Characterizations of lignocellulose waxes and study of their effects on enzymatic saccharification for biofuel production

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Abstract Wax is considered to be by-products of biofuel production from lignocellulosic biomass and agricultural processing plants. Although, attempts to find applications of waxes obtained from these sources have been made, yet, little proportions of cases were successfully practical in industrial applications due to lacking of wax characterization data. This research aims to characterize wax properties of three types of lignocellulose biomass; Rice straw (RS), Sugarcane bagasse (SB) and Napier grass (NG); obtained from Soxhlet extraction technique with hexane solvent. The chemical and physical properties of waxes were analysed by Fourier Transform Infrared analysis (FT-IR) analysis and Differential Scanning Calorimeter (DSC) suggesting the similarity of properties between NG and SB waxes. To test the effect of wax on biofuel production, both dewaxed and non-dewaxed biomass were pretreated with diluted alkali and enzymatically hydrolysed by cellulase. The results showed that the combination of dewaxing and alkali pretreatment increased the cellulose proportions in biomass compared to alkali pretreatment for 21.8%, 10.1% and 16.1% in RS, NG and SB, respectively. Additionally, the combined dewaxing and pretreatment improved the saccharification yields of RS, NG and SB for 5.84%, 35.03% and 26.10%, respectively. Altogether, the existence of wax clearly showed the negative effect on biofuel production, thus dewatering process should be further considered to be included to improve the biorefining process.

1. Introduction
Due to awareness of greenhouse effect happening worldwide, Thai government and private sectors put efforts in development of renewable energy production from biomass, especially lignocellulose biomass obtained from agricultural processing plants and agricultural activities. Many types of agricultural wastes and by-products, i.e. rice straw, sugarcane bagasse, palm oil shell, cassava pulp, corn cobs, as well as dedicated energy crops, i.e. napier grass were used as raw materials for biofuel productions in a lab scale and a pilot scale in various forms, including biogas and bioethanol [1]. Based on several reports on these biofuel productions, wax that obtained as unused by-products caused the practical cleaning problem and reduced heat transfer efficiency of the reactors. Currently, the use of wax was overlooked due to its relatively low proportion in total biomass weight (0.5% - 4% w/w) [2]. Many discussions and works were brought out to find applications of these by-products, however, very few cases were success...
in industrial scale production due to lacking of economically feasible separation process, and actual potential properties of wax obtained from different biomass.

Natural wax found in plants and animals is categorized as an ester molecule consisting of long alkyl chains of fatty acids, alcohols, ketones and aldehydes. Wax and its derivatives have been applied as additives in value-added products, such as cosmetic ingredients for lip-sticks, mascara formulations, and many others. Additionally, based on an analysis of wax composition, a polyacrylamide (Policosanol) was also present, which this substance has antihypertensive properties, LDL fat reduction, triglycerides reduction, chloride reduction in the bloodstream and heart disease prevention. Regarding to the current price of policocanol as a therapeutic supplement at 620 USD/kg, it is very attractive to promote the use of wax in various applications [3]. In biofuel production side, wax has natural property as a coating agent and an anti-wetting agent. Previously, a study showed that the existence of wax reduced the ethanol yield in sorghum grain from 106.2 g/L to 86.1 g/L [4]. Thus, wax prohibits the accessibility of hydrolyze enzyme to interact with cellulose fibrils in biomass, resulting in reduction in hydrolysis efficiency, and biofuel yields ultimately.

In general, production of biofuels from lignocellulosic biomass consists of 4 main steps including pretreatment, hydrolysis, fermentation and product recovery. The critical steps in practical and economical aspects are pretreatment and hydrolysis that contribute to 40-60% of overall process cost. Many studies demonstrated that the efficiency of hydrolysis of lignocellulose could be improved by pretreatment to alter recalcitrant structure of biomass and remove hydrolysis inhibitors, such as lignin [5-9]. On the other hand, there are only 2 research works focused on the effect of wax on the biofuel production, and both works selected the sorghum plant as the model biomass [9-10]. The main purposes of this study were the chemical and physical characterization of waxes obtained from three types of lignocellulosic biomass, including Rice straw (RS), Napier grass (NG), and Sugarcane bagasse (SB). The effects of dewaxing on enzymatic hydrolysis were demonstrated by observing the released reducing sugars that subsequently converted to different forms of biofuels. The knowledge gained from this study could highlight the importance of wax in biorefining process and biofuel production.

