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Effects of Liquid Hot Water Pretreatment on Enzymatic Hydrolysis and Physicochemical Changes of Corncobs

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Abstract Liquid hot water (LHW) pretreatment is an efficient chemical-free strategy for enhancing enzymatic digestibility of lignocellulosic biomass for conversion to fuels and chemicals in biorefinery. In this study, effects of LHW on removals of hemicelluloses and lignin from corncobs were studied under varying reaction conditions. LHW pretreatment at 160 °C for 10 min promoted the highest levels of hemicellulose solubilization into the liquid phase, resulting into the maximized pentose yield of 58.8% in the liquid and more than 60% removal of lignin from the solid, with 73.1% glucose recovery from enzymatic hydrolysis of the pretreated biomass using 10 FPU/g Celluclast™. This led to the maximal glucose and pentose recoveries of 81.9 and 71.2%, respectively, when combining sugars from the liquid phase from LHW and hydrolysis of the solid. Scanning electron microscopy revealed disruption of the intact biomass structure allowing increasing enzyme's accessibility to the cellulose microfibers which showed higher crystallinity index compared to the native biomass as shown by x-ray diffraction with a marked increase in surface area as revealed by BET measurement. The work provides an insight into effects of LHW on modification of physicochemical properties of corncobs and an efficient approach for its processing in biorefinery industry.

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Keywords Corncob · Enzymatic hydrolysis · Lignocelluloses · Liquid hot water · Pretreatment

Introduction

The fluctuation of crude oil price and the increasing concerns on greenhouse gas release to global warming are main drivers on finding renewable resources for production of fuels and chemicals. Lignocellulosic biomass has been considered as an alternative sustainable renewable feedstock for the promising biorefinery industry. Lignocelluloses typically comprise three major biopolymers: (1) cellulose, a linear homopolymer of D-glucose formed into a highly organized crystalline fibrous structure which are associated with (2) hemicellulose, an amorphous branched heteropolymer of pentoses, hexoses, and sugar acids, which acts as an interconnecting substance, and (3) lignin, a heteropolymer of phenolic alcohols (*p*-coumaryl, coniferyl, and sinapyl alcohols), which acts as a protective shield giving strength to plant cells [1]. These biopolymers are organized into a multi-component complex structure of the plant cell wall, which is highly recalcitrant to physical, chemical, and enzymatic hydrolysis.

Pretreatment is a pre-requisite step in sugar platform biorefinery. The main purposes of pretreatment include removals of hemicelluloses and/or lignin, decrystallization of cellulose, and reduction in degree of polymerization. These effects, overall, result in increasing accessible surface areas and digestibility of the cellulose microfibers, leading to higher released sugars from the enzymatic hydrolysis step. Several chemical, thermal, and biological pretreatment methods have been extensively studied on woody biomass and agricultural by-products with differences in their advantages and limitations on technical and economic aspects [2–4]. Autohydrolysis by liquid hot water (LHW) is considered one of the most promising pretreatment strategies owing to its high efficiency and low cost compared with other leading pretreatment technologies, i.e., diluted acid, lime, ammonia fiber explosion, and ammonia recycling percolation [5]. In LHW, water is used as the sole solvent at high temperatures under pressurized conditions. Under these conditions, hydronium ions are generated in situ by ionization of water, leading to the release of acetic acid from hemicelluloses, which in turn auto-catalyzes the solubilization of hemicelluloses in an acidic environment and leads to degradation of carbohydrates [6]. This results in an increased accessibility to cellulose microfibers while the hemicellulose are further hydrolyzed and then isolated from biomass [7]. Addition of external acids and alkalis in catalytic LHW or organic solvents has led to various options on increasing efficiency and modifying selectivity of the process, making it one of the most attractive pretreatment approaches at present [8, 9].

