



Mild O₂-aided alkaline pretreatment effectively improves fractionated efficiency and enzymatic digestibility of Napier grass stem towards a sustainable biorefinery

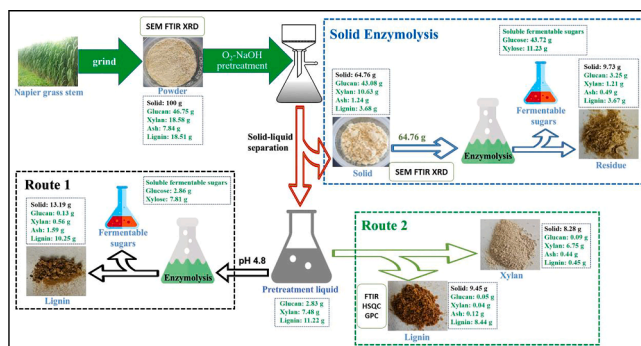
Weipeng Song, Lincai Peng*, Danish Bakhshyar, Liang He, Junhua Zhang

BiomassChem Group, Faculty of Chemical Engineering, Kunming University of Science and Technology, Kunming 650500, Yunnan, China

HIGHLIGHTS

- A mild and reliable O₂-aided alkaline pretreatment of Napier grass stem was designed.
- O₂-NaOH pretreatment improves fractionated efficiency and enzymatic digestibility.
- Pretreatment proceeded at low temperature of 80 °C and dilute NaOH solution of 1%.
- High glucan retention (92%) and lignin removal (over 80%) maximized saccharification.
- Isolated lignin had a well-preserved structure with great potential for valorization.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
 Napier grass
 Pretreatment
 Fractionation
 Enzymatic digestibility
 Lignin

ABSTRACT

Napier grass is a promising energy source on account of its strong adaptability and high productivity. Herein, an O₂-aided alkaline pretreatment with mild operating conditions was developed to modify Napier grass stem structure for improving its fractionated efficiency and enzymatic digestibility. Compared with the conventional alkaline pretreatment, it could be proceeded at lower temperature (80 °C) and dilute NaOH solution (1%) to remove over 80% lignin and retain 92% cellulose. The recovered lignin possessed typical structures of native lignin and well-preserved molecular weight, anticipating feasible potential in preparation of biomaterials or aromatic chemicals. Coupled with the enzymatic hydrolysis managements of solid remain and hydrolysate after the pretreatment, the recovery yields of glucose and xylose based on the raw material feeds reached 89.7% and 90.2%, respectively. This contribution demonstrates a highly-reliable strategy to fractionate Napier grass stem for maximizing fermentation sugar production and valorizing lignin toward sustainable biorefinery processes.

1. Introduction

Growing demand of global energy and environmental concerns have

led increased efforts to seek renewable sources to replace consumptive fossil resources. Using abundant and inedible lignocellulosic biomass as the feedstock for sustainable production of fuels and chemicals

* Corresponding author.

E-mail address: penglincai@kust.edu.cn (L. Peng).

<https://doi.org/10.1016/j.biortech.2020.124162>

Received 15 August 2020; Received in revised form 17 September 2020; Accepted 18 September 2020

Available online 24 September 2020

0960-8524/© 2020 Elsevier Ltd. All rights reserved.

represents one promising alternative strategy (Chandel et al., 2018). Napier grass (*Pennisetum purpureum*), a perennial grass family crop, is widely distributed in tropical and subtropical regions as well as adaptable to grow in southern China. It has great potential as an energy source by reason of its fast growth (ability to be harvested up to four times per year), high biomass productivity (25–40 tons dry matter per hectare annually), minimal management with low nutrient inputs, and strong adaptability to the environment (Takara and Khanal, 2015). The leaf of Napier grass is suitable for use as animal fodder because of its abundant protein content and softness. By contrast, the highly lignified stem is underutilized (Perez-Boada et al., 2014; Phitsuwan et al., 2016). Napier grass stem has recently attracted considerable interest as a potential lignocellulosic feedstock for biofuel production (e.g., ethanol, butanol, and methane) due to its high carbohydrate content of over 60% (Takara and Khanal, 2015; He et al., 2017; Reddy et al., 2018; Narinthorn et al., 2019). However, lignocellulosic recalcitrance remains a key challenge towards the enzyme-based biorefinery industry. The efficient pretreatment capable of deconstructing the recalcitrance of lignocellulosic material is considered as a foundational step to accomplish valorization based on the above-pursued goal (Kumari and Singh, 2018; Sankaran et al., 2020).

Over the past years, diverse pretreatment techniques dealing with lignocellulosic biomass have been developed (e.g., physical, biological, acid, alkaline, organic and deep eutectic solvents, and irradiation-linked pretreatments), which have successfully employed to improve fractionated efficiency and enzymatic digestibility of the raw materials (Sankaran et al., 2020; Xu et al., 2020; Yoo et al., 2020). Among them, alkaline pretreatment has triggered massive investigation in previous literatures (Singh et al., 2015; Kim et al., 2016), and proposed as one of highly attractive alternatives because the process can be conducted under industrially relevant conditions similar to those for fiber pulping (Pakkanen and Alen, 2013; da Silva Perez and Van Heiningen, 2015). During the alkaline pretreatment, the alkali-labile linkages in lignin-carbohydrate complex are readily broken down to disrupt cell wall by solubilizing hemicelluloses and lignin, thus leading to the exposure of cellulose to enzymes. Phitsuwan et al. (2016) evaluated four common alkaline pretreatments on Napier grass stem involving NaOH, Ca(OH)₂, NH₃, and alkaline H₂O₂. NaOH pretreatment was found to be significantly superior to other three investigated alkaline pretreatments, judging by the lignin removal and enzymatic digestibility. However, 70.8% of glucan recovery in the solid remain after NaOH pretreatment is non-dominant, and the reaction can be accomplished with the requirement of a relatively high temperature (121 °C). Tsai et al. (2018) attempted to use a concentrated NaOH solution (10%) for Napier grass stem pretreatment at 90 °C resulting in a 63.0% delignification and only 62.1% glucan recovery, and a moderate glucose yield of 75.4% was obtained from enzymatic hydrolysis of the pretreated substrate. Accordingly, development of a mild alkali-related pretreatment process to improve fractionated efficiency (i.e., high lignin removal and intact glucan retention) and enzymatic digestibility of Napier grass stem is highly desirable towards a sustainable biorefinery.

Oxygen delignification in alkaline solution has been proved to be feasible and rewarding for non-wood fiber pulping based on economic, environmental, and energy consumption considerations (Salmela et al., 2008; Yue et al., 2016). Inspired by this, Lin's group developed a cooking process with active oxygen and solid alkali (MgO) for clean pulping and biomass pretreatment at around 160 °C, which displayed a high delignification rate (85–95%) without obvious degradation of cellulose for cornstalk and bagasse. The obtained cellulose-rich material is suitable for producing fermentable sugars and other high-value chemicals (Pang et al., 2012; Xie et al., 2013; Jiang et al., 2017). When NaOH was used instead of MgO, bagasse pretreatment can be conducted at lower temperatures to obtain a comparable delignification rate and enzymatic hydrolysis yield (Bi et al., 2016). Very recently, Wang's group proposed a two-step process involving liquid hot water and O₂-NaOH (or O₂-Na₂CO₃) pretreatments on reed, which showed

benefits in terms of xylan recovery, lignin removal, and enzymatic digestibility (Xia et al., 2020; Lu et al., 2020). These positive findings portend that the accession of excess O₂ will likely be helpful to develop a mild and reliable pretreatment process for Napier grass stem valorization.

In this contribution, we report, for the first time, the O₂-aided alkaline pretreatment of Napier grass stem that allows the reaction to proceed under mild conditions (i.e., 80 °C and 1% NaOH) with a high fractionated efficiency for maximizing fermentation sugar production and valorizing lignin. Chemical composition of the solid remain after the pretreatment, structural characterization of the solid remain and isolated lignin, and enzymatic digestibility of the solid remain and pretreated hydrolysate were systematically investigated for evaluating the effectiveness of O₂-NaOH pretreatment and explaining its fractionated mechanism. The exposed results were also well compared with sole NaOH pretreatment to better understand the superiority of molecular O₂ existence.

