CHAPTER TWO

EXPERIMENTAL

2.1 *Jatropha* Oil Extraction

2.1.1 Materials

All chemicals used in the present study were of analytical grade reagents and they were used without further purification. Enzymes Alcalase® 2.4 L and Celluclast® 1.5 L, are products of Novozymes® and they were purchased from Science Technics Sdn Bhd, Petaling Jaya, Selangor, Malaysia. Alcalase® 2.4 L was reported to contain mostly alkaline protease and endo protease with specific enzyme activity of 2.4 AU-A/g while for Celluclast® 1.5 L which contains mostly Cellulase with enzyme activity of 700 EGU/g.

2.1.2 Methodology

2.1.2.1 Solvent Extraction Process (Soaking Method)

Jatropha curcas seeds were dried overnight at 70°C to remove any moisture present in the seeds. Ground and pulverised *Jatropha curcas* seeds to uniform size were soaked in hexane for a specific period of time at room temperature. The extracted oil was obtained upon filtration and evaporation under reduced pressure and at temperature of 70°C. Rotary evaporator Buchi R-114, oven Memmert UFB 500 and laboratory blender Waring 8010 S were used in the current study. Yield was calculated after removal of residual solvent. The conditions used were tabulated in Table 2.1.

Table 2.1 : Solvent extraction (soaking method) of *Jatropha curcas*

Experiment	Ratio of *Jatropha* seed to hexane (g/mL)	Duration (hours)	Solvent
1	1:1	16	Hexane
2	1:2	16	Hexane
3	1:3	16	Hexane
4	1:4	16	Hexane
5	1:5	16	Hexane

2.1.2.2 Solvent Extraction Process (Soxhlet Method)

Solvent extraction (soxhlet method) of *Jatropha curcas* oil was carried out using Soxhlet apparatus in accordance with Palm oil Research Institute Malaysia (PORIM) test method (Siew *et al*., 1995). 10 g of powdered and de-shelled seeds of *Jatropha curcas* was weighed to the nearest 1 mg into the extractor and the latter was plugged with cotton wool while hexane was added into the flask. The extraction was carried out by using 150 mL of hexane for 6 hours in the presence of 1 mg of pumice stone. The extract was dried at atmospheric pressure at 100ºC for 2 hours. The flask was cooled in the desiccators to ambient temperature for 30 minutes and then weighed to the nearest 1 mg.

2.1.2.3 Enzymatic Assisted Aqueous Oil Extraction

Jatropha curcas seeds were dried overnight at 70°C to remove moisture content in the seeds. The de-shelled seeds were ground by using a laboratory blender, Waring 8010 S with medium speed for 5-6 minutes and sieved manually to uniform fine powder (less than 1 mm). Figure 2.1 illustrated the sample preparation of pulverised *Jatropha* seeds for further analyses.