Corncob is one of the most abundant agricultural residue in Thailand with the annual availability around 1.1 million tons. Most are used as animal feed while the rest is disposed which results in environmental problem [10]. Various pretreatment methods using chemicals (e.g., acid and alkalis) and physicochemical methods (e.g., steam explosion and CO_2 explosion pretreatment) have been reported for increasing its enzymatic digestibility with variation in their efficiencies and cost-effectiveness, related to chemical and energy consumptions [11–14]. However, no study on the application on the chemical-free LHW pretreatment on corncobs has been reported. In this work, the implementation of LHW on improving digestibility of corncobs and its effects on physical and chemical characteristics of the biomass were studied. The work gives insights into the efficiencies of LHW as an eco-friendly alternative pretreatment strategy for this potent starting material in biorefinery industry.

Materials and Methods

Materials

Corncobs were obtained from Suwan farm, Nakorn Ratchasrima province, Thailand. The biomass was physically processed using a cutting mill (Retsch SM2000, Hann, Germany) and sieved to particle size of 250–420 μm (0.21–0.35 mesh). The processed biomass was then used for experimental studies. The biomass contained 42.6% cellulose, 28.0% hemicellulose, 15.3% lignin, and 6.7% ash on a dry-weight basis according to the standard NREL method [15].

Autohydrolysis Pretreatment

Pretreatment was performed in a multi-reactor system consisting of a 6 × 50-mL reactor in a temperature-controlled jacket with a vertical shaking system to provide optimal mixing. The individual stainless steel reactor vessel was installed with a thermocouple to record the temperature inside the reactor. Corncobs were pretreated using deionized water under a set of conditions with varying temperatures (140–180 °C) with the heating rate of 20 °C/min and residence times (5, 10, and 20 min) while substrate loading was fixed at 10% (*w*/*v*) and the initial pressure was at 25 bar under nitrogen. The reactor was quenched in a water bath after heating at the desired conditions. The pretreated solid was separated from the liquid fraction by filtration and then thoroughly washed with tap water on a Bushner funnel. The sample was dried at 60 °C before subjecting to enzymatic hydrolysis to evaluate the pretreatment efficiency.

Enzymatic Hydrolysis

The pretreatment efficiencies were assessed based on digestibility of the pretreated solid residues using a commercial cellulase. The hydrolysis reactions of 1-mL total volume contained 5% (*w*/*v*) pretreated substrate with 10 FPU/g *Trichoderma reesei* cellulase (Celluclast™ 1.5L; Novozymes AS, Bagvaerd, Denmark) supplemented with 330 IU/g *Aspergillus niger* β-glucosidase (Novozym 188; Novozymes AS) and 120 IU/g based on endoxylanase activity of *Humicola inosolens* hemicellulase (Optimash® BG; Danisco AS, Copenhagen, Denmark) in 50 mM sodium acetate buffer, pH 5.0. The reactions were incubated at 50 °C for 72 h with vertical mixing at 30 rpm. The experiments were done in triplicate. Filter paper unit (FPU) for cellulase was analyzed according to a standard method [16]. β-Glucosidase activity was determined using *p*-nitrophenyl-β-D-glucopyranoside as the substrate [17]. One international unit (IU) was defined as the amount of enzyme which produced 1 μmol of reducing sugar or *p*-nitrophenolate in 1 min. The fermentable sugar profiles were analyzed on a Waters e2695 high-performance liquid chromatograph, equipped with a differential refractometer, using a Biorad Aminex HPX-87P column [18]. Sugar yields were reported based on the amount of sugars obtained from enzymatic hydrolysis of the solid residues on a dry-weight basis. Sugar recoveries were reported as the percentage of glucose or pentose recovered based on the available cellulose (×1.11) and hemicellulose (×1.13) in the native biomass.

Scanning Electron Microscopy Analysis

The native and pretreated biomass microstructures were analyzed by scanning electron microscope (SEM) using a JSM-6301F Scanning Electron Microscope (JEOL, Tokyo, Japan). The samples were dried and coated with gold for analysis. An electron beam energy of 5 kV was used for analysis.

