The combined pretreatment using ultrasonication with different chemical pretreatments to enhance lignin production and enzymatic hydrolysis efficiency

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Abstract. Sugarcane bagasse is a lignocellulosic biomass and an abundant agricultural residue in Thailand. Therefore, it has been studied to maximize and convert it into high value bioproducts. However, due to its complex structure, pretreatment processes are important for enhancing the efficiency of component recovery. This study presents the combined ultrasonication and chemical pretreatment methods, specifically alkaline pretreatment and deep eutectic solvent (DES), to investigate their effects on lignin yield and enzymatic hydrolysis efficiency. The study examined the use of ultrasonication with 1M sodium hydroxide (Power rate 60%, 30 min) and the process sequence between ultrasonication before alkaline pretreatment (UBA) and ultrasonication after alkaline pretreatment (UAA) under the same conditions. Results showed that UBA obtained the highest lignin yield of 94%. The enzyme hydrolysis efficiency has higher reduced sugar yields from 44 to 189-190 mg/g biomass. In terms of DES pretreatment using a mixture of choline chloride-ethylene glycol-lactic acid (ChCl-EG-LA), different solid-liquid ratios 1:20 (20DES) and 1:40 (40DES). Lignin yields are 30% and 12%, respectively, which were less than those of ultrasonic combined with alkaline. The enzyme hydrolysis efficiency of 20DES has reducing sugar yields 186.79 mg/g biomass ,while 40DES has highest reducing sugars yields 186.79 mg/g biomass.

1 Introduction

The globe is currently dealing with the issue of rising greenhouse gases, which are causing global temperature increases and climate change, primarily because of human activities that consume fossil fuels. As a result, alternative energy sources have become an important role in replacing fossil fuels because it is clean energy that is never exhausted and does not pollute the environment. Agricultural waste materials are one of the interesting alternative energy sources represents a sustained renewable, widespread and low-cost source [3].

Sugarcane bagasse (SCB), a lignocellulosic biomass and agricultural residue from the sugar industry, is abundantly available in Thailand. It is commonly used as fuel in power generation, animal feed, or for composting. Lignocellulosic biomass mainly consists of three natural polymers: cellulose, hemicellulose, and lignin. These components can convert it into high-value products through a biorefinery process [5]. This process resembles petroleum refining, except it uses biomass as the feedstock. It allows for the fractionation of biomass into its key components to produce high-value products such as bioethanol from cellulose and hemicellulose, or natural adhesives and biochemicals from lignin.

Due to the complex molecular structure of lignocellulosic biomass, some factors make biomass recalcitrant like degree of lignification, heterogeneous structure, and complex nature of the cell wall. Hence, the pretreatment process becomes a crucial role in enhancing the accessibility of each component and improving the overall yield that direct conversion to bioethanol is often inefficient. Pretreatment methods are generally categorized into four types: physical, chemical, physicochemical, and biological [1].

Among these, alkaline pretreatment is widely employed due to its simplicity and efficiency in removing lignin, hemicellulose, and reducing cellulose crystallinity. That makes the biomass more amenable to downstream processing. However, it often requires large amounts of chemicals, raising environmental concerns [2].

To solve this problem, Deep Eutectic Solvents (DES) have emerged as an eco-friendly alternative. Its green solvents, biodegradability, low toxicity, easy to synthesize, and low cost of production [18]. DES are mixtures comprising hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) that form a eutectic mixture with lower melting points than their constituents [14]. The types of DES depend on different types of HBDs, including polyalcohol, acids, alkaline, and ternary DESs. Ternary DESs constructed with HBA, acid, and different contents of diols as HBD inhibited the formation of lignin and the quality of the lignocellulosic components

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[16]. Though their high viscosity can affect the efficiency of biomass decomposition.

To further enhance chemical pretreatment effectiveness, integration with physical methods such as ultrasonication has gained interest. Ultrasonication generates cavitation bubbles and shear forces in the liquid medium, which promote better penetration of solvent molecules into the biomass structure, thereby improving pretreatment efficiency [1].

