Enzymatic Response to Structural and Chemical Transformations in *Hibiscus sabdariffa* var. *altissima* Bark and Core during Phosphoric Acid Pretreatment

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To investigate the potential of *Hibiscus sabdariffa* var. *altissima* (Thai kenaf) biomass as a feedstock for bioethanol production, Thai kenaf bark and core were pretreated at a moderate temperature with different phosphoric acid (H_3PO_4) concentrations. It was revealed that there was a higher glucan content in the Thai kenaf bark (57.97% ± 0.36%) compared with that in the core (43.10% ± 0.15%). The H_3PO_4 pretreatment resulted in a reduction in the lignin content and total removal of hemicellulose. This exposed the cellulose to attack by cellulase enzymes and resulted in an increased enzymatic digestibility. A high glucose concentration (GC; 7.02 g/L) and hydrolysis efficiency (HE; 95.79%) were achieved with 75% H_3PO_4 for the bark after 72 h of enzymatic hydrolysis. However, these values were not that different from those of the 70% H_3PO_4-pretreated bark (6.89 g/L and 95.43%, respectively). Nevertheless, the Thai kenaf core pretreated with 75% H_3PO_4 recorded a higher GC (6.30 g/L) and HE (91.67%) after 72 h of enzymatic hydrolysis. The scanning electron microscopy and X-ray diffraction analyses revealed the destruction of the surface structure and an increase in the porosity and crystallinity index of the Thai kenaf biomass, which corresponded to an increased enzymatic digestibility.

Keywords: Bark; Core; Hibiscus sabdariffa var. altissima; Phosphoric acid pretreatment; Thai kenaf

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INTRODUCTION

Bioethanol is regarded as a promising renewable transportation fuel (Hoşgün *et al*. 2017). Compared with fossil fuels, bioethanol combustion yields low greenhouse gas emissions and prevents environmental pollution (Lewandowska *et al*. 2016). Currently, bioethanol is commercially produced from sugar- and starch-based crops (Smichi *et al*. 2014). The leading global producers, including Brazil and the United States, produce bioethanol from sugarcane and corn, respectively (Avci *et al*. 2013). However, the excessive use of food-related crops for bioethanol production may result in high food prices (Sun *et al*. 2016). An alternative feedstock for bioethanol production is lignocellulosic materials.

Lignocellulosic biomass, the most abundant renewable resource on Earth, includes non-edible forest, agricultural, and agroindustrial residues (Xu and Huang 2014). These materials are rich in structural carbohydrates (cellulose and hemicellulose) that are crossed-linked and strongly bonded to lignin to form complex structures (Galbe and Zacchi 2012). Carbohydrates can be hydrolysed to monomer sugar units by enzymes and fermented to bioethanol (Yoon *et al*. 2015). The complex structures of lignocellulosic biomasses make them resistant to enzymatic hydrolysis, which results in low sugar yields (Nieves *et al*. 2011). Therefore, the conversion of lignocellulosic biomass to bioethanol requires a pretreatment step prior to enzymatic hydrolysis and fermentation (Kim *et al*. 2016).

Pretreatment of the biomass is very important in breaking down the recalcitrant structure of biomass to enhance the bioconversion process (Qing *et al.* 2017). Several pretreatment methods, classified as physical, chemical, biological, or a combination thereof, have been developed for conversion (Qing *et al*. 2017; Kusi *et al*. 2018). Chemical pretreatment with acid has been widely studied and reported to be one of the most effective methods (Foston and Ragauskas 2010). The major disadvantage of acid pretreatment is the formation of inhibitors, including furfural, hydroxymethylfurfural, and acetic acid. The production of these inhibitors hinders enzymatic hydrolysis and fermentation processes (Lee *et al*. 2015). Nevertheless, studies have shown that phosphoric acid (H_3PO_4) is less toxic and produces low concentrations of inhibitors (Nieves *et al*. 2016). Zhang *et al*. (2007) showed that concentrated phosphoric acid (>82%) pretreatment at a moderate temperature (50 °C) enhanced the enzymatic hydrolysis efficiency of switch grass, Douglas fir, corn stover and hybrid poplar biomass.

