A Study of Feasibility of Pretreatment Process to Utilize Lignocellulosic Biomass as Materials for Biodiesel Production

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Abstract

Biomass is the most abundant renewable resource in the world and has potential to use as alternative materials to fossil resources for production of chemicals and fuels. For the effective conversion from biomass to biofuels or other chemicals, it requires high efficient hydrolysis of cellulose to glucose or fermentable sugars. In this study, lignocellulosic biomass, rice straw, rice husk, and water hyacinth were pretreated with different chemicals, or pretreated with microwave heating, or with combination of chemicals and microwave heating. Pretreated biomass was saccharified by using commercial cellulase enzymes and released sugar contents were measured. The combination of two pretreatment methods exhibited a synergy effect with 71.77% of the enzymatic sugar conversion. To study the possibility to utilize sugars from saccharified biomass, the *de novo* biosynthesis of fatty acid ethyl esters (FAEEs) in *Acinetobacter spp* were observed. The key biochemical reaction is the esterification between fatty acyl Co-A and ethanol using diacylglycerol acyltransferase (DGAT). The highest FAEE production up to 1,040±51 mg/l was found in *A.baylyi* culture that use biomass hydrolysate as a sole carbon source.

1. Introduction

The continuous growing of industry worldwide leads to the increasing of energy demand to support their production processes. Natural petroleum is the main resource to generate energy, however its price is increasing substantially. To increase the security in energy situation, it is important to develop the alternative option of petroleum fuels.

Biodiesel is one of renewable biofuels to replace petroleum diesel that contributes to the reduction of pollution emission and increasing of energy supply. Biodiesel is fatty acid alkyl esters (FAAE), which includes fatty acid methyl ester (FAME) and fatty acid ethyl ester (FAEE), and is conventionally produced through the transesterification reaction between methanol (or ethanol) and triacylglycerol (TAG). Likewise, FAAE is also synthesized by esterification process between fatty acid and alcohol [1] as shown in Fig.1. TAG is mainly obtained from oilseed plants and microalgae, therefore sustainable supply of TAG is a major bottleneck for current biodiesel production.

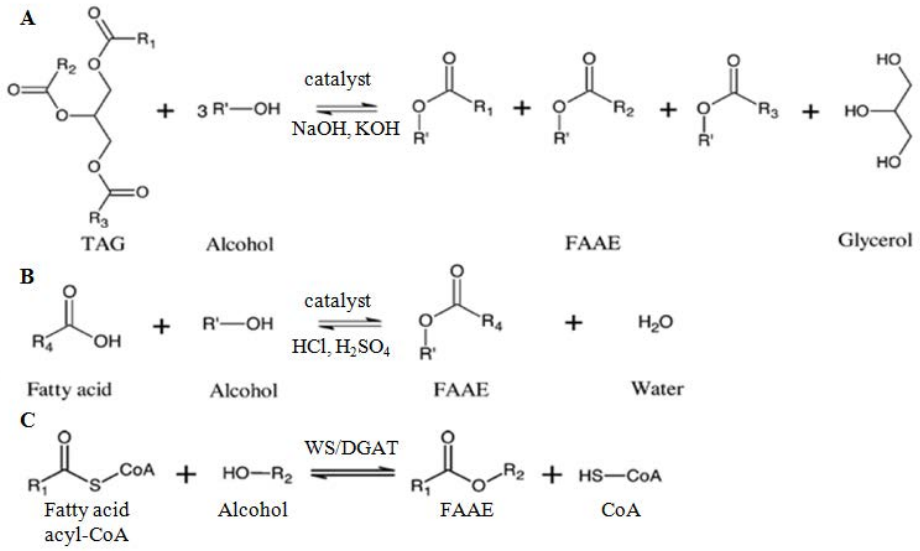


Fig.1. FAAEs are produced through (a) transesterification and (b) esterification reaction. (a) FAAE is produced by transesterification reaction between TAG and alcohol using NaOH or KOH as catalyst. (b) Fatty acid reacts with alcohol to produce FAAE via esterification process using acid as catalyst. (c) Fatty acid acyl-CoA reacts with alcohol to produce FAAE via esterification process using DGAT enzyme as catalyst.

