorporating both sugar production and inhibitor formation during the hot-water extraction process. Furthermore, different types of biomass possess different structures and compositions, which would give rise to different characteristics of pretreatment. An advanced pretreatment process is required to be tailored to the unique compositional and structural features of lignocellulosic biomass [1].

Rice straw is one major agricultural residue and one potential lignocellulosic substrate for fuel ethanol production. Until now, the only work on HCW pretreatment of rice straw was mainly restricted to the analysis of solubilization by pretreatment [24], and no detailed study has been reported to our knowledge. The present work examined in detail the characteristics of HCW pretreatment of rice straw including sugar production and inhibitor formation in the liquid fraction as well as enzymatic hydrolysis and fermentation of the pretreated material.

Materials and Methods

Rice Straw

Rice straw (*Oryza sativa* cv. Binan-mochi) used in this work was obtained from a local farmer in Hiroshima prefecture, Japan in November 2006. Rice straw was cut to 5 cm, air-dried, and then milled by a cutter mill (Fritsch, Germany) to 2 mm. After mesh sieving, particles of 250–420 μm were obtained and used for HCW pretreatment.

HCW Pretreatment

The HCW reactor (PARR 4565, PARR Instrument Company, Moline, IL, USA) was a stainless steel autoclave with a volume of 100 ml equipped with a mechanical stirrer and a PARR 4842 temperature and tachometer controller. Three grams of rice straw (250–420 μm) and 30 ml of deionized water were loaded in the reactor. The water content of rice straw was measured by a Mettler–Toledo halogen moisture analyzer before weighing and taken into account in subsequent calculations. The initial pressure of the reactor was kept at 2.0 MPa with nitrogen gas, and the reactor was agitated at a speed of 56 rpm. The reactor was heated by an electric mantle heater at a rate of approximately 4.5 °C/min. The

pretreatment temperature was controlled at 140, 160, 180, 200, 220 and 240 °C, and the pretreatment time was set as 10 or 30 min, initiating when a target temperature was reached. Upon completion of the set time, the reactor was cooled by immersing into room temperature water immediately and cooled down to 80 °C within 5 min. The severity parameters corresponding to different HCW pretreatment conditions are calculated as follows and shown in Fig. 1:

$$R_0 = t \exp[(T - 100)/14.75] \quad (1)$$

where t is the reaction time (min), and T is the hydrolysis temperature (°C) [25]. After pretreatment (at about 40 °C), the solid fraction and the liquid fraction were separated by filtration with the solid residue washed by deionized water several times. The solid fraction was then lyophilized, and the liquid fraction including washing water was stored at −80 °C.

Analytical Methods

The composition of raw material and solid fractions was analyzed according to the analytical procedure of the National Renewable Energy Laboratory (NREL) [26]. Acid-insoluble residue in the analysis of lignin content was taken as acid-insoluble lignin. The ash content was determined by placing 1.5-g sample in a muffle furnace at 600 °C for 24 h, cooling in a desiccator, and then weighing for ash.

Sugars and degradation products in the liquid fraction samples were analyzed by following the NREL procedure [27]. Monomeric sugars in the liquid fraction were analyzed by high-performance liquid chromatography (HPLC; Jasco, Japan) using a Biorad Aminex HPX-87P column (300×7.8 mm) equipped with a refractive index detector [27]. The mobile phase was degassed deionized water with a flow rate of 1.0 ml/min. The column temperature was 80°C. Total sugars consisting of monomeric and oligomeric sugars in the liquid fraction were analyzed by sulfuric acid hydrolysis method followed by HPLC determination [27].

Degradation products in the liquid fraction were analyzed by HPLC using a Biorad Aminex HPX-87H column (300×7.8 mm) equipped with a refractive index detector [27]. The mobile phase was 0.01 N sulfuric acid with a flow rate of 0.6 ml/min. The column temperature was 60 °C.

Analysis was carried out in duplicate, and results are expressed as the mean values. Relative standard deviations in all cases were within 5%.