In the tropical regions of Southeast Asia, such as Thailand, there are many potential lignocellulosic materials that can be used for bioethanol production. *Hibiscus sabdariffa* var. *altissima* (Thai kenaf) is among a variety of fibre crops that have been extensively studied and cultivated for the textile industry in Thailand. The plant is also used for the production of other products, such as ropes and sacks, for domestic and international markets. Interest in these fibre crops have dwindled because of low exports in the textile industry. However, these fibre plants may also be used as energy crops for bioethanol production. They are non-food plants, inexpensive to cultivate (as they grow well in low quality soils), drought-tolerant, and have a high dry matter yield (Fathima and Balasubramanian 2006). The conversion of these fibre crops to bioethanol will not only provide an alternative feedstock, but also help in developing rural areas in Thailand and provide a sustainable source of income for local farmers. The potential of Thai kenaf bark and core as a feedstock for bioethanol production was therefore studied.

EXPERIMENTAL

Materials

Dried Thai kenaf KK50, a cross between KK2515-238 and THS-22, was provided by Khon Kaen Field Crop Research Centre (Khon Kaen, Thailand). The bark was separated from the core and cut into smaller pieces. The samples were then milled with a wood-miller (SM 100, Retsch, Haan, Germany), passed through a 150-µm to 300-µm screen, and stored at room temperature in air-tight plastic bags until further analysis.

Methods

Chemical composition

The chemical composition of the Thai kenaf biomass, including the cellulose, hemicellulose, acid insoluble lignin (AIL), and acid soluble lignin (ASL) contents, before and after pretreatment was determined according to Sluiter *et al*. (2012). The ash content of the untreated biomass sample was determined according to Sluiter *et al*. (2008). Monomer sugars were analysed by high-performance liquid chromatography (HPLC; LC-20AD system, Shimadzu Co. Ltd., Kyoto, Japan) with a Bio-Rad Aminex HPX-87P column (Hercules, CA) and refractive index detector (RID-10A, Shimadzu). The column was operated at 80 °C with an injection volume of 20 µL per sample. Filtered HPLC-grade water was used for the elution at a flow rate of 0.6 mL/min.

Phosphoric acid pretreatment

The phosphoric acid pretreatment of the Thai kenaf biomass was performed in 50-mL polypropylene tubes by mixing 3 g of dry biomass with 24 mL of 70%, 75%, 80%, or 85% (v/v) H_3PO_4. The mixture in the tube was then covered and subjected to a temperature of 60 °C for 60 min using a water bath (Eyela Pro Cool Bath NCB-3300, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The reaction was stopped by adding approximately 30 mL of acetone to the slurry with thorough mixing. The supernatant was discarded after centrifugation at 2500 rpm for 10 min. The solid part was re-suspended in acetone and centrifuged again. The supernatant was again poured out. This process was repeated three times, after which the solid part was washed thoroughly with distilled water until the pH reached 7.0.

Crystallinity

The crystallinity of the raw and pretreated Thai kenaf biomass was determined by X-ray diffraction (XRD) using a PANalytical X'pert Pro PW 3040/60 diffractometer (Almelo, Netherlands). The biomass was first washed three times with acetone and air-dried at ambient temperature. The dried biomass was ground, passed through a 150-µm mesh screen, and scanned from 2θ values of 10° to 40°. The biomass crystallinity index (CrI) was determined using Eq. 1:

$$CrI = (I_{002} - I_{am}) / I_{002} \times 100\% \quad (1)$$

where I_{002} and I_{am} are the intensities at a 2θ of 22.2° and 18.0°, respectively.

Scanning electron microscopy

The morphological characteristics of the raw and pretreated Thai kenaf biomass were observed with scanning electron microscopy (SEM) using a LEO 1455VP (Zeiss, Gottingen, Germany). The biomass was mounted on aluminum stubs after being freeze dried. The samples were coated with gold and analysed with SEM.

Enzymatic hydrolysis

Enzymatic hydrolysis was performed following the method described by Siripong *et al*. (2016) with slight modifications. The raw and pretreated Thai kenaf biomass were enzymatically hydrolyzed in a 50-mL Erlenmeyer flask containing 0.1 g of biomass (dry basis), 0.05 M sodium citrate buffer (pH = 4.8), and 0.1 mL of 2% sodium azide (w/v) with a total reaction volume of 10 mL. An enzyme mixture contained cellulase (celluclast 1.5 L, Sigma-Aldrich, St. Louis, USA) and β-glucosidase (Oriental Yeast Co. Ltd., Tokyo, Japan) at a loading of 30 filter paper units/g and 60 U/g of dry biomass, respectively. The reaction mixture was then incubated on a rotary shaker (Innova 4340, New Brunswick Scientific Company, New Jersey, USA) at 50 °C and 150 rpm for 72 h. The hydrolysates were sampled (20 µL) periodically (12 h, 24 h, 48 h, and 72 h) for monomer sugars analysis using HPLC. The hydrolysis efficiency was determined using Eq. 2,

$$HE\ (\%) = [(Glucose\ released\ (g) \times 0.9) / Glucan\ in\ initial\ biomass\ (g)] \times 100\% \quad (2)$$

where *HE* is the hydrolysis efficiency.

