CHAPTER THREE

RESULTS AND DISCUSSION

3.1 OIL EXTRACTION PROCESS

3.1.1 Solvent Extraction Process (Soaking Method)

The soaking method for oil extraction from the seed is one of the most common method of oil extraction (Dunford, 2008). In the present study, the soaking method on *Jatropha curcas* seeds was carried out for 16 hours to investigate the effect of the ratio of mass of *Jatropha* oil seeds to the volume of the hexane used. The ratio of mass of *Jatropha* oil seeds, (g) to volume of hexane used, (mL) of 1:1, 1:2, 1:3, 1:4 and 1:5 were investigated (Experiments 1,2,3,4 and 5 respectively). The experimental design of the solvent extraction process using soaking method is discussed in section 2.1.2.1. The results of the oil yield on *Jatropha* oil extraction *via* the soaking method are tabulated in Table 3.1.

Table 3.1 : Solvent extraction *via* soaking method of *Jatropha curcas*

Experiment	Ratio of *Jatropha* seed to hexane (g/mL)	Duration (hours)	Solvent	Oil Yield, % (m/m)
1	1:1	16	Hexane	12.9
2	1:2	16	Hexane	12.2
3	1:3	16	Hexane	13.0
4	1:4	16	Hexane	15.6
5	1:5	16	Hexane	13.0

The result showed that the highest percentage of oil yield, 15.6% (m/m) was obtained in sample of Experiment 4 when the ratio of mass *Jatropha* oil seeds, (g) to volume of hexane used, (mL) is 1:4.

As the ratio was increased to 1:5, the percentage of the oil yield was decreased to 13.0% (m/m) due to the maximum contact between the solvent and the seeds not being achieved. The limitations of the experiment included low temperature condition; as the extractions were carried out in room temperature and no solvent flow throughout the experiments. Percentage of free fatty acid, FFA (as per oleic acid) in the oil obtained from this method was found to be low, in the range of 2 - 2.5% (Table 3.2).

Table 3.2 : Percentage of FFA, % (as oleic acid) for each oil sample extracted using chemical extraction

Experiment	Percentage of FFA, %
1	2.0
2	2.3
3	2.2
4	2.3
5	2.2

3.1.2 Solvent Extraction Process (Soxhlet Method)

In the solvent extraction method, hexane is the favoured solvent; hexane-based processes have been in industrial operation for a long time (Rosenthal *et al*., 1996). In the present study, soxhlet extraction was carried out according to Palm Oil Research Institute Malaysia (PORIM) test method (Siew *et al*., 1995) as described in section 2.1.2.2. The product with 52.98% (m/m) of oil yield was obtained from the experiment with free fatty acid content (as oleic acid) of 2.45%.

The same procedures were repeated in prolonged duration at 8 hours and at 10 hours, to observe the effect on oil yield. The results of percentage of *Jatropha* oil and free fatty acid content (% as oleic acid) were tabulated in Table 3.3.

Table 3.3 : Solvent extraction (soxhlet method) of *Jatropha curcas*

Experiment	Duration (hours)	Oil Yield, % (m/m)	Percentage of free fatty acids, % (as oleic acid)
S1 (A)	6	52.98	2.45
S1 (B)	8	52.96	2.51
S1 (C)	10	52.97	2.58

Based on the results tabulated in Table 3.3, the percentages of free fatty acids, % (as oleic acid) increased as duration of extraction increased. However, duration of extraction did not have significant difference on the yield of *Jatropha* oil in percentage, in comparison of Experiment S1 (A), S1 (B) and S1 (C). The oil yield was higher than that from solvent extraction *via* soaking method. Although the oil yield obtained was high, but required the higher amount of hexane used (industrial scale), could be a contributing factor to the industrial emissions of volatile organic compounds (VOCs). The production of VOCs in the conventional process is particularly bothersome since these can react in the atmosphere with other pollutants to generate ozone and other photochemical oxidants which can be dangerous to human wellbeing and can be the reason of destruction to crops (Rosenthal *et al.*, 1996).

3.1.3 Enzymatic Assisted Aqueous Oil Extraction.

An alternative pre-treatment to facilitate the release of oil from the seed could be enzymatic degradation. In this way, the partial hydrolysis of the seed cell structures with appropriate enzymes would increase permeability, which would in turn increase mass transfer (Rosenthal, 1996).

Enzymatic assisted aqueous oil extraction (EAAOE) has been extensively studied. It has received much attention, and viewed as an alternative method to extract oil from oil-bearing seeds as discussed in Section 1.3.4. Not only this green technology is beneficial to people health but EAAOE is also environment friendly. In the present project, improvements in terms of extraction yield and extraction rate were expected to be achieved. Therefore, the preparation of the seed before EAAOE was essential to maximise oil recovery as discussed in Section 2.1.2.3.

3.1.3.1 Selection of Enzymes

In most of the EAAOE reported, the enzymes used were usually varies such as protease, cellulase, pectinase, xylanase and amylase (Shah *et al*., 2005; Winkler *et al*., 1997). In current study, two commercially available enzymes, namely, Alcalase® 2.4 L (alkaline protease) and Celluclast® 1.5 L (cellulase) were engaged. The selection of protease and cellulase were corresponded with the morphology and composition of the kernel. Oil in the kernel of *Jatropha* was dispersed in matrices of protein and cellulosic cells. In order to release the oil, both protein and cellulosic complexes must be broken down. In this respect, the Alcalase®, a protease, was stipulated to be involved in the hydrolysis of protein in the cell membranes, lipid body membranes and in the cytoplasm of the oilseeds while the Celluclast®, a cellulase, will break the structure of cotyledon cell walls present in the kernel. Rosenthal *et al.* (1996) reported the interruption of the cytoplasmic network which is mainly composed of proteins by proteolytic enzymes, therefore making the internal structure less strictly bound and dense thus enabling easier elimination of protein and lipid in the cell. In current research, the *Jatropha* oil was extracted with the single addition of enzyme, Alcalase® and Celluclast® respectively in view of the effectiveness of enzymes selected.

The experimental design of single addition of enzyme in the EAAOE is showed in Table 2.2. The percentage of oil yield in single addition of enzyme added in the EAAOE is tabularised in Table 3.4.

Table 3.4 : Single addition of enzyme in the EAAOE

Experiment	Enzyme	pH	Oil Yield, % (m/m)
6	Celluclast®	4.5	31.9
7	Celluclast®	5.0	34.7
8	Celluclast®	6.0	26.4
9	Alcalase®	7.0	35.7
10	Alcalase®	8.0	40.6
11	Alcalase®	9.0	30.5

From the Table 3.4, it was found that in the single addition of enzyme in EAAOE, the oil yield was highest at pH 5.0 and pH 8.0 for Celluclast® and Alcalase® respectively. It was observed that as pH of the enzyme mixtures was increased, the oil yield was decreased. Therefore, EAAOE must be done at the optimum pH to achieve high oil yield. These results were further compared with the combination and step-wise addition of enzymes in EAAOE as illustrated in Figure 3.1.

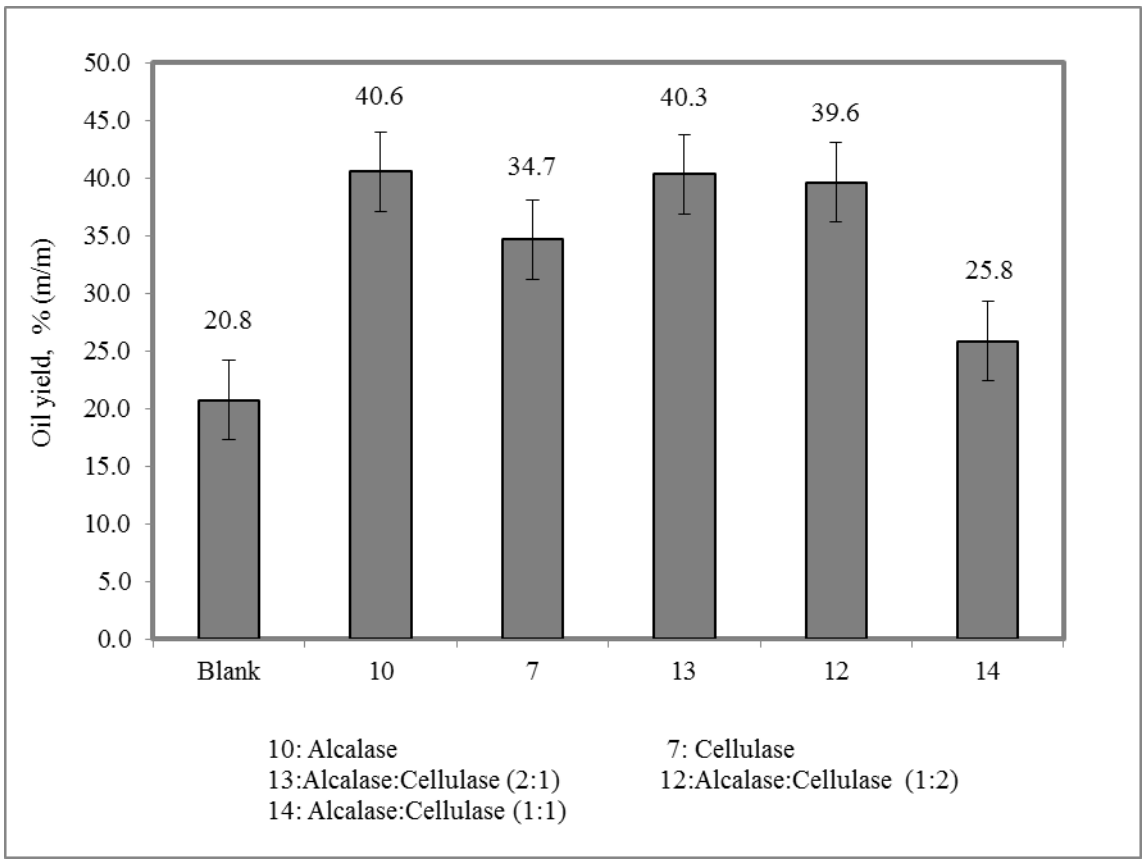


Figure 3.1 : Comparison of percentage (%) on oil yield by singular and combination enzymatic extractions

A blank sample was conducted as a control of enzymatic extraction where the *Jatropha* de-shelled seeds were extracted without enzyme addition. The oil yield was found to be 20.8% (m/m). The efficiency of both enzymes was tested under similar extraction parameters at pH 8.0 for Alcalase® and pH 5.0 for Celluclast® and it was found that Alcalase®-extracted oil yield is higher, 40.6% (m/m) than that of Celluclast®-extracted oil, 34.7% (m/m) (Figure 3.1, Experiment 10 and 7).

A binary enzyme system made up of both Alcalase® and Celluclast® at ratio of 1:2 and 1:1 (Figure 3.1, experiment 13 and 12), implied that oil-bearing cells were bound more strongly to its cellulosic materials than to the protein cells. When the concentration of Alcalase® and Celluclast® was increased by two-fold, the oil yield was higher than that when equal amount was used (Figure 3.1, experiment 14). It was unexpectedly found that the combination of both enzymes did not increase the oil yield more than in the singular enzymatic extraction. Results were comparable with the prior observation by Winkler *et al.* (1997) that combination of both proteases with hemicellulases and/or cellulases enzymes did not further increase the oil yield.

On the other hand, step-wise enzyme addition experiments conducted in the present study have provided important information on the enzymatic mechanism in the oil extraction as illustrated in Figure 3.2. A comparison between the one-step and step-wise addition of both enzymes at the same concentration has found that the cellulosic materials should be broken down first and followed by protein hydrolysis to facilitate oil releasing in the extraction process. Oil yield increased from 17.8 % (m/m) (Figure 3.2, Experiment 15) to 25.5 % (m/m) (Figure 3.2, Experiment 17) when Celluclast® was added first and followed by Alcalase®. This finding is useful when oil and protein extractions are targeted at the same time. Clearly from these experiments, it was learnt that the protein cell had been broken down during the step-wise enzymatic extraction, in which the oil-bearing cells were microscopically dispersed, should precede the destruction of cellulosic structure.

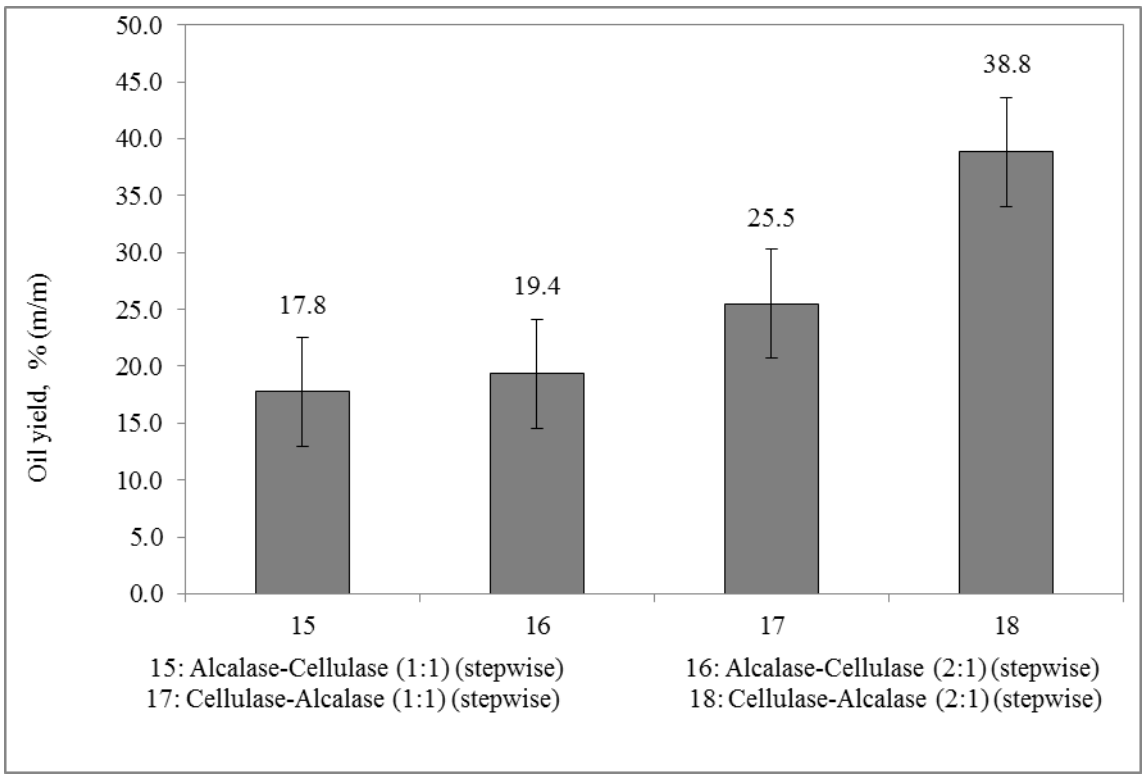


Figure 3.2 : Comparison of percentage (%) on oil yield by step-wise addition enzymatic extractions

3.1.3.2 Effect of Enzyme Concentration

The effect of enzyme concentration on oil extraction yield was studied under various extraction parameters as summarised in Table 2.5 and the results are exhibited in Figure 3.3. As discussed in section 3.1.3.1, a blank sample was conducted as a control of enzymatic extraction where the *Jatropha* de-shelled seeds were extracted without enzyme addition and the oil yield was found to be 20.8% (m/m). A comparison of the total oil yield between two different types of enzymes, Alcalase® and Celluclast® used in the enzymatic aqueous oil extraction is shown in Figure 3.3. The oil yield obtained by of Alcalase®, 58.7% (m/m) was comparable to that using Celluclast®, 56.7% (m/m) which could be clearly seen after the optimum ratio of the mass of enzyme to *Jatropha* seeds was achieved at 6% (m/m) both for Alcalase® and Celluclast®. The increment of enzyme concentration beyond 6% (m/m) resulted in formation of viscous emulsion and rendered in low oil yield.

