CHAPTER III

EXPERIMENTAL

3.1 Materials and equipment

3.1.1 Chemicals

1. Hexane; commercial grade
2. Sodium hydroxide (NaOH); Carlo Erba
3. Methanol (CH_3OH); Merck KGaA Darmstadt, Germany
4. Ethyl acetate ($CH_3COOCH_2CH_3$); analytical grade
5. Acetic acid (CH_3COOH); analytical grade
6. Sulfuric acid (H_2SO_4); analytical grade
7. Chloroform-d ($CDCl_3$): NMR spectroscopy grade; Merck KGaA Darmstadt, Germany
8. Sodium nitrate ($NaNO_3$); analytical grade
9. Ammonium chloride (NH_4Cl); analytical grade
10. Hydrochloric acid (HCl); analytical grade
11. Potassium carbonate (K_2CO_3); analytical grade
12. Malt extract; Himedia
13. Yeast extract; Himedia
14. Peptone; Himedia
15. Gelatin
16. Glucose
17. Sucrose
18. Potato
19. Starch (Cassava)
20. Molasses
21. Urea

3.1.2 Equipment

1. Autoclave
2. Rotary evaporator
3. Reflux condenser set
4. Soxhlet apparatus
5. Hotplate stirrer with magnetic stirrer set
6. Separatory funnel stand and clamps
7. Thermometer
8. Volumetric flask, Erlenmeyer flask and round bottom flask
9. Beaker
10. Filter paper
11. Ultrasonic bath
12. Microplate reader

3.1.3 Culture media

1. MEB (Malt extract broth)
   - Malt extracts 20 g
   - Glucose 20 g
   - Peptone 1 g
   - Distilled water 1 L
2. PDB (Potato dextrose broth)
   - Potato, peeled and diced 200 g
   - Glucose 20 g
   - Distilled water 1 L
3. YES (Yeast extract sucrose broth)
   - Yeast extracts 20 g
   - Sucrose 150 g
   - Distilled water 1 L

3.2 An endophytic fungus *Fusarium* sp. ZcSK01

In this research an endophytic fungus *Fusarium* sp. isolated from rhizomes of *Zingiber cassumunar* Roxb. was used as a source of oil for biodiesel production using the surface sterilization method. The fungus has been identified as *Fusarium* sp. by the means of morphology. [35]

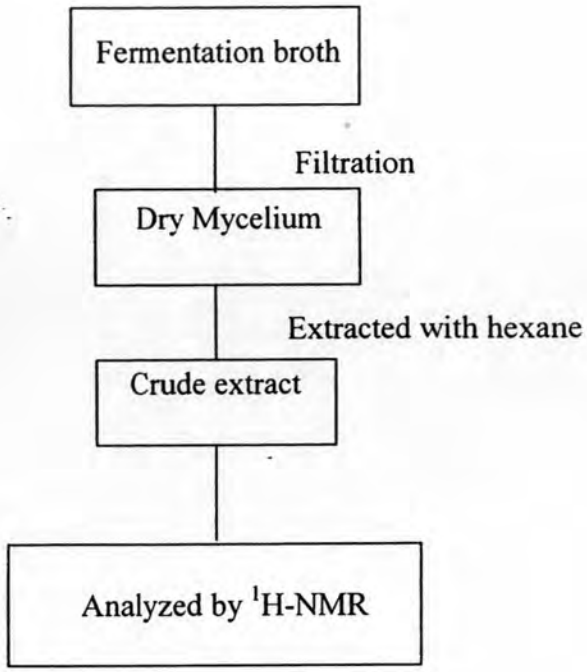
3.3 Cultivation and extraction of endophytic fungus

3.3.1 Cultivation

The endophytic fungus was grown on MEA at room temperature (25-30°C) for 14 days. The agar was then cut into 8 mm diameter with a flamed cork hold borer. Three pieces of agar cultures were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of culture media (each 30 flasks) and statically incubated at room temperature for 30 days. Comparison of all media was shown in appendix A.

