Research Square

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Alkaline organosolv pretreatment of different sorghum stem parts for enhancing the total reducing sugar yields and p-coumaric acid release

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Research

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Abstract

Background : The sorghum stem can be divided into the pith and rind parts with obvious differences in cell type and chemical composition, thus arising the different recalcitrance to enzyme hydrolysis and demand for different pretreatment conditions. The introduction of organic solvents in the pretreatment can reduce over-degradation of cellulose and hemicellulose, but significance of solvent addition in pretreatment of different parts of sorghum stem is still unclear. Valorization of each component is critical for economy of sorghum biorefinery. Therefore, in this study, NaOH-ethanol pretreatment condition for different parts of the sorghum stem was optimized in order to maximize p -coumaric acid release and total reducing sugar recovery.

Result: Ethanol addition improved p -coumaric acid release and delignification efficiency, but significantly reduced hemicellulose deconstruction in NaOH-ethanol pretreatment. The pith and rind require different NaOH-ethanol pretreatment conditions for maximal p -coumaric acid release and xylan preservation. The p - coumaric acid release yields by optimized NaOH-ethanol pretreatment reached 94.07%, 97.24% and 95.05% from pith, rind and whole stem, respectively, which increased by 8.16%, 8.38% and 8.39% compared to those of NaOH pretreated samples. Comparatively, xylan was more resistant to be dissolved from the rind than from the pith in the same NaOH-ethanol conditions. Adding xylanase significantly enhanced the enzymatic saccharification of pretreated residues. The total reducing sugar yields after NaOH-ethanol pretreatment and enzymatic hydrolysis reached 84.06%, 82.29% and 84.09% for pith, rind and whole stem, respectively, which increased by 29.56%, 23.67% and 25.56% compared to those of NaOH pretreated samples.

Conclusion: These results indicated that NaOH-ethanol is effective for the efficient fractionation and pretreatment of sorghum biomass. This work will help to understand the differences of different parts of sorghum stem under NaOH-ethanol pretreatment, thereby improving the full-component utilization of sorghum stem. Keywords: Sorghum stem; NaOH-ethanol pretreatment; p -coumaric acid; enzymatic hydrolysis.

Background

Lignocellulosic biomass is an important feedstock for the production of biofuels and biobased chemical products [1]. Cellulose, hemicellulose and lignin are the main biochemical components of lignocellulose. These three components are strongly intermeshing and bonded together (covalently or non-covalently) to form lignocellulosic matrix. Structural features make lignocellulosic biomass difficult to be deconstructed and digested, and result in relatively low digestibility of lignocellulosic feedstock [2]. In order to improve the enzymatic digestibility of lignocellulosic biomass, various pretreatment methods have been investigated based on the properties of each lignocellulosic feedstock [3]. Common pretreatment methods include acid treatment [4], alkali treatment [5], organosolv treatment [6], automated hydrolysis treatment [7], steam explosion treatment [8], etc. Among these pretreatment methods, alkali pretreatment is widely used due to the advantages of mild conditions, non-toxic, and efficient delignification [9, 10].

In general, the purpose of the pretreatment is to remove lignin and hemicellulose, thereby increasing the enzymatic hydrolysis efficiency of cellulose [11]. Xylan is the main hemicellulose component occupying 20–30% of biomass of the hardwoods (dicots) and herbaceous plants [12]. Effective utilization of xylan can improve the rational utilization of sugars in lignocellulosic biomass, which leads to the production of high-yield fermentable sugar/ethanol [13, 14]. However, xylan is susceptible to pretreatment processes and easy to be degraded due to its amorphous and branched structure [15].

Sorghum is one of the main bioenergy crops, the biorefinery of its soluble sugar and bagasse residue for biofuels and biochemicals has attracted much attention in recent years [16–19]. Beside soluble sugar and lignocellulose residue, sorghum stem also contains significant amounts of hydroxycinnamic acid, such as p-coumaric acid (pCA) [20]. PCA is widely used in the health and pharmaceutical industries because of a variety of physiological functions. Its phenolic acid structure can eliminate free radicals in the body and has a preventive effect on diseases closely related to oxidative stress damage [21]. PCA is also a precursor for the production of value-added aromatic chemical products [22]. In lignocellulose, pCA is attached to lignin via an ester linkage [23].

The ester bond can be broken under mild alkaline conditions [24]. In traditional alkaline pretreatment, however, half or even more amount of the hemicellulose is also dissolved [10, 20]. The dissolved hemicelluloses in the liquid streams were difficult to be recovered and reutilized [25], therefore reducing the total sugar utilization. Organosolv pretreatment is attractive due to its efficient lignin fractionation and convenience of recycling [26]. The use of organic solvents (such as ethanol) instead of water during alkaline pretreatment avoids the over-deconstruction of cellulose and hemicellulose, as well as the formation of conventional inhibitors [27].

Previous studies have reported enzymatic recalcitrance in different parts of internodes from sugar cane due to cell type and chemical composition [28]. The sorghum pith region contained parenchyma cells that were not extensively lignified. In contrast, the rind region contained highly lignified vessels and fibers and was very recalcitrant to enzymatic hydrolysis [28]. Although it was frequently reported that the introduction of organic solvents in the pretreatment can reduce over-degradation of cellulose and hemicelluloses, the significance of organic solvents addition in pretreatment of different parts of sorghum is still unclear. In this study, NaOH-ethanol pretreatment was described to facilitate the release of pCA in different parts of sorghum stem, but minimize the degradation of sugar polymers (cellulose and hemicellulose) as much as possible, and therefore enhance the total reducing sugar recovery. The processing conditions (NaOH loading, ethanol content, processing time and temperature) were optimized using response surface methodology. Structural characterizations of raw and pretreated sorghum sample were investigated by Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM) and X-ray diffraction (XRD).

Results And Discussion

Composition of raw sorghum samples

The composition of raw pith, rind and whole stem on a dry matter basis are shown in Table 1. The content of esterified *p*CA in pith was 2.21%, which was slightly higher than that in rind (2.08%). The content of cellulose (40.64%) and lignin (24.04%) in rind was slightly higher than that in pith (37.20% and 21.48%, respectively). The total carbohydrates including glucan, xylan, and arabinan in rind and pith reached 60.29% and 58.25%, respectively. Variations in sorghum stem composition from previous researches were observed in this study, which may be due to the differences in the geographical location, fertilization, heterogeneity of feedstock of the samples and several other environmental factors [20, 32].

