A STUDY OF ENZYMATIC REACTION IN BIODIESEL CONVERSION USING FRESH AND WASTE COOKING OIL

By

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ABSTRACT

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Tay Khim Sim

In recent years, soaring energy demand due to rapid industrialization growth has led to fast depletion of fossil fuel and prompted the scientific community to invest considerable effort in searching for renewable substitutes. The desirable alternative fuels should be environmental friendly, non-toxic, biodegradable and renewable. Fatty acid methyl ester (FAME), which is commonly called biodiesel, is a promising alternative fuel with all of the above desirable attributes. Commercialization of biodiesel is however facing major hurdle due to high cost of production, primarily from the raw material cost.

An attractive solution to the problem of high material cost may be the utilization of waste cooking oil (WCO) as an economical source for biodiesel production. Under the presence of high free fatty acids in WCO, enzyme-

catalyzed transesterification represents an efficient method without the drawbacks of low yield when chemical transesterification is used. In this study, the catalyst used was commercial available lipases Thermomyces Lanuginosus (TLIM) and also a self-prepared variant by immobilizing TL in chitosan beads using a binary immobilization method. Both WCO and fresh cooking oil (FCO) were used as feedstock and together with biodiesel produced from the reaction were characterized by the determination of acid value, saponification value, and iodine value. The parameters varied in the experiments were molar ratio of methanol (MeOH) to oil, reaction temperature, and lipase concentration. All reactions were carried out in the presence of 1:1 (v/v) solvent tert-butanol to oil. Duration required for reaction was initially explored by testing up to 72 h and most of the tests were later fixed for a normal reaction cycle of 24 h which was found to be sufficient. For each test, the oil, lipase and solvent were heated in a conical flask placed in an incubator shaker running at 200rpm and MeOH was added in three successive steps. The determination of methyl esters (ME), triglycerides (TG), diglycerides (DG), monoglycerides (MG) and glycerol were carried out using gas chromatography. The results showed that an increase in the number of moles of MeOH resulted in an increase in ME content and optimum content of ME was observed at reaction temperature of 40ºC. With sufficient pre-conditioning, the reaction on WCO using self-prepared chitosan immobilized TL could attain a ME content of around 35% which was close to that obtained using commercial TLIM. Under the condition of MeOH to oil molar ratio of 6:1, 1% (w/w) of lipase to oil

weight and 40°C in a normal cycle duration of 24 h. Also, the activity of chitosan immobilized TL could still manage to maintain at 76% after 12 cycles.

Response Surface Methodology (RSM) based on the 5-level-3-factor central composite design had been used to identify the factors that influence the transesterification of FCO and WCO using chitosan immobilized TL. The parameters and their range of values employed in the experiments were molar ratio of MeOH to oil between 1:1 and 6:1, reaction temperature between 30 and 50°C, and concentration between 0.5 and 1.5% (w/w) of lipase to oil. Analysis of variance on the regression models show that reaction temperature, lipase concentration and MeOH:oil molar ratio were all significant variables in affecting ME content. The R^2 values of the models for FCO and WCO were 0.9793 and 0.9442 respectively. The P-value for the FCO and WCO regression models are lower than 0.05 indicating these model terms are significant.

Special Dedication of This Grateful Feeling to My…

Beloved parents;

Mr. Tay Hai Kim @ Chern Hai Chin

Mdm. Chen Siew Mey @ Chin Ah Moy

Loving brothers and sisters;

Yong Haur, Khim Kiang, Yong Kiat, Khim Wang, Khim Tiang, Yong Liang

For Their Love, Support and Best Wishes.

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APPROVAL SHEET

This thesis entitled "**A STUDY OF ENZYMATIC REACTION IN BIODIESEL CONVERSION USING FRESH AND WASTE COOKING OIL**" was prepared by **TAY KHIM SIM** and submitted in partial fulfillment of the requirements for the degree of Master of Engineering Science at Universiti Tunku Abdul Rahman.

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SUBMISSION OF THESIS

It is hereby certified that **TAY KHIM SIM** (ID No: **08UEM08126**) has completed this report entitled "A STUDY OF ENZYMATIC REACTION IN BIODIESEL CONVERSION USING FRESH AND WASTE COOKING OIL" under supervision of Dr. THAN CHEOK FAH from the Department of Mechanical and Material Engineering, and Dr LEE KHIA MIN from the Department of Civil Engineering, Faculty of Engineering and Science.

I understand that University will upload softcopy of my thesis in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____

(TAY KHIM SIM)

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

(TAY KHIM SIM)

Date: 26/6/2012

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LIST OF ABBREVIATIONS AND SYMBOLS

ASTM	American Society for Testing and Materials
FAME	Fatty Acid Methyl Ester
WCO	Waste Cooking Oil
Etc.	Et cetera
MG	Monoglycerides
DG	Diglycerides
TG	Triglycerides
Et. al	and others
PAH	Polycyclic aromatic hydrocarbon
CPO	Crude Palm Oil
TAG	Triacylglycerol
FFA	Free Fatty Acid
MeOH	Methanol
TLIM	Immobilised Thermomyces Lanuginosa
GC	Gas Chromatography
SEM	Scanning Electron Microscope
HHV	High Heating Value
KV	Kinematic Viscosity
IV	Iodine Value

CN	Cetane Number
SV	Saponification Value
AC	Ash Content
CR	Carbon Residue
MW	Molecular Weight
ME	Methyl Ester
KOH	Potassium Hydroxide
AV	Acid Value
KHP	Potassium Hydrogen Phthalate
BSA	Borun Serum Albumin
FID	Flame Ionisation Detector

CHAPTER 1.0

INTRODUCTION

1.1 Renewable Energy Sources

Fast depletion coupled with higher demand and limited availability of fossil fuel due to large population and industrialization growth all around the world accelerates worldwide effort to search for alternative sources of energy to replace the fossil fuels. In addition, worldwide concerns on the potential impact of global climate change, declining air and water quality, and serious human health considerations are inspiring the development of alternative fuels which can reduce greenhouse gases that cause global warming.

In 1893s, Rudolf Diesel tested peanut oil as fuel for his engine for the first time. Before World War II, biodiesel was implemented in South Africa to power heavy-duty vehicles. In 1930s and 1940s, vegetables oils were used as fuels. In August, 1982, the first International conference held in Fargo, North Dakota included active discussion on plant and vegetable oils as fuels (Ma & Hanna, 1999). The agenda of the meeting were the cost, engine performance, durability, additives and preparation specification of alternative fuels. Furthermore,

vegetable oil was envisaged to play an important role as the feedstock for producing alternative fuels.

Various new and renewable resources of energy have been investigated and explored recently such as biodiesel, bioalcohol, biomass, solar energy, hydoenergy etc. The preferred alternatives fuel is biodiesel as it can be implemented readily in existing engines without major changes.

1.2 Background of Biodiesel

Biodiesel has attracted increasing world attention as an alternative fuel. It is a non-petroleum based fuel consisting of long chain alkyl esters. Biodiesel is renewable, biodegradable, non-toxic, and essentially free of sulfur and aromatics. Biodiesel can be used alone in pure form or blended with petroleum diesel at any concentration. It has become an alternative fuel source in standard diesel engines and applies in different areas such as in railroad, aircraft or as heating oil.

The American Society for Testing and Materials (ASTM) defines biodiesel as monoalkyl esters of long chain fatty acids derived from a renewable lipid feedstock, such as vegetable oil or animal fat (Marchetti & Errazu, 2008; Kasteren & Nisworo, 2007). Biodiesel can be synthesized from different types of virgin oil feedstock. In the United States, soybean oil is normally used. On the

other hand, the principal raw material in Europe is rapeseed oil while sunflower oil is preferred in South America. The feedstock of biodiesel synthesis in Malaysia is mainly palm oil whereas coconut oil is mostly used in the Philippines. The conversion of oil into biodiesel through transesterification process reduces the molecular weight and viscosity of oil to that close to diesel fuels.

Biodiesel is normally characterised for their physical and fuel properties based on standard methods such as ASTM by determining its density, iodine value, acid value, viscosity, cloud points and pour points, saponification value, gross heat of combustion and volatility. The standard density for biodiesel fuel should between 0.860 and 0.900 g/cm^3 at 15ºC. Density is important in airless combustion systems because it influences the efficiency of atomisation of the fuel. Iodine value is a determination of unsaturation degree of the fuel. It is an important measure of the oxidative stability of oil. The ASTM standard of acid number for pure biodiesel is 0.8 mgKOH/g. High acid number will cause degradation of rubber parts in older engines resulting in filter clogging. The viscosity of biodiesel fuel is in the range of 1.9 – 6.0 mm^2/s based on ASTM method.

1.2.1 Advantages of Biodiesel

One of the advantages of biodiesel is renewability where the oil sources may be derived from animal fats, corn, soybeans or other crops. Renewable fuel can thus be produced domestically that can ensure reasonable price stability without shortage. Besides, biodiesel is more environmental friendly with less carbon emissions and greenhouse effect than conventional diesel fuel since it is made from natural sources (Chhetri, Watts & Islam, 2008). Little sulfur production from biodiesel combustion reduces the risk of acid rain.

In addition, biodiesel has higher flash point (about 150ºC) than diesel fuel (about 50ºC), thus it is less volatile, safer to store and transport the fuel. Despite of higher flash points of biodiesel which is safer in transport and storage, it produces less carcinogenic polycyclic aromatic hydrocarbon (PAH) and nitrated PAH (Saifuddin & Chua, 2004) because it is biodegradable and non-toxic. More than 90% of biodiesel can be biodegraded within 21 days. Biodiesel may be produced under normal laboratory conditions, mild conditions of pH, temperature and pressure (Saifuddin & Raziah, 2008).

In the United States and Europe, biodiesel is being applied in many trucks, buses and cars. Though biodiesel fuel has lower energy content per unit volume compared with standard diesel fuel, it is miscible with petroleum where it can blend at any ratio with diesel and gives comparable performance and durability as

petroleum diesel fuel. The blending of biodiesel fuel with conventional diesel can dilute its environmental impact (Hartman, 2010).

1.2.2 Limitations of Biodiesel

Biodiesel has higher viscosity and lower volatility than fossil diesel. However, problems usually appear only after the engine has been operating for a longer periods of time. Coking and trumpet formation on the injectors occur to such an extent that fuel atomisation does not occur properly or is even prohibited in consequence of plugged orifices, carbon deposits, oil ring sticking, thickening and gelling of the lubricating oil (Ma & Hanna, 1999). The inefficient mixing of oil with air thus leads to incomplete combustion. In addition, biodiesel with poorer low-temperature flow properties than fossil diesel could cause engine operating problems because biodiesel fuel will be crystallised at low-temperature (below 0ºC). Oxidative and thermal polymerisation of vegetable oils causes deposition on injectors and gum forming. Biodiesel has relatively high cloud point, pour point and cold-filter plugging point (Srivastava & Prasad, 2000).

A major hurdle in biodiesel production is the high cost of the feedstock of oil used. Besides, the production from food-grade vegetable oil is expensive and ethically unacceptable. Thus, alternative feedstock is desirable to substitute

refined oil such as using crude vegetable oil, soap stocks, acid oils or waste oil (Marchetti, Miguel & Errazu, 2007).

1.2.3 Overview of Biodiesel Synthesis Methods

Various methods have been proposed to synthesise biodiesel by chemical and enzymatic transesterification. Conventionally, methyl esters synthesis is accomplished through chemical transesterification which can use acid catalyst, alkali catalyst or a two-stage acid and alkali catalysis.

Biodiesel production using chemical transesterification is normally used due to lower cost and high conversion ratio of triacylglycerols (TAG) to methyl esters in a short time (Nie, Xie, Wang & Tan, 2006). However, there are several drawbacks to these approaches. For alkali-catalysed transesterification, high water content will decrease the biodiesel yield while at high free fatty acid (FFA) condition, the formation of soaps would require large amount of water for purification. This not only leads to overall production cost increase but also pollution of water. Besides, the glycerol recovery is difficult and involves high energy consumption (Nie *et al.*, 2006; Ting, Huang, Giridhar & Wu, 2008; Yagiz, Kazan & Akin, 2007). On the other hand, acid-catalysed transesterification can endure with high water content and high FFA conditions which solve the drawbacks from alkali-catalysed transesterfication. However, higher reaction

temperature and longer reaction time are needed in order to obtain high conversion ratio of biodiesel (Ting *et al.*, 2008).

Recently, enzymatic synthesis has emerged as a potential method in biodiesel production. Enzymatic hydrolysis process can tolerate high FFA oils and be carried out under mild temperature, and produce high quality of biodiesel under strict selectivity of enzymes. Although the cost of enzymes is high, it can be cost effective by repeated use of enzymes and high price of by-product glycerol without complex treatment (Ting *et al.*, 2008; Ting, Tung, Giridhar & Wu, 2006). Though chemical transesterification process is cheaper than enzymatic synthesis, the later causes lower pollution of the natural environment which should be taken into consideration (Antczak, Kubiak, Antczak & Bielecki, 2009).

Immobilisation of enzyme can improve stability, avoid product contamination and render reusable to reduce the cost of expensive enzymes. Fixation of enzymes leads to better resistance to thermal denaturation and provides highly active, chemically and thermal stable heterogeneous biocatalysts compared with native enzymes. Several studies concerning the production of biodiesel from fresh vegetable oils such as canola oil (Dizge & Keskinler, 2008), palm oil (Knezevic, Mojovic & Adnadjevic, 1998), sunflower oil (Dizge & Keskinler, 2008), soybean oil (Ting *et al.*, 2008), cotton seed oil (Royon, Daz, Ellenrieder & Locatelli, 2007), and waste oil (Halim, Kamaruddin & Fernando,

2009; Maceiras, *et al.*, 2009; Halim & Kamaruddin, 2008) as feedstock for enzymatic production of biodiesel have been reported previously.

Lipases are the most widely used enzymes able to catalyse the transesterification, esterification and aminolysis in organic solvent systems (Villeneuve, Muderhwa, Graille & Haas, 2000). Thus, fats that contain TAG and FFA can be enzymatically converted to biodiesel in a one-step process. Lipase can be activated at oil-water interphase and are capable of preserving their catalytic activity in non-aqueous and biphasic systems and in micellar solution. However, its low stability and activity or selectivity coupled with the high cost prohibits its use in industrial hydrolytic reactions (Ting *et al.*, 2006; Antczak *et al.*, 2009).

Recently, researchers have attempted immobilisation of enzymes by using cheaper support materials to reduce the cost of conversion. The materials considered suitable for enzyme support should have high affinity for proteins, availability of reactive functional groups, mechanical stability, rigidity, feasibility of regeneration and high loading capacity (Krajewska, 2004). Due to environment concern, the enzyme support should be non-toxic and biodegradable. Some non-expensive materials have been used as enzyme support such as wood cellulignin, rice husk and rice straw, chitin, amberlite and chitosan (Foresti & Ferreira, 2007).

1.3 Biodiesel Perspective in Malaysia

Malaysia is one of the largest producers of palm oil in the world and palm oil production has increased tremendously from 2.57 million metric tons in year 1980 to 14.96 million tons in year 2005 (Chew & Bhatia, 2008). Crude palm oil, used palm oil and palm oil-based fatty acid mixture are the three potential feed stocks for biofuels production in Malaysia. Palm oil is a favorable feedstock for biodiesel production due to its most productive oil bearing plant with highest yield around 4000 kg per hectar compared with other vegetable oils. Besides, oil palm is less dependable on the vagaries of weather compared to other crops. However, the price of CPO in year 2011 was at RM3384/ton is too expensive if used as biodiesel feedstock.

According to the Malaysian Biodiesel Association, biodiesel industry in Malaysia is at a stagnant stage where the production is almost zero. The latest statistics from the Malaysian Palm Oil Board showed that biodiesel export in July 2010 was at an alarming low level of 137 tonnes which is 95% drop from 2518 tonnes a month earlier. Although the production of palm oil in Malaysia is high, most of the producers are actively involved in the extraction and sale of palm oil phytonutrients and oleochemical products. The operations of biodiesel production cannot be maintained due to the high cost of production and the lack of the much needed incentives and subsidies from the government. Therefore, the biodiesel production by using crude palm oil is not being focused most of the time.

In order to reduce the cost of biodiesel feedstock, WCO from restaurants and household may be considered due to the possibility to acquire from restaurants and household at low cost. In Malaysia, our brief survey of a well known fast food franchise chain company indicates that oil consumption of every outlet varies slightly. For a typical outlet, 17L of palm oil is consumed per day. Using this as average consumption, 300 outlets of the fast food company throughout Malaysia roughly will consume about 1,861,500L of oil per year. Besides, there are many other fast food restaurants, hawker stalls and household usage of oils throughout the country where the production of WCO is unaccountable. Therefore, the availability of WCO and low cost to acquire them can be attractive as raw material in biodiesel production. More importantly, the recycling of WCO can decrease the burden of government to deal with disposal problems and minimise environment impact.

1.4 Potential of WCO as Feedstock for Biodiesel Conversion

The Energy Information Administration of United States estimated that about 100 million gallons of WCO is produced per day in USA, where the average *per capita* WCO was reported as 9 pounds *per* year. This is similar to another study in Canada where a total of 33 million Canadian populations consumed approximately 135,000 tons/year of edible oils (Chhetri et *al.*, 2008). According to Peiro *et al.* (2008), it was estimated that in Europe 5 kg of used

cooking oils are generated per inhabitant, totaling 2.5 million metric tons per year and approximately 2.5 billion pounds of waste restaurant fats were collected annually in United States. Most of the inexpensive feedstocks are devoted mostly to industrial uses and feedstock for animal feed (Canakci, 2007).

In Asia, WCO collected in the third largest city of Guangzhou in China was estimated to be over 20 thousand tons every year (Wang, Ou, Liu & Zhang, 2007). According to Meng, Chen and Wang (2008), China consumed approaching 22 million tons of edible oils annually. More than 4.5 million tons of used oil and grease were generated by the country each year, roughly half of which could be collected through the collection and recycling system. Hence 2 million tons of used oils could be used for biodiesel production line. Besides, In Malaysia, an estimated of 50,000 tons of used frying oils, both vegetable oils and animal fats are disposed off yearly without treatment as wastes (Loh, Choo, Cheng & Ma, 2006).

Careless disposal of WCO creates negative impacts on the environment. Many countries lack proper management of the waste and the disposal problems are a significant challenge. Although some of the waste can be used for soap production and animal feeding, but the major part is discarded into the environment (Chhetri *et al.*, 2008). Thus, the replacement of raw material biodiesel with WCO could minimise pollution and also greatly help in reducing the cost of biodiesel production.

1.5 Objective and Scope of Study

Biodiesel conversion employing various transesterification methods has been studied extensively in the past decades using fresh vegetable oils such as canola oil, palm oil, rapeseed oil etc. However, transesterification using WCO has not been studied as widely compared to FCO. Hence, this study aims to explore further biodiesel conversion using WCO enzymatic transesterification. The binary immobilisation method is used for the attachment of the enzymes on chitosan beads. Chitosan, is recognised by its excellent properties for lipase support such as biodegradable to harmless products, non-toxicity, and great affinity for proteins (Foresti & Forreira, 2007). Being a by-product from the seafood processing industry, it can be obtained cheaply and abundantly from chitin. This would result in overall cost reduction and make the process environment friendly (Ting *et al.*, 2006).

Hence, this study has the objectives of using both low cost feedstock and environment friendly catalyst for producing the biodiesel. Optimisation of the reaction is investigated at various conditions such as the amount of lipase, molar ratio of methanol (MeOH) to raw oil, and reaction temperature. Enzymatic reaction on biodiesel production using WCO is determined using lipase *Thermomyces Lanuginosa* immobilised on chitosan powder via the binary immobilisation method. In addition, the characteristics of pre and post treatment stages of biodiesel are also investigated by acid value, saponification value, and

iodine value, and gas chromatography (GC). The structure of chitosan beads are also examined by using scanning electron microscopy (SEM).

1.6 Outline of Thesis

Chapter 1 contains brief background information on biodiesel production methods and also the scope of this study.

Chapter 2 contains literature review on previous biodiesel production methods, in particular on lipase catalyzed transesterification and lipase immobilization methods. Effects of various parameters investigated in previous studies on biodiesel conversion are also discussed.

Chapter 3 contains discussion on preparation of chitosan beads and TL immobilization. Methods of qualitative and quantitative analyses of the oil feedstock and transesterification products, and the experimental design are studied.