3.3.2 Extraction

The culture broth was filtered through filter paper (Whatman No.1). The mycelia were dried in oven at 80°C and extracted with hexane in ether ultrasonic bath or using a Soxhlet apparatus. The hexane extract was evaporated under reduced pressure to afford a yellow crude material. The crude extracts were analyzed by ^{1}H-NMR. The extraction procedure is shown in Scheme 3.1.



Scheme 3.1 Extraction of the mycelia

3.4 Influence of cultivation parameters on oil production

3.4.1 Determination of suitable method for extraction of oil from mycelium

MEB medium was selected to study cultivation time and the suitable method for extract. The agar was then cut into 8 mm diameter with a flamed cork hold borer into 250 mL Erlenmeyer flasks containing 100 mL of MEB medium and stopped by a cotton cap and then statically incubated at room temperature for 30 days. The method for extraction was compared in 2 methods in following:

1. The extraction method was extracted by soaking. The time of extraction was varied 1, 2 and 3 days, respectively.

2. The extraction method was extracted by Soxhlet apparatus for oil production. The extraction via using a Soxhlet apparatus was carried out for 6 h.

3.4.2 Determination of growth profile of the endophytic fungus

The agar was then cut into 8 mm diameter with a flamed cork hold borer into 250 mL Erlenmeyer flasks containing 100 mL of MEB medium and stopped by a cotton cap. The culture was incubated at room temperature under static condition for

30 days. The fungal mycelia were harvested every 3 days. Mycelium and broth were separated by filtering through Whatman no. 93 filter paper. The mycelia were dried in oven at 80°C and then weighted. The mycelia dry weights were calculated from the difference between dry weights of filter paper before and after harvested mycelium of cultures. Determination of the growing has been done 4 replicates.

3.4.3 Influence of the amount of malt extract in MEB medium on oil production

The amount of malt extract in MEB medium was varied 0, 1, 5, 10, 20, 30 and 40 g/L respectively for oil production. The fermentations were performed in 250 mL Erlenmeyer flasks containing 100 mL of culture media and stopped by a cotton cap. The culture was incubated at room temperature under static condition for 30 days.

3.4.4 The effect of various carbon sources in MEB on oil production

In order to determine the effect of different carbon source on oil production, carbon source were used for oil production including glucose, sucrose, cassava and molasses in the following:

Table 3.1 The effect of different carbon source

Amount in media (g/1 L) / Experimental	Carbon source	Malt extract	Peptone
CS_1	20	20	1
CS_2	20	20	1
CS_3	20	20	1
CS_4	10	20	1

Remark : CS_1 was experiment of glucose as carbon source

CS_2 was experiment of sucrose as carbon source

CS_3 was experiment of cassava as carbon source

CS_4 was experiment of molasses as carbon source

The fermentations were performed in 250 mL Erlenmeyer flasks containing 100 mL of culture media. The culture was incubated at room temperature under static condition for 30 days.

3.4.5 The effect of various nitrogen sources in MEB on oil production

The aim of this work was to study how the nitrogen source affects the oil production. In order to determine the effect of different nitrogen sources on oil production, nitrogen source were used for oil production including peptone, sodium nitrate, ammonium chloride, urea and yeast extract in the following:

Table 3.2 The effect of different nitrogen source

Amount in media (g/1 L) / Experimental	Nitrogen source	Malt extract	Glucose
NS_1	0	20	20
NS_2	0.85	20	20
NS_3	0.50	20	20
NS_4	0.60	20	20
NS_5	1.10	20	20
NS_6	1.00	20	20

Remark : NS_1 was experiment of no nitrogen source

NS_2 was experiment of sodium nitrate as nitrogen source

NS_3 was experiment of ammonium chloride as nitrogen source

NS_4 was experiment of urea as nitrogen source

NS_5 was experiment of yeast extract as nitrogen source

NS_6 was experiment of peptone as nitrogen source

The fermentations were performed in 250 mL Erlenmeyer flasks containing 100 mL of culture media and stopped by a cotton cap. The culture was incubated at room temperature under static condition for 30 days.

3.5 Hydrolysis of cellulose

3.5.1 Detannin with base solution [36]

Bagasse 50 g was treated with 10%NaOH solution 200 mL at 60°C for 6 h. Then the solution was filtered to separate the solvent and bagasse. Bagasse was washed with distilled water until washing water was neutral.