Comparison of NaOH-ethanol pretreatment of different sorghum stem parts

The effect of different variables on the release of *p*CA and the recovery of xylan in residues from different sorghum parts were investigated. As shown in Table 2, the release of *p*CA from of the sorghum was strongly related to the NaOH and ethanol concentrations, however, the degree varied among different stem parts. At a low NaOH concentration (i.e. 0.5%), the release of *p*CA was very low, but significantly higher *p*CA was released from the pith than from the rind under the same conditions. As the NaOH concentration increased and the ethanol content exceeded 40% (v/v), the release of *p*CA from the rind slightly exceeded over that from the pith under the same conditions. NaOH concentration is also the key factor affecting the xylan dissolution, and more xylan was dissolved as NaOH concentration increased. In general, xylan was more resistant to be dissolved from the rind than from the pith in the same NaOH-ethanol conditions. This may be related to the difference in cell types and chemical composition in different sorghum stem parts. The pith is rich in parenchyma cells and the rind contains more vascular bundles. Parenchyma cells in pith have bigger lumens and thinner cell walls, and vascular bundles in rind are composed of tightly packed vessel elements [33]. On other hand, the easier dissolution of xylan from pith than those from rind under the same conditions may be partly attributed to the lower lignin content in the sorghum pith than in the rind [28]. Interestingly, there was a low linear correlation between the release of *p*CA and the recovery of xylan in residues for both pith and rind (Additional file 1: Fig. S1). Indeed, there is little chemically structural connection between *p*CA and xylan as most of *p*CA is attached to lignin with ester bond [34].

The regression coefficients of the model were determined by analysis of variance (ANOVA) in Additional file 1: Table S1. All models were significant at the $P < 0.0001$ level, which shows that all models were valid and do not lack fit, so this indicates that the model can be used to predict response. Several optimized solutions for model prediction were selected based on maximum *p*CA release and maximum xylan recovery in residues from different sorghum stem parts. As shown in Table 3, the actual value was close to predicted value, which verified the reliability of the model. Compared with pith, the NaOH concentration in rind was higher, and the treatment time was shorter. The possible reason is that the structure of the rind is denser, so higher NaOH concentration was needed to release *p*CA.

Considering the maximum release of *p*CA and the maximum recovery of xylan in residues from different sorghum stem parts, the following conditions were selected for NaOH-ethanol pretreatment on the pith, rind and whole stem: 1.63% NaOH, 70% ethanol, 66 °C, 3.18 h; 1.90% NaOH, 70% ethanol, 69.8 °C, 1.00 h and 1.46% NaOH, 70% ethanol, 70 °C, 2.19 h, respectively. NaOH pretreatment was also carried out in same conditions (except ethanol) to further understand the significance of ethanol in NaOH-ethanol pretreatment. The solid recovery, *p*CA release and content of each component in solid residues are shown in Table 4. In general, the solid recovery, *p*CA release and the glucan and xylan content of NaOH-ethanol pretreated residues were relatively higher than the corresponding NaOH pretreated residues, but the lignin content was lower than the NaOH pretreated residues. These results suggested that ethanol addition promoted the release of *p*CA and delignification to a certain extent, but obviously prevented the deconstruction of xylan and cellulose. Compared to NaOH pretreatment, the release yields of *p*CA in pith, rind and whole stem by NaOH-ethanol pretreatment were increased by 8.16%, 8.38% and 8.39%, respectively (Table 4). Meanwhile, the delignification rates were increased by 5.57%, 11.47% and 11.04%, respectively (Fig. 1). The glucan recoveries of pith, rind and whole stem after NaOH-ethanol pretreatment reached 89.78%, 94.85% and 93.19%, which increased by 10.76%, 7.97% and 7.29% compared to NaOH pretreatment. The xylan recoveries of pith, rind and whole stem after NaOH-ethanol pretreatment reached 76.80%, 88.46% and 85.01%, which increased by 47.75%, 15.11% and 35.97% compared to NaOH pretreatment (Fig. 1). The solid recovery of the NaOH-ethanol pretreated pith, rind and whole stem were 6.71%, 3.31% and 6.50% higher than those of NaOH pretreatment, respectively, which was mainly due to the improved recovery of glucan and xylan (Table 4). Particularly, the dissolution of xylan in pith was more serious in NaOH pretreatment condition compared to NaOH-ethanol pretreatment, indicating that addition of ethanol in NaOH solution could effectively minimize the deconstruction of xylan in pith (Fig. 1). Overall, the addition of ethanol to the NaOH pretreatment released almost all of esterified *p*CA while retaining most of cellulose and hemicellulose in the solid residue.

Enzymatic hydrolysis of solid residues by Cellic® CTec2

The enzymatic hydrolysis yields of raw and pretreated materials with Cellulase Cellic® CTec2 and β-glucosidase were shown in Fig. 2. The glucose enzymatic hydrolysis yield of NaOH-ethanol pretreated pith, rind and whole stem were 84.29%, 71.22% and 80.48%, respectively, which were 88.95%, 194.54% and 160.20% higher than that of untreated pith, rind and whole stem, respectively (Fig. 2A). Similarly, the xylose enzymatic hydrolysis yields of NaOH-ethanol pretreated pith, rind and whole stem were 78.33%, 62.70% and 75.53%, respectively, which were 59.89%, 87.78% and 117.23% higher than untreated pith, rind and whole stem, respectively (Fig. 2B). The enzymatic hydrolysis efficiency of the NaOH-ethanol pretreated residues was also obviously higher than that of NaOH pretreated residues. The glucose enzymatic hydrolysis yields of NaOH-ethanol pretreated pith, rind and whole stem were 7.39%, 15.45% and 12.18% higher than that of the NaOH pretreated pith, rind and whole stem, respectively (Fig. 2A). The xylose enzymatic hydrolysis yields of NaOH-ethanol pretreated pith, rind and whole stem were 6.43%, 10.86% and 17.61% higher than NaOH pretreated pith, rind and whole stem, respectively (Fig. 2B). This may be attributed to more effective in delignification by NaOH-ethanol pretreatment compared to that of

NaOH pretreatment, because lignin is one of the major factors inhibiting enzymatic saccharification [35]. This result was consistent with the previous finding reported by Huang et al. [36]. They described that the introduction of ethanol into alkaline peroxide pretreatment enhanced the delignification of bamboo and thus improved its enzymatic hydrolysis efficiency. In addition, the improvement of the enzymatic saccharification efficiency of the NaOH-ethanol pretreated residues might be also partly brought by its higher release of *p*CA compared to NaOH pretreatment, since it has been reported that the presence of phenolic acids in residues has a negative effect on the enzymatic hydrolysis of lignocellulose [37]. In summary, alkali organosolv pretreatment not only reduces the over-degradation of cellulose and hemicellulose, but also enhances the delignification of biomass, resulting in enhanced enzymatic digestion of biomass [26, 38].