2. Materials and methods

2.1. Materials

Napier grass was harvested from Yunnan Province, China. Leaf was firstly removed from Napier grass. The remaining stem was submitted to drying process in oven 105 °C for 24 h, then milled and collected 40–60 mesh powder with a classifying sieve for use in this work. The chemical composition of Napier grass stem based on a dry weight was glucan 46.75%, xylan 18.58%, lignin 18.51% and ash 7.84%. Commercial cellulase (Celluclast 1.5 L, from *Trichoderma reesei* ATCC 26921) and β-glucosidase (Novozyme 188, from *Aspergillus niger*) were supplied by Novozymes Corporation (Denmark).

2.2. O₂-NaOH pretreatment of Napier grass stem

The pretreatments of Napier grass stem were carried out in a 100 mL stainless reactor with an oil-bath heating and magnetic agitation. In a typical run for O₂-NaOH pretreatment, 3 g of dry raw material (solid/liquid mass ratio, 1:20), a given amount of NaOH, and 60 mL H₂O were charged into the reactor, and then O₂ was admitted into the sealed reactor. The reactor was brought to the desired temperature by external heating and continuously stirred at 400 rpm for the pretreatment. Effect of O₂ pressure, NaOH dosage, temperature, and time were evaluated. In contrast, the NaOH pretreatments were performed by varying NaOH dosage, temperature, and time. A detailed experimental design was presented in Table 1. After pretreatment, the solid remain was separated from pretreatment liquor by filtration, and then washed with deionized water until the supernatant filtrate reached neutral and dried in an oven at 105 °C. The pretreated hydrolysate was collected for further analysis.

2.3. Separation of xylan and lignin from pretreated hydrolysate

For the isolation of xylan, an ethanol precipitation method was used as a previous reported (Choi et al., 2019). Briefly, three times volume of dehydrated ethanol was added to one volume of the pretreated hydrolysate. The formed mixture was centrifuged at 8000 rpm for 10 min to obtain precipitation. Then, the precipitation was washed with ethanol several times to remove the residual NaOH until the pH value of solution was neutral, finally to obtain xylan.

Lignin in the filtrate was firstly precipitated by adjusting pH to 2 with H₂SO₄. The isolated crude lignin dissolved in 90% (w/w) acetic acid, and the soluble fraction was slowly introduced into deionized water to precipitate. Afterward, the precipitate was washed with deionized water (pH = 2, adjusted with 2 mol/L H₂SO₄) until the odor of acetic acid disappeared, and then was freeze-dried to obtain the resulting lignin (Qian et al., 2019). The isolated lignins from the O₂-NaOH pretreatment and NaOH pretreatment were defined as OAL and AL, respectively.

Table 1
Experimental design for O₂-NaOH and NaOH pretreatments of Napier grass stem.

Entry	Pretreatment method	O ₂ pressure, MPa	NaOH dosage, %	Temperature, °C	Time, min	
1	O ₂ -NaOH pretreatment	0.1	1.0	80	60	
2		0.2				
3		0.3				
4		0.4				
5		0.5				
6		0.5	0	80	60	
7			0.5			
8			1.0			
9			1.5			
10			2.0			
11	NaOH pretreatment	0.5	1.0	50	60	
12				60		
13				70		
14				80		
15				90		
16		0.5	1.0	80	30	
17					60	
18					90	
19					120	
20					150	
21				1.0	80	60
22				1.5		
23				2.0		
24				2.5		
25				3.0		
26				3.5		
27			1.0	90	60	
28				100		
29				110		
30				120		
31				130		
32			1.0	80	90	
33					120	
34					150	
35					180	
36					210	

2.4. Structural characterization of pretreated Napier grass stem and isolated lignin

The Napier grass stem samples before and after pretreatment were characterized by scanning electron microscopy (SEM), attenuated total reflectance infrared spectroscopy (ATR-FTIR), and X-ray diffraction (XRD). The structural characterization of isolated lignin was determined using ATR-FTIR, two dimensional heteronuclear single quantum coherence nuclear magnetic resonance (2D HSQC NMR), and gel permeation chromatography (GPC). SEM images were visualized using a Tescan Vega 3 instrument operated at 20 kV. ATR-FTIR spectra were recorded on a Nicolet Magna 750 FTIR spectrophotometer using a diamond ATR accessory, and data were collected from 4000⁻¹ and 500 cm⁻¹ at a resolution of 0.5 cm⁻¹. XRD patterns were obtained with a Rigaku D/MAX 2200 diffractometer using Cu Ka ($\lambda = 0.154$ nm) radiation source operated at 40 kV and 100 mA. Crystallinity index (CrI) was calculated according to the method described by Segal et al. (1959). 2D HSQC NMR spectra were acquired on a Bruker 600 MHz spectrometer fitted with a cryogenically cooled 5 mm TCI gradient probe with inverse geometry (proton coils closest to the sample), and Bruker's Topspin 3.1 (Mac) software was used for spectral processing. Molecular weight distribution of the acetylated lignins was analyzed using an Agilent 1100 GPC equipped with an ultraviolet detector at 254 nm on a PL-gel mixed-E column (300 mm \times 7.5 mm).

2.5. Enzymatic hydrolysis

The pretreated Napier grass stem samples (2 wt% substrate) were subjected to enzymatic hydrolysis at cellulase and β -glucosidase loadings of 25 FPU/g and 15 IU/g substrate in sodium phosphate buffer (0.05 mol/L, pH 4.8). The formed mixture was incubated at 50 °C and 180 rpm in a 100 mL Erlenmeyer flasks with a 50 mL work volume. Samples of 0.2 mL were periodically withdrawn during hydrolysis for the determination of glucose and xylose. In addition, the enzymatic hydrolysis of soluble glucan and xylan in the pretreated hydrolysate was performed as the above described method. The difference was that 12% H₂SO₄ was used to adjust the pH value of the pretreatment liquid from 13.1 to 4.8.

2.6. Chemical composition analysis

The chemical composition of Napier grass stem and pretreated samples were determined according to the National Renewable Laboratory Analytical Procedure using the standard two steps of acid hydrolysis (Sluiter et al., 2008). Quantitative analyses of monomeric sugars were performed on a Shimadzu high-performance liquid chromatography (HPLC-10AVP) equipped with a refractive index detector and a Copsil NH₂ column (250 mm \times 4.6 mm). A solution of deionized water and acetonitrile at the volume ratio of 3:7 was used as the mobile phase with a flow rate of 1.0 mL min⁻¹. Each experiment was performed at least twice, and the average values were adopted.

Glucan recovery, xylan removal, and lignin removal after pretreatment were calculated as follows:

$$\text{Glucan recovery} = (\text{glucan content in pretreated sample} \times \text{solid remain}) / \text{glucan content in raw material}$$

$$\text{Xylan removal} = (\text{xylan content in raw material} - \text{xylan content in pretreated sample} \times \text{solid remain}) / \text{xylan content in raw material}$$

$$\text{Lignin removal} = (\text{lignin content in raw material} - \text{lignin content in pretreated sample} \times \text{solid remain}) / \text{lignin content in raw material}$$

Yields of glucose and xylose after enzymatic hydrolysis were defined as follows:

$$\text{Glucose yield} = \text{glucose amount in hydrolysate} / \text{potential amount of glucose loaded in the feed}$$

$$\text{Xylose yield} = \text{xylose amount in hydrolysate} / \text{potential amount of xylose loaded in the feed}$$