Statistical analysis

Sampling and all of the analyses were performed in triplicate and the data was presented as the mean with the standard deviation (SD). The data collected was analysed by an analysis of variance using SPSS software (version 17.0, Statsoft Inc., Tulsa, USA). A comparison of the treatment means was performed using Tukey's Test.

RESULTS AND DISCUSSION

Chemical Composition of the Thai Kenaf Bark and Core

The chemical composition analysis of the Thai kenaf biomass showed significantly high contents of glucan, ASL, ash, and ethanol extractive in the bark compared with that in the core. However, the contents of xylan, AIL, and protein in the core were significantly higher than that in the bark (Table 1). The glucan content of both the bark and core was higher than that reported for several sources of lignocellulosic biomass, including corn stover (37.1% ± 0.03%) (Avci *et al*. 2013), rice straw (35.9%) (Yang *et al*. 2012), barley straw (35.9%) (Park and Kim 2012), and *Pennisetum purpureum* stem (26.0%) (Phitsuwan *et al*. 2016). The high glucan content of the Thai kenaf biomass was an indication of its potential as a feedstock for bioethanol production.

Table 1. Chemical Composition of the Thai Kenaf Bark and Core

Component	Bark (% DW)	Core (% DW)
Glucan	57.97 ± 0.36[a]	43.10 ± 0.15[b]
Xylan	11.94 ± 0.07[b]	19.76 ± 0.16[a]
AIL	7.37 ± 0.21[b]	16.79 ± 0.21[a]
ASL	4.45 ± 0.01[a]	4.04 ± 0.01[b]
Protein	0.34 ± 0.01[b]	1.03 ± 0.02[a]
Ash	3.75 ± 0.02[a]	2.17 ± 0.08[b]
Extractive	6.89 ± 0.57[a]	5.65 ± 0.06[b]

Values are the mean ± SD; Means in the same row with different letters differ statistically at $P < 0.05$; % DW is the percent dry weight

Effect of the Phosphoric Acid Pretreatment on the Thai Kenaf Biomass

To enhance the efficiency of enzymatic conversion of cellulose to glucose, phosphoric acid pretreatment was performed on the Thai kenaf biomass. The effect of different phosphoric acid concentrations on the chemical composition, cellulose crystallinity, and cell wall morphology of the Thai kenaf biomass was analysed.

Effect on the chemical composition

Pretreatment of the lignocellulosic biomass with acid results in the hydrolysis of hemicellulose and destruction of the lignin structure (Foston and Ragauskas 2010). These changes make cellulose available for attack by hydrolytic enzymes and thus enhance the efficiency of enzymatic hydrolysis (Galbe and Zacchi 2012). The phosphoric acid pretreatment of the Thai kenaf biomass resulted in significant changes in the chemical composition. Increasing the phosphoric acid concentration resulted in a significant decrease in the xylan, AIL, and ASL contents in both the bark and core (Tables 2 and 3). The xylan was completely removed when the biomass was pretreated with 75%, 80%, and 85% H_3PO_4. This resulted in a significant increase in the glucan content in both the bark (Table 2) and core (Table 3). Pretreatment with 85% H_3PO_4 caused a more than 50% reduction in the total solids content in both the bark and core. However, more than 50% of the glucan was retained in both Thai kenaf biomass types after pretreatment under various conditions. The reduction in the total solids content was attributed to the removal of various components, including lignin and polysaccharides, as was reported in the study by Kacem *et al*. (2016). The effect of the phosphoric acid pretreatment in this study was more pronounced than the decrease and complete removal of hemicellulose from the Thai kenaf biomass. Lee *et al*. (2015) reported that acid pretreatment mainly results in the removal of hemicellulose and partial digestion of cellulose. The acid concentration has a direct influence on the degree of hemicellulose removal during acid pretreatment. A higher acid concentration during pretreatment results in the removal of a large amount of the

solid portion (Lewandowska *et al.* 2016). Yoon *et al.* (2015) reported the removal of 30.6% of the cellulose and 59.7% of the xylan from the bark of goat willow after pretreatment with 85% H_3PO_4.