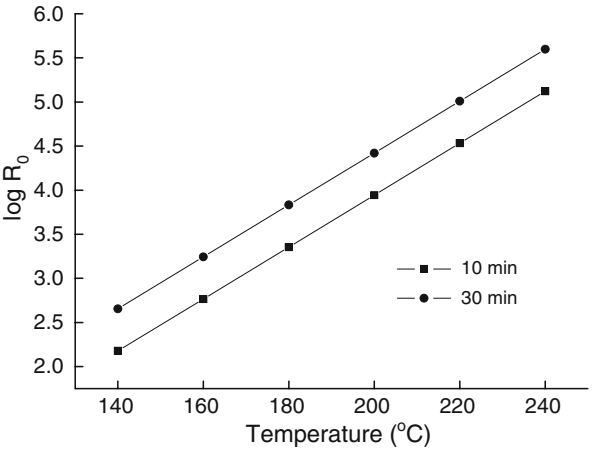

Fig. 1 Severity parameters of HCW pretreatment calculated according to Overend and Chornet [25]

Solubilization of rice straw by pretreatment was calculated as the percentage of raw material dissolved by pretreatment by measuring dry weight of the solid fraction. pH in the liquid fraction was measured by a pH meter at room temperature.

Enzymatic Hydrolysis

Enzymatic hydrolysis of raw material and HCW solid fractions was carried out in 50 mM acetate buffer (pH 5.0) at 45 °C for 72 h. A substrate concentration of 2% was used to avoid possible substrate inhibition. Two enzyme loadings of Acremonium cellulase (Meiji Seika, Japan, specific activity determined as 307.7 FPU/g protein), i.e., 40 and 10 FPU/g substrate, were adopted. In addition, 5 IU/g substrate of Novozyme 188 (β-glucosidase, 617 IU/ml) and 2%(*v*/*v*)/g substrate of Optimash BG (β-glucanase/xylanase, Genencor, 11255 CMCU/g protein) were supplemented to improve the efficiency of enzymatic hydrolysis according to our previous work. Controls containing no substrate were performed simultaneously to eliminate the deviation in hydrolysis results caused by any sugars existing in enzyme preparations. Production of monomeric sugars was analyzed by HPLC as described above. The sugar yields were defined as the percentage of total sugars available in raw material or solid fraction enzymatically converted to monomeric sugars. Enzymatic hydrolysis was performed in duplicate, and results are presented as the average. Standard deviations were less than 5%.

Separate Hydrolysis and Fermentation

Separate hydrolysis and fermentation (SHF) was carried out to check the fermentability of pretreated rice straw, with the solid fraction from pretreatment at 180 °C for 30 min as the substrate. Hydrolysis was performed in a 13-ml vial magnetically stirred with a reaction volume of 8 ml. The substrate concentration was 5%, and the enzyme loadings were 40 FPU/g substrate of Acremonium cellulase, 5 IU/g substrate of Novozyme 188 and 2%(*v*/*v*)/g substrate of Optimash BG. Hydrolysis was carried out in 50 mM acetate buffer (pH 5.0) at 45 °C for 72 h. Upon completion of hydrolysis, 0.8 ml of YPD preculture of *Saccharomyces cerevisiae* type II (Sigma) was inoculated, and fermentation was carried out at 30 °C for 72 h. For comparison, the solid fraction together with about one-fourth strength of the hydrolysate from the same pretreatment was also tested for SHF. pH was checked and adjusted to the same value for the two cases before and after saccharification. Samples from hydrolysis and fermentation were analyzed by HPLC using a Biorad Aminex HPX-87P column under the same conditions as above.

Scanning Electron Microscopy

The morphology of rice straw untreated, pretreated under different HCW conditions, and enzymatically hydrolyzed was observed by a scanning electron microscope (HITACHI S-3400N).

Results and Discussion

Raw Material

The composition of rice straw used in this work was determined to be (on a dry weight basis): 36.40% glucan, 19.15% xylan, 3.04% galactan, 1.59% arabinan, 16.51% acid

insoluble lignin, 1.71% acid soluble lignin, 1.45% acetyl, and 15.65% ash. The contents of most carbohydrates, such as glucan and xylan, and lignin were comparable to those of corn stover, whereas the contents of arabinan and acetyl group were less than half of those in corn stover [2]. In addition, mannan was not detected in the raw material. It is noticed that the ash content was much higher compared with many kinds of lignocellulosic biomass especially woody biomass [3]. The compositional differences may suggest a noteworthy structural distinction and would give rise to unique characteristics of HCW pretreatment of rice straw.