3. Results and discussion

3.1. Efficiency comparison of O₂-NaOH pretreatment and NaOH pretreatment

Napier grass stem pretreated by combining O₂ and NaOH was systematically investigated under various operating conditions. Fig. 1 presents the results of chemical composition (glucan, xylan, and lignin) of pretreated Napier grass stem samples and pretreatment efficiency (glucan recovery, xylan removal, and lignin removal). As can be seen from Fig. 1a, the coexistence of O₂ played a positive role in the delignification process, and the removal of lignin showed a rising trend gradually with the increase of O₂ pressure. A high lignin removal of 80.1% could be accomplished after O₂-NaOH pretreatment in the presence of 0.5 MPa O₂, whereas only 54.7% lignin was removed with a sole NaOH pretreatment. Under O₂-aided alkali environments, the covalent bonding between the sugar group and lignin molecule may be easier to break based on the oxidization of benzene ring and side chain of lignin to increase its hydrophilicity, thus resulting in more lignin dissolution (Wu et al., 2019). In addition to this, O₂-NaOH pretreatment led to higher glucan recovery in the solid remain, which was increased from 83.3% to 92.1% after introducing 0.5 MPa O₂ into NaOH system. This efficiency has an integrated interest in the subsequent enzymatic saccharification for the accumulation of glucose. The improved protection of glucan-based solid fractions under O₂ atmosphere can be attributed to the

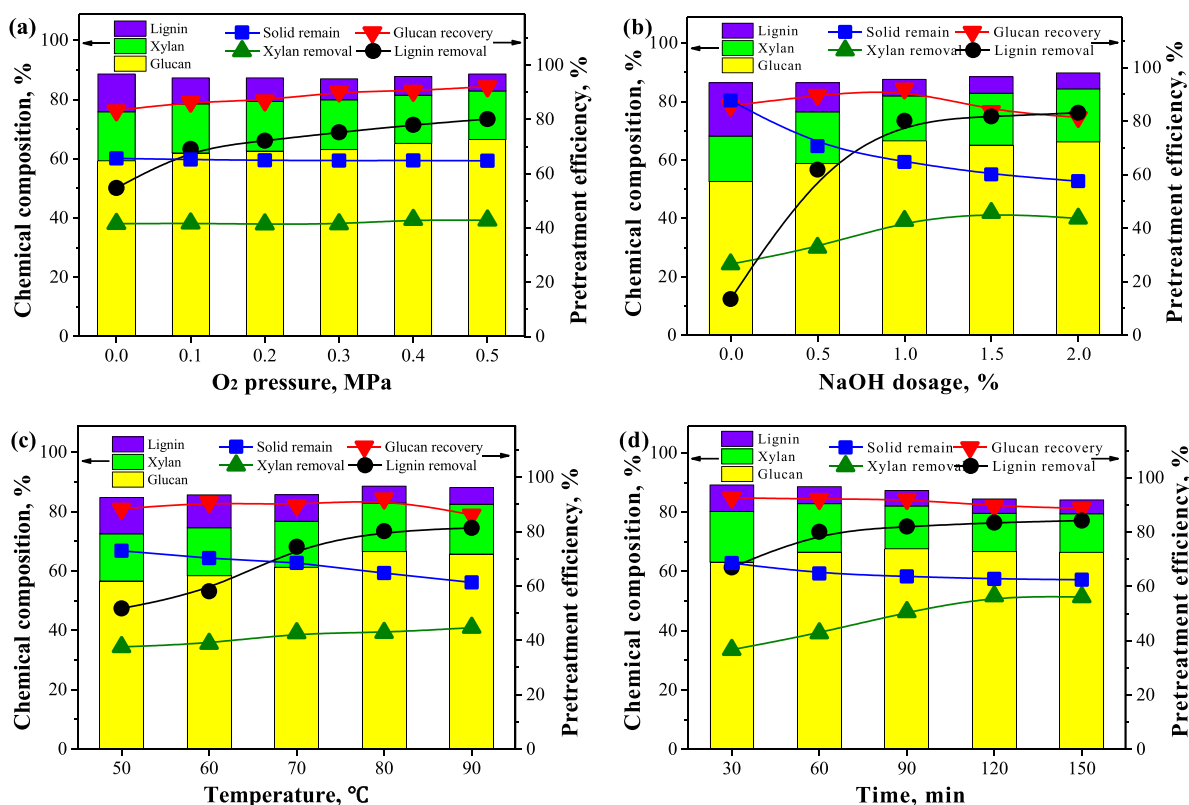


Fig. 1. Chemical composition and pretreatment efficiency of Napier grass stem with O₂-NaOH pretreatment. (a) Effect of O₂ pressure (1% NaOH, 80 °C, 60 min); (b) Effect of NaOH dosage (0.5 MPa O₂, 80 °C, 60 min); (c) Effect of temperature (1% NaOH, 0.5 MPa O₂, 60 min); (d) Effect of time (1% NaOH, 0.5 MPa O₂, 80 °C).

oxidization of terminal reductive groups in cellulose chain decreasing its alkaline peeling reactions. The above findings suggest that the coexistence of O₂ can effectively improve the selectivity of fractionated components to realize highly separation of lignin and cellulose, thus affording a favorable precondition in the biorefinery process of Napier grass stem.

The effects of other pretreatment variables including NaOH dosage, temperature, and time were further investigated using O₂-aided NaOH system. The pretreatment efficiency was highly sensitive to NaOH dosage (Fig. 1b). With the increase of NaOH dosage, the removal of lignin and xylan both increased first, and then maintained nearly constant. However, there was a decline trend in glucan recovery as NaOH dosage rose above 1%, signifying that the glucan tends to be hydrolyzed into water-soluble fragments. Temperature and time for O₂-NaOH pretreatment showed a positive impact on the removal of lignin and xylan (Fig. 1c–d). By contrast, lignin removal was more sensitive to pretreatment temperature, while the extension of time was more conducive to the degradation of xylan. Considering the cost, energy consumption, delignification rate, and glucan retention, a mild O₂-aided alkaline pretreatment using a combination of 0.5 MPa O₂ and 1% NaOH at 80 °C for 60 min can be competent to fractionate Napier grass stem towards an enzyme-based lignocellulosic biorefinery process. Based on it, a scale-up test in 15 L reactor was conducted for Napier grass stem pretreatment.

Table 2 shows that the results in relation to solid remain, glucan recovery, and removal of xylan and lignin were in highly agreement with the results using the 100 mL reactor, reflecting the potential of O₂-NaOH pretreatment for practical implementation.

To better understand the superiority of O₂-NaOH pretreatment, the experiments were also carried out using sole NaOH pretreatment by intensifying process conditions to improve fractionated efficiency of Napier grass stem. Indeed, increased NaOH dosage and pretreatment temperature can both effectively promote the removal of lignin (Fig. 2a–b). In contrast, pretreatment time had a limited effect on facilitating the fractionated efficiency, and only 63.2% lignin was removed after 210 min (Fig. 2c). An improved delignification rate of 77.7% can be implemented at elevated temperature of 130 °C, which is close to that of the O₂-NaOH pretreatment (80.1%). However, it must be pointed out that the degradation of glucan gradually increased with the intensification of pretreatment conditions, and only 80% intact glucan could be retained in the solid remain. Besides, the cost relying on high NaOH loading or elevated temperature to achieve comparative delignification rate becomes higher than the usage of pressured O₂. Nevertheless, considering that O₂-aided alkaline pretreatment could result in higher lignin removal and glucan retention under mild conditions, and thereby increase feedstock utilization and processing efficiency, which may afford cost advantages over the traditional method.

Table 2
Scale-up test for the O₂-NaOH pretreatment of Napier grass stem.

Reactor	Solid remain, %	Glucan, %		Xylan, %		Lignin, %	
		Content	Recovery	Content	Removal	Content	Removal
15 L ^a	63.2	67.4	91.1	17.5	40.4	6.2	78.7
100 mL ^b	64.8	66.5	92.1	16.4	42.8	5.7	80.1

^a 0.6 kg raw material, 12 L H₂O, 1% NaOH, 0.5 MPa O₂, 80 °C, 60 min.

^b 3 g raw material, 60 mL H₂O, 1% NaOH, 0.5 MPa O₂, 80 °C, 60 min.