Table 2. Chemical Composition of the Phosphoric Acid-pretreated Thai Kenaf Bark

Composition (%)	Untreated (%)	70% H_3PO_4	75% H_3PO_4	80% H_3PO_4	85% H_3PO_4
Glucan	57.97 ± 0.36^{d}	59.07 ± 0.49^{d}	64.92 ± 0.49^{c}	72.08 ± 0.39^{b}	82.58 ± 0.34^{a}
Xylan	11.94 ± 0.07^{a}	1.51 ± 0.07^{b}	N.D.	N.D.	N.D.
AIL	7.37 ± 0.21^{a}	6.19 ± 0.12^{b}	4.80 ± 0.12^{c}	4.31 ± 0.08^{d}	2.08 ± 0.09^{e}
ASL	4.45 ± 0.02^{a}	2.35 ± 0.03^{b}	1.89 ± 0.03^{c}	1.56 ± 0.03^{d}	1.40 ± 0.02^{e}
Components Retained (%)					
Solid Residue	100^{a}	82.45 ± 0.17^{b}	68.76 ± 0.15^{c}	60.11 ± 0.08^{d}	40.51 ± 0.16^{e}
Glucan	100^{a}	84.02 ± 0.09^{b}	77.00 ± 0.10^{c}	74.74 ± 0.10^{d}	57.70 ± 0.16^{e}
Xylan	100^{a}	10.42 ± 0.12^{b}	N.D.	N.D.	N.D.
AIL	100^{a}	69.25 ± 0.15^{b}	44.73 ± 0.14^{c}	35.12 ± 0.14^{d}	11.44 ± 0.11^{e}
ASL	100^{a}	43.58 ± 0.09^{b}	29.25 ± 0.11^{c}	21.13 ± 0.11^{d}	12.74 ± 0.13^{e}

Values are the mean ± SD; Means in the same row with different letters differ statistically at $P < 0.05$; N.D. – not determined

Table 3. Chemical Composition of the Phosphoric Acid-pretreated Thai Kenaf Core

Composition (%)	Untreated (%)	70% H_3PO_4	75% H_3PO_4	80% H_3PO_4	85% H_3PO_4
Glucan	43.10 ± 0.15^{e}	46.81 ± 0.16^{d}	54.57 ± 0.21^{c}	71.74 ± 0.13^{b}	77.58 ± 0.25^{a}
Xylan	19.76 ± 0.16^{a}	5.14 ± 0.76^{b}	N.D.	N.D.	N.D.
AIL	16.79 ± 0.21^{a}	15.07 ± 0.08^{b}	12.04 ± 0.34^{c}	9.37 ± 0.24^{d}	5.50 ± 0.24^{e}
ASL	4.04 ± 0.01^{a}	2.33 ± 0.03^{b}	1.80 ± 0.02^{c}	1.33 ± 0.03^{d}	1.03 ± 0.02^{e}
Components Retained (%)					
Solid Residue	100^{a}	85.44 ± 0.18^{b}	66.93 ± 0.19^{c}	49.80 ± 0.15^{d}	37.85 ± 0.12^{e}
Glucan	100^{a}	92.81 ± 0.13^{b}	84.74 ± 0.16^{c}	82.89 ± 0.11^{d}	68.11 ± 0.09^{e}
Xylan	100^{a}	22.23 ± 0.18^{b}	N.D.	N.D.	N.D.
AIL	100^{a}	76.67 ± 0.18^{b}	47.99 ± 0.14^{c}	27.79 ± 0.16^{d}	12.41 ± 0.17^{e}
ASL	100^{a}	49.36 ± 0.09^{b}	29.83 ± 0.13^{c}	16.42 ± 0.16^{d}	9.66 ± 0.12^{e}

Values are the mean ± SD; Means in the same row with different letters differ statistically at $P < 0.05$; N.D. – not determined

Effect on the cellulose crystallinity

The CrI determined by XRD analysis provides information about the crystalline and non-crystalline fractions of lignocellulosic biomass (Phitsuwan *et al.* 2016). The XRD analysis of the untreated and pretreated Thai kenaf biomass showed two main diffraction peaks (Figs. 1 and 2) at 16° and 22°, which related to cellulose II (non-crystalline cellulose) and cellulose I (crystalline cellulose), respectively.

It was observed that the CrI of cellulose the biomass increased after pretreatment with 70%, 75%, and 80% H_3PO_4 (Table 4). The XRD profile showed that the crystalline structure of cellulose in Thai kenaf biomass preteated at these conditions (70%, 75%, and 80% H_3PO_4) was more evident than that of 85% H_3PO_4 and untreated. The CrI of cellulose in biomass pretreated with 85% H_3PO_4 on the other hand was lower compared to the other H_3PO_4 concentrations and untreated biomass (Table 4).