FAAEs are also produced through *in vivo* esterification reaction between fatty acid acyl-CoA and alcohol using diacylglycerol acyltransferase (DGAT) enzyme as a catalyst (Fig.1). DGAT is the key enzyme for biosynthesis of storage lipids found in *Acinetobacter baylyi* [2]. In living organisms, fatty acids and their derivatives are generally produced as they are the important organic molecules that affect the regular function of cells, for example the structure of cell membrane. Fatty acids are synthesized from glucose through multi-step metabolic pathways [3]. Interestingly, starting from glucose as a material, ethanol is also produced through anaerobic fermentation giving a possibility to produce FAAE *in vivo* using glucose as raw material [4].

Considering to the whole plants, approximately 70% of total dry mass is lignocellulose that is mainly consisted of three types of polymers including cellulose, hemicellulose, and lignin [5]. Cellulose is a homopolymer of glucose, while hemicellulose is heteropolymer of hexose and pentose

sugars [6]. Because sugars are available in lignocellulose biomass, it is interesting to develop the strategy to utilize lignocellulose biomass for biodiesel production instead of TAGs. This idea provide possibility to overcome the bottle-neck of limited availability of plantseeds oil, and to potentially reduce the waste produced in the fields or agricultural-related industries.

Altogether, the usage of sugar monomer derived from lignocellulosic biomass is possible. However, conversion of lignocellulose to sugars with high yield to make total operation cost becomes feasible for industrial scale is still challenging. In general, the rate-limiting step of biofuel production from lignocellulosic biomass is hydrolysis step, therefore the pretreatment process is recommended before continuing to hydrolysis. Pretreatment process helps to loosen the microfibrils of cellulose to provide the chance for cellulolytic enzymes to attach the substrate surfaces [7].

Therefore, this study aims to focus on the feasibility study of utilization of lignocellulosic biomass as material for biodiesel production through fermentation process. First, rice straw, rice husk, and water hyacinth were selected as representative of lignocellulosic biomass as they are agricultural wastes that are abundant in each year. These three types of biomass were pretreated by different chemicals and microwave heating and efficiency of each pretreatment methods were evaluated. Second, the hydrolysates of biomass containing sugars were tested as carbon sources for fermentation of *Acinetobacter spp* for FAEE or biodiesel production.

2. Materials and Methods

Materials and chemicals

Rice (*Oryza sativa*) straw, and rice husk, were obtained from local rice field in Mahasarakham province, Thailand. Water hyacinth (*Eichhornia crassipes*) were obtained from river in Bangkok, Thailand. All wet biomass was dried in hot air oven at 60 ^{o}C until dried weight is constant, milled and sieved with a 10-mesh aluminium sieves. Sieved samples were kept in sealed bags at 4 ^{o}C prior to use. Cellulase from *Trichoderma reesei* (Celluclast® 1.5L), cellobiase from *Aspergillus niger* (Novozyme 188), were purchased from Signa-Aldrich (St. Louis, MO, USA). Other chemicals used in this study were purchased from Ajax (Bangkok, Thailand).