3.5.2 Hydrolysis of cellulose with acid solution

After detannin, the cellulose 50 g from 3.5.1 was hydrolyzed by 10% hydrochloric acid 500 mL for 6 hour. Then, the solutions were neutralized by addition of sodium hydroxide 50%.

3.5.3 Sugar analysis [36]

The reducing sugar was determined by dinitrosalicylic acid method (DNS method). The technique was prepared by using glucose to generate a standard curve. DNS reagent (See details in Appendix A) in 0.1 mL was added to 0.1 mL of sample in eppendorf. The mixture was placed in boil water for 10-15 min to develop the red-brown color. 30 μL of 40% potassium sodium tartrate solution was added to stabilized the color. The reaction was cooled at room temperature and measured the absorbance with a microplate reader at 540 nm.

3.5.4 Oil production from the fungus using the hydrolyzed-cellulose solution as a carbon source

MEB medium was selected to study growth of fungus in hydrolyzed solutions for medium. The hydrolyzed-cellulose solutions were performed using cultures broths of fungus. It was cultured in MEB medium containing malt extract 5 g, yeast extract 1.1 g, hydrolyzed solution 500 mL and distilled water 500 mL.

3.6 Biodiesel production

3.6.1 One-step alkali base catalyzed transesterification

1. The variation of methanol to oil ratio

Firstly, in the transesterification process, different methanol to oil ratios 10:1, 20:1, 30:1 and 40:1 were used to investigate their influence on the methyl ester yields of the oils with NaOH to oil ratio 1% as catalyst. All the reactions were carried out in the reaction glass tubes, which were immersed in a glass water bath placed on the plate of magnetic stirrer of 400 rpm. The temperature and the reaction time for all process were maintained at 65°C and for 2 hour, respectively. The reaction was monitored by TLC developed by hexane:ethylacetate (90:10 v/v) and visualized by vanillin solution. After cooling down to room temperature the reaction mixture was transferred to separatory funnel and left overnight. The methyl ester layer and the glycerol layer were separated. The methyl ester layer (top phase) was washing with hot water until washing water was neutral. The methyl ester (5 mg) was subjected to ^{1}H-NMR analysis.

2. The variation of NaOH to oil ratios

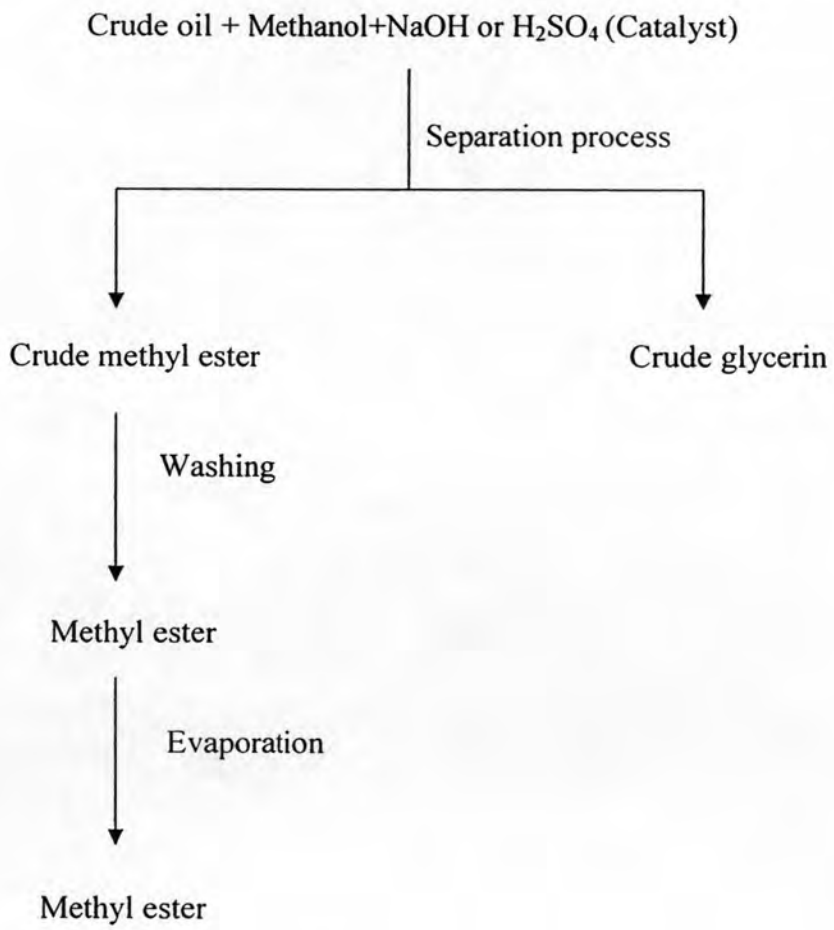
The different amount of catalyst (NaOH; 0.5%, 1.0% and 1.5%w/w) were used to investigate their influence on the methyl ester yields of the oils with methanol to oil ratio 40:1. The conditions for the variation of NaOH to oil ratios are also processed by variation of methanol to oil ratio. The reaction was monitored by TLC developed by hexane:ethylacetate (90:10 v/v) and visualized by vanillin solution. After cooling down to room temperature the reaction mixture was transferred to separatory funnel and left overnight. The methyl ester layer and the glycerol layer were separated. The methyl ester layer (top phase) was washing with hot water until washing water was neutral. The methyl ester (5 mg) was subjected to ^{1}H-NMR analysis.