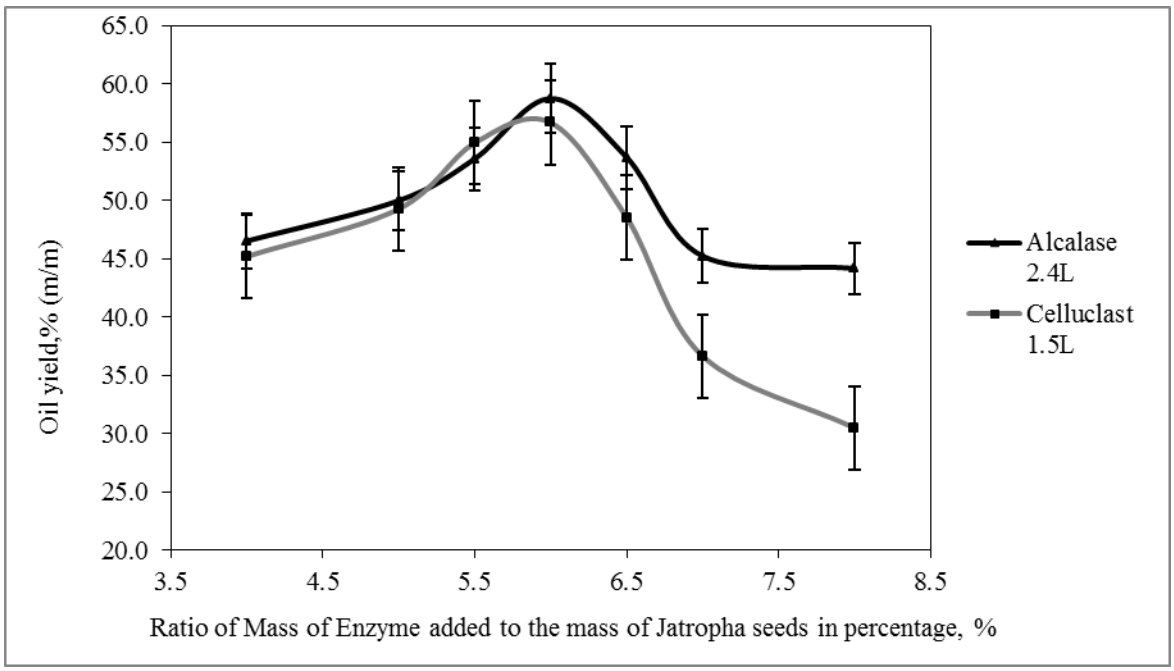


Figure 3.3 : Effect of using enzymes individually in the increment of mass of enzyme added into EAAOE

3.1.3.3 Effect of pH

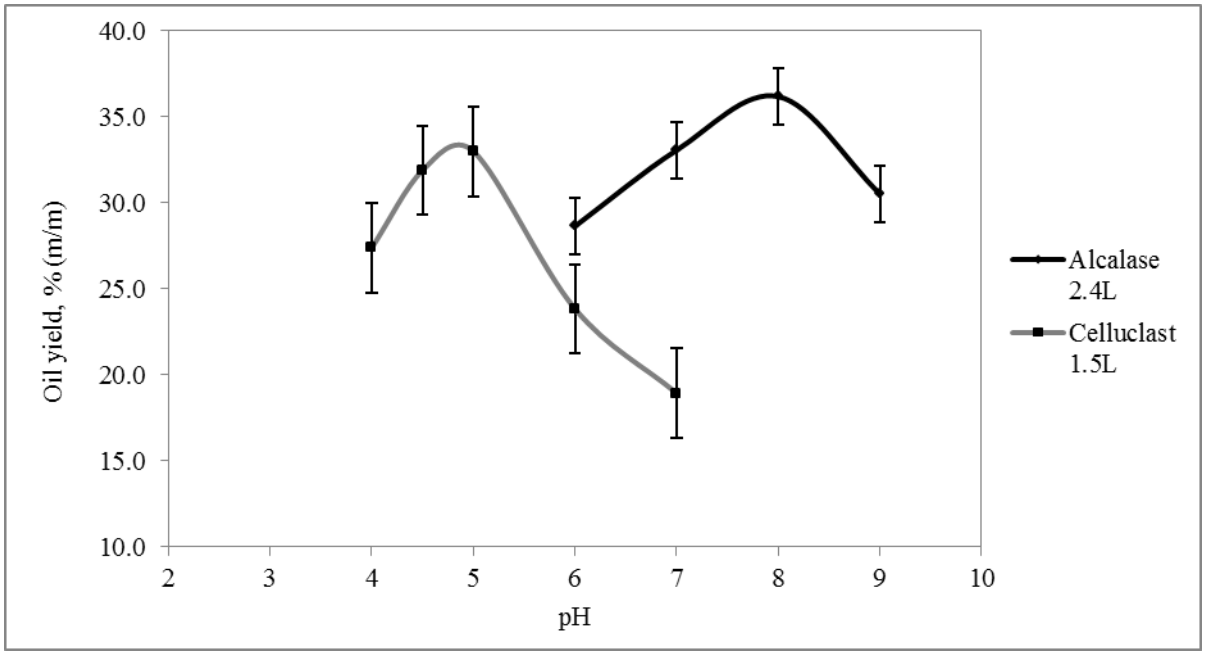


Figure 3.4 : Study of the pH of each enzyme into EAAOE with condition of temperature 50°C and the duration of 16 hours incubation time

The optimum pH values for Alcalase® and Celluclast® activity fall in the range of pH 7.0 to 9.0 and pH 4.0 to 6.0, respectively (Novozymes, 2009). In the current work, the enzymatic extraction was carried out in the duration of 16 hours at different pH values in order to examine the effect of pH on oil yield. The reaction parameters are described as in Table 2.6. The correlation between the pH used in the extraction with the oil yield obtained is shown in Figure 3.4. From Figure 3.4, the optimum pH for Celluclast® and Alcalase® are pH 5.0 (33.0% m/m) and pH 8.0 (36.2% m/m), respectively. The *Jatropha* oil extracted by Alcalase® at pH 8.0 results in oil with free fatty acid content of 2.12% while oil extracted by Celluclast® at pH 5.0 has free fatty acid content at 2.28%.

There was no correlation between the acidity of oil with the pH engaged for the extraction. A comparison of acid value of oil extracted by Soxhlet extraction further confirmed the current observation as the *Jatropha* oil extracted by solvent extraction with free fatty acid value of 2.45%. The change of pH values throughout the experiments was found to be within 1.0 unit from the respective optimum pH of the enzymes. However, no buffer was added into the mixture because the resultant pH was still within the pH range for optimum performance of both enzymes. In addition, the addition of buffer was found to hinder oil recovery due to the sample amount used in the experiments were small and emulsion formation caused by prolonged and continuous agitation of extraction mixtures.

3.1.3.4 Effect of Varying Extraction Duration and Particle Size

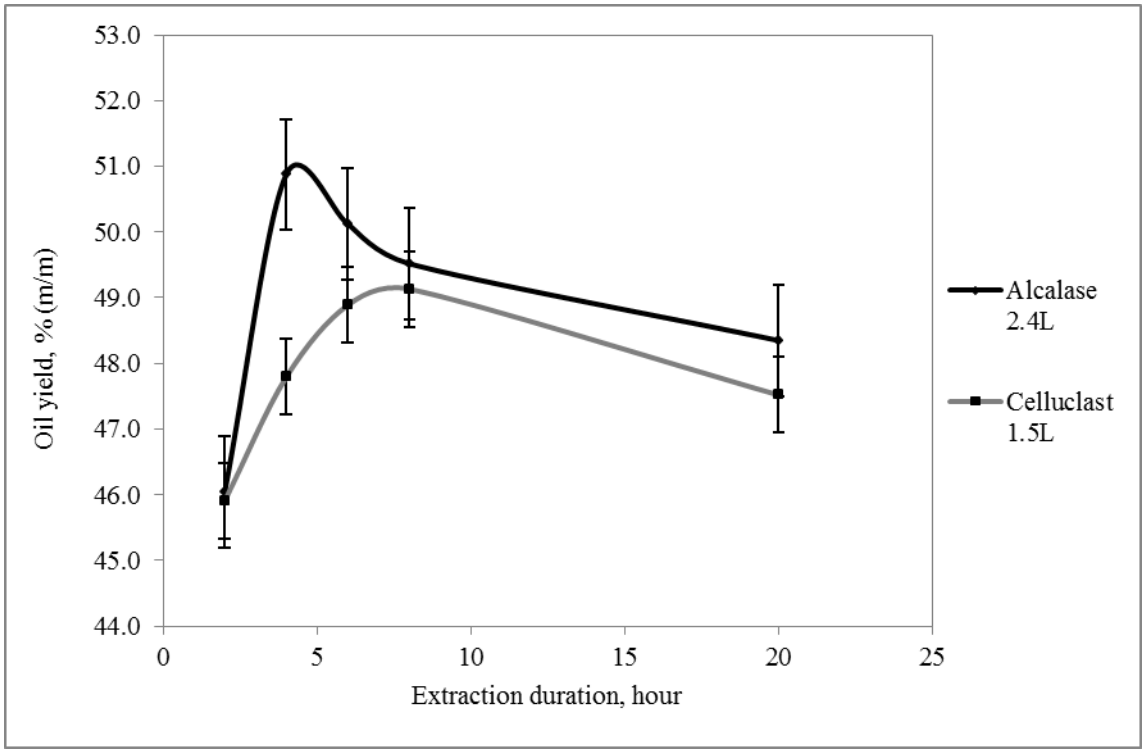


Figure 3.5 : Effect of incubation time in EAAOE

The experimental condition to study the effect of varying extraction duration is tabulated in Table 2.7. As illustrated in Figure 3.5, the oil extraction from fine powdered *Jatropha curcas* seeds for 4 hours (Alcalase®) and 8 hours (Celluclast®) managed to generate oil yield with optimised value of about 51% (m/m). There was significant increase in the oil yield when uniform and small particle size (less than 1 mm) of *Jatropha curcas* seeds was used due to increased total surface area and dispersal of oil released. The yield increased by 1.3% (m/m) and duration was reduced to 4 hours from total 8 hours (Alcalase®) and 1.6% (m/m) in 12 hours reduction of total 20 hours (Celluclast®). In addition, the uniform size of the powdered seeds has resulted in consistent and reproducible results which made study of other comparative extraction variables on yield possible.

In terms of effect of extraction duration, Alcalase®- assisted extraction produced oil yield of 51% (m/m) in 4 hours extraction; higher than that after 8 hours extraction, 50% (m/m). Meanwhile, Alcalase® needed 8 hours to extract the highest oil yield of 49% (m/m). It suggests that extended extraction duration was not required in enzymatic extraction as it caused reduction in oil yield. The deterioration of oil yield might be due to the emulsion formation induced by prolonged agitation of extraction mixture.

3.1.3.5 Effect of Extraction Temperature

Enzymatic extractions were conducted at optimum pH for both enzymes, pH 5.0 for Celluclast® and pH 8.0 for Alcalase® in the duration of 8 hours as summarised in Table 2.8. 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. Temperature had significant effect on total yield of *Jatropha* oil in EAAOE. The correlation between temperatures of extraction process with the oil yield obtained was demonstrated in Figure 3.6.

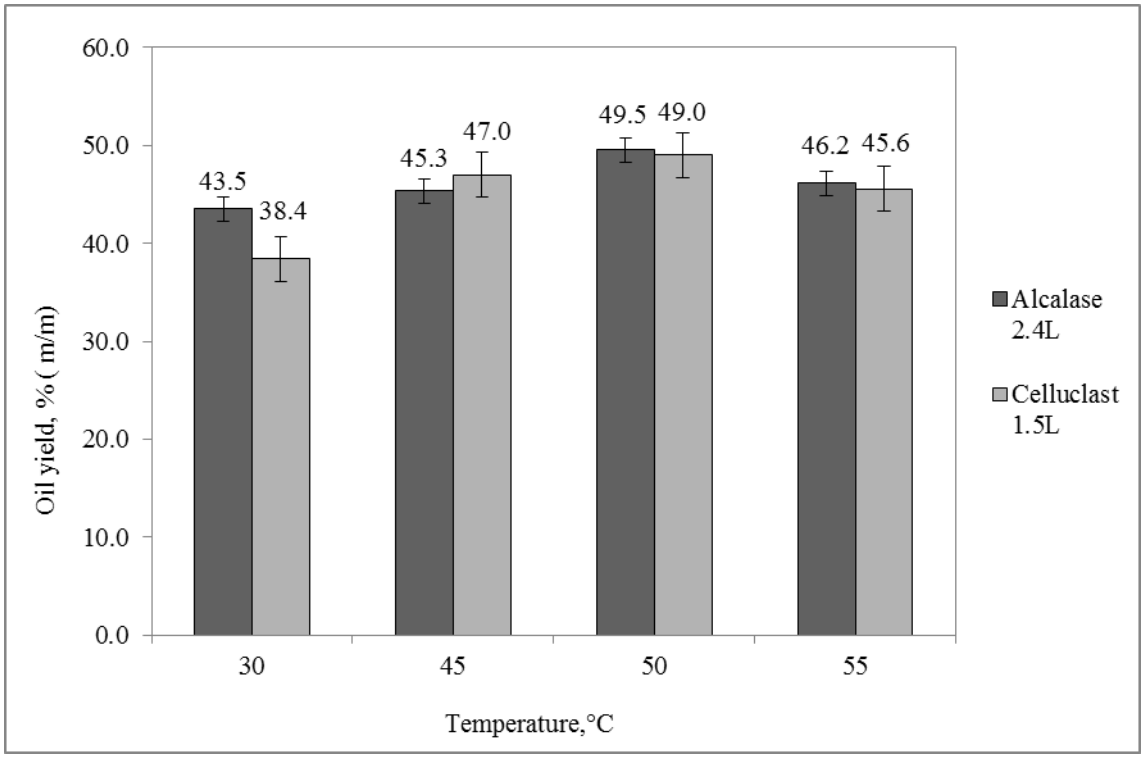


Figure 3.6 : Effect of varying incubation temperature in EAAOE

Based on Figure 3.6, as extraction temperature increased, oil yield rose from 43.5% (m/m) and 38.4% (m/m) to a maximum value of 49.5% (m/m) and 49.0% (m/m) for Alcalase® and Celluclast®, respectively. Further temperature increase beyond 55°C reduces the oil yield to 46.2% (m/m) and 45.6% (m/m). The process temperature seems to be a critical parameter because decreasing the temperature merely by 5°C to 45°C significantly lessened the total oil extracted. Increasing the temperature led to reduced oil recovery presumably because the enzyme becomes thermo-inactivated (Sharma *et al*., 2002[b]; Gupta and Gupta, 1993). The optimum temperature conditions for maximum EAAOE of *Jatropha* oil yield and quality was obtained at 50ºC. The findings were in agreement with Shah *et al.* (2005) *i.e.* the optimum oil yield obtained was at 50ºC for alkaline protease and cellulase/hemicellulase enzymes.