2. Materials and methods

2.1. Extraction of plants wax

Rice straw (RS), Napier grass (NG), and Sugarcane bagasse (SB) were obtained from local farms located in the central part of Thailand. These fresh samples were dried at 80 °C in hot-air oven, then ground to pass a 1.0 mm sized-sieve and kept in the desiccators at room temperature prior to use. The wax was extracted from plants according to the procedure described by Athukorala methods [11] with minor modifications. Ground plants (five g) was extracted thoroughly with hexane (250 mL) in a Soxhlet extraction apparatus for six h [12-14]. Waxes compounds were collected after the solvent was evaporated in a vacuum oven for further analysis. Determinations of fiber compositions, including cellulose, hemicelluloses, and lignin were conducted using Van Soest methods [15].

2.2. Characterization of wax

2.2.1. Differential scanning calorimeter (DSC). Physical properties of crude plants waxes were analyzed by using Differential scanning calorimeter (TA Instruments DSC Model Q100) to measure their melting temperature (Tm). An amount of 3–4 mg of the sample was crimped in the aluminum pan and loaded along with reference pan without a sample. The heating rate was 10 o C/min, and heating temperature was carried from 30°C to 100°C.

2.2.2. Fourier transform infrared analysis (FT-IR). The structural constituents and chemical changes of functional groups of each biomass sample were analysed by using FT-IR instrument (Perkin Elmer Spectrum 1). Absorbance spectra of samples in chloroform were recorded using KBr windows. The spectra scanning ranged from 4000 to 400 cm-1 at resolution 4 cm-1.
2.3. **Alkaline pretreatment**

The pretreatment of ground plant samples and dewaxed plant samples were carried out in an autoclave machine with operating temperature at 121 °C for 30 min. Samples were pretreated with 2.0% NaOH solutions at a solid loading of 10% (w/v) in the screw-capped glass bottle. The pretreated sample was cooled down at room temperature, and the solid and the liquid fractions were separated using a funnel and a filter paper. The pretreated samples were washed several times with deionized water until neutral pH, and then the solid residue was dried in the oven at 65 °C, and was kept in dried form for further analysis and hydrolysis.

2.4. **Enzymatic hydrolysis**

The untreated, dewaxed and pretreated samples were enzymatically hydrolyzed with biomass loading at 5% w/v of dried material in 50 mM sodium citrate buffer at pH 4.8. Enzyme cocktails containing 400 μl of 2% sodium azide and 350 μl of CelluClast 1.5L® (Sigma-Aldrich, USA) and 100 μl of β-glucosidase (Megazyme, USA) per gram of tested biomass were added. The mixture was kept in a shaking incubator (150 rpm) at 45 °C for 72 h [16-18]. The total reducing sugar concentration was determined according to the 3,5-Dinitrosalicylic acid (DNS) assay using D-glucose as a standard.

3. **Results and discussion**

3.1. **Effect of dewaxing on modification of fiber compositions**

Naturally, wax deposits on plant surfaces and prevents water activities, a.k.a. aqueous alkaline solution for pretreatment in this study. NaOH pretreatment on lignocellulosic biomass was shown to remove lignin, a hydrolysis inhibitor, and thus to increases proportions of cellulose and hemicellulose contents of pretreated biomass [19]. To prove whether dewaxing has an impact on pretreatment efficiency and modification of fiber compositions, each type of biomass, including RS, NG and SB, was divided to four groups, including untreated, dewaxed, pretreated and dewaxed+pretreated samples.

**Table 1.** The contents of cellulose, hemicellulose, and lignin in RS, NG and SB after dewaxing and alkaline pretreatment.

| Samples | Treatment          | Cellulose (%)     | Hemicellulose (%) | Lignin (%)  |
|---------|--------------------|-------------------|------------------|-------------|
| RS      | Untreated          | 47.062 ± 1.682    | 25.354 ± 1.908   | 6.003 ± 1.128 |
|         | Dewaxed            | 46.967 ± 0.637    | 23.331 ± 0.164   | 10.717 ± 0.238 |
|         | Pretreated         | 55.327 ± 0.270    | 16.125 ± 3.916   | 4.278 ±0.376  |
|         | Dewaxed + Pretreated| 77.140 ± 3.139    | 8.028 ± 3.574    | 1.300 ± 0.521 |
| NG      | Untreated          | 40.416 ± 0.594    | 25.348 ±1.091    | 15.101 ± 0.608 |
|         | Dewaxed            | 38.402 ± 0.686    | 24.945 ± 1.658   | 14.751 ± 0.323 |
|         | Pretreated         | 64.780 ± 2.208    | 13.396 ± 0.917   | 2.959±0.264   |
|         | Dewaxed + Pretreated| 74.872± 0.708    | 11.693 ± 2.692   | 2.563 ± 0.376 |
| SB      | Untreated          | 57.669 ± 0.784    | 28.842 ± 0.665   | 12.381 ± 0.409 |
|         | Dewaxed            | 57.732 ± 1.461    | 25.421 ± 1.381   | 11.145 ± 0.241 |
|         | Pretreated         | 64.135 ± 0.728    | 12.501 ± 1.862   | 3.122 ± 0.06  |
|         | Dewaxed + Pretreated| 80.225 ± 0.669   | 11.324 ± 0.428   | 2.899 ± 0.206 |