Effect of xylanase on enzymatic hydrolysis of solid residues by Cellic® CTec2

Since relatively high xylan was retained in NaOH-ethanol pretreated residues, the saccharification of cellulose may be still impeded by the highly reserved xylan in the lignocellulosic matrix [39]. Therefore, the effect of xylanase on enzymatic hydrolysis of solid residues was studied by adding xylanase to enzymatic hydrolysis system. Three different dosages of xylanase (5 BXU, 10 BXU and 15 BXU/g substrate) were selected for the enzymatic hydrolysis of NaOH-ethanol pretreated solid residues, and the results were shown in Fig. 3. After the addition of xylanase, the enzymatic hydrolysis yields of glucose and xylose were significantly improved with the increase of xylan dosage (Fig. 3). This was attributable to the increased accessibility of cellulose to cellulose when xylanase was degraded by xylanase. As shown in Fig. 4, when the dosage of xylan was 15 BXU/g substrate, the glucose enzymatic hydrolysis yields of NaOH-ethanol pretreated pith, rind and whole stem were increased to 97.93%, 88.71% and 91.98%, respectively (Fig. 4A), and the xylose enzymatic hydrolysis yields were also increased to 99.34%, 88.11% and 94.79% (Fig. 4B). Similarly, the glucose enzymatic hydrolysis and xylose enzymatic hydrolysis yields were also increased when xylanase was added during the enzymatic hydrolysis of NaOH pretreated sample. The glucose enzymatic hydrolysis yields of the NaOH pretreated pith, rind and whole stem were enhanced to 90.36%, 78.86% and 84.00%, respectively, and the xylose enzymatic hydrolysis yields were enhanced to 92.43%, 78.22% and 86.41%, respectively (Fig. 4A and Fig. 4B). Comparatively, the improvement of glucose and xylose enzymatic hydrolysis yields by xylanase was more significant for NaOH-ethanol pretreated samples than those of NaOH pretreated samples, probably due to higher preserved xylan in former samples. These results confirmed the promoting effect of xylanase on enzymatic hydrolysis of solid residues, which was consistent with the previous report of negative effects of retaining xylan on enzymatic hydrolysis of cellulose [40].

The $total_{Glu.}$ yields and $total_{Xyl.}$ yields of various pretreated materials after adding xylanase (15 BXU/g substrate) were calculated. In above condition, the $total_{Glu.}$ yields of NaOH-ethanol pretreated pith, rind and whole stem were 87.92%, 84.14% and 85.72%, respectively (Fig. 4C), which were 20.03%, 21.45% and

17.49%, respectively, higher than those of NaOH pretreated samples. The total$_{Xyl.}$ yields were 76.29%, 77.94% and 80.58% (Fig. 4D), which were 58.77%, 29.66% and 49.17%, respectively, higher than those of NaOH pretreated samples. Taken together, the total reducing sugar yields (glucose and xylose) after enzymatic hydrolysis reached 84.06%, 82.29% and 84.09% for NaOH-ethanol pretreated pith, rind and whole stem, respectively, which were 29.56%, 23.67% and 25.56% higher than the corresponding NaOH pretreated samples. The substantial increase of total reducing sugar yields was attributed to the improvement in both solid recovery and enzymatic hydrolysis yields when using NaOH-ethanol pretreatment. It was worth noting that the total$_{Xyl.}$ yield of the NaOH pretreated pith was lower than that of the untreated pith, because nearly half of the xylan was degraded during the NaOH pretreatment.

Purification of *p*CA

Macroporous adsorption resins have been widely used in chemical and medicinal industries, especially for extraction, separation and purification of biochemical products, due to its advantages such as low cost, higher efficiency and simpler operation [41, 42]. In this study, macroporous adsorption resin D101 was used to purify *p*CA. The adsorption rate of D101 resin was about 96%, and the desorption rate was about 91%, which indicates that most of the *p*CA was recovered after purification.

Structural characterization of untreated and pretreated sorghum sample

FTIR spectrum analysis

The aromatic structure strength and functional group identification of the untreated and treated samples were investigated using FTIR techniques. FTIR spectra of the untreated and treated samples are shown in Additional file 1: Fig. S2. The wavelength assignments of the lignin, cellulose and hemicellulose related bands are summarized in Additional file 1: Table S2. Absorption spectra at 1732 cm^{-1} is due to the ester-linked acetyl, feruloyl and *p*-coumaroyl groups on hemicellulose and/or lignin [43]. The band of the pretreated material at 1732 cm^{-1} almost completely disappeared, indicating that almost all of the ester-linked phenolic acid was released into the liquid. The bands at 1106 cm^{-1}, 1254 cm^{-1} and 1513 cm^{-1} are characteristic bands of lignin [44]. These lignin-related bands were significantly reduced after treatment, indicating that most of the lignin was removed during pretreatment. After pretreatment, the characteristic band of the β-anomer or the β-linked glucose polymer increased significantly at 899 cm^{-1}, indicating a significant increase in the cellulose content in the treated residue [45]. In summary, FTIR spectroscopy results show that the pretreatment can break the ester bond of *p*CA to lignin and remove most of the lignin.

SEM analysis

In order to observe the changes in the substrate surface, SEM was applied to investigate the morphological features and surface characteristics of the raw and the pretreated pith, rind and whole stem. As can be seen from Additional file 1: Fig. S3, the surface of the raw material was relatively smooth. The surfaces of the pretreated residue were altered with evident coarse surface and porous areas. Available surface area of the cellulose fiber structure is essential for enzymatic hydrolysis of lignocellulosic materials [46]. Both NaOH-ethanol and NaOH pretreatment destroyed the recalcitrant structure of the lignocelluloses, increased the surface area and porosity of biomass, which accelerated the saccharification process.