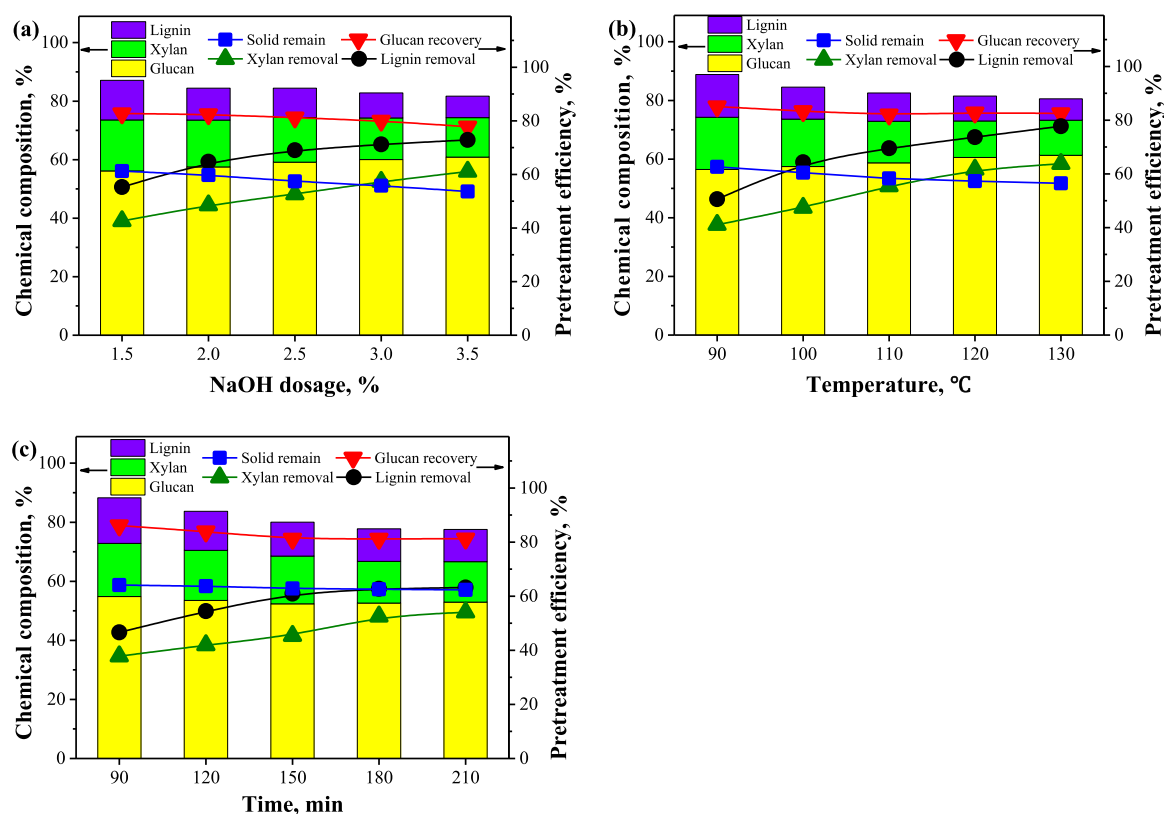


Fig. 2. Chemical composition and pretreatment efficiency of Napier grass stem with NaOH pretreatment. (a) Effect of NaOH dosage (80 °C, 60 min); (b) Effect of temperature (1% NaOH, 60 min); (c) Effect of time (1% NaOH, 80 °C).

The mild O₂-aided alkaline pretreatment can serve as a reliable and promising fractionated strategy for the development of enzyme-based biorefinery process from Napier grass stem.

3.2. Structural characterization of pretreated Napier grass stem

To gain a visual insight into the pretreatment efficiency, the structural changes of Napier grass stem with different pretreatment methods were characterized by SEM, ATR-FTIR, and XRD techniques, respectively.

The SEM images of Napier grass stem before and after pretreatment are presented in [Supplemental Material](#). After 1% NaOH pretreatment, the dense and rigid surface of Napier grass stem was partially destroyed, and a series of folds appeared with an uneven surface topography. O₂-aided NaOH pretreatment gave rise to fragmentary structures with some cracks. With the increase of O₂ pressure from 0.1 MPa to 0.5 MPa, the surface of fibers became tattered with a loose structure, resulting in the exposure of internal layer of fibers. Only increasing NaOH dosage to 3.5% in the absence of O₂, rugged and fluffy surfaces can be appeared after pretreatment, but few internal structure of fibers was exposed ([Satari et al., 2019](#)). Elevating temperature to 130 °C without O₂ atmosphere could improve the fibers swelling and softening, whereas the inherent structure of fibers had not fundamentally destroyed after pretreatment. These discrepant ultrastructural features indicate that the deconstruction of Napier grass stem with O₂-NaOH pretreatment is more significant than that with sole NaOH pretreatment. The exposure of internal layer fibers with a loose structure increases the contact area and accessibility of enzyme to the substrate, which has identified as one of key factors to improve the enzymatic digestibility of lignocellulosic biomass ([Alam et al., 2019](#)).

The variations of surface chemical functionality of Napier grass stem after pretreatment were analyzed by ATR-FTIR spectra (See [Supplemental Material](#)). The stretching vibration of O–H and C–H groups at

3348 cm⁻¹ and 2899 cm⁻¹ was related with the hydrogen bonding and aliphatic moieties in cellulose. The intensities of the two peaks after O₂-NaOH pretreatment were weaker than those after sole NaOH pretreatment, meaning that the presence of O₂ facilitates the disruption of intramolecular hydrogen bond and crystalline structure of cellulose ([Xie et al., 2013](#); [Chen et al., 2020](#)). The absorption peaks at 1731 cm⁻¹ and 1253 cm⁻¹ were disappeared or significantly weakened after all tested pretreatments, probably attributing to the cleavage of ester group and ether linkages from hemicellulose and lignin ([Wang et al., 2019](#); [Zhong et al., 2020](#)). The absorption signals at 1604 cm⁻¹ and 1512 cm⁻¹ associated to the aromatic skeletal vibrations in lignin decreased after pretreatment ([Cheng et al., 2018](#)). Moreover, the intensities of these two peaks with O₂-NaOH pretreatment or harsh pretreatment conditions (e. g., 3.5% NaOH dosage, and 130 °C) showed weaker than those with 1% NaOH pretreatment at 80 °C, which are consistent with certain amounts of lignin removal in the chemical composition analysis (Figs. 1–2). The signals at 1320 cm⁻¹ (representing the lignin containing syringyl and guaiacyl) and 833 cm⁻¹ (possibly associated with the vibration of syringyl C–H bond in lignin) were weakened or disappeared after pretreatment ([Harrison et al., 2013](#)). The absorption peaks at 1164 cm⁻¹, 1054 cm⁻¹, and 897 cm⁻¹ are contributed by C–O–C, C–O, and C–H stretches in cellulose and hemicellulose ([Dias et al., 2020](#)). The peak intensity especially at 897 cm⁻¹ markedly strengthened after pretreatment, which was resulted from the removal of most lignin in Napier grass stem to form carbohydrate-rich solid residue.

The crystallinity of cellulose has highly impact on the enzymatic digestibility. XRD patterns in [Supplemental Material](#) show that the key crystalline peaks of all samples were assigned to representative cellulose I. The intensity of the peaks and crystallinity index (CrI) have both increased after pretreatment due to the removal of amorphous components such as hemicellulose and lignin. It is worth noting that the removal of lignin with O₂-NaOH pretreatment (80.1%) was apparently higher than that with sole NaOH pretreatment (54.7%) under identical

operating conditions, but the CrI for O₂-NaOH pretreatment was lower. This indicates that the coexistence of O₂ available promotes the breakage of hydrogen bonds in cellulose chains to destroy its crystalline region, thus resulting in the reduction of CrI. This observation is in line with the results of FTIR analysis, in which the stretching vibration of O-H and C-H groups at 3348 cm⁻¹ and 2899 cm⁻¹ weakened after O₂-NaOH pretreatment. In addition, severe pretreatment operations at higher NaOH dosage or temperature were conducive to the destruction of crystalline region in cellulose, and increased NaOH dosage to 3.5% showed better decrystallization of cellulose than elevated pretreatment temperature to 130 °C.