(i) *Jatropha* seeds

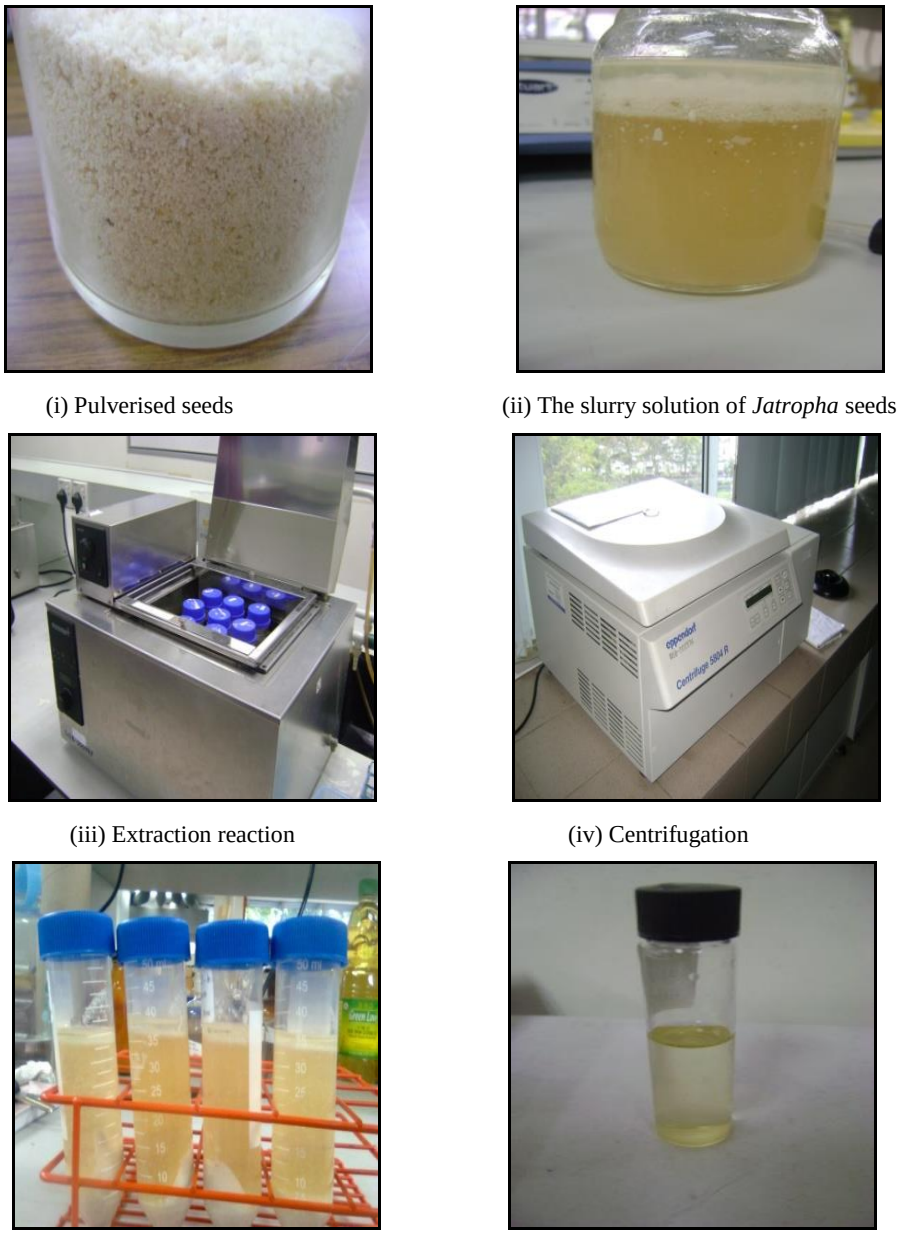
(ii) Cross-section of *Jatropha* seeds

(iii) De-shelled seeds

(iv) Pulverised seeds

Figure 2.1 : Sample preparation of pulverised *Jatropha* seeds

Pulverised *Jatropha* kernels were suspended in distilled water in the presence of Alcalase®. The slurry solution obtained, as in Figure 2.2(ii) was further subjected to constant heat and stirring in the waterbath shaker; Memmert WNB 22 MOO with shaking device (Figure 2.2(iii)). The oil layer in the upper phase of oil-aqueous layer (Figure 2.2(v)) was collected and weighed after centrifugation process by centrifuge machine; Eppendorf 5804R (Figure 2.2(iv)).



(i) Pulverised seeds

(ii) The slurry solution of *Jatropha* seeds

(iii) Extraction reaction

(iv) Centrifugation

(v) Oil-aqueous layer

(vi) *Jatropha* Oil

Figure 2.2 : Methodology of EAAOE

The experiments were also conducted by replacing Alcalase® with Celluclast®. A control experiment (blank) was carried out in the absence of enzyme. All experiments were done in duplicates and the mean data was reported. The experimental condition for single type addition of enzyme assisted aqueous oil extraction (EAAOE) was tabulated in Table 2.2.

Table 2.2 : EAAOE study using different type of enzyme

Experiment	Enzyme	Ratio of *Jatropha* seed to hexane (g/mL)	pH	Duration, (hours)	Solvent
6	Celluclast®	1:6	4.5	16	Hexane
7	Celluclast®	1:6	5.0	16	Hexane
8	Celluclast®	1:6	6.0	16	Hexane
9	Alcalase®	1:6	7.0	16	Hexane
10	Alcalase®	1:6	8.0	16	Hexane
11	Alcalase®	1:6	9.0	16	Hexane

The reaction temperatures were set at 50°C. The pH of the mixtures was adjusted according to the desired pH of enzymes used, pH 8.0 for enzyme Alcalase® and pH 5.0 for enzyme Celluclast® using pH meter; Cyberscan pH510 and buffer solution of 0.1 N sodium hydroxide (NaOH) or 0.1 N acid hydrochloric (HCl).

Table 2.3 shows the experimental condition for the combination addition of enzymes in EAAOE. When a combination of both enzymes was used, pre-determined amount of enzymes were added in a single step or added in a step-wise manner. The reaction temperatures were set at 50°C, seed to water ratio of 1:6 and pH 7.0. Similar to the experiments done in single addition of enzyme in EAAOE, the pH of enzymes was set using 0.1 N NaOH or 0.1 N HCl.

Table 2.3 : Combination addition of enzyme in EAAOE

Experiment	Enzymes	Ratio of *Jatropha* seed to hexane (g/mL)	pH
12	Alcalase® : Celluclast®	1:2	4.5
13	Alcalase® : Celluclast®	2:1	8.0
14	Alcalase® : Celluclast®	1:1	7.0

In the step-wise enzymatic oil extraction, the experiments were carried out in a similar manner as the above experiments (Table 2.3) in terms of the preparation of the powdered *Jatropha* seeds. The experimental measurement for the step-wise addition of enzymes are tabulated in Table 2.4. In contrast to the non step-wise, the oil extraction processes were carried out in 16 hours where the first enzyme was added in the oil extraction in the first 8 hours followed by the addition of second enzyme in the next 8 hours. The experiments were conducted for both equal and non-equal ratio of mass of enzyme to mass of de-shelled seeds. Similar to single addition of enzyme in EAAOE, the reaction temperatures were set at 50°C, seed to water ratio was adjusted to 1:6. The pH of the mixtures were adjusted according to the respective pH of enzymes used using 0.1 N NaOH or 0.1 N HCl.

Table 2.4 : Step-wise addition of enzyme in EAAOE

Experiment	Enzymes	Ratio of *Jatropha* seed to hexane (g/mL)	pH (1^{st} 8 hours)	pH (2^{nd} 8 hours)
15	Alcalase®- Celluclast®	1:2	8.0	5.0
16	Alcalase®- Celluclast®	2:1	8.0	5.0
17	Celluclast®- Alcalase®	1:1	5.0	8.0
18	Celluclast®- Alcalase®	2:1	5.0	8.0

Study on the effect of enzyme concentration was done as tabulated in Table 2.5. The reaction temperatures were set similar as step-wise enzyme addition experiments (Table 2.4), at 50°C for duration of 8 hours. The pH of the mixtures were adjusted according to the respective pH of enzymes used, pH 8.0 for Alcalase® and pH 5.0 for Celluclast®, using 0.1 N NaOH or 0.1 N HCl

Table 2.5 : The effect of enzyme concentration added to EAAOE

Experiment	Enzymes	Mass of enzyme used, mg	Ratio of *Jatropha* seed to enzyme, % (m/m)
19	Alcalase®	200	4
20	Alcalase®	250	5
21	Alcalase®	300	6
22	Alcalase®	350	7
23	Alcalase®	400	8
24	Celluclast®	200	4
25	Celluclast®	250	5
26	Celluclast®	300	6
27	Celluclast®	350	7
28	Celluclast®	400	8

Table 2.6 tabulates the experimental condition to determine the effect of pH in the EAAOE. The mass of enzyme added in the experiments was 250 mg. Similarly to the study of the enzyme concentration, the reaction temperatures were set at 50°C, the seed to water ratio of 1:6 in the extraction duration of 6 hours. The pH of the mixtures were adjusted using 0.1 N NaOH or 0.1 N HCl.