3.1.3.6 Effect of Ultrasonication during Extraction

Contrary to reported positive effect of ultrasonication on extraction yield (Shah *et al*., 2005), current research found that higher oil yield was observed when no ultrasonication step was engaged as illustrated in Figure 3.7.

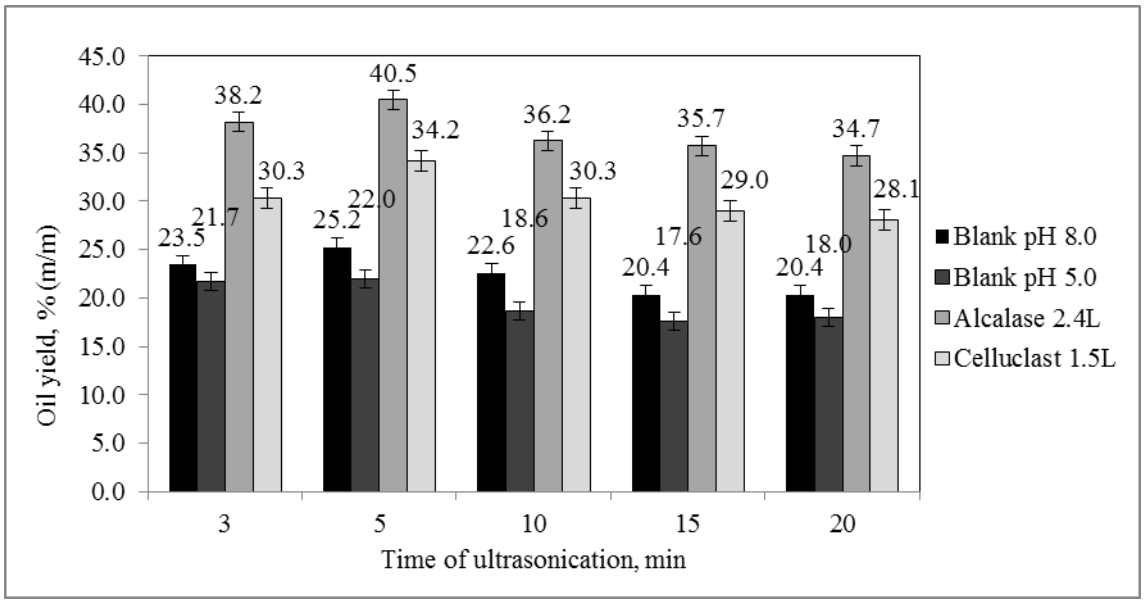


Figure 3.7 : Effect of ultrasonication in EAAOE

The reaction condition to study the effect of ultrasonication process was tabulated as in Table 2.9. In the present study, blank samples were done for 3, 5, 10, 15 and 20 minutes of ultrasonication at pH 5.0 and pH 8.0 respectively. All parts of the equipment and glassware in contact with the blank sample were enzyme-free. The highest oil yield of blank samples was 25.2% (m/m) and 22.0% (m/m) for pH 8.0 and pH 5.0 respectively and the lowest oil yield of 20.4% (m/m) and 17.6% (m/m) respectively. The comparison results of blank samples and EAAOE samples treated with ultrasonication justified the effect of ultrasonication extraction process on *Jatropha* oil yield, albeit low oil yield.

The incorporation of 5 minutes of ultrasonication during the enzymatic oil extraction achieved highest oil yield, *i.e.* Alcalase®, 40.5% (m/m) and Celluclast®, 34.2% (m/m). From the graph observation, addition of enzymes; Alcalase® and Celluclast® improved the oil yield from ultrasonication extraction. Moreover there was no linear correlation between the oil yield and ultrasonication duration. Further to that, the oil yield decreased to 34.7% (m/m) and 28.1% (m/m) for Alcalase® and Celluclast®, respectively, when ultrasonication was applied up to 20 minutes. The effect of ultrasonication process in the enzymatic oil extraction was further investigated using polarising microscope as illustrated in Figure 3.8.

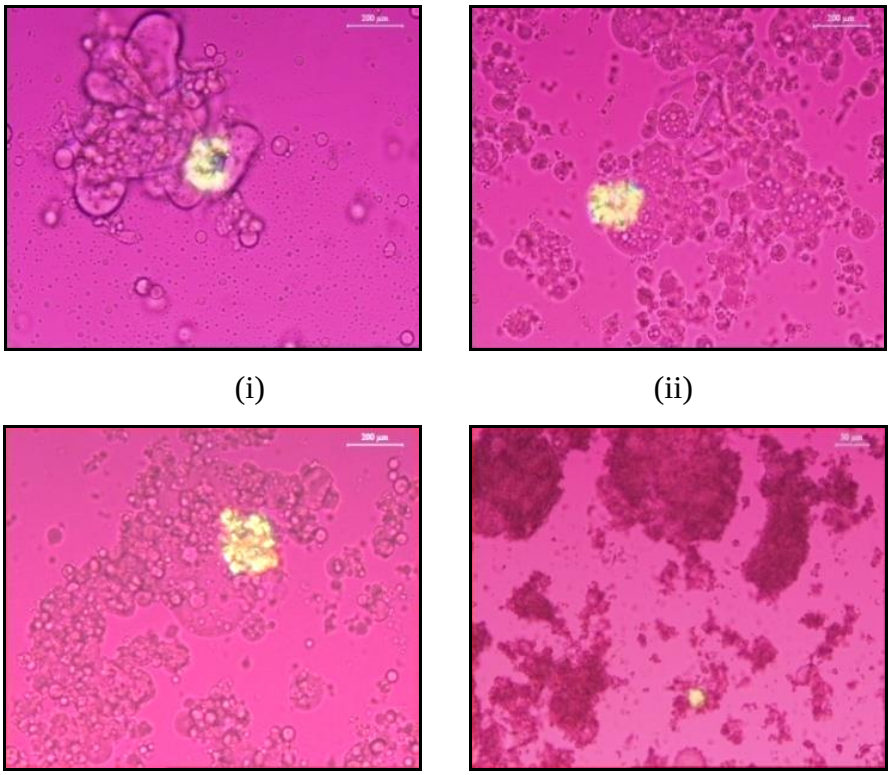


Figure 3.8 : Pictures of ultrasonication effect on EAAOE studied under polarising microscope at (i) 3 minutes, (ii) 5 minutes, (iii) 10 minutes and (iv) 20 minutes

The observation examined that ultrasonication process had disrupted the cell wall of the seed body structure by shear forces. Consequently, the intracellular particles were released out of the cell walls or subcellular organelles envelope mechanically. The observation under polarising microscope of current work was concurring with Cravotto *et al.* (2008), as they reported that ultrasonic waves generate bubbles in the solvent, the bubbles burst near the cell walls which produce shock waves, causing the contents (i.e. lipids) to be released into the solvent. It was suggested that ultrasonication process up to 20 minutes had damaged the oil inside both protein and cellulosic complexes of *Jatropha curcas* seeds and subsequently, led to declination in enzyme activity. In the present study of ultrasonication process, the maximum yield of 74% (m/m) was obtained in the condition of ultrasonication for 5 minutes followed by EAAOE using an alkaline protease at pH 9.0.

3.1.3.7 Effect of water:seed ratio

Water was used in the present study as the extraction medium. However, the use of a large amount of water will generate large amount of effluent. Therefore, EAAOE should be conducted in minimal amount of water without compromising the extraction yield. The experimental condition to study the effect of water:seed ratio is tabulated in Table 2.10. The result of this effect can be seen in the Figure 3.9 as the highest oil yield (49% m/m) was obtained when 6 mL of water was used for every gram of enzymes. It was observed that as the volume of water increased more than 30 mL, the percentage of oil yield started to decrease to 48% (m/m) for both Alcalase® and Celluclast®. Optimal amount of water was crucial for the right dispersion matrix of enzymes and pulverised kernel seeds

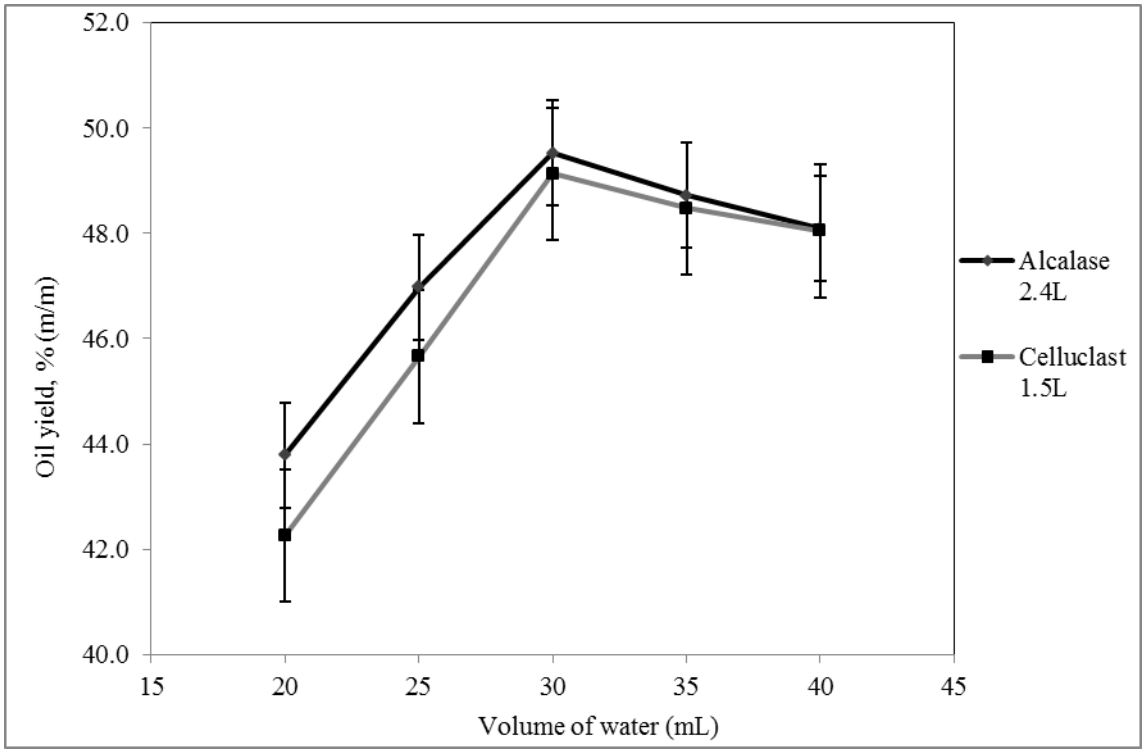


Figure 3.9 : Correlation between studies of water: seed ratio and percentage of oil yield, % obtained

3.1.3.8 Oil recovery from oil-water mixture

Upon aqueous enzymatic extraction at 50°C, an oil-water mixture was obtained. The failure to separate and recover the oil will affect the quantification of overall oil yield. Thus, an efficient way to recover the oil from such mixture was required. The processes for the oil recovery were including centrifugation condition, hexane-aided separation and freezing process of the aqueous layer.

In the present work, the recovery of the oil extracted using Alcalase® (at pH 8; pH was adjusted by using 0.1 N NaOH and 50°C) was enhanced by subjecting the centrifugation process at 4°C and 8000 rpm, coupled with hexane-aided separation and subsequent 2 hours freezing of the aqueous layer. The experimental conditions in this study were described in Table 2.11.

The proposed steps have improved the overall yield to 49.6% (m/m) (Table 3.5, Experiment 83). There was an improvement of 6% (m/m) when prolonged overnight cooling duration of 2-phase oil-water layer achieved after the centrifugation process (Table 3.5, Experiments 76 and 77). Oil-water mixtures from two different experiments were centrifuged at 25°C and 4°C in order to observe the effect of the temperature of centrifugation process on the oil recovery. The temperature of 4°C and 25°C were selected for comparing centrifugation process at between chill temperature and room temperature. The oil yield was slightly higher when the temperature of the centrifugation process was reduced down to 4°C.

Table 3.5 : 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	Oil yield[§], % (m/m)
76	25	8000	30	5	-	37.9
77	25	8000	30	5	Overnight	43.6
78	25	8000	30	5	Overnight	46.8
79	4	8000	30	5	Overnight	49.6
80	4	8000	30	5	-	45.8
81	4	8000	30	-	Overnight	39.2
82	4	8000	30	-	-	37.9
83	4	8000	30	5	2	49.6

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

Hexane-aided separation was important as significant increment in oil yield was observed. The presence of hexane has eased the handling and separation of the oil by lowering its viscosity since the experiments were conducted in small scale. Although the use of hexane did improve the oil yield in the current study, it is not propose to be used in large scale.

The reason that hexane aided separation was incorporated in this study is for accurate quantification of the oil yield. Oil which was extracted and present in the oil-water layer but not successfully isolated due to the limitation of the recovery step will cause lower oil yield and provide false interpretation. The amount of solvent used in the current study was very low in comparison with solvent extraction process which necessitates large amount of solvent. The usage of hexane to aid oil-water phase separation in EAAOE may not be necessary when the process is conducted at a larger scale.

3.2 Transesterification

Figure 3.10 illustrates the transesterification reaction between triacylglycerols in *Jatropha* oil by methanol in the presence of sodium hydroxide (NaOH) catalyst.

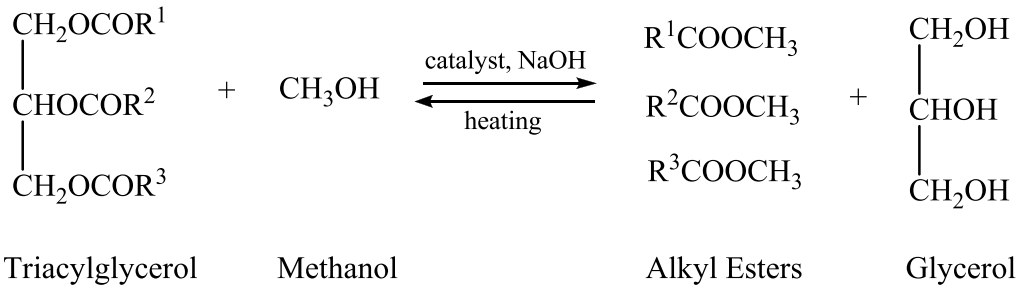


Figure 3.10 : Transesterification reaction between triacylgycerols in *Jatropha* oil by methanol in the presence of NaOH catalyst

The materials and the set-up of transesterification process were described in Section 2.3. Two separated layers of esters and glycerol were formed after the completion of transesterification reaction. The glycerol layer was decanted and the esters layer was purified and dried as discussed in Section 2.3.2.1. The esters layer was washed with warm distilled water of 50-55°C carefully to remove out the excessive methanol and catalyst until neutral decanted aqueous layer was obtained on addition of phenolphthalein, indicated that the esters was no longer alkaline.

The washing process of esters was done slowly and cautiously to reduce the formation of emulsion (soap layer) in between esters and aqueous layers. Emulsion build up will affect the percentage of esters yield. Saturated sodium chloride (NaCl) solution was added to break any emulsion formed from the washing step.

3.2.1 Double-stage Transesterification

A base-catalysed transesterification is the most commonly and preferable method used for methyl ester conversion, because the process can be accomplished in a short reaction time under mild reaction conditions. In addition, the possibility of saponification formation will be reduced due to oil properties which should contain low FFA and moisture content. Saponification formation will decrease the yield of methyl ester production (Sivasamy *et al*., 2009). In the present study, double-stage transesterification was carried out to produce biodiesel from crude *Jatropha* oil with high free fatty acid (FFA) content (10.22%). A high FFA in the oil deactivates the catalyst NaOH, and the addition of excess amount of NaOH as compensation, consequently high quantity of emulsion can form in the reaction, which increased viscosity, lead to the formation of gels and the difficulty associated with glycerine separation and loss in ester yield (Berchmans and Hirata, 2008). Due to its high FFA content, the crude *Jatropha* oil was processed in two stages of transesterification reaction.