Pretreatment procedure

For chemical pretreatment, one gram of milled biomass was mixed with 20 ml of chemicals for pretreatment, including sodium hydroxide, hydrochloric acid, ammonium hydroxide, and urea and incubated in rotary shaker at 50 ^{o}C for 24 hr. After incubation, the slurry biomass was filtrated through a Whatman filter paper (#1004-110) with a Buchner funnel. After washing with distilled water 200 ml for 4 times, the samples were dried in oven at 60 ^{o}C until dried weight is constant, then kept in a sealed bag at 4 ^{o}C prior to digestibility evaluation. For microwave pretreatment, one gram of milled biomass was mixed with 20 ml of distilled water, then put in household microwave oven. The power of microwave exposure was adjusted and samples were exposed with different power for 5 min. After pretreatment, biomass was filtrated through a Whatman filter paper, dried, and kept in a sealed bag prior to use. For sequential pretreatment, biomass was pretreated with sodium hydroxide for 24 hr, and directly exposed with microwave. Sequential pretreated biomass was washed, dried and kept prior enzymatic hydrolysis.

Enzymatic hydrolysis
Hydrolysis of the pretreated rice straw, rice husk and water hyacinth was performed in a 50 mM sodium citrate buffer (pH 4.8) with commercial cellulase (Celluclast 1.5L) loading 20 FPU/g substrate and supplemented with 100 CBU/g substrate of beta-glucosidase (Novozyme 188). 0.5 g of pretreated biomass was mixed with 20 ml of buffer as a reaction mixture and incubated in rotary shaker at 50 ^{o}C for 48 hr with an agitation rate of 150 rpm. After 48 hr digestion, the reaction mixture was filtrated to separate the leftover biomass from the liquid fraction. The liquid fraction remained was stored in refrigerator until analysis for reducing sugars by dinitrosalicylic (DNS) method [8].

Fermentation using biomass hydrolysates
Acinetobacter baylyi (courtesy provided by Assoc. Alisa Vangnai [9]) and *Acinetobacter calcoaceticus* (ATCC31012) were streaked onto Nutrient agar (NA) plates and incubated at 30 ^{o}C for 16 hr. single colony was picked and inoculated into 10 ml of Nutrient broth medium in 50 ml flasks, and the flask were incubated at 30 ^{o}C in a rotary shaker for 16 hr. Then 1 ml of culture was inoculated in 100 ml minimal control medium (containing 15 mM KH_2PO_4, 8 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4.7H_2O$, and 10 mM succinic acid, pH 7.0) and incubated at 30 ^{o}C in a rotary shaker for 20 hr. To test the ability of *Acinetobacter* to utilize saccharified sugars derived from biomass hydrolysates as carbon source, minimal medium was modified by adding filtrated (through 0.2 μm porous membrane) biomass hydrolysates to substitute succinic acid. The cells were collected by centrifugation at 5,000 rpm for 5 min and pellet was kept, the culture was extracted for gas chromatography analysis (GC).

Measurement of FAEEs production
For fatty acid ethyl esters extraction based on standard method [10], culture pellet was mixed thoroughly with 10 ml of organic solvent containing chloroform and methanol (2:1 ratio by v/v). After dispersion, the whole mixture was agitated 15 min in an orbital shaker at room temperature. The homogenate was centrifuged to recover the liquid phase, and washed with 0.2 volume of 1% NaCl solution. The separated lower phase was recovered and concentrated by rotory evaporator. Samples were analyzed by GC (Shimadzu GC-2010 Plus) using DB-wax column (Agilent, 30 m in length, with 0.25 mm ID and 25 μm film thickness). The following temperature program was applied: 1 min at 40 ^{o}C, 15 min ramp to 280 ^{o}C, and constant at 280 ^{o}C for 10 min. 0.1 mg of nonadecanoic acid methyl ester was added as an internal standard, and the quantity of fatty acid ethyl ester was calculated by reference to the internal standard.

3. Results and Discussion

Optimization of enzyme concentration for hydrolysis of rice straw, rice husk, and water hyacinth
Initial experiments were done to select the optimal condition of each pretreatment method and to compare the efficiency of different pretreatment methods. Here, the concentration of commercial cellulase enzyme was optimized for the hydrolysis of pretreated samples. First, to find the optimal time for hydrolysis, unpretreated rice straw, rice husk, and water hyacinth were hydrolyzed with different loads of cellulase enzymes and incubation times (Fig. 2). The contents of released reducing sugars were increased linearly until 24 hr, and its increment rate reduced considerably afterward. Based on hydrolysis yield and time consuming, the incubation time of 48 hr was selected to use in comparative study to evaluate the efficiency of different pretreatment methods. It is also found that the hydrolysis yield of 20 FPU/g-substrate enzyme loading is increase significantly compared to 4 FPU/g-substrate (Fig. 2).