XRD analysis

The CrI was calculated according to X-ray diffractograms. XRD analysis of untreated and pretreated sorghum samples are shown in Additional file 1: Fig. S4. In raw materials, the CrI of the pith, rind and whole stem were 30.29%, 34.61% and 30.84%, respectively. After NaOH-ethanol pretreatment, the CrI of pith, rind and whole stem were increased to 63.01%, 43.27% and 54.41%, respectively. Similarly, the CrI of NaOH pretreated pith, rind and whole stem were also increased to 58.14%, 42.17% and 45.86%, respectively. Lignocellulosic crystallinity was considered as an important characteristic for enzymatic digestibility [47]. According to previous reports, the CrI of lignocelluloses was inversely related to the amorphous substances in cell wall where degradation of hemicelluloses and disordered fractions of cellulose, and delignification can all make the CrI increase [39]. Therefore, higher CrI of NaOH-ethanol pretreated residue may be due to the higher level of lignin removal and the recovery of cellulose compared to NaOH pretreated residue.

Overall mass balance

The process of pretreatment and enzymatic saccharification of sorghum samples are shown in Fig. 5. NaOH-ethanol pretreatment was performed under the following conditions: 1.63% NaOH, 70% ethanol, 66 °C, 3.18 h; 1.90% NaOH, 70% ethanol, 69.8 °C, 1.00 h; and 1.46% NaOH, 70% ethanol, 70 °C, 2.19 h for pith, rind and whole stem, respectively. The solid residues were enzymatically hydrolyzed with cellulose cocktail (15 FPU/g substrate Cellic® CTec2, 30 CBU/g substrate β-glucosidase and 15 BXU/g substrate xylanase). Enzymatic saccharification of pretreated pith, rind and whole stem yielded 6.98 g, 7.54 g and 7.13 g of glucose, respectively, which accounted for 84.37%, 83.46% and 84.02% of the glucose in sorghum pith, rind and whole stem, respectively. Meanwhile, enzymatic saccharification produced 3.23 g, 3.04 g and 3.18 g of xylose, respectively, which accounted for 76.72%, 77.21% and 79.16% of the xylose in sorghum pith, rind and whole stem, respectively. After acidification, the NaOH-ethanol treated liquid fractions of pith, rind and whole stem contained 0.41 g, 0.40 g and 0.40 g of *p*CA, respectively. After

purification by resin column, the recovered *p*CA were 0.36 g, 0.35 g and 0.36 g, which accounted for 81.45%, 84.13% and 84.51% of the esterified *p*CA in sorghum pith, rind and whole stem, respectively.

Moreover, the hemicellulose obtained from the liquid fractions of the pretreated pith, rind, and whole stem were 0.74 g, 0.38 g and 0.51 g, respectively, which contained 42.64%, 44.29% and 40.97% of xylan, respectively. The hemicellulose was collected and subjected to enzymatic hydrolysis for production of XOS. EpXYN1 displayed the best enzymatic hydrolysis efficiency, and the total XOS (xylobiose to xylohexaose) yields on basis of xylan from the pretreated pith, rind and whole stem were 31.19%, 32.71% and 30.28%, respectively (Additional file 1: Table S3). The results suggested that the dissolved xylan during the NaOH-ethanol pretreatment process can also be utilized for XOS production.

Conclusions

In this study, NaOH-ethanol pretreatment was used to treat different parts of the sorghum (pith and rind) to enhance the release of pCA and the recovery of total reducing sugars. The pith and rind require different pretreatment conditions due to structure chemical composition differences. Under optimized conditions, the rind acquired higher pCA release and xylan recovery than the pith. Compared to NaOH pretreatment, higher release of pCA and recovery of hemicellulose were obtained in NaOH-ethanol pretreatment. The total reducing sugar yields was enhanced after NaOH-ethanol pretreatment and enzymatic hydrolysis because of the improvement of enzymatic hydrolysis and sugar polymers (glucan and xylan) recovery. All the results suggested that NaOH-ethanol pretreatment is a promise pretreatment method of sorghum biorefinery for product fermentable sugars and value-added products.

Materials And Methods

Materials

Sorghum stem was obtained from Hengshui, Hebei Province, China. The pith and rind were separated by manual peeling with a sharp knife and washed three times with tap water to remove field dirt. After drying at 65 °C, sorghum samples were ground and passed through 40-mesh sieve. Cellulase Cellic® CTec2 and β-glucosidase (Novozyme 188) were purchased from Novozymes China (Shanghai, China). Commercial xylanase (X2629-100g) was purchased from Sigma-Aldrich (Shanghai, China). The xylanase EpXYN1, EpXYN3 and XynII were prepared according to our previous researches [29, 30]. Other chemicals and reagents used in this study were analytical grade.

NaOH-ethanol pretreatment of different sorghum stem parts

The NaOH-ethanol pretreatment experiments were carried out with different temperature (60-70 °C) and time (1-4 h) in a plastic centrifuge. One gram of sorghum stem was suspended in 20 ml of mixed solution

of NaOH (0.5-2%, w/v) and ethanol (10-70%, w/v). Addition of $NaHSO_3$ (100 mg/L) prevents oxidation of the phenolic acid. The whole slurry was separated by vacuum filtration and washed by equal volume of water, then the liquids were combined. The treated solid was washed with water until the filtrate reached a neutral pH and then dried at 65 °C for the following experiments. The pH of NaOH-ethanol extracted liquid fraction was adjusted to 1 ~ 2 with HCl, and then 1.5 times the volume of absolute ethanol was added to precipitate hemicellulose and allowed to stand at 4 °C for 24 h. The precipitate was collected as extracted xylan by centrifugation and dried at 65 °C for the following experiments. The liquid fraction was rotary evaporated to recover ethanol, and then the *p*CA was analyzed by high-performance liquid chromatography (HPLC, Agilent 1260, USA).