X-Ray Diffraction Analysis

Crystallinity of the native and pretreated biomass was determined by x-ray diffraction using an X'Pert PRO diffractometer (PANalytical, Almelo, The Netherlands). The samples were scanned in a range of $2\theta = 10$–$30°$ with a step size of 0.02° at 500 kV, 30 mA and radiation at Cu Kα ($\lambda = 1.54$ Å). Crystallinity was calculated according to the following equations for crystallinity index (Eq. 1) [19].

$$\mathrm{CrI}(\%) = \left[\frac{I_{002} - I_{amorphous}}{I_{002}}\right] \times 100 \tag{1}$$

in which I_{002} is the intensity for the crystalline portion of biomass (i.e., cellulose) at 2 = 22.4 and $I_{\mathrm{amorphous}}$ is the peak for the amorphous portion (i.e., cellulose, hemicellulose, and lignin) at 2 = 18.0.

BET Surface Area Measurement

The method of Brunauer, Emmett, and Teller (BET) was used to determine the total surface area of materials. Raw and pretreated biomass samples were analyzed for the BET surface area using a Belsorp-max TPDpro (BEL Japan, Tokyo, Japan) with thermal conductivity detector (Semi-diffusion type, 4-element W-Re filament) at the National Nanotechnology Center, Thailand.

Results and Discussion

Autohydrolysis Effects on Sugar Releases in Liquid Fraction

In this study, corncobs were pretreated under LHW conditions in the absence of promoters using varying temperatures (140–180 °C) and residence times (5–20 min). The percentages of sugar yields released into the liquid fractions during the pretreatment step are presented in Fig. 1. Autohydrolysis caused substantial solubilization and hydrolysis of hemicelluloses in the feedstock to monomeric sugars under the experimental conditions. A trend of increasing sugar yields was observed with increasing pretreatment temperature and time. Higher accumulation of pentoses (xylose and arabinose) in the liquid phase was found with the maximal yield of 124.9 mg/g, equivalent to 58.8% pentose recovery from the biomass pretreated at 160 °C for 10 min. Longer operation times at this temperature led to lower sugar yield yields, probably due to degradation of sugars to dehydrated products. In contrast, low amounts of glucose were found in the liquid fraction under the operating conditions, owing to the highly resistant nature of the cellulose microfibers under the experimental conditions. Similar trends on the accumulations of furfural and 5-dydroxymethyl furfural (HMF) in the liquid phase were observed (Fig. 2). Furfural from dehydration of the released pentoses from hemicellulose was found as the major degraded product, while HMF from degradation of hexoses, particularly glucose, was observed only in a low amount due to limited solubilization of glucan. The maximum HMF and furfural