During the first transesterification process, 4.9 g of freshly prepared sodium hydroxide (NaOH) solution was introduced in the reactor which was summation of the amount NaOH used for neutralisation of FFA and 0.5% of the weight of the initial amount of *Jatropha* oil, 250 g. The mixture with molar ratio of oil to methanol of 1:21 was refluxed in 3 hours at temperature range of 65°C to 68°C with stirring. The mixture was allowed to settle for 2 hours after the reaction was completed, and top layer of methanol-water mixture was removed. The FFA value was reduced to 1.25% after first step of transesterification process. The methyl esters obtained was proceeded with re-transesterification process for another 1 hour and 30 minutes with similar oil to methanol molar ratio of 1:21 and at temperature range of 65°C to 68°C.

After the completion of double stage of transesterification, the FFA value was reduced to 0.24%. The percentage of methyl ester was calculated after washing, filtered and drying process and the yield was 34.2% (mass of methyl ester/ mass of oil). Double-stage transesterification has a disadvantage as its required longer duration of reaction time as the experiment was completed in 6 hours and 30 minutes. The progress of the transesterification processes were monitored by thin layer chromatography as shown in Figure 3.11.

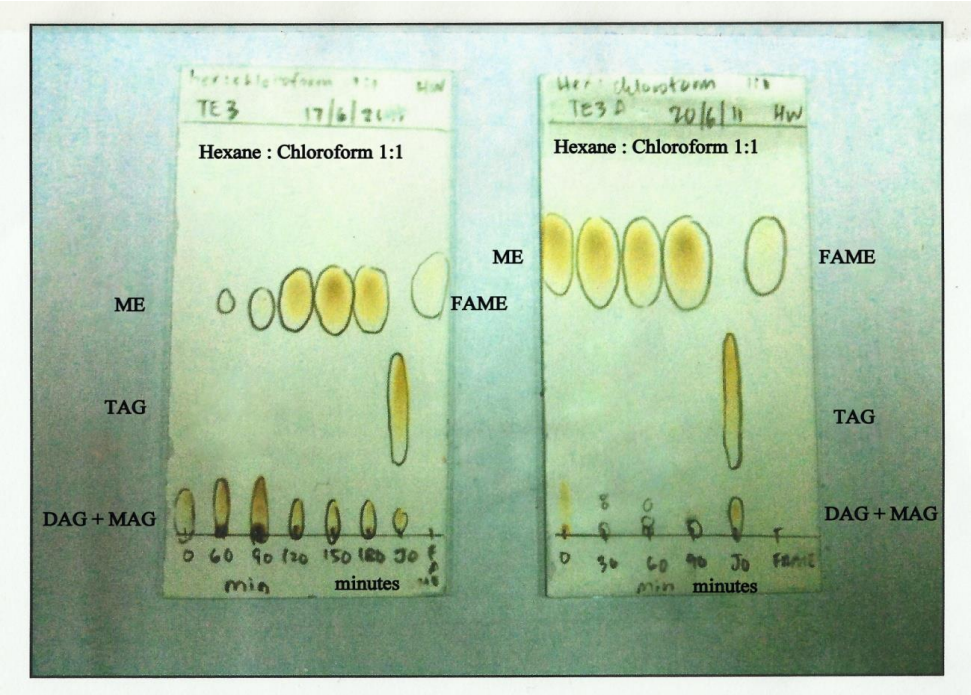


Figure 3.11 : Thin-layer chromatography of double transesterification reaction

The TLC plates spotted with sample aliquots were developed in mobile phase of saturated chromatographic tanks of hexane/chloroform eluants (1:1 v/v) and iodine vapour was used as a detection reagent as indicated in Section 2.3.3. TLC plate labelled with TE3 represents the first step of transesterification process and TE3A was labelled on the second plate on the right hand side which represent second step of transesterification process.

The spots of methyl esters, mono-, di-, and triacylglycerols were labelled as ME, MAG, DAG and TAG respectively. Besides, *Jatropha* oil and standard fatty acid methyl esters were labelled as JO and FAME in that order. The separation of the methyl esters and acylglycerols fraction was shown as in Figure 3.11. Based on the observation of TE3 plate, the highest retention factor, R_f value, 0.58 represented methyl esters (ME) at 180 minutes, followed by the trace of MAG, DAG and TAG left in the reaction mixture with R_f of 0.11. Clearly distinguishable separation between methyl esters (R_f = 0.77) and small trace of acylglycerols mixture (R_f = 0.02) was shown in TE3A plates after re-transesterification process at 90 minutes. TLC is an enviable option as compared with methods of using gas chromatography and high performance liquid chromatography to monitor the progress of transesterification process mainly due to its simplicity, fast detection, sensitive, less equipments and low operation cost (Fontana *et al*., 2009).

3.2.2 Esterification as Pre-treatment of High Free Fatty Acid content

Base catalysed transesterification is not recommended for oil of high FFA content (higher than 2 mg KOH/ g oil) for it deactivates the catalyst and causes to soap formation. As a result, yield decreases (Alptekin and Canakci, 2010). As double-stage required longer duration time of transesterification process for *Jatropha* oil methyl ester (JOME) conversion, alternative method such as esterification reaction using acid catalyst reduced the duration time of JOME conversion. In the present research, the duration of the experiment was done in 4 hours and 50 minutes. The FFA value of *Jatropha* oil before acid pre-treatment and transesterification reaction was 10.3%. As discussed in Section 2.3.2.2, the *Jatropha* oil and methanol were refluxed in oil bath within temperature range of 110°C to 120°C, with similar oil to methanol molar ratio of 1:21.

After the acid pre-treatment, the reaction was immediately preceded with transesterification process. 1.46 g of sodium hydroxide (NaOH) dissolved in methanol was added in the reactor while stirring. The mixture was allowed to settle for 2 hours and top layer of methanol-water mixture was removed subsequent to the completion of the transesterification reaction. The percentage of methyl ester was calculated after washing, filtered and drying process and the yield was 62.1% (m/m). The FFA value of JOME was decreased to 0.26% after the reactions. Figure 3.12 illustrated the thin layer chromatography plates which monitored the progress of esterification pre-treatment and transesterification reaction.

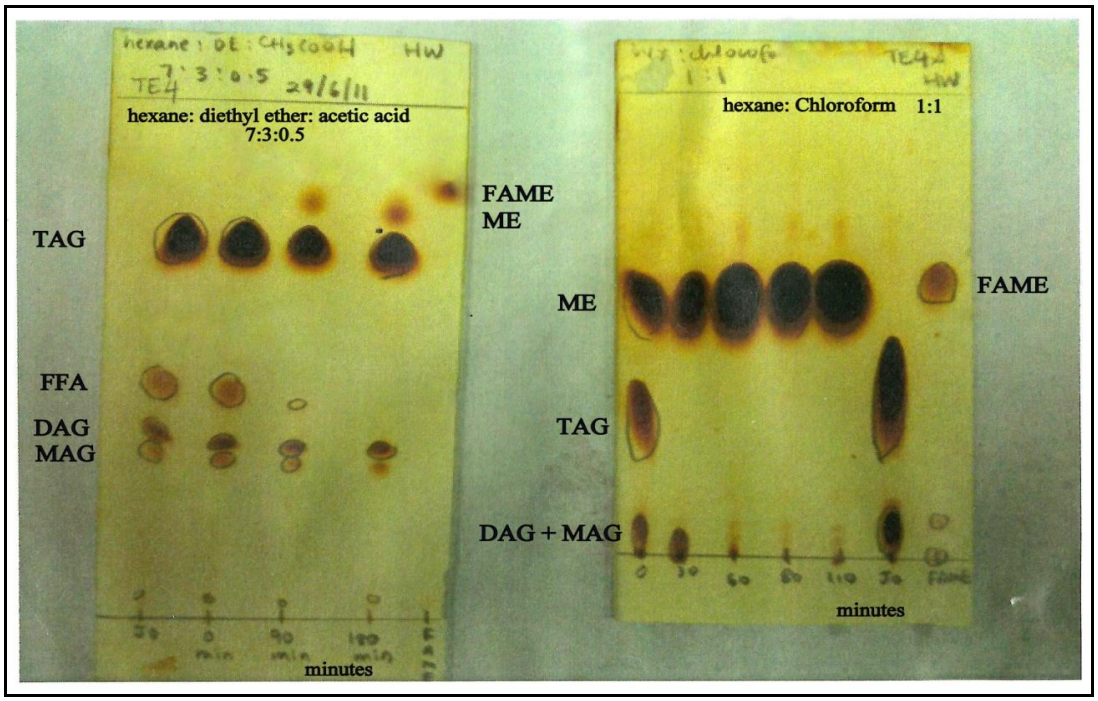


Figure 3.12 : Thin-layer chromatography of direct esterification and transesterification reaction

The TLC plates spotted with sample aliquots were developed in mobile phase of saturated chromatographic tanks of hexane/diethyl ether/acetic acid eluants (7:3:0.5 v/v/v) and hexane/chloroform eluants (1:1 v/v) for TE4 and TE4A respectively. Iodine vapour was used as a detection reagent for visualisation as indicated in Section 2.3.3.

TLC plate labeled with TE4 represents the acid pre-treatment process and TE4A was labeled on the second plate on the right hand side which represent following transesterification process. Similarly to double-stage transesterification in Section 3.2.1, the spots of methyl esters, mono-, di-, and triacylglycerols were labeled as ME, MAG, DAG and TAG respectively similarly to TE3 and TE3A plates. In addition, *Jatropha* oil, free fatty acid and standard fatty acid methyl esters were labelled as JO, FFA and FAME in that order. TE4 plate displays separation between acylglycerols, FFA and methyl esters. At 180 minutes, FFA spots (retention factor, R_f = 0.46) was disappeared completely which indicated that the esterification process was completed. In the TE4A plate, noticeable separation between methyl esters (R_f = 0.73) and small trace of acylglycerols mixture (R_f = 0.06) was observed in transesterification process within 110 minutes.

3.3 Analysis of *Jatropha* oil

3.3.1 Determination of Free Fatty Acid Content

The free fatty acid (FFA) content analysis can verify the amount of FFA in *Jatropha* oil, as a consequence of the hydrolysis of the ester bond between the fatty acid and glycerol molecule (Siew *et al*., 1995). It was determined in accordance with Palm Oil Research Institute Malaysia (PORIM) official test methods as indicated in Section 2.2.1 (Siew *et al*., 1995). All experiments were done in triplicates and the mean data was reported. Table 3.6 tabulates the FFA analysis of *Jatropha* oil extracted from solvent extraction (soaking method and soxhlet method), enzymatic assisted aqueous oil extraction (EAAOE) and commercial *Jatropha* oil.

Table 3.6 : FFA analysis of *Jatropha* oil

Experiment	Sample	FFA, % as oleic acid
J1	Commercial *Jatropha* oil (Nurazira, 2009)	4.22
J2	Extracted *Jatropha* oil (solvent extraction : soxhlet method) (Nurazira, 2009)	2.76
S1	Extracted *Jatropha* oil (solvent method : soxhlet method)	2.48
E1	Extracted *Jatropha* oil (EAAOE: Alcalase)	2.15
E2	Extracted *Jatropha* oil (EAAOE: Cellulase)	2.30

The FFA content of *Jatropha* oil extracted by using soxhlet method, experiment S1 was compared to the FFA content of *Jatropha* oil extracted by using similar method, experiment J2 (Nurazira, 2009) due to the similar supply of the *Jatropha curcas* seeds used in the current work. The results in Table 3.6 shows that the percentage of FFA of *Jatropha* oil for both experiments were consistent with 2.76% and 2.48% respectively. The FFA content of *Jatropha* oil extracted by using EAAOE method, namely experiment E1 and E2 were slightly lower than *Jatropha* oil extracted from soxhlet method. Overall, the FFA content of experiment S1, E1, E2 was significantly low in comparison with the reported FFA content (4.22%) of commercial *Jatropha* oil (Nurazira, 2009).

3.3.2 Determination of Iodine Value

In the current study, iodine value (IV) of *Jatropha* oil was analysed in conformity with Palm Oil Research Institute Malaysia (PORIM) official test methods as indicated in Section 2.2.2 (Siew *et al*., 1995). All experiments were done in duplicates and the mean data was reported in Table 3.7.

Table 3.7 : Iodine value analysis of *Jatropha* oil

Experiment	Sample	IV
J1	Commercial *Jatropha* oil (Nurazira, A., 2009)	99.4
J2	Extracted *Jatropha* oil (solvent extraction: soxhlet method) (Nurazira, A., 2009)	94.9
S1	Extracted *Jatropha* oil (solvent extraction : soxhlet method)	95.7
E1	Extracted *Jatropha* oil (EAAOE: Alcalase)	96.5
E2	Extracted *Jatropha* oil (EAAOE: Cellulase)	95.1

IV is the measure of degree of unsaturation level of fats and oils (Siew *et al*., 1995). Higher unsaturation level of fats and oils has higher IV. High IV of *Jatropha* oil is caused by high content of unsaturation fatty acid such as oleic acid and linoleic acid (Akbar *et al*., 2009). IV of the studied *Jatropha* oil, namely experiment S1, E1 and E2 were 95.7, 96.5 and 95.1 correspondingly. In comparison with IV studied by Nurazira (2009) which used similar *Jatropha curcas* seed, it was found that there were no significant different between experiment J2 and experiment S1. In addition, the studied *Jatropha* oil contained quite low IV in contrast with commercial *Jatropha* oil, 99.4 reported by Nurazira (2009).

3.3.3 Determination of Peroxide Value

The peroxides (primary oxidation products) are correlated with the extent of oxidation of oils and fats. Hydroperoxides are unstable and will develop secondary oxidation products (ketones, aldehydes, epoxides, alcohols). The *Jatropha* oil is subjected to oxidation in contact with the oxygen in the air. Once this process advances at ambient temperatures, the initial hydroperoxides are produced by the addition of oxygen to a carbon atom adjacent to a carbon-carbon double bond.

As a result, it can caused undesirable aromas in the oil and generation of off flavours. The extent of this level of oxidation can be evaluated by the peroxide value (PV) (Shanta and Decker, 1994). In the present study, PV of *Jatropha* oil was analysed in accordance with Palm Oil Research Institute Malaysia (PORIM) official test methods as discussed in Section 2.2.3 (Siew *et al*., 1995). The results of the PV determination of the *Jatropha* oil extracted were shown in Table 3.8. All experiments were done in duplicates and the mean data was reported.

Table 3.8 : Peroxide value analysis of *Jatropha* oil

Experiment	Sample	PV (meq/kg)
S1	Extracted *Jatropha* oil (solvent method: soxhlet method)	2.30
E1	Extracted *Jatropha* oil (EAAOE: Alcalase)	1.83
E2	Extracted *Jatropha* oil (EAAOE: Cellulase)	1.77

In the present study, freshly prepared *Jatropha* oil was used for all experiments in the PV analysis. *Jatropha* oil extracted from solvent extraction via soxhlet method (Experiment S1) has a peroxide value of 2.30 meq/kg. Significant differences can be observed for the experiments from different method of extraction between solvent extraction and EAAOE. The PV of experiments E1 and E2 were lower than experiment S1. The low peroxide value indicated that there was no primary oxidation at the time of this analysis. The peroxide value can increase during the time the oil are stored.