The solid recovery, *p*CA release, xylan recovery, glucan recovery, and delignification were calculated according to the following equations, respectively:

Solid recovery yield (%) = 100 × regenerated residue / raw material

(1)

*P*CA release yield (%) = 100 × amount of released *p*CA after pretreatment / amount of *p*CA in raw material

(2)

Xylan recovery yield (%) = 100 × (0.88 × amount of xylose in the residue) / amount of xylan in raw material

(3)

Glucan recovery yield (%) =100 × (0.9 × amount of glucose in residue) / amount of glucan in raw material

(4)

Delignification yield (%) = 100 × (amount of lignin in raw material - amount of lignin in residue) / amount of lignin in raw material

(5)

Enzymatic hydrolysis

Enzymatic hydrolysis of solid residue by Cellic® CTec2 with or without xylanase

The pretreated residue was enzymatically hydrolyzed in a conical flask with phosphate buffer (pH 4.8), and the substrate was added at 2% (w/v). Sodium azide (10mM) was also added to the solution to

prevent microbial contamination. The enzymatic hydrolysis was conducted at 50 °C for 72 h in the presence of Cellic® CTec2 cellulase (15 FPU/g substrate), β-glucosidase (30 CBU/g substrate) with/without xylanase (15 BXU/g substrate) in a shaking incubator at 150 rpm.

The glucose enzymatic hydrolysis yield, xylose enzymatic hydrolysis yield, total glucose yield, and total xylose yield were calculated according to equations as follows:

Glucose enzymatic hydrolysis yield (%) = 100 × (0.9 × amount of released glucose after enzymatic hydrolysis) / amount of glucan in residue

(6)

Xylose enzymatic hydrolysis yield (%) = 100 × (0.88 × amount of released xylose after enzymatic hydrolysis) / amount of xylan in residue

(7)

Total glucose yield ($Total_{Glu.}$, %) = 100 × (0.9 × amount of glucose in residue × glucose hydrolysis yield) / amount of glucan in raw material

(8)

Total xylose yield ($Total_{Xyl.}$, %) = 100 × (0.88 × amount of xylose in residue × xylan hydrolysis yield) / amount of xylan in raw material

(9)

Enzymatic hydrolysis of extracted xylan

The enzymatic hydrolysis of extracted xylan from the liquid fraction of NaOH-ethanol pretreated samples was carried out in phosphate buffer with a final solid loading of 5% (w/v) and 20 BXU/g substrate of xylanase. Sodium azide (10mM) was added to the solution to prevent microbial contamination. The enzymatic hydrolysis process was incubated at optimum pH and optimum temperature for each enzyme (EpXYN1 and EpXYN3 at pH 4.8, 50 °C; XynII at pH 7.0, 60 °C) at 150 rpm in a shaking incubator for 24 h. The xylooligosaccharides (XOS) in the supernatant was analyzed using ion chromatography (Dionex ICS-3000, USA).

Experimental design and statistical analysis

The various process parameters involved in NaOH-ethanol pretreatment of different sorghum stem parts were evaluated using Box–Behnken design of Design Expert 11. The variables selected were NaOH loading (0.5-2%, w/v), ethanol content (10-70%, v/v), temperature (60-70 °C) and time (1-4 h). The

analytical responses were *p*CA release yield (%) and xylan recovery yield (%). A total of 29 runs were designed and 5 replicates were performed at the center point.

Analytical methods

The contents of esterified phenolic acid (*p*CA and ferulic acid) in the sorghum pith, rind and whole stem were analyzed according to the method described by Jiang et al. [20]. The structural components of glucan, xylan, arabinan and lignin of raw and pretreated samples were determined using the two-step acid hydrolysis method according to the National Renewable Energy Laboratory standard procedure [31]. Glucose, xylose and arabinose were determined using HPLC (Agilent 1100, USA) consisting of refractive index detector and a column of Bio-Rad Aminex HPX-87H column (300 mm×7.8 mm; USA). Column temperature was 55 °C using 5 mM H_2SO_4 as the mobile phase with flow rate of 0.6 mL/min. All experiments were performed in triplicate and the average data was reported.

Functional group changes of the raw material and the pretreated residue were examined by FTIR spectroscopy (Bruker VERTEX 80V, Germany). The sample was scanned in the range from 4000 cm^{-1} to 400 cm^{-1}. SEM was used to observe the microstructure of the samples before and after pretreatment. Sample photographs were taken at a magnification of 1000 using FEI Quanta 200 (USA). The crystal structure of the lignocellulosic materials was analyzed using XRD. X-ray diffractometer (Rigaku Ultima IV, Japan) uses Cu-Kα (k = 1.54 Å) as a radiation source. The scan range was 5° ~ 50° and the scan rate was 10°/min. The refractive index (CrI) of the crystalline sample was calculated from the XRD peak according to the following equation:

$$CrI = 100\% \times (I_{002} - I_{am}) / I_{002} \quad (10)$$

Where I_{002} is the intensity of the crystallinity peak at 2θ ≈ 22.5°, and I_{am} is the intensity of amorphous cellulose between lattice planes of 101 and 002 at 2θ of 18.7°.

Overall mass balance

The overall mass balance of each process stage was described as Fig. 5. First of all, 20 g of the each sample (sorghum pith, rind or whole stem) was separately pretreated by NaOH-ethanol solution (400 mL) as described in Fig. 5. The liquid fraction was separated from the solid fraction by vacuum filtration after cooling. Subsequently, the solid fraction was washed with water until neutral pH was achieved followed by drying at 65 °C to enzymatic hydrolysis. The pH of liquid fraction was adjusted to 1 ~ 2 with HCl, and 1.5 times the volume of absolute ethanol was added to the supernatant and stored at 4 °C for 24 h. Xylan precipitate was separated by centrifugation and dried for enzymatic hydrolysis, then ethanol was recovered using a rotary evaporator. Distilled water was added to the remaining liquid to replenish the

original volume, and centrifuge to remove lignin. Macroporous adsorption resins D101 was used to adsorb *p*CA in the supernatant and eluted with 70% (v/v) ethanol. *P*CA in the elute was detected by HPLC.

