



Efficient removal of lignin with the maintenance of hemicellulose from kenaf by two-stage pretreatment process



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ABSTRACT

The enhancement of lignocellulose hydrolysis using enzyme complexes requires an efficient pretreatment process to obtain susceptible conditions for the enzyme attack. This study focuses on removing a major part of the lignin layer from kenaf (*Hibiscus cannabinus*) while simultaneously maintaining most of the hemicellulose. A two-stage pretreatment process is adopted using calcium hydroxide, $\text{Ca}(\text{OH})_2$, and peracetic acid, PPA, to break the recalcitrant lignin layer from other structural polysaccharides. An experimental screening of several pretreatment chemicals, concentrations, temperatures and solid–liquid ratios enabled the production of an optimally designed pretreatment process for kenaf. Our results showed that the pretreatment process has provide 59.25% lignin removal while maintaining 87.72% and 96.17% hemicellulose and cellulose, respectively, using 1 g of $\text{Ca}(\text{OH})_2/\text{L}$ and a 8:1 (mL:g) ratio of liquid– $\text{Ca}(\text{OH})_2$ at 50 °C for 1.5 h followed by 20% peracetic acid pretreatment at 75 °C for 2 h. These results validate this mild approach for aiding future enzymatic hydrolysis.

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1. Introduction

Lignocellulosic biomass refers to plant biomass with an outer recalcitrant lignin layer and lignin tightly bound with complex carbohydrate polymers (hemicellulose and cellulose). Hemicellulose and cellulose can be further hydrolysed to glucose and various other sugars that are valuable for subsequent fuel ethanol production via fermentation. Plant biodegradation in nature is a very slow process because of the inaccessibility of hydrolytic enzymes to the carbohydrate polymers due to differences in lignin and substrate crystallinity in various biomass materials. Therefore, a suitable pretreatment process for different types of biomass to partially fractionate polymer complexes must be optimally developed (Yang & Wyman, 2004; Yu, Jameel, Chang, & Park, 2011) to enhance the degree of delignification of the substrate. Such pretreatment processes typically require elevated temperature and pressure combined with an acid or base catalysis to yield lignocellulosic materials that are more susceptible to enzyme attack (Berlin, Maximenko, Gilkes, & Saddler, 2007; Kim & Lee, 2005).

A wide variety of pretreatment processes that include enzymatic hydrolysis of the crystalline cellulose layer of the carbohydrate polymers have been studied over the past 30 years. These pretreatments include steam explosion, ammonia fibre explosion (AFEX), harsh acid–alkali and organosolv pretreatment (Mosier et al., 2005), ozone delignification (García-Cubero, González-Benito, Indacochea, Coca, & Bolado, 2009), sodium chlorite delignification (Hubbell & Ragauskas, 2010) and oxygen delignification (Sierra-Ramírez, García, & Holtzapple, 2011). However, most of these treatments involve the removal of both lignin and hemicellulose and only maintain cellulose content for further enzymatic saccharification into simple sugars. Most of these processes suffer from slow cellulose digestion, low sugar yields and severe reaction conditions (high temperature and/or high pressure), which require the use of expensive equipment. These pretreatments thus have high processing cost, have limited effectiveness, and may generate side products that can inhibit subsequent fermentations (Lau & Dale, 2010). Recent attention was directed to degrading hemicellulose into several valuable products, such as animal feed, xylitol for sugar alternatives, and prebiotics. The partial removal of lignin from lignocellulose significantly improves the subsequent enzymatic hydrolysis of cellulose and hemicelluloses to fermentable sugars (Duncan et al., 2010). Therefore, an efficient lignocellulose pretreatment process with modest reaction conditions is greatly needed to

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reduce sugar degradation, improve cellulose-hemicellulose hydrolysis, decrease operating cost and initial capital investments (Zhang et al., 2007), and increase the efficiency of pretreatment methods for industrial use (Yin et al., 2011).

Kenaf (*Hibiscus cannabinus*) is an annual plant with a woody base that grows up to 1.5–3.5 m tall. The stems are 1–2 cm in diameter. In Malaysia, the cultivation of the kenaf tree is expanding due to its short time to harvest (2–6 months). The development of agricultural practices for sustainable production holds high potential in diverse industrial areas, including paper, biofuels, automobile parts, construction and packaging materials, animal feed and environmental cleaners. Kenaf correspondingly serves as quality animal feed because of its high content of structural carbohydrate ($\approx 89\%$).

In this study, we proposed a two-stage process of alkaline–acid pretreatment to improve delignification progression in order to facilitate the enzymatic hydrolysis of hemicellulose in kenaf. The effect of the pretreatment conditions on the percentage of lignin removal was screened and investigated to achieve a high percentage of cellulose-hemicellulose polymers retained in the pretreated-kenaf substrate. To the best of our knowledge, no such work has ever been reported on lignocellulosic kenaf.