It is possible to delay the rate of oxidative rancidity of oil and fat during handling and storage. It was reported by Berger (1994) that there are four main influences on the rate of autoxidation. The first engages the level of contact with air. Oxidation cannot happen without oxygen, and appropriate storage and handling circumstances can decrease the contact between the oil and air.

The second influence is temperature. The rate of reaction can be slowed down by avoiding overheating during storage or extraction processes. Berger (1994) reports that the rate of reaction of oxygen with oil and fat can enhance twice with every 10°C increase in temperature. The third factor that can accelerate the rate of oxidation is the presence of catalysts such as metals and traces of already oxidised oil and fat. Contact with copper or iron should be avoided, and cleanliness can prevent contact between non-oxidised and oxidised oil and fat. Finally, light can stimulate photo oxidation, thus exposure with light should be minimised as much as possible.

3.3.4 Determination of Saponification Value

In the present study, saponification value (SV) of *Jatropha* oil was analysed by referring to Palm Oil Research Institute Malaysia (PORIM) official test methods as indicated in Section 2.2.4 (Siew *et al*., 1995). All experiments were done in duplicates and the mean data was reported in Table 3.9.

Table 3.9 : Saponification value analysis of *Jatropha* oil

Experiment	Sample	SV
S1	Extracted *Jatropha* oil (solvent method: soxhlet method)	200.9
E1	Extracted *Jatropha* oil (EAAOE: Alcalase)	207.9
E2	Extracted *Jatropha* oil (EAAOE: Cellulase)	205.1

SV analysis of the studied oil was 200.9, 207.9 and 205.1 for Experiment S1, E1 and E2 respectively. SV provides information regarding the characteristic of the fatty acids of the *Jatropha* oil and particularly concerning the solubility of their soaps in water. The higher the SV of a fat free from moisture and unsaponifiable matter, the more soluble the soap that can be made from it.

3.3.5 Determination of Moisture Content

In the present study, moisture content of *Jatropha* oil was analysed as discussed in Section 2.2.5 by using Karl Fischer Volumetric Titration. All experiments were done in duplicates and the mean data was reported in Table 3.10.

Table 3.10 : Moisture content analysis of *Jatropha* oil

Experiment	Sample	Moisture content, %
S1	Extracted *Jatropha* oil (solvent method: soxhlet method)	0.12
E1	Extracted *Jatropha* oil (EAAOE: Alcalase)	0.11
E2	Extracted *Jatropha* oil (EAAOE: Cellulase)	0.11

Based on results in Table 3.10, there was no significant difference in the moisture content between the *Jatropha* oils from both solvent extraction and EAAOE methods. The moisture content reported in all experiments was relatively lower than 0.2% as reported for *Jatropha* oil (Tint and Mya, 2009). The low moisture content exhibits that the oil was in enhanced condition and could not be easily subjected to contamination or rancidity (Fellows, 1997).

It is essential that moisture content have significant consequence on the transesterification of acylglycerols with alcohol using catalyst (Goodrum, 2002). The presence of moisture affects the transesterification reaction to partly transform to saponification reaction, which form soap and consequently lowering the yield of methyl esters. Saponification also causes complication in the separation process of methyl ester and glycerol in view of the fact that it enhances the viscosity and form gels (Berchmans and Hirata, 2008).

3.3.6 Determination of Density Value

The estimation of the density value analysis of oil is essential in the design of unit processes such as distillation, heat exchangers, piping and reactors (Rodenbush *et al.*, 1999). It was determined as indicated in Section 2.2.6. All experiments were done in three measurements and the mean data was reported. Table 3.11 tabularises the density value analysis of *Jatropha* oil extracted from solvent extraction (soaking method and soxhlet method), enzymatic assisted aqueous oil extraction (EAAOE), commercial *Jatropha* oil, refined bleached and deodorised (RBD) palm olein and commercial olive oil.

Table 3.11 : Density value analysis of *Jatropha* oil

Experiment	Sample	Density (g/cm^3)	Specific Gravity
J1	Commercial *Jatropha* oil (Nurazira, 2009)	0.9205	0.9232
J3	Commercial *Jatropha* oil (BIONAS)	0.9127	0.9154
S1	*Jatropha* oil extracted by solvent method (Soxhlet extraction)	0.9119	0.9146
E1	*Jatropha* oil extraction by EAAOE (Alcalase®)	0.9130	0.9157
E2	*Jatropha* oil extracted by EAAOE (Celluclast®)	0.9134	0.9161
C1	RBD Palm Olein	0.9096	0.9123
C2	Commercial cooking oil	0.9091	0.9118
C3	Commercial olive oil	0.9105	0.9132

Density value (DV) analyses of the studied oil were 0.9119, 0.9130 and 0.9134 g/cm^3 for Experiment S1, E1 and E2 respectively. There were no significant differences in comparison with commercial *Jatropha* oil (Experiment J1 and J3), 0.9205 g/cm^3 and 0.9127 g/cm^3. DV of RBD palm olein, commercial cooking oil and commercial olive oil were done to compare with the *Jatropha* oil extracted in current work. Based on the results in Table 3.11, it was observed that the DV of *Jatropha* oil was in similar range with the RBD palm olein and commercial oils. Specific gravity (SG) is a way of relating the density of oil to the density of water. It was important to determine the SG of the Jatropha oil because the SG of Jatropha oil methyl ester should be reduced to a significant extent when compared with the SG of Jatropha oil (Kwye and Oo, 2009).

3.3.7 Fatty Acid Composition Analysis of *Jatropha* Oil by Gas Chromatography

Fatty acid composition (FAC) analysis was another essential quantification performed in the study. Chain length and number of double bonds denote the physical characteristics of both fatty acids and triacylglycerols (Mittelbach and Remschmidt, 2004). The main types of fatty acids that can be present in a triacylglycerols based on component acids are palmitic (16:0), oleic (18:1) and linoleic (18:2) with other acids such as linolenic (18:3) and stearic (18:0) at lower level (Gunstone, 2008). FAC of *Jatropha* oil was analysed by gas chromatography flame ionisation detector (GC-FID). The flame ionisation detector (FID) was chosen in FAC analysis as FID has high sensitivity and stability with almost organic compounds, a low dead volume, a fast response time (Christie, 1990). The sample preparation of *Jatropha* oil methyl ester derivatives, conditions and temperature programming of GC-FID was discussed in section 2.2.7. All experiments were done in duplicates and the mean data was reported. Figures 3.13, 3.14, 3.15 and 3.16 depict FAC profiles of FAME standard and *Jatropha* oil from solvent extraction (Experiment S1) and EAAOE methods, Alcalase® (Experiment E1) and Celluclast® (Experiment E2).

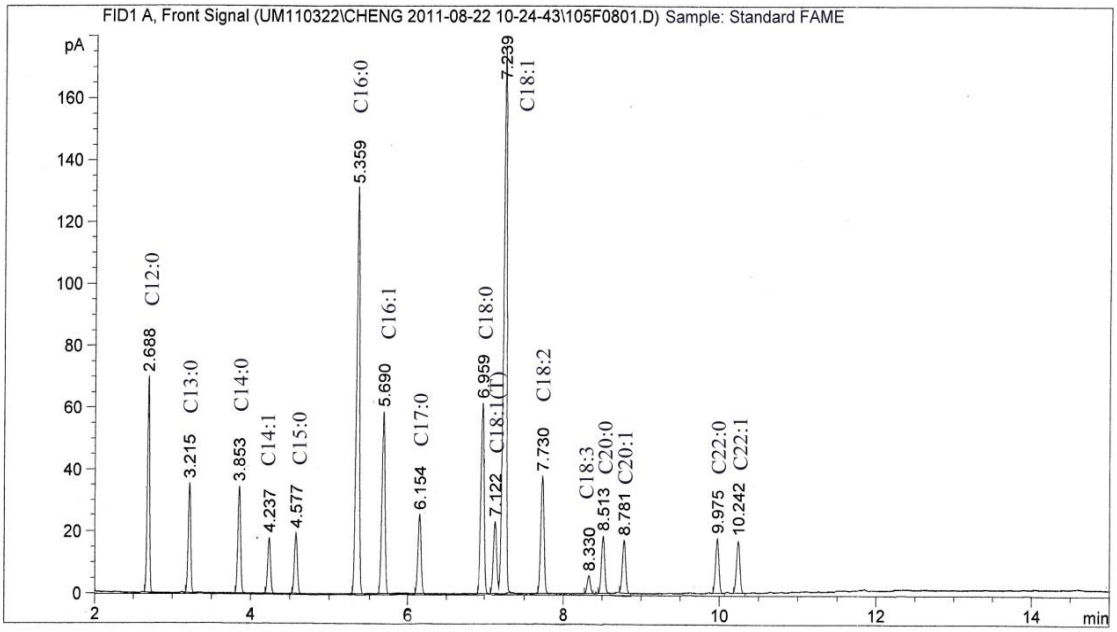


Figure 3.13 : GC chromatogram of FAME standard

Besides GCMS profiles of each peak of fatty acids which were used for confirmation tools, the identification of the peaks was achieved by means of comparing the reaction times of *Jatropha* oil with the retention times of fatty acid methyl ester (FAME) standard analysed under the same conditions (Figure 3.13). The range of fatty acids of FAME standard from lauric acid (C12:0) to erucic acid (C22:1) was eluted within 12 minutes of analysis time.

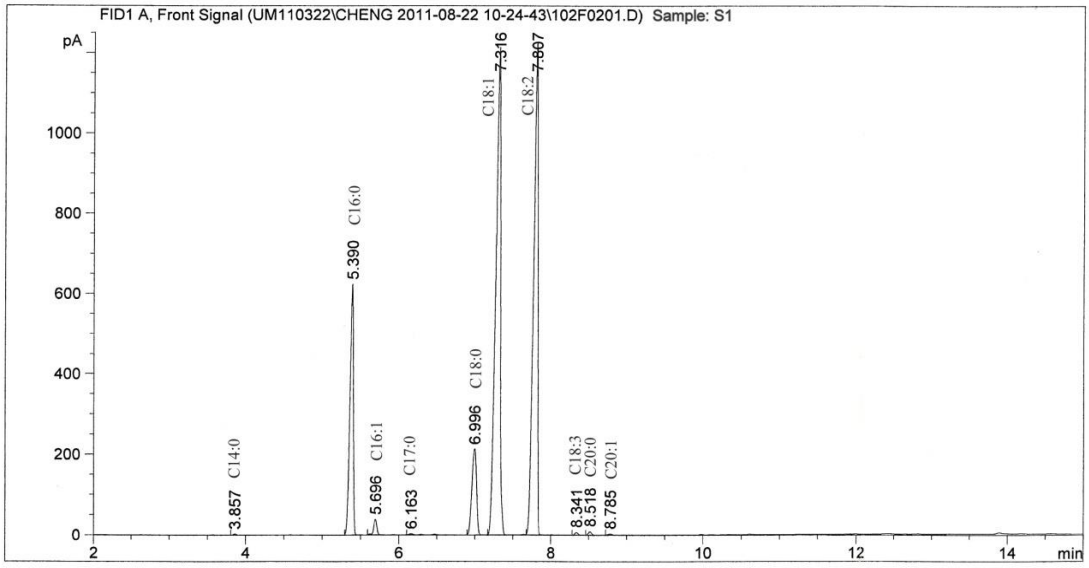


Figure 3.14 : GC chromatogram of experiment S1

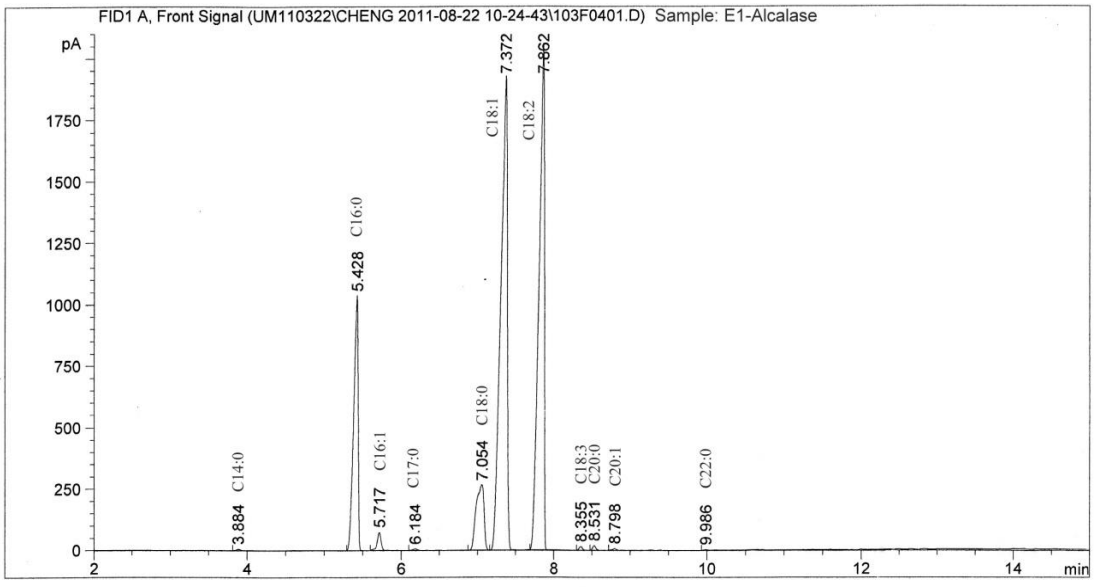


Figure 3.15 : GC chromatogram of experiment E1

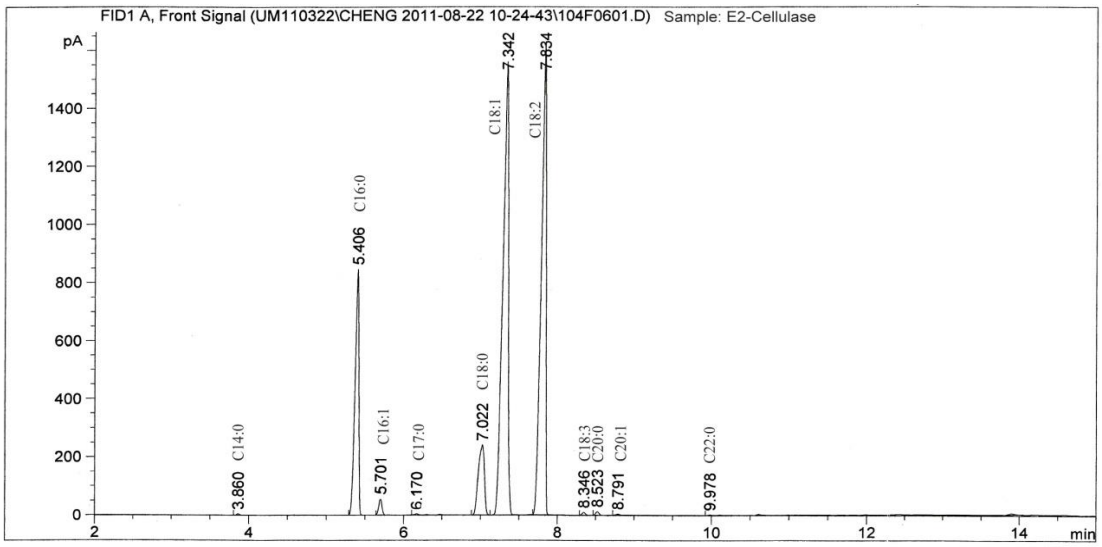


Figure 3.16 : GC chromatogram of experiment E2

Table 3.12 tabulates the FAC analysis of *Jatropha* oil extracted from solvent extraction (soaking method and soxhlet method), enzymatic assisted aqueous oil extraction (EAAOE) and commercial *Jatropha* oil.