Abbreviations

*P*CA: *P*-coumaric acid; FTIR: Fourier transform infrared; SEM: Scanning electron microscopy; XRD: X-ray diffraction; HPLC: High-performance liquid chromatography; FPU: Filter paper units; CBU: Cellobiase units; BXU: Birch xylan units; XOS: xylooligosaccharides; CrI: Refractive index.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this manuscript and its Additional file.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DL researched and drafted the manuscript. LL provided some experimental materials and guided the experimental process. SD interpreted the experimental data and reviewed the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Composition of raw sorghum pith, rind and the whole stem.

Glucan (%)	Xylan (%)	Arabinan (%)	Lignin (%)	Ester linked *p*CA (%)	Ester linked ferulic acid (%)
37.20 ± 0.19	18.51 ± 0.10	2.54 ± 0.06	21.48 ± 0.23	2.21 ± 0.14	0.38 ± 0.07
40.64 ± 0.08	17.33 ± 0.13	2.32 ± 0.11	24.04 ± 0.27	2.08 ± 0.22	0.43 ± 0.04
38.22 ± 0.21	17.70 ± 0.07	2.47 ± 0.09	23.35 ± 0.31	2.13 ± 0.17	0.41 ± 0.09

Table 2 Factors (NaOH loading, ethanol content, temperature, and time) and the responses (released *p*CA and recovered xylan in residues) of the Box–Behnken design used for response surface methodology.

			Response					
ıol (%)	Temperature (°C)	Time (h)	Released *p*CA (%)			Recovered xylan in residues (%)		
			Pith	Rind	Whole	Pith	Rind	Whole
10	70	2.5	66.23	67.64	67.57	79.78	74.35	75.21
70	65	1	83.99	83.74	83.82	76.06	86.71	84.69
40	65	2.5	79.66	85.56	81.68	80.42	82.21	80.28
10	65	2.5	85.05	88.45	88.29	63.95	72.78	62.18
40	60	4	79.22	86.40	79.70	73.22	81.10	82.49
10	65	4	84.90	87.36	87.19	65.39	77.34	77.80
70	60	2.5	75.00	82.14	78.72	81.84	84.69	79.63
40	65	1	85.44	88.31	87.60	65.11	80.53	70.17
40	70	2.5	45.47	25.97	45.02	79.98	82.44	81.01
40	65	4	32.36	31.59	33.02	89.17	82.95	80.13
40	65	2.5	80.27	84.54	82.89	81.03	82.07	81.02
70	65	2.5	36.49	21.49	32.92	88.92	85.44	88.17
40	60	2.5	86.89	92.30	90.52	64.88	79.12	68.76
70	65	4	84.15	86.52	85.85	83.71	82.64	81.43
10	65	2.5	33.08	32.00	34.48	82.73	85.31	73.70
70	70	2.5	90.14	91.14	90.73	79.94	86.96	87.58
40	65	2.5	78.96	84.25	82.47	80.76	83.11	79.87
40	65	1	31.53	19.42	29.79	85.12	89.10	83.22
40	70	2.5	87.14	93.68	90.17	64.70	74.80	70.11
70	65	2.5	94.97	98.07	98.44	67.98	84.89	68.77
40	65	2.5	79.28	87.44	80.13	79.82	82.89	80.05
10	60	2.5	57.88	56.28	57.51	70.35	75.86	75.80
40	60	1	67.08	66.70	71.29	80.95	83.15	78.85
40	60	2.5	28.14	22.31	28.02	84.60	83.07	86.24
10	65	1	53.48	52.89	55.36	77.08	73.69	74.36
40	65	2.5	81.77	85.79	83.11	79.91	82.58	80.74
40	70	4	84.80	91.44	90.94	74.72	79.11	76.34
40	70	1	77.19	79.06	78.81	72.52	82.80	79.47
40	65	4	97.12	95.41	95.30	65.36	74.10	65.80

Table 3 Confirmation of the predicted optimum condition with the experimental results.

	NaOH (%)	Ethanol (%)	Temperature (°C)	Time (h)	Released *p*CA (%)		Recovered xylan in residues (%)	
					Predicted values	Actual values	Predicted values	Actual values
Pith	1.63	70	66.0	3.18	94.59	94.07	77.28	76.85
	1.72	70	63.4	2.29	94.00	94.22	76.20	76.01
	1.51	70	65.2	3.30	92.63	91.76	77.69	76.32
Rind	1.90	70	69.8	1.00	98.99	97.24	88.73	88.46
	1.80	70	62.1	1.50	97.28	97.05	87.56	86.47
	1.55	70	68.8	1.95	96.00	94.74	86.79	84.99
Whole Stem	1.46	70	70.0	2.19	95.13	95.05	85.35	85.04
	1.52	70	70.0	2.00	95.90	94.77	83.68	82.96
	1.39	70	70.0	3.00	92.07	92.31	82.85	82.12

Table 4 The release of *p*CA during pretreatment, the recovery of the solid residue and the content of each component in solid residues. NaOH-ethanol pretreatment was performed under the following conditions: 1.63% NaOH, 70% ethanol, 66 °C, 3.18 h; 1.90% NaOH, 70% ethanol, 69.8 °C, 1.00 h and 1.46% NaOH, 70% ethanol, 70 °C, 2.19 h for the pith, rind and whole stem, respectively. NaOH pretreatment was performed under the same conditions, except that 70% ethanol was not included.

	Materials	Solid recovery (%)	Released *p*CA (%)	Composition			
				Glucan (%)	Xylan (%)	Arabinan (%)	Lignin (%)
NaOH-ethanol	Pith	59.48 ± 0.54	94.07 ± 1.34	56.15 ± 0.72	23.90 ± 0.27	2.95 ± 0.14	5.40 ± 0.16
	Rind	75.85 ± 0.71	97.24 ± 2.11	50.82 ± 0.17	20.21 ± 0.93	2.45 ± 0.07	10.81 ± 0.09
	Whole stem	68.52 ± 0.59	95.05 ± 1.57	51.98 ± 1.04	21.96 ± 0.12	2.73 ± 0.20	6.32 ± 0.21
NaOH	Pith	55.74 ± 1.16	86.97 ± 0.79	54.10 ± 0.35	17.26 ± 1.12	2.64 ± 0.23	7.49 ± 1.06
	Rind	73.42 ± 0.78	89.72 ± 1.96	48.63 ± 1.86	18.14 ± 0.89	2.12 ± 0.11	13.39 ± 0.95
	Whole stem	64.34 ± 0.94	87.69 ± 1.63	51.60 ± 1.03	17.21 ± 0.67	2.34 ± 0.18	9.67 ± 0.64