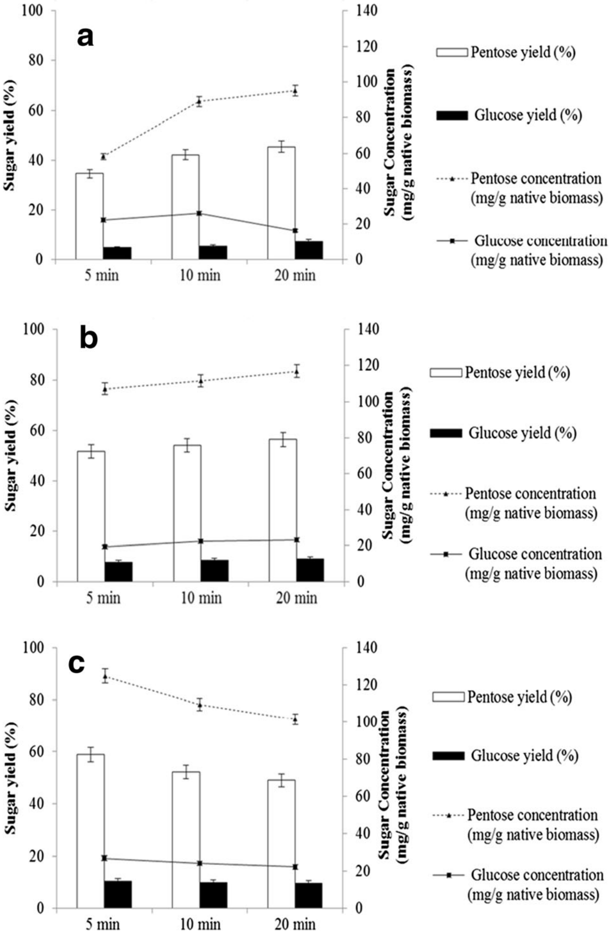


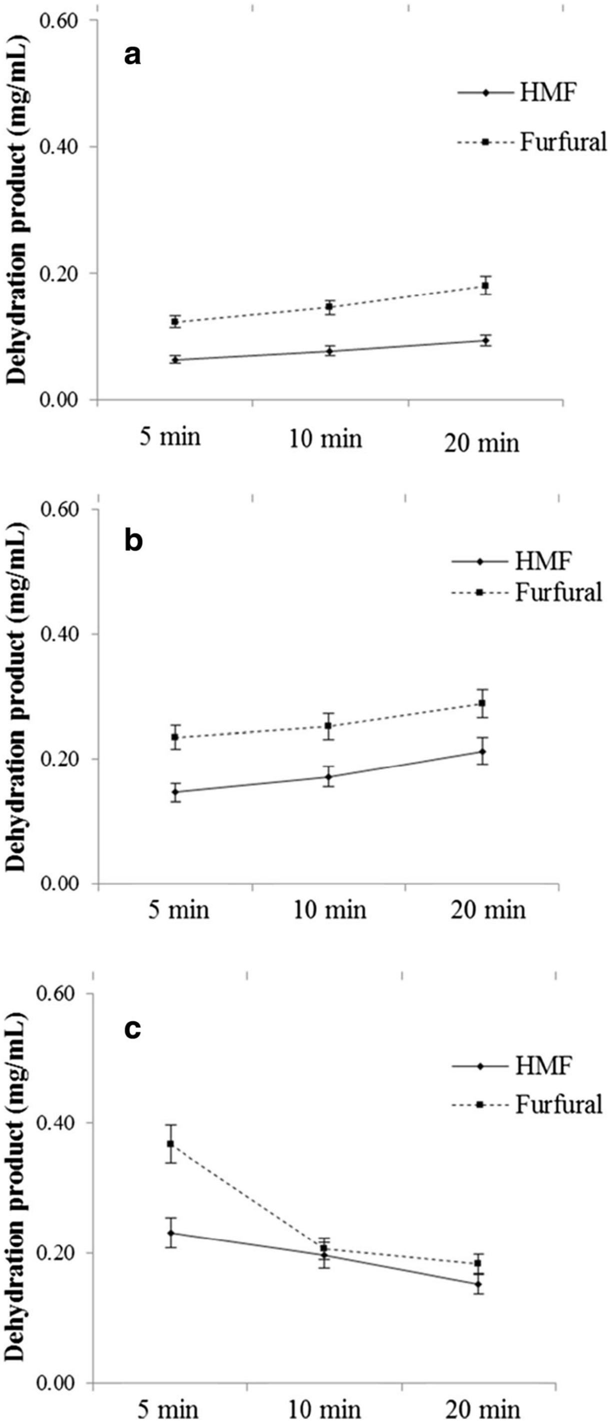
Fig. 1 Effects of LHW pretreatment on sugar releases into the liquid phase. Reactions contained 10% *w*/*v* corncobs and heated at the respective temperature for 5–20 min. **a** 140 °C; **b** 160 °C; **c** 180 °C

concentrations of 0.23 and 0.37 mg/mL, respectively, equivalent to 0.03 and 0.04 mg/g biomass, respectively, were detected in the hydrolysate of corncobs pretreated 180 °C for 5 min. Formation of furan-derivative products as well as organic acids (acetic acid, *p*-hydroxybenzaldehyde, and hydroxybenzoic acid) originated from the degradation of hemicellulose and lignin in LHW process have been reported with increasing reaction stringency [20–22]. The concentration of HMF and furfural found in this study was below the inhibitory levels for fermentable microbes (2 mg/mL) [23]. Furthermore, lignin removal of 60% from the raw material was found under the optimal condition (160 °C, 10 min) while majority of ash was still in the solid phase under the experimental conditions.

