1. Introduction

Ethanol production from lignocellulose is attracting the attention of biomass researchers because lignocellulose do not compete with food crops, and exists abundantly1)–4). Topics that have been the focus of research in recent years include pretreatment5),6), enzyme development7), production or detoxification of fermentation inhibitors8),9), development of ethanol producing microorganisms10) and reactor setup11), and system analysis and development12),13). In the ethanol production process, promoting cellulose hydrolysis to produce glucose is a challenge. Due to its rigid crystalline structure, enzymes are only able to hydrolyze cellulose at a very slow rate. To circumvent this problem, two-stage hydrolysis is conducted, where hydrothermal pretreatment is followed by enzymatic hydrolysis and ethanol fermentation in order to compare their reaction characteristics. The effect of hydrothermal pretreatment temperature was studied. Furfural, acetic acid, formic acid, and vanillin were studied in the context of being fermentation inhibitors. Differences in glucose production were observed when different feedstocks were used in the hydrothermal pretreatment step, whereas both inhibitor production and its effect on fermentation were the same regardless of feedstock.

Keywords
Biomass, Ethanol, Eucalyptus, Empty fruit bunch, Inhibition, Hydrothermal pretreatment

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Petchpradab et al.15) employed a reaction network to explain the changes observed during rubber wood treatment. They observed that without treatment, the enzymes were unable to hydrolyze cellulose. However, after hydrothermal pretreatment, the same enzymes were able to convert part of the cellulose into glucose. The phenomenon occurring in hot, compressed water should be the combined effects of lignin and hemicellulose removal, reduction in crystallinity, and hydrolysis itself. The authors assumed this change to be a first order reaction. The reaction network is shown in Fig. 1, where C denotes original cellulose which is not hydrolyzed by a chosen enzyme, C* denotes cellulose which can be hydrolyzed by the enzyme, G denotes glucose, and D denotes further decomposed products from glucose. They succeeded to determine the reaction rate constant for each reaction in the network so that the model could reproduce the experimental results. However, it is unclear whether these reaction rate constants can be applied to other feedstocks. It has also been reported that hydrothermal pretreatment produces fermentation inhibitors such as furfural16). However, it is not clear whether the amount of fermentation inhibitors produced is the same for all varieties of biomass. The information regarding the dependence of feed-
stock species on hydrothermal pretreatment characteristics is essential for reactor design and development. Comparison of the two different biomass species in terms of their hydrothermal pretreatment characteristics should provide us with a potential starting point in this regard. The purpose of this study is to compare the hydrothermal pretreatment characteristics of eucalyptus and empty fruit bunch (EFB) using the same reaction apparatus and procedure so that effect of feedstock species can be elucidated.

2. Experimental

2.1. Overall Procedure

The lignocellulosic feedstocks, eucalyptus and EFB were hydrothermally pretreated. The product was then saccharized by the enzyme, and further fermented by yeast for ethanol production. Detail of the each step is shown in the following subsections.

EFB was collected from an oil palm mill (FELCRA Nassaruddin Bota) at Perak. Eucalyptus was obtained from the sawmill which was located in Nakhonnayok prefecture, Thailand. The composition of each feedstock is shown in Table 1. The particle size is a little bit different between EFB and eucalyptus, but it is known that the effect of particle size does not affect the reaction characteristics so much.

2.2. Hydrothermal Pretreatment

The reactor was an autoclave with inner diameter of 8 cm and depth of 20 cm. A glass beaker was placed in the autoclave, filled with a specific amount of water and feedstock, and heated. After reaching the target temperature and maintaining it for 10 min, the autoclave was cooled down. The content was then recovered and the glucose concentration was analyzed using high-performance liquid chromatography (HPLC). The concentration of fermentation inhibitors was also determined with furfural and acetic acid analyzed using a gas chromatograph apparatus equipped with a flame ionization detector (GC-FID). Formic acid and vanillin analyzed using HPLC (LC-20, Shimadzu) equipped with an RI detector and gold amino column (Thermo Scientific) operated at 40 °C with 80 % acetonitrile mixture of distilled water as a mobile phase at a flow rate of 1.2 mL/min. The GC/FID analysis has been carried out with an Shimadzu model GC-2014. The capillary column that has been selected for this analysis was BPX5 with 30 m, 0.25 mm, and 0.25 mm of its length, internal diameter and film thickness, respectively. The operating parameters were set as follows. The oven temperature was held at 35 °C for 2 min. Then, the temperature was raised to 250 °C with the heating rate of 20 °C/min and held for 20 min. Meanwhile, the injector and detector temperatures were set constant at 280 °C. Helium with nitrogen of 99.999 % was used as a carrier gas. The flow rate of the purified helium was set to 1.5 mL/min. After all the set temperatures were reached and the GC/FID system was ready for sample injection, 0.4 µL of the sample was injected into a HP5 fused silica (5 % phenyl polysilphenylene-siloxane) of the BPX5 capillary column. The split ratio was set to 60 and the total analysis time was 32.75 min. The experimental conditions are shown in Table 2.