3.6.2 One-step acid catalyzed transesterification

1. Sulfuric acid catalyzed transesterification

In the transesterification process, methanol to oil ratios 10:1 and H_2SO_4 to oil ratio 1% w/w were produced biodiesel production. The conditions for sulfuric acid catalyzed esterification are also processed by variation of methanol to oil ratio in one step base catalytic process. The reaction was monitored by TLC developed by hexane:ethyl acetate (90:10 v/v) and visualized by vanillin solution. After cooling down to room temperature the reaction mixture was transferred to separatory funnel and left overnight. The methyl ester layer and the glycerol layer were separated. The impurities in methyl ester layer (top phase) were removed by washing with hot water until washing water was neutral. The methyl ester (5 mg) was subjected to ^{1}H-NMR analysis.

The flow diagram for base-catalyzed and acid catalyzed process on the laboratory scale was showed in Scheme 3.2.



Scheme 3.2 Base catalyzed and acid catalyzed transesterification

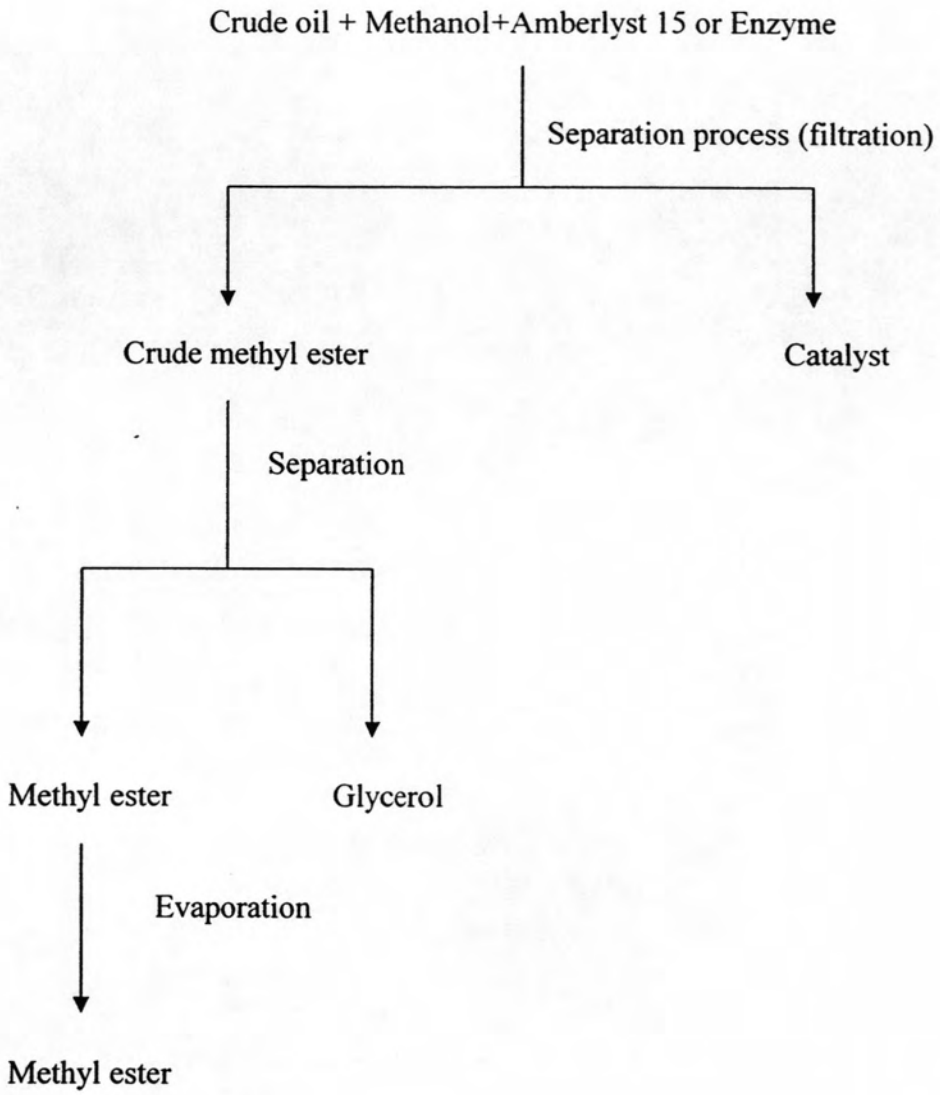
2. Amberlyst 15 catalyzed transesterification