Figures

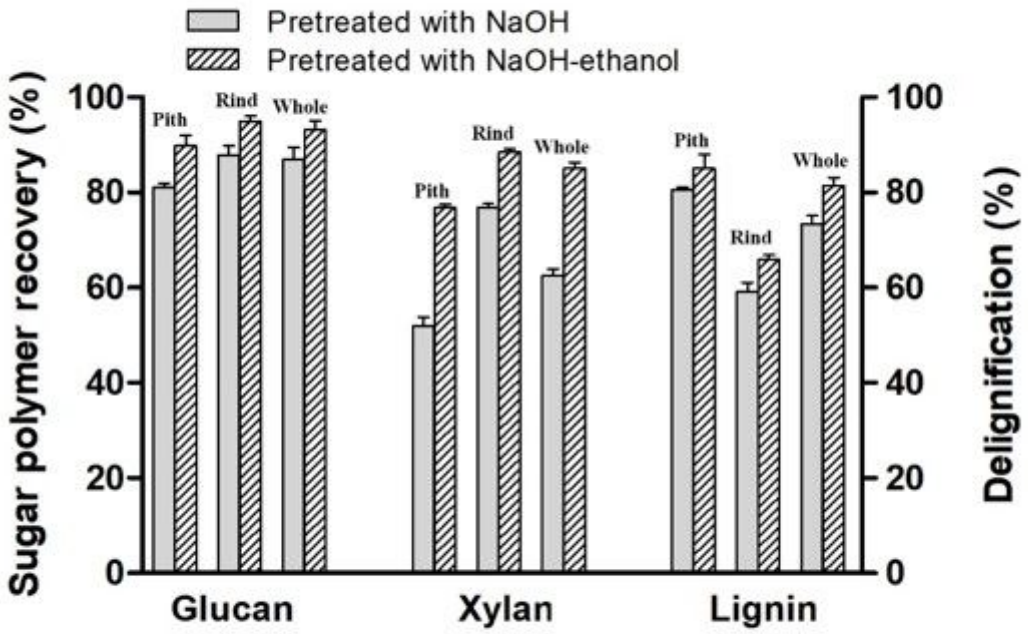

Figure 1

Sugar recovery (glucan and xylan) and lignin removal after pretreatment. NaOH-ethanol pretreatment was performed under the following conditions: 1.63% NaOH, 70% ethanol, 66 °C, 3.18 h; 1.90% NaOH, 70% ethanol, 69.8 °C, 1.00 h; and 1.46% NaOH, 70% ethanol, 70 °C, 2.19 h for pith, rind and whole stem, respectively. NaOH pretreatment was performed under the same conditions, except that 70% ethanol was not included.

Fig. 2

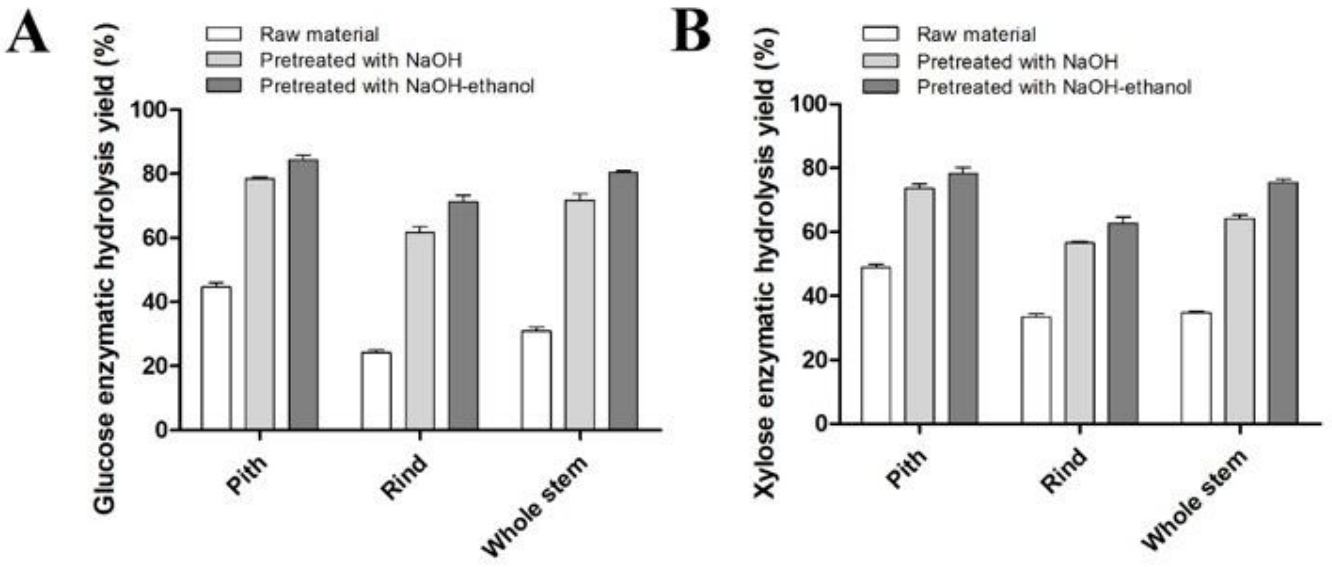

Figure 2

Glucose (A) and xylose (B) enzymatic hydrolysis yields of raw and pretreated materials. The sample (2%, w/v) was hydrolyzed at 50 °C for 72 h using Cellic® CTec2 (15 FPU/g substrate) and β-glucosidase (30 CBU/g substrate).