3.3. Structural characterization of isolated lignin

ART-FTIR spectra in Supplemental Material show that there were similar characteristics of two isolated lignin from O₂-NaOH pretreatment and NaOH pretreatment, respectively. A wide absorption signal at 3395 cm⁻¹ was associated with the stretching vibrations of phenolic and alcoholic hydroxyl groups in the lignin samples. The intensity of peak at 1708 cm⁻¹ related to C-O stretching vibration for OAL was stronger than that for AL, probably due to the intensive oxidation of lignin fractions in the presence of O₂ (Zhang et al., 2020). The peaks at 1597 cm⁻¹, 1507 cm⁻¹, 1463 cm⁻¹, and 1422 cm⁻¹ were the stretching vibrations of aromatic skeleton, indicating that the isolated lignin had an unbroken benzene ring structure. Moreover, the absorption signals at 1328 cm⁻¹ (assigned to C-O stretching of syringyl and guaiacyl rings), 1264 cm⁻¹ (belonged to C = O stretching of guaiacyl ring), 1227 cm⁻¹ (ring breathing with C-C, C-O and C = O stretching), 1126 cm⁻¹ (associated with the hydroxycinnamic acid ester or ether linkages), and 1031 cm⁻¹ (C-H vibrational stretching of guaiac ring) were observed (Jiang et al., 2018; Morales et al., 2018).

To further understand structural features of Napier grass stem lignin and distinctions between O₂-NaOH pretreatment and NaOH pretreatment for delignification process, 2D HSQC NMR was used to characterize the aliphatic and aromatic regions and semi-quantitative calculate the relative abundance of various moieties in lignin, as summarized in Table 3. The cross-signals in the aromatic region mainly corresponded to syringyl (S), guaiacyl (G) units, and p-coumarates (PCA). The S/G ratio in OAL (1.8) was higher than that in AL (1.3), suggesting that S unit is easier to dissolve out from the raw material under alkali environment with molecular O₂. PCA is cross-linked to the sidechain of lignin, and its content in OAL was more than that in AL. This indicates that the coexistence of O₂ can be conducive to the breakage of PCA-related sidechain linkages in cell wall (Jiang et al., 2018). The signals of the interunit linkages involving β-O-4' (A), β-5' (B) and β-β (C) were presented in the sidechain region of lignin samples. The intensity signal of β-5' cross-peak was highly weak with a low-level content. The cross-peak of β-O-4' substructures as the main sidechain linkages increased after the interfusion of O₂ for alkaline pretreatment (OAL). O₂ can react with phenolic lignin structures in alkali environments (Gierer, 1997). It is thus speculated that the β-O-4' structures which are partly related to phenolic structures could be broken by the attacking of O₂ to increase the

Table 3

Semi-quantification results of various moieties for the isolated lignin samples relative to AL and OAL determined by 2D HSQC NMR.

Lignin interunit linkage	δ_C/δ_H , ppm	Content, %	
		AL	OAL
p-Coumarates (PCA)	130.4/7.5	24.5	30.0
Syringyl (S)	104.3/6.7	55.9	64.6
Guaiacyl (G)	111.4/6.9	44.1	35.4
β-O-4' substructures (A)	60.2/3.4	32.7	56.3
β-5' substructures (B)	73.6/3.9	1.7	1.7
β-β substructures (C)	71.7/3.3	40.7	40.5
S/G ratio	-	1.3	1.8

hydrophilic functional groups of lignin molecules, leading to the dissolution of added lignin fractions (Deng et al., 2019). According to our characterization findings and the previous literatures (Rovio et al., 2011; Kuitunen et al., 2011; Yue et al., 2016; Ding et al., 2018), the possible reaction mechanisms for the sidechain cleavage and aromatic ring opening in the O₂-aided alkaline delignification process are proposed as follows. The sidechain oxidation reaction mainly occurs at the linkage position of the lignin structural unit with double bonds such as α-hydroxyl and carbonyl structures. The phenolic structures can be attacked by oxygen-containing electrophiles, resulting in benzene oxidation and ring opening to form water-soluble carboxylic acids.

In addition to the structural features, the molecular weight of lignin is key to further chemical valorization or polymeric material preparation. Table 4 presents the values of weight-average (M_w), number-average (M_n), and polydispersity index (PDI) of the acetylated lignin samples measured by GPC. The released lignin from O₂-NaOH pretreatment (OAL) showed slightly lower M_w and M_n than that from sole NaOH pretreatment (AL). This result can match with the above-mentioned characterizations, in which the presence of O₂ facilitates the sidechain cleavage and aromatic ring opening to lessen the molecular fragments of lignin in a certain extent. Advantageously, OAL had a narrower PDI of 1.76, indicating a relatively homogeneous size of the lignin fractions. Combined with the above observations, the O₂-aided alkaline released lignin has great potential as a precursor for the production of functional biomaterials and/or valuable chemicals.

3.4. Evaluation of enzymatic digestibility for pretreated Napier grass stem

The efficiency of O₂-NaOH pretreatment for Napier grass stem was evaluated based on the enzymatic digestibility of solid residues to produce monosaccharides, and the yield of glucose with the extension of enzymolysis time at different pretreatment conditions is shown in Fig. 3. The presence of molecular O₂ during alkaline pretreatment had a positive effect on enzymatic efficiency of pretreated substrates (Fig. 3a). Compared with sole NaOH pretreatment, the glucose yield from Napier grass stem after 0.5 MPa O₂-aided NaOH pretreatment was improved by around 20% with the same enzymolysis times. The effectively improvement of enzymatic digestibility for pretreated substrates could be attributed to the increase of lignin removal and loose of cell wall structure, thus resulting in more cellulase accessible active sites of cellulose. Alkali environment was a primary driving force in the delignification process for improving enzymatic hydrolysis efficiency of lignocellulose. The high glucose yield over 90% can be accomplished when the NaOH dosage reached 1% for pretreatment (Fig. 3b). Pretreatment temperature exhibited a stronger influence on the subsequent enzymatic digestibility than pretreatment time (Fig. 3a-b). A steady increase in the glucose yield was observed when the pretreatment temperature raised from 50 °C to 80 °C, while this change was inconspicuous after the pretreatment time of 60 min. Further, the contour plot showing the effect of lignin and xylan removal on glucose yield is provided in Fig. 4. It can be found that the enzymatic hydrolysis yield was directly related to the delignification rate of Napier grass stem, and had little to do with xylan removal. By and large, the more lignin removal, the higher glucose yield. About 80% lignin removal of Napier grass stem using O₂-NaOH pretreatment led to over 90% glucose yield from enzymatic hydrolysis process. On the other hand, the hemicellulose remained in the pretreated solid residues could also be hydrolyzed to monosaccharide (mainly xylose) accompanied by cellulose hydrolysis. The

Table 4

Molecular weights of weight-average (M_w) and number-average (M_n), and polydispersity index (PDI) for the isolated lignin samples relative to AL and OAL.