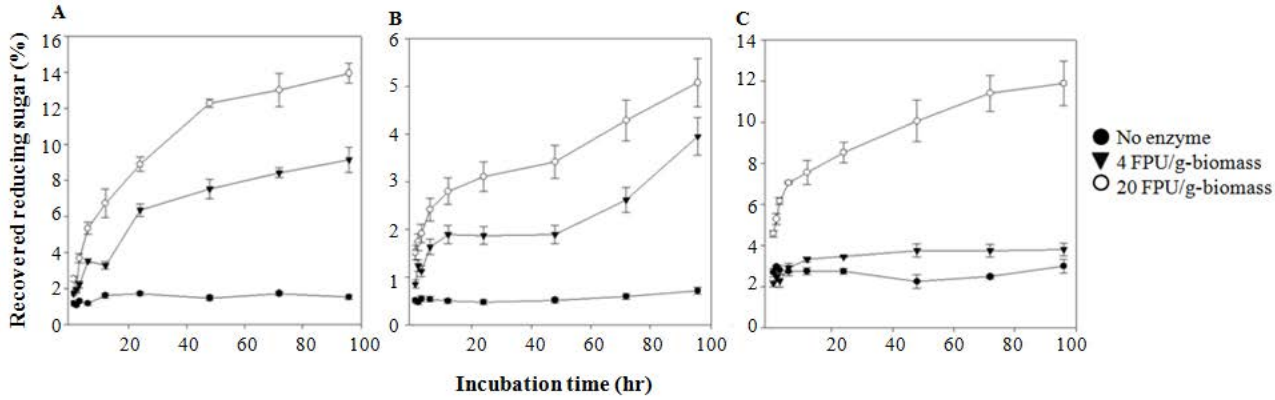


Fig. 2. Production of reducing sugars at different enzyme concentration s during hydrolysis of unpretreated (a) rice straw, (b) rice husk, and (c) water hyacinth samples by Celluclast 1.5L from *Trichoderma reesei*. Each experiments were performed triplicates.

Similar trends of hydrolysis were found in rice straw, rice husk, and water hyacinth digestion. However, the maximum reducing sugars released from rice straw, rice husk, and water hyacinth were different (13.95%, 5.07%, and 11.89% respectively). According to other studies as shown in Table 1 that reported the contents of cellulose, hemicellulose, and lignin in rice straw, rice husk, and water hyacinth (Table 1), the contents of cellulose and hemicellulose of these three types of biomass are not really different, but lignin content of rice husk (26-31%) is relatively high compared to rice straw (10-15%) and water hyacinth (10-16%). The lower contents of recovered reducing sugars from rice husk might caused by the inhibition of high lignin contents on cellulase enzyme activity as reported in other studies [11-13]. Clearly, without pretreatment the percent reducing sugar recovery no more than 13.95%. Therefore, this evidence suggested that it is necessary to perform pretreatment before hydrolysis. Furthermore, rice husk might need to be pretreated with the method that can remove lignin more than rice straw and water hyacinth.

Biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Rice Husk	25-35	18-21	26-31	[14, 15]
Rice Straw	35-40	25-30	10-15	[14, 16]
Water Hyacinth	20-31	22-33	10-16	[17, 18]

Table 1. The composition of cellulose, hemicellulose and lignin in rice straw, rice husk, and water hyacinth.