Production of Sugars in Liquid Fraction

The production of sugars, including monomeric sugars and total sugars, in the liquid fraction from HCW pretreatment was displayed in Fig. 2. It is seen that the production of monomeric sugars, i.e., free glucose, xylose, galactose, and arabinose, under each pretreatment condition tested was trivial, and soluble oligosaccharides constituted almost all the components of total sugars. The production of total glucose accounted for 4.4–4.9% of glucan in raw material at 180 °C and below and decreased with elevated temperature (Fig. 2a). Total glucose may mainly result from some easily solubilized polysaccharides in rice straw and hardly from crystalline cellulose, which can be degraded usually at higher

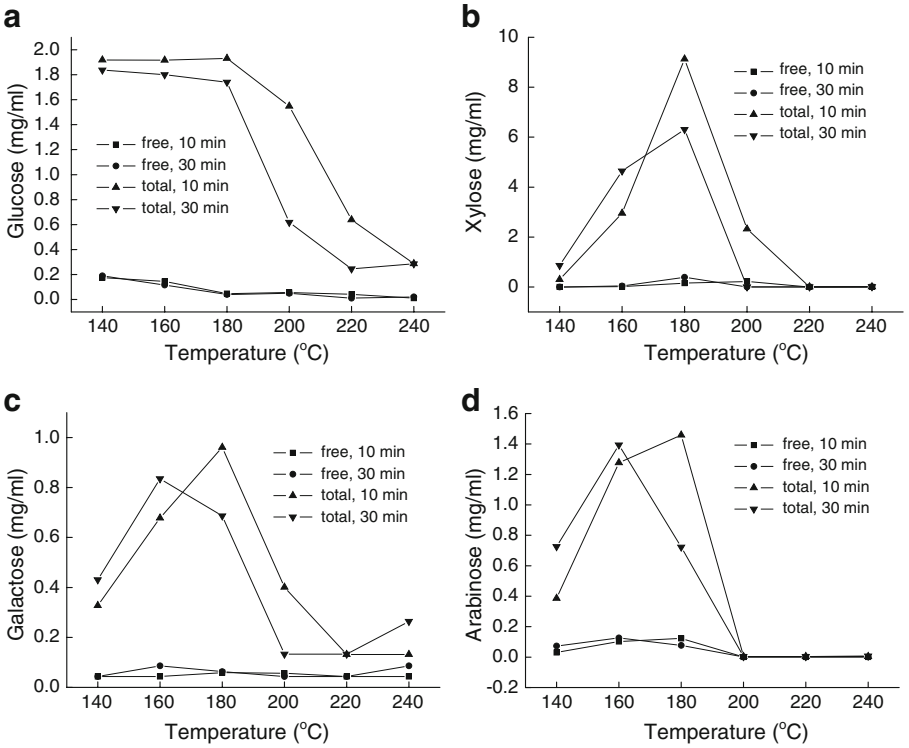

Fig. 2 Production of free and total sugars in liquid fraction from HCW pretreatment of rice straw. **a** Glucose, **b** xylose, **c** galactose, **d** arabinose. Sugars were analyzed by following the NREL procedure [27]. Analysis was performed in duplicate, and results are expressed as the mean values. Standard deviations were within 5%

temperatures (~230 °C). A similar result was also reported in the filtrate from hot-water-pretreated olive tree residues [23]. Total xylose production increased with increasing temperature, peaked at 180 °C, and decreased to zero at higher temperatures due to degradation (Fig. 2b). The maximum production accounted for 43.3% of xylan in raw material for 10-min pretreatment and 29.8% for 30-min pretreatment. Xylooligosaccharides were found to be the major soluble sugar generated from HCW pretreatment of rice straw owing to the solubilization of hemicellulose. The production profiles of total galactose and total arabinose were similar, sugar production peaking at 160 °C for 30-min pretreatment and at 180 °C for 10-min pretreatment, but their amounts were much lower than that of total xylose (Fig. 2c,d). In addition, no mannose or mannan was detected in the liquid fraction, as is in agreement with the compositional analysis of raw material.

It is found that pretreatment temperature basically dominated the pattern of sugar production for an HCW process, whereas the length of pretreatment time exerted an influence on the amount of the produced sugars. Pretreatment for 10 min tended to generate a higher amount of sugars than pretreatment for 30 min. A 30-min process gave threefold of R_0 of a 10-min process for a given temperature, and solubilized oligosaccharides can be degraded to monomeric sugars and then further to smaller byproducts during a longer process, thus affecting the amount of sugars remained in the liquid fraction.

Formation of Inhibitors in Liquid Fraction

HCW pretreatment can produce byproducts from the disruption of lignocellulosic structure and the degradation of pentoses, hexoses, and lignin, which would be inhibitory to subsequent enzymatic hydrolysis and ethanol fermentation. The formation of some inhibitors in liquid fraction from HCW pretreatment of rice straw is shown in Fig. 3.