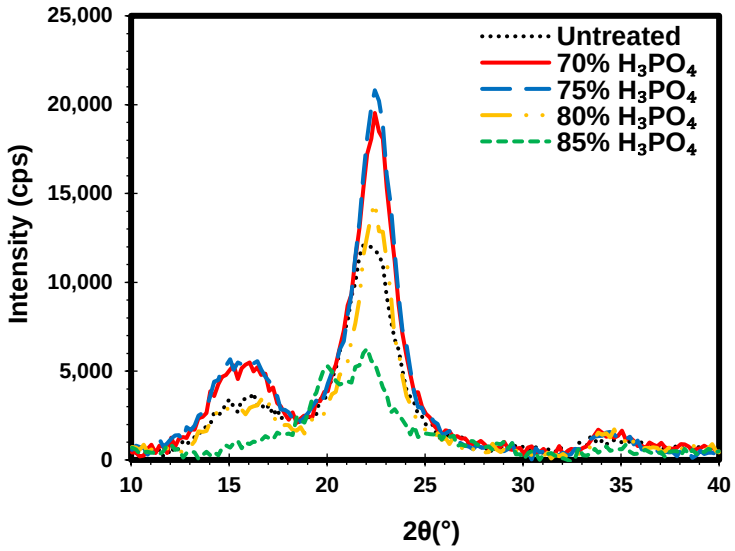

Fig. 1. XRD profile of the Thai kenaf bark

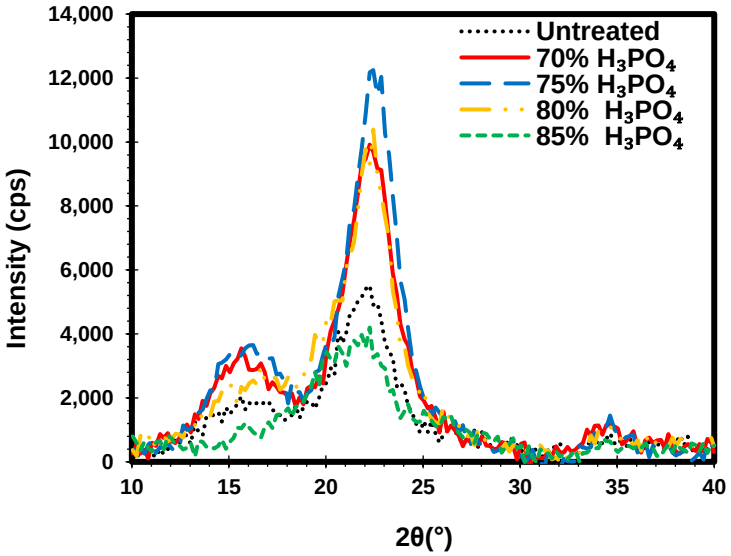

Fig. 2. XRD profile of the Thai kenaf core

The cellulose structure in biomass pretreated at this condition (85%) was decrystallized and converted to amorphous form. Decrystallization of cellulose in switchgrass and napier grass after pretreatment with concentrated phosphoric acid (83% to 85%) have been reported by Sathitsuksanoh *et al.* (2011) and Takata *et al.* (2013), respectively. The present study confirms the effectiveness of concentrated phosphoric acid (85%) in destroying the crystalline cellulose structure in biomass unlike concentrations of 80% and below. However, although the CrI of cellulose in biomass pretreated at 70%, 75%, and 80% H_3PO_4 was high, it was found that the HE and GC were greatly increased after hydrolysis (Figs. 5 and 6). Similar phenomenon was observed in earlier studies by different researchers (Hideno *et al*. 2013; Siripong *et al*. 2016).

Table 4. CrI of the Untreated and Pretreated Thai Kenaf Bark and Core Biomass

	Untreated	70% H_3PO_4	75% H_3PO_4	80% H_3PO_4	85% H_3PO_4
Thai Kenaf Bark	83.29%	88.95%	89.92%	86.66%	77.50%
Thai Kenaf Core	76.99%	83.82%	85.18%	83.65%	69.76%

Effect on the biomass surface structure

The morphological characteristics of the pretreated Thai kenaf biomass were observed with SEM. The results showed substantial changes in the surface structures of both the pretreated bark (Fig. 3) and core (Fig. 4).

