CHAPTER III

EXPERIMENTAL

3.1 Materials and equipments

3.1.1 Chemicals

1. Methanol (CH_3OH); analytical grade; Merck
2. Hexane (C_6H_6); analytical grade; Merck
3. Isopropanol; analytical grade; Merck
4. *tert*-Butanol; for synthesis grade; Merck
5. Sulfuric acid 96%; analytical grade; Fisher Scientific
6. Sodium hydroxide; analytical grade; CARLO ERBA reagents
7. Novozyme 435; S.M. Chemical Suppliers Co., Ltd.
8. Vanillin solution (containing vanillin (1%) and conc.H_2SO_4 (4.5%) in ethanol)
9. Rice bran oil soapstock; Thai edible oil Co., Ltd
10. Acid oil of rice bran oil; Surin bran oil Co., Ltd.
11. Rice bran oil fatty acid; Surin bran oil Co., Ltd.
12. Anhydrous sodium sulphate; analytical grade; Merck
13. Chloroform-D ($CDCl_3$); NMR spectroscopy grade; Merck KGaA Darmstadt,Germany

3.1.2 Equipments

1. Hotplate stirrer with magnetic stirrer set
2. Reflux condenser set
3. Thermometer
4. Vessel vial, round bottom flask, volumetric flask and erlenmeyer flask
5. Separatory funnel stand and clamps
6. Beaker
7. Suction vacuum pump set
8. Filter paper
9. Centrifuge

10. Rotary evaporator

11. pH-indicator strips; pH 0-14 Universal indicator; Merck

3.2 Neutralization of rice bran oil soapstock (RBOSS)

Neutralization of RBOSS was performed by addition of a 30%v/v solution of conc. sulfuric acid in water. RBOSS (50 grams) was heated to 90°C in water bath for 10 minutes. To a stirred RBOSS at 90°C, 30% v/v sulfuric acid (2.98 grams) was added. After stirring and heating at 90°C for 45 minutes, the mixture was poured into a separation funnel. The upper layer was separated and washed with water until pH was neutral. The organic layer was dried with anhydrous Na_2SO_4 to give acid oil (21.5 grams, 43%).

3.3 Chemical properties of acid oil and rice fatty acid

3.3.1 Fatty acid compositions were determined by EN14103

3.3.2 Acid value was determined by ASTM D664

3.4 Biodiesel production from RBOSS using enzyme catalyzed process

RBOSS (3 grams) and methanol (2:1 molar ratio of methanol to FFA) was placed in a 20 mL screw-capped vessel and the esterification was in a presence of 10%wt Novozyme 435 under shaking (90 oscillations/min) at 60°C. The reaction was monitored by TLC developed by hexane:EtOAc (90:10 v/v) and visualized by vanillin/H_2SO_4 solution. The mixture was cooled to room temperature and extracted with hexane. The hexane phase was separated and dried over anhydrous Na_2SO_4. After evaporation of the solvent, a dark green-brown oil residue was obtained (1.08 grams, 36%).

3.5 Biodiesel production from acid oil using Novozyme 435

Acid oil (3 grams) was added into the 20 mL screw-capped vessel. The reaction was carried out by shaking (90 oscillations/min) and the optimized condition was investigated below at a required amount of methanol and Novozyme 435. The reaction was monitored by TLC developed by hexane:EtOAc (90:10 v/v) and visualized by vanillin/H_2SO_4 solution. Conversion of fatty acid to FAME (or amount of FAME in the product) was analyzed by ^{1}H-NMR. After the reaction was completed the reaction mixture was separated under centrifuge at 3000 rpm for 10 minutes. The weight of methyl ester (0.1 gram) was subjected to determine of acid value and 5 mg of methyl ester was subjected to ^{1}H-NMR analysis for determination of fatty acid methyl ester (FAME).