Table 3.12 : FAC profile of *Jatropha* oil using GC-FID and GCMS

Composition of Fatty Acid (as % methyl ester)	Solvent extraction (soxhlet method) Experiment S1	Enzymatic Assisted Aqueous Oil Extraction (EAAOE)	
		Alcalase Experiment E1	Cellulase Experiment E2
Oleic (C18:1)	40.4	40.0	39.9
Linoleic (C18:2)	36.4	35.9	37.2
Palmitic (C16:0)	14.8	15.9	15.1
Stearic (C18:0)	7.0	6.8	6.6
Palmitoleic (C16:1)	0.9	0.9	0.9
Arachidic (C20:0)	0.2	0.2	0.2
Linolenic (C18:3)	0.2	0.2	0.2
Myristic (C14:0)	0.1	0.1	0.1
Margaric (C17:0)	0.1	0.1	0.1
cis-Arachidic (C20:1)	0.1	0.1	0.1
Behenic (C22:0)	-	0.03	0.03

Table 3.12 shows major long chain fatty acids present in the *Jatropha* oil which are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). *Jatropha* oil contains high percentage of unsaturated fatty acid which was about 77.9%, 77.0% and 77.8% for Experiment S1, E1 and E2 respectively. There was no significant difference in comparison of FAC analysis between solvent extraction *via* soxhlet method and EAAOE method. Oleic acid showed the highest percentage of FAC composition followed by linoleic acid in all experiments. Therefore, *Jatropha* oil can be categorised as oleic–linoleic oil.

3.3.8 Summary of Characterisation of *Jatropha* Oil

The quality of *Jatropha* oil extracted from enzymatic extraction was evaluated and summarised in Table 3.13.

Table 3.13 : Oil quality analysis of *Jatropha* oil

Characterisation	J1[a]	J3	S1	E1	E2
State	Liquid at room temperature, (28-31)°C				
Solubility	Soluble in organic solvents, insoluble in water				
FFA, % as oleic acid	4.22	10.33	2.48	2.15	2.30
Iodine Value	99.4	97.5	95.7	96.5	95.1
Peroxide Value (meq/kg)	-	3.84	2.30	1.83	1.77
Saponification Value	-	202.2	200.9	207.9	205.1
Density Value (g/cm^3)	0.9205	0.9209	0.9119	0.9130	0.9134
Moisture Content (%)	-	0.16	0.12	0.11	0.11
Total saturated fatty acid (%)	-	22.3[b]	22.1	23.0	22.2
Total mono unsaturated fatty acid (%)	-	42-43.1[b]	41.4	40.9	41.2
Total polyunsaturated fatty acid (%)	-	34-36[b]	36.5	36.1	36.6

Notes: [a]Source adopted from Nurazira (2009)
[b]Source adopted from product specification provided by BIONAS

The *Jatropha* oil obtained by EAAOE method was analysed by specific manners to determine its physicochemical characteristics that indicate its quality in comparison with quality specifications required by the European standard for biodiesel, EN 14214 and American standard ASTM D6751. In general, the evaluation showed that the *Jatropha* oil extracted by EAAOE was comparable to solvent extraction (experiment S1) and the properties are in the acceptable range of *Jatropha* oil quality in accordance with Palm Oil Research Institute Malaysia (PORIM) Test Method (Siew *et al*., 1995). In addition, the evaluation was in line with some previous studies on the physicochemical properties of *Jatropha* oil. Salimon and Waled (2012) reported that the physicochemical properties of *Jatropha* oil cultivated in Malaysia showed iodine value of 103.2, free fatty acid content of 1.68% and saponification value of 197.8.

In the previous research by Nzikou *et al.* (2009), the iodine value, free fatty acid content and saponification value of *Jatropha* oil extracted *via* soxhlet method were 102.43, 2.24% (% as oleic acid) and 167 respectively. The results of the above authors agree with those of the present work. The values of free fatty acid content of both enzymes in EAAOE were very low and the values obtained for the water content are far below the limits imposed by the quality standard for biodiesel EN 14214. In addition, the high content of unsaturated fatty acids in both experiment E1 and E2 creates advantages such as increasing the prospect of utilising the biodiesel in winter, which is of great significance for the countries with cold weather as well as better engine performance due to higher energy content.

3.3.9 Determination of Phorbol Esters in *Jatropha* Oil by High Performance Liquid Chromatography Flame Ionisation Detector and Liquid Chromatography Mass Spectrometry.

The content of toxic compound, namely phorbol esters in *Jatropha* oil was determined by using High Performance Liquid Chromatography-Flame Ionisation Detector (HPLC) and Liquid Chromatography Mass Spectrometry (LCMS). The methodology of HPLC measurements and sample preparation of methanol extracts from *Jatropha* oil are indicated in Section 2.2.8. The sample injection volume of phorbol ester standard was 10 µl with concentration of 500 ppm. Figure 3.17 depicts the peak of pure standard of phorbol esters; phorbol 12-myristate 13-acetate.

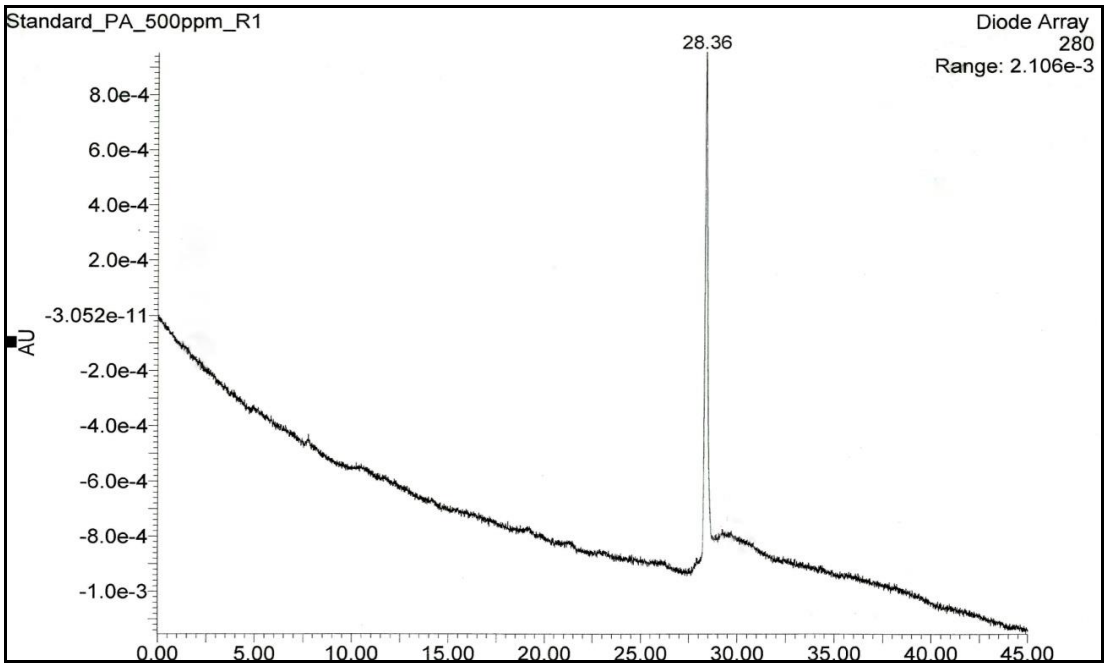


Figure 3.17 : HPLC chromatogram of Phorbol 12-myristate 13-acetate

Based on Figure 3.17, the strong peak was appeared at retention time 28 to 30 minutes represent phorbol 12-myristate 13-acetate compound. Further peak identification and quantification were made by comparing the retention time between the sample (Figure 3.18, 3.19 and 3.20) and the standard compound, phorbol 12-myristate 13-acetate. In the present study, the sample injection volume of sample, methanol extracts from *Jatropha* oil were 1 µl with concentration of 200 ppm (Figure 3.18, 3.19 and 3.20).

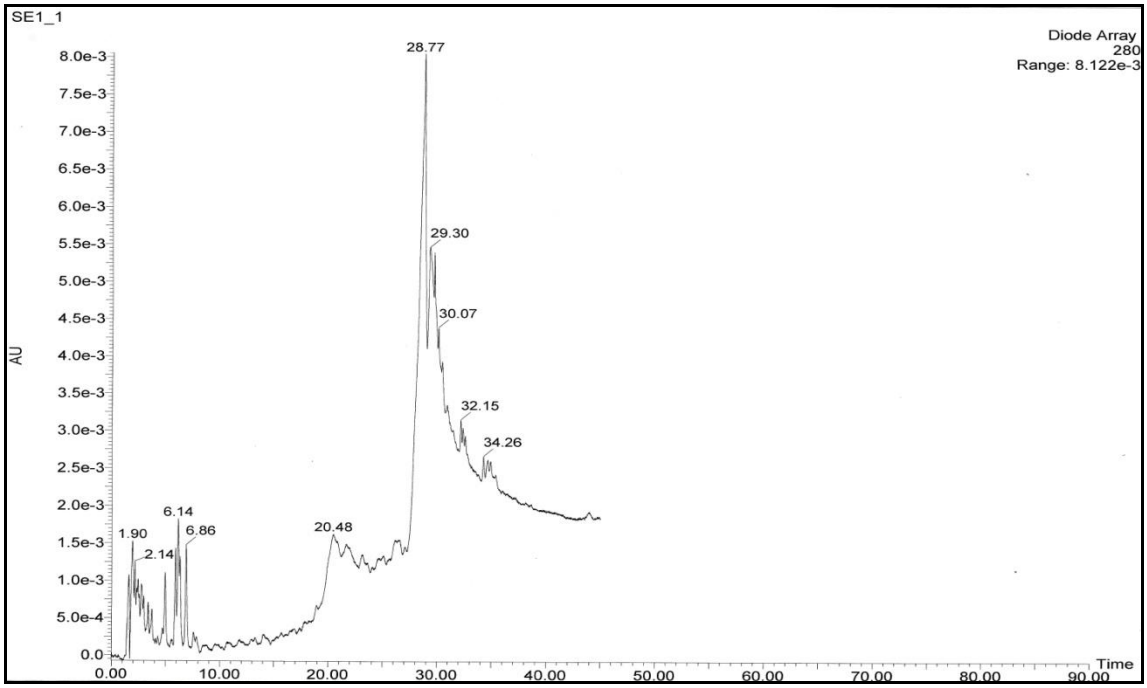


Figure 3.18 : HPLC chromatogram of experiment S1

In Figure 3.18, several unidentified peaks were observed at retention times 2 to 10 minutes. These unidentified peaks were dismissed and not included in the calculations as the retention times of unidentified peaks of this region were different in comparison with retention time of standard of phorbol esters; phorbol 12-myristate 13-acetate. Similar observation of several unidentified peaks was detected in HPLC chromatogram of experiment E1 and E2.

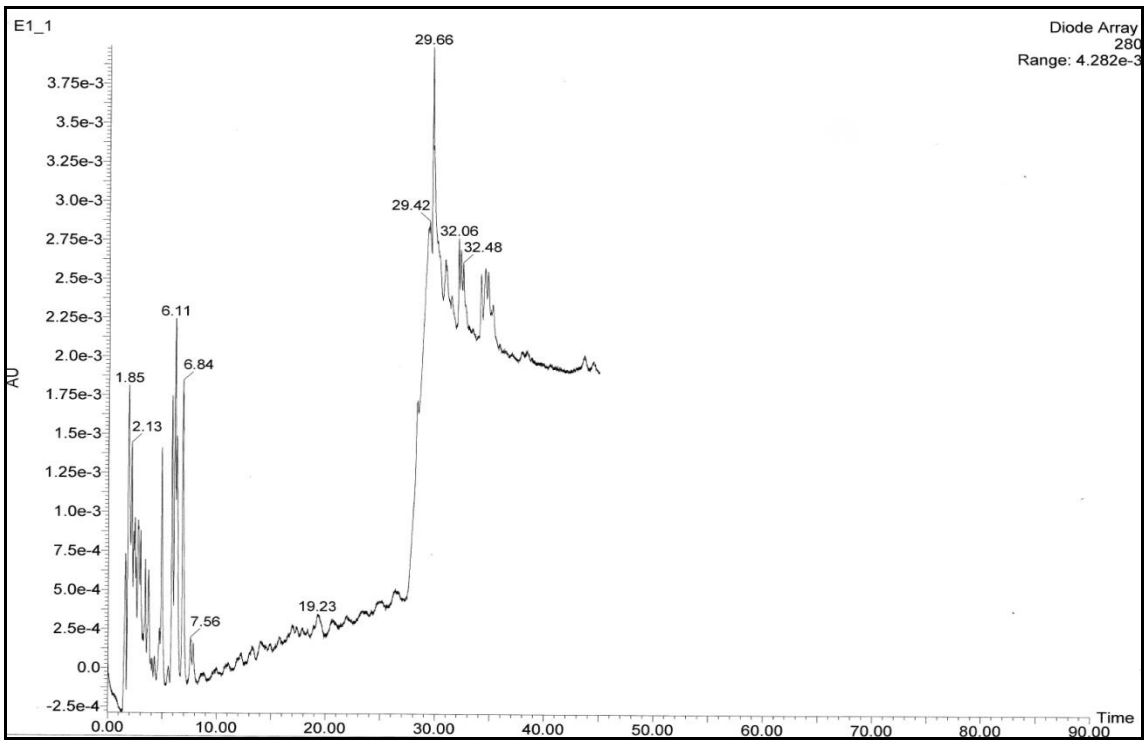


Figure 3.19 : HPLC chromatogram of experiment E1

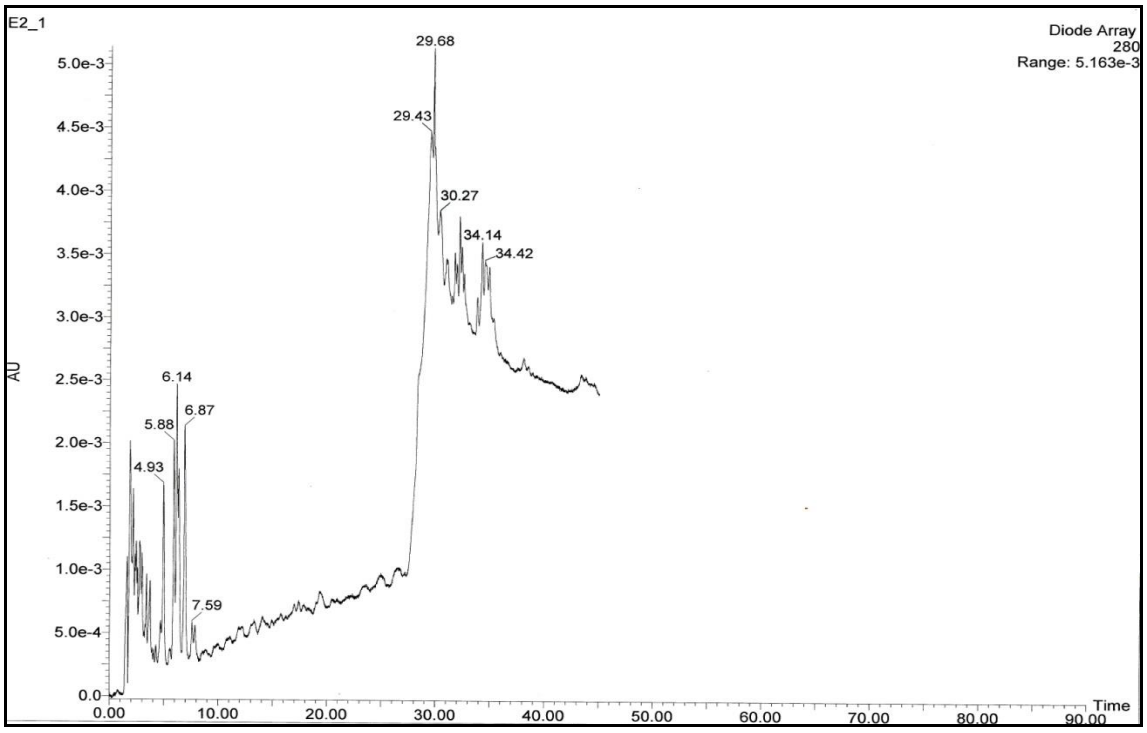


Figure 3.20 : HPLC chromatogram of experiment E2

The presence of phorbol ester compounds in all the experiments was eluted in between 28 to 38 minutes. The percentages of phorbol esters content were calculated and tabulated in Table 3.14. The *Jatropha* oil extracted from both solvent extraction and EAAOE method contained 0.2-0.4% phorbol esters.