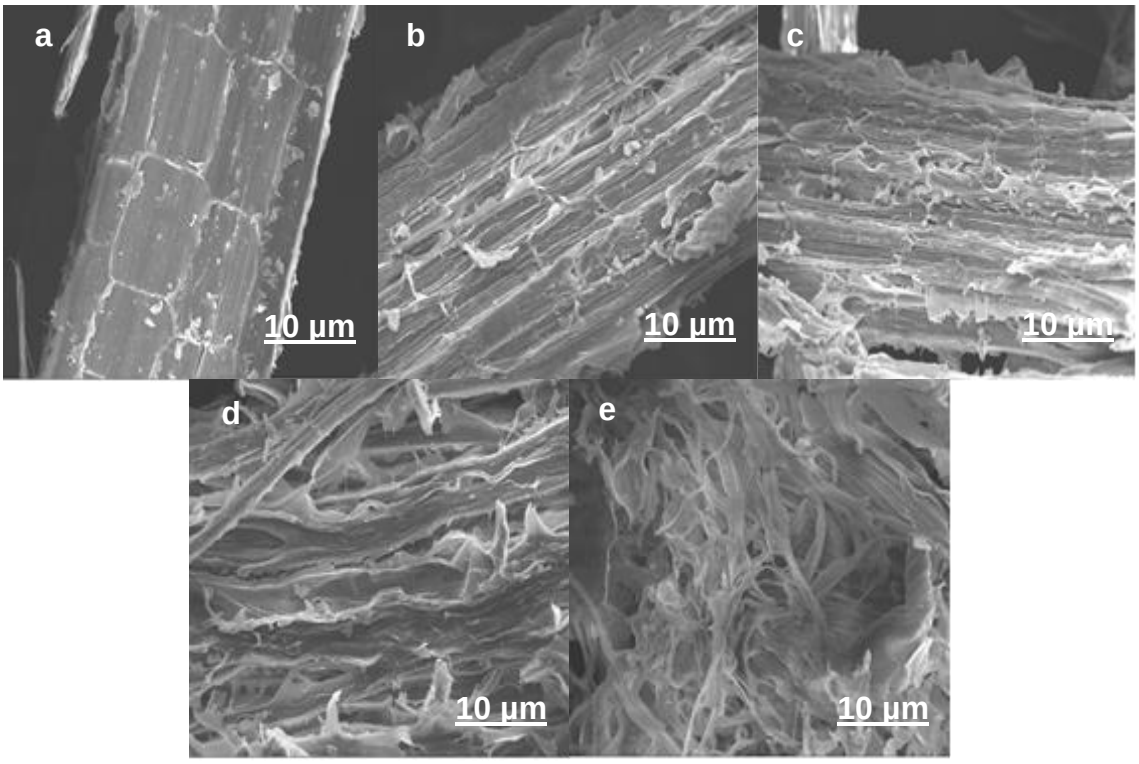

Fig. 3. SEM images of the untreated and pretreated Thai kenaf bark: (a) untreated; (b) 70% H_3PO_4; (c) 75% H_3PO_4; (d) 80% H_3PO_4; and (e) 85% H_3PO_4