Chapter 4 contains the findings and discussion of this study.

Chapter 5 contains the conclusions from this study and recommendations for future work.

References and appendices are attached at the end of this thesis.

CHAPTER 2.0

LITERTURE REVIEW

2.1 Refine Oils and Fats

Basically, oils and fats are mainly water-insoluble, hydrophobic substances in plants and animals which are made up of one mole of glycerol and three moles of fatty acids and commonly named as triglyceride (TG). The structure of TG is shown in Figure 2.1 where the R1, R2 and R3 represent the carbon chains of the fatty acid which may be the same or different. Fatty acids vary in carbon chain length and numbers of unsaturated bonds (Ma & Hanna, 1999). Table 2.1 shows the fatty acid composition of vegetable oil samples (Demirbas, 2005). Natural vegetable oils and fats are extracted from crude oil or fats that contain free fatty acids, phospholipids, sterols, water, odorants and other impurities. Free fatty acids and moisture reduce the efficiency of transesterification in converting the feedstock into biodiesel (Canakci, 2007).

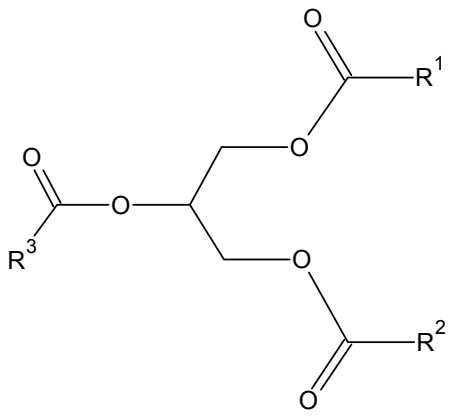

Figure 2.1: Structure of triglycerides

Table 2.1: Fatty acid composition of vegetable oils (Demirbas, 2005)

Sample	16:0	16:1	18:0	18:1	18:2	18:3	Others
Cottonseed	28.7	0	0.9	13.0	57.4	0	0
Poppyseed	12.6	0.1	4.0	22.3	60.2	0.5	0
Rapeseed	3.5	0	0.9	64.1	22.3	8.2	0
Safflowerseed	7.3	0	1.9	13.6	77.2	0	0
Sunfflowerseed	6.4	0.1	2.9	17.7	72.9	0	0
Sesameseed	13.1	0	3.9	52.8	30.2	0	0
Linseed	5.1	0.3	2.5	18.9	18.1	55.1	0
Wheat grain	20.6	1.0	1.1	16.6	56.0	2.9	1.8
Palm	42.6	0.3	4.4	40.5	10.1	0.2	1.1
Corn Marrow	11.8	0	2.0	24.8	61.3	0	0.3
Castor	1.1	0	3.1	4.9	1.3	0	89.6
Soybean	13.9	0.3	2.1	23.2	56.2	4.3	0
Bay laurel leaf	25.9	0.3	3.1	10.8	11.3	17.6	31.0
Peanut kernel	11.4	0	2.4	48.3	32.0	0.9	4.0
Hazelnut kernel	4.9	0.2	2.6	83.6	8.5	0.2	0
Walnut kernel	7.2	0.2	1.9	18.5	56.0	16.2	0
Almond kernel	6.5	0.5	1.4	70.7	20.0	0	0.9
Olive kernel	5.0	0.3	1.6	74.7	17.6	0	0.8
Coconut	7.8	0.1	3.0	4.4	0.8	0	65.7

Table 2.2 shows the physical and chemical properties of some vegetable oils (Demirbas, 2003). According to Srivastava and Prasad (2000), kinematic viscosity of vegetable oils varies in the range 30 to 40mm^2/s at 38ºC. Large molecular mass and chemical structure will lead to high viscosity. Cetane numbers are always in the range of 32 to 40 while Iodine Value ranges from 0 to 200 depending upon degree of unsaturation. The high heating value (HHV) of the vegetable oils are in the range of 39 to 40MJ/kg which is low compared to diesel fuel that is around 45MJ/kg. HHV can be decreased by about 10% in the presence of chemically bound oxygen in vegetable oils. High values of ash content will cause the problems of cocking of injectors and fuel filters clogging. According to Felizardo *et al.* (2006), EN 14214 (2003) establishes a maximum value of 0.02% for sulphated ash.

Table 2.2: Physical and chemical properties of vegetable oils (Demirbas, 2003)

Sample	KV	CR	CN	HHV	AC	IV	SV
Cottonseed	33.7	0.25	33.7	39.4	0.02	113.20	207.71
Poppyseed	42.4	0.25	36.7	39.6	0.02	116.83	196.82
Rapeseed	37.3	0.31	37.5	39.7	0.006	108.05	197.07
Safflowerseed	31.6	0.26	42.0	39.5	0.007	139.83	190.23
Sunfflowerseed	34.4	0.28	36.7	39.6	0.01	132.32	191.70
Sesameseed	36.0	0.25	40.4	39.4	0.002	91.76	210.34
Linseed	28.0	0.24	27.6	39.3	0.01	156.74	188.71
Wheat grain	32.6	0.23	35.2	39.3	0.02	120.96	205.68
Corn Marrow	35.1	0.22	37.5	39.6	0.01	119.41	194.14
Castor	29.7	0.21	42.3	37.4	0.01	88.72	202.71
Soybean	33.1	0.24	38.1	39.6	0.006	69.82	220.78
Bay laurel leaf	23.2	0.20	33.6	39.3	0.03	105.15	220.62
Peanut kernel	40.0	0.22	34.6	39.5	0.02	119.55	199.80
Hazelnut kernel	24.0	0.21	52.9	39.8	0.01	98.62	197.63
Walnut kernel	36.8	0.24	33.6	39.6	0.02	135.24	190.82
Almond kernel	34.2	0.22	34.5	39.8	0.01	102.35	197.56
Olive kernel	29.2	0.23	49.3	39.7	0.008	100.16	196.83

Note: KV (kinematic viscosity, mm^2/s at 311K); CR (carbon residue**,** wt.%); CN (Cetane number); HHV (high heating value, MJ/kg); AC (ash content, wt.%); IV (Iodine value, Cg I/g oil); SV (saponification value, mg KOH/g oil)

2.2 Methods of Fuel Conversion from Triglycerides

Though vegetable oils can be used as fuel for compression ignition engines, there are problems associated with their high viscosities, low volatilities and polyunsaturated character. Four ways to reduce the viscosity of vegetable oils

are dilution, emulsification, pyrolysis and transesterification. Among the four derivatives, transesterification is the most popular method where the product is biodiesel.

2.2.1 Dilution

Dilution is one way to reduce the high viscosity of vegetable oils. Through blending with diesel fuel, viscosity of vegetable oils can be lowered. In 1980, Caterpillar Brazil used pre-combustion chamber engines with blending of 10% vegetable oil to maintain total power without any alterations or adjustments to the engine. On the other hand, the performance of engine during a longer term was prematurely terminated when using 100% crude soybean oil (Ma & Hanna, 1999). This is because of the high viscosity of oil which is about 10-20 times higher than diesel fuel and low volatility which leads to incomplete burning and formation of deposits in the fuel injectors (Demirbas, 2003).

There are several researches on dilution of vegetable oils such as sunflower oil and soybean oil with diesel fuel (Srivastava & Prasad, 2000). However, problems appear only after the engine operated for a longer periods of time. Two severe problems were oil deterioration and incomplete combustion. Oxidation and polymerisation during storage will cause gum formation, high viscosity, acid composition, and FFA content. The gum which did not combust

completely resulted in carbon deposits, coking of injectors on piston and head of engine, and thickening of lubricant.

2.2.2 Microemulsion

Microemulsions are defined as colloidal equilibrium dispersions of optically isotropic fluid microstructures with dimensions of range 1-150 nm formed spontaneously from two immiscible liquids and one or more ionic or non-ionic amphiphiles. A microemulsion can be made of vegetable oils with an ester and dispersant, an alcohol and a surfactant with or without diesel fuels (Srivastava & Prasad, 2000). Microemulsions are isotropic, clear, or translucent thermodynamically stable dispersions of oil, water, surfactant, and often a small amphiphilic molecule called co-surfactant (Balat & Balat, 2008). Both the ionic and non-ionic microemulsions of aqueous ethanol in soybean oil have good characteristics in short term performance (Ma & Hanna, 1999). A microemulsion of MeOH with vegetable oils can perform nearly as well as No. 2 diesel fuels (Srivastava & Prasad, 2000).

Some of the fuels in diesel engines were tested by the Engine Manufacturers Association (EMA). Microemulsions with alcohol content have lower volumetric HHV than diesel fuels but have high latent heat of vaporisation and tend to cool the combustion chamber which would reduce nozzle coking. A

reported engine test on microemulsion fuel containing 50% No. 2 Fuel, 25% degummed and alkali-refined soybean oil, 5% ethanol, and 20% 1-butanol passed the 200h EMA test, but carbon lacquer deposits on the injector tips, in-take valves and tops of the cylinder liners were the major problems (Balat & Balat, 2008). Another test on microemulsion fuel consisting of soybean oil: MeOH: 2-octanol: Cetane improver (52.7:13.3:33.3:1) showed the accumulation of carbon around the orifices of the injector nozzles and heavy deposits on exhaust valves (Srivastava & Prasad, 2000).

2.2.3 Pyrolysis

Pyrolysis is the direct thermal decomposition of the organic matrix in the absence of oxygen or at limited oxygen supplies to cleavage the chemical bonds into smaller molecules with the aid of catalyst. It is popular for converting biomass to solid, liquid, and gaseous fuels. The advantages of pyrolysis are its simple and inexpensive construct. Depending on the operating conditions, pyrolysis can be divided into three subclasses which are conventional pyrolysis, fast pyrolysis and flash pyrolysis. It produces gas mixture, carbon rich solid residue and bio-oil. Thermal decomposition of TG produces bio-oil with an aqueous phase containing low molecular weight oxygenated organic compounds and a non-aqueous phase containing oxygenated compounds (Chew & Bhatia, 2008). Figure 2.2 shows the mechanism of pyrolysis. Many structures and

multiplicity of possible reactions are formed in thermal decomposition of TG. Generally, thermal decomposition of these structures proceeds through either a free-radical or carbonium ion mechanism.

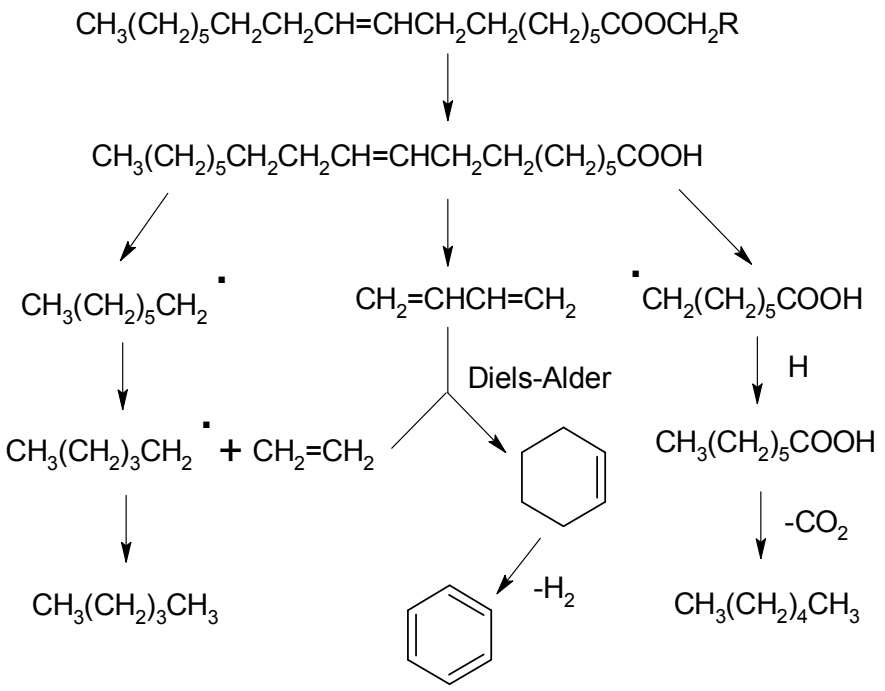

Figure 2.2: Thermal cracking mechanism

Different types of TG had been used to produce different types and amounts of liquid product since World War I and II. The first thermal cracking of vegetable oil was conducted in an attempt to synthesise fuel from vegetable oil. In 1947, thermal cracking of tung oil calcium soap was reported where tung oil was saponified with lime and generate crude oil which was refined to produce diesel fuel and small amounts of gasoline and kerosene (Demirbas, 2003). According to Xu, Jiang, Sun and Chen (2010), pyrolysis of soybean oil was carried out at

temperature ranging from 480 to 520ºC. By using a basic catalyst, it is possible to obtain oils with good cold-flow properties and high heat value. For pyrolysis of palm oil, it was found that the products contained higher percentage of saturated fatty acid and large heavy fractions (Maher & Bressler, 2007). Thermal cracking of vegetable oils produced acceptable amounts of sulphur, water and sediment and copper corrosion values but unacceptable ash, carbon residue amounts and pour point (Srivastava & Prasad, 2000).

There are other researches on maximising the yield by using different types of oil such as tung oil (Demirbas, 2003), soybean oil (Lima *et al.*, 2004), sunflower oil (Balat & Balat, 2008), copra oil and palm oil (Lima *et al.*, 2004). Catalysts have been used in many studies, but generally are metallic salts to obtain paraffin and olefins similar to those present in petroleum sources.

One of the main drawbacks of bio-oil is that the composition of pyrolytic oils is similar to the feedstock and is different from petroleum derived fuels and chemicals. The characteristics of bio-oil can vary greatly depending on the starting material. Besides, bio-oil as fuel has low HHV which is 40% less than fuel oil, high viscosity and high substantial solids contents (Maher & Bressler, 2007).

2.2.4 Transesterification

Transesterification is the chemical reaction between TG of vegetable oils or animal fats with alcohol with the aid of a catalyst. When TG reacts with alcohol, the three fatty acid chains are released from the glycerol skeleton and combine with alcohol to form esters and co-product glycerol as given below.

Triglycerides + Alcohol ---→ Esters + Glycerol

$$TG + 3ROH \xrightarrow{\text{catalyst}} GL + 3R'CO_2R \qquad (2.1)$$

The stepwise reactions may be represented as follows:

$$TG + ROH \underset{k_4}{\overset{k_1}{\rightleftharpoons}} DG + R'CO_2R \qquad (2.2)$$

$$DG + ROH \underset{k_5}{\overset{k_2}{\rightleftharpoons}} MG + R'CO_2R \qquad (2.3)$$

$$MG + ROH \underset{k_6}{\overset{k_3}{\rightleftharpoons}} GL + R'CO_2R \qquad (2.4)$$

Through transesterification, large and branched TG are broken down into smaller molecules and shorter chain length. The esters will have lower molecular weight (MW) which is approximately one-third of TG, and lower viscosity that is one-eighth of TG. The transesterification requires a catalyst to break the oil molecules and an alcohol to combine with the separated esters. A catalyst is used to improve the reaction rate and yield. The types of catalyst chosen depend on the nature of oil used. The reversible reaction shows that excess amount of alcohol will lead to the formation of esters with the help of catalyst to increase the reaction rate and yield. Primary or secondary alcohols with 1-8 carbon atoms are chosen to be used in transesterification such as MeOH, ethanol, propanol, and butanol (Demirbas, 2003).

There are several ways to undergo transesterification such as alkali catalyst, acid catalyst, biocatalyst, heterogeneous catalyst or without catalyst in the reaction state (Ranganathan *et al.*, 2008). The variables that affect the esters yield during transesterification are molar ratio of alcohol to vegetable oil, catalyst type, reaction temperature, reaction time and water content.

2.2.5 Comparison of Methods

Among the four derivatives which are dilution, micro-emulsification, pyrolysis and transesterification, dilution and microemulsions techniques can

lower the viscosity of oil but the problem of the fuel products on engine performance still exist. Pyrolysis needs extreme reaction condition which is above 500ºC and expensive equipment to undergo the reaction. Transesterification is the most popular method due to its thermal stability and the fuel product is similar to the conventional diesel fuel. It can reduce the viscosity of vegetables oils and maintains the HHV and CN similar to petroleum fuel. Short and long term engine testing showed that biodiesel produced by transesterification has performed well in engines.

2.3 Biodiesel Conversion by Transesterification Methods

2.3.1 Alkali Catalysed Transesterification

During the initial process of alkali-catalysed transesterification, Alcoxy is formed with alcohol with the aid of catalyst which then reacts with TG to form esters and co-product glycerol. At the end process, two phases of solution are formed where glycerol settles at the bottom part. The common catalyst used is sodium hydroxide or potassium hydroxide and the alcohol is either MeOH or ethanol. MeOH is preferred because of its lower cost and has physical and chemical advantages in view of the fact that it is polar and shorter chain length.

The advantages of using alkali process are lower temperature which is below 60ºC and least corrosive condition. However, high water content will

decrease the biodiesel yield while at high FFA condition, the formation of soaps would require large amount of water for purification. This leads to overall cost increase and also pollution of water. Besides, the glycerol recovery is difficult and requires high energy consumption (Yagiz *et al.*; 2007, Ranganathan *et al.*, 2008; Halim *et al.*, 2009). The glycerol that contains alkali has to be treated as waste (Shimada Watanabe, Sugihara & Tominaga, 2002). Alkali metal alkoxides are more efficient compared to acidic catalyst. Transmethylations occur approximately 4000 times faster in the presence of alkaline catalyst than acidic catalyst (Srivastava & Prasad, 2000).

2.3.2 Acid Catalysed Transesterification

Acid-catalysed transesterification can endure with high water content, and high free fatty acid conditions which solve the drawbacks from alkali-catalysed transesterfication and higher yield is obtained. However, higher reaction temperature and longer reaction time are needed in order to obtain high conversion ratio of biodiesel. Besides, acid with corrosive characteristics may cause damage to the equipment (Ranganathan *et al.*, 2008). The most commonly used acids are sulfuric acid and sulfonic acid.

2.3.3 Enzymatic Catalysed Transesterification

Utilisation of lipase as catalyst in transesterification for biodiesel conversion has greater potentials than chemical transesterification. Lipase-catalysed transesterification has become more attractive due to the easier glycerol separation and simpler purification process. Furthermore, by using lipase as catalyst, the process is more tolerant of water content in oil and biodiesel yield is increased without soap formation. Therefore effective and cheap lipase ensuring cost efficient, large-scale transesterification is of great interest and being pursued worldwide.

There are several studies using different lipases such as *Candida Antarctica*, *Pseudomonas Cepacia* and *Thermomyces Lanuginosus* (Martin & Otero, 2008) either in free form or immobilized on kaolinites, ceramics, silica, etc. (Yagiz *et al.*, 2007). Lipases immobilised in suitable supports can be reused without separation. The operating temperature is normally below 50ºC which is low compared to other techniques. However, there are some disadvantages such as inhibition effects between enzyme and alcohol and the high cost of enzymes which tends to limit its use in large scale production of biodiesel. There are several reports on heterogeneous catalysts such as amorphous zirconia and aluminuim. Support materials play an important role in that they not only reduce the cost of immobilised enzyme but also provide large surface area for least

diffusion limitation to enzymatic reactions (Dizge, Ayginer, Imer, Bayramoglu, & Tanriseyen, 2008).

2.3.4 Non-catalytic Transesterification

A newly developed process for biodiesel production by non-catalytic transesterification of raw oils with MeOH that allows simple process and high yield had been reported. In a proposed one-step method, FAME can be generated by esterification reaction of the free fatty acids with high yield and without saponified products. Additionally, the separation and purification are simple. However, this method requires extreme temperature and conditions of 350ºC and 43MPa, which will cause breakdown of unsaturated fatty acids. A two-step method with mild reaction at 270ºC and 7MPa which will not cause breakdown of unsaturated fatty acids or other changes was also reported. In the study of Saka and Isayama (2009), the highest FAME yield of 97% was derived after processing for 45 min at 350ºC and 20MPa using molar ratio of rapeseed oil to methyl acetate of 1:42.

2.3.5 Comparison of Methods

Among transesterification processes for biodiesel fuel production, alkaline-catalysed transesterification is the most widely used in industry. This is because of their high conversion of TG to ME with moderate reaction conditions and fast reaction rate. However, there are several drawbacks in the process such as large amount of alkali wastewater production for purification, difficult glycerol recovery and alkali catalyst separation process, time consuming, and soap formation. Although acid- catalysed process is effective in ME production, but its slow reaction rate, high temperature and usage of high amount of alcohol are the main obstacles for biodiesel production.