2.3. Enzymatic Hydrolysis

Buffer fluid and enzyme (Cellulase from Trichoderma reesei ATCC 26921: Sigma-Aldrich) were added to 100 mL of hydrothermal pretreatment product. This mixture was shaken for 48 h (our unpublished previous study showed that 48 h should be sufficient time to allow for hydrolysis of the product of hydrothermal pretreatment), and the change in glucose concentration was determined by HPLC. Table 3 shows the conditions for this enzymatic hydrolysis.

2.4. Ethanol Fermentation

Yeast (Saccharomyces cerevisiae, Sigma-Aldrich Type II) was added to 150 mL of the enzymatic hydrolysis product and this mixture was then shaken in order to promote ethanol fermentation. After 54 h, the product was analyzed for glucose concentration which has not been consumed. The conditions for ethanol fermentation are shown in Table 4.

3. Results and Discussion

3.1. Glucose Production by Hydrothermal Pretreatment

Figure 2 shows the change in the glucose yield with treatment temperature, and clearly indicates that glucose yield initially increases with temperature until...
reaching a maximum; further increase in temperature will reduce the yield. This result is reasonable, considering that hydrolysis of cellulose is enhanced with increasing temperature, but further decomposition takes place at higher temperatures. However, the amount of glucose obtained is much higher for eucalyptus than for EFB, implying that cellulose in eucalyptus is hydrolyzed more easily. This difference may be caused by the difference in cellulose crystallinity or degree of polymerization, but it is beyond the scope of this study.

3.2. Effect of Hydrothermal Pretreatment on Enzymatic Hydrolysis

Figure 3(a) shows the change of glucose yield over time during enzymatic hydrolysis for EFB. The yield of glucose increases with time during enzymatic hydrolysis, indicating the effectiveness of hydrothermal pretreatment on the cellulose treatment. The glucose yield increases more for hydrothermal pretreatment at lower temperatures.

Figure 3(b) shows the change of glucose yield over time during enzymatic hydrolysis for eucalyptus. Unlike EFB, the increase in glucose yield over time is quite limited, and almost no cellulose is hydrolyzed enzymatically. This would seem to indicate that the behavior of cellulose is completely different in EFB and eucalyptus. The reason for this difference is not clear at this stage, but higher hemicellulose content and lower cellulose content in eucalyptus may play a role.

3.3. Fermentation Inhibitor Production via Hydrothermal Pretreatment

Only furfural, acetic acid, formic acid, and vanillin were observed in this study. Figure 4 shows the inhibitor concentration after hydrothermal pretreatment except vanillin. The yield of furfural increased with treatment temperature, with peak concentration greater than 1 g/kg at 215 °C. Further increases in temperature resulted in a sudden reduction in furfural yield. Increase in temperature results in fast reaction rate, and thus improvement of the yield. However, furfural is known to decompose when treated longer in hydro-
thermal condition\(^{19)}\), which explains the decrease in furfural yield when the temperature is increased past a certain point.

Meanwhile, the yield of acetic acid monotonously increases with temperature, to 1-1.6 g/kg at 230-240 \(\degree C\). Acetic acid is known to be relatively stable under hydrothermal conditions, and is often observed in supercritical water oxidation processes, so its accumulation is predictable. It is also known that acetic acid inhibits yeast growth, but does not inhibit ethanol fermentation\(^{22)}\). Thus, its effect on ethanol production can be limited.

Like acetic acid, formic acid concentration changes monotonously with temperature. In previous studies, furfural, acetic acid, and formic acid affected the yeast growth at 0.5, 0.9, and 0.7 g/kg, respectively\(^{23)}\). Thus, the concentration observed here is expected to affect the yeast growth in the fermentation stage. The change in vanillin concentration with temperature, as shown in Fig. 5, shows also a monotonous increase with temperature.

It should be noted that the changes in the yield of the inhibitor with temperature are mostly the same for both eucalyptus and EFB, indicating the possibility that inhibitor generation from these lignocellulosics may be generalized. For future studies, more feedstock should be treated hydrothermally.

3.4 Effect of Inhibitor on Ethanol Fermentation

Figure 6 shows the change in glucose concentration over time due to the consumption of glucose for ethanol fermentation. When complete conversion of glucose to ethanol is achieved, glucose concentration should be zero. However, glucose concentration is well above zero even after 54 h. This implies that the effect of the inhibitors is pronounced, and that the activity of the yeast is, in fact, inhibited. It should be noted that for hydrothermal pretreatment of EFB at 160 \(\degree C\), where production of acetic acid and furfural is negligible, glucose concentration is significantly decreased. However, with low concentrations of furfural and acetic acid, glucose conversion is far from completion. Vanillin is still found at this low temperature; therefore, it is probable that other lignin-derived products\(^{23)}\) affect the fermentation process. However, it is not possible to detect and identify all of them. Regardless, fermentation proceeds much better at 160 \(\degree C\) when a low concentration of inhibitors is present.