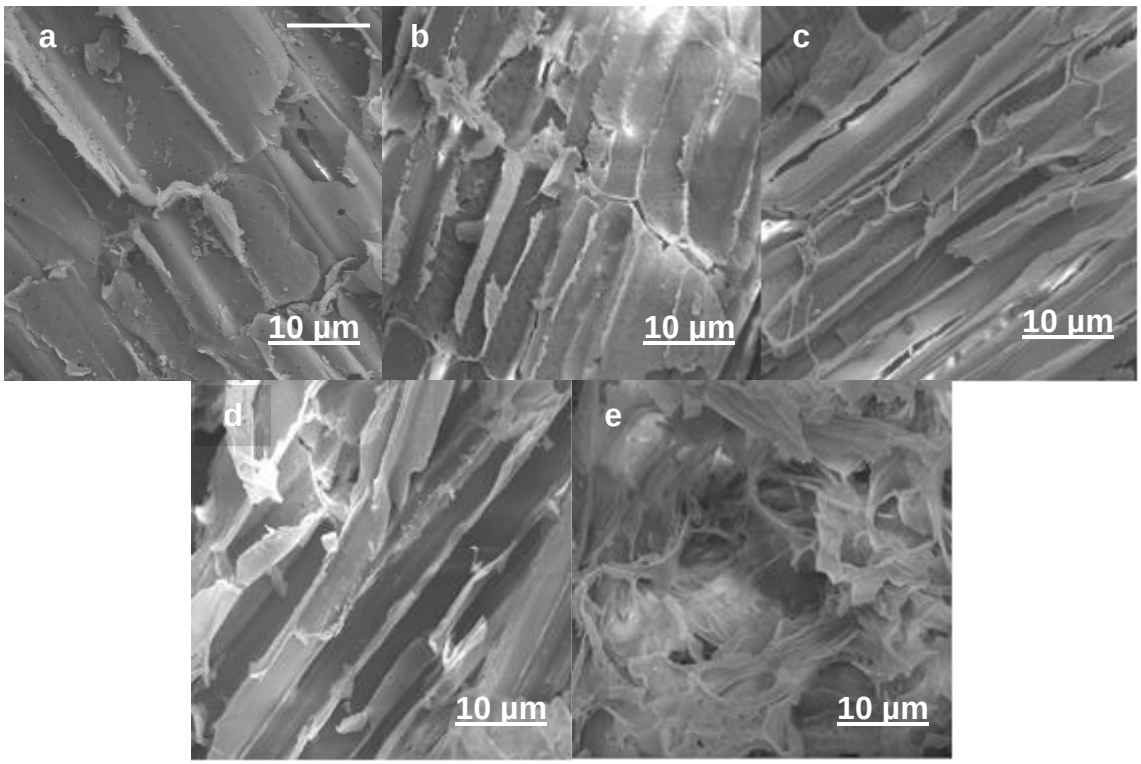

Fig. 4. SEM images of the untreated and pretreated Thai kenaf core: (a) untreated; (b) 70% H_3PO_4; (c) 75% H_3PO_4; (d) 80% H_3PO_4; and (e) 85% H_3PO_4

The surface structure of the untreated bark (Fig. 3a) was intact with an organized and even structure, while that of the core (Fig. 4a) was smooth with few cracks. These structural characteristics of the untreated Thai kenaf biomass hindered the access of cellulase to cellulose fibrils (Wang *et al*. 2014). The structure of the untreated Thai kenaf biomass was extensively altered after the phosphoric acid pretreatment. The intact structure of the untreated bark was destroyed by the pretreatment process. The fibres gradually separated from each other until they became highly disorganized (Figs. 3b, 3c, 3d, and 3e). Pretreatment of the core also resulted in a rough surface and more cracks (Figs. 4b, 4c, 4d, and 4e).

With an increase in the phosphoric acid concentration, the surface destruction became more pronounced. Pretreatment with 85% H_3PO_4 resulted in the total collapse of the structure and production of more fragments (Figs. 3e and 4e). The results of the SEM analysis confirmed the results from the XRD analysis. The structural changes after the pretreatment were an indication of the depolymerization of the Thai kenaf biomass and were attributed to the removal of hemicellulose and destruction of the lignin structure by phosphoric acid (Siripong *et al.* 2016). These changes provided greater access of the cellulose to hydrolytic enzymes.

Enzymatic Hydrolysis

The effect of the phosphoric acid pretreatment on the digestibility of the exposed cellulose was analysed by the enzymatic hydrolysis of the untreated and pretreated Thai kenaf bark (Fig. 5) and core (Fig. 6). The results showed a sharp increase in the glucose concentration (GC) and hydrolysis efficiency (HE) in the first 12 h, after which there was a steady increase up to 72 h. The pretreated biomass samples for both the bark and core recorded significantly high GC and HE values compared with that of the untreated samples. High GC and HE values under all of the conditions for both the bark and core were recorded at 72 h of hydrolysis.