2. Materials and methods

2.1. Substrate preparation

Fresh whole kenaf stem (core + bast, 3–4 months old) was kindly supplied by a kenaf processing company in Bachok, Kelantan (North-East Malaysia), and was oven dried for 24 h (105°C) until it was a constant weight. The moisture content of the kenaf stem was approximately 10% (w/w). Dried kenaf stem with a moisture content of 10% (w/w) was then hand chopped into small pieces and ground using a mechanical grinder to obtain particle sizes of 40–60 mesh. The dried-ground kenaf stem was eventually kept in a sealed plastic container prior the pretreatment processes described below.

2.2. Chemical analysis of substrate

The initial chemical content of the kenaf stem was characterised starting with the extraction method prior to holocellulose (defined as the sum of cellulose and hemicellulose contents), alpha-cellulose, hemicellulose determination and the Klason lignin test. Ash and pectin content was determined. Dry weights were determined by oven drying a sample of the feedstock for 48 h at 100°C , according to the NREL protocol for determining total solids in biomass, LAP-001 (Sluiter & National Renewable Energy, 2008). Acid insoluble lignin content (Klason Lignin) was determined by a modified version of the method described in TAPPI T222, for acid-insoluble lignin in wood and pulp (TAPPI, T-222, 1988), by two-stage sulphuric acid hydrolysis. The chemical composition of the kenaf stem was determined before and after each pretreatment process. All analyses were carried out on a dry weight basis (Sluiter, Ruiz, Scarlata, Sluiter, & Templeton, 2010).

2.3. Screening and optimisation of 1st-stage pretreatment conditions

The dried, ground whole kenaf stem was weighed into several different shake flasks for the screening of different types of chemicals, chemical loadings, liquid-to-solid ratios, and temperatures. In the first step, eight different types of chemicals and their concentrations were screened. One-gram dry weight of solid kenaf and 3 mL of aqueous solution tested were mixed together, which was equivalent to an initial dry solid material concentration of 0.33 wt.%. The chemical pretreatments were performed at 60°C in a water bath shaker for 1.5 h. After the reaction ended, the solid residues were

washed thoroughly with distilled water several times using a sieve until neutral pH and were pressed to remove excess water. The effect of chemical pretreatment on the removal of lignin was investigated using both acid and alkaline chemicals. All of the screening processes were carried out one factor at a time (OFAT) in duplicate. The range of type of chemicals (NaOH , CaCO_3 , $\text{Ca}(\text{OH})_2$, HCl , H_2SO_4); chemical loading (w/v)% (0.5, 0.75, 0.87, 1.0, 6.0, 10.0); liquid-to-solid ratio (mL:g) (4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1); temperature, $^\circ\text{C}$ (40, 50, 60, 80, 100) were screened. The best-chosen value of the initial parameter was used for subsequent parameter screening. The time was fixed at 1.5 h for all the experiments. After every screening of each parameter, the kenaf substrate was washed thoroughly with distilled water through a sieve to remove the excess chemical bound to the pretreated substrate, and the substrate was then pressed to remove excess water.

2.4. Preparation of peracetic acid (PPA)

PPA was prepared by the reaction of acetic acid and 30% hydrogen peroxide, with a volume ratio of 1.5:1 at room temperature for 72 h. To hasten the reaction, 1.5% (w/w) of sulphuric acid was added as a catalyst (Zhao, Peng, Cheng, & Liu, 2009).

2.5. 2nd-stage pretreatment

The first stage pretreated kenaf substrate was treated with 20% (v/v) PPA in a water bath shaker at 75°C for 2 h with a liquid-to-solid ratio of 1.1 (mL:g). The substrate was then thoroughly washed with distilled water several times until neutral pH and oven dried to a constant weight. Finally, further chemical analyses were performed on the lignin, cellulose and hemicellulose remaining after the pretreatment.

The electron micrograph of the pretreated and untreated kenaf samples was taken by a scanning electron microscope (SEM). All the samples were initially pre-coated with a thin layer of nano-sized gold powder to enhance the contrast of the images.

2.6. Enzyme saccharification

The solid kenaf biomass was hydrolysed with xylanase at an initial sample concentration of 3% (w/v) in 20 mL of 50 mM sodium acetate buffer (pH 5.0). Recombinant xylanase from *Trichoderma reesei* expressed in *Pichia pastoris* was used for the hydrolysis (400 U enzyme loading). The enzymatic reactions were incubated in a reciprocating water bath shaker at 200 rpm for 24 h at 50°C . The supernatants were centrifuged and removed for sugar content analysis. The hydrolysis of hemicellulose and the enzymatic saccharification were calculated (Sharma, Kalra, & Kocher, 2004):

Hemicellulose hydrolysis (%)

$$= \frac{\text{The amount of xylose produced} \times 0.88}{\text{The amount of hemicellulose in raw kenaf}} \times 100$$

where 0.88 is the correction factor to compensate for the addition of a water molecule during hydrolysis.

2.7. Analytical methods

Total cellulose and hemicellulose in raw and various pretreated samples were determined using the American Society for Testing and Materials (ASTM) standard method. Sample recoveries after various pretreatments were determined based on dry weight. Enzymatic hydrolysis products (glucose and xylose) were determined using high performance liquid chromatography (HPLC), equipped with a Rezex RSO-Oligosaccharide Ag+ 4% guard column (60 mm $\times$ 10.00 mm, Phenomenex) in line with a

Table 1

Chemical composition of whole kenaf stem (core + bast).