In the case of Amberlyst 15 catalyst are also processed by using the same method as in sulfuric acid catalyst. The reaction was monitored by TLC developed by hexane:ethylacetate (90:10 v/v) and visualized by vanillin reagent. After cooling down to room temperature the reaction mixture was filtered catalyst. The methyl ester (5 mg) was subjected to ^{1}H-NMR analysis.

3.6.3 One-step enzymetic catalyzed transesterification (Novozyme 435)

In the transesterification process, biodiesel was produced by using methanol to oil ratios 2:1 and enzyme to oil ratio 10% w/w. The conditions for enzymetic catalyzed transesterification are also processed by variation of methanol to oil ratio in one step base catalyzed process. The reaction was monitored by TLC developed by hexane:ethylacetate (90:10 v/v) and visualized by vanillin solution. After cooling down to room temperature the reaction mixture was filtered catalyst. The methyl ester (5 mg) was subjected to ^{1}H-NMR analysis.

The flow diagram for 2 step catalyzed process on the laboratory scale was showed in Scheme 3.3.



Scheme 3.3 Heterogenious catalyzed transesterification

3.6.4 Synthesis of methyl ester via 2-step catalyzed process

The first step was acid esterification and pretreatment for removing FFA in the oil, which is mainly a pretreatment process, which could reduce the FFA.