The production of acetic acid increased at higher temperatures, with the greater amounts for 30-min pretreatment than for 10-min pretreatment at each temperature (Fig. 3a), indicating that more significant breakdown of lignocellulosic structure occurred at higher temperatures and longer treatment time. The similar effect of temperature on acetic acid formation was also observed in other work [19]. Furfural production achieved the maximum (2.8 mg/ml) at 200 °C for both 10-min and 30-min processes (Fig. 3b), corresponding to complete or nearly complete degradation of xylose and arabinose. Furthermore, 30-min pretreatment caused greater production of furfural at temperatures lower than 200 °C and more rapid degradation of furfural at temperatures higher than 200 °C compared with 10-min pretreatment. The formation of 5-hydroxymethyl-2-furaldehyde (HMF) was also enhanced at higher process severity (HMF can be further degraded under harsh conditions such as 240 °C for 30 min) and was largely consistent with the decomposition of glucose and galactose (Fig. 3c). Formic acid, which can be produced from the degradation of furfural and HMF, was also detected in the liquid fraction (Fig. 3d). However, the maximal production of HMF and formic acid was only about one third of that of acetic acid and furfural, suggesting that acetic acid and furfural may have a greater impact on the following biochemical transformation processes. Additionally, no production of levulinic acid, a degradation product of HMF, was found during HCW pretreatment under all conditions tested, implying that furfural may be the major source of formic acid.

Solubilization of Rice Straw

The solubilization of rice straw by HCW pretreatment is shown in Fig. 4. It is clear that higher process severity gave rise to greater solubilization of rice straw, which was no less

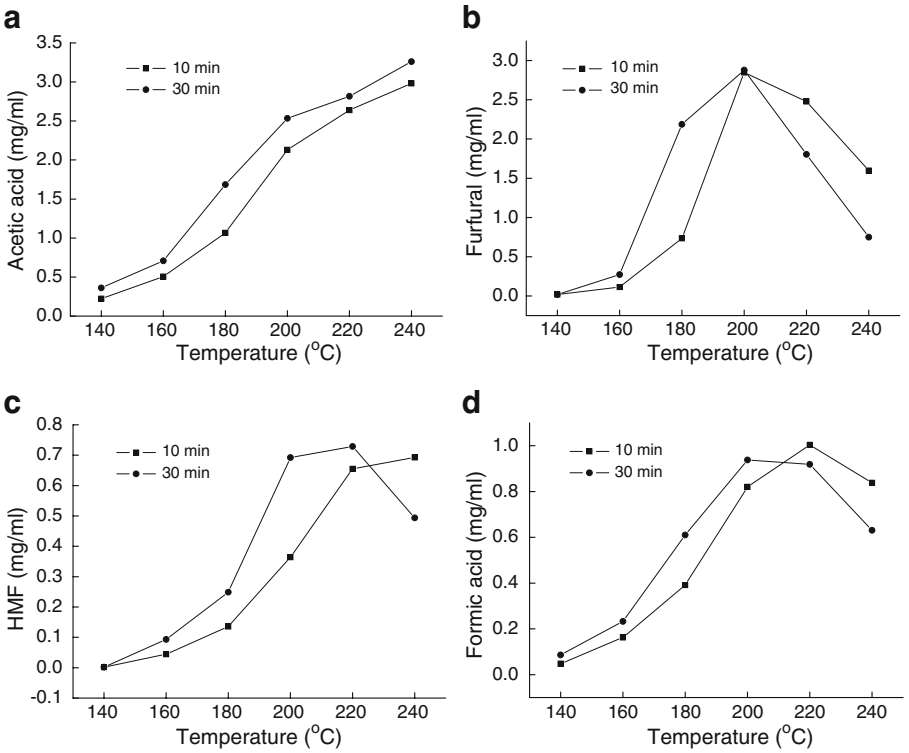

Fig. 3 Formation of inhibitors in liquid fraction from HCW pretreatment of rice straw. **a** Acetic acid, **b** furfural, **c** HMF, and **d** formic acid. Degradation products were analyzed by following the NREL procedure [27]. Analysis was performed in duplicate, and results are expressed as the mean values. Standard deviations were within 5%

than 38.3% at 180 °C and above. Solubilization from 180 to 220 °C changed slightly, corresponding to no more production of sugars within the temperature range (Fig. 2). Solubilization displayed similar tendency for both 10- and 30-min pretreatment, though being somewhat greater for 30 min treatment at each temperature. The little difference in solubilization between 10- and 30-min pretreatment from 180 to 220 °C indicated the insignificance of processing time at these temperatures.