Enzymatic Hydrolysis of the Solid Residues

The influences of LHW on enzymatic digestibility of the solids obtained under varying pretreatment conditions were determined based on sugars released after enzymatic hydrolysis using a commercial enzyme mixture. The native biomass was relatively resistant to enzymatic hydrolysis resulting in a low sugar yield (41.3 mg/g pretreated biomass). Increasing

Fig. 2 Degradation products formation during LHW pretreatment of corncobs. Reactions contained 10% *w*/*v* corncobs and heated at the respective temperature for 5–20 min. **a** 140 °C; **b** 160 °C; **c** 180 °C



pretreatment temperature and time led to higher sugar yield from enzymatic hydrolysis of the solid residues (Fig. 3). Glucose and pentoses (xylose and arabinose) were detected as the major sugars from hydrolysis of cellulose and hemicellulose in the pretreated corncobs. Increasing glucose yield from the pretreated solid residues was found with increasing temperature and time at the low temperature range. The highest glucose yield of 794.1 mg/g, equivalent to 73.1% glucose recovery from the native corncob, was obtained using LHW pretreatment at 160 °C for 10 min. Further increases in the reaction temperature and residence time resulted in lower sugar yields due to partial hydrolysis of glucan into the liquid phase under the conditions with higher stringencies. Variation in the pentose yield was found under the experimental