Composition	% Total dry weight ^a (w/w) ± SD
Extractives	9.94 ± 0.02
Holocellulose	89.12 ± 0.02
Alpha-cellulose	37.29 ± 0.02
Hemicellulose	51.83 ± 0.04
Klason lignin	14.38 ± 0.01
Ash	4.69 ± 0.01
Pectin	15 ± 0.03

^a All the numbers are based on the average of three replicates of initial oven-dried sample. SD is the standard deviation of the replicates.

Rezex RSO-Oligosaccharide Ag+ 4% column (200 mm × 10.00 mm, Phenomenex analytical column) coupled to a Waters Associates refractive index (RI) detector, and eluted at 80 °C with deionised water flowing at 0.25 mL/min. The reducing sugar concentration was determined according to the Somogyi-Nelson method.

3. Results and discussion

3.1. Chemical composition

According to Rowell and Stout (1998), differences in the chemical composition and fibre properties of plant tissue are governed by the stage in the growing season and the type of soil used. As the harvesting period lengthens, the cellulose composition in the plant increases and crystallises. The lignocellulose structure would gradually become more resistant to degradation. Although the increase of plant age gives a positive effect on decreasing the extractive contents, it increases the lignin content and slightly decreases the holocellulose composition (Ang, Leh, & Lee, 2010) especially the amorphous hemicellulose layer which has become one of the valuable and important substrates nowadays. Hence, it may be advantageous to harvest the kenaf crop less than 5 months after planting depending on the harvesting conditions (Webber Iii & Bledsoe, 2002). Kenaf crop is usually harvested by the month of three and above due to its maturity. Chemical composition analysis carried out for 5–6 months-old kenaf showed high percentage of lignin (19.0–20.3%) and cellulose (46.0–52.1%) with low holocellulose content (75.8–78.3%) (Ibrahim, Daud, & Law, 2011; Jinshu Shi, Barnes, Horstemeyer, Wang, & Hassan, 2011). In contrast, the prematurely harvested kenaf (1–2-month-old) contained low lignin, cellulose and hemicelluloses composition. This is due to the incomplete formation of the middle lamella in the cell wall and that the parallel bundles of fibrils that were oriented as an angle with respect to the fibre axis, that gradually decreased with growth (Rowell & Stout, 1998). Previous analysis carried out for 3.5–4-month-old hardwood kenaf shows that the composition of holocellulose, cellulose and lignin are (71–89)% (31–64)% and (14–34)%, respectively (Abdul Khalil, Yusra, Bhat, & Jawaid, 2010). Therefore, whole kenaf substrate harvested at 3–4 months was used for this study due to its high hemicellulose content (up to 50%) with moderate lignin content. The initial chemical composition of the kenaf stem analysed in this study is detailed in Table 1.

3.2. Effect of pretreatment with various types of chemicals on the delignification of kenaf

The objective of the chemical screening process is to obtain the highest percentage of lignin removal while maintaining the highest percentage of both hemicellulose and cellulose in the kenaf substrate. The removal of lignin will improve the performance of further enzymatic hydrolysis by reducing condensed lignin which can adsorb protein from aqueous solutions and also by reducing non specific adsorption of the enzymes (Yang & Wyman, 2004). Residual

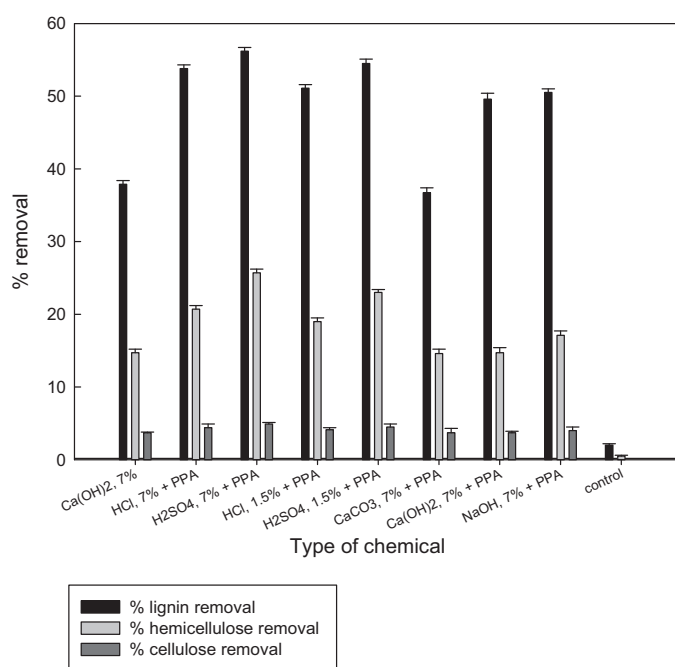


Fig. 1. Screening for the effects of different chemicals on delignification.