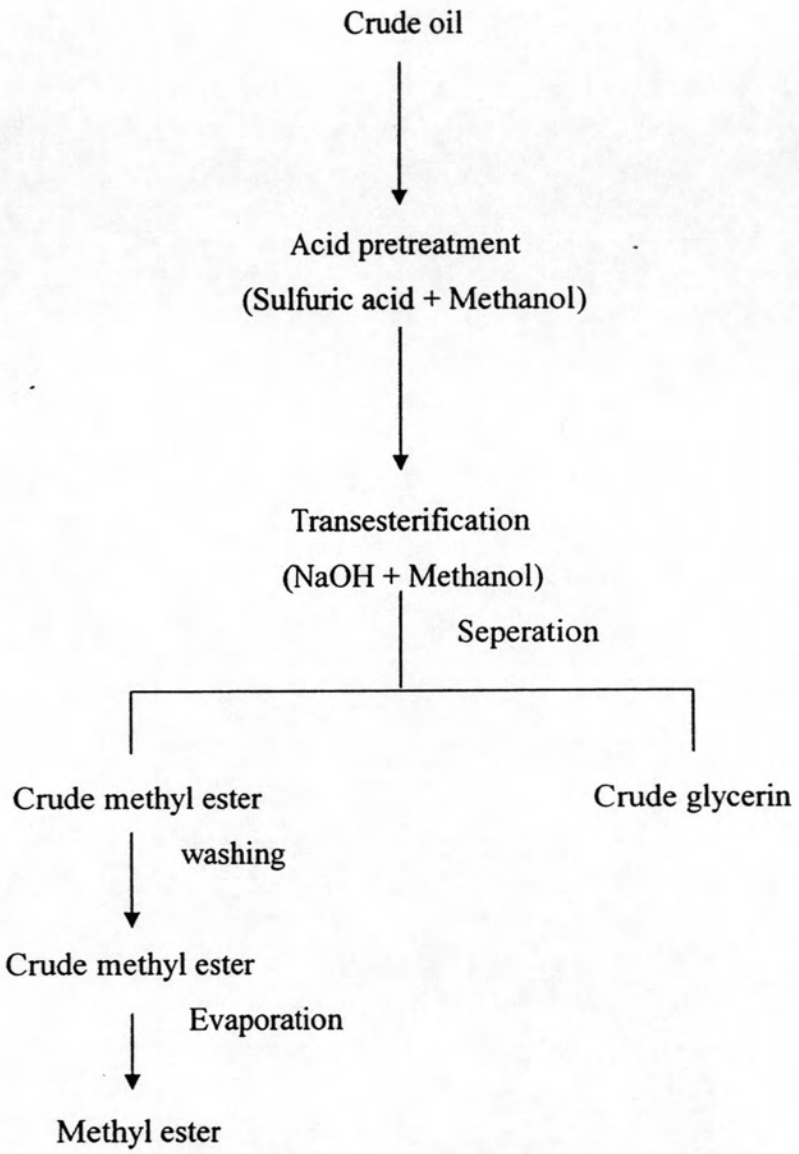
1. Acid pretreatment

On this step, the oil was poured into the reaction glass tubes and heated. The solution of concentration H_2SO_4 acid (1.0% based on the oil weight) in methanol was heated at 65°C and then added into the reaction glass tubes. The methanol to oil ratios were investigated their influence on FFA of oil. After one hour of reaction, the mixture was allowed to settle for 2 hours and the methanol–water fraction at the top layer was removed. The optimum condition having the lowest FFA was used for the transesterification reaction.

2. Base catalyzed transesterification.

In the second step, optimum condition for NaOH to oil ratio and methanol to oil ratio were investigated. Firstly, the oil product that has been pretreated from the first step was poured into the reaction glass tubes and heated at 65°C. The solution of NaOH in methanol at 1.0% w/w of the oil were heated to 65°C prior to addition and then added to the heated oil. The reaction mixture was heated and stirred again at 65°C and 400 rpm for 2 hours. The reaction was monitored by TLC developed by hexane:ethylacetate (90:10 v/v) and visualized by vanillin solution. After cooling down to room temperature the reaction mixture was transferred to separatory funnel and left overnight. The methyl ester layer and the glycerol layer were separated. The methyl ester layer (top phase) must be conducted to remove impurities by washing with hot water until washing water was neutral. The methyl ester (5 mg) was subjected to ^{1}H-NMR analysis.

The flow diagram for 2 step catalyzed process on the laboratory scale was showed in Scheme 3.4.



Scheme 3.4 Two step catalyzed process

3.7 Properties of biodiesel

The physical properties of biodiesel were determined as shown in Table 3.3.

Table 3.3 Tested method of purified methyl ester

Property	Method
Methyl ester, %wt.	EN 14103
Viscosity	ASTM D445
Specific Gravity kg/l @ 15° C	ASTM D4052
Density, lb/gal @ 60° C	ASTM D 1298
Flash point	ASTM D93
Free glycerin, %wt.	EN 14105
Pour point (° C)	ASTM D97
Cloud point (° C)	ASTM D2500