Enzyme-catalysed and non-catalytic transesterifications might become more well-liked for biodiesel production because of fewer drawbacks. The main hurdle for enzyme-catalysed is their high cost of enzyme catalysts while for non-catalysed transesterification is their extreme temperature and pressure condition. However, both the methods have simple separation and purification steps to obtain ME yield and are more environmental friendly methods.

2.4 Enzyme Immobilisation Method

There are some problems in the usage of enzymes such as high cost of isolation and purification of free enzymes, the instability of structures once isolated from their natural environments, and a trace of substances that can act as inhibitors. These problems can shorten the lifetime of enzymes. Like chemical catalyst, most enzymes can dissolve in water, contaminate the product and hard to recover in the active form from the reaction for reuse. Thus, immobilisation is one of the most successful methods to overcome the above limitation. Enzymes may be attached to cellular membranes; when dispersed on the surface of carrier particles, the systems stabilise the structure and activities of enzymes. Compared with free enzymes, immobilised enzymes are more robust and more resistant to environment changes. The immobilised enzyme has higher mechanical stability, rigidity, feasibility of regeneration and high loading capacity (Foresti & Forreira, 2007). In addition, it is non-toxic, and reusable to undergo continuous operation of enzymatic process as it is easy to recover both the enzymes and product (Krajewka, 2004).

There are several methods to immobilise the enzymes such as adsorption or precipitation onto hydrophobic materials, covalent attachment to functional groups, entrapment in polymer gels, and adsorption in macroporous anion exchange resins (Sharma, Chisti & Banerjee, 2001). Ting *et al.* (2006) found that catalytic activity of immobilised enzyme is much better than free lipase.

2.4.1 Cross-linking Method

Cross-linking is the most effective immobilization method where the enzyme is covalently bonded to a matrix through a chemical reaction. The active sites of enzymes are not affected. The enzyme forms a covalent link with active groups of support material either through the reactive groups on side chains of its amino acids such as lysine, arginine, histidine, tyrosine or with terminal amino or with terminal amino and carboxyl groups of the polypeptide chains. The reagents used for immobilising enzyme by cross-linking are diazobendidine, dinitrophenyl sulphone, diphenyl, disulphonic acid or glutaraldehyde. However, the inflexibility of the covalent bonds precludes the self-healing properties exhibited by chemoadsorbed self-assembled monolayers. The spacer molecules like poly (ethylene glycol) can help to reduce the steric hindrance by the substrate.

2.4.2 Adsorption Method

Adsorption is the slowest immobilization method without a chemical reaction because the enzyme is only attached to the outside of an inert material. The active site of the immobilised enzyme may be blocked by the matrix or bead which reduces the activity of the enzyme. The enzymes may be immobilised by adsorption to several types of materials where the adsorbents should be chosen such that enzymes are bound firmly with minimum inactivation.

2.4.3 Entrapment and Microencapsulation

In entrapment method, the enzymes are trapped in insoluble beads or microspheres such as calcium alginate. However, the insoluble substance hinders the arrival of the substrate and the exit of products. One of the methods to prepare the immobilised enzyme is through the process of phase separation. As both the phases of the solution are not miscible with one another the minor phase will form droplets within the major phase when mixed. The enzyme is entrapped within the droplets. Another method is by chemical polymerisation for the entrapment of enzyme.

Microencapsulation is the least developed immobilisation technique. The artificial cells enclosed by a membrane and large molecules such as enzymes are not able to pass through the synthetic membrane whereas small molecules such as the substrates and products can pass through it. By microencapsulation, enzyme does not chemically interact with the polymer and thus denaturation is avoided. The limiting parameters of using this technique are the mass transfer and diffusion problems. The entrapped enzymes are better used with small substrates since only small particles are able to pass through the membrane and reach the active sites (Villeneuve *et al.*, 2000).

2.5 Enzymes Reusability

There are two reasons for reduced lipase activity after several usages. First, the glycerol absorbed on the surface of immobilised lipase constrains the contact of substrate and enzyme molecules. The second reason is due to the toxicity of MeOH to enzyme. In order to solve the problem, Chen *et al*. (2009) suggested the use of solvent such as acetone to remove the glycerol and by modifying the enzyme with chemical or biological measures or adopting a novel technology of enzymatic immobilisation.

In the study of Chen and Wu (2003), washing with 2-butanol or *tert*-butanol was used to regenerate the deactivated immobilised lipase. Alternatively, Foresti and Forreira (2007) used ethanol to wash the immobilised lipase in order to remove any substrate or product retained in the matrix. Then the washed immobilised enzymes were dried at 50ºC for 1 h and introduced into fresh medium. After five uses at 45ºC, the lipase *Candida Antarctica* immobilised in chitosan powder accounted for more than 90% of its activity.

In the research of Gao, Tan, Nie, and Wang (2006), enzyme activity of *Candida sp*. 99-125 was maintained at 85.1% after 19 times of reuse compared with the first use. The immobilised lipase was filtered and consecutively reused after each reaction. Activity retention of 10.5% after seven reuses (Knezevic *et al.*, 1998), and 74% after ten reuses (Hung, Giridhar, Chiou and Wu, 2003) were

reported in the literature. In comparison, the lipase activity by binary immobilised lipase was reported to be higher.

2.6 Factors Affecting Biodiesel Conversion

2.6.1 Solvent Selection

The role of organic solvent in transesterification reaction is to increase the reaction rate through improved mutual solubility of hydrophobic TG and hydrophilic alcohols. Appropriate organic solvents are used to dissolve MeOH in oil. The optimisation of organic solvent ensures good solubility of substrates and maintains enzymatic activity. Organic solvents protect enzymes from denaturation caused by high concentrations of alcohols. There are three types of organic solvents which are hydrophilic, hydrophobic and ionic liquids. The yield of biodiesel synthesis in numerous organic solvents is dependent on lipase preparation and composition of fats. The solvents usually used in the system are *n*-hexane (Chen *et al.*, 2009), *tert*-butanol (Royon *et al.*, 2007), acetone (Halim & Kamaruddin, 2008), 1,4-dioxane, benzene, chloroform, or tetrahydrofuran (Iso *et al.*, 2001), *n*-heptane, petroleum ether as well as cyclohexane (Antczak *et al.*, 2009).

In the study of lipase-catalysed transesterification, He *et al.* (2008), compared among five commercial lipases with whole cell *Rhizopus chinensis* in

solvent-free system and reaction with the mixture of soybean oil and MeOH. The ME yields obtained for Novozyme 435, *Pseudomonas cepacia*, *Pseudomonas cepacia* immobilised on ceramic, *Candida rugosa*, porcine pancreas and *Rhizopus chinensis* are 30, 27, 27.4, 5, 24.1 and 28.4% respectively.

In the study of Nie *et al.* (2006), high ester yield was observed at 96% in *n*-hexane solvent and 94% in *n*-heptane and cyclohexane. On the other hand, polar solvents acetone gave ester yield of 40% only. This result shows the suitability of non-polar solvent in biodiesel conversion. A solvent system with the mixture of WCO, MeOH, solvent hexane and water catalysed with lipase *Candida sp*. 99-125 was pumped into the reactor. With increasing solvent, it was noted that the biodiesel yield increased from 44 to 73%. The solvent increased the substrate molecule contact with the lipase which improved the mass transfer between substrate and products. As the concentration of short-chain alcohols was decreased by the solvents, the toxic effect of alcohol was reduced on immobilised lipase. However, as the hexane content was increased to 20%, the FAME concentration decreased because the opportunity for reaction between substrate and lipase molecules was reduced (Chen *et al*., 2009).

Tert-butanol is the most popular solvent chosen as co-solvent in the transesterification reaction because of its non-toxic characteristics and relative low cost. Lipase catalysed transesterification study by Halim and Kamaruddin (2008) in organic solvent *tert*-butanol showed the highest FAME yield at 61%

with lipase Novozyme 435, Lipozyme TLIM and Lipozyme RMIM. The positive effect might be due to the ability of *tert*-butanol to dissolve both MeOH and glycerol. On the other hand, Minal (2005) showed that in the presence of *tert*-butanol resulted in the ME yield of 84% with mixture of lipase Lipozyme TLIM and Novozyme 435. Furthermore, under the conditions of 28% (v/v) *tert*-butanol, 6:1 MeOH to oil molar ratio, cotton seed oil and Novozyme 435, an oil conversion of 90% was observed after 10 h reaction at 50ºC (Royon *et al.*, 2007).

In *tert*-butanol system which is a moderate polar solvent, it was demonstrated that it could eliminate the negative effect of MeOH and glycerol by-product on enzymatic transesterification for biodiesel production (Orrego *et al.*, 2010). Glycerol is hydrophilic and insoluble in oil, so it is easily adsorbed onto the surface of immobilised lipase and decreases its operational stability (Dizge & Keskinler, 2008). As both MeOH and by-product glycerol are soluble in *tert*-butanol solvent, so no glycerol is adsorbed onto the surface of lipases. Thus, with the aid of *tert*-butanol, lipase stability may be enhanced (Halim *et al.*, 2009). Moreover, *tert*-butanol will not denature the enzymes and is an efficient medium to regenerate enzyme (Chen & Wu, 2003; Royon *et al.*, 2007).

2.6.2 Selection of Alcohol and Molar Ratio of Substrates

Alcohols that are used for biodiesel production comprised of MeOH, ethanol, propanol, isopropanol, 2-propanol, and isobutanol. Alcohols with high molecular mass have higher density and boiling temperature. MeOH and ethanol are popular and mainly used for biodiesel production due to lower cost but they are stronger denaturing agents than longer aliphatic alcohol and could inactivate enzymes. Furthermore, the rate of lipase-catalysed reaction increases with the length of hydrocarbon chain of alcohol. Antczak *et al.* (2009) found that the selection of alcohol for enzymatic biodiesel synthesis correlated with the properties of enzymatic preparation such as types of enzymes used, reaction system (with or without organic solvent), and time course.

In order to shift the transesterification reaction forwards, either excess amount of alcohol or removal of one or more of the products from the reaction mixture may be used. A molar ratio of 6:1 is commonly used in industrial process to obtain higher ME yield than 98% by weight (Srivastava & Prasad, 2000). Shimada *et al.*, (2002) found that immobilised *Candida Antarctica* lipase was inactivated in mixture containing more than 1.5 molar equivalents of MeOH in oil in solvent free system. MeOH was completely consumed in methanolysis of vegetable oil with less than 1/3 molar equivalent of MeOH for the stoichiometric amount using immobilised *C. Antarctica* lipase but reaction rate decreased significantly by adding greater than ½ molar equivalent of MeOH. Results

showed that immobilised lipase was irreversibly inactivated by contact with insoluble MeOH in the oil. Shimada *et al.* (2002) attempted methanolysis by three step additions of 1/3 molar equivalent of MeOH. After 48 h with addition of three 1/3 molar equivalent of MeOH, the conversion yields reached 97.3 and 90.4% for vegetable oil and waste oil respectively. Based on the result, the inhibitory effect of MeOH was large at the initial stage, but when oil conversion increased, inhibition decreased because solubility of MeOH in TG was low but high in FAME. Another inhibition due to glycerol attachment on the catalyst was low at the beginning and became larger at high oil conversions. Nie *et al.* (2006) found that the conversion remained constant when MeOH was added stepwise more than three times or continuously added. Thus, the best conversion ratio obtained was 95% after three steps addition where each step of 1/3 molar equivalent of MeOH was added for every 10 h.

On the other hand, Halim and Kamaruddin (2008) established that FAME yield increased with increasing MeOH to oil molar ratio viz. 58% (3:1), 72% (4:1), 73% (6:1) and 75% (8:1). The 4:1 ratio was considered as optimum since the FAME yield did not show significant increase beyond that. On the other hand, in solvent free system, immobilised enzyme *Candida sp* denatured when MeOH to oil molar ratio was at 1:1.

Maceiras *et al.* (2009) found that increase of molar ratio from 1:1 to 25:1 increased 40% of methyl esters yield and the optimum molar ratio of MeOH to

waste frying oil was 25:1 with 89.1% of ME yield by enzymatic catalysed transesterification using Novozyme 435. However, Martin and Otero (2008) found that different types of lipase needed different molar ratio of MeOH. The conversion using Novozyme 435 increased from 31% (1:1), 83% (2:1) to 86% (6:1) while for Lipozyme TLIM, conversion varied at 75% (1:1), 81% (2:1) and 64% (6:1). The results showed that non-specific lipase Novozyme 435 is more robust in the presence of alcohol and produces quantitative conversions of oil to biodiesel. It is necessary for sequential addition of molar equivalent of alcohol in Lipozyme TLIM which is known to be sn-1,3-specific due to the deactivation by low molecular weight alcohol.

On the other hand, Dizge and Keskinler (2008) reported that for MeOH to oil ratio of higher than ten and lower than five, MeOH concentrations would decrease the ME yield. Although increase in the number of moles of MeOH will increase the ester production, an opposite result set in once a maximum level was reached due to enzyme inactivation. Based on the result in Royon *et al.* (2007), best yields were obtained at 3.6:1 molar ratio of MeOH: oil in the presence of *tert*-butanol.

2.6.3 Selection of Lipase

From past studies, only a few lipases had been found to be capable of efficient biodiesel conversion and some of them were able to work in both organic solvent and solvent-free systems. Lipases have been divided into three types in terms of regioselectivity which are sn-1,3-specific, sn-2-specific and nonspecific. Lipases characterised by the narrow regiospecificity are not applicable to biodiesel conversion and the majority of lipases used showed both wide substrate specificity and regiospecificity.

In the study of Nie *et al.* (2006), lipase *Candida sp. 99-125* was immobilised by adsorbing onto a textile membrane. The batch methanolysis was carried out with a mixture of salad oil, n-hexane, water, lipase and MeOH. The mixture was incubated on an orbital shaker at 40ºC and 180rpm where the ME yields was 96% under optimal conditions. Besides, continuous methanolysis was conducted using columns packed with immobilised enzyme with the reaction mixture of petroleum ether solvent, water, salad oil and MeOH and 93% ME yield was obtained. In addition, free and immobilised *Candida. Antarctica* lipase solutions were prepared by adding lipase to distilled water. When immobilised lipases were used, they were soaked in water for 30 min to activate the enzyme. Then, the mixture of lipase, different volumes of MeOH, and simulated palm oil were added to the reactor at 45ºC (Al-Zuhair, Javaraman, Krishnan & Chan, 2009). The experiment that took place in *n*-hexane solvent system gave ME yield

of 5.9% while in solvent-free system, equilibrium yield was about 40%. The results showed that the solvent diluted and posed additional mass transfer resistance. Chen and Wu (2003) used *Candida Antarctica* to undergo transesterification reaction with soybean oil and MeOH in an incubator for 30 min at 30ºC with shaking at 200rpm. On the other hand, Halim *et al.* (2009) carried out the transesterification reaction in a continuous packed bed reactor. The mixture comprised of waste cooking palm oil, MeOH, Novozyme 435 and *tert*-butanol as solvent was fed upwards through the column using a peristaltic pump at 40ºC. The optimum conditions of 10.53cm packed bed height and 0.57ml/min flow rate gave 79.1% FAME yield.

In the work of Dizge and Keskinler (2008), the lipase *T. Lanuginosus* was immobilised in polyurethane foams using polyglutaraldehyde as cross-linking agent. *T. Lanugonosus* lipase was mixed with calcium acetate buffer containing polyethylene glycol, surfactant Span 80, isocyanate, and polyglutaraldehyse solution. The prepared immobilised *T. Lanuginosus* was used for transesterification process in refined canola oil. The reaction mixture of canola oil, lipase, MeOH to oil molar ratio at 6:1, and water at 40ºC, 180rpm and for 24 h achieved 90% ME yield. Opimisation of lipase *Thermomyces* by using Taguchi methodology was reported by Dizge *et al.* (2008). The immobilisation was carried out by circulating enzyme solution in calcium acetate buffer at 26ºC for 25 h with gentle shaking at 250rpm. The reaction between the immobilised lipase, oil and MeOH was fixed at 65ºC for 24 h. MeOH was added to the mixture in three steps

to avoid strong MeOH inhibition. After the reaction, the immobilised enzyme was removed from the products by filtration and washed with *tert*-butanol. The prepared biocatalyst was characterised by Scanning Electron Microscope (SEM). Biodiesel yield from powdered immobilised enzyme was 97%.

A binary immobilisation method was studied by Ting *et al.* (2006) where soybean oil was used as feedstock and lipase *Candida Rugosa* as catalyst. Hydrolysis of soybean oil with the immobilised lipase was carried out in a flask at constant agitation for formation of emulsion of oil and water. Later, Ting *et al.* (2008) directly used the oil phase that contained the fatty acids as the feedstock for acid catalysed transesterification at 65ºC and 99% conversion of biodiesel was obtained after 12 h. Another immobilisation of lipase *Candida rugosa* in support chitosan by cross-linking method with glutaraldehyde was done by Shao, Meng, He and Sun (2008) and methyl conversion of 63.6% was obtained at MeOH molar ratio of 4:1 at 45ºC. In the study of Hung *et al.* (2003), binary immobilisation method was used to prepare immobilised *Candida rugosa*. Three different methods were investigated and the most efficient method was found to be the lipase first immobilised to hydroxyl groups of chitosan followed by immobilisation with amino groups by cross-linking with glutaraldehyde.

In the study of Foresti and Forreira (2007), lipases from *Candida rugosa, Pseudomonas fluorescens* and *Candida antarctica B* were immobilised onto chitosan and glutaraldehyde-pretreated chitosan powders. First, the

glutaraldehyde-pretreated chitosans were prepared by suspending the chitosan powder in glutaraldehyde/phosphate buffer solution under stirring at room temperature for 30 min. Then, the supports were filtered and washed with distilled water and stored at 50ºC for 12 h. Lipases were added to phosphate buffer solutions with strong stirring for 30 min to solubilise the lipase. Then, the lipase solution was contacted with chitosan powder and glutaraldehyde-pretreated chitosans for around 7 h. Finally, the immobilised catalysts were titrated, washed with distilled water and dried at 50ºC for 12 h. The prepared biocatalysts were then used in direct esterification of oleic acid and ethanol. 75% of fatty acid conversion was achieved after 7 h of reaction and characterisation of chitosan-immobilised catalysts was carried out using SEM.

Martin and Otero (2008) studied alcoholysis of four types of enzymes which were non-specific enzyme Novozyme 435, and sn-1(3)-regiospecific lipase Lipozyme TLIM, Lipozyme RMIM and Lipase *Pseudomonas S.* in stopper batch reactors at different temperatures ranging 25 to 60ºC at 200rpm. The reaction mixtures comprised of oil, varying quantities of ethanol or MeOH, hexadecane as internal standard and different amount of enzymes. The reaction achieved 80% conversion in 7 h at different conditions. These 3 enzymes were also tested in the work of Halim and Kamaruddin (2008) at 40ºC, 160rpm and oil to MeOH ratio of 1:3. There are also studies using mixtures of enzymes for transesterification. Wang, Du, Liu, Li and Dai (2006) used 3% of TLIM and 2% of Novozyme 435 where biodiesel yield achieved 97%.

2.6.4 Lipase Amount

Ordinarily, FAME conversion increases in proportion with an increase of lipase concentration within a certain range but remains constant at higher concentration. In the research of He *et al.* (2008), highest ME yield was obtained only with 8% of lipase based on oil weight in the reactions. Low amount of lipase was chosen due to the cost. The determination of the effect of enzyme content in the range of 5 to 35% on the transesterification reaction at 40ºC was carried out by Chen *et al.* (2009) using WCO. FAME content increased initially when increasing the content of enzyme due to more substrate molecules were able to adsorb onto the active site of lipase. However, the increment in FAME content declined as enzyme content rose from 25 to 35% due to excessive enzyme.

On the other hand, highest FAME yield was found with 10% dosage of enzyme by Maceiras *et al.* (2009). Besides, 3% (w/w) of enzyme was sufficient to achieve highest FAME yield in the research of Ognjanovic *et al.* (2008). Halim and Kamaruddin (2008) reported that there was not much effect on FAME yield when lipase quantity increased further from 10 to 15%.