Table 3.14 : Percentage of phorbol esters content in *Jatropha* oil

Experiment	Percentage of phorbol esters content, %
S1	0.4
E1	0.2
E2	0.3

Mass spectrometry (MS) provides valuable information such as molecular mass (via mass to charge *(m/z)* ratio), molecular structural information and quantitative data, all at high sensitivity (Thompson, 2008).

In the current work, the molar mass of phorbol esters was determined by using liquid chromatography mass spectrometry (LCMS). The atmospheric pressure ionisation (API) technique used was electrospray ionisation (ESI). Figure 3.21 illustrates the chromatogram of methanol extracts from *Jatropha* oil (Experiment E1) with ESI scan from 32 to 36 minutes.

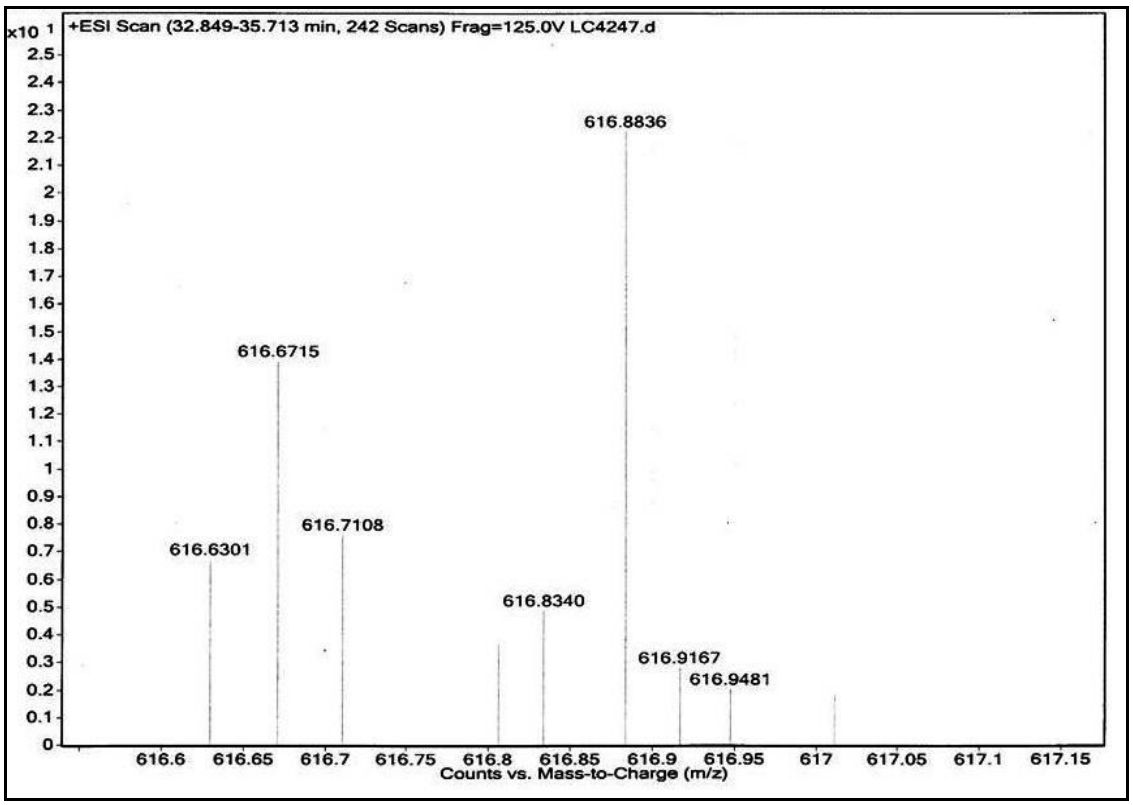
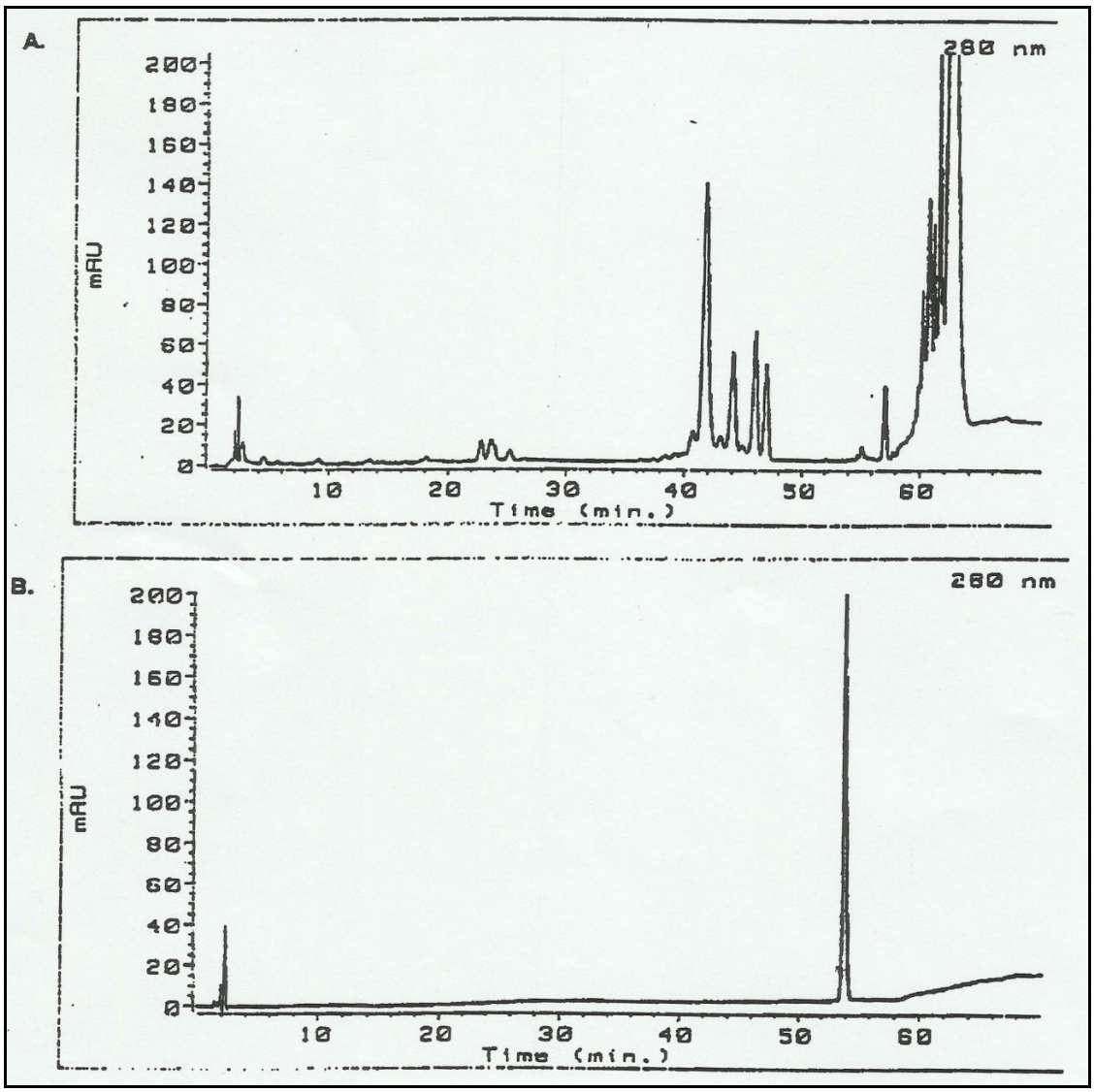


Figure 3.21 : LCMS chromatogram of E1

Based on Figure 3.21, highest peak at 616.8 m/z represents molecular mass of phorbol 12-myristate, 13-acetate ester. From LCMS results, it was determined that the presence of phorbol esters in the sample of experiment E1. Similar results were obtained from both LCMS chromatograms of experiment E2 and experiment S1. The present work was compared to previous study by Wink *et al.* (1997). In their finding, *Jatropha* oil contains up to 1- 2% phorbol esters (retention time between 40 to 48 minutes) which use similar sample preparation of *Jatropha* oil and phorbol esters standard, TPA as an external standard for quantification as shown in Figure 3.22.



Notes: They were using reversed phase column C18 (LiChrospher 100, endcapped 5 µm), 250 x 4 mm I. D. (Lichrocart). Solvent systems of (A) 1.75 mL o-phosphoric acid (85%) in 1 litre distilled water, (B) acetonitrile and (C) tetrahydrofuran. Gradient: Start with 60% A and 40% B; for 10 minutes decreased A to 50% and increased B to 50%; for the next 30 minutes a linear gradient to 25% A and 75% B; then increased B to 100% within next 15 minutes. The column washed with 100% and adjusted to initial condition. Separation was performed at room temperature, 22°C with flow rate of 1.3 mL/min.

Figure 3.22 : (A) HPLC profile from *Jatropha* oil and (B) HPLC profile of a pure phorbol ester, phorbol-12-myristate 13-acetate (TPA) (Adopted from Wink *et al*., 1997)

3.4 Analysis of *Jatropha* Oil Methyl Ester

3.4.1 Determination of Ester Content

The ester content of *Jatropha* was determined by using Gas Chromatography-Flame Ionisation Detector (GC-FID). The methodology of ester content measurements and sample preparation of *Jatropha* oil methyl ester were indicated in Section 2.3.7. The sample injection volume of FAME standard and *Jatropha* oil methyl ester were 1 µl with concentration of 50000 ppm. Figure 3.23 portrays the peak of the FAME standard (Sigma-Aldrich) which was used as a reference in the analysis.

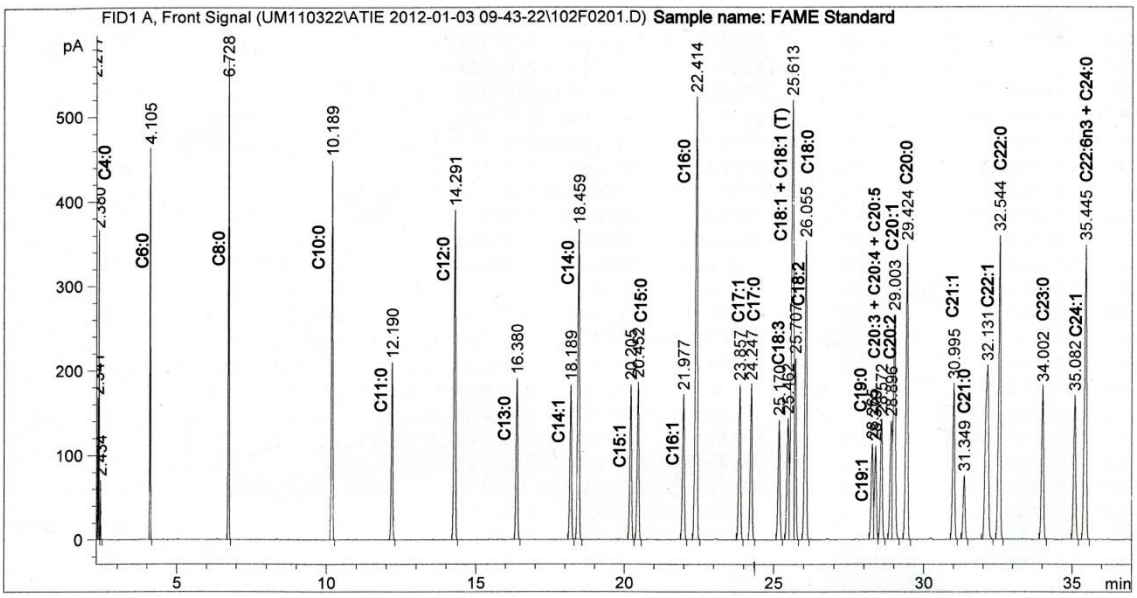


Figure 3.23 : GC chromatogram of FAME standard for ester content determination

The assignment of the peaks in the chromatogram of FAME standard was confirmed by mass spectrometry (GC-MS). Further peak identification and quantification were made by comparing the peak area between the sample of experiments TE3 (double-stage transesterification) and TE4 (direct esterification and transesterification) (Figure 3.24 and 3.25) and the FAME standard.