M. M. Kininge and P. R. Gogate investigated the enhancement of sugarcane bagasse delignification by comparing the performance of an ultrasonic horn and an ultrasonic bath. It was found that the efficacy of the ultrasonic horn is superior compared to the ultrasonic bath. The optimized condition was 1 M sodium hydroxide at biomass loading ratio 1:20 (1 h, 70°C, power 100 W and 70% duty cycle) yielding 67.30% as the delignification extent, which resulted of delignification 67.30% [12] but ultrasonic horn only suitable for small quantity ultrasonic probes are therefore attractive because they have high power and can be further upscaled in the future [9].

This research focuses on the combined pretreatment using ultrasonication with two different chemical pretreatments, specifically alkaline 1 M sodium hydroxide, with the process sequence between ultrasonication before alkaline pretreatment (UBA) and ultrasonication after alkaline pretreatment (UAA) under the same conditions and DES (ChCl-EG-LA), different solid-liquid ratios 1:20 (20DES) and 1:40 (40DES). The objective is to investigate their effects on lignin yield and enzymatic hydrolysis efficiency.

2 Materials and methods

2.1 Materials

Sugarcane bagasse (SCB) was supplied by sugar industry, Thailand. After drying overnight in an oven at 60°C, SCB was milled and sieved to mesh size of ≤ 0.5 mm. and stored in a desiccator. The chemicals used in this study consist of sodium hydroxide (NaOH, purity 99%), sulfuric acid (H_2SO_4, purity 98%), Choline chloride (ChCl), Ethylene glycol (EG), lactic acid (LA), Ethanol, the commercial enzyme solution CTech 2 (cellulase), sodium acetate.

2.2 Combined pretreatment of SCB

2.2.1 Combined pretreatment of SCB using ultrasonication-alkaline pretreatment

The SCB was combined with 1 M sodium hydroxide (NaOH) at a solid-liquid loading of 5% (w/v). In which the first sample (ultrasonication after alkaline pretreatment, UAA) was heated on a hot plate at 80°C and stirred for 30 minutes at 250 rpm before being conducted with an ultrasonic probe (JY98-IIIDN, USA) on-time of 2 s and an off-time of 4 s at a power rate 60% for 30 min. The second sample (ultrasonication before alkaline pretreatment, UBA) was under the same conditions but performed with ultrasonication before being heated on a hot plate.

After the pretreatment liquid and solid fractions were separated by using a Vacuum pump. The solid residue was washed with distilled water and dried overnight at 60°C. The liquid fractions or black liquor were precipitated with 6 M H_2SO_4 dropwise while stirring until pH 4. The solution was left stirring for 1 hour until final pH 4. The lignin precipitate was collected by centrifugation at 5000 rpm for 20 minutes. Lignin was subsequently washed with DI water and dried overnight at 50°C.

2.2.2 Combined pretreatment of SCB using ultrasonication-DES

The ternary deep eutectic solvent (TDES) was synthesized from ChCl: EG: LA at a molar ratio is 1:1:2. The NATDES was stirred at 500 rpm at 80°C until it turned homogeneous.

The SCB was combined with NATDES at different solid-liquid loadings of 1:20 and 1:40 (w/w) and exposed to an ultrasonic probe with an on-time of 10 s and off-time of 5 s at a power rate of 50% for 20 min. Soxhlet extraction at 120°C and stirred for 3 h at 500 rpm. The solid residue was washed with a mixture of water and ethanol until a neutral pH was achieved and dried overnight at 60°C. DI water was added to the filtrate afterward to precipitate lignin. The precipitated lignin was collected after centrifugation at 5000 r/min for 20 min. Lignin was subsequently washed with DI water and dried overnight at 50°C.