Lignin	M_w	M_n	PDI
AL ^a	4115	2261	1.82
OAL ^b	3808	2152	1.76

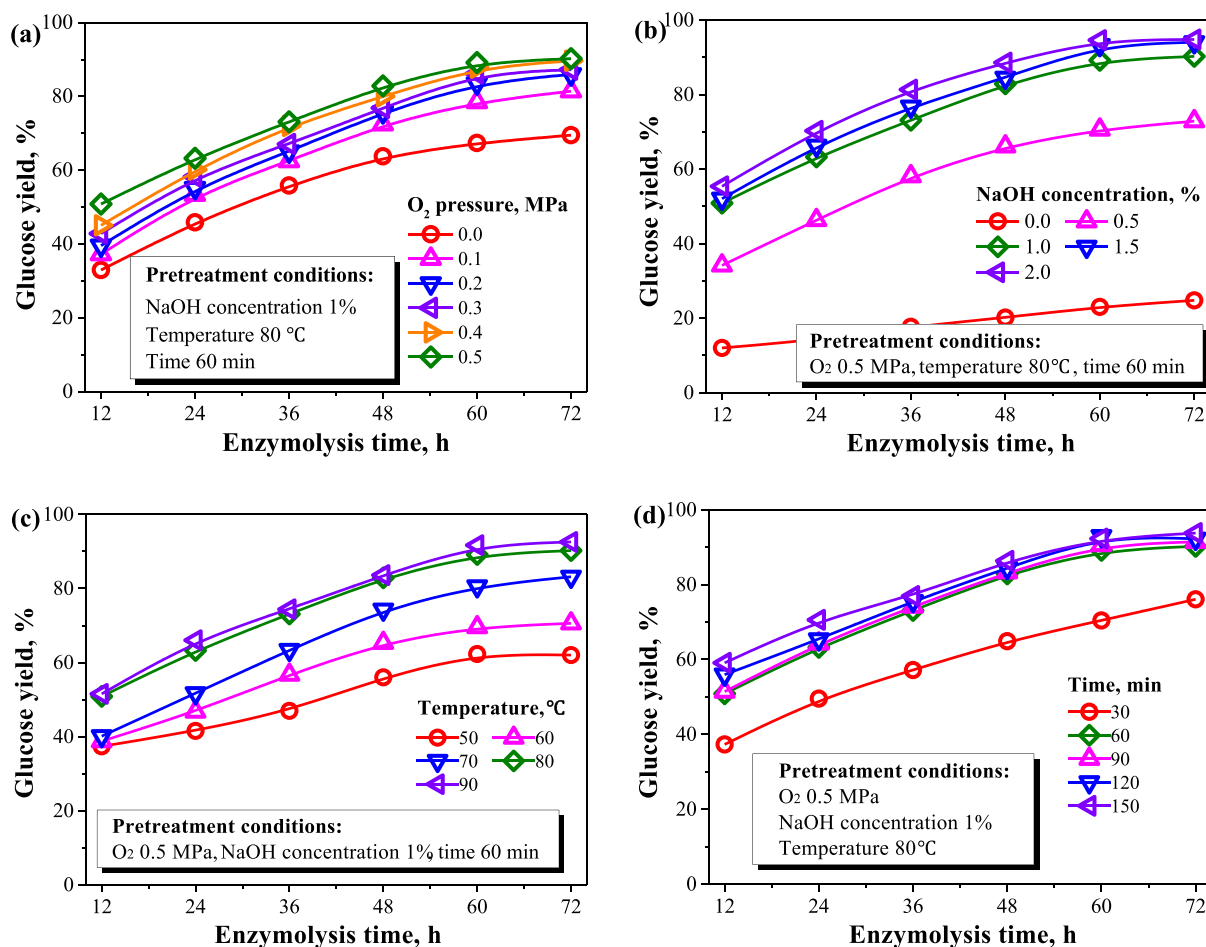


Fig. 3. Comparison of glucose yield from enzymatic hydrolysis for pretreated Napier grass stem under various O₂-aided alkaline pretreatment conditions. Enzymatic hydrolysis conditions: substrate concentration 2%, cellulase 25 FPU/g substrate, β -glucosidase 15 IU/g substrate, pH 4.8, 50 °C.

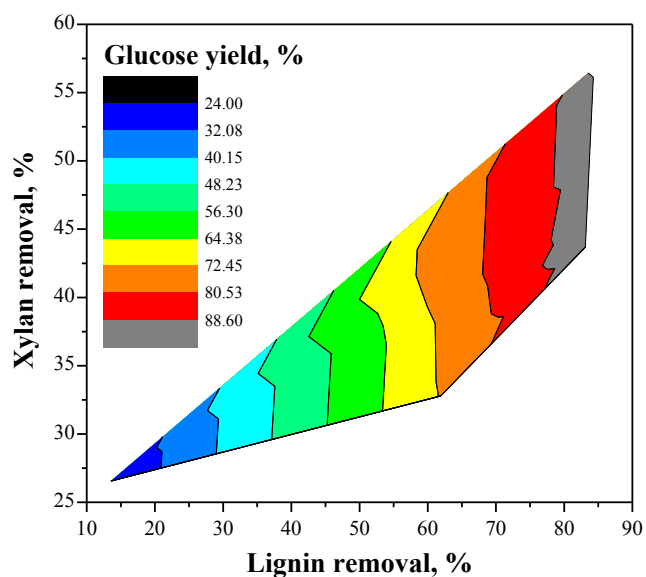


Fig. 4. Contour plot showing the effect of lignin and xylan removal on glucose yield (data from Fig. 3 at an enzymolysis time of 72 h).

similar effect trends of pretreatment variables on the xylose yield appeared, in comparison of these on the glucose yield. A significantly higher xylose yield of 92.9% after 72 h enzymatic hydrolysis for O₂-

NaOH pretreatment (0.5 MPa O₂, 1% NaOH, 80 °C, 60 min) was achieved than that of 75.5% for sole NaOH pretreatment (1% NaOH, 80 °C, 60 min).

3.5. Flowchart and results based on O₂-NaOH pretreatment of Napier grass stem

A sustainable biorefinery process claims to take full advantage of cellulose, hemicellulose and lignin in lignocellulosic biomass for producing functional materials, platform chemicals and biofuels. In this work, we made efforts to develop a viable fractional method as the precondition of biorefinery towards Napier grass stem valorization. Herein, the flowchart and results describing fermentable sugars production and pretreated hydrolysate management from Napier grass stem based on our proposed O₂-NaOH pretreatment are shown in Fig. 5. The solid substrate from the O₂-NaOH pretreated Napier grass stem can be effectively converted into soluble fermentable sugars via enzymatic hydrolysis, in where 43.72 g glucose and 12.91 g xylose were obtained from 100 g the raw material. In this enzymatic hydrolysis step, the recovery rates of glucose and xylose based on the raw material were 84.2% and 53.2%, respectively. The residual unhydrolyzed solid mainly composed of glucan (3.25 g), xylan (1.21 g) and lignin (3.67 g). For the pretreated hydrolysate, the existing soluble polysaccharides were attempt to hydrolyze enzymatically into fermentable sugars after adjusting the pH value of hydrolysate from 13.1 to 4.8 (Route 1). It is found that the soluble polysaccharides in the hydrolysate were mostly transformed into glucose (91.0% yield) and xylose (91.9%) even in the presence of bulk lignin. Coupled with the enzymatic hydrolysis

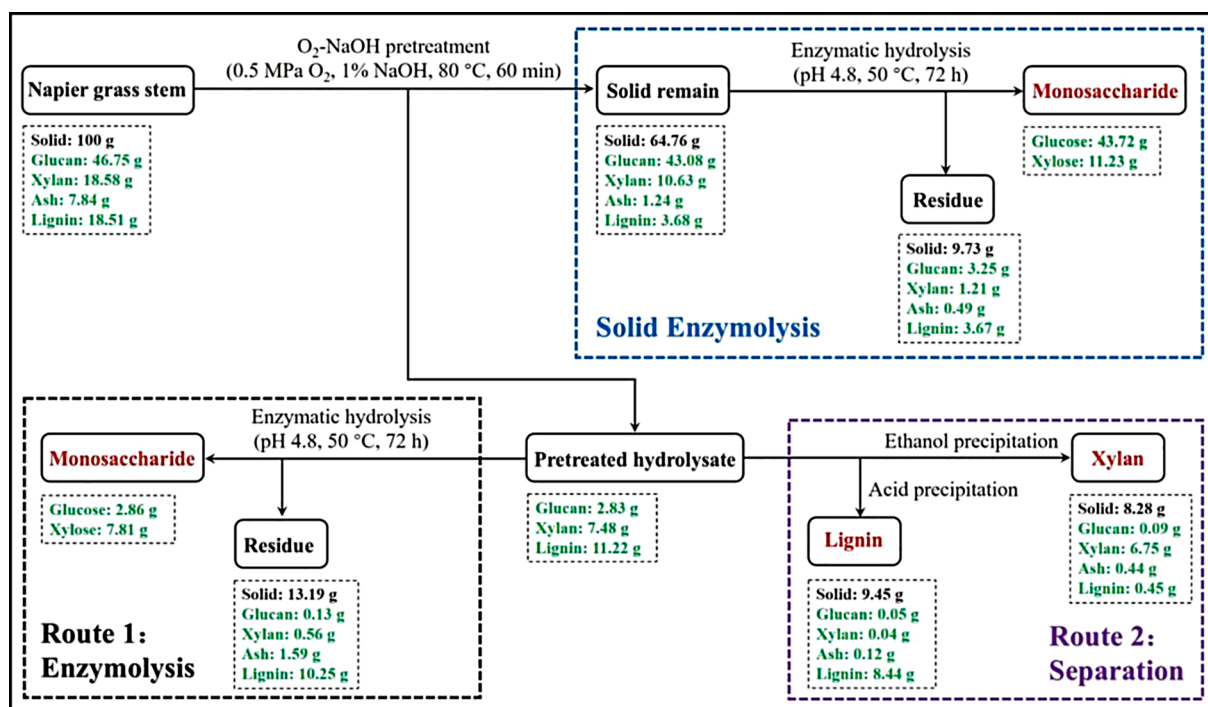


Fig. 5. Flowchart and results based on the O₂-NaOH pretreatment of Napier grass stem.