2.6.5 Reaction Time

The time for deactivation or depletion of substrate depends on the types of enzymes and other reaction conditions. Royon *et al.* (2007) reported oil conversion of more than 90% when using *C. Antarctica* lipase in enzymatic methanolysis of vegetable oils after 7 h, but they used 30% of catalyst while Shimada *et al*. (2002) spent 34 h with 4% (w/w) immobilised lipase and 96.8% of oil conversion was attained. Ognjanovic, Saponjic, Bezbradica and Knezivic (2008) found the yields of ME were constant after 24 h with *C. Antarctica*, indicating that optimum reaction time was reached and biodiesel yield of 99.6% obtained. Royon *et al.* (2007) found that 100% conversion was reached by using 1.7% (w/w) of Novozyme 435 catalyst at 50ºC in 24 h with the aid of *tert*-butanol as solvent. Du, Xu, Liu, and Zheng (2004) obtained a yield of 92% of ME in 12 h with large amount of enzyme Novozyme 435 which was 30% based on oil weight. However, Maceiras *et al.* (2009) who also used Novozyme 435 only required 4 h to achieve constant ME yield involving different MeOH to oil molar ratio, enzyme dosage of 10% based on oil weight and at 50ºC. For Yagiz *et al.* (2007), the immobilisation of enzyme *T. Lanuginosus* on hydrotalcite reached steady state after 5 h. Dizge *et al.* (2008) studied using commercial soluble lipase of *T. Lanuginosus* in reaction time of 5 and 24 h. They concluded that the biodiesel yields were 86.9% at 5 h and 97.0% after 24 h reaction.

2.6.6 Effect of Oil Purity

Investigation by Felizardo *et al.* (2006) showed that biodiesel produced from WCO with lower acid value had a better purity than higher acidic WCO by as much as 14%. Besides, in Dizge *et al.* (2008) three different kinds of oils used for investigating the effectiveness of the transesterification reaction were sunflower oil, soybean oil and WCO. The yields obtained for sunflower, soybean and WCO were 63.8, 55.5 and 50.9% respectively for 5 h at 65ºC with excess MeOH.

The three-step batch methanolysis reaction in the work of Shimada *et al.* (2002) showed that 90.4% conversion was obtained from WCO with immobilised *C. Antarctica* while 95.9% conversion was obtained from vegetable oil. The difference may be attributed to the presence of oxidised fatty acid compounds in waste oil. When vegetable oil is used for frying, some of the fatty acids are converted to epoxides, aldehydes, polymers and others oxidised compound by oxidation or thermal polymerisation. Hence, the percentage of conversion from WCO is lower because lipase did not recognise the oxidised compounds.

For FAME analysis, Maceiras *et al.* (2009) found that through transesterification process of WCO and Novozyme 435, the product mainly contained oleic acid (58.7%) followed by linoleic acid (26.6%), palmitic acid (9.5%), and stearic acid (4.6%). Approximately 60% of the fatty acids were found

to be monounsaturated (C18:1) while 27% were poly-unsaturated fatty acids (C18:2, C18:3). Besides, there were roughly 11% of saturated fatty acids which are palmitic acid and stearic acid. 2% of the products were unidentified. On the other hand, Chen *et al.* (2009) established that the FAME components in WCO with *Candida sp.* were mainly methyl palmitate, methyl stearate, methyl linoleate and methyl oleate.

2.6.7 Reaction Temperature

Reaction temperature is one of the important parameters in enzymatic transesterification. High temperature can produce higher formation but may lead to denaturation of enzymes. On the other hand, long reaction time is needed at low temperature. Optimum temperatures for transesterification reactions catalysed by various lipases in solvent free system are in the range of 30 to 50ºC while in solvent system are in the range of 30 to 55ºC.

Martin and Otero (2008) found that optimum temperature for Lipozyme TLIM and Lipozyme RMIM was 25ºC or less while higher conversions at increased temperature were obtained for Novozyme 435. Investigation of Nie *et al.* (2006) showed that at the temperature range from 27 to 50ºC, highest yield was observed at 40ºC with immobilised lipase *Candida sp.* in synthesis. For lower temperature, better result was obtained if the reaction time was extended to 60 h

while for temperature above 40ºC, a decrease in conversion ratio was observed. Yagiz *et al.* (2007) found that immobilised lipase *Thermomyces Lanuginosus* on hydrotalcite could be used up to two times at 45ºC with little loss of activity and enzyme activity retained 36% after seven cycles. However, for the reaction at 55ºC, the enzyme lost its activity after one usage and only 14% of enzyme activity remained at the end of the seventh cycle. He *et al.* (2008) suggested that for energy saving, 30 to 40ºC is the most suitable temperature range which also can avoid lipase denaturation. Thus, they had chosen 30ºC as the reaction temperature and 86.7% of ME yield was obtained in n-heptane.

In the studies of Ting *et al.* (2006), there was no significant loss of activity of immobilised lipase from 25 to 40ºC. Their results showed that thermal stability of binary immobilised lipase decreased at higher temperature while free lipase was stable only up to 35ºC and beyond this temperature, catalytic activity decreased. In the report of Dizge and Keskinler (2008), 40ºC was found to be the optimal temperature for biodiesel production with the *T. Lanuginosus*. Above 40ºC, ester production decreased while FFA increase was observed. Foresti and Forreira (2007) observed similar relationship between transesterification rate and temperature. Reaction rate was slow at low reaction temperature below 35ºC while tests at 65ºC revealed incipient denaturation effects. At high temperature of 75ºC, lipase unfolding and biocatalyst denatured. The catalyst developed highest activity at 45ºC and showed drastic reduction in its catalytic activity at 65ºC. Chen *et al.* (2009) also found the lipase activity increased sharply when

temperature was enhanced from 35 to 40ºC under the most suitable reaction time. When temperature was increased from 40 to 50ºC, lipase activity started to increase gradually. Temperature higher than 60ºC will volatilise the MeOH and solvent. Besides, high temperature can affect the thermal stability of lipase and usage life. Gao *et al.* (2006) showed that optimum reaction temperature of immobilised lipase was 40ºC.

2.7 Response Surface Methodology (RSM) Model Fitting

A five-level-two-factor centre composite rotatable design CCRD was employed in the study of Halim *et al.* (2009). The two independent factors chosen for the optimisation of transesterification of WCO were packed bed height and substrate flow rate. FAME yield was taken as the response of the design experiments and its relationship with the two independent variables was studied. The results obtained were analysed by ANOVA to assess the goodness of fit. The computed F value was higher than theoretical F value indicating the regression model is reliable in predicting the FAME yield. The non-significant value of lack of fit "Prob > F" more than 0.05 showed that the quadratic model was valid for the study. The optimum conditions of packed bed height and substrate flow rate were 10.53cm and 0.57ml/min respectively and 79.1% FAME yield was determined.

On the other hand, a RSM analysis of immobilised *Candida Rugosa* lipase on chitosan catalysed preparation of biodiesel from rapeseed soapstock with MeOH was carried out in the research of Shao *et al.* (2008). The four parameters in the study were MeOH and oil ratio, enzyme amount, water content and reaction temperature. The coefficient of determination was 92.86% while the probability value (Prob > F less than 0.05) demonstrated very high significance regression model was obtained. The optimum conditions for biodiesel production with 63.6% ME yield were MeOH to oil molar ratio of 4:1, enzyme amount of 8%, water content of 6% and reaction temperature at 45ºC.

CHAPTER 3.0

MATERIALS AND METHODOLOGY

3.1 Raw Materials

FCO was obtained from refined, bleached and deodourised palm oil; WCO was obtained from local restaurants; Chitosan; Sodium Hydroxide (NaOH), Bradford Reagent, n-heptane, 0.1 N Sodium thiosulphate, glutaraldehyde (GTA), and Toluene from R&M Marketing; 99.8% Acetic Acid from Bendosen; Ethanol (EtOH), Methanol (MeOH), Potassium Hydroxide (KI), Wij's Reagent and 37% Hydrochloric acid from Merck; 1-ethyl-3-(3-dimethylaminopropyl) Carboimide Hydrochloride (EDC) were purchased from Fluka; *Thermomyces Lanuginosa* (Lipozyme TL 100L), Immobililsed *Thermomyces Lanuginosa* (Lipozyme TLIM), *Tert*-Butanol, Potassium Iodide, and Cyclohexane were obtained from QRec; Deionised water (DI), Bovine Serum Albumin (BSA) from Bio Ins; Potassium Hydrogen Phthalate (KHP) and Isopropanol were obtained from Systerm; FAME mix C_{14}-C_{22}, ASTM D6584 Internal Standard Solution Standard Kit, Methyl Heptadecanoate, and N-methyl-N-(trimetylsilyl)trifluoro-acetamide were supplied from Supelco.

3.2 Preparation of Chitosan Beads

A 3% chitosan solution was prepared by adding 3g of chitosan powder into 97g of 1% acetic acid solution. Then, the chitosan solution was added drop-wise into 1N NaOH in 26% (v/v) of EtOH to produce spherical beads with diameter range of 1-2mm under stirring. After the solution was stirred continuously overnight, spherical beads were filtered out and washed with DI water until neutrality. Finally, the beads were stored in DI water at 4°C until use. Figure 3.1 summarises the sequence in the preparation process (Ting *et al.*, 2006).

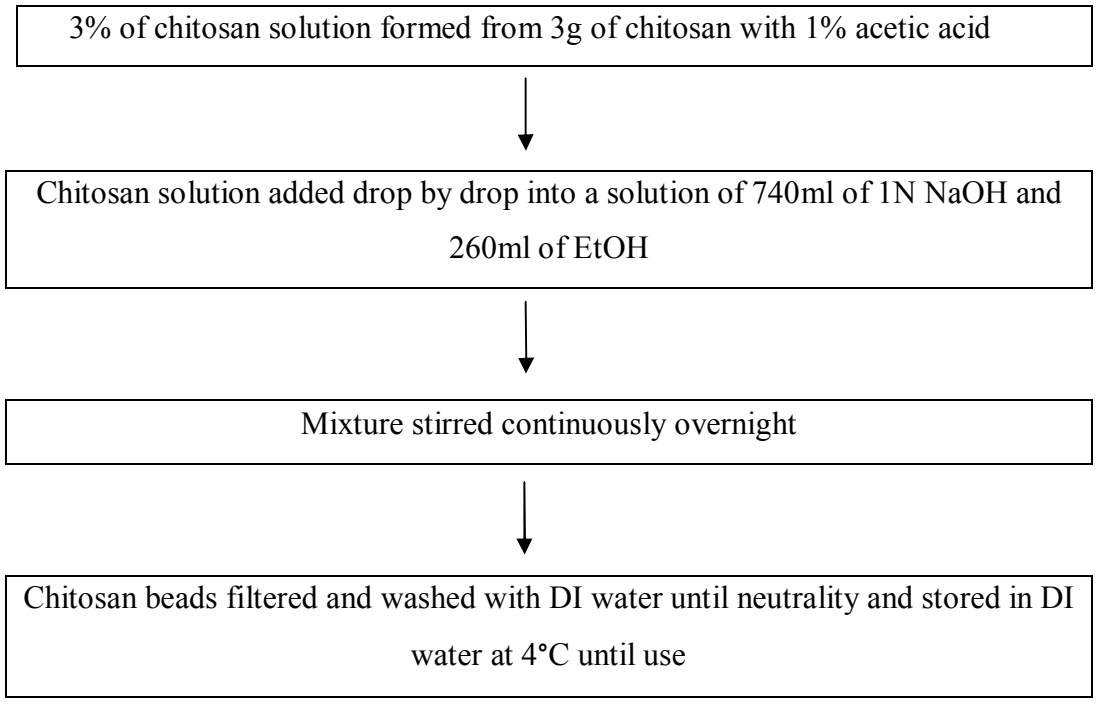

Figure 3.1: Flow chart for chitosan beads preparation.

3.3 Immobilisation of Enzyme

Immobilisation of lipase to chitosan beads was carried out by the binary methods modified by Ting *et al.* (2006). 1g of chitosan beads was mixed with 3ml of 0.25% (w/v) EDC for the activation of its hydroxyl groups. After reacting for 15 min, the beads were filtered and transferred to 3ml of 0.5% (w/v) lipase solution. The supernatant was decanted after 1 h and the beads were added into 3ml of 0.005% (v/v) GTA solution to mix for 40 min. Then the supernatant was removed and 3ml of 0.5% (w/v) lipase in DI water was added to the beads and allowed to react for 45 min. The beads were then washed thrice in DI water. Finally, the beads were resuspended in DI water and stored at 4°C until use. Figure 3.2 summarises the sequence in the immobilisation process.

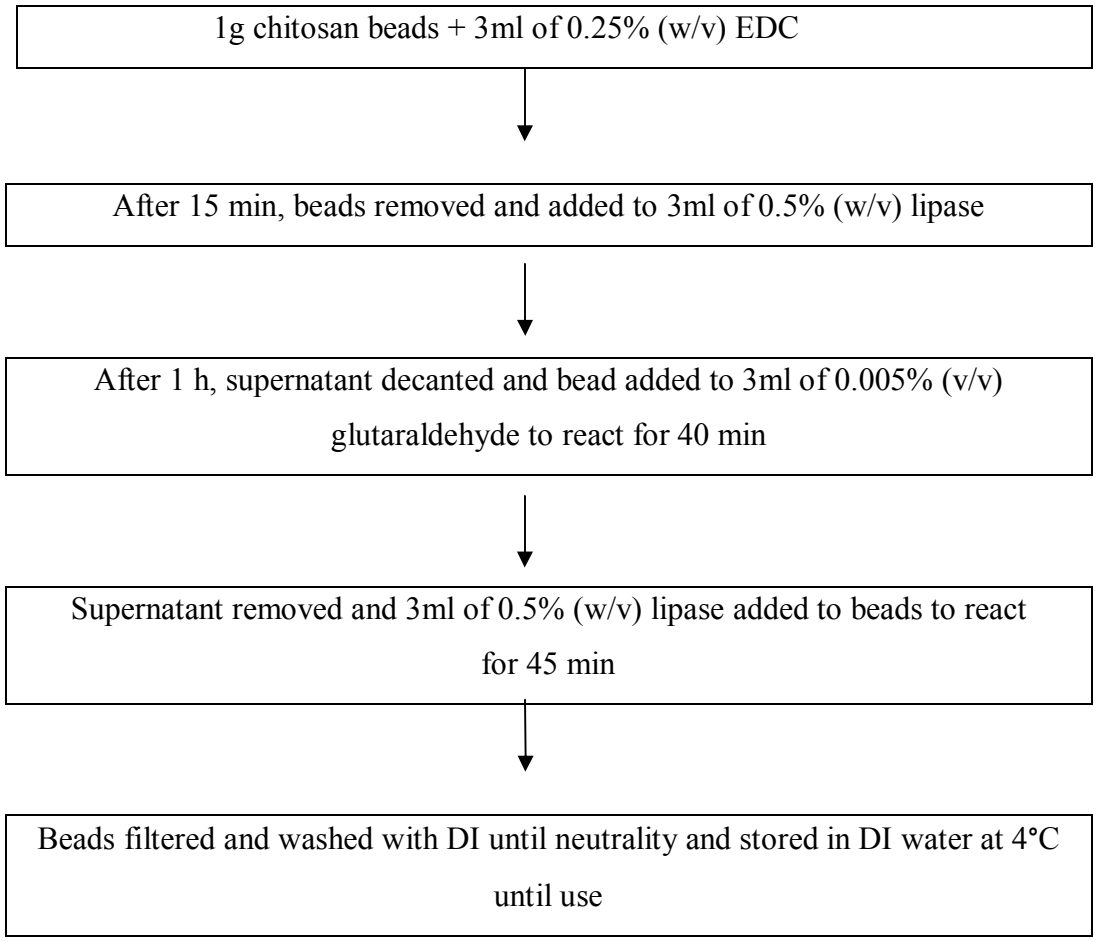

Figure 3.2: Flow chart for immobilisation of enzyme.

3.3.1 Protein Assay

The total protein taken for immobilisation and protein present in the supernatant after immobilisation were measured by Bradford Assay using BSA as standard. The amount of protein bound on the support was found by subtracting the unbound protein from total protein.

The sample was taken from the supernatant of the lipase solution and 100µl was added into a 1.5ml centrifuge tube. Then, 1ml of Bradford Reagent was added into the tube and vortex. The analyses were read at absorbance range of 500-700nm by using a UV-Vis spectrophotometer.

3.3.2 Topography of Beads

A scanning electron microscope (SEM) is a microscope that uses electrons instead of light to form an image. It can be used to get information about a sample surface topography. The images of the sample surface are scanned with a high energy beam of electrons in a raster scan pattern. Magnification in a SEM can be controlled over a range of up to 6 orders of magnitude from about 10 to 500,000 times. The beads were examined before and after reaction using this equipment.

3.3.3 Estimated Cost of Beads Preparation

Table 3.1 shows the breakdown of materials cost to prepare immobilised TL on chitosan beads. The unit cost is estimated to be RM0.432 per gram while the commercial Lipozyme TLIM is RM 0.44 per gram. Although both cost almost the same, environmental issue may be another concern. Commercial Lipozyme TLIM is immobilised in particulate mesoporous silica carriers which are harmful

to human health. On the other hand, the self prepared immobilised TL is immobilised in the waste product of the seafood processing industry - chitosan. The present preparation method not only results in cost reduction but is also environment friendly.

Table 3.1: Cost breakdown for preparing 1g of chitosan beads immobilised in TL

Substances	Consumed	Cost (RM)	Unit Price (RM)
Chitosan powder	3g	(1.44 x 3)/50g	0.086
0.25% EDC	3ml	(10.875/100) x 3ml	0.33
0.5% lipase	6ml	(0.25/100) x 6ml	0.015
0.005% Glutaraldehyde	5µl	0.001	0.001
		TOTAL	0.432 per gram

3.4 Transesterification Reaction Methodology

In this study, two types of oil chosen as the feedstock of biodiesel conversion were FCO and WCO. The latter was collected from local restaurants and filtered to remove any food residue. Besides, two types of immobilised enzymes were used as the catalyst of transesterification reaction namely commercial TLIM and the self-prepared immobilised TL binding on chitosan beads as outlined in the previous sections.

Transesterification reactions from FCO and WCO as feedstock using TLIM and self-prepared immobilised TL as catalyst were compared. Reactions were carried out at different temperatures from 30 to 50ºC, molar ratio of MeOH to oil from 1 to 6, and the amount of enzymes used was from 0.5 to 1.5% based on the weight of oil. *Tert*-butanol was also added as solvent in a 1:1 volume ratio to oil.

The enzymatic transesterificatiton was carried out in a 100ml conical flask in an incubator shaker running at 200rpm for 24 h. The mixture comprised of the desired amount of oil, MeOH, solvent *tert*-butanol, and enzymes. The total amount of MeOH was divided into three equal portions and added step-wise until the experimental molar ratio was reached. Table 3.2 shows 20 experimental runs on WCO and FCO based on design of experiment using central composite design with one replication. After the transesterification reaction, the used immobilised enzymes were decanted and reused with fresh medium. The response of RSM was the FAME content obtained at each combination of different parameters.

Table 3.2: Experimental design for transesterification study

Run	MeOH Ratio	Temperature (°C)	Enzyme (%)
1	2.01	34.05	0.70
2	3.50	30.00	1.00
3	3.50	40.00	1.00
4	3.50	40.00	1.00
5	2.01	45.95	1.30
6	3.50	50.00	1.00
7	4.99	45.95	0.70
8	4.99	34.05	0.70
9	2.01	45.95	0.70
10	3.50	40.00	1.00
11	6.00	40.00	1.00
12	3.50	40.00	1.00
13	1.00	40.00	1.00
14	4.99	34.05	1.30
15	3.50	40.00	1.50
16	3.50	40.00	1.00
17	2.01	34.05	1.30
18	3.50	40.00	0.50
19	3.50	40.00	1.00
20	4.99	45.95	1.30

3.5 Methods of Characterising Feedstock Oils and Products

3.5.1 Acid Value (AV)

An acid value test is used to determine the acidic constituents in petroleum products and lubricants that are soluble in mixtures of toluene and isopropanol (IPA). This is a standard testing method based on American Society for Testing and Materials (ASTM) D-664 and was used to test the oil samples before reaction and the products after reaction.