Table 2.6 : The effect of pH on the extraction yield

Experiment	Enzymes	pH
29	Alcalase®	9.0
30	Alcalase®	8.0
31	Alcalase®	7.0
32	Alcalase®	6.0
33	Celluclast®	7.0
34	Celluclast®	6.0
35	Celluclast®	5.0
36	Celluclast®	4.5
37	Celluclast®	4.0

The experimental design to study the effect of varying extraction duration is tabulated in Table 2.7. The mass of enzyme added in the experiments was 250 mg. The reaction temperatures were set similarly as above, at 50°C. The pH of the mixtures were adjusted according to the respective pH of enzymes used, pH 8.0 for Alcalase® and pH 5.0 for Celluclast®, using 0.1 N NaOH or 0.1 N HCl

Table 2.7 : The effect of varying the extraction time

Experiment	Enzymes	Duration, hours
38	Alcalase®	2
39	Alcalase®	4
40	Alcalase®	6
41	Alcalase®	8
42	Alcalase®	20
43	Celluclast®	2
44	Celluclast®	4
45	Celluclast®	6
46	Celluclast®	8
47	Celluclast®	20

Temperature is one of the important parameter that affects the oil yield in the EAAOE. The experimental conditions to study the correlation between temperatures of extraction process with the oil yield are exhibited in Table 2.8. The reaction duration of the experiments involved in this study was within 8 hours. The mass of enzyme added in the experiments was 250 mg. The pH of the mixtures were adjusted according to the respective pH of enzymes used, pH 8.0 for Alcalase® and pH 5.0 for Celluclast®, using 0.1 N NaOH or 0.1 N HCl.

Table 2.8 : The effect of varying the temperature in EAAOE

Experiment	Enzymes	Temperature, °C
48	Alcalase®	30
49	Alcalase®	45
50	Alcalase®	50
51	Alcalase®	55
52	Celluclast®	30
53	Celluclast®	45
54	Celluclast®	50
55	Celluclast®	55

Ultrasonic bath machine; Wise Clean Ultrasonic Cleaner Digital was used to determine the effect of incorporation of ultrasonication process in the EAAOE. The condition to study the effect of ultrasonication process was demonstrated as in Table 2.9. The mass of enzyme added in the experiments was 250 mg. The pH of the mixtures were adjusted according to the respective pH of enzymes used, pH 8.0 for Alcalase® and pH 5.0 for Celluclast®, using 0.1 N NaOH or 0.1 N HCl. Blank samples were run at pH 5.0 and pH 8.0, at the same conditions without adding enzymes which act as a control in the experiments. The EAAOE was further preceded in the duration of 8 hours at 50°C. The effect of ultrasonication process was further investigated using polarising microscope; Leica polarising microscope (magnifying lens diameter size of 50D) at 3 minutes, 5 minutes, 10 minutes and 20 minutes.

Table 2.9 : The effect of incorporation of ultrasonication process in EAAOE

Experiment	Enzymes	Time of ultrasonication process, minutes
56	Alcalase®	3
57	Alcalase®	5
58	Alcalase®	10
59	Alcalase®	15
60	Alcalase®	20
61	Celluclast®	3
62	Celluclast®	5
63	Celluclast®	10
64	Celluclast®	15
65	Celluclast®	20

Besides temperature, pH, extraction time, enzymes selection and so on, the ratio of water to seed was one of the important factor that affected the oil yield in EAAOE. The experimental condition to study the effect of water:seed ratio was illustrated in Table 2.10. The reaction duration of the experiments involved in this study was within 8 hours. The mass of enzyme added in the experiments was 250 mg. The reaction temperatures were set similar as above, at 50°C. The pH of the mixtures were adjusted according to the respective pH of enzymes used, pH 8.0 for Alcalase® and pH 5.0 for Celluclast®, using 0.1 N NaOH or 0.1 N HCl.

Table 2.10 : The effect of volume of water added in the suspension of *Jatropha* seeds

Experiment	Enzymes	Volume of water, mL	Ratio volume of water to mass of seeds, (mL/g)
66	Alcalase®	20	1 :4
67	Alcalase®	25	1 :5
68	Alcalase®	30	1 :6
69	Alcalase®	35	1 :7
70	Alcalase®	40	1 :8
71	Celluclast®	20	1 :4
72	Celluclast®	25	1 :5
73	Celluclast®	30	1 :6
74	Celluclast®	35	1 :7
75	Celluclast®	40	1 :8

2.1.2.4 Oil Recovery from Oil-Water Mixture

The effects of centrifugation temperature, solvent-aided oil separation and chilling process were studied using the enzyme Alcalase®. The oil-water mixture was subjected to the centrifugation process and then hexane was added into the mixture to form an oil-hexane layer and an aqueous layer. The mixture was further cooled to freeze the aqueous layer and ease the oil-hexane layer separation. Finally, hexane was removed by a rotary evaporator. The experimental design and condition in this study are shown in Table 2.11.

Table 2.11 : The effect of recovery step variables on overall extraction oil yield

Experimental	Centrifuge temperature, T / °C	Centrifuge speed, rpm	Centrifuge Duration, min	Volume of hexane added, mL	Cooling duration, hours
76	25	8000	30	5	-
77	25	8000	30	5	Overnight
78	25	8000	30	5	Overnight
79	4	8000	30	5	Overnight
80	4	8000	30	5	-
81	4	8000	30	-	Overnight
82	4	8000	30	-	-
83	4	8000	30	5	2

Notes:
Experiment 76, 77: enzyme Alcalase®, incubation temperature,T = 30°C, duration of incubation, t = 8 hours, pH 8.0;
Experiment 78, 79, 80, 81, 82, and 83: enzyme Alcalase®, incubation temperature, T = 50°C, duration of incubation, t= 8 hours, pH 8.0

2.1.2.5 Extraction Yield

Oil recovery from the enzymatic assisted aqueous oil extraction (EAAOE) was calculated using Equation 2.1 as the percentage of *Jatropha* oil obtained with respect to total oil content. The total oil content was determined by solvent extraction method using Soxhlet apparatus according to Palm Oil Research Institute Malaysia (PORIM) official test method (Siew *et al*., 1995).