operations of pretreated solid remain and hydrolysate, the recovery rate of glucose and xylose reached 89.7% and 90.2%, respectively. Due to the hydrolysis of polysaccharides, the lignin was handily separated from the pretreated hydrolysate for further valorization. On the side, the xylan and lignin in the hydrolysate were collected step by step using ethanol precipitation, followed by acid precipitation method (Route 2) (Choi et al., 2019; Sun et al., 2001). The separated lignin had relatively high purity (89.3%), which has been structurally characterized hereinbefore. The obtained xylan (6.75 g) can serve as a potential source to develop functional oligosaccharide having many beneficial biomedical and health effects (Shen et al., 2016; Naidu et al., 2018). Considering process sustainability and valorization of compounds, Route 2 for the management of hydrolysate may be a more promising candidate from an industrial perspective.

4. Conclusions

O₂-aided alkaline pretreatment of Napier grass stem showed distinct advantages over sole alkaline pretreatment, including lignin removal, glucan recovery, energy consumption, and enzymatic digestibility. It could be proceeded at low temperature (80 °C) and dilute NaOH solution (1%) to remove over 80% lignin and retain 92% cellulose. The recovered lignin possessed well-preserved native structures with great potential for valorization. The recovery yields of glucose and xylose reached 89.7% and 90.2% from combined management of pretreated solid residue and hydrolysate via enzymatic hydrolysis. This study opens a reliable option to fractionate and saccharify Napier grass stem towards biorefinery processes.

CRedit authorship contribution statement

Weipeng Song: Methodology, Investigation, Visualization, Writing - original draft. **Lincai Peng:** Conceptualization, Validation, Formal analysis, Resources, Writing - review & editing, Supervision, Funding acquisition. **Danish Bakhshyar:** Investigation. **Liang He:** Methodology, Supervision. **Junhua Zhang:** Formal analysis, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (21965018), and Yunnan Ten Thousand Talents Plan Young & Elite Talents Project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biortech.2020.124162>.

References

- Alam, A., Zhang, R., Liu, P., Huang, J., Wang, Y., Hu, Z., Madadi, M., Sun, D., Hu, R., Ragauskas, A.J., Tu, Y., Peng, L., 2019. A finalized determinant for complete lignocellulose enzymatic saccharification potential to maximize bioethanol production in bioenergy *Miscanthus*. *Biotechnol. Biofuels* 12, 99.
- Bi, S., Peng, L., Chen, K., Zhu, Z., 2016. Enhanced enzymatic saccharification of sugarcane bagasse pretreated by combining O₂ and NaOH. *Bioresour. Technol.* 214, 692–699.
- Chandel, A.K., Garlapati, V.K., Singh, A.K., Antunes, F.A., da Silva, S.S., 2018. The path forward for lignocellulose biorefineries: Bottlenecks, solutions, and perspective on commercialization. *Bioresour. Technol.* 264, 370–381.
- Chen, Q., Wang, X., Huang, H., Cao, S., Chen, L., Huang, L., Ma, X., 2020. Turkey Red oil - An effective alkaline extraction booster for enhanced hemicelluloses separation from bamboo kraft pulp and improved fock reactivity of resultant dissolving pulp. *Ind. Crop. Prod.* 145, 112127.
- Cheng, B., Zhang, X., Lin, Q., Xin, F., Sun, R., Wang, X., Ren, J., 2018. A new approach to recycle oxalic acid during lignocellulose pretreatment for xylose production. *Biotechnol. Biofuels* 11, 324.
- Choi, J.H., Park, S.Y., Kim, J.H., Cho, S.M., Fang, S.K., Hong, C., Choi, I.G., 2019. Selective deconstruction of hemicellulose and lignin with producing derivatives by sequential pretreatment process for biorefining concept. *Bioresour. Technol.* 291, 121913.
- da Silva Perez, D., van Heiningen, A., 2015. Prediction of alkaline pulping yield: equation derivation and validation. *Cellulose* 22, 3967–3979.