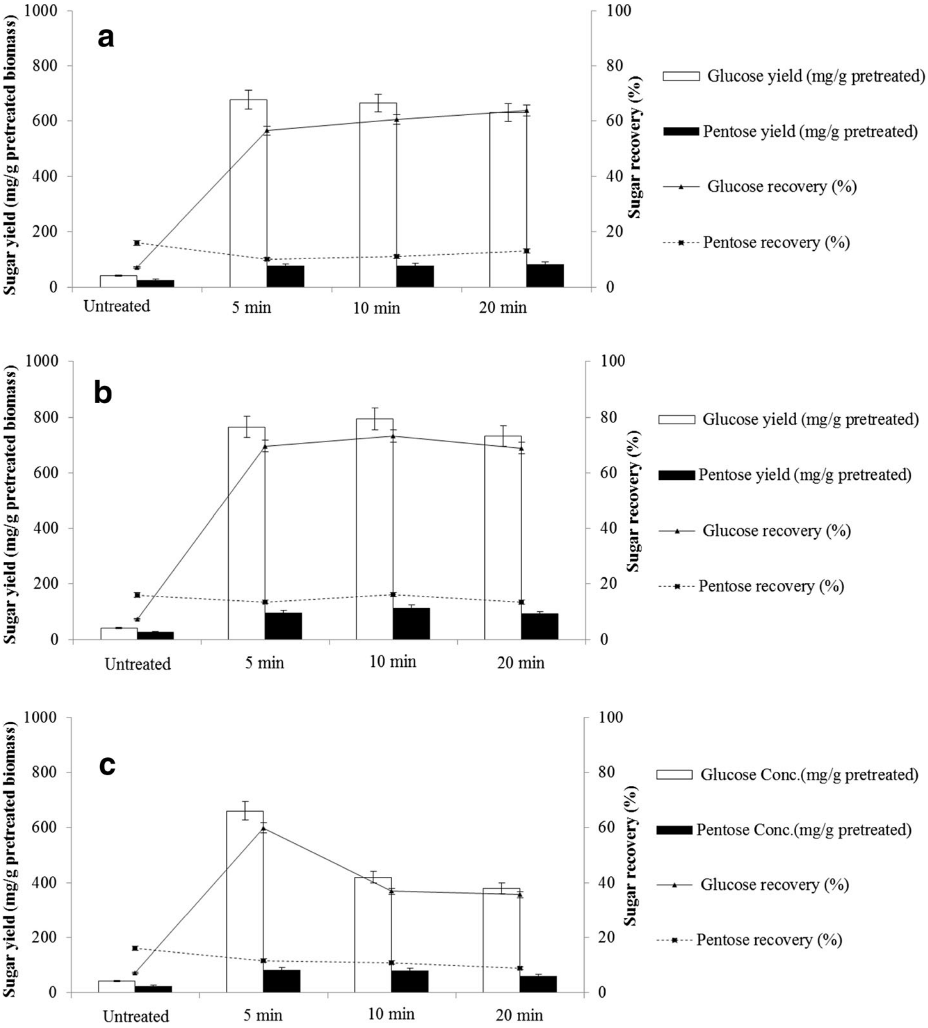


Fig. 3 Effects of LHW pretreatment on enzymatic digestibility of the solid residues. Reactions contained 10% *w*/*v* corncobs and heated at the respective temperature for 5–20 min. **a** 140 °C; **b** 160 °C; **c** 180 °C. The reactions contained 5% (*w*/*v*) pretreated substrate with 10 FPU/g Celluclast™ 1.5L supplemented with 330 IU/g of β-glucosidase (Novozym 188) and 120 IU/g xylanase activity (Optimash® BG) in 50 mM sodium acetate buffer, pH 5.0, and incubated at 50 °C for 72 h. The experiments were done in triplicate

conditions (37.2–67.8 mg/g). Combined with the released glucose and pentose into the liquid phase under the respective conditions (160 °C, 10 min), this led to the maximal glucose and pentose recoveries of 81.9 and 71.2%, respectively, from the native biomass.

Physicochemical Analysis of Pretreated Solid Residues

Physical structure modifications of corncobs pretreated by LHW under the conditions for maximal sugar yields were analyzed by SEM (Fig. 4). The native corncobs showed an intact surface with no apparent cracks and cavities. However, the microstructures of corncobs are disrupted by LHW pretreatment which affected the removals of hemicellulose and surface lignin. This resulted in peeling of the surface lignin allowing increasing accessibility to the inner cellulose microfibers correlated to the higher enzymatic digestibility. These changes in the biomass microstructures were similar to those observed in various agricultural residues pretreated by various methods [24, 25].

Crystallinity and surface area of the pretreated corncob are also considered important factors influencing the efficiency of enzymatic hydrolysis. Increased crystallinity (CrI) (73.6%) was observed for corncobs pretreated by LHW under the optimal condition (160 °C, 10 min) compared with native biomass (61.2%) (Fig. 5). The increases in CrI were related to effects of LHW on removal of the amorphous xylan and lignin fractions, while it showed less effect on the disruption of the highly crystalline cellulose. Increases in biomass

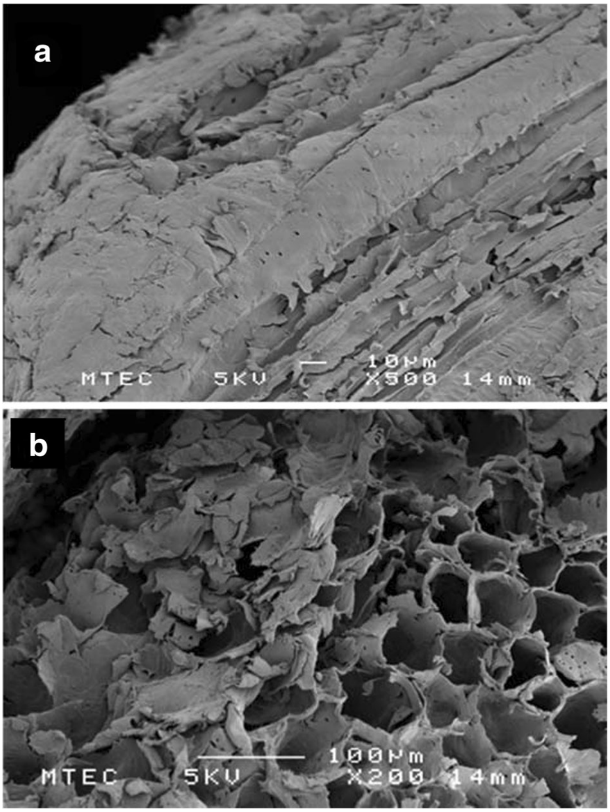


Fig. 4 Scanning electron micrographs of native and pretreated corncobs. **a** Native corncobs; **b** corncobs pretreated by LHW at 160 °C for 10 min