Equation 2.1

Oil recovery, % (m/m) = [Oil extracted by EAAOE / Total oil content] x 100%

2.2 Analysis of Physicochemical Properties of *Jatropha* Oil

In order to evaluate the properties of *Jatropha* oil physically and chemically, several test methods were performed according methods report by Siew *et al.* (1995).

2.2.1 Determination of Free Fatty Acid Content

The free fatty acid (FFA) content of *Jatropha* oil extracted was determined (Siew *et al*., 1995). The FFA can be described as the number of potassium or sodium hydroxide (in milligrams) required to neutralise the free acids in one gram of sample. The acidity is the content of FFA conventionally expressed as a percentage (m/m) of oleic acid is indicated in Equation 2.2.

Equation 2.2

$$\text{FFA\% as oleic acid (C18:1)} = \frac{28.2 \times N \times V}{W}$$

Where N = normality of NaOH solution;

V = volume of NaOH solution used in mL;

W= weight of sample;

Oleic acid was used in the calculation of the FFA content in *Jatropha* oil due to the high composition (% methyl ester) of oleic acid in the fatty acid composition of *Jatropha* oil as discussed in Section 1.2.3.1. 5 g ± 0.01 g of *Jatropha* oil sample for FFA content determination was weighed into conical flask. 50 mL of the neutralised sample was added into the conical flask; which was prepared by boiling 50 mL of isopropanol in a flask and was neutralised by drop-wise addition of 0.1 N NaOH solution till a faint, but permanent pink colour was obtained. The conical flask of *Jatropha* oil sample with neutralised solvent was heated on the hot plate to about 40°C.

The sample was gently shaked while titrated with 0.1 N standard NaOH solution until the appearance of the first permanent pink colour. The colour was persisted for 30 seconds before the reading was taken for the further calculation as in Equation 2.2.

0.1 N standard NaOH was standardised with potassium hydrogen phthalate (KHP). KHP was dried in an oven at 120°C for 2 hours and was allowed to cool in dessicator before use. 0.4 g ± 0.001 g of the KHP was weighed directly into a conical flask. 50 mL of distilled water and phenolphthalein indicator were added. The mixture was placed on the hot plate and swirled until the KHP salt has completely dissolved. The solution was further titrated with NaOH to the first appearance of a permanent light pink colour. Normality of the NaOH was calculated as in Equation 2.3.

Equation 2.3

$$\text{Normality of the alkali, NaOH} = \frac{M \times 10^3}{V \times 204.2}$$

Where M = is the mass, in grams, of phthalate taken; and

V = is the volume, in millilitres, of NaOH

2.2.2 Determination of Iodine Value

Iodine value (IV) is a parameter to quantify the unsaturation level of fats and oils. IV is defined as the number of grams of iodine absorbed by 100 g of the fat under the test conditions. A solution of iodine monochloride in a mixture of acetic acid and cyclohexane was added to a test portion. The excess of iodine monochloride was reduced by adding potassium iodide solution and water after 30 minutes reaction, and titration of the liberated iodine with standard sodium thiosulphate solution (Siew *et al*., 1995). IV can be calculated using Equation 2.4.

Equation 2.4

$$\text{Iodine value} = \frac{12.69N\,(V_2 - V_1)}{W}$$

Where N is the exact normality of the sodium thiosulphate solution used;

V_2 is the volume, in millilitres, of the sodium thiosulphate solution used for the blank test;

V_1 is the volume, in millilitres, of the sodium thiosulphate solution used for the determination;

W is the weight, in grams, of the test portion.

0.2 g of *Jatropha* oil was weighed to the nearest 0.0001 g in the glass weighing vial. The vial was placed in a 500 mL flask. 20 mL of cyclohexane was added to dissolve the oil. 25 mL of Wijs solution was added, stopper was inserted, gently shaked and placed the vial in the dark for 1 hour. Subsequently, 20 mL of the potassium iodide solution and 100 mL of water was added. The mixtures were titrated with the standard sodium thiosulphate solution ($Na_2S_2O_3$) until the yellow colour almost disappears. 2 mL of the starch indicator was added and the titration was continued until the blue colour disappeared with vigorous shaking. The analysis was done in duplicates and blank sample was carried out simultaneously. 0.1 N standard $Na_2S_2O_3$ solution was standardised with potassium dichromate ($K_2Cr_2O_7$). 0.16-0.22 g of finely ground and dried potassium dichromate was weighed into a 500 mL flask by difference from a weighing bottle, was dissolved in 25 mL of water. 5 mL of concentrated hydrochloric acid (HCl) and 20 mL of potassium iodine solution were added, allowed to stand for 5 minutes and 100 mL of distilled water was added. The mixture was titrated with $Na_2S_2O_3$ solution, shaking continuously until the yellow colour has almost disappeared. Normality of $Na_2S_2O_3$ solution was calculated as in Equation 2.5.

Equation 2.5

$$\text{Normality of } Na_2S_2O_3 \text{ solution} = \frac{20.394 \times W}{V}$$

Where W = Mass of potassium dichromate, $K_2Cr_2O_7$

V = Volume of $Na_2S_2O_3$ solution in millilitres.

2.2.3 Determination of Peroxide Value (PV)

The peroxide value (PV) is a measure of milliequivalents of active oxygen per kilogram of oil or fats which oxidise potassium iodide under the conditions of the test (Siew *et al*., 1995). PV of *Jatropha* oil can be calculated using Equation 2.6.