Effect of microwave pretreatment

Many pretreatment methods have been developed to find the suitable methods for each biomass types, including chemical, biological, and thermophysical pretreatment. In this study, we aimed to apply microwave irradiation for pretreatment process because it accelerates molecule collision to create thermal condition, which could be counted as thermophysical pretreatment [19]. Microwave irradiation has potential to be desirable pretreatment, because it is possible to optimize the operation conditions and has relatively short retention time compared to conventional heating. However, the economically energy consumption need to be evaluated to decide whether it is reasonable for practical biofuel production.

In this study, household microwave was used as a microwave irradiation generator. Based on the different compositions of each biomass types, we hypothesized that different powers of microwave

will suitable to each types of biomass. After microwave irradiation to rice straw, rice husk, and water hyacinth, the pretreated biomass was placed until its tempearature cools down to room temperature, then proceed to saccharification reaction. As shown in Fig. 3, the results showed that the microwave power at 400, 200, and 200 watts gives the highest amount of recovered reducing sugars in rice husk, rice straw, and water hyacinth respectively (9.92%, 19.97%, and 16.65%).

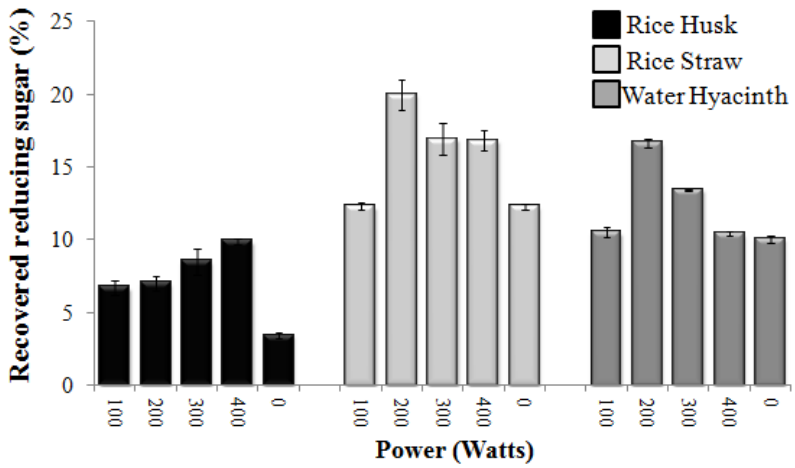
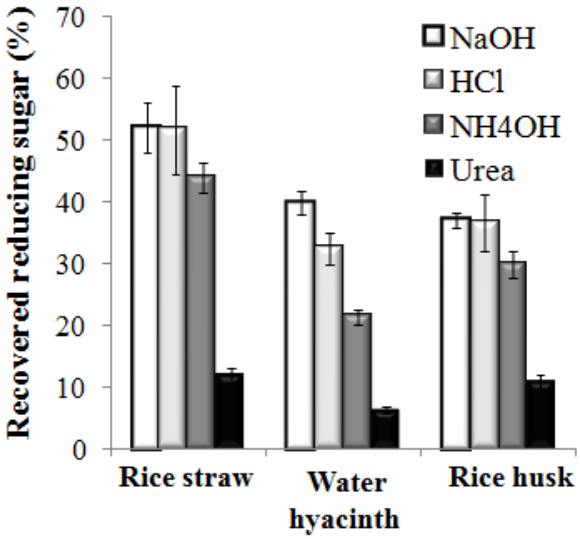


Fig. 3. Production of reducing sugars from microwave pretreated rice husk, rice straw, and water hyacinth. The powers of microwave were varied with 5 min exposure time. Each experiments were performed triplicates.