Based on this method, equal volumes of IPA and toluene were mixed in a conical flask as titration solvent. A few drops of phenolphthalein were added into the flask. The titration solvent was neutralised with 0.1N KOH in IPA. The mixture was poured into another conical flask that contained oil sample. Finally, titrated with 0.1N KOH in IPA until pink end point and the volume of 0.1N KOH used was recorded. Same procedures as to prepare the standard mixture but KHP was first dried in an oven at 110ºC for 2 h and then cooled down to room temperature in a dessicator. After that, 0.4085g KHP was weighed and added to a conical flask.

The following formulae are used in the standard calculation.

$$\text{AV} = \frac{56.1 \times M \times F \times (A - B)}{W} \quad (3.1)$$

$$\text{F} = \frac{1000 W_{KHP}}{204.23 V_{eq}} \quad (3.2)$$

where

M is the molar concentration of KOH (moles/l)

F is the factor of 0.1N KOH

A is volume of KOH used to reach end point equivalent to basic buffer (ml)

B is the volume corresponding to A for blank titration (ml)

W is the mass of sample (g)

W_{KHP} is the amount in grams of KHP in 50ml of KHP standard solution

V_{eq} is the amount of titrant consumed at the equivalent point.

204.23 is the molecular weight of KHP

3.5.2 Iodine Value (IV)

Iodine value is the determination of the amount of unsaturated fatty acids. As they form double bonds with iodine compounds, the higher the iodine value, the more unsaturated fatty acid bonds are present in fats. The iodine value of fats and oils is based on the Wij's method.

For a simple analysis, 0.3 g of oil sample was mixed with 25ml of Wij's solution and 20ml iodine value solvent. Flasks were stored in the dark for an hour at 25 ± 5ºC. Upon removing the flasks from dark, 20ml of 10% KI solution and 150ml of DI water were added. The solution was then titrated against 0.1M of $Na_2S_2O_3$ until yellow colour. A few drops of starch indicator were added and the

solution turned blue. After that, the titration was continued with 0.1M of $Na_2S_2O_3$ until colourless. On the other hand, a blank solution was prepared following the above procedures but without the addition of sample. The sample must be titrated within 30 min of reaction termination.

Since $1cm^3$ of 0.1M $Na_2S_2O_3$ is equivalent to 0.01269g of iodine, the calculation of IV is carried out by the formula below.

$$\text{IV} = \frac{(B-S)\times M \times 12.69}{wt} \qquad (3.3)$$

where B is the titration of blank (ml), S is the titration of test sample (ml), M is the molarity of $Na_2S_2O_3$, wt is the weight of oil in grams.

3.5.3 Saponification Value (SP)

Saponification value is the number of milligrams KOH required to saponify 1g of the oil sample. 5g of the sample were added into a 500ml Erlenmeyer flask. After that, 50ml of 0.5 N KOH were added into the flask and the solution boiled for saponification. Finally, the solution was titrated with 0.5N HCl with the addition of phenolphthalein as indicator.

Calculation procedure is shown as below.

$$\text{SP} = \frac{56.1(B-S)\times N}{g} \qquad (3.4)$$

where B is the titration of blank (ml), S is the titration of test sample (ml), N is Normality of HCl and g is grams of sample

3.5.4 Composition of Products

Gas chromatography (GC) may be used for separating and analysing compounds that can be vaporised without decomposition. It represents the main spectrometer to determine the ME and MG, DG, TG and glycerol composition. The FAME contents in the reaction were quantified using a Perkin Elmer Clarus 500 GC equipped with flame ionisation detector (FID). Determination of FAME was based on European Standard EN14103. The column was ZB-FFAP with dimension of 60m length x 0.25mm ID x 0.25µm film thickness. Helium was used as the carrier gas at 1ml/min. The oven temperature was set at 200 to 260ºC at 2ºC/min. The injection was done in split ratio 15 while the injector and detector temperatures were set at 220 and 250ºC respectively.

Methyl heptadecanoate was used as internal standard. 100mg of standard mixture was diluted into 5 different concentrations of solutions to prepare the standard curve. Approximately 25mg of standard solution or oil sample were mixed with methyl heptadecanoate in a 1ml vial. Then, the mixture was topped up with n-heptane. The analysis was done by injecting 1µl sample into the column.

The methyl ester content C, expressed as a mass fraction in percent, is calculated following standard calculation procedure.

$$C = \frac{(\sum A) - A_{El}}{A_{El}} \times \frac{C_{El} \times V_{El}}{m} \times 100\% \qquad (3.5)$$

where

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

A_{El} is the peak area corresponding to methyl heptadecanoate;

C_{El} is the concentration, in mg per ml, of the methyl heptadecanoate solution being used;

V_{El} is the volume, in ml, of the methyl heptadecanoate solution being used;

m is the weight of the sample in mg.

The determination of free and total glycerol and mono-, di-, and triglyceride contents was based on British Standard (BS:EN, 14105:2003). The GC was equipped with an on-column injector and FID. The column used was ZB-5HT inferno with dimension of 15m length x 0.32mm ID x 0.1µm film thickness connected with a 2m length x 0.53mm ID guard column. The carrier gas was helium set at a pressure of 11.6psi. The column temperature was programmed at 50ºC for 1 min, up to 180ºC at 15ºC/min, at 7 ºC/min up to 230ºC, at 10ºC/min up to 370ºC and held for 5 min. The injection was on-column while the injector and detector were set at 370ºC.

Five different concentrations of standard mixture containing glycerol, monoolein, diolein and triolein were purchased from Sigma Aldrich. 100µl of the standard mixture was added into a 1ml vial. Then, 10µl of 1000µg/ml butanetriol (internal standard 1), 10µl of 800µg/ml tricaprin (internal standard 2), and 10µl of N-methyl-N-(trimethylsilyl)trifluoro-acetamide (MSTFA) were added into the vial. The sample mixture was shaken and stood at room temperature for 20 min. Then, 0.8ml of n-heptane was added to the vial and shaken. Finally, 1µl of sample was injected for analysis. For the oil determination, the standard mixture was replaced with oil sample and mixed with the internal standard for examination. The calculation of the mass of TG, DG, MG and ME in one gram of oil is based on the standard calibration graphs (APPENDIX A3).

CHAPTER 4.0

RESULTS AND DISCUSSION

4.1 Binary Immobilisation

The structure of chitosan is shown in Figure 4.1. Chitosan possesses amino and hydroxyl groups and during immobilisation the reactive hydroxyl groups of chitosan are cross-linked with the amino groups of the enzyme using multifunctional reagents. However, the amino groups are rarely utilised in the immobilisation of enzymes. Enhancement of the protein bound is increased if the amino groups are also utilised in the immobilisation through a binary method.

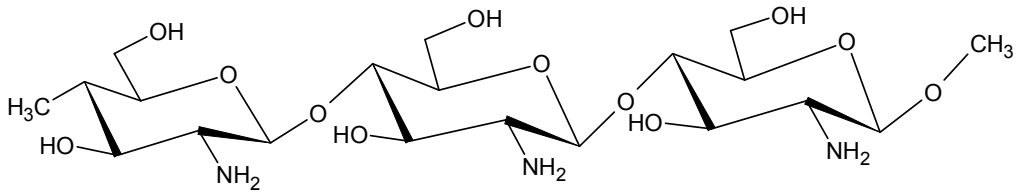

Figure 4.1: Structure of chitosan

Binary immobilisation method is an efficient method where almost all the lipases may be attached to the chitosan beads. In the study by Hung *et al.* (2003), lipase was immobilised to chitosan beads by first linking to the hydroxyl groups

of chitosan activated with EDC followed by cross-linking more lipase to the amino groups of chitosan using GTA (Hung *et al.*, 2003). A schematic illustration of the binding site of lipase to the chitosan support is shown in Figure 4.2.

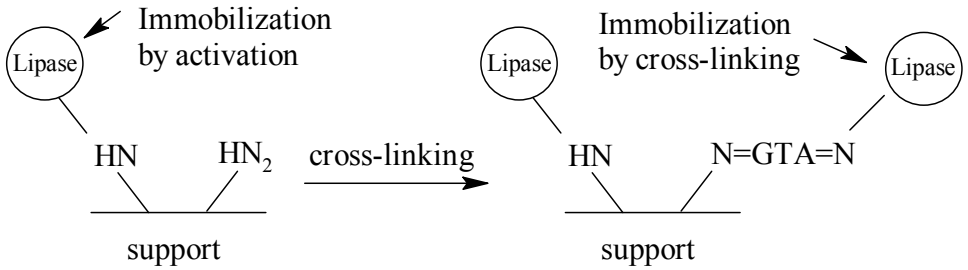

Figure 4.2: Schematic illustration of the binding immobilisation of lipase to the support (GTA: glutaraldehyde)

Ions are removed from the hydroxyl groups of chitosan and the resulting molecule reacts with EDC to form an unstable complex consisting of chitosan and acylisourea. Then, the alkyl group of chitosan which is formed by the release of acylisourea in the presence of the enzyme binds to the amino group of the enzyme to form the immobilised lipase. EDC and GTA are generally used as the protein cross-links. According to Hung *et al.* (2003), EDC which reacts preferentially and used for the activation of the hydroxyl groups of chitosan would make it polyanionic and when the enzyme is coupled with a polyanionic support, the optimum pH would shift in the alkaline direction. The hydroxyl groups of chitosan which are activated by EDC will react with the amine groups of lipase. On the other hand, more lipase molecules are cross-linked through its amino groups to chitosan by using GTA. During cross-linking, the aldehyde group of

GTA reacts with the amino group of chitosan or the enzyme to form imino group (–CH=N–) (Royon *et al.*, 2007). The binding yield is enhanced through both the application of cross-linkers.

4.1.1 Present Method of Immobilisation

The amount of immobilised enzyme on the beads was determined using Bradford method by measuring the initial and final concentrations of the protein supernatant after immobilisation. UV-Vis spectrophotometer was used for protein determination and getting a standard calibration curve of BSA. Protein loading of lipase was expressed in mass of protein on the chitosan beads. The absorbance of protein was carried out at the range of 500-700 nm wavelengths. Figure 4.3 shows the standard curve of protein assay by using BSA as standard. The R^2 coefficient value is 0.9943 which shows the excellent linear relationship between absorbance and concentration of BSA.

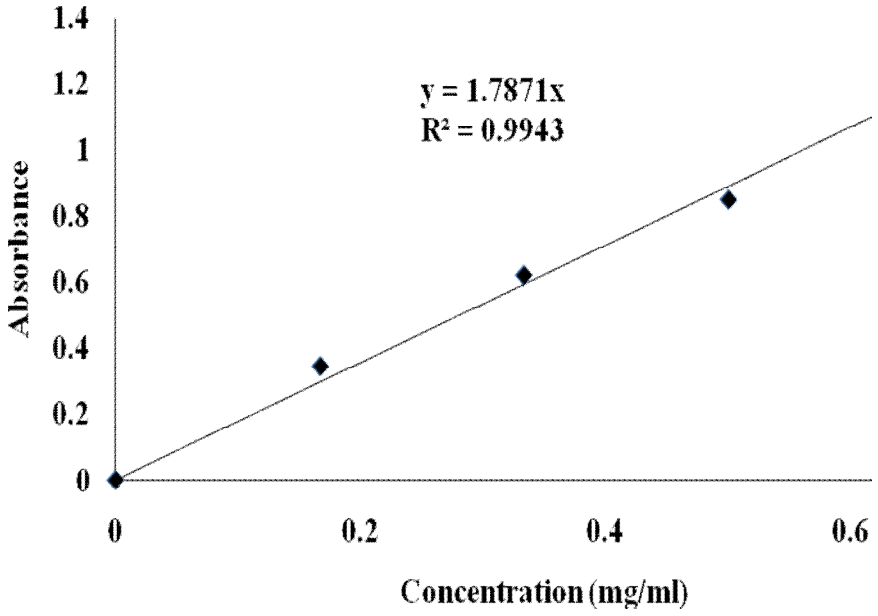

Figure 4.3: Protein Assay Standard curve from different concentrations of BSA

Two steps of 5mg/ml of lipase solution were immobilised on 1g of chitosan support. The first lipase solution used was for EDC activation while the second lipase solution was for glutaraldehyde (GTA) crosslinking. The supernatants of the solutions were collected to investigate the residue of lipase unattached to the support. Table 4.1 shows the amount of lipase left after the reaction with 1g of chitosan from 5mg/ml lipase solution. The absorbance of the first supernatant was 0.036; based on the equation in standard curve, the protein residue was 0.02mg/ml. This means that 4.98mg/ml of lipase concentration or 14.94mg of lipase had been attached in 1g of chitosan. Following that, the second supernatant showed that all the lipases were attached to the chitosan support. Thus,

in 1g of chitosan beads, there were 29.94mg/g of lipase attached on it. The total protein loading in 1g of chitosan is shown in Table 4.2.

Table 4.1: Lipase residue after reaction with 1g of chitosan from 5mg/ml lipase solution

Sample	Absorbance	Lipase residue (mg/ml)
Supernatant 1	0.036	0.02
Supernatant 2	0.000	0.00

Table 4.2: Lipase concentration and protein loading in 1g of chitosan

	Lipase Concentration (mg/ml)	Protein loading (mg/g-chitosan)
Activation of EDC	4.98	14.94
Crosslinking with GTA	5.00	15.00
Total	9.98	29.94

Figure 4.4 shows four SEM microphotographs of the chitosan powder, chitosan beads, chitosan immobilised lipase TL and chitosan immobilised lipase TL after transesterification reaction respectively. As can be seen in Figure 4.4(a) and (b), the surface of the chitosan bead was less grainy than the powder form but still appeared rough. After immobilisation, the surface became smoother due to lipases attached on the beads (Figure 4.4(c)). However, the surface of the chitosan beads after biodiesel reaction seemed to be degraded as shown in Figure 4.4(d).

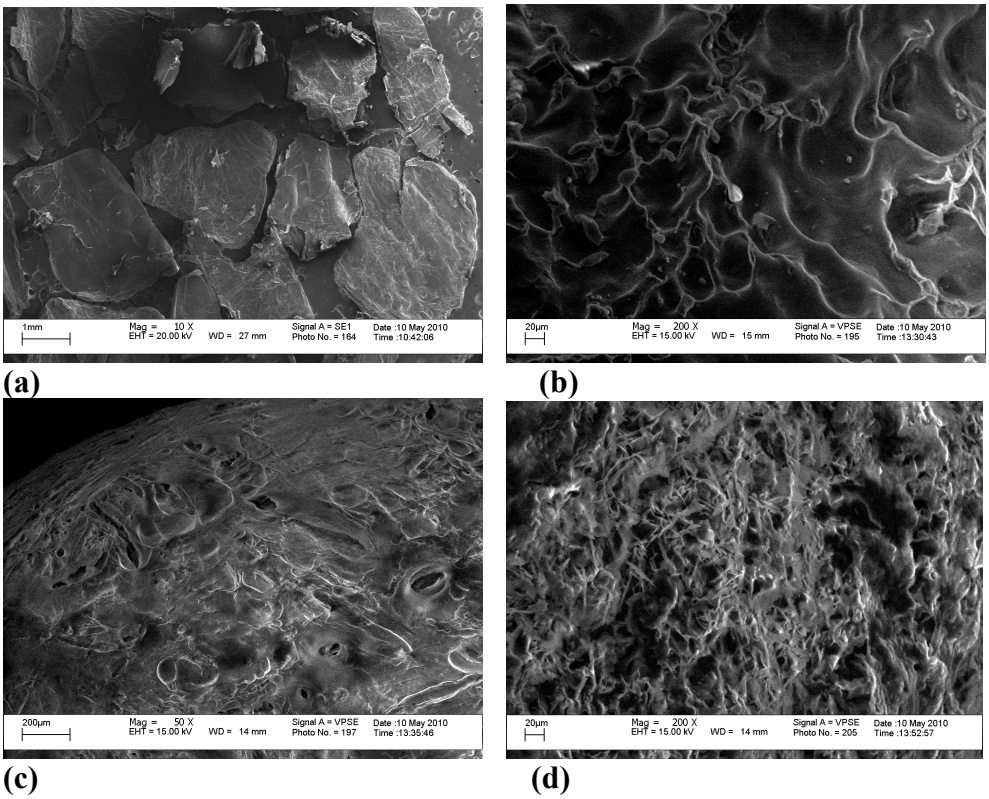

Figure 4.4: SEM photographs of (a) chitosan powder, (b) chitosan beads before immobilisation, (c) chitosan beads after immobilisation and (d) chitosan beads after biodiesel reaction.

4.2 Characterisation of Oil Feedstock and Products

The properties of the oil feedstock and ME were determined based on standard methods. In this study, the characterisations were based on AV, IV, density and saponification. The results are shown in Table 4.3.

Table 4.3: Characteristics of WCO, FCO and esterified WCO and FCO using chitosan immobilised TL lipase

Sample	AV mg KOH/g	Density kg/m^3	Saponification mg/g	IV $gI_2/100g$
WCO	4.96	834.12	197.86	51.12
Esterifieds WCO	0.79	861.10	200.79	25.66
FCO	0.34	844.43	195.71	55.86
Esterifieds FCO	0.47	864.56	191.34	17.48

Based on ASTM standard value, the desired AV is below 0.8mg KOH/g of oil (Issariyakul *et al.*, 2006). AV of crude WCO was very high at first which was 4.96mg/g; however the AV was reduced substantially to around 0.79mg/g after transesterification. The large decrement of AV from WCO to esterified WCO shows that the enzymatic transesterification process was performing accordingly. On the other hand, the AV of FCO was around 0.34mg/g but increased slightly after transesterification to 0.47mg/g. Although there was a slight increment of AV from FCO to esterified FCO, the AV was still below the standard value of 0.8mg/g.

Density will directly affect the engine performance characteristics. The desired biodiesel fuel should have a density between 860 and 900kg/m^3 (Felizardo *et al.*, 2006). In our works, the densities of FCO and WCO measured at laboratory condition of approximately 28°C were 844 and 834kg/m^3 respectively. However, the densities of esterified WCO and FCO increased to 861 and 864kg/m^3 after esterification. Fuel injection equipment operates on a volume metering system;

hence a higher density of biodiesel will deliver a slightly greater mass of fuel in the volume metering equipment (Demirbas, 2005). Regarding to Alptekin & Canakci (2008), the density of biodiesel is greater but energy content is lower compared to diesel fuel. This negative property showed that more fuel needed to inject into the combustion chamber in order to gain same power of engine.

SV is the amount of alkali necessary to saponify a definite quantity of sample and is expressed as the mg of potassium hydroxide (KOH) required to saponify 1 g of sample. The smaller the length of fatty acid chains, the higher the saponification number. The SV of esterified WCO and FCO were 200.79 and 191.34mg/g respectively while WCO and FCO showed 197.86 and 195.71mg/g respectively. There was no significant difference in SV before and after esterification which implied that the lengths of fatty acid chains did not have momentous difference.

High IV reflects the characteristics of the oxidative stability of oil, shorter shelf life, lower quality and high content of bisallylic methylene carbons. The IV of WCO and FCO were 51.12 and 55.86gI_2/100g respectively which were higher than esterified WCO and FCO at 25.66 and 17.48gI_2/100g respectively. This phenomenon indicates that higher content of polyunsaturated fatty acid was found in oil than in methyl esters (Liu, Xin and Yan, 2009). Table 4.4 shows the fatty acid composition of FCO, WCO, esterified WCO and FCO. The results show higher amounts of unsaturated fatty acid in the original oils and higher saturated

fatty acid in esterified oils which resulted in the lower IV value after transesterification. Thus, the present reaction has successfully overcome the steric hindrance from polyunsaturated fatty acids and converting them to saturated ones. Besides, based on Bhagwat *et al.* (2005), some enzymes are unable to accept bulky compounds. The steric bulk of the substituent group in the alcohol and the chain length also affect the reaction rate. Thus, using tert-butanol as co-solvent in the present reaction helps to reduce the inhibitory effect of methanol (Ranganathan *et al.*, 2008).

Table 4.4: Fatty acid composition of experimental oils before and after reaction

Sample	C14:0	16:0	18:0	18:1	18:2
FCO	1.00	42.60	4.40	42.10	10.10
Esterified FCO	1.14	48.89	5.02	37.65	7.29
WCO	2.12	33.70	13.08	44.10	7.00
Esterified WCO	1.03	51.38	5.35	36.12	6.03

4.3 Products of Transesterication Reaction

4.3.1 Qualitative Analysis and Content of FAME

Figure 4.5 shows the GC-FID chromatogram of the biodiesel produced by transesterification process of WCO using MeOH to oil ratio of 5:1, 0.7% (w/w) of the amount of lipase based on oil weight, *tert*-butanol solvent system of the same

weight as oil, and stirring at 200rpm at 46°C after twenty four hours. The FAME contained mainly the ME of myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) respectively. The major proportion of FAME was found to be palmitic acid ME. Table 4.5 shows the retention time and contents of each FAME in the study.