2.3 Enzyme hydrolysis

Enzymatic hydrolysis of untreated and pretreated SCB was conducted using commercial enzymes CTech 2 (Cellulase). The enzyme activity was estimated to be 161.03 FPU/ml. The enzyme loading of 20 FPU/g substrate in sodium acetate buffer (50 mM, pH = 5) in assey tube 50 ml. All tubes were mixture in shaking incubator (JSSI-100C) with 200 rpm at 50°C for 72 h. Stop the enzyme reaction by boiling in water at 100 for 5-10 min. Withdraw 2 ml of supernatant into the eppendorfs centrifuge at 1300 rpm for 5 min and collect the supernatant into HPLC vial to determine the sugar concentration.

2.4 Characterization of SCB

2.4.1 Quantification of total lignin and carbohydrates

The components of SCB, i.e., cellulose, lignin, hemicellulose, extractives, and ash, were determined through compositional analysis by the standard protocol of the National Renewable Energy Laboratory (NREL) [4]. The lignin removal % was determined with the following equation:

$$\text{Lignin removal (\%)} = \left[1 - \frac{\%\text{Lignin}_{\text{pretreated SCB}} \times W_{\text{solid recovery}}}{\%\text{Lignin}_{\text{untreated SCB}} \times W_{\text{untreated SCB}}}\right] \times 100 \quad (1)$$

The acid soluble lignin was determined through the UV-Visible spectrophotometer (Genesys 10S, Thermo Scientific) absorbance measurement at 280 nm.

The carbohydrate composition of SCB was determined by high performance liquid chromatography (HPLC) using a BioRad Aminex HPX-87H column and 0.005 mol/L sulphuric acid as mobile phase.

2.4.2 Fourier transform infrared spectroscopy (FTIR)

The untreated, pretreated SCB and lignin samples were characterized by FTIR (Nicolet iS50, Thermo Scientific) scans within the wavenumber range of 4000 to 400 cm^{-1}, employing a spectral resolution of 4 cm^{-1}. This was conducted utilizing the attenuated total reflection mode (ATR).

2.4.3 X-Ray (XRD)

The untreated and pretreated SCB were characterized by XRD to determine the crystallinity between angles 2θ range of 5 to 51° at 0.04 step. The crystallinity index (CrI) was calculated by equation:

$$\text{CrI (\%)} = \left[\frac{I_{002} - I_{am}}{I_{002}}\right] \times 100 \quad (2)$$

I_{002} : The crystalline region $2\theta = 22°$ and I_{am} : The amorphous region $2\theta = 18°$.

3 Results and discussion

3.1 Composition analysis of SCB

Untreated SCB consists of cellulose 35.95%, hemicellulose 21.07% and lignin 26.89% as shown in Table 1. Generally, SCB is composed of approximately 35-45% cellulose, 26-35% hemicellulose, and 11-25% lignin [5]. In comparison, cellulose was within the general range, while hemicellulose and lignin were lower and higher than the range. This is because the composition of SCB varies from diverse sources and strains. Pretreated SCB can be seen that the composition of cellulose increased from the untreated SCB, while the hemicellulose and lignin decreased, which is in accordance with the main objective of pretreatment to decrease hemicellulose and lignin and increase the cellulose content [7].

Ultrasonication-alkaline pretreated SCB in this study produced a relatively high lignin yield, with UBA achieving 94.37%, which was higher than UAA at 72.38%. The calculation of lignin removal, based on equation (1), predicted a value of approximately 71%, which was lower than the lignin yield, indicating that the lignin product still contains other extractive substances. In previous studies, I. F. Mota et al. pretreated SCB with 6 % w/w NaOH for 1 h at 90°C, achieving 72.8% lignin removal [8]. The lignin removal reported in that study was comparable to the present work employing combined pretreatment, despite the lower concentration of the chemical used. Because the intense shear forces resulting from ultrasonication can modify SCB surface structure, which is expected to enhance the adsorption of solvent and allow better access of solvent to SCB. But of course, if the ultrasonic pretreatment sequence is combined after the alkaline pretreatment (UAA), the amount of lignin yield will be lower as seen in Table 1, since NaOH plays a crucial role in the solubilization of lignin, as the basicity of the solvent [15], and ultrasonication may not be able to assist in breaking down the structure.