Effect of chemical pretreatment

Based on the results of microwave pretreatement, the enhancement of saccharification efficiency is not really high compared to unpretreated biomass control (Fig. 3). We therefore selected chemicals that were reviewed somewhere else to be used in pretreatment process [20]. In this study, sodium hydroxide (NaOH), hydrochloric acid (HCl), ammonium hydroxide (NH_4OH), and urea have been comparatively tested with rice straw, rice husk, and water hyacinth at different concentration. First, different concentration of each chemicals (0.5%-2.5% w/w for NaOH, 0.5%-2.5% w/w for HCl, 10%-30% w/w for NH_4OH, and 7.5%-17.5% w/w for urea) were tested to find the optimal concentration that gives the highest contents of recovered reducing sugars. After pretreatment by each chemicals, biomass were washed with distilled water, and saccharified by commercial cellulase enzymes. The reducing sugars released from saccharified biomass were measured to evaluate the efficiency of pretreatment.

As shown in Fig. 4, NaOH pretreatment gives the highest contents of recovered reducing sugars from rice straw, rice husk, and water hyacinth, compared to other chemicals. Here, the optimal concentration of each chemicals for treatment was also shown in Fig. 4. Comparing to NaOH, and HCl, NH_4OH and urea still have relatively lower efficiency although the high concentration of these chemicals was used. Surprisingly, comparing to results in Fig. 1, the sugar recovery from pretreated rice husk is increased up to similar level of water hyacinth, when NaOH, HCl, and NH_4OH were used. So, pretreatment of rice husk using these three chemicals is literally recommended.



Chemicals	Rice straw (%)	Rice husk (%)	Water hyacinth (%)
NaOH	2.5	2.5	2.5
HCl	2.5	2.5	2.5
NH_4OH	30	30	25
Urea	15	15	17.5

Fig. 4. Production of reducing sugars from four different chemicals pretreated rice husk, rice straw, and water hyacinth (Left panel). The optimal concentrations of different chemicals that enhance the highest saccharification efficiency of each biomass were presented (Right panel). Each experiments were performed triplicates.

Effect of sequential pretreatment

Based on the results of microwave, and chemical pretreatment, we selected the optimal condition of each pretreatments to combine in this experiments. First we pretreated rice straw, rice husk, and water hyacinth in 1% of NaOH for 24 hour as described in the materials and methods. We selected 1% NaOH for first step pretreatment because NaOH is the best chemicals among other tested chemical (Fig. 4). Additionally, although 2.5% concentration NaOH pretreatment has the highest efficiency, there are significant biomass loss during washing step (23.22%±4.5% of starting biomass weight) as it required more repeated washing compared to other concentration.

After 1% NaOH pretreatment, we directly exposed optimal-power microwave on pretreated biomass (200, 400, and 200 watts for rice straw, rice husk, and water hyacinth, respectively) for 5 min. Then sequential pretreated biomass were washed and saccharified. And contents of reducing sugar were measured as shown in Fig. 5. The results showed that sequential pretreatment of microwave and 1% NaOH have synergy effects on enhancement of saccharification. For example, in rice straw, 19.96%, 40.4% and 71.77% of biomass were changed to reducing sugars from microwave, 1% NaOH, and sequential pretreatment respectively. Likewise, the rice husk, and water hyacinth also get %reducing sugar up to 52.35% and 61.86%, respectively. Therefore, the result of this experiment suggested the pretreatment method that is applicable to different types of biomass. Although, the efficiency of this pretreatment method is not higher than other studies, but the cost of chemicals, and complexity of method are practical for further downstream process.