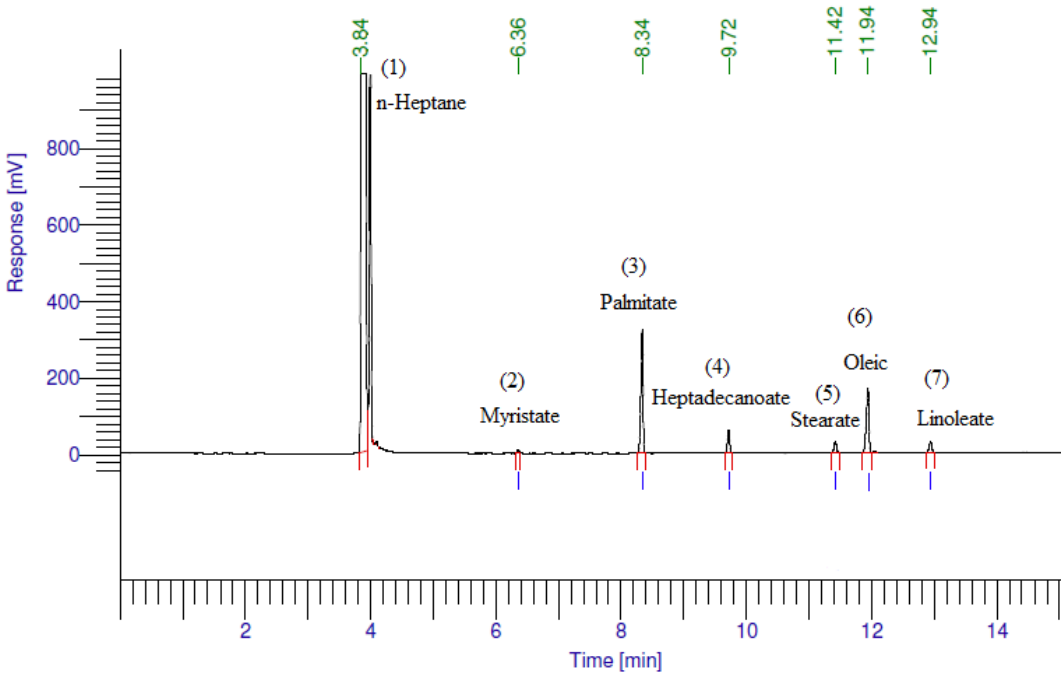

Figure 4.5: GC-FID chromatogram of the biodiesel produced by enzymatic transesterification process after 24 h. The peaks 1-7 indicate n-heptane (solvent), myristic acid methyl ester, palmitic acid methyl ester, heptadecanoic acid methyl ester (Internal standard), stearic acid methyl ester, oleic methyl ester, and linoleic acid methyl ester respectively.

Table 4.5: Retention times and content of each fatty acid methyl ester in biodiesel produced by the transesterification process

FAME	C14:0	C16:0	C18:0	C18:1	C18:2
Retention time (min)	6.40	8.40	11.53	12.05	13.06
Contents (%)	1.17	52.85	6.41	34.21	5.36

Transesterification of FCO and WCO were explored. The FAME content of FCO and WCO for 3 cycles (each cycle is 24 h) are compared in Figure 4.6. ME content is the percentage of ME defined in EN 14103 and calculated by equation 3.5; alternatively this is also estimated by suming all major C_{14}-C_{24} esters using the calibration graphs and divided by the sample mass. The trends of ME content for both FCO and WCO are almost the same.

For the first cycle, the ME contents for FCO and WCO were stable after 8 h which managed to reach 21 and 19% respectively. The methanol was added to the solution every 2 h consecutively 6 h. After 24 h, the lipase was reused with another batch of fresh medium and continued for second cycle. The ME content increased with lipase reuse and reached the highest amount of about 35% for both esterified WCO and FCO after 24 h. For the third cycle of transesterification, the FAME contents for WCO and FCO were stable and managed to reach the plateau of at least 33%.

The highest FAME contents of about 35% were achieved for WCO and FCO after 3 cycles of esterification, hence FAME content of WCO was

compatible to FCO. Higher acid value of WCO and the presence of impurities in the oil feedstock may retard the transesterification of substrate and produce low content of the product.

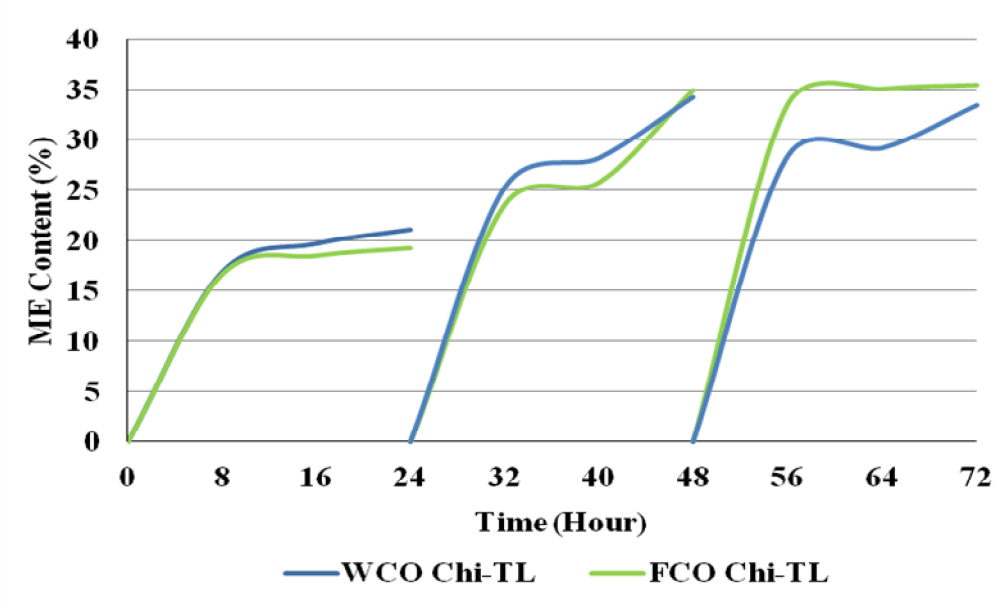

Figure 4.6: ME content of FCO and WCO by using chitosan immobilised TL with the condition of MeOH to oil molar ratio 6:1, 1:1 (v/v) tert-butanol based on the volume of oil, 1% (w/w) of lipase to oil weight, and with stirring 200rpm at 40°C for 72 h.

4.3.2 Glycerol, TG, DG, and MG Contents

GC chromatogram provides the qualitative analysis of the contents of glycerol, TG, DG, and MG. The retention times are shown in Table 4.6. Figure 4.7 shows the results of biodiesel produced from WCO at MeOH to oil ratio of

6:1, 1% (w/w) of the amount of lipase based on oil weight, *tert*-butanol solvent system of the same weight as oil, and stirring at 200rpm at 40°C after 24 h. The main component in the product sample was ME. A small amount of DG and a noticeably larger amount of MG were still present in the ester products which imply that the transesterification reaction was partially completed. The TGs in the oil feedstock had been converted to MEs and MG predominantly. The presence of glycerol could inactivate the enzymes.

Table 4.6: Retention times and content of TG, DG, MG, and ME in biodiesel produced by the transesterification process

	ME	**MG**	**DG**	**TG**
Retention time (min)	9.00	15.65	24.5	30.5
Contents (%)	47.07	16.64	2.40	33.88

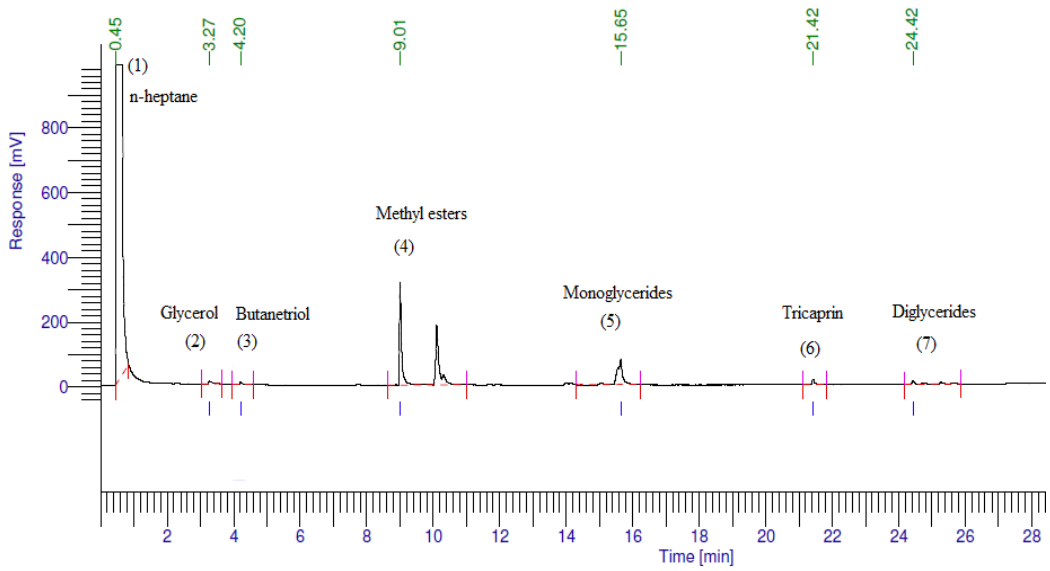

Figure 4.7: GC chromatogram of the biodiesel produced by enzymatic transesterification process after 24 h. The peaks 1-7 indecated n-heptane (solvent), glycerol, Butanetriol (Internal standard 1), methyl esters, MG, tricaprin (Internal standard 2) and DG respectively.

4.3.3 Comparison of FAME Contents between Chitosan Immobilised Lipase and Commercial TLIM

Transesterification reactions were compared using 1% (w/w) chitosan immobilised TL and 10% (w/w) commercial immobilised TLIM based on oil weight for both FCO and WCO. The reactions were carried out with MeOH/oil molar ratio of 6:1, 1:1 (v/v) *tert*-butanol/oil, with stirring at 200rpm and 40°C. At the end of each batch (24 h), the reaction mixture was filtered and filtrate collected for further investigation. Then, the immobilised lipases were introduced into fresh medium.

As can be seen in Figure 4.8, commercial TLIM generally produced higher ME content compared with the self-prepared chitosan immobilised enzyme. Although the ME content for 1% chitosan TL in the first cycle was quite low at around 20%, but in the 2nd cycle, it was able to produce within 5% different from the commercial TLIM at the end of the cycle. In the third cycle, both chitosan immobilised enzyme and commercial TLIM showed about the same ME contents of around 35% at the end of the cycle.

As chitosan beads were stored in DI water before being used in the first cycle, ME content was low because lipases showed maximal activity with water. When water was depleted with subsequent reuse, the produced FFA was transformed to methyl esters. Thus, in order to obtain good ME content, the prepared lipases should be immersed in solvent before reaction to increase the lipase activity (Royon *et. al*, 2007). Since 1% of the self-prepared chitosan immobilised enzyme produced comparable ME content as 10% of commercial TLIM and both unit costs were about the same, the present product seems cost-effective compared to commercial TLIM.

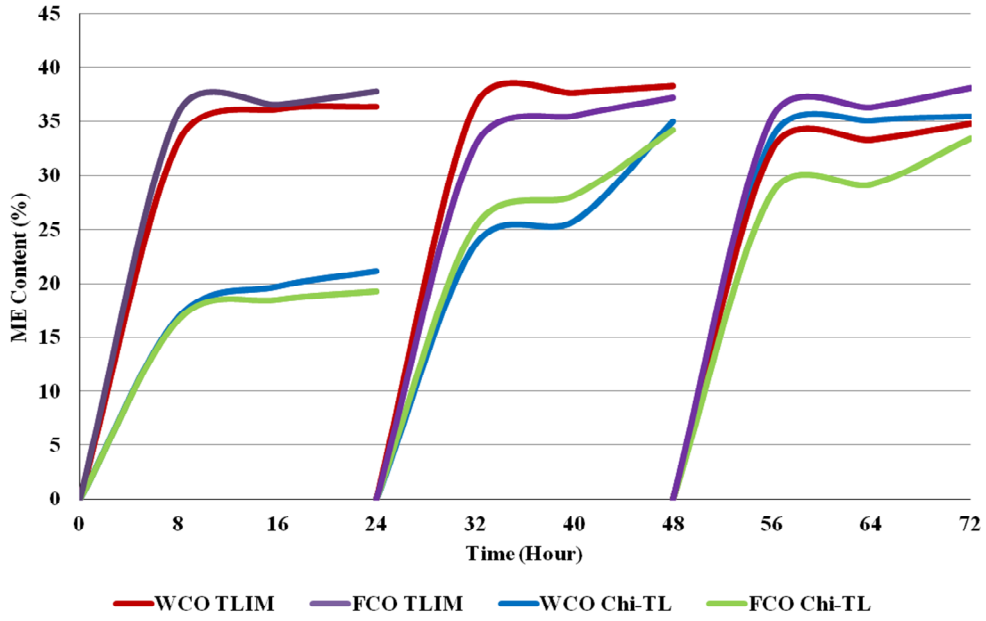

Figure 4.8: ME content of FCO and WCO by using 1% (w/w) chitosan immobilised TL and 10% (w/w) TLIM based on oil weight with the condition of MeOH/oil molar ratio 6:1, 1:1 (v/v) tert-butanol/oil, with stirring 200rpm, at 40°C for 72 h (3 cycles).

4.3.4 Effect of Methanol to Oil Ratio

MeOH is generally used for large scale industrial biodiesel production. MeOH to oil molar ratio is one of the most important parameters in biodiesel production. In general, higher molar ratios result in ester conversion in a shorter time but the short-chain alcohols like MeOH may damage lipases on account of its intense polarity and the hydrophilic property. When immobilised lipase contact with MeOH that is insoluble in oil, the enzyme activity could be distorted. Shimada *et al.* (2002) and Gao *et al.* (2006) showed that the stepwise alcoholysis

system was helpful and the three-step batch methanolysis was the most effective. With the addition of organic solvent to the reaction mixture, the solubility of alcohol is increased and hence the enzymes are protected from inactivation.

In theory, the conversion of oil to ME requires at least three molar equivalents of MeOH. The value depends on the properties of WCO and the type of catalyst used. Usually, in solvent systems, a slight excess of alcohol is necessary to achieve satisfactory content in the transesterification process. Royon *et al.* (2007) obtained best contents at 3.6:1 molar ratio of MeOH: oil in the presence of *tert*-butanol. Thus, MeOH to oil ratios were set in the range of 1 to 6. Although molar excess of alcohol over fatty acids contained in TGs will increase transesterification content, the main problem of excess MeOH is the strong denatured properties which can inactivate enzymes. In order to reduce the inactivation of enzymes by MeOH, the desired amount of alcohol can be added stepwise.

Figures 4.9 and 4.10 show the effect of increasing MeOH:oil ratio for a cycle of 24 h. Amount of ME increased when increasing the MeOH:oil ratio for both the FCO and WCO. The inhibitory effect of MeOH is large at the beginning of the reaction, but with increasing oil conversion it decreases because of MeOH solubility is higher in the product ME than in TGs. The highest amount of ME at about 200mg/g of oil could be obtained at oil/MeOH ratio of 1:6. Through three successive stepwise additions of MeOH, an increase in the number of moles of

MeOH resulted in an increase in the ester production. However, as the formation of esters reaches a maximum level, further increases in the MeOH concentrations could result in a decrease in the formation of esters due to enzyme inactivation. The formation of glycerol byproduct can also physically block the entrance of support pores, thus affecting ester content.

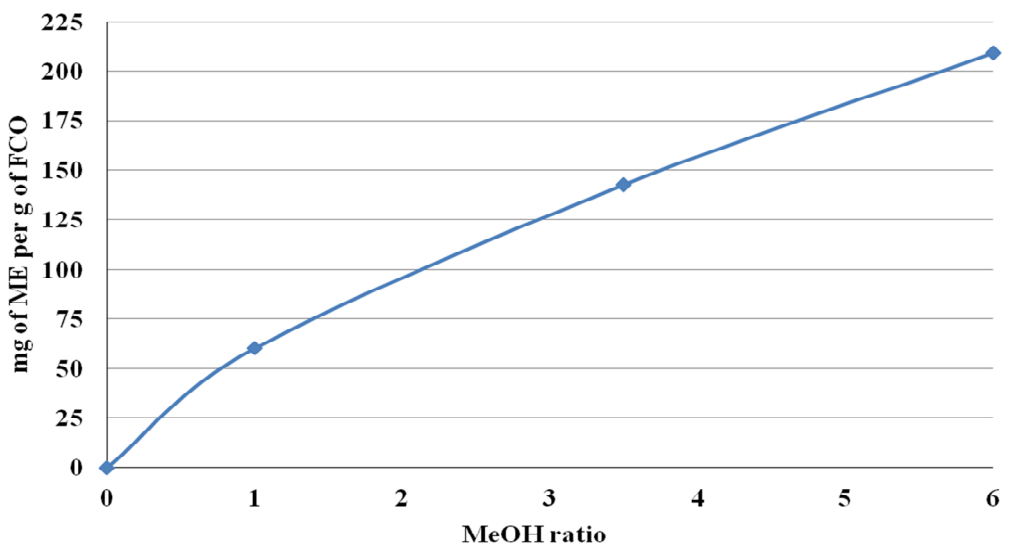

Figure 4.9: Effect of MeOH in FCO with the condition of 1% (w/w) chitosan immobilised TL enzyme, 1:1 tert-butanol to oil, and with stirring 200rpm at 40°C for 24 h.

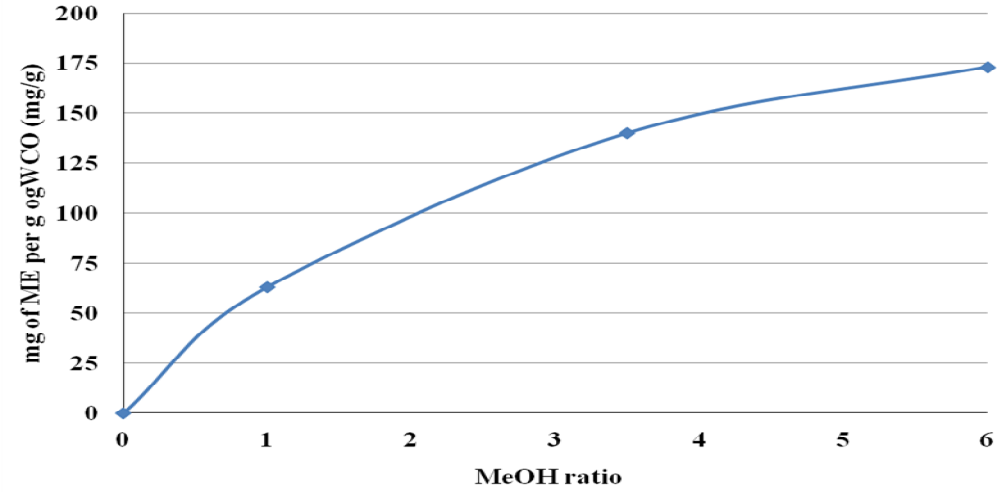

Figure 4.10: Effect of MeOH in WCO with the condition of 1% (w/w) chitosan immobilised TL enzyme, 1:1 tert-butanol to oil, and with stirring 200rpm at 40°C for 24 h.

Figure 4.11 shows the comparison of ME contents for FCO and WCO by using chitosan immobilised TL (for the first cycle) and commercial TLIM. The ME content obtained using commercial immobilised TLIM was higher compared with chitosan immobilised TL at all MeOH:oil ratios. However, there was not much difference in ME contents between FCO and WCO. The content of ME generally increased at higher MeOH to oil ratio.