Ultrasonication-DES pretreated with difference solid-liquid ratios of 1:20 (20DES) can obtain more lignin yield than 1:40 (40DES), which were 30.63% and 11.9%, respectively, though were lower than those obtained from ultrasonication-alkaline pretreated. The lignin removal was higher than the corresponding lignin yield, which were 63.33% and 76.98%, respectively. The relatively low lignin yield can be attributed to the dissolution of lignin into the solution, as the presence of diols acting as HBD inhibited lignin reformation [16].

From the lignin removal, 40DES achieved the highest lignin removal but exhibited the lowest lignin yield, while ultrasonication-alkaline pretreatment had less lignin removal; however, UBA gave the highest lignin yield.

Table 1. Effect of pretreatment on composition of untreated and pretreated SCB.

Pretreatment conditions	Biomass composition			Solid recovery (%)	Lignin removal (%)	Lignin yield (%)
	Cellulose (%w/w)	Hemicellulose (%w/w)	Lignin (%w/w)			
Untreated SCB	35.95	21.07	26.89	-	-	-
UAA SCB	68.10	18.27	15.06	60	71.44	72.38
UBA SCB	70.33	16.17	14.59	52.35	71.6	94.37
20DES SCB	63.73	9.77	15.40	63.33	63.74	30.63
40DES SCB	70.95	10.99	14.20	43.6	76.98	11.9

3.2 FTIR

FTIR was performed on untreated and pretreatment SCB to analyze the functional groups, as shown in Figure 1. The band at 3200-3500 cm^{-1} represents O–H stretching of intramolecular hydrogen bond and the peak at 2890 cm^{-1} indicates the stretching vibration of the C–H functional groups in cellulose [14-15]. The peak at 1730 cm^{-1} represented the C=O stretching vibrations of the acetyl group of hemicellulose or carbonyl ester of p-coumaric lignin [11]. The peak at 1160 cm^{-1} represented the C–O–C bonds of carbohydrate [6]. The peak at 1030 cm^{-1} represented the C-O bonds of cellulose, and the intensification of this peak after combined pretreatment translated to a higher composition of cellulose [10]. The peak at 893 cm^{-1} represented polysaccharide content, particularly cellulose, due to the removal of lignin and hemicellulose [6]. Pretreated SCB showed higher concentrations than the untreated SCB, especially the ultrasonic-DES pretreatment showed higher peak intensities of 3334, 1730, 1160, and 893 cm^{-1} because it was a mild pretreatment process that still retained the functional groups of lignin and hemicellulose in structure.

In terms of lignin samples obtained from the combined pretreatment from Figure 2. the peak of lignin from combined pretreatments looked like the standard of lignin. The band 1508-1600 cm^{-1} represented the aromatic skeletal C—C linkages from the lignin aromatic ring [15]. The peak at 1458 cm^{-1} represents the C-H deformation and aromatic vibration of lignin [6]. The peak at 1420 cm^{-1} is C=C stretching with C-H in plane deformation aromatic skeleton. The peak at 1138 cm^{-1} is C-H in plane aromatic deformation G-ring, which was observed in the ultrasonic–alkaline. The lignin obtained from ultrasonic-alkaline and ultrasonic-DES pretreatment are different types of lignin due to different peak characteristics. Lignin from ultrasonic-DES has peak intensity of 1597 cm^{-1} which is lignin aromatic ring and 832 cm^{-1} representing polysaccharide content, particularly cellulose.