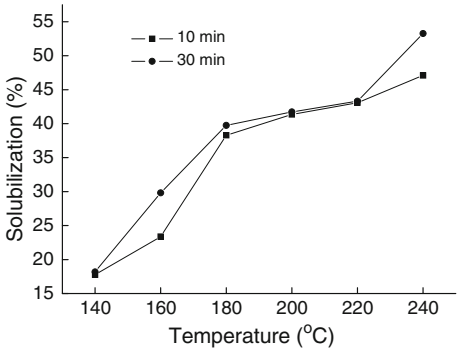

Fig. 4 Solubilization of rice straw by HCW pretreatment, expressed as the percentage of raw material dissolved by pretreatment on the basis of dry weight

The solubilization of rice straw caused a pH change in the liquid fraction (Fig. 5). pH was lowered by the release of organic acids during pretreatment, reached the lowest at 200 or 220 °C, and slightly increased thereafter probably due to the decomposition of organic acids at high temperatures. It is thought that a higher temperature together with its relative slower heating up process gave rise to more serious destruction of lignocellulosic structure and the resultant formation of more organic acids than a lower temperature, contributing primarily to the pH change. The inherent buffering capacity of protein components in rice straw is also suggested to play a role in maintaining pH. The pH profile corresponded largely with the production of acetic acid and formic acid (Fig. 3a,d). Moreover, the pH profile that 30-min pretreatment preceded 10-min pretreatment in terms of temperature can be attributed to the higher severity of the 30-min process.

The solubilization by HCW gave rise to a great change in the composition of rice straw. Xylan and acetyl group were remarkably removed from raw material by pretreatment at 180 °C, with xylan contents of only 2.78% (30 min) and 7.96% (10 min) and acetyl contents of only 0.30% (30 min) and 0.66% (10 min) in solid fractions. The xylan content was decreased to nearly one seventh of that in raw material by pretreatment for 30 min. Galactan and arabinan were completely dissolved after pretreatment at 180 °C. In contrast, the glucan content was increased to more than 50% by pretreatment at 180 °C (52.97% for 30 min and 52.47% for 10 min) thanks to the removal of hemicellulose and other components. In fact, the highest content of glucan in solid fraction was 53.0% at 180 °C for 30-min pretreatment and 54.3% at 200 °C for 10-min pretreatment, respectively.

Enzymatic Hydrolysis

The sugar yields by enzymatic hydrolysis of solid fractions from HCW pretreatment under different conditions are given in Fig. 6. The glucose yields increased at elevated temperature, regardless of pretreatment time (10 or 30 min) and enzyme loadings (10 or 40 FPU/g), and was no less than 85% at 180 °C and above for 30-min pretreatment and at 200 °C and above for 10-min pretreatment (Fig. 6a). At the same enzyme loading, longer pretreatment time brought about a higher glucose yield at each temperature. At the same pretreatment time, higher enzyme loading gave rise to a higher glucose yield at each temperature. The increased cellulose digestibility as a function of pretreatment temperature and time, also noticed by Cara et al. [23], may be attributed to the solubilization of hemicellulose during pretreatment. In fact, the starting point of the approximate plateau of high enzymatic digestibility at 180 °C coincided with the maximum production of hemicellulosic sugars in the liquid fraction (Fig. 2). Compared with the glucose yields by enzymatic hydrolysis of raw material (7.8% for 10 FPU/g and 17.0% for