After dewaxing process by Soxhlet extraction, the wax yields obtained from RS, NG and SB were 0.564%, 1.678% and 0.577% w/w of dried biomass, respectively. The fiber composition analysis, including cellulose, hemicellulose and lignin was conducted for each sample (Table 1). The results from
three types of plants showed that dewaxing did not really change the compositions of cellulose, hemicellulose and lignin compared to untreated sample. After alkaline pretreatment, the lignin content was removed and therefore cellulose was increased compared to untreated and dewaxed samples. For example, in NG, the cellulose content increased from 40.416% to 64.780%, while the lignin content decreased from 15.101% to 2.959%. This result supported the fact that alkaline pretreatment helps to remove lignin from the biomass. In case of dewaxed + pretreated sample, the cellulose contents of RS, NG and SB were further increased than pretreated samples, and the lignin contents were also more reduced (Table 1). For example, the cellulose contents of RS, NG and SB were increased to 77.140%, 74.872% and 80.225%, respectively. Altogether, the changes in fiber compositions suggested that dewaxing has the positive effect on pretreatment by uncovering the fiber complexes to interact with alkaline and water molecules.

3.2. Effects of dewaxing on enzymatic saccharification
In previous section, dewaxing was demonstrated to improve pretreatment efficiency. Likewise, in this section, the effect of dewaxing on enzymatic saccharification was assessed. To fully function, cellulase enzymes should be able to gain access to lignocellulose fibrils. The more hydrolysis interactions, the more reducing sugars were released. The contents of reducing sugars were quantitatively measured using DNS assay as described in the method section.

Comparing between pretreated and dewaxed + pretreated sample, all types of tested biomass in this study, including RS, NG and SB, showed improvement on saccharification in dewaxed + pretreated sample. The reducing sugars of dewaxed + pretreated samples of RS, NG and SB were increased for 5.83%, 35.03% and 26.10%, respectively compared to pretreated samples (Table 2). The increased yield of enzymatic saccharification could be expected to improve the biofuel yield in downstream process. This result could be explained that after wax was removed from the biomass, the accessibilities of alkaline and cellulase to cellulose fibrils were gained to remove lignin inhibitor and to hydrolyse cellulose subsequently.

| Samples            | Released reducing sugars (mg/ml) |
|--------------------|---------------------------------|
| Pretreated sample  |                                 |
| RS                 | 17.111                          |
| NG                 | 12.438                          |
| SB                 | 15.262                          |
| Dewaxed + Pretreated sample |             |
| RS                 | 18.110                          |
| NG                 | 16.795                          |
| SB                 | 19.246                          |

3.3. Physical and chemical properties of RS, NG and SB waxes
DSC was used to investigate phase transitions in the plant wax and to estimate its melting points, which is one of the most important physical property of wax. The phase transition of melting is endothermic and therefore reflects a negative heat flow. The decomposition temperature ($T_d$) was determined during DSC analysis, the maximum temperature should not exceed its decomposition point. All the waxes were found to have a $T_d$ above 100 °C, so that a temperature of 80 °C was set to be the maximum tested temperature. The thermograms for the three biomass waxes, including RS, NG and SB, were shown in figure 1. The RS, NG and SB waxes possessed very similar phase transitions as their main peaks were positioned at 77 °C.
Figure 1. DSC thermograms of three biomass waxes, including RS, NG and SB.

Figure 2. FT-IR spectra of three biomass waxes, including RS, NG and SB.