Equation 2.6

$$\text{Peroxide value} = \frac{(V_s - V_b)\ N \times 1000}{W}$$

Where V_s is the volume in millilitres, of the sodium thiosulphate solution of normality N, used for the determination;

V_b is the volume, in millilitres, of the sodium thiosulphate solution used for the blank test;

W is the weight, in grams, of the test portion;

N is the normality of the sodium thiosulphate solution.

2 g of *Jatropha* oil was weighed to the nearest 0.0001 g and transferred into the 250 mL conical flask. 30 mL of the acetic acid-chloroform solution (3:2 v/v) was added into the flask. The flask was swirled until the sample was dissolved in the solution. 0.5 mL of saturated potassium iodide was added by using graduated pipette. The solution was swirled again for 1 minute and 30 mL of distilled water was added.

A few drops of starch solution were added in the solution. The solution was titrated with 0.01 N sodium thiosulphate solution ($Na_2S_2O_3$) which added gradually, with constant and vigorous shaking. The titration was continued, the flask was shaken vigorously near the end-point to liberate all the iodine from the chloroform layers. The $Na_2S_2O_3$ solution was added dropwise until the blue color disappeared. At the same time, parallel with PV analysis of *Jatropha* oil, a blank sample was carried out. The blank titration should not exceed 0.1 mL of the 0.01 N $Na_2S_2O_3$ solution.

0.1 N standard $Na_2S_2O_3$ solution was standardised with 0.01 N potassium dichromate ($K_2Cr_2O_7$) solution. 4.90 g of dried $K_2Cr_2O_7$ was dissolved in distilled water and made up to 1 litre in a volumetric flask. 25 mL of the standard $K_2Cr_2O_7$ solution was pipetted into a conical flask. 5 mL of concentrated sulphuric acid (H_2SO_4) and 10 mL of potassium iodide (KI) solution (10% w/v) was added and swirled homogeneously.

The mixture was allowed to stand for 5 minutes and 10 mL of distilled water was added. The mixture was further titrated with $Na_2S_2O_3$ solution, shaking continuously until the yellow colour has almost disappeared. 1 mL of starch indicator was added and titration was continued until the blue colour has just disappeared. Normality of $Na_2S_2O_3$ solution was calculated as in Equation 2.7.

Equation 2.7

$$\text{Normality of } Na_2S_2O_3 \text{ solution} = \frac{25 \times N}{V}$$

Where N = Exact normality of the standard potassium dichromate, $K_2Cr_2O_7$ solution

V = Volume of $Na_2S_2O_3$ solution in millilitres.

2.2.4 Determination of Saponification Value

Saponification value (SV) is expressed by the number of potassium hydroxide required to saponify one gram of fat under the conditions specified. It is a measurement of the average molecular weight of all the fatty acids present. The acylglycerols present are splitted by alcoholic alkali and any free fatty acids are neutralised. Excess alkali is back-titrated with hydrochloric acid (HCl) in the presence of an indicator, phenolphthalein solution (Siew *et al*., 1995). SV is given by Equation 2.8.

Equation 2.8

$$\text{Saponification value} = \frac{56.1\ N\ (Vb-Vs)}{W}$$

Where V_b is the volume, in millilitres of the HCl solution used for the blank;

V_s is the volume in millilitres of the HCl used for the determination of the sample;

N is the normality of the HCl;

W is the weight, in grams of the test portion.

2 g of *Jatropha* oil was weighed to the nearest 0.005 g and transferred into a conical flask. The volume of HCl required for the determination will be about half that required for the blank determination. 25 mL of the ethanolic potassium hydroxide solution and some boiling aids were added. The reflux condenser was connected and boiled for at least 60 minutes, swirling the contents of the flask from time to time. The flask and condenser were allowed to cool slightly and washed the inside of the condenser with a little distilled water. 1 mL of phenolphthalein solution was added and titrated with 0.5 N HCl until the pink colour of the indicator disappears. A blank determination was conducted simultaneously with the test sample using the same procedure.

2.2.5 Determination of Moisture Content

Water content is one of the important quality parameters of the oil products. Since *Jatropha* oil produced will be converted to *Jatropha* oil methyl esters *via* transesterification process, the presence of small amount of water may inhibit the conversion. Thus, moisture content in the oil must be analysed in the present study. The moisture content analysis was performed by Karl Fischer Volumetric Titration using Hydranal methanol rapid as solvent medium and Hydranal-Composite 5 as titration agent. 40 ml of Hydranal Methanol Rapid was added into the titration vessel for pre-titration. Sample is ready to be added when the drift value is lower than 5. Afterward, 1 g of sample was injected into the titration vessel for titration.

2.2.6 Determination of Density Value

Density is a fundamental physical property of matter, as every element generally has a distinctive density that can correlate with other properties to characterise the element itself. In the present study, the density of *Jatropha* oil was analysed by using digital density meter DMA 4500 (brand: Anton Paar, Austria). 4 mL of *Jatropha* oil was injected by using 5 cc/mL disposable syringe into the u-shape capillary tube of the density meter. The oil was injected slowly and carefully to avoid the formation of air bubbles inside the capillary tube. Samples were equilibrated to within 0.01°C of the desired temperature, 25°C. The density value of *Jatropha* oil was measured with an uncertainty of ± 0.05 g/cm^3 as the temperature has been stabilised. The capillary tube was washed with hexane until the reading at the density meter decrease to 0.012 g/cm^3 for the next sample injection. Reported values were an average of three measurements.