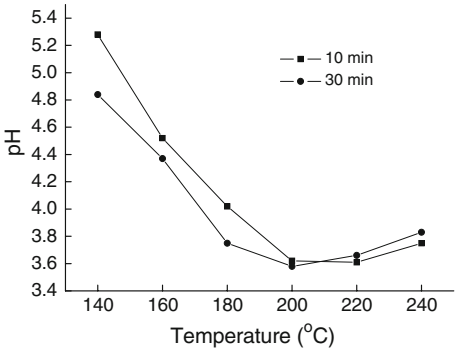

Fig. 5 pH in liquid fraction from HCW pretreatment of rice straw

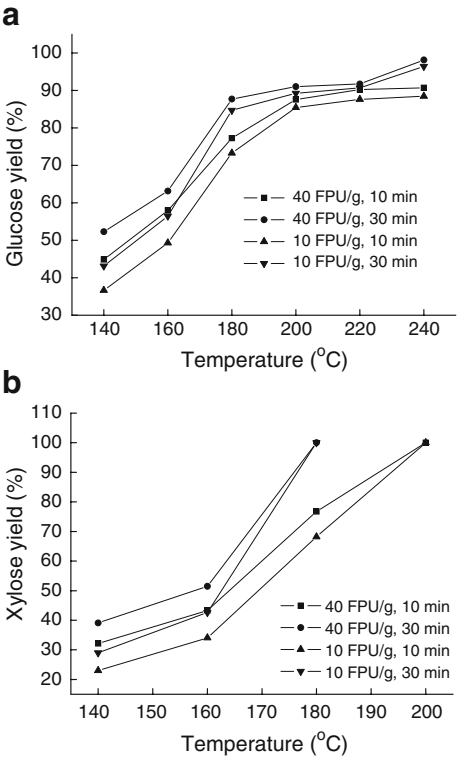

Fig. 6 Sugar yields by enzymatic hydrolysis (pH 5.0, 45 °C, 72 h) of HCW pretreated rice straw. **a** Glucose, **b** xylose. Yields were defined as the percentage of total sugars available in solid fraction converted to monomeric sugars. Enzymatic hydrolysis was performed in duplicate, and results are expressed as the average. Standard deviations were less than 5%

40 FPU/g), it is clear that HCW significantly improved the efficiency of enzymatic hydrolysis of rice straw. While temperature, pretreatment time, and enzyme loading exerted a similar effect on the xylose yields by enzymatic hydrolysis, xylose was completely recovered at 180 °C for 30-min pretreatment and at 200 °C for 10-min pretreatment (Fig. 6b), as is in good contrast with the xylose yields of raw material (11.4% for 10 FPU/g and 16.6% for 40 FPU/g).

Considering sugar yields by enzymatic hydrolysis and process severity, it is recommended that pretreatment at 180 °C for 30 min can be the most cost-effective HCW process of rice straw. The solid fraction derived from the pretreatment (180 °C, 30 min) was therefore adopted for examination of the efficiency of ethanol fermentation.

Figure 7 depicts solubilization of HCW pretreated rice straw by enzymatic hydrolysis at a cellulase loading of 40 FPU/g. Solubilization here was expressed as the percentage of solid

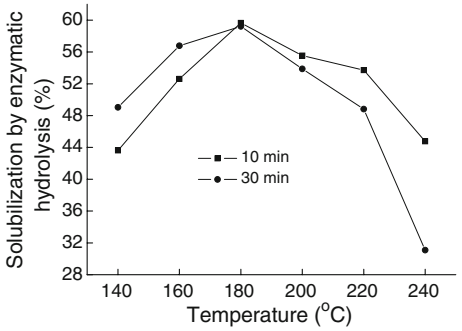

Fig. 7 Solubilization of HCW pretreated rice straw by enzymatic hydrolysis (pH 5.0, 45 °C, 72 h) at a cellulase loading of 40 FPU/g, expressed as the percentage of solid fraction dissolved by enzymatic hydrolysis on the basis of dry weight

fraction dissolved by enzymatic hydrolysis on the basis of dry weight. Solubilization peaked at 180 °C for both 10- and 30-min pretreatment, implying the significance of 180 °C for an HCW process. The profile of solubilization can be explained by sugar production from enzymatic hydrolysis and the fact that rice straw pretreated at higher temperatures contained higher content of lignin and other components that cannot be enzymatically hydrolyzed. Solubilization of pretreated rice straw by enzymatic hydrolysis at 10 FPU/g possessed a similar trend (data not shown).

Separate Hydrolysis and Fermentation

Figure 8 illustrates SHF of rice straw pretreated at 180 °C for 30 min. The glucose yield by enzymatic hydrolysis with a substrate concentration of 5% was 80.3% (a little lower than that with 2% substrate concentration shown above), whereas xylose was totally recovered from hydrolysis (Fig. 8a). Glucose enzymatically produced was rapidly converted to ethanol within 8 h during the subsequent fermentation. Approximately 100% of theoretical yield of ethanol was obtained for glucose (Fig. 8b). It was found that the hydrolysate added together with the solid fraction, resulting in the following final concentrations of inhibitors in the SHF system, i.e., acetic acid 0.43 mg/ml, furfural 0.55 mg/ml, HMF 0.063 mg/ml, and formic acid 0.15 mg/ml, had almost no effect on hydrolysis and fermentation of pretreated rice straw (Fig. 8a,c).