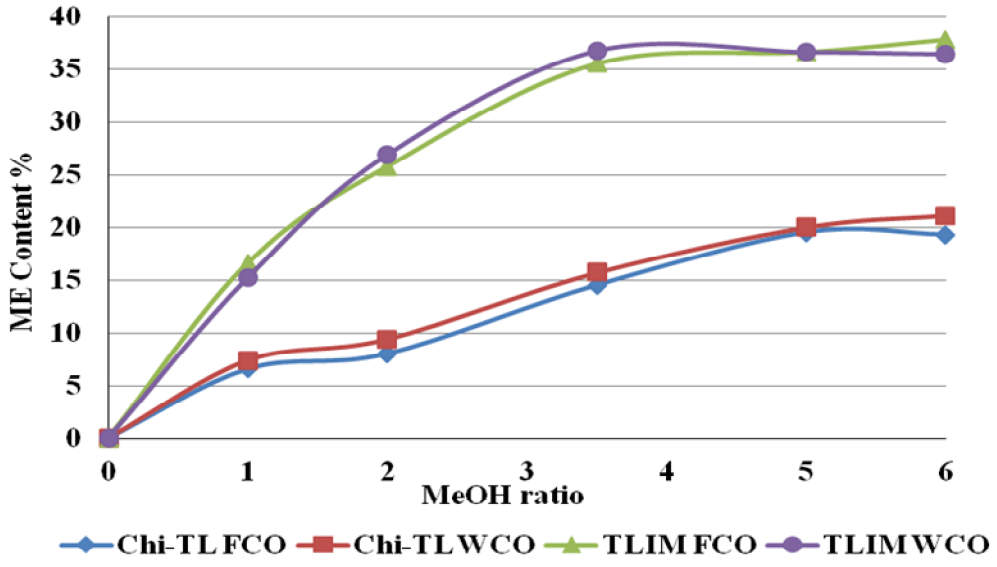

Figure 4.11: Effect of MeOH/Oil molar ratio on transesterification reaction with 1% (w/w) chitosan immobilised lipase and 10% (w/w) TLIM, 1:1 tert-butanol to oil, and with stirring 200rpm at 40°C for 24 h by using FCO and WCO

4.3.5 Effect of Temperature

Generally, the rate of reaction is slow when the temperature is below 25°C while further increment in temperature above 50°C causes no increase in reaction since higher temperature inactivates the lipase. The immobilised lipase has better stabilisation and resistance to heat that induces inactivation. Based on literature survey, reaction temperatures from 30 to 50ºC were used in a series of experiments on FCO and WCO.

The difference in ME contents from FCO and WCO due to the effect of temperature can be seen in Figure 4.12 while the change in compositions of the reaction mixture for WCO at different temperatures is shown in Figure 4.13. It was found that the enzyme lost its activity and denatured dramatically when the temperature was above 50ºC. The ME content started to decrease above 40ºC while the concentration of DG tended to increase. Thus, the optimum temperature for enzymatic transesterification was observed at 40ºC for the test conditions used. The existence of an optimum temperature condition can be due to the interplay between operational stability of the biocatalyst and the rate of transesterification. However, the desired temperature depends on the molar ratio of alcohol to oil, type of organic solvent and thermostability of enzymatic preparation.

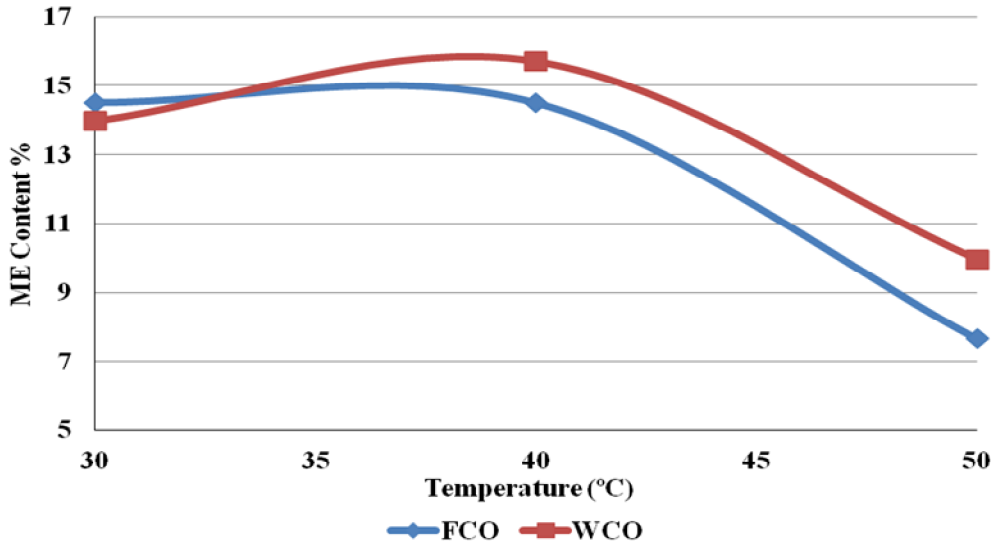

Figure 4.12: Effect of temperature to transesterification in FCO and WCO with the condition of MeOH ratio of 3.5 to oil, 1:1 *tert*-butanol to oil, 1% (w/w) chitosan immobilised TL to oil with stirring at 200rpm for 24 h.

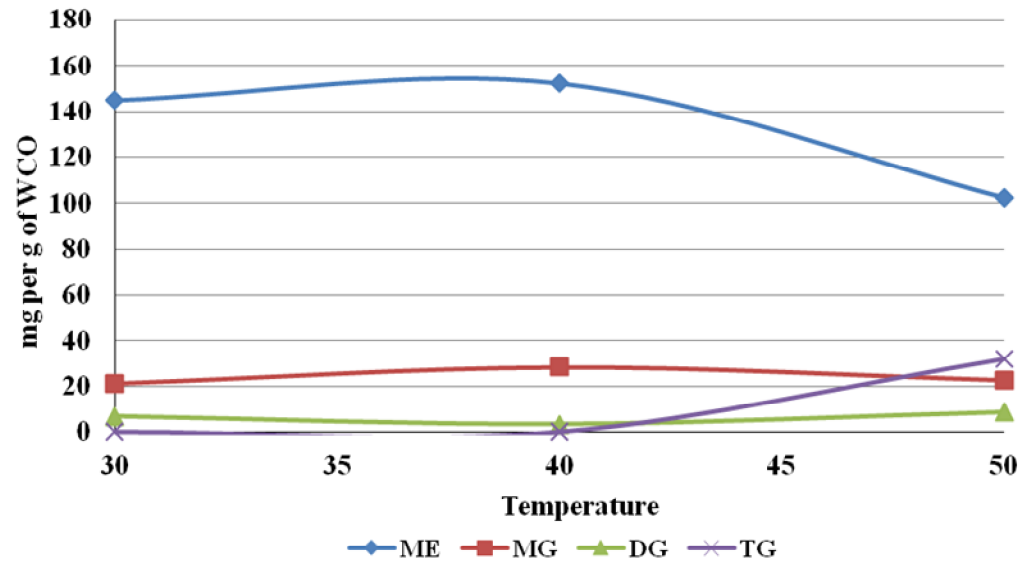

Figure 4.13: Composition of reaction mixture at different temperature in WCO with the condition of MeOH ratio of 3.5 to oil, 1:1 *tert*-butanol to oil, 1% (w/w) chitosan immobilised TL to oil with stirring at 200rpm for 24 h.

4.3.6 Effect of Enzyme Dosage

Based on the findings of Maceiras *et al*. (2009) the ME contents increased gradually for lipase concentration below 10% but decreased at higher concentration. They suggested that the liquid volume was insufficient to carry out the reaction and unable to completely suspend the solid catalyst when an excess of enzyme over the required amount was used.

In order to investigate the effect of the dosage of enzyme on methanolysis of WCO and FCO, the amount of chitosan immobilised TL was varied from 0 to

10% of the weight of oil at constant condition of 1:1 *tert*-butanol, MeOH:oil ratio at 3.5, stirring at 200rpm and 40°C. Based on the results shown in Figure 4.14, ME content increased with increase in lipase concentrations but then dropped and remained constant at higher lipase concentration. There was no improvement in the reaction by increasing the amount of catalyst from 4 to 10%. Although for a higher amount of catalyst, the reaction was noticeably faster but all the experiments reached the same ME content at the end of reaction cycle. This is because when the enzyme content was under 4%, the oil content was dominant. When the enzyme content rose from 4 to 10%, the FAME content remained constant as the liquid phase was not sufficient to completely suspend the solid catalyst. By considering the cost of industrial production, the addition of lipase at 0.5 to 1.5% of the oil mass was chosen in the subsequent reactions.

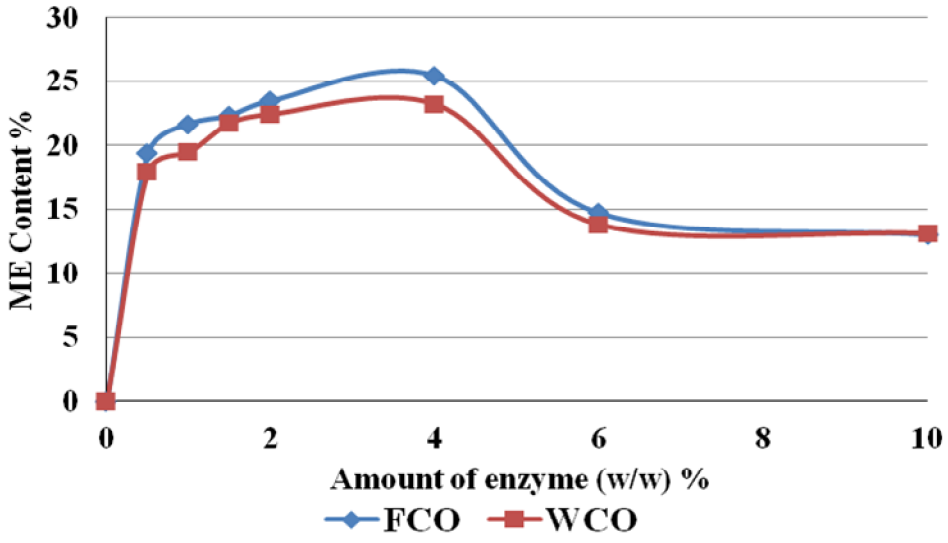

Figure 4.14: ME content of biodiesel FCO and WCO with different dosage of chitosan immobilised TL at 40°C, MeOH to oil ratio of 3.5 with stirring at 200rpm for 24 h.

4.3.7 Reaction Time

The transesterification reaction using chitosan immobilised TL on WCO and FCO was monitored for 72 h. Figures 4.15 and 4.16 show the composition of reaction mixture for FCO and WCO respectively. Once the reaction started, the amount of TGs decreased sharply due to conversion to DG and MG. After 24 h, the amount of TG and DG were almost zero while ME content increased gradually as MG continued to decrease. The results indicate that the optimum reaction time was 24 h since after that time the ME content was practically constant.

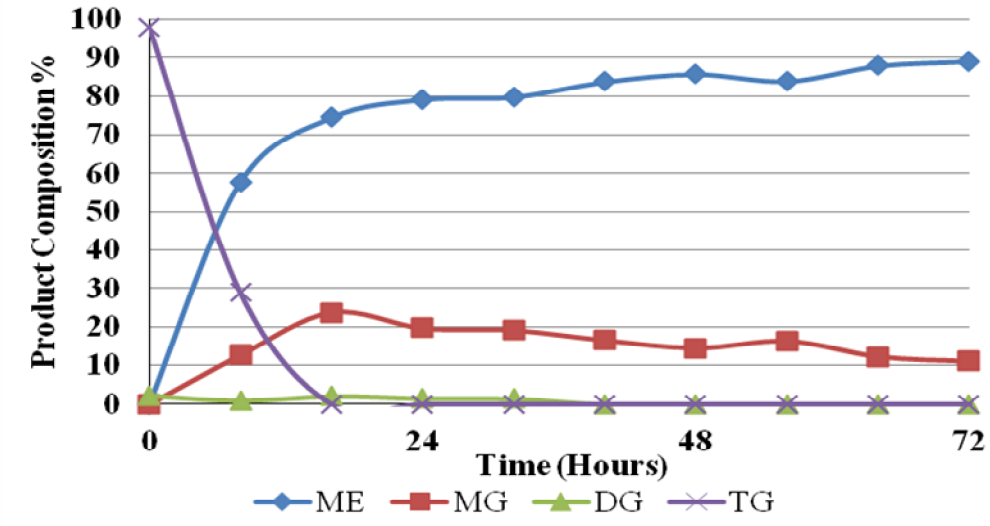

Figure 4.15: Product composition of reaction mixture of FCO at 72 h in the condition of MeOH ratio to oil at 6:1, 1:1 *tert*-butanol to oil volume, 1% (w/w) chitosan immobilised TL to oil weight at 40°C.

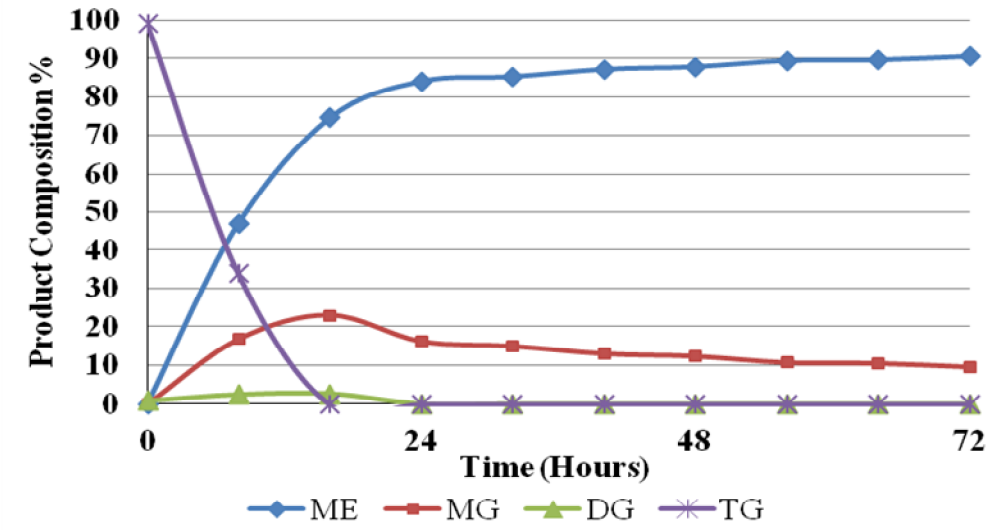

Figure 4.16: Product composition of reaction mixture of WCO at 72 h with the condition of MeOH ratio to oil at 6:1, 1:1 (v/v) *tert*-butanol to oil volume, 1% (w/w) chitosan immobilised TL to oil weight at 40°C.

4.3.8 Enzyme Regeneration

Operational stability and the regeneration of binary immobilised lipase are important for economical use of enzyme in repeated batch or continuous transesterification reaction. After the completion of reaction, immobilised enzyme can be separated by the simple process of decantation, and does not require special method of separation. The immobilised lipase may be filtered and consecutively reused after each reaction.

Operational stability of the self-prepared chitosan immobilised lipase and its regeneration capability were assessed and the results are shown in Figure 4.17 based on the ME content. The reaction was carried out for 12 cycles where each cycle was 24 h. As reported in section 4.3.3 it was found that there was a significant increase in ME content from the 1st to 3rd cycles in which the highest content of around 36% was achieved and maintained for the next 3 cycles. The operational stability of the chitosan immobilised lipase was thus longer than 12 cycles.

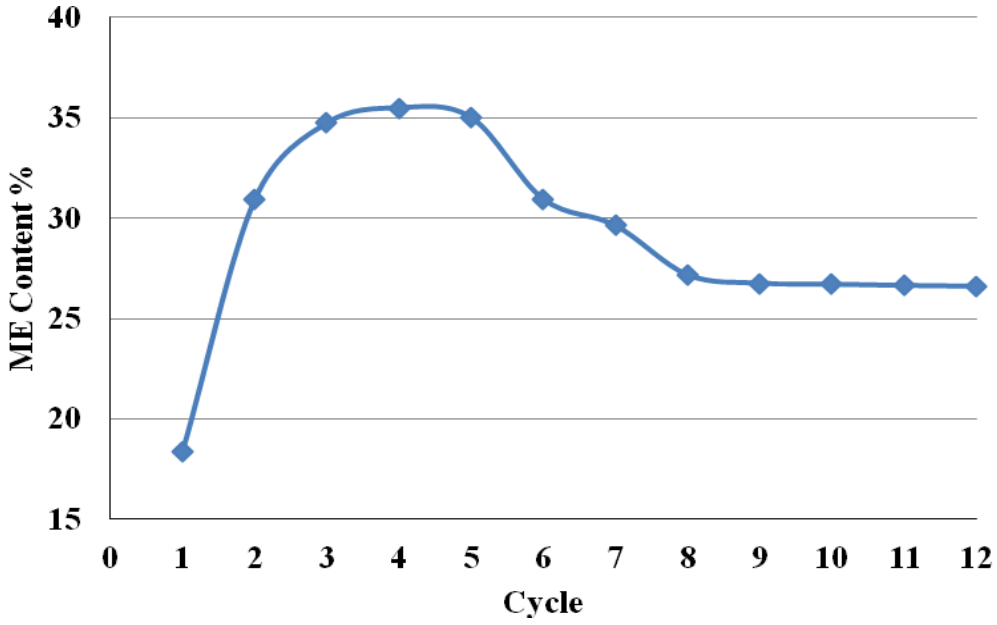

Figure 4.17: Operational stability of chitosan immobilised lipase during twelve batch reactions in the transesterification of WCO, 0.7% (w/w) of the enzyme amount to oil weight, MeOH/oil molar ratio at 5:1, 200rpm stirring at 46°C.

Figure 4.18 shows the residual activity in percentage during 12 cycles of the reactions. From the begining, the enzyme activity was low which only performed 50% of its activity. Optimum enzyme activity was obtained at cycle 4 and continued to perform well at the next cycle. Subsequently, the enzyme activity started to decrease. After 12 cycles of reactions, the activity of immobilised enzymes still managed to retain 76% of the highest activity achieved at the 4^{th} cycle. This result shows that the immobilisation method could provide a stable enzyme structure over long run. The byproduct of glycerol which can block the immobilised enzyme may be the main reason for the deteriorating enzymatic activity. For fresh immobilised lipase, lower activity may be due to restricted penetration of substrate into the preparation. Therefore fresh immobilised enzyme should be pre-treated by immersing in a solvent before reaction to achieve better result. In order to solve the problems, further steps may be needed such as washing the enzyme with solvent after every cycle.

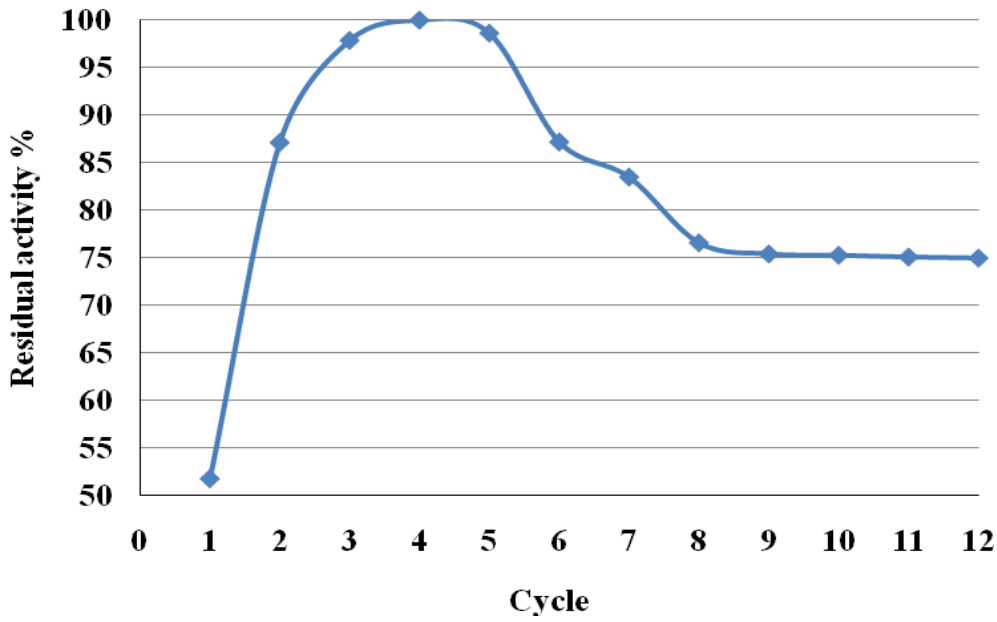

Figure 4.18: Residual activity of chitosan immobilised lipase during twelve batch reactions in the transesterification of WCO, 0.7% (w/w) of the enzyme amount to oil weight, MeOH/oil molar ratio at 5:1, 200rpm stirring at 46°C.