The similarity of concentration increase exhibited by the inhibitors used in this study indicates that it may be possible to use a single inhibitor as a general representative. Considering its monotonous increase with temperature and ease of detection, formic acid was selected as the general indicator of inhibitor production by hydrothermal pretreatment.
Figure 7 shows the relationship between formic acid concentration and glucose conversion during the fermentation process, which was obtained by plotting glucose conversion shown in Fig. 6 against the corresponding formic acid concentration. For both eucalyptus and EFB, a higher concentration of inhibitors being present resulted in suppressed fermentation. As previously stated, glucose should be completely converted when no inhibitors exists; in other words, conversion should be unity for zero concentration of formic acid. Please note that the behavior of formic acid represents the behavior of all other inhibitors, and thus for zero concentration of formic acid means zero concentration of other inhibitors, too. With an increase in inhibitor concentration (represented by the formic acid concentration here), the conversion of glucose during fermentation is reduced. It is clear that the relationship is essentially identical for both eucalyptus and EFB, and can be expressed by identical graphs. This result is interesting from the viewpoint of practical application, but will require more in-depth study in order to be useful in the elucidation of the inhibition mechanism.

3. 5. Overall Comparison between Eucalyptus and EFB

The comparison of the characteristics of glucose production, inhibitor production, and fermentation inhibition between eucalyptus and EFB lead to several conclusions. Reactions that proceeds via hydrothermal pretreatment is completely different depending on the choice of feedstock. In this study, EFB did not release considerable amount of glucose, and enzymatic hydrolysis was effective for increasing the glucose yield. In contrast, eucalyptus was able to readily release glucose using hydrothermal pretreatment, but enzymatic hydrolysis did not promote the same increase in the yield of glucose. However, inhibitor production shows rather similar behavior irrespective of feedstock, and is about 1 g/kg of furfural and acetic acid at 220°C under the conditions employed here. A further increase in temperature results in the decomposition of furfural, but the concentration of acetic acid remains high. The release of vanillin is almost identical for both of these feedstocks. The effects of the inhibitor on fermentation does not differ between the two feedstock studied here. This can be understood when we consider furfural and acetic acid can both be produced from hemicellulose24), which is dissolved at a rather early stage of the hydrothermal pretreatment. Once dissolved, the reaction
rate will remain constant for different feedstocks. The same holds true for vanillin, which is produced from lignin. Once inhibitors are produced, their effect on yeast activity should be the same irrespective of the feedstock because there are no differences between the inhibitors from eucalyptus and those from EFB. If this hypothesis is correct, future studies regarding the hydrothermal pretreatment of lignocellulosic biomass should be concentrated on the reaction in hydrothermal pretreatment because it differs for different feedstocks, and in the case of fermentation and inhibitor generation, a generalized characterization should be possible. Our immediate goal is to conduct a similar study with a broader range of feedstocks to determine whether this assumption is correct.

4. Conclusion

Eucalyptus and EFB were both subjected to hydrothermal pretreatment followed by enzymatic hydrolysis and ethanol fermentation, and the effects of hydrothermal pretreatment condition were studied. The concentrations of furfural, acetic acid, formic acid, and vanillin were determined, as they are known fermentation inhibitors. Formic acid was selected as a representative inhibitor, meaning that the production of inhibitors could be generally represented by an increase in the concentration of formic acid. Differences in glucose production were observed when different feedstocks were used in the hydrothermal pretreatment step, however, both inhibitor production and its effect on fermentation was the same regardless of the feedstock employed.

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References

要　旨
ユーポリとアブラヤシ空果核のエタノール発酵のための水熱前処理に関する比較検討

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リグノセルロース系バイオマスからの効率的なエネルギー生産はエネルギー利用の分野で最も重要な研究トピックの一つとなっている。水熱前処理を行うことによって、リグノセルロース系バイオマスから効率良くエタノールを生産できることを知らされているが、原料種類がこのエタノール生産に及ぼす影響については明らかとなっていない。そこで本研究では、ユーポリとアブラヤシ空果核について水熱前処理、酵素加水分解、さらにエタノール発酵し、両者の反応特性を比較した。水熱前処理の温度の効果を検討し、発酵阻害物質であるフルフラール、酢酸、ギ酸、そしてパニリンの濃度変化を確認した。両原料の違いは水熱前処理におけるグルコース生産に見られ、阻害物質の生産ならびにその発酵への影響は同じであった。