lignin on the surface of lignocelluloses slows hydrolysis rates and decreases digestibility (Zhang et al., 2007). Initially, several types of chemicals were screened under different pH conditions. Fig. 1 shows the results obtained after kenaf pretreatment with several types of chemicals. Ca(OH)₂ with the addition of 20% PPA exhibited the highest percentage of lignin removal (36.8%) and retained most of the hemicellulose (85.3%) and cellulose (96.3%) in the kenaf. Additionally, Ca(OH)₂ has the advantage of low cost compared with other harsh chemicals, making it a good choice for the pretreatment of kenaf. The PPA loading for the best enzymatic hydrolysis yield, considering the cost and effectiveness, was found to be 21% based on the oven-dried weight of sugar cane (Teixeira, Linden, & Schroeder, 1999). This is because both kenaf and sugar cane are categorised as hardwood species due to their similar wood structure. Furthermore, the addition of PPA to the kenaf treatment process has been shown to increase the percentage of lignin removal by 12% (as shown in Fig. 1). PPA, which specifically cleaves aromatic nuclei in lignin, generating dicarboxylic acids and their lactones, is a promising lignin-selective reagent and is recognised as a powerful oxidising agent (Sun, Tomkinson, Zhu, & Wang, 2000). Even though the PPA addition has been shown to improve the percentage of lignin removal in kenaf substrate, the percentage of delignification is still low in kenaf compared to that when sugarcane bagasse is used as a substrate (Zhao et al., 2009). This low percentage of delignification could be due to the strong fibrous structure in kenaf (Rahman, 2010), which hinders the delignification process compared to other lignocellulosic biomasses. Moreover, some studies have categorised kenaf as having both hardwood and softwood characteristics, which means the lignin layer in kenaf is higher than the common range for hardwood (Akil et al., 2011). This thick layer of lignin overcoating the kenaf substrate requires the delignification process to be more robust than that for sugar cane. Therefore, to further improve the removal of lignin in kenaf, acidic treatments using HCl and H₂SO₄ were also tested. Acidic treatments with HCl and H₂SO₄ at 1.5 and 7% (w/v), respectively, showed excellent lignin removal of more than 50% compared to the alkaline treatments. Regardless of the high percentage of hemicellulose removed (>20%), because of the intensive post-treatment required after acidic treatments and their harsh environmental effects we did not select

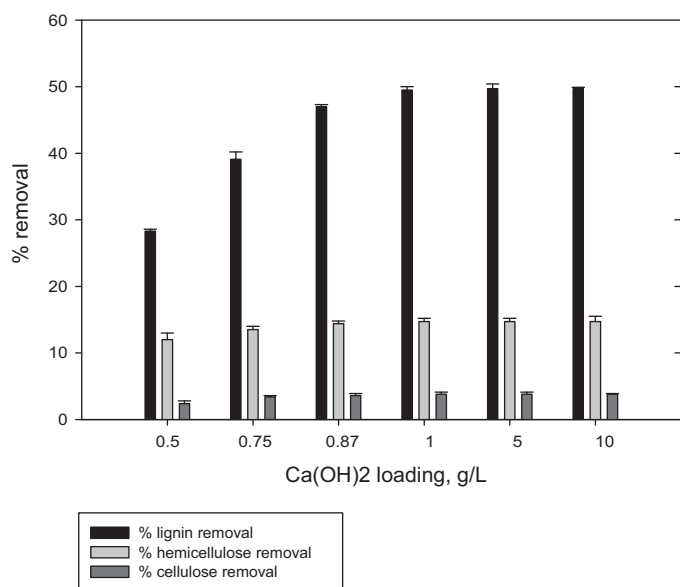


Fig. 2. Screening the effects of the $\text{Ca}(\text{OH})_2$ concentration on delignification.

acidic treatment for the pretreatment process in this study. The percentage of lignin removal in kenaf using NaOH was similar to that obtained by $\text{Ca}(\text{OH})_2$. Conversely, the percentage of hemicellulose removed was also high (17.1%). Hence, $\text{Ca}(\text{OH})_2$ was chosen as the best pretreatment method for kenaf in this study. Kenaf soaked in only distilled water was used as the control, and the results showed insignificant removal of lignin, hemicellulose and cellulose.

3.3. Effect of pretreatment with various alkaline concentrations on the delignification of kenaf

Alkaline pretreatment is well known for its efficiency in the delignification of lignocellulose as well as its environmentally friendliness in allowing further enzymatic hydrolysis (Yamashita, Shono, Sasaki, & Nakamura, 2010) with fewer by-products. Fig. 2 shows the results of lignin, hemicellulose and cellulose removal (in percentage) with different $\text{Ca}(\text{OH})_2$ concentrations ranging from 0.5 to 10 g/L prior to the addition of a fixed PPA concentration (20%). The pretreatment temperature and liquid-to-solid ratio (mL:g) were initially held constant at 60 °C and 3:1, respectively, for 1.5 h at this screening stage. A significant increase in the percentage of lignin removal, from 28.3% at 0.5 g/L to 49.5% at 1.0 g/L of $\text{Ca}(\text{OH})_2$ plus 20% PPA, was observed. However, no significant increase was observed in lignin removal at $\text{Ca}(\text{OH})_2$ concentrations above 1.0 g/L. This result is due to the very limited solubility of $\text{Ca}(\text{OH})_2$ in water, which is generally known to be 1.6 g/L at 20 °C. Thus, the concentration of 1.0 g/L tested in this study is the maximum solubility, and any additional $\text{Ca}(\text{OH})_2$ powder in the water, even with the temperature increased up to 90 °C, is not soluble (data not shown). Therefore, no further lignin removal was observed above the maximum solubility value of $\text{Ca}(\text{OH})_2$ in water. Complete lignin removal is impossible to obtain with alkaline pretreatment due to the recalcitrant structure of lignin attached to the holocellulose matrix. The main objective for this optimum $\text{Ca}(\text{OH})_2$ concentration (1.0 g/L) on kenaf is to obtain maximum degradation of ester bonds, which leads to the alteration of the lignin structure, a reduction of the lignin–hemicellulose complex, cellulose swelling, and the partial decrystallisation of cellulose (Cheng et al., 2010) within the lignocellulosic cell wall matrix. A small percentage of the hemicellulose and cellulose content was also removed from the kenaf after the pretreatment, possibly due to the partial