2.2.7 Determination of Fatty Acid Composition

2.2.7.1 Preparation of Methyl Ester Derivatives of Fatty Acids

Fatty acid composition (FAC) measures the percentage of the individual fatty acids (as in methyl esters), whether present as free fatty acids or in the esterified form in the triacylglycerol molecules in oil (Kuntom *et al*., 2005). The analysis was done by gas chromatography fitted with flame ionised detector (GC-FID). The preparation of methyl esters derivatives of fatty acids was by base-catalysed transesterification process where theoretically acylglycerols are transesterified very rapidly in anhydrous methanol in the presence of a basic catalyst (Christie, 1989). Approximately 35 mg of *Jatropha* oil was dissolved in 1 mL of dry toluene in a test tube. 3 mL of 0.5 M sodium methoxide in anhydrous methanol was added to the solution and the reaction mixture was maintained in a water-bath at 50°C for 15 minutes. 0.1 mL of glacial acetic acid was then added and followed with 5 mL of water. The required esters were extracted into hexane (2 x 5 mL) using a separating funnel. Subsequently, hexane layer was dried over anhydrous sodium sulphate (Na_2SO_4). The solvent was further removed under reduced pressure on a rotary evaporator.

2.2.7.2 FAC Analysis by Gas Chromatography - Flame Ionisation Detector

GC analyses were performed on a Shimadzu, GC-2010A Series equipped with flame ionisation detector (FID). 1 µL of the esters solution obtained was further injected to gas chromatography – flame ionisation detector (GC-FID) for fatty acid composition (FAC) analysis of *Jatropha* oil. The injection of standard solution of FAME mixture purchased from Sigma-Aldrich was carried out as an external standard for FAC analysis. The temperature of oven, injection port and detector were set at 140°C, 240°C and 260°C. The pressure of carrier gas, nitrogen was set at 12.9 psi and total flow rate of 111 mL/min.

The polar column, BPX-70 (70% Cyanopropyl polysilphenylene – siloxane) used has inner diameter of 0.25 mm ID with film thickness of 0.25 µm and length of 30 m. The maximum temperature of the column was 260°C and the column flow at 2.1 mL/min. The injection mode was split with split ratio of 50:1 and split flow at 105.8 mL/min. As for the temperature programming, an initial temperature of 140°C was hold for 2 minutes and was then programmed at 8°C per minute until the final temperature was 220°C and held for another 5 minutes. Total run time was 17 minutes.

2.2.7.3 FAC Analysis by Gas Chromatography - Mass Spectrometry

The 1 µL of the esters solution obtained was further injected to gas chromatography – mass spectrometry (GCMS) analysis. Similar to GC-FID analysis, the temperature of oven, injection port and detector were set at 140°C, 240°C and 260°C. The pressure of carrier gas, nitrogen was set at 12.9 psi and total flow rate of 111 mL/min. The polar column, BPX-70 (70% Cyanopropyl polysilphenylene – siloxane) used has inner diameter of 0.25 mm ID with film thickness of 0.25 µm and length of 30 m. The maximum temperature of the column was 260°C and the column flow at 2.1 mL/min. The injection mode was split with split ratio of 50:1 and split flow at 105.8 mL/min. As for the temperature programming, an initial temperature of 140°C was hold for 2 minutes and was then increased at 8°C per minute until the final temperature was 220°C and held for another 5 minutes. Total run time was 17 minutes.

2.2.8 Determination of Phorbol Esters in *Jatropha* Oil by High Performance Liquid Chromatography

Two types of solvent system were prepared, namely, formic solution (1.75 mL formic acid in a 1 L of distilled water) and HPLC gradient grade of acetonitrile. Both solvent systems were filtered by using 0.2 µm nylon membrane filter and degassed by using ultrasonicator bath. The WATERS GI HPLC pump, WATERS 2998 HPLC Auto Diode Array Detector, WATERS 2767 HPLC autosampler manager, and software Mass Lynx 4.1 were used. The analytical column used was reverse phase C18 (Chromolith® end-capped 5 µm, 4.6 x 100 mm I. D., Merck). General parameters for HPLC were including flow rate of 1 mL/min, column temperature at 30°C, pressure at 2000-4000 psi. The absorbance of phorbol esters was recorded at 280 nm.

The solvent gradient system begun with 40% of formic solution and 60% of acetonitrile followed by reducing formic solution to 25% and increasing the acetonitrile to 75% in the next 25 minutes and finally to 100% acetonitrile for the next 20 minutes. The condition of acetonitrile at 100% was maintained for 2 minutes. The total run time was 46 minutes. The column was adjusted back to initial conditions (40% of formic solution and 60% of acetonitrile) and was maintained for 5 minutes before proceeding with the next sample injection.

2.2.8.1 Extraction of Phorbol Esters from *Jatropha* Oil

The method of phorbol esters extraction was adapted from Gaudani *et al.* (2009). 10 g of *Jatropha* oil was extracted from *Jatropha curcas* seeds as discussed in earlier Section 2.1.2.2 and Section 2.1.2.3. 250 mL of n-hexane was added gently to the oil until a homogenised mixture has formed. The mixture was then transferred into a separating funnel in order to increase the volume and surface area. 200 mL of methanol was added drop wise to the mixture at similar rate for every extraction (40-45 drops per minute).

After the completion of drop wise addition process of methanol, subsequently the mixture was left for 3 hours. The lower layer of methanol extract was collected drop wise without disturbing the upper layer of hexane in a clean separate beaker. The process was repeated four times to achieve complete phorbol esters extraction from the oil. The methanol extract was combined together from repeated extraction process and was placed in the water bath at 65°C to remove the methanol from the extracted material. The remaining methanol was flushed with nitrogen and the mass of extracted material was recorded. 1 mL of methanol was added and vortex thoroughly. The mixture was filtered by using 0.2 nylon filters and transferred into 1.5 mL HPLC vial. The filtrate was injected into HPLC detector (WATERS 2998 HPLC Auto Diode Array Detector) and the analytical column is reverse phase C18 (Chromolith® end-capped 5 µm, 4.6 x 100 mm I. D., Merck).