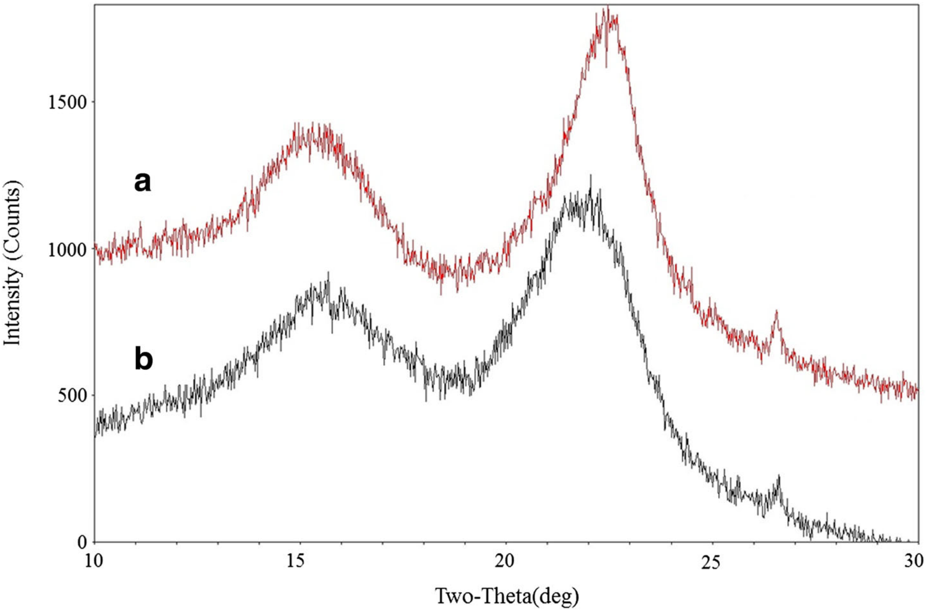


Fig. 5 X-ray diffraction profiles of the native (**a**) and pretreated (**b**) biomass

crystallinity were reported in various biomasses pretreated by LHW in the absence [26] and presence of acid/alkali promoters [8, 9] and also other pretreatment methods, e.g., aqueous ammonia pretreatment [27].

Pretreatment by LHW resulted in increasing surface area compared to the native corncobs (3.4 m^2/g). The highest surface area of 7.1 m^2/g was found with the reaction of 160 °C for 10 min. This resulted in increased enzyme accessibility to the cellulose fibers, leading to higher enzymatic susceptibility of the solid fraction. This was correlated to the highest glucose yield from hydrolysis of the biomass pretreated under these respective conditions.

Enzymatic hydrolysis of lignocelluloses can be influenced by various factors, including cellulose crystallinity and swelling, lignin content, and the complex shield of the lignin–carbohydrate complex which restricts the enzyme's accessibility to the polysaccharide fractions [4]. The LHW pretreatment of corncobs reported in this study showed good performance on improving digestibility of the biomass with the glucose recovery of 73.1% from hydrolysis of the pretreated solid. This was associated with the increased accessibility to the cellulose fibers due to removals of the non-cellulosic components and higher surface area of the pretreated substrate. The sugar recoveries could be further increased by combining those in the liquid phase from the LHW pretreatment step and from enzymatic hydrolysis of the solid. This resulted in the maximal sugar recovery for 81.9 and 71.2% for glucose and pentose, respectively, under the optimal conditions (160 °C, 10 min). The sugar recoveries in this study were comparable to those previously reported on pretreatment of corncobs using other pretreatment technologies (Table 1), e.g., sulfite pretreatment and dilute sulfuric acid pretreatment, which gave glucose recovery in the range of 78.1–81.2% [28, 29] and higher than that reported in some works using dilute phosphoric acid (<65%) and electron beam irradiation method in the absence of catalyst (<50%) [30, 31]. The yield obtained in our work was slightly lower than what has been reported using costly deep eutectic solvents [32]. The results thus