Fig. 3

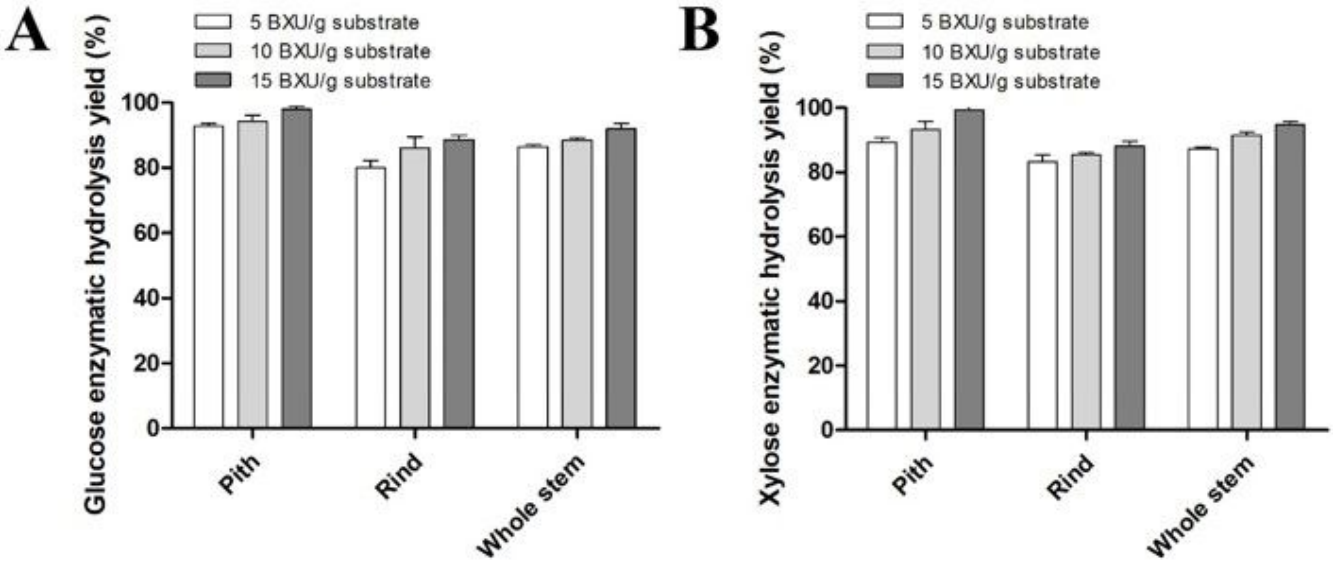

Figure 3

Effect of different dosages of adding xylanase on enzymatic hydrolysis of NaOH-ethanol pretreated materials. The sample (2%, w/v) was hydrolyzed at 50 °C for 72 h using cellulose cocktail (15 FPU/g substrate Cellic® CTec2, 30 CBU/g substrate β-glucosidase and 5-15 BXU/g substrate xylanase).

Fig. 4

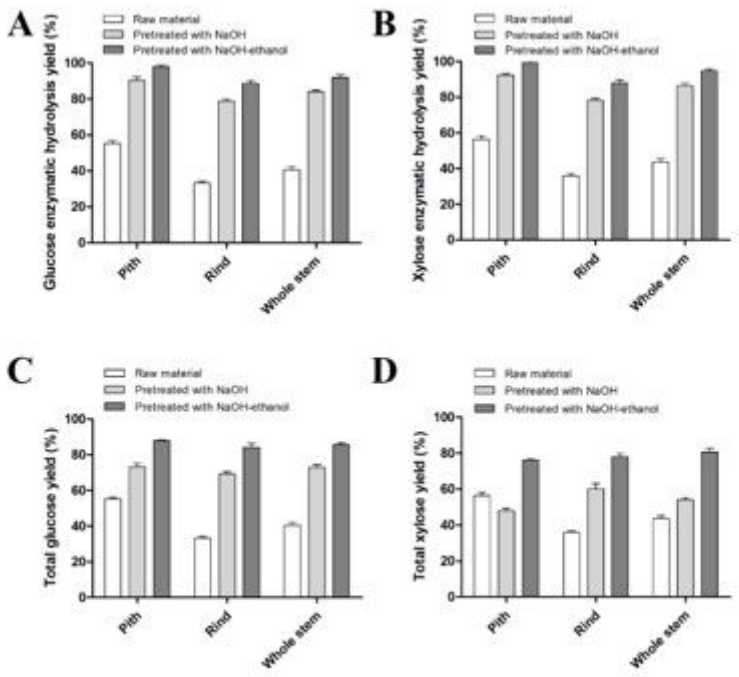

Figure 4

Comparison of enzymatic hydrolysis yields (glucose (A) and xylose (B)) and total yields of sugar (glucose (C) and xylose (D)) of raw, NaOH pretreated and NaOH-ethanol pretreated materials with adding xylanase. The sample (2%, w/v) was hydrolyzed at 50 °C for 72 h using cellulose cocktail (15 FPU/g substrate Cellic® CTec2, 30 CBU/g substrate β-glucosidase and 15 BXU/g substrate xylanase).

Fig. 5

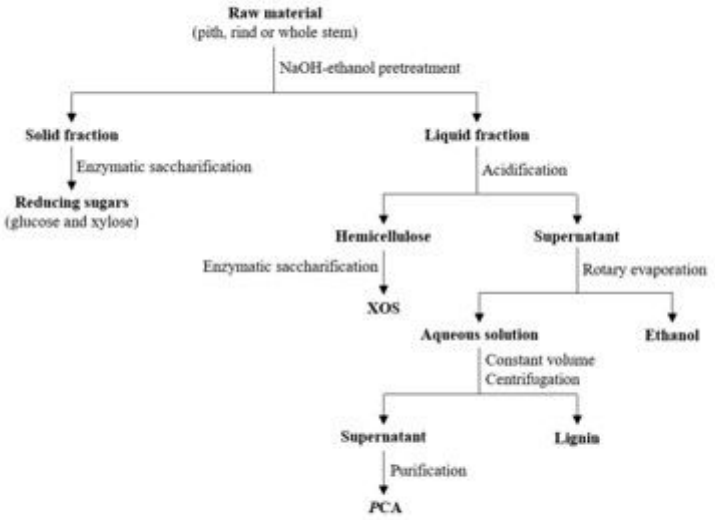

Figure 5

Overall mass balance for the process of NaOH-ethanol pretreatment and enzymatic saccharification. The overall mass balance was investigated with 20 g of the each raw material (pith, rind or whole stem). The conditions of pretreatment were as follows: 1.63% NaOH, 70% ethanol, 66 °C, 3.18 h; 1.90% NaOH, 70% ethanol, 69.8 °C, 1.00 h, and 1.46% NaOH, 70% ethanol, 70 °C, 2.19 h for pith, rind and whole stem, respectively.

Supplementary Files

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