2.2.9 Determination of Phorbol Esters from *Jatropha* Oil by Liquid Chromatography – Mass Spectrometry

Two types of solvent system were prepared, namely, formic solution (1.75 mL formic acid in a 1 L of distilled water) and HPLC gradient grade of acetonitrile. Both solvent systems were filtered by using 0.2 µm nylon membrane filter and degassed by using ultrasonicator bath. The Agilent 6530 Accurate-Mass Q-TOF liquid chromatography mass spectrometry (LCMS) paired with Agilent's 1200 series LCs, Agilent Jet Stream Electrospray Ionization (ESI) source, and software Agilent MassHunter workstation software – quantitative analysis were used. The analytical column used was reverse phase C18 (Purospher® STAR end-capped 5 µm, 4.6 x 100 mm I. D., Merck). General parameters for LCMS were including flow rate of 0.25 mL/min, column temperature at 40°C, pressure limit up to 500 bar.

The solvent gradient system begun with 40% of formic solution and 60% of acetonitrile followed by increasing the acetonitrile to 100% in the next 30 minutes and reducing the acetonitrile to 60% for the next 15 minutes. The condition of acetonitrile at 100% was maintained for 1 minute. The total run time was 46 minutes. The column was adjusted back to initial conditions (40% of formic solution and 60% of acetonitrile) and was maintained for 5 minutes before proceeding with the next sample injection.

2.3 Transesterification Process

2.3.1 Materials

Crude *Jatropha* oil was purchased from Bionas Sdn. Bhd. Methanol, dichloromethane, hexane, diethyl ether and glacial acetic acid, sodium hydroxide (NaOH), anhydrous sodium sulphide (Na_2SO_4), and sulphuric acid (H_2SO_4) were supplied from Systerm. Thin layer chromatography (TLC) plates pre-coated with silica 60 F_{254} were purchased from Merck. Triacontane and N, N-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Sigma-Aldrich.

2.3.2 Types of Transesterification

2.3.2.1 Double-Stage Transesterification

Double-stage transesterification reactions were carried out to produce *Jatropha* oil methyl esters from *Jatropha* oil with high content of free fatty acids (FFA). The first stage of transesterification reaction was simple batch-wise reaction and the set-up included a three-necks round bottom flask equipped with a condenser and a thermometer. Figure 2.3 depicts the reaction set up of transesterification reaction.

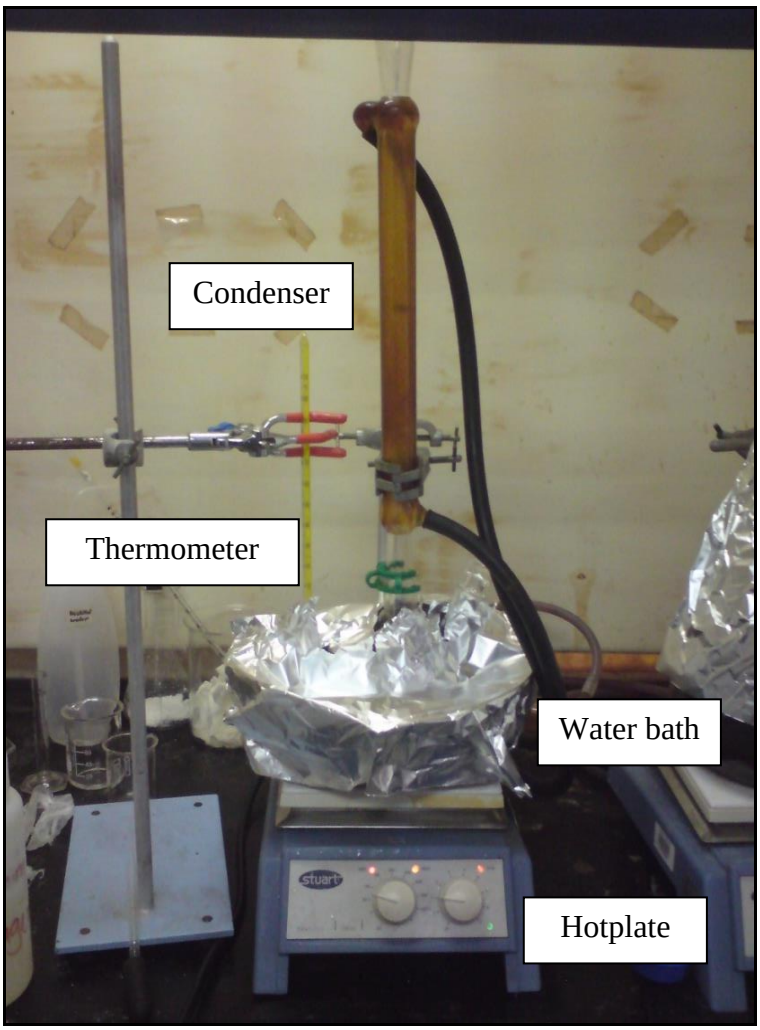


Figure 2.3 : Reaction set up of transesterification reaction

The flask was submerged in the water bath which was placed on magnetic stirrer heating plate. Molar ratio of oil to methanol for the transesterification process was 1:21. The catalyst, sodium hydroxide (NaOH) was dissolved in methanol and the solution was added to the reaction mixture at the pre-set reaction temperature, 65°C to 68°C. The catalytic amount of NaOH was calculated as summation of 0.5% of the total mass of *Jatropha* oil and the amount needed for neutralisation of free fatty acids. The reaction progress of methyl ester conversion was monitored by thin layer chromatography (TLC) as described in Section 2.3.3. Upon completion of transesterification reaction, the reaction mixtures were transferred into a separating funnel to separate esters from glycerol layers.

The glycerol layer was decanted and the esters layer was purified and dried. The esters layer was washed with warm distilled water of 50-55°C and saturated sodium chloride (NaCl) solution using a separating funnel. The ester was further dried using anhydrous sodium sulphate (Na_2SO_4) and vacuum and weight. The second stage transesterification process was conducted on the esters yielded from the first stage transesterification. Similar procedure as the first stage transesterification was used.