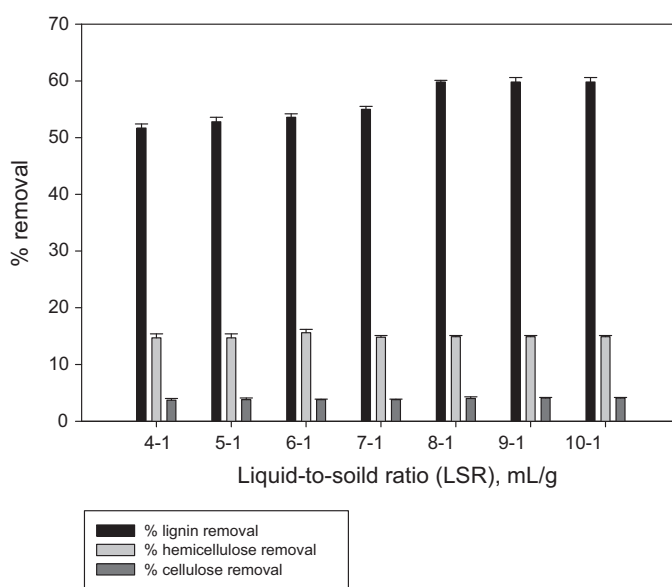


Fig. 3. Screening of the effect of the liquid-to-solid ratio on delignification.

decrystallisation of cellulose and partial hemicellulose salvation caused by alkaline hydrolysis (Zhao et al., 2009).

3.4. Effect of pretreatment with various liquid-to-solid ratios on the delignification of kenaf

The liquid-to-solid ratio of the sugarcane bagasse in alkaline solution during pretreatment was shown to have a significant impact on the yield of reducing sugar after enzymatic hydrolysis (Zhao et al., 2009). However, a number of lignocellulosic substrates showed that at increasing substrate concentrations, the corresponding yield decreased in a trend that cannot be explained by current models and knowledge of enzyme–substrate interactions (Kristensen, Felby, & Jørgensen, 2009). Therefore, screening of the kenaf solid-to-liquid ratio in this study was carried out with an alkaline concentration of 1.0 g/L, as optimised previously (Fig. 2), at 60 °C for 1.5 h. As shown in Fig. 3, increasing the liquid-to-solid ratio to 8:1 (1.0 g kenaf substrate soaked in 8 mL of buffer solution) for kenaf pretreatment provided almost 60% lignin removal, which is considered high. However, increasing the liquid-to-solid ratio above 8:1 afforded no substantial improvement in lignin removal. A liquid-to-solid ratio below 3:1 (data not shown) provided very low lignin removal (<15%). This low removal with a lower liquid-to-solid ratio probably results because there is an insufficient amount of alkaline solution within the shake flask to allow full contact of the alkaline solution with the compact kenaf biomass, thus hindering the hydrolysis of the lignin layer surrounding the substrate's surface. The amount of solid in the shake flask at a ratio below 3:1 was observed to be rigidly static; however, there was an insignificant amount of free alkaline liquid present, thus lessening the surface area of the substrate in contact with the $\text{Ca}(\text{OH})_2$ ions. Interestingly, results obtained from this study reveal that increasing the liquid-to-solid ratio from 4:1 to 10:1 provides no apparent change in the hemicellulose and cellulose removal in kenaf. Thus, the liquid-to-solid ratio only affects the removal of the lignin layer, and the holocellulose composition in kenaf is maintained. Pretreatment of kenaf with only distilled water solution at a liquid-to-solid ratio of 10:1 was carried out as a control, and the result showed minimum lignin removal from kenaf (data not shown).