Morphology of HCW-Pretreated Rice Straw

The scanning electron microscopy (SEM) micrographs of rice straw untreated and treated under different conditions are presented in Fig. 9. The raw material displayed a regular and compact surface structure (Fig. 9a), and pretreatment destroyed lignocellulosic structure significantly, with higher severity resulting in more serious structural breakdown (Fig. 9b–e). Many vascular structures appeared after pretreatment at 180 °C especially for 30 min in comparison with pretreatment at 140 °C and pretreatment at 240 °C for 30 min apparently eliminated most of these cellulosic structures. The attack by cellulase effectively disintegrated cellulosic structures remained after pretreatment, with a glucose yield of 87.7% achieved (Fig. 9f). In contrast, if raw material was enzymatically hydrolyzed without pretreatment, enzymes can only attack the surface of rice straw obtaining a glucose yield of 17.0%. The situation differed markedly from pretreatment followed by enzymatic hydrolysis (Fig. 9g).

Conclusions

This work has studied the characteristics of pretreatment of rice straw by hot-compressed water including the compositional features of the liquid fraction and enzymatic hydrolysis of pretreated material.

It was found that oligosaccharides constituted almost all the components of total sugars in the liquid fraction. The maximum production of total glucose accounted for 4.4–4.9% of glucan in raw material, and the highest production of total xylose at 180 °C accounted for 43.3% of xylan in raw material for 10-min pretreatment and 29.8% for 30-min pretreatment. The production of acetic acid indicated more significant breakdown of lignocellulosic structure occurring at higher temperature and longer pretreatment time. Furfural production peaked at 2.8 mg/ml at 200 °C for both 10- and 30-min processes,

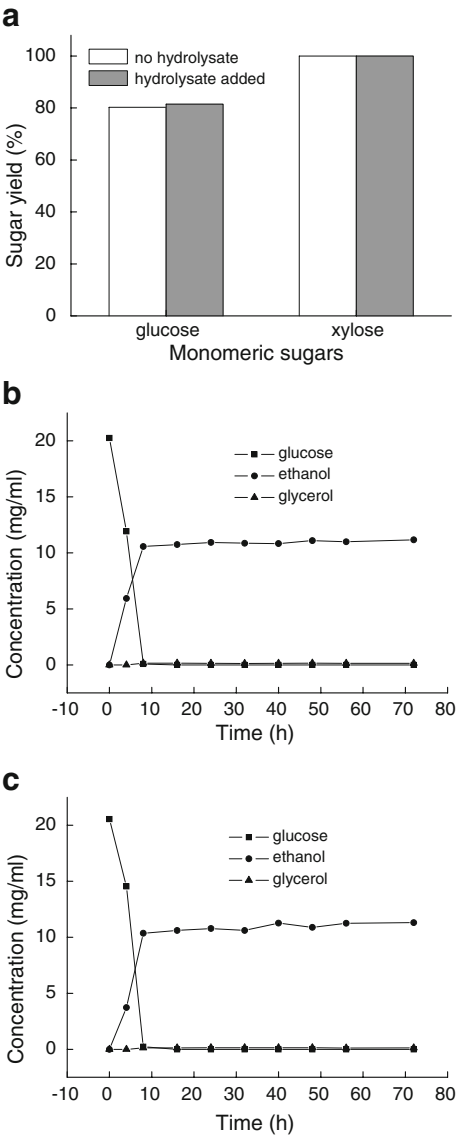

Fig. 8 Separate hydrolysis (cellulase 40 FPU/g, pH 5.0, 45 °C, 72 h) and fermentation of rice straw pretreated at 180 °C for 30 min. **a** Sugar yields by enzymatic hydrolysis; **b** fermentation of hydrolyzed solid fraction; **c** fermentation of hydrolyzed solid fraction in the presence of hydrolysate

corresponding to complete or nearly complete degradation of pentoses. The glucose yield by enzymatic hydrolysis of pretreated rice straw increased with elevated temperature and was no less than 85% at 180 °C and above for 30-min pretreatment and at 200 °C and above for 10-min pretreatment. Approximately 100% of theoretical ethanol yield was obtained for glucose in SHF. SEM micrographs confirmed the significant disruption of lignocellulosic structure and the enhancement of enzymatic hydrolysis by pretreatment.

In terms of sugar recovery, inhibitor formation and process severity, a temperature of 180 °C for a time of 30 min can be taken as the most cost-effective process for pretreatment of rice straw by hot-compressed water.