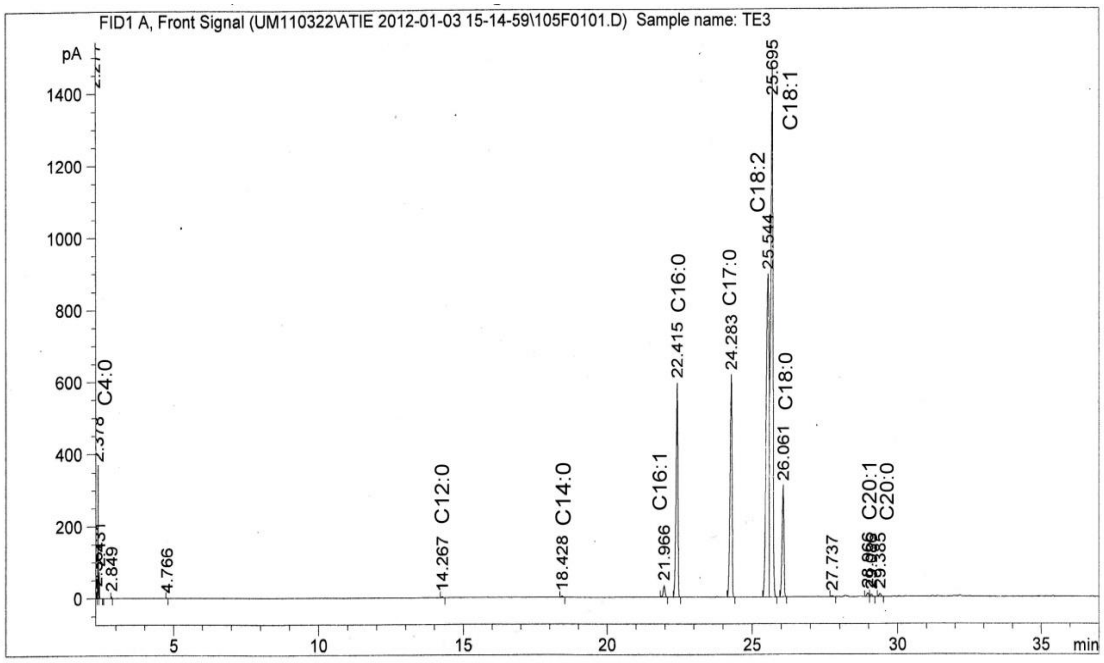


Figure 3.24 : GC chromatogram of experiment TE3 for ester content determination

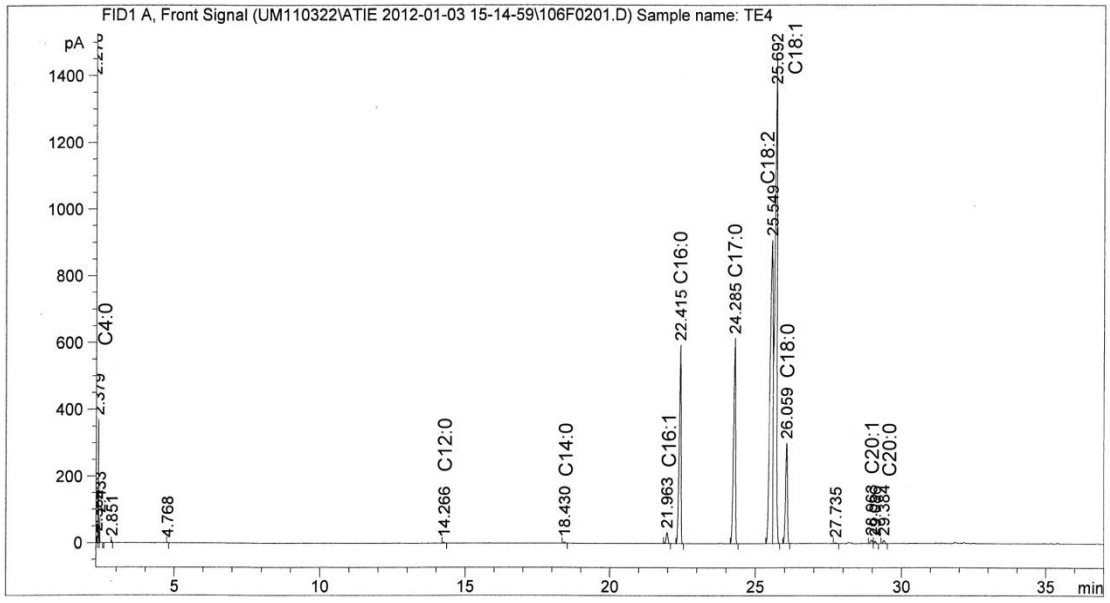


Figure 3.25 : GC chromatogram of experiment TE4 for ester content determination

In the present study, the sample injection volume of sample, *Jatropha* oil methyl ester were 1 µl with concentration of 50000 ppm. The ester content of experiments TE3 and TE4 was tabulated in Table 3.15.

Table 3.15 : Ester content of *Jatropha* oil methyl ester

Experiment	Description	Ester content (% FAME)
TE3	Double-stage transesterification	128
TE4	Direct esterification and transesterification reaction	129

Values of ester content were recalculated after the experimental conditions and purity of the internal standard were verified. The internal standard solutions (IS) of Methyl Heptadecanoate solution was freshly prepared for every measurement. The result shows that the ester contents were unacceptable and should be discarded as the average of two determinations of ester content for both experiments was higher than 100%; 28% greater when quantified using the equation 2.9 as indicated in Section 2.3.7.2.

EN 14103 is suitable to quantify the ester content in biodiesel free of heptadecanoate ester (C17:0), because it is employed as IS. For that reason, EN 14103 cannot be applied to the analysis of methyl ester in current work because C17:0 was found in *Jatropha curcas*. In previous work done by Gasparini *et al.* (2011), they proposed an improved method, based on EN 14103 capable to determine ester content in tallow biodiesel. Modification of the original equation from EN 14103 by introducing a correction factor (F), resulted in another equation more adequate to analyse the ester content in tallow biodiesel and its blends. Thus, it was strongly suggest that further studies should attempt to modify the equation in EN 14103 to be compatible with natural contents of heptadecanoic acid ester in oils and fats.

3.4.2 Determination of Free Fatty Acid Content

The Free fatty acid (FFA) content analysis of *Jatropha* oil methyl ester were determined in conformity with Palm Oil Research Institute Malaysia (PORIM) official test methods as indicated in Section 2.2.1 (Siew *et al*., 1995). All experiments were done in triplicates and the mean data was reported. Table 3.16 tabulates the FFA analysis of *Jatropha* oil methyl ester of experiment TE3 and experiment TE4.

Table 3.16 : FFA analysis of *Jatropha* oil methyl ester

Experiment	Description	FFA, % as oleic acid
TE3	Double-stage transesterification	0.24
TE4	Direct esterification and transesterification	0.26

As shown in Table 3.16, the FFA of methyl esters prepared from both methods, experiments TE3 and TE4 were well within the limits of American Standards and Testing Materials, (ASTM D6751) and European Standards (EN 14214) as both standards recommend FFA content of not more than 0.5% in biodiesel. Higher values of FFA in biodiesel lead to corrosion of rubber parts and cause deposits in the engine.

Murugesan *et al*. (2009) recommends FFA content of less than 3% in the feedstock for biodiesel production while Gerpen (2005) suggests the value of not more than 5%. A high FFA value in the feedstock consumes the catalyst during base catalysed transesterification reaction to produce biodiesel. It also leads to saponification which lowers the yield and increases formation of emulsions in the product making it difficult to separate biodiesel from glycerols (Gupta *et al*., 2004).

3.4.3 Determination of Moisture Content

Moisture content of *Jatropha* oil methyl ester was analysed as discussed in Section 2.2.5. All experiments were done in triplicates and the mean data was reported in Table 3.17.

Table 3.17 : Moisture content analysis of *Jatropha* oil methyl ester

Experiment	Description	Moisture content, %
TE3	Double-stage transesterification	0.045
TE4	Direct esterification and transesterification	0.043

Based on Table 3.17, the moisture content were 0.045% and 0.043% for experiments TE3 and TE4 respectively. The maximum amount of allowable water content in biodiesel as specified in ASTM standard D6751 is 0.050%vol (ASTM Standards, 2003). Moisture content indices for both methyl esters were well within the permitted limit. High moisture content in biodiesel can cause problems such as water accumulation and microbial growth in fuel handling, storage, and transportation equipment.

3.4.4 Determination of Density Value

The density value of *Jatropha* oil methyl esters were determined as indicated in Section 2.2.6 and Section 2.3.6. All experiments were done in three measurements and the mean data was reported. Table 3.18 tabularises the density value analysis of *Jatropha* oil methyl esters.

Table 3.18 : Density value analysis of *Jatropha* oil methyl ester

Experiment	Description	Density (g/cm^3)	Specific Gravity
TE3	Double-stage transesterification	0.8872	0.8894
TE4	Direct esterification and transesterification	0.8709	0.8763

The density values of *Jatropha* oil methyl ester were 0.8872 g/cm^3 and 0.8709 g/cm^3 for experiment TE3 and TE4 respectively. The density values for both experiments reduced to a significant extent when compared with the density of *Jatropha* oil. It falls within the acceptable range (860 to 900 kg/m^3) of European Standards for biodiesel fuels (EN14214) as indicated in Table 1.10

3.4.5 Determination of Pour Point

The observation of pour point value for *Jatropha* oil and *Jatropha* oil methyl ester were tabulated in Table 3.19. The appearance of the samples was observed at 15°C (9°C above the expected pour point). In every multiple of 3°C below starting temperature, the test jar was removed and tilted to determine if there was movement of the oil in the test jar.

Table 3.19 : Observation of *Jatropha* oil and *Jatropha* oil methyl ester for pour point determination

Temperature (°C)	*Jatropha* oil	*Jatropha* oil methyl ester
15	Cloudy solution was slightly formed at the bottom of the test jar	There was no change in physical appearance of the sample
12	The formation of cloudy solution was increased, nearly quarter of the test jar	Cloudy solution was slightly formed at the bottom of the test jar
9	The formation of cloudy solution remain unchanged as compared to sample appearance at 12°C	The formation of cloudy solution remain unchanged as compared to sample appearance at 12°C
6	The oil sample was started to freeze and solidify, The oil sample was still able to flow when the test jar was tilted.	The formation of cloudy solution was increased, nearly half of the test jar.
3	The oil sample was solidified but was still able to flow when the test jar was tilted.	The oil sample was started to freeze and solidify, The oil sample was still able to flow when the test jar was tilted.

‘**Table 3.19**, continued’

0	Similar result at 3°C was observed.	No flow movement was observed and *Jatropha* oil methyl ester sample was solidified.
-3	No flow movement was observed and *Jatropha* oil methyl ester sample was solidified.	-

Based on the observation of the experiments given in the Table 3.19, the pour point of *Jatropha* oil and *Jatropha* oil methyl ester were 0°C and 3°C respectively as the pour point determined by this analysis was recorded at temperature 3°C above the temperature wherein the *Jatropha* oil and *Jatropha* oil methyl ester appeared solid. The pour point of biodiesel from *Jatropha* oil was low (3°C) due to the higher content of unsaturated fatty acid in crude *Jatropha* oil. The result was found to be within the specified limit (-15 to 10°C) of biodiesel standard, ASTM D6751 as described in Table 1.7. Furthermore, the result of pour point shows that *Jatropha* oil methyl ester has low temperature flow characteristic as compared to palm oil methyl ester which has pour point of 15ºC (Choo *et al*., 2005). Biodiesel from *Jatropha* oil was suitable for the tropical area and also moderate temperature area.

3.4.6 Summary of Characterisation of *Jatropha* Oil Methyl Ester

The quality of *Jatropha* oil methyl ester from double-stage transesterification reaction (experiment TE3) and transesterification reaction with prior direct esterification as a pre-treatment of high FFA were evaluated and summarised in Table 3.20. In general, the evaluation showed that the methyl ester of experiment TE3 and TE4 meet the European specifications of EN 14124 and the American standard test method of ASTM D6751.

Table 3.20 : Quality analysis of *Jatropha* oil methyl ester

Analysis	Double-stage transesterification reaction (Experiment TE3)	Direct esterification (pre-treatment to high free fatty acid) and transesterification reaction (Experiment TE4)
Colour	dark yellow	light yellow
Total reaction time (hour)	6 hours 30 minutes	4 hours 50 minutes
Free Fatty acid (FFA) content (% as oleic acid)	0.24	0.26
Moisture content (%)	0.045	0.043
Density value at 25°C (g/cm^3)	0.8872	0.8709
Specific gravity	0.8894	0.8763
Pour point (°C)	3	3
Percentage of total methyl ester obtained (% m/m)	34.2	62.1

The presence of high free fatty acids content can considerably reduce the yield of methyl esters. Two-step process, acid pre-treatment esterification process and subsequently base-catalysed transesterification process was preferable. The finding in the present work was consistent with the previous studies (Berchmans and Hirata, 2008; Veljkovic *et al.*, 2006; Ghadge and Raheman, 2005). The conversion of free fatty acids to fatty acid methyl esters by an acid catalysed esterification and followed by transesterification reaction using alkaline catalyst in the second step was favourable. Berchmans and Hirata (2008) reported that crude *Jatropha curcas* oil with high content of free fatty acids was esterified with acid pretreatment process which lowers the free fatty acid content to less than 1%. The second stage, alkali base catalysed transesterification process gave 90% methyl ester yield. In contrast with finding by Berchmans and Hirata (2008), the total methyl ester obtained in the present work was lower due to methyl ester dissolution in the glycerol phase and triacylglycerols saponification.

3.5 Instrumental Analysis

The instrumental methods of chemical analysis in the present research were summarised as in Table 3.21.

Table 3.21 : Instrumental methods of chemical analysis and application

Instrument	Application
Polarising microscope	Study of the effect of ultrasonication in the EAAOE
Karl Fischer Volumetric Titration	Determination of moisture content
Density meter	Determination of density value
Gas chromatography- flame ionization detector (GC-FID) and mass spectrometer (GCMS)	Determination of fatty acid composition Determination of ester content
High performance Liquid Chromatography (HPLC) and mass spectrometer (LCMS)	Determination of toxic compound (phorbol esters) in the *Jatropha* oil

Overall, learning outcomes of instrumental analysis in the current work of enzymatic extraction and transesterification of *Jatropha curcas* oil were summarised as below:

- Ability to demonstrate an efficient understanding of the physical and chemical theory used in instrumental analysis and in sample preparation prior to instrumental analyses in accordance to the standard practice.
- Assessment in identification of the sources of errors and interferences in chemical and instrumental analysis in data analysis.
- Ability to assess quality compliance to international standards (*i.e.* ASTM D6751 and EN 14214).
- Lastly, distinguish between qualitative and quantitative measurements and compare and critically select methods for corresponding analyses e.g. analysis using chromatography and mass spectrometry.

CONCLUSION

The assessment of oil quality extracted using enzymatic assisted aqueous oil extraction (EAAOE) was explored. The enzymes used were Alcalase® 2.4L and Celluclast® 1.5L. The enzyme presence facilitated the recovery of the oil. Parameters involved in EAAOE which included selection of enzymes, pH, temperature, duration of incubation time, effect of ultrasonication, concentration of enzyme added, amount of water added in the seed suspension and oil recovery from oil-water mixture were optimised. The optimised conditions for EAAOE of *Jatropha* oil are as follows: extraction of oil with 6mL/ 1g water/oil; temperature, 50°C, pH 5.0 and pH 8.0 for Celluclast® and Alcalase®, and duration of incubation time, 4 hours(Alcalase®) and 8 hours (Celluclast®) and concentration of enzyme added 6% (m/m). The enhancement of oil recovery were achieved by subjecting the centrifugation process at 4°C and 8000 rpm, coupled with hexane-aided separation and subsequent 2 hours freezing of the aqueous layer. The average total extraction yield of 58%, m/m of total oil of *Jatropha curcas* was achieved under the optimised conditions of EAAOE.

The content of the toxic phorbol esters in *Jatropha* oil was elucidated using high performance liquid chromatography and liquid chromatography - mass spectrometry. The result showed that the phorbol esters content in *Jatropha* oil extracted using the EAAOE method consists of 0.2% and 0.3% for the enzyme Alcalase® and Celluclast® respectively. The oil extracted from EAAOE exhibited good physicochemical properties and is useful as biodiesel feedstock in industrial application. Good recovery in this environmentally friendly process shows that it is promising to avoid solvents, which are harmful to the environment as well as to the remaining protein cake.

At the moment, the cost of the enzyme is a major factor that will prevent adoption of this technology. Nevertheless, increasing environmental concerns coupled with the development of more efficient downstream processing technology for enzymes is expected to make this a feasible and preferable alternative for oil extraction in the near future.

The selection method for high free fatty acids (FFA) transesterification is direct esterification as a pre-treatment of high FFA prior to transesterification reaction. In comparison to double-stage transesterification process, it was found that the first method gave higher total methyl ester yield percentage, 62.1% m/m which is near to 30% greater than the latter transesterification method. In addition, acid pre-treatment in esterification shortens the total duration of reaction time. Last but not least, the quality of *Jatropha* oil methyl ester was well within permitted specifications of American standard ASTM D6751 and European standard EN 14214.