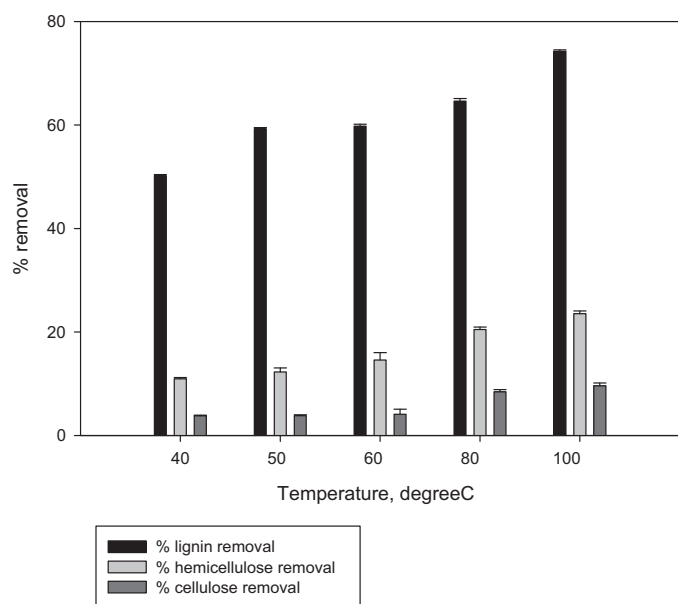


Fig. 4. Screening of the effect of temperature on delignification.

3.5. Effect of pretreatment with various temperatures on the delignification of kenaf

Increasing the temperature in pretreatments for delignification has been shown to increase the percentage of lignin removal. In this present study, increasing the temperature from 40 °C to 100 °C significantly increased the percentage of lignin removal to more than 70% in kenaf, as shown in Fig. 4. However, the temperature increase also removed a high percentage of holocellulose, with the hemicellulose being critically removed after the pretreatment process, when compared to cellulose. At 40 °C, about 50% of the lignin was removed from the kenaf within 1.5 h of pretreatment. Furthermore, at 50 °C, more than 59% of the lignin was removed with only 15% holocellulose removal. High temperatures are expected to cause the structure of the kenaf to swell and expand, eventually increasing the surface area in contact with $\text{Ca}(\text{OH})_2$. This enlargement of the inner surface area makes the carbohydrates accessible to further hydrolytic treatment (Mosier et al., 2005). A further increase in temperature to 60 °C caused 18% holocellulose removal, with only a slight increase in lignin removal. Thus, to maintain less than 20% holocellulose removal, 50 °C was chosen as the best temperature for kenaf pretreatment in this study. The lower temperature used provided better results than those in the work performed by Bøe, Johnsen, and Hoff (2012), where the highest degree of delignification obtained in Norway spruce and bagasse was ~66% using high temperature (240 °C) and organosolv pretreatment (Bøe et al., 2012). Other studies have shown that increasing the temperature from 37 to 60 °C provided only 45% lignin removal in aspen wood (Yin et al., 2011).

3.6. Carbohydrate composition after pretreatment

The percentage of holocellulose content in raw kenaf was 89.12%, corresponding to 37.29% cellulose and 51.83% hemicellulose. While the cellulose and hemicellulose contents were only reduced by 3.83% and 12.29%, respectively, after the pretreatment, the lignin content was significantly reduced from 14.38% to 5.86%. Fig. 5 shows the summary of lignin, hemicellulose and cellulose contents in raw kenaf, pretreated kenaf and their percent removal, respectively. In this study, 59.25% of the lignin content was successfully removed from kenaf, which is higher than the 45% lignin

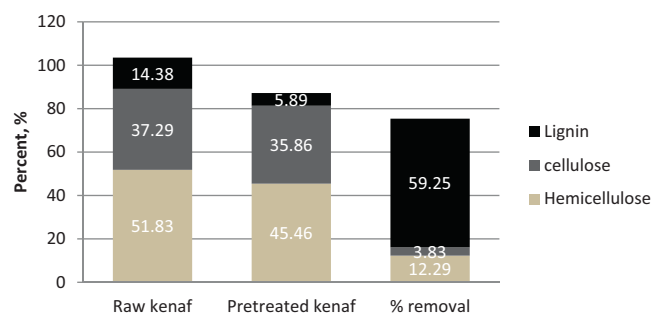


Fig. 5. Comparison of lignin, hemicellulose and cellulose content (%) in raw kenaf and pretreated kenaf and % removal of each composition after pretreatment.

removal reported from aspen wood using enzymatically generated peracetic acid (Yin et al., 2011). A few comparison related to percentage of lignin removal from several literatures is summarised in Table 2. The observed large amount of lignin removal in kenaf probably resulted from the $\text{Ca}(\text{OH})_2$ -PPA pretreatment; this combination of chemicals is believed to selectively remove lignin but preserve both hemicellulose and cellulose (Zhao et al., 2009). The 12.29% hemicellulose removal from kenaf after the $\text{Ca}(\text{OH})_2$ -PPA pretreatment is far less than the 45.61% hemicellulose removal from bamboo after steam explosion pretreatment (Yamashita et al., 2010). The results showed an improvement in the pretreatment process of lignocellulosic biomass for further enzymatic saccharification of hemicellulose to valuable sugars. However, the stage of their growing season must be taken into consideration, as xylan, ash and iron content in kenaf have been reported to decrease as the plant matures (Rowell & Stout, 1998).

The successful implementation of enzymatic hydrolysis technology on different biomasses requires competent analysis of complex biomass pretreatment streams. During the pretreatment process, a percentage of its composition will be removed depending on the pretreatment intensity. Therefore, before undergoing further enzymatic hydrolysis, remaining lignin, cellulose and hemicellulose content in the substrate has to be determined to develop an accurate enzyme mixture for its maximum degradation.