3.5.1 Optimization of process parameter

3.5.1.1 Effect of reaction temperature

In this section, the effect of temperature on the ester content and on the esterification of acid oil was investigated with its reaction temperature varying at 30°C, 40°C and 50°C. The operation conditions during the whole reaction process were fixed at 2:1 molar ratio of methanol to FFA, 10%wt of Novozyme 435 and reaction time for 24 hrs.

3.5.1.2 Effect of molar ratio of methanol to FFA

In this section, the effect of molar ratio of methanol to FFA on the ester content and on the esterification of acid oil, the reaction temperature used was similar to that in Section 3.5.1.1, was investigated with its molar ratio of methanol to FFA varying at 1:1, 2:1 and 3:1. The operation conditions during the whole reaction process were fixed at 10%wt of Novozyme 435 and reaction time 24 hrs.

3.5.1.3 Effect of amount of Novozyme 435

In this section, the effect of amount of Novozyme 435 on the ester content and on the esterification of acid oil, the reaction temperature used was similar

to that in Section 3.5.1.1 and the molar ratio of methanol to FFA used was similar to that in Section 3.5.1.2, was investigated with its amount of Novozyme 435 varying at 5%, 7.5% and 10%. The operation condition during the whole reaction process was fixed at reaction time 24 hrs.

3.5.2 The transesterification of remained triglyceride in methyl ester of acid oil by Novozyme 435

The remained triglyceride in the product from the Novozyme 435 catalyzed esterification of acid oil was investigated. The operation conditions during the whole reaction process were fixed at the molar ratio of methanol to FFA at 1:1, reaction time of 30 hrs and 2 grams of the esterified product (from 3.5.1).

3.6 Biodiesel production using enzyme and base catalyzed process

3.6.1 First step: enzyme catalyzed esterification

Acid oil (25 grams) was added into the 100 mL round bottom flask. The reaction was carried out by shaking (90 oscillations/min) and the optimized condition was investigated below at a required amount of methanol and Novozyme 435 and reaction temperature followed optimal condition from 3.5.1 at reaction time 2 hrs.

3.6.2 Second step: base catalyzed transesterification

In the second step, optimal ratio of methanol to oil ratio were investigated, different methanol to TG ratios of 5:1, 7:1, 9:1 and 11:1 (see appendix C). Firstly, the first step product was added into the round bottom flask and heated to 65°C for 20 minutes. Then a solution of 0.8% NaOH w/w of the first step product in methanol was added. The reaction mixture was stirred with magnetic stirrer at 65°C for 1 hr. The reaction was monitored by TLC developed by hexane:EtOAc (90:10 v/v) and visualized by vanillin/H_2SO_4 solution. After cooling down to room temperature the reaction mixture was transferred to a separatory funnel and washed with water until the washing water was neutral. The methyl ester layer was separated and evaporated by a

rotary evaporator to remove a residue of methanol. The methyl ester (0.1 gram) was subjected to determine of acid value and 5 mg of methyl ester was subjected to ^{1}H-NMR analysis for determination of fatty acid methyl ester.

3.6.3 Biodiesel production from rice fatty acid using enzyme and base catalyzed process

In the biodiesel production from rice fatty acid (10 grams) via 2 step process, the reaction condition was similar to the procedure in Section 3.6.1 and in Section 3.6.2.

3.7 Biodiesel production from acid oil using acid and base catalyzed process

All reactions were carried out in a 50 mL round-bottom flask equipped with a reflux condenser and placed in water bath with a temperature controller and magnetic stirrer and the acid value and FAME content of all reaction products were determined by titration and ^{1}H-NMR analysis, respectively.