2.3.2.2 Acid-Catalysed Esterification as a Pre-treatment of *Jatropha* Oil with High Free Fatty Acid

Esterification reaction was done prior to transesterification to produce *Jatropha* oil methyl ester from *Jatropha* oil with high content of free fatty acids (FFA). The reaction set up was described similarly as in Section 2.3.2.1 and Figure 2.3. During acid-catalysed esterification, oil bath was used to immerse the reaction flask and the temperature was set at 110-120°C. Molar ratio of oil to methanol was 1:21. The catalyst, concentrated sulphuric acid (H_2SO_4) was added to the reaction mixture. The concentration of sulphuric acid added was one percent of total mass of fatty acid in the oil samples. The reaction progress of esterification process of free fatty acid was also monitored by thin layer chromatography (TLC) as described in Section 2.3.3. The esterification process was completed once the free fatty acid was not detected in TLC. Sodium hydroxide (NaOH) which dissolved in methanol was added to the reaction for the transesterification process. The amount of NaOH added was calculated based on the mass of NaOH needed to neutralise the catalytic amount of H_2SO_4 and 0.5% of mass of oil for catalytic reaction. The reaction progress of transesterification was monitored by TLC as described in Section 2.3.3 while washing and drying of the product were carried as described in Section 2.3.2.1.

2.3.3 Monitoring Reaction Progress of Transesterification Process by Thin Layer Chromatography

Thin Layer Chromatography (TLC) was one of analytical methods used in monitoring the progress of transesterification reactions. TLC was carried out when small amount of aliquots of reaction mixture, commercial *Jatropha* oil and standard solution of FAME mixture (Sigma-Aldrich) were examined on precoated silica gel 60 F_{254}, 5 x 10 cm glass plates with 0.25 mm thickness (Merck, Darmstadt, Germany). The TLC plates spotted with sample were developed in saturated chromatographic tanks with hexane/diethyl ether/acetic acid system (7:3:1, v/v/v) and hexane/chloroform (1:1, v/v) at room temperature. The spots of the sample on the TLC plate were visualised by using iodine vapour.

2.3.4 Determination of Free Fatty Acid Content

The free fatty acid (FFA) content of *Jatropha* oil methyl ester was determined in accordance with that reported by Siew *et al*. (1995) as discussed in Section 2.2.1.

2.3.5 Determination of Moisture Content

Moisture content in the methyl esters must be analysed in the present study. The moisture content analysis was performed as discussed in Section 2.2.5.

2.3.6 Determination of Density Value

In the present study density value in the methyl esters must be analysed and compared with *Jatropha* oil. The analysis was performed as discussed in Section 2.2.6.

2.3.7 Determination of Pour Point

Petrotest instrument D12279 (Berlin) (Figure 2.4) was used in determining pour point of *Jatropha* oil and *Jatropha* oil methyl ester as indicated in standard test method for pour point of petroleum products (ASTM D97-04).

Figure 2.4 : Petrotest instrument D12279

Samples were heated to 45°C in a waterbath to ensure that the oil will flow smoothly into the test jar. The test jar containing samples was transferred into a waterbath without stirring at 24°C. The disk was placed at the bottom of the jacket; the gasket was placed around the test jar, 25 mm from the bottom. The test jar was inserted in the jacket to avoid direct contact with the cooling medium. The appearances of the samples were observed at 9°C above the expected pour point of *Jatropha* oil and for every multiple of 3°C below starting temperature. The temperature of the waterbath was decreased to 0°C when samples reached 27°C and - 18°C bath when the samples temperature reached 9°C.

2.3.8 Determination of Ester Content by using Gas Chromatography - Flame Ionisation Detector

Gas chromatography - flame ionisation detector (GC-FID) analysis was done to analyse the total ester content of *Jatropha* oil. GC analyses were performed on a Shimadzu, GC-2010A Series equipped with flame ionisation detector (FID). Heptane (analytical GC reagent grade) was used in ester content determination purchased from Merck. While methyl heptadecanoate was used as an internal standard in the analysis purchased from Sigma-Aldrich.

2.3.8.1 Parameters and Temperature Programming

The temperature of oven, injection port and detector were set at 60°C, 250°C and 310°C. The pressure of carrier gas, nitrogen, was set at 8.9 psi and split flow rate at 95.4 mL/min. The non-polar column, BPX-5, has inner diameter of 0.25 mm with film thickness of 0.25µm was used. An initial temperature of 60°C was hold for one minute and was then increased at 11°C per minute until the temperature reached 110°C. Then, the temperature was increased at 5°C/min until the final temperature reached 300°C and then held for another 10 minutes.

2.3.8.2 Sample Preparation and Quantification of Ester Content

About 250 mg of sample was weighed and dissolved in 5mL of of methyl heptadecanoate solution (10 mg/mL). The sample was then injected into gas chromatography – flame ionised detector (GC-FID). Ester content was determined to evaluate the total percentage of methyl ester conversion from *Jatropha* oil as outlined in the European Standard; BS EN14103: 2003 (E) and calculated using the following Equation 2.9.

Equation 2.9

$$C = \frac{(\sum_A) - A_{E_1}}{A_{E_1}} \times \frac{C_{E_1} \times V_{E_1}}{m} \times 100\%$$

Where

$\sum_A$ is the total peak area from the methyl ester in C_{14} to that in $C_{24:1}$

A_{E1} is the peak area correspondingly to the methyl heptadecanoate

C_{E1} is the concentration, in milligrams per millilitres, of the methyl heptadecanoate solution being used

V_{E1} is the volume, in millilitres, of the methyl heptadecanoate solution being used

m is the mass, in milligrams, of the sample

In the current research, the sample preparation of *Jatropha* oil methyl ester was described as in Table 2.12.

Table 2.12 : Sample preparation of *Jatropha* oil methyl ester for ester content determination

Experiment	TE3	TE4
Description	Double-stage transesterification	Direct esterification (pre-treatment of high free fatty acid (FFA)) and transesterification process
Mass of sample (mg)	250.11	250.29
Volume of methyl heptadecanoate solution (mL)	5.0	5.0
Concentration of sample in methyl heptadecanoate solution (ppm)	50022	50058