FT-IR analysis was conducted to monitor the alterations of chemical compositions and chemical interactions of substances. Extracted wax samples obtained from RS, NG and SB were analysed by using FT-IR and the absorption spectrums in the wavenumber ranging between 4000-400 cm\(^{-1}\) were observed (Figure 2). Similar to DSC analysis, three types of waxes also have similar spectral patterns, although the magnitudes of absorption peaks were differentiated. It could be seen that NG and SB waxes had similar magnitudes of peaks suggesting the similar chemical properties of these two waxes, which were in well agreement with saccharification results in Table 2. The bands at 2918 cm\(^{-1}\) and 2849 cm\(^{-1}\) were assigned to the asymmetric and symmetric CH\(_2\) stretching vibrations, which were wax characteristic for an aliphatic fraction [20]. The band at 1713 cm\(^{-1}\) was represented to carbonyl stretching in fatty acids. The band at 1463 cm\(^{-1}\) was referred to the bending of CH\(_2\) in lignin or wax. The bands at 1600 and 1510 cm\(^{-1}\) were characteristic for lignin fraction [21]. The bands at 1377, 1170, 1130, 1055 and 999 cm\(^{-1}\) were also matched to lignin [22]. These FT-IR peaks indicated the presence of fatty acid ester and lignin in wax.

3.4. Structural constituents and chemical changes
The chemical changes and structural constituents of A) untreated, B) dewaxed, C) pretreated and D) dewaxed+pretreated samples of RS, NG and SB were investigated by using FT-IR spectroscopy (Figure 3). Comparing between untreated and dewaxed samples in three types of biomass, the patterns and magnitudes of peaks were similar suggested that dewaxing did not engage in any chemical alteration. This observation was agreed well with the analysis of fiber composition in Table 1 that dewaxing did not cause any change in contents of cellulose, hemicellulose and lignin.

Figure 3. FT-IR spectra of (a) RS, (b) NG and (c) SB biomass, including A) Untreated, (B) Dewaxed, (C) Pretreated and (D) Dewaxed+Pretreated.

On the other hands, NaOH pretreatment caused the reductions of peak magnitudes at 1,734 cm\(^{-1}\), 1,432 cm\(^{-1}\), 1,376 cm\(^{-1}\), 1,247 cm\(^{-1}\), 1,162 cm\(^{-1}\), 1,059 cm\(^{-1}\) and 719 cm\(^{-1}\) (Figure 3). It could be explained the presence of NaOH in biomass dissolve the cellulose, and split and reform of new inter- and intramolecular hydrogen bonds between cellulose fibrils [23]. Moreover, the magnitude of bands at 1,377 cm\(^{-1}\) and 1,463 cm\(^{-1}\), represented CH\(_2\) scissoring motion in crystalline cellulose [24] were reduced in NaOH-pretreatment sample and this reduction was even bigger in dewaxed+pretreated sample. This result indicates that NaOH pretreatment decreased the crystalline cellulose and exposed more surface areas of amorphous cellulose for enzymatic interactions that subsequently leaded to enhancement of reducing sugar productions. Furthermore, a band at 897 cm\(^{-1}\), representing the \(\beta\)-glucoside linkage belonging to cellulose and hemicellulose, was more intense in dewaxed+pretreated sample than the others, which correlated to the fiber composition analysis in Table 1. Ether bonds at 1,250 cm\(^{-1}\) peak disappeared after pretreatment meaning that after pretreatment, these linkages between lignin and carbohydrates were disrupted, e.g. loss of lignin [25]. The peak at 1,739 cm\(^{-1}\), representing aromatic C=C stretching of the aromatic ring of lignin [26], was diminished. It is firmly concluded that the
disappearance of lignin peaks at 1,236 cm\(^{-1}\) and 1,650 cm\(^{-1}\) were due to removal of lignin coating on the surface of cellulose fibers of pretreated samples [27].

4. Conclusion
In this study, dewaxing process of RS, NG and SB showed clear improvement of pretreatment efficiency and enzymatic saccharification. Although the analysis of physical and chemical characters of waxes by using FT-IR and fiber composition analysis suggested the similarity of untreated and dewaxed samples, the combination of dewaxing and pretreatment showed clear modification of biomass as following mechanisms. First, combined dewaxing and pretreatment removed lignin inhibitor, and increased the proportion of cellulose in biomass. Second, it altered the inter- and intramolecular interactions of cellulose fibril complexes. Third, it transformed cellulose from the crystalline to amorphous structure. The synergistic effects of dewaxing and pretreatment in promotion of biofuel productions from different biomass demonstrated in this study could help to establish a feasible biorefining process in the future.

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