The effect of molar ratio of methanol to TG to the percentage of conversion of esterification and transesterification was studied as follow:

3.7.1 First step: acid catalyzed esterification

In the first step, optimal ratio of methanol to oil ratio were investigated, different methanol to TG ratios of 3:1,5:1, 7:1, 9:1, 11:1 and 13:1 (see appendix C). Firstly, the acid oil was added into the round bottom flask and heated to 65°C for 20 minutes. Then a solution of 1% H_2SO_4w/w of the first step product in methanol was added. The reaction mixture was stirred with magnetic stirrer at 65°C for 1 hr. The reaction was monitored by TLC developed by hexane:EtOAc (90:10 v/v) and visualized by vanillin/H_2SO_4 solution. After cooling down to room temperature the reaction mixture was transferred to a separatory funnel and washed with water until the washing water was neutral. The methyl ester layer was separated and evaporated by a rotary evaporator to remove a residue of methanol. The methyl ester (0.1 gram) was

subjected to determine of acid value and 5 mg of methyl ester was subjected to ^{1}H-NMR analysis for determination of fatty acid methyl ester.

3.7.2 Second step: acid catalyzed esterification

In the second step, optimal ratio of methanol to oil ratio were investigated, different methanol to TG ratios of 3:1, 5:1, 7:1, and 9:1 (see appendix C). Firstly, the first step product was added into the round bottom flask and heated to 65°C for 20 minutes. Then a solution of 1% H_2SO_4 w/w of the first step product in methanol was added. The reaction mixture was stirred with magnetic stirrer at 65°C for 1 hr. The reaction was monitored by TLC developed by hexane:EtOAc (90:10 v/v) and visualized by vanillin/H_2SO_4 solution. After cooling down to room temperature the reaction mixture was transferred to a separatory funnel and washed with water until the washing water was neutral. The methyl ester layer was separated and evaporated by a rotary evaporator to remove a residue of methanol. The methyl ester (0.1 gram) was subjected to determine of acid value and 5 mg of methyl ester was subjected to ^{1}H-NMR analysis for determination of fatty acid methyl ester.

3.7.3 Third step: base catalyzed transesterification

In the third step, the optimum molar ratio of methanol to TG follows section 3.6.2. Firstly, the second step product was added into the round bottom flask and heated to 65°C for 20 minutes. Then a solution of 0.8% NaOH w/w of the first step product in methanol was added. The reaction mixture was stirred with magnetic stirrer at 65°C for 1 hr. The reaction was monitored by TLC developed by hexane:EtOAc (90:10 v/v) and visualized by vanillin/H_2SO_4 solution. After cooling down to room temperature the reaction mixture was transferred to a separatory funnel and washed with water until the washing water was neutral. The methyl ester layer was separated and evaporated by a rotary evaporator to remove a residue of methanol. The methyl ester (0.1 gram) was subjected to determine of acid value and 5 mg of methyl ester was subjected to ^{1}H-NMR analysis for determination of fatty acid methyl ester.

3.7.4 Biodiesel production from rice fatty acid using acid and base catalyzed process

In the biodiesel production from rice fatty acid (10 grams) via 3 step process, the reaction condition was similar to the procedure in Section 3.7.1, 3.7.2 and 3.7.3.

3.8 Repeated use of Novozyme 435

When Novozyme 435 was used as a catalyst for conversion of FFA to FAME, the activity was increased by repeating the reaction. Therefore, pretreatment of Novozyme 435 was performed in a 20 mL screw-capped vessel by shaking (90 oscillations/min) and reaction mixture, which was composed of given amounts of acid oil 1 gram, 2:1 molar ratio of methanol to FFA. Immobilize lipase (Novozyme 435) 10%wt of rice fatty acid reaction time 2 hrs. After the reaction was completed, 5 mg of sample where subjected to ^{1}H-NMR and sample was sucked, the reaction was left. Novozyme 435 was washed by tert-butanol filtering and drying on suction pump for 30 minutes in the first method and there was no washing for Novozyme 435 by using tert-butanol in the second method. The esterification was repeated 9 cycles of both methods by transferring the lipase to fresh substrate mixture.

3.9 Properties of biodiesel

The percentage of methyl ester, flash point and oxidation stability tested by the Department of Energy Business, Ministry of Energy, following EN14103, ASTM D93 and EN14112.