- Deng, Z., Xia, A., Liao, Q., Zhu, X., Huang, Y., Fu, Q., 2019. Laccase pretreatment of wheat straw: effects of the physicochemical characteristics and the kinetics of enzymatic hydrolysis. *Biotechnol. Biofuels* 12, 159.
- Dias, D.d.S., Otoni, C.G., da Silva, R.R., Meneguim, A.B., Capparelli Mattoso, L.H., Barud, H.S., Ribeiro, C.A., 2020. Large scale manufacturing of puree-only edible films from onion bulb (*Allium cepa* L.): Probing production and structure-processing-property correlations. *Ind. Crop. Prod.* 145, 111847.
- Ding, N., Song, X., Jiang, Y., Luo, B., Zeng, X., Sun, Y., Tang, X., Lei, T., Lin, L., 2018. Cooking with active oxygen and solid alkali facilitates lignin degradation in bamboo pretreatment. *Sustain. Energy Fuels* 2, 2206–2214.
- Gierer, J., 1997. Formation and involvement of superoxide (O_2^- /HO $_2^\bullet$) and hydroxyl (OH $^\bullet$) radicals in TCF bleaching processes: A review. *Holzforschung* 51, 34–46.
- Harrison, M.D., Zhang, Z., Shand, K., O'Hara, I.M., Doherty, W.O.S., Dale, J.L., 2013. Effect of pretreatment on saccharification of sugar cane bagasse by complex and simple enzyme mixtures. *Bioresour. Technol.* 148, 105–113.
- He, C.R., Kuo, Y.Y., Li, S.Y., 2017. Lignocellulosic butanol production from *Napier* grass using semi-simultaneous saccharification fermentation. *Bioresour. Technol.* 231, 101–108.
- Jiang, B., Zhang, Y., Gu, L., Wu, W., Zhao, H., Jin, Y., 2018. Structural elucidation and antioxidant activity of lignin isolated from rice straw and alkali-oxygen black liquor. *Int. J. Biol. Macromol.* 116, 513–519.
- Jiang, Y., Zeng, X., Luque, R., Tang, X., Sun, Y., Lei, T., Liu, S., Lin, L., 2017. Cooking with active oxygen and solid alkali: A promising alternative approach for lignocellulosic biorefineries. *ChemSusChem* 10, 3982–3993.
- Kim, J.S., Lee, Y.Y., Kim, T.H., 2016. A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. *Bioresour. Technol.* 199, 42–48.
- Kuitunen, S., Kalliola, A., Tarvo, V., Tamminen, T., Rovio, S., Liitia, T., Ohra-aho, T., Lehtimaa, T., Vuorinen, T., Alopaeus, V., 2011. Lignin oxidation mechanisms under oxygen delignification conditions. Part 3. Reaction pathways and modeling. *Holzforschung* 65, 587–599.
- Kumari, D., Singh, R., 2018. Pretreatment of lignocellulosic wastes for biofuel production: A critical review. *Renew. Sust. Energ. Rev.* 90, 877–891.
- Lu, J., Liu, H., Song, F., Xia, F., Huang, X., Zhang, Z., Cheng, Y., Wang, H., 2020. Combining hydrothermal-alkaline/oxygen pretreatment of reed with PEG 6,000-assisted enzyme hydrolysis promote bioethanol fermentation and reduce enzyme loading. *Ind. Crop. Prod.* 153, 112615.
- Morales, A., Gullon, B., Davila, I., Eibes, G., Labidi, J., Gullon, P., 2018. Optimization of alkaline pretreatment for the co-production of biopolymer lignin and bioethanol from chestnut shells following a biorefinery approach. *Ind. Crop. Prod.* 124, 582–592.
- Naidu, D., Hlangothi, S., John, M., 2018. Bio-based products from xylan: A review. *Carbohydr. Polym.* 179, 28–41.
- Narinhorn, R., Choorit, W., Chisti, Y., 2019. Alkaline and fungal pretreatments for improving methane potential of *Napier* grass. *Biomass Bioenergy* 127, 105262.
- Pakkanen, H., Alen, R., 2013. Alkali consumption of aliphatic carboxylic acids during alkaline pulping of wood and nonwood feedstocks. *Holzforschung* 67, 643–650.
- Pang, C., Xie, T., Lin, L., Zhuang, J., Liu, Y., Shi, J., Yang, Q., 2012. Changes of the surface structure of corn stalk in the cooking process with active oxygen and MgO-based solid alkali as a pretreatment of its biomass conversion. *Bioresour. Technol.* 103, 432–439.
- Perez-Boada, M., Prieto, A., Prinsen, P., Forquin-Gomez, M.P., del Rio, J.C., Gutierrez, A., Martinez, A.T., Faulds, C.B., 2014. Enzymatic degradation of Elephant grass (*Pennisetum purpureum*) stems: Influence of the pith and bark in the total hydrolysis. *Bioresour. Technol.* 167, 469–475.
- Phitsuwan, P., Sakka, K., Ratanakhanokchai, K., 2016. Structural changes and enzymatic response of *Napier* grass (*Pennisetum purpureum*) stem induced by alkaline pretreatment. *Bioresour. Technol.* 218, 247–256.
- Qian, M., Lei, H., Villota, E., Mateo, W., Zhao, Y., Huo, E., Zhang, Q., Lin, X., Huang, Z., 2019. Optimization of delignification from *Douglas* fir sawdust by alkaline pretreatment with sodium hydroxide and its effect on structural and chemical properties of lignin and pyrolysis products. *Bioresour. Technol.* 8, 100339.
- Reddy, K.O., Maheswari, C.U., Dhlamini, M.S., Mothudi, B.M., Kommula, V.P., Zhang, J., Zhang, J., Rajulu, A.V., 2018. Extraction and characterization of cellulose single fibers from native african napier grass. *Carbohydr. Polym.* 188, 85–91.
- Rovio, S., Kuitunen, S., Ohra-aho, T., Alakurtti, S., Kalliola, A., Tamminen, T., 2011. Lignin oxidation mechanisms under oxygen delignification conditions. Part 2: Advanced methods for the detailed characterization of lignin oxidation mechanisms. *Holzforschung* 65, 575–585.
- Salmela, M., Alén, R., Vu, M.T.H., 2008. Description of kraft cooking and oxygen-alkali delignification of bamboo by pulp and dissolving material analysis. *Ind. Crop. Prod.* 28, 47–55.
- Sankaran, R., Cruz, R., Pakalapati, H., Show, P.L., Ling, T., Chen, W.-H., Tao, Y., 2020. Recent advances in the pretreatment of microalgal and lignocellulosic biomass: A comprehensive review. *Bioresour. Technol.* 298, 122476.
- Satari, B., Karimi, K., Kumar, R., 2019. Cellulose solvent-based pretreatment for enhanced second-generation biofuel production: A review. *Sustain. Energy Fuels* 3, 11–62.
- Segal, L., Creely, J.J., Martin, A.E., Conrad, C.M., 1959. An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. *Text. Res. J.* 29, 786–794.
- Shen, R., Li, H.Q., Zhang, J., Xu, J., 2016. Effects of impurities in alkali-extracted xylan on its enzymatic hydrolysis to produce xylo-oligosaccharides. *Appl. Biochem. Biotechnol.* 179, 740–752.
- Singh, J., Suhag, M., Dhaka, A., 2015. Augmented digestion of lignocellulose by steam explosion, acid and alkaline pretreatment methods: A review. *Carbohydr. Polym.* 117, 624–631.
- Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Determination of structural carbohydrates and lignin in biomass. Laboratory analytical procedures, NREL/TP-510-42618.
- Sun, R.C., Sun, X., Wen, J.L., 2001. Fractional and structural characterization of lignins isolated by alkali and alkaline peroxide from barley straw. *J. Agr. Food Chem.* 49, 5322–5330.
- Takara, D., Khanal, S.K., 2015. Characterizing compositional changes of *Napier* grass at different stages of growth for biofuel and biobased products potential. *Bioresour. Technol.* 188, 103–108.
- Tsai, M.H., Lee, W.C., Kuan, W.C., Sirisansaneeyakul, S., Savarajara, A., 2018. Evaluation of different pretreatments of *Napier* grass for enzymatic saccharification and ethanol production. *Energy Sci. Eng.* 6, 683–692.
- Wang, H., Zuo, M., Ding, N., Yan, G., Zeng, X., Tang, X., Sun, Y., Lei, T., Lin, L., 2019. Preparation of nanocellulose with high-pressure homogenization from pretreated biomass with cooking with active oxygen and solid alkali. *ACS Sustain. Chem. Eng.* 7, 9378–9386.
- Wu, J., Chandra, R., Saddler, J., 2019. Alkali-oxygen treatment prior to the mechanical pulping of hardwood enhances enzymatic hydrolysis and carbohydrate recovery through selective lignin modification. *Sustain. Energy Fuels* 3, 227–236.
- Xia, F., Gong, J., Lu, J., Cheng, Y., Zhai, S., An, Q., Wang, H., 2020. Combined liquid hot water with sodium carbonate-oxygen pretreatment to improve enzymatic saccharification of reed. *Bioresour. Technol.* 297, 122498.
- Xie, T., Lin, L., Pang, C., Zhuang, J., Shi, J., Yang, Q., 2013. Efficient enzymatic hydrolysis of the bagasse pulp prepared with active oxygen and MgO-based solid alkali. *Carbohydr. Polym.* 94, 807–813.
- Xu, H., Peng, J., Kong, Y., Liu, Y., Su, Z., Li, B., Song, X., Liu, S., Tian, W., 2020. Key process parameters for deep eutectic solvents pretreatment of lignocellulosic biomass materials: A review. *Bioresour. Technol.* 310, 123416.
- Yoo, C.G., Meng, X., Pu, Y., Ragauskas, A.J., 2020. The critical role of lignin in lignocellulosic biomass conversion and recent pretreatment strategies: A comprehensive review. *Bioresour. Technol.* 301, 122784.
- Yue, F., Lan, W., Hu, S., Chen, K.L., Lu, F., 2016. Structural modifications of sugarcane bagasse lignins during wet-storage and soda-oxygen pulping. *ACS Sustain. Chem. Eng.* 4, 5311–5318.
- Zhang, Q., Deng, Y., Tan, X., Wang, W., Yu, Q., Chen, X., Miao, C., Guo, Y., Zhang, Y., Zhuang, X., Yuan, Z., 2020. Biphasic fractionation of rice straw under mild condition in acidified 2-phenoxyethanol/water system. *Ind. Crop. Prod.* 145, 112091.
- Zhong, L., Yang, L., Wang, C., Ji, X., Yang, G., Chen, J., Lyu, G., Xu, F., Yoo, C.G., 2020. NaOH-aided sulfonate pretreatment for effective fractionation and utilization of willow (*Salix matsudana* cv. Zhuliu). *Ind. Eng. Chem. Res.* 10.1021/acs.iecr.0c01208.