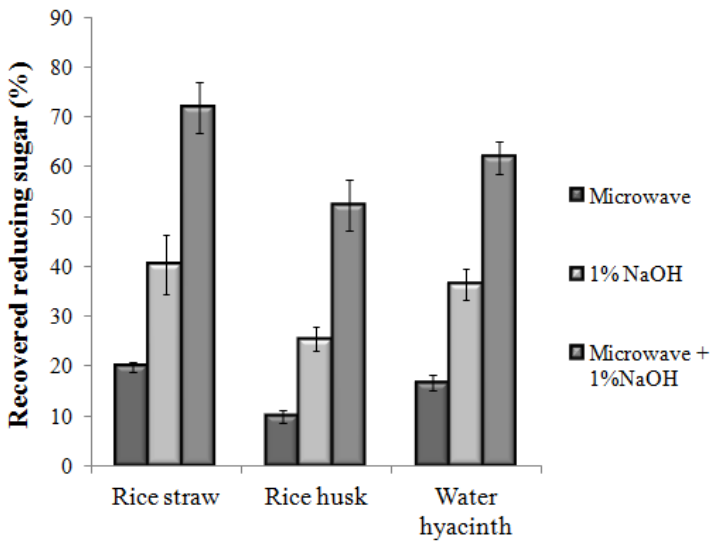


Fig. 5. Production of reducing sugars from sequential pretreated rice husk, rice straw, and water hyacinth. Each experiments were performed triplicates.

Fermentation of hydrolysate by Acinetobacter spp.

To study the feasibility of utilization of sugars derived from biomass, we applied the property of *Acinetobacter* bacteria that produce endogenous DGAT enzyme to catalyze the esterification reaction between fatty acid and ethanol to produce FAEE or biodiesel [2, 21]. First, the hydrolysate from saccharified pretreated rice straw was supplemented into minimal medium at different ratio to substitute the carbon source because rice straw hydrolysate has higher sugar content compared to rice husk and water hyacinth. After culturing the *A.baylyi* and *A.calcoaceticus* in medium for 20 hr, the cells were harvested, and their dry weights were measured. The FAEEs of cultures were extracted and quantified as described in materials and methods (Table 2).

Considering the dry weight yields, *Acinetobacter spp* cultured in nutrient rich media has the higher dry weight compared to cultures that used hydrolysated as carbon source. Interestingly, the higher amount of biodiesel was produced by cultures that used hydrolysates as carbon source. This observed results might be explained that the nutrient composition in nutrient rich media might not suitable for FAEE production. For example, if there is enough carbon and nitrogen source, bacteria prefer to undergo to cell proliferation, and will not accumulate lipid within their cells. On the other hand, in minimal media, it might not have enough nitrogen source to allow cell division, so cells accumulate lipid within the cells and those lipid become substrates of FAEEs.

Medium	Total dry weight (g/l)	Total biodiesel production (g/l)
A.baylyi		
Nutrient rice media	2.89±0.07	0.62±0.05
Minimal media + 50% hydrolysates	2.59±0.02	0.83±0.02
Minimal media + 25% hydrolysates	2.35±0.05	1.04±0.05
A.calcoaceticus		
Nutrient rice media	2.67±0.03	0.40±0.11
Minimal media + 50% hydrolysates	2.69±0.06	0.75±0.07
Minimal media + 25% hydrolysates	2.33±0.07	0.81±0.09

Table 2. The total dry weight and biodiesel production of *A.baylyi* and *A.calcoaceticus*. Each experiments were performed triplicates.

4. Conclusion

In this study, we developed the sequential pretreatment method and evaluated its efficiency to enhance saccharification of three different types of lignocellulosic biomass (rice straw, rice husk, and water hyacinth) by commercial cellulase enzymes. The synergistic effect of two different pretreatment methods was observed which giving up to 71.77% biomass recovery. Although the efficiency is not higher than other reports, but our sequential pretreatment method is simple and cheap. Additionally, we also studied the feasibility for utilization of lignocellulosic biomass hydrolysate as carbon source for *Acinetobacter* bacteria. Using minimal medium supplemented with 25% hydrolysates, *A.baylyi* culture can produce FAEEs up to 1,040±0.05 mg/l. This preliminary study supported the idea to produce biodiesel from lignocellulosic biomass to substitute the use of petroleum. However, further experiments are need to be done to analyze the hydrolysated compositions, so the optimization of medium composition will be possible to maximize the FAEE production. Additionally, the cost of pretreatment process, for example energy consumption, will be need to calculate to evaluate whether it is reasonable for production.

Acknowledgements

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