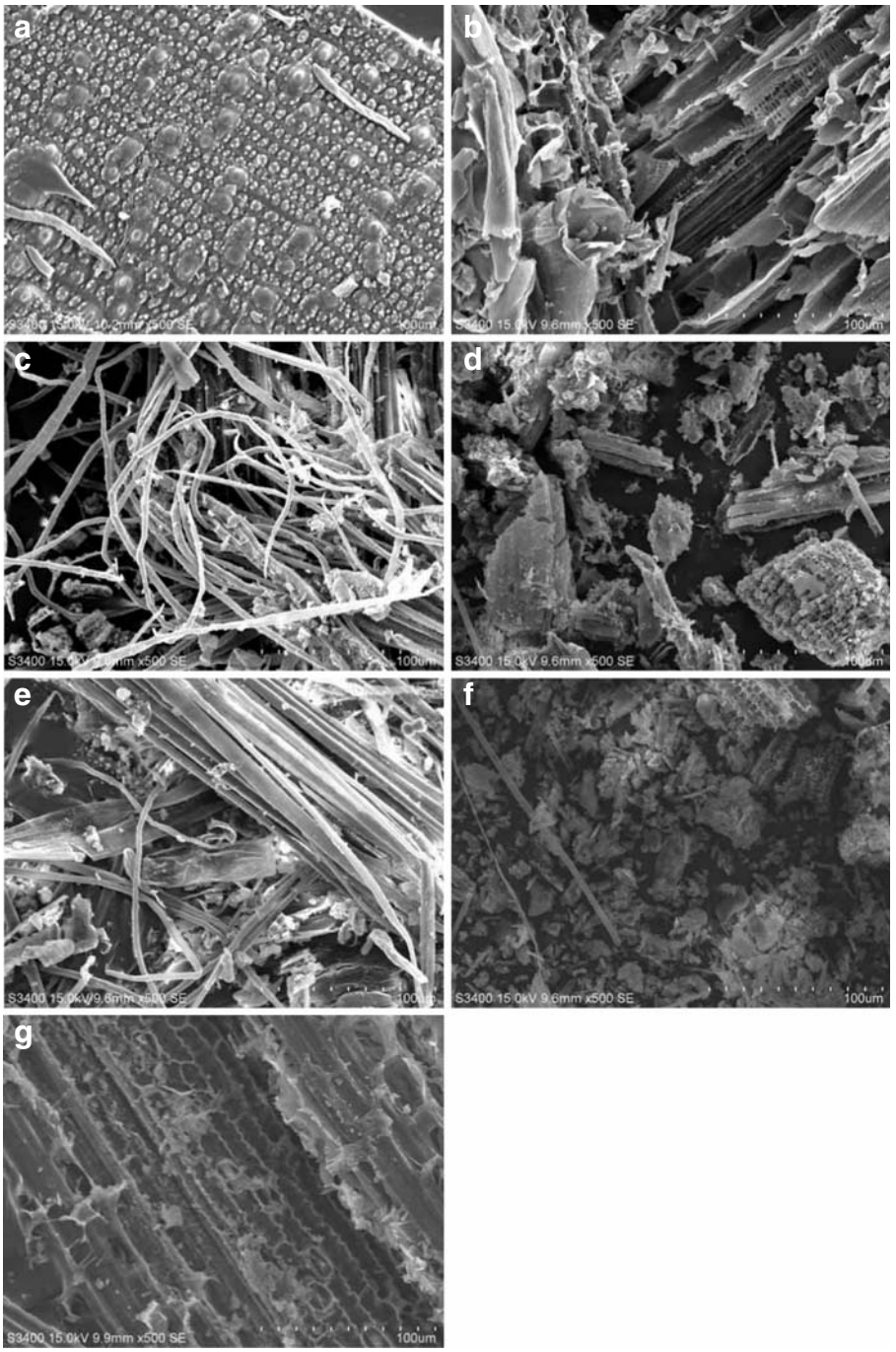

Fig. 9 SEM micrographs of rice straw untreated, pretreated and enzymatically hydrolyzed (×500 magnification). **a** Raw material; **b** pretreated at 140 °C for 30 min; **c** pretreated at 180 °C for 30 min; **d** pretreated at 240 °C for 30 min; **e** pretreated at 180 °C for 10 min; **f** pretreated at 180 °C for 30 min and hydrolyzed by 40 FPU/g of cellulase; **g** hydrolyzed by 40 FPU/g of cellulase

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