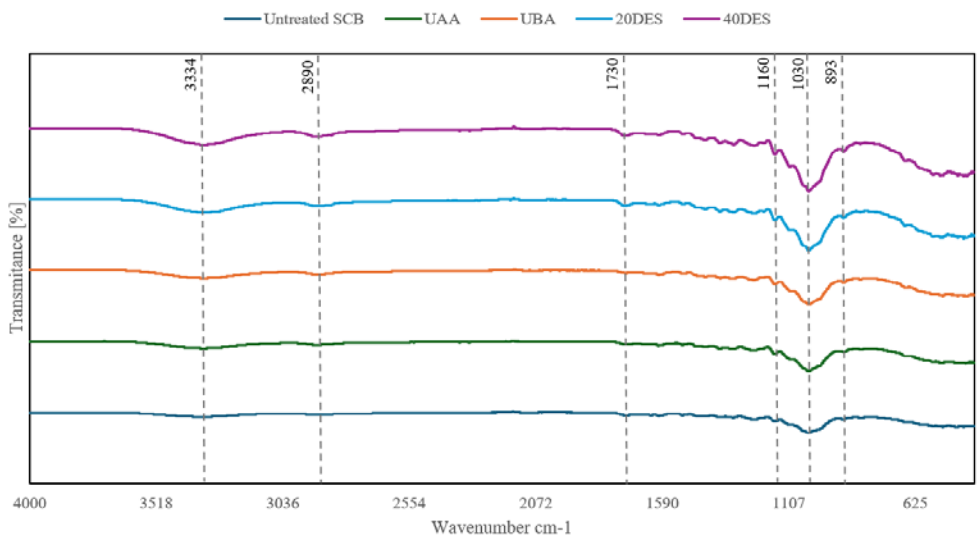

Fig. 1. FTIR spectra of raw and SCB pretreatment.

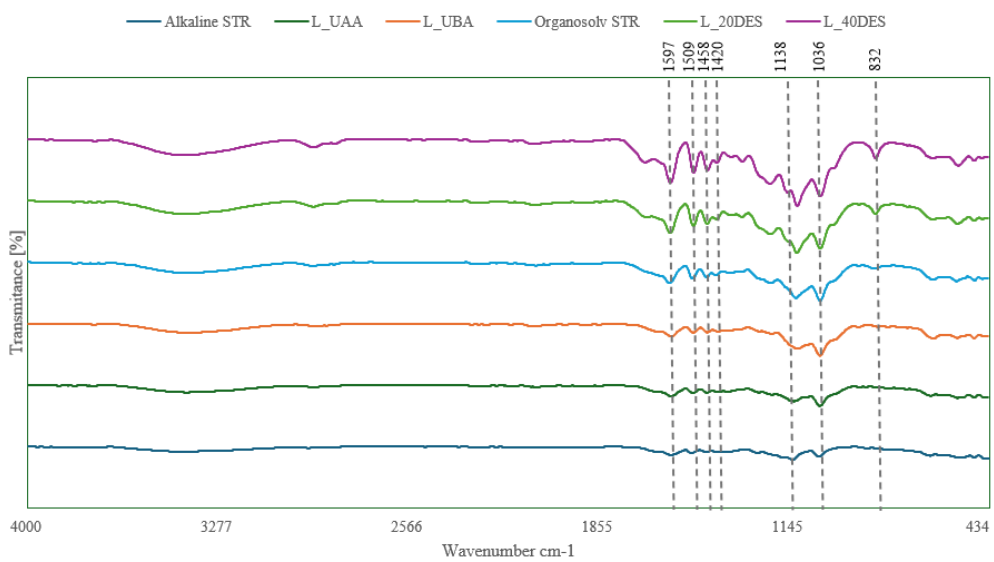

Fig. 2. FTIR spectra of standard lignin and lignin from combined pretreatment

3.3 XRD

The XRD analysis results of untreated and pretreated SCB are shown in Figure 3(a). According to equation (2). observed the CrI shown in Figure 3(b) of untreated was 24%, while the CrI of UBA, UBB, 20DES and 40DES were 44%, 51%, 49% and 56%. After pretreatment, the CrI value increased because the pretreatment removed the amorphous lignin, resulting in higher crystallinity, which affects the enzymatic hydrolysis [12].