3.7. Enzymatic hydrolysis of kenaf stem before and after pretreatment

With the 59.25% of the lignin content removed, the portion of carbohydrate content in the delignified lignocellulosic kenaf generally increased. Removal of lignin has been demonstrated to improve further enzymatic hydrolysis (Chang & Holtzapple, 2000; Mooney, Mansfield, Touhy, & Saddler, 1998). In this study, the high percentage of lignin removal from kenaf after pretreatment lead to an improvement in the hydrolysis product compared to the untreated kenaf. The lignin barrier in untreated kenaf has typically caused concerns regarding three aspects of enzymatic hydrolysis: lignin blocks the accessibility of enzymes, lignin non-productively adsorbs enzymes, and the lignin-carbohydrate complex likely limits enzymatic hydrolysis. According to Jørgensen, Kristensen, and Felby (2007), cellulose and hemicellulose are embedded in lignin, and lignin acts as a physical barrier to protect cellulose and hemicellulose against microbial or chemical degradation. Furthermore, lignin has the ability to bind with enzymes, and without any delignification, up to 60–70% of the total added enzymes can be bound to lignin after complete hydrolysis of the cellulose and hemicellulose fraction in lignocellulose (Jørgensen et al., 2007).

Untreated samples of raw kenaf have very poor enzymatic hydrolysis because of the complex, compact and unopened structure of kenaf, which limits the access of enzymes to breakdown its carbohydrate polymers. The untreated kenaf (raw kenaf) produced

Table 2

Summary of the percentage of lignin removal from different hardwood and softwood substrates and pretreatment methods.

Substrate	Pretreatment	% Lignin removal	% Hemicellulose and cellulose removal	Reference
Kenaf	Ca(OH) ₂ –peracetic acid (PPA)	59.25	Hemicellulose = 12.28% Cellulose = 3.83%	This study
Aspen wood	Peracetic acid	45	ND ^a	Yin et al. (2011)
Sugarcane bagasse	NaOH–peracetic acid	96.9	Hemicellulose + Cellulose = 23.4%	Zhao et al. (2011)
Aspen wood	Peracetic acid	40	ND ^a	Duncan et al. (2010)
Corn stover	Aqueous ammonia	55–74	Hemicellulose = 15%	Kim and Lee (2005)
Maize stems	Peroxymonosulfuric acid, peroxyformic acid, peracetic acid, and hydrogen peroxide	47.1–91.3	Hemicellulose > 20%	Sun et al. (2000)
Corn stover	Batch and flowthrough	12.1–68.4	Hemicellulose = 25.4–100%	Yang and Wyman (2004)
Sugarcane bagasse	Alkali (NaOH)–peracetic acid	95.62	Hemicellulose + Cellulose = 26.18%	Zhao et al. (2009)
Forest biomass	Alkaline-sodium chlorite	20–40	ND ^a	Yu et al. (2011)

^a ND = not determined.

221.67 mg/g of reducing sugar compared to 811.33 mg/g of reducing sugar released from the pretreated kenaf. Meanwhile, regarding xylose production, HPLC analysis showed that 807 mg/g xylose was released from the pretreated kenaf while 339 mg/g xylose was released from the raw kenaf. This significant 2.4-fold increase in xylose yield is a successful hydrolysis result. Reducing sugar and xylose production were significantly higher in the pretreated kenaf compared to the untreated kenaf. Furthermore, the percentage of enzymatic saccharification obtained from the pretreated kenaf is 73.02% compared to only 19.95% obtained from the untreated kenaf (raw kenaf). Thus, a 3.7-fold was obtained by pretreating the kenaf prior to enzymatic processing. Meanwhile, the hemicellulose hydrolysis for the treated and untreated kenaf are 80.7% and 33.9%, respectively. Both the percentage of enzymatic saccharification and hemicellulose hydrolysis were significantly higher in the pretreated kenaf compared to the untreated kenaf. In this study, the pretreatment step by Ca(OH)₂–PPA provided a better enzymatic

hydrolysis environment for kenaf by maintaining high content of cellulose and hemicellulose for enzymatic hydrolysis compared to other pretreatment methods (Fig. 1). Without this step, the high sugar production would have been difficult to achieve. Thus, the result obtained has proven that the two-stage pretreatment process using Ca(OH)₂–PPA has drastically improved the enzymatic hydrolysis in kenaf by providing higher source of cellulose and hemicellulose.