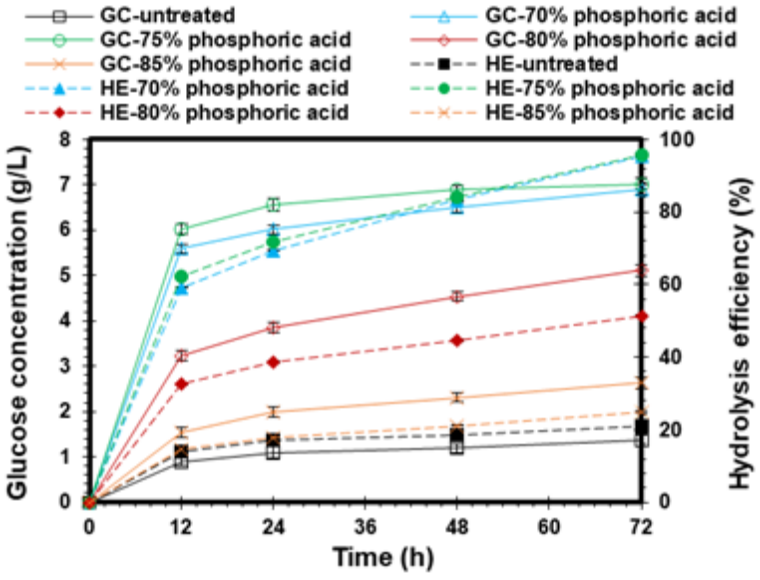

Fig 5. Hydrolysis efficiency of the pretreated Thai kenaf bark

The highest GC and HE for the bark (7.02 g/L and 95.79%, respectively) were observed with the 75% H_3PO_4 pretreatment. However, these values were not particularly different from those of the biomass pretreated with 70% H_3PO_4 (6.89 g/L and 95.43%, respectively). The Thai kenaf core pretreated with 75% H_3PO_4 recorded significantly higher GC and HE values after enzymatic hydrolysis (6.30 g/L and 91.67%, respectively).

It was observed that although the biomass pretreated with 70% H_3PO_4 retained more glucan, high xylan, AIL, and ASL contents were retained (Tables 2 and 3), especially in the core, which negatively affected the GC and HE. The highest HE for both the bark (95.79%) and core (91.67%) recorded in the biomass pretreated with 75% H_3PO_4 in this study were greater than that reported for 80% H_3PO_4-pretreated *A. aspera* (86.2%), *S. acuta* (82.2%) (Siripong *et al*. 2016), wheat straw (68.7%), oak chips (50.2%), bamboo (39.3%), Jerusalem artichoke stalk (59.6%), spruce chips (36.3%), and corn (43.5%) (Wang *et al*. 2014).

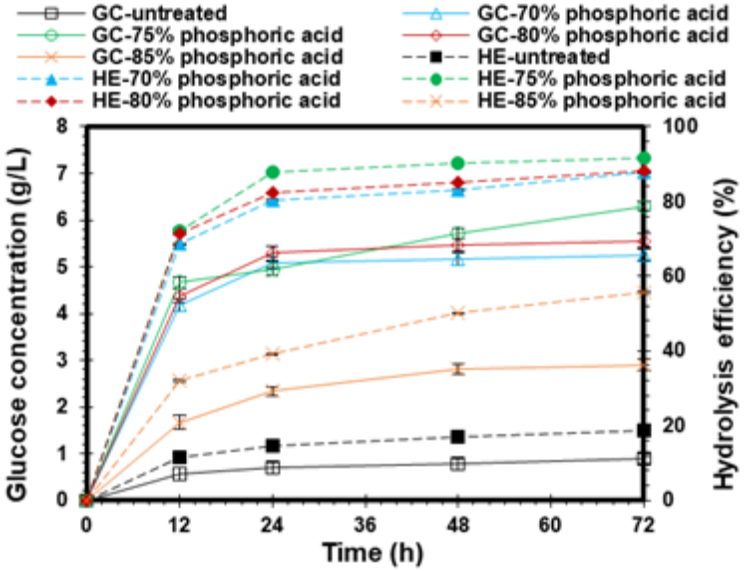

Fig 6. Hydrolysis efficiency of the pretreated Thai kenaf core

The phosphoric acid pretreatment resulted in a significant increase in the GC and HE, although the CrI values were high after the pretreatment. The significant removal of hemicellulose and lignin, which exposed the crystalline cellulose, enhanced the efficiency of the enzyme activities during hydrolysis. The removal of hemicellulose and destruction of the lignin structure increased the susceptibility of cellulose to enzymes. The phosphoric acid pretreatment of the Thai kenaf biomass resulted in the partial delignification and complete removal of hemicellulose, which promoted exposure of cellulose fibres to attack by cellulase and resulted in an increased enzymatic digestibility.

CONCLUSIONS

1. This study has shown the potential of Thai kenaf biomass as a feedstock for bioethanol production and established effective pretreatment conditions for attaining a high sugar yield and enhanced efficiency during enzymatic hydrolysis.
2. To obtain a high sugar yield, low energy consuming pretreatment processes using 70% and 75% H_3PO_4 were needed for Thai kenaf bark and core, respectively. These pretreatment conditions greatly improved the enzymatic digestibility by reducing the lignin and totally removing the hemicellulose, which exposed the cellulose fibres of both the Thai kenaf bark and core to enzyme attack.

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