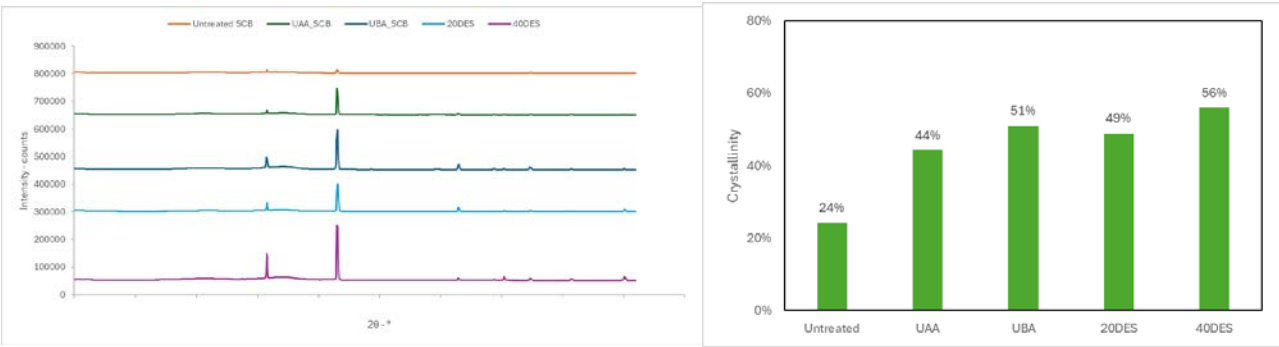

Fig. 3. (a) XRD and (b) crystallinity of untreated and pretreated SCB.

3.4 Enzymatic hydrolysis

Following enzymatic hydrolysis, the comparison among untreated, UAA, UBA, 20DES, and 40DES, as shown in Figure 4, revealed reducing sugars yield of 44.01, 189.44, 190.48, 186.79, and 194.46 mg/g biomass, respectively. The results clearly demonstrate that reducing sugars yield increased after pretreatment, consistent with the CrI obtained from XRD analysis. Moreover, the type of pretreatment influenced the composition of sugars released. Ultrasonic–alkaline pretreatment produced three sugars—glucose, xylose, and arabinose—with glucose at approximately 143–150 mg/g biomass. In contrast, ultrasonic–DES pretreatment produced two sugars, glucose and xylose, with 40DES achieving the highest glucose yield at 162 mg/g biomass.

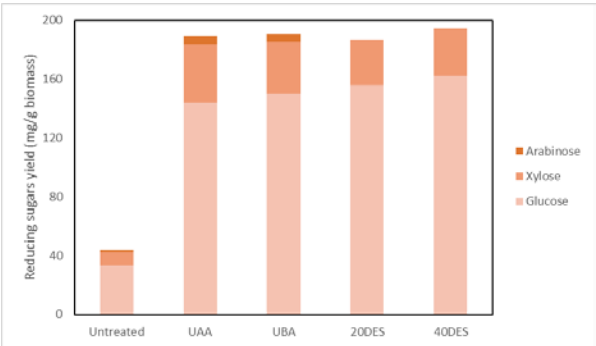

Fig. 4. Reducing sugars yield of untreated and pretreatment SCB.

4 Conclusions

The combined pretreatment using ultrasonication with different chemical pretreatments affected the lignin yield and enzyme hydrolysis efficiency. In terms of lignin yield, UBA achieved the highest yield at 94%. That means the sequence between ultrasonication before and after alkaline pretreatment had a significant effect on lignin yield. Nevertheless, lignin removal calculations suggested that the extracted lignin required further purification to eliminate impurities before utilization. In the part of ultrasonication-DES, lignin yields were less than 50%, attributable to the selected type of DES inhibiting lignin formation and causing most of the lignin to remain dissolved in solution. However, the solid-liquid ratio also affects lignin yield and using different chemicals that impact the type of lignin yield. Regarding enzymatic hydrolysis, pretreatment enhanced reducing sugar yields, with 40DES producing the highest yield at 194.46 mg/g biomass. Future studies should focus on analyzing the structural properties, identifying the most suitable agent for ultrasonic–DES to maximize lignin yield, evaluating the techno-economic analysis, and environmental impacts.

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