Table 1 Comparison on glucose recovery obtained from corncobs using different pretreatment methods

Pretreatment	Pretreatment conditions	Enzymatic hydrolysis	Sugar recovery
LHW (this study)	10% solid loading, 160 °C, 10 min	5% (*w*/*v*) substrate with 10 FPU/g Celluclast™ 1.5L supplemented with 330 IU/g of β-glucosidase (Novozym 188) and 120 IU/g xylanase activity (Optimash® BG) in 50 mM sodium acetate buffer, pH 5.0, and incubated at 50 °C for 72 h	Glc = 81.9%
Sulfite pretreatment [28]	7.6:1% solid loading, sulfite charge 7.1%, 156 °C, 1.4 h	8% (*w*/*v*) substrate with 30 FPU/g cellulase ZC-1700 in 0.1 M sodium acetate buffer, at 45 °C for 72 h	Glc + cellobiose = 79.3%
Dilute sulfuric acid pretreatment [29]	10% solid loading, 0.5% H_2SO_4, 125 °C, 0.5 h	8% (*w*/*v*) substrate with 30 FPU/g cellulase ZC-1700 in 0.1 M sodium acetate buffer, at 45 °C for 72 h	Glc + cellobiose = 78.1%
Dilute acid pretreatment [30]	5 g of corncob in reaction mixture of 325 mL, 1.75% (*w*/*w*) H_3PO_4, 140 °C, 10 min	0.5 g pretreated biomass in 15 mL of 0.05 N citric acid–sodium citrate buffer (pH 4.8) at 50 °C for 48 h	Glc < 65%
Electron beam irradiation [31]	1 g of corncobs with 2% (*v*/*v*) of H_2SO_4, 121 °C, 15 min	10% (*w*/*v*) pretreated biomass with 10 FPU/g of *Acremonium* cellulase in 50 mM citrate buffer (pH 5) for 48 h	Glc < 50%
Deep eutectic solvent pretreatment [32]	Solid ratio of 16:1 (g/g), 80 °C, 15 h using Ch-Cl imidazole solvents	6% (*w*/*v*) substrate with Cellic CTec 2 enzymes in 0.1 M sodium acetate buffer, pH 4.8, and incubated at 50 °C for 80 h	Glc = 86.0%

suggested high efficiency of the LHW pretreatment process reported in our study, although this cannot be directly compared due to slight variation in the enzymatic hydrolysis conditions.

LHW has been considered advantageous to other chemical and physicochemical methods as it is chemical-free and generally allowed less accumulation of inhibitory by-products owing to the bulk water used in the pretreatment process compared to acid-catalyzed hydrothermal pretreatment and steam explosion [33]. This can allow saving on the neutralization, washing or detoxification step of the solid residues before the subsequent enzymatic and fermentation steps, and on waste treatment. The highest levels of inhibitory by-products obtained by LHW in this study were relatively lower than that obtained using microwave-assisted hydrothermal pretreatment (11.6 and 1.8 mg/g for furfural and HMF, respectively [34]). The results thus suggest the advantages of the developed LHW process for pretreatment of corncobs in sugar platform biorefinery.

Conclusion

The chemical-free LHW pretreatment was shown as an effective method for pretreatment of corncobs. This led to the pentose and glucose yields of 71.2 and 81.9%, respectively, combining those from enzymatic hydrolysis of the solid and in the liquid from the pretreatment step with trace amount of inhibitory by-product formation which was below the inhibitory level for ethanogenic yeasts. The increase in biomass digestibility was associated with removals of the non-cellulose components and increase in accessible surface area of the biomass. The work indicates the potential for implementation of LHW as an effective method for improving conversion efficiency of corncobs in sugar platform biorefinery.

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