In another study, high enzymatic conversion was also achieved when 15% lignin content was reduced in forest biomass, which correspond to 0.3–0.35 g/g accessible pore volume (Yu, Jameel, Chang & Park, 2011). A study carried out by Zhao, Wu, and Liu (2011) showed that the enzymatic conversion of sugarcane bagasse is 70% (Zhao et al., 2011). Meanwhile, enzymatic hydrolysis of bamboo resulted in 64.3% of saccharification (Yamashita et al., 2010). A total of 97% and 92.04% saccharification was obtained from aspen wood (Yin et al., 2011) and sugarcane bagasse (Zhao et al., 2009),

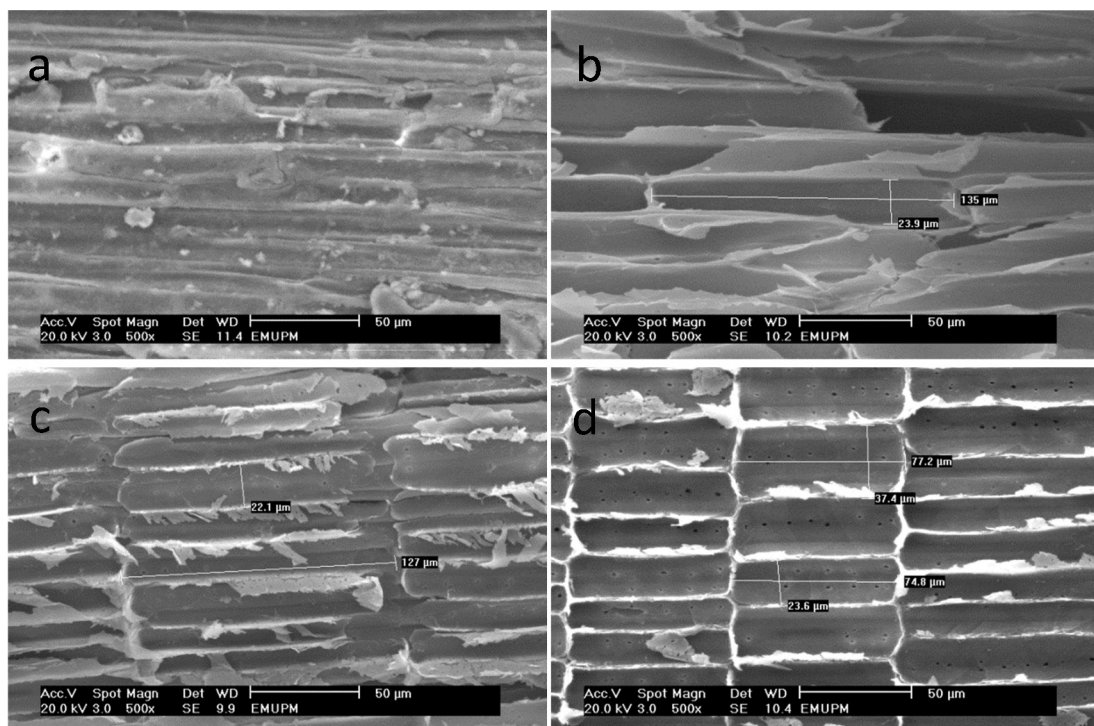


Fig. 6. Scanning electron microscopic (SEM) image of different types of lignocellulosic kenaf pretreatments at 500× magnification: (a) untreated kenaf, (b) Ca(OH)₂ treated kenaf only, (c) PPA treated kenaf only, (d) combined Ca(OH)₂–PPA treated kenaf.

respectively. The difference in percentage of saccharification in each substrate depends on several parameters including the type of enzyme used. Several parameters have to be optimised during the enzymatic hydrolysis in order to increase the hydrolysis ratio for every substrate. The important parameters to be considered are enzyme loading (Kristensen et al., 2009), reaction time, substrate loading, temperature, solid-to-liquid ratio (Zhao et al., 2009), pH (Yin et al., 2011) and etc. The non optimised condition for enzymatic hydrolysis will result in low percentage of enzymatic saccharification and low percentage of hemicellulose hydrolysis. In general, a complete enzymatic saccharification of lignocellulosic biomass requires a blend or a mixture of enzyme to act synergistically degrading the carbohydrate structure.

Fig. 6 shows the micrograph of the kenaf stem before and after pretreatment. It shows that the combination of $\text{Ca}(\text{OH})_2$ and PPA treatment on kenaf stem does effectively remove the lignin layer (outer layer) that protects the stem from any rupture. This figure clearly shows how the combined pretreatment with $\text{Ca}(\text{OH})_2$ -PPA results in higher enzymatic saccharification compared to the other three conditions. The opened-up structure of kenaf shown in Fig. 6d shows the easy access of hydrolytic enzymes for lignocellulosic hydrolysis compared to the rigid structure shown in Fig. 6a. Pretreatment using only $\text{Ca}(\text{OH})_2$ (Fig. 6b) and only PPA (Fig. 6c) managed to hydrolyse only a small part of the kenaf outer structure. Lignin removal has also been reported to improve cellulose and hemicellulose accessibility effectively by creating pores and by breaking the lignin-carbohydrate complex (Mooney et al., 1998; Yang & Wyman, 2004).

4. Conclusion

The combined $\text{Ca}(\text{OH})_2$ -PPA pretreatment proposed in this study has been demonstrated to be the best pretreatment method for kenaf, as indicated by the significantly increased percentage of lignin removal due to the destruction of the lignin layer and the preserved holocellulose content, especially hemicellulose, which facilitates further enzymatic hydrolysis. This mild pretreatment process provided better and more efficient enzymatic hydrolysis of kenaf and yielded higher C5 and C6 sugar production for further applications.

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