4.4 Response Surface Methodology (RSM)

RSM was used to identify the factors that influenced the transesterification of FCO and WCO. The five-level-three-factor central composite design (CCD) requires 20 experiments consisting of 8 factorial points, 6 axial points and 6 central points. The variables were reaction temperature (30-50ºC), lipase concentration (0.5-1.5% based on oil weight), and the MeOH:oil molar ratio (1:1-6:1). These variables have been commonly used for modeling the transesterification reactions. The relationship between the response (FAME

content) and the three independent factors was studied. The experimental sequence was randomised in order to minimise the effects of other uncontrolled factors. The results of the experimental design for transesterification of FCO and WCO are shown in Table 4.7. The coefficients of the full regression model equation and their statistical significance were determined and evaluated using Design-Expert 7.1.3 software.

The goodness of fit of the model was evaluated by coefficient of determination (R^2) and the analysis of variance (ANOVA). ANOVA analysis is necessary to determine the significance of a quadratic model, in which the regression sum of square is subdivided into one part that attributes to linear regression and another part that attributes to the quadratic model. The positive sign in front of the terms indicates synergistic effect while negative sign indicates antagonistic effect. P values are used to check the significance of each coefficient and the interaction strength of each parameter. They are inversely proportional to the significance of the corresponding coefficient. Adequate precision is used to measure the signal to noise ratio where a ratio greater than 4 is desirable. The coefficient of variation (CV) which is the ratio of the estimated standard error to the mean value of the observed response in percentage is a measurement of reproducibility of the model. If the CV is not greater than 10%, the model is considered reasonably reproducible (Halim *et. al*, 2009). The analysis for response surface reduced quadratic model on esterified FCO and WCO is shown

in Table 4.8 while ANOVA for the regression models and respective model terms for FCO and WCO are given in Tables 4.9 and 4.10 respectively.

Table 4.7: Results of the experimental design for transesterification of FCO and WCO

Run	MeOH Ratio	Temperature (°C)	Enzyme (%)	Response (FCO)	Response (WCO)
1	2.0	34.1	0.70	11.773	11.561
2	3.5	30.0	1.00	14.504	13.966
3	3.5	40.0	1.00	14.494	15.704
4	3.5	40.0	1.00	14.862	14.581
5	2.0	46.0	1.30	5.692	5.69
6	3.5	50.0	1.00	7.669	9.962
7	5.0	46.0	0.70	19.99	17.803
8	5.0	34.1	0.70	17.994	19.236
9	2.0	46.0	0.70	10.75	9.372
10	3.5	40.0	1.00	14.695	13.807
11	6.0	40.0	1.00	19.289	21.128
12	3.5	40.0	1.00	15.403	17.185
13	1.0	40.0	1.00	6.561	7.385
14	5.0	34.1	1.30	19.568	16.029
15	3.5	40.0	1.50	13.285	13.736
16	3.5	40.0	1.00	14.286	13.635
17	2.0	34.1	1.30	12.257	10.12
18	3.5	40.0	0.50	19.387	17.925
19	3.5	40.0	1.00	15.494	15.703
20	5.0	46.0	1.30	14.58	14.406

Table 4.8: Analysis for Response Surface Reduced Quadratic Model for esterified FCO and WCO

Sample	CV %	R^2	Adj R^2	Pred R^2	Adeq Precision
Esterified FCO	5.58	0.9793	0.9642	0.8963	28.523
Esterified WCO	8.14	0.9442	0.9185	0.8773	22.582

Table 4.9: ANOVA for the regression model and respective model terms for esterified FCO

Source	Sum of Squares	Df	Mean Square	F value	p-value Prob>F	
Model	322.53	8	40.32	64.90	<0.0001	Significant
A – Oil:MeOH	206.20	1	206.20	331.93	<0.0001	
B – Temperature	35.68	1	35.68	57.44	<0.0001	
C – Enzyme %	25.53	1	25.53	41.10	<0.0001	
AB	2.64	1	2.64	4.25	0.0637	
BC	19.61	1	19.61	31.57	0.0002	
A^2	4.17	1	4.17	6.72	0.0251	
B^2	20.34	1	20.34	32.75	0.0001	
C^2	6.43	1	6.43	10.35	0.0082	
Residual	6.83	11	0.62			
Lack of Fit	5.65	6	0.94	3.97	0.0761	Not significant
Pure Error	1.19	5	0.24			

Table 4.10: ANOVA for the regression model and respective model terms for esterified WCO

Source	Sum of Squares	Df	Mean Square	F value	p-value Prob>F	
Model	283.64	6	47.27	36.69	<0.0001	Significant
A – Oil:MeOH	212.29	1	212.29	164.77	<0.0001	
B – Temperature	19.72	1	19.72	15.30	0.0018	
C – Enzyme %	25.80	1	25.80	20.03	0.0006	
AB	1.59	1	1.59	1.23	0.2872	
BC	0.74	1	0.74	0.57	0.4624	
B^2	23.51	1	23.51	18.24	0.0009	
Residual	16.75	13	1.29			
Lack of Fit	7.59	8	0.95	0.52	0.8057	Not significant
Pure Error	9.16	5	1.83			

Equations 4.1 and 4.2 are the final regression models for FCO and WCO respectively.

The final model in terms of coded value is:

$$Y= +14.85 + 3.89A – 1.62B – 1.37C + 0.57BC – 0.54A^2 -1.19B^2 + 0.67C^2 \quad (4.1)$$

$$Y = +14.81 + 3.94A -1.2B -1.37C -0.27 B^2 \quad (4.2)$$

where Y is the ME content (%), A is molar ratio of MeOH to oil, B is Temperature (ºC) and C is the amount of enzyme (%).

In Table 4.9, it may be seen that the P-value for the FCO regression model is smaller than 0.0001 which implies the model is significant while the P-values of the model terms involving A, B, C, BC, A^2, B^2 and C^2 are lower than 0.0500, thus indicating these model terms are significant. The R^2 value obtained is 0.9793 which means 97.93% of the variability in the response could be explained by the model.

In Table 4.10, the P-value for the WCO regression model is smaller than 0.0001 which implies that the model is significant while the P-values of the model terms involving A, B, C and B^2 are lower than 0.0500, thus indicating these model terms are significant. The R^2 value obtained is 0.9442 which means 94.42% of the variability in the response could be explained by the model.

The effect of parameters on the content within the experimental space may be visualised by the response plots of the equation. The three dimensional (3D) response surfaces and the contour plots are shown in Figures 4.19 to 4.22 as a function of the interactions of any two of the variables. Figure 4.19 shows the predicted values of FAME content from FCO as a function of the reaction temperature and the methanol:oil molar ratio with 1% amount of enzyme. At any designated molar ratios, the FAME content increased with the temperature up to 40ºC and decreased thereafter. The best content at around 16.53% has been achieved at the substrate molar ratio of 4. Although excess alcohol is required for higher reaction rate, but due to the insolubility in TGs, undissolved MeOH will

inhibit the enzymatic methanolysis at higher molar ratios. Figure 4.20 shows the results for the interaction of the enzyme concentration versus the temperature at MeOH:oil molar ratio of 3.5. Methyl ester content decreased with enzyme amount more than 1% may be due to the decrease of enzyme activity caused by lipase aggregation (Shao *et al.*, 2008). Optimum ME content appeared to occur at temperature below 40ºC and enzyme concentration around 1%.

Similar trend was observed for WCO. Figure 4.21 shows the contour plots and 3D response surfaces of the predicted FAME content from WCO as a function of reaction temperature and methanol:oil molar ratio at 1% amount of enzyme. The FAME content increased when the temperature and MeOH:oil molar ratio increased but the trend reversed when the temperature reached 40ºC and molar ratio above 4. The lowest contents can be expected at high MeOH:oil molar ratios and high temperature. Better content was found at lower temperature probably due to the higher inactivation effect of nucleophile on the lipase at higher temperatures. Figure 4.22 shows the interaction between enzyme concentration and temperature on FAME content at MeOH:oil molar ratio of 3.5. It appears that the FAME content increased with temperature at low amount of enzyme.

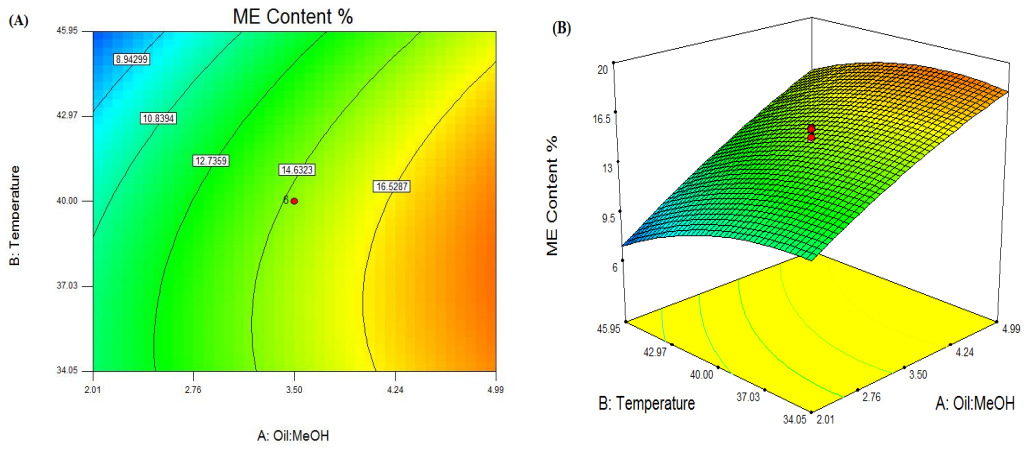

Figure 4.19: Contour plot (A) and response surface curve (B) for FCO showing predicted response surface of ME content as a function of methanol:oil molar ratio and temperature at 1% amount of enzyme.

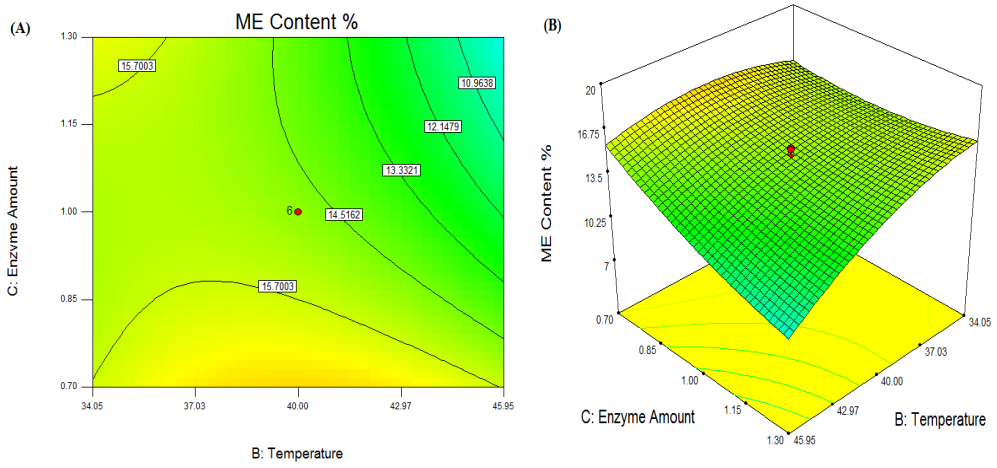

Figure 4.20: Contour plot (A) and response surface curve (B) for FCO showing predicted response surface of FAME content as a function of amount of enzyme and temperature at MeOH:oil ratio of 3.5.

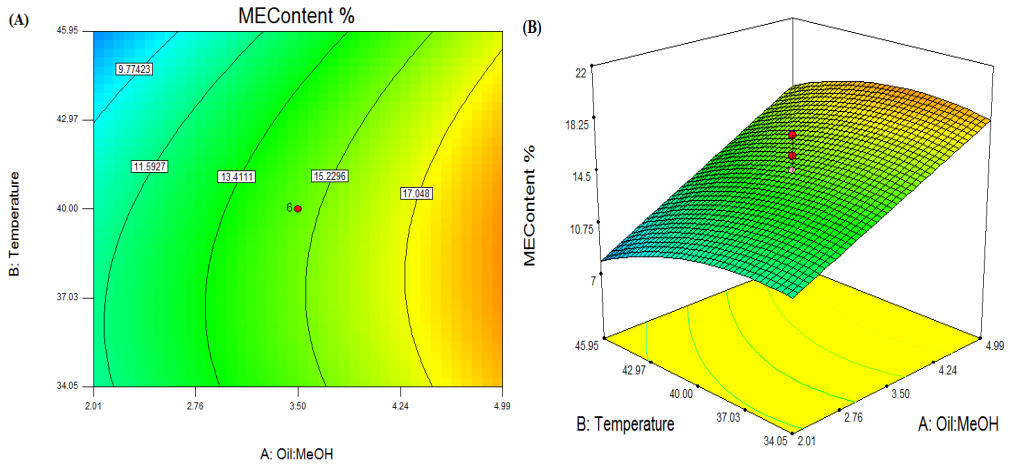

Figure 4.21: Contour plot (A) and response surface curve (B) for WCO showing predicted response surface of FAME content as a function of methanol:oil molar ratio and temperature at 1% amount of enzyme.

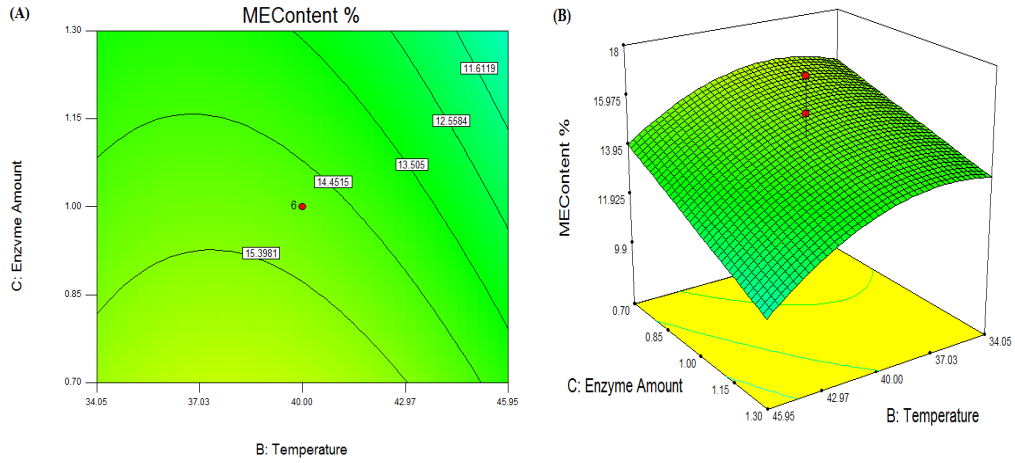

Figure 4.22: Contour plot (A) and response surface curve (B) for WCO showing predicted response surface of FAME content as a function of amount of enzyme and temperature at MeOH:oil ratio of 3.5.

Generally, both the response surfaces obtained were of convex nature giving an optimum operation condition. These results reveal the possibility of selecting reaction conditions to obtain maximum FAME content of WCO and FCO in a solvent system. The presented contour and 3D plots are desirable with several optimal combinations to obtain high FAME content. However, the convexity might not be high enough which means the response optimized value based on the combination effects of methanol:oil molar ratio, enzyme amount and temperature may not vary widely from the single variable optimized conditions.

Next, an optimum reaction condition for FAME synthesis from FCO was selected for testing at MeOH to oil molar ratio of 5.0, 0.71% of enzyme based on oil weight, reaction temperature of 41.3ºC, 1:1 *tert*-butanol based on volume of oil and stirring at 200rpm for 24 h. At this condition, the predicted ME content based on the regression model would be 20.1%. Another condition of FAME synthesis from WCO was selected at MeOH to oil molar ratio of 5.0, reaction temperature of 38.9ºC, 0.7% of enzyme based on oil weight, 1:1 *tert*-butanol based on volume of oil and stirring at 200rpm. The predicted ME content based on the regression model would be 20.2%. In order to assess the model prediction at the above conditions, two new sets of experiments were carried out. The results are compared in Figure 4.23. The measured ME contents for FCO and WCO at the end of the 24 h duration were 20.1 and 19.8% respectively. The results agreed with the predicted ME contents and hence show that the regression models are reliable.

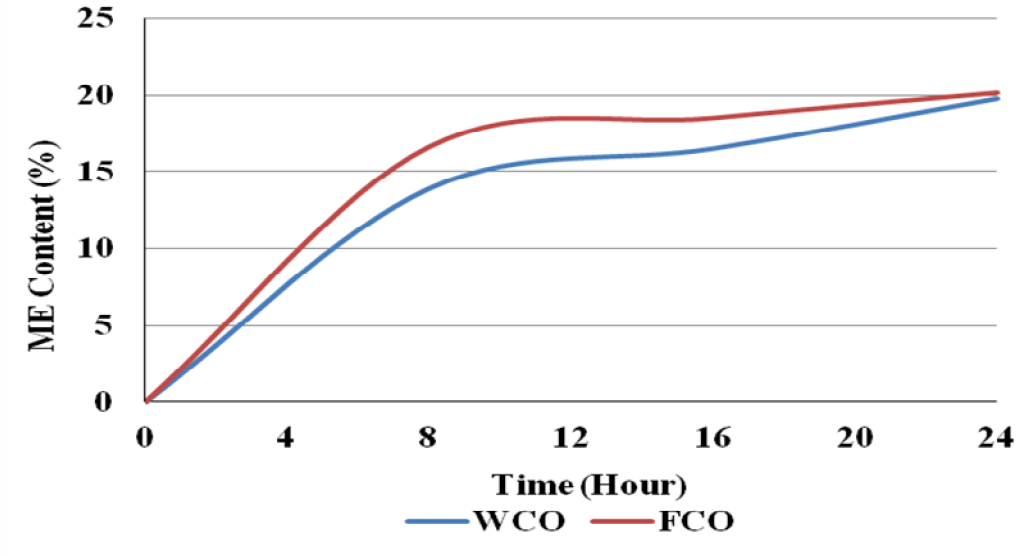

Figure 4.23: ME content from WCO and FCO at the selected condition at 1:1 tert-butanol based on volume of oil with stirring at 200rpm for 24 h.

CHAPTER 5.0

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In this study, the lipase transesterification process of WCO and FCO was successfully investigated. Free lipase TL was immobilised on chitosan beads through a binary immobilisation method. Quantitative determinations of FCO and WCO based on the acid value, saponification value, iodine value and the topography of the chitosan immobilised TL were carried out. Besides, qualitative determination on ME content and masses of TG, DG, MG and glycerol were analysed by GC. Effect of molar ratio of MeOH to oil, reaction temperature, time course, enzyme dosage and solvent on ME content was investigated.

It was found that almost all the lipases were successfully attached to the chitosan beads by the binary immobilisation method. Through this process, the hydroxyl groups of the chitosan activated by EDC and react with the amine groups of lipase. More lipase molecules were cross-linked through its amino groups to chitosan by using glutaraldehyde. The binding yield was enhanced through both the application of cross-linkers.

The presence of *tert*-butanol increased the effectiveness of enzyme since MeOH and by-product glycerols are soluble in *tert*-butanol solvent. With less likelihood of glycerol being adsorbed onto the surface of lipases, lipase stability was enhanced. The optimum temperature for the enzymatic transesterification was observed at 40ºC. Higher temperature will cause denaturation of enzymes while low temperature will give slow reaction. The optimum duration of the reaction was 24 h as there was no appreciable increase of ME content beyond 24 h. In addition, the activity of chitosan immobilised TL still managed to retain 76% after 12 cycles of 24 h.

From the comparison of the 10% (w/w) commercial TLIM and 1% (w/w) self-prepared chitosan immobilised TL based on oil weight, the activity of commercial TLIM was found to be higher in the first and second cycles. However, the chitosan immobilised TL was able to produce the same ME content of 35% as the commercial TLIM under the condition of MeOH to oil molar ratio of 6:1, 1:1 (v/v) *tert*-butanol to oil with stirring at 200rpm and 40°C for 72 h. The initial low ME content from chitosan immobilised TL was mainly due to the presence of DI water in the freshly prepared lipase and hence they should be immersed in solvent before reaction to increase the lipase activity.

Regression models for transesterification of FCO and WCO were obtained using the RSM and 5-level-3-factor central composite design. The P-values of several parameters for the models were smaller than 0.05, indicate that the model

terms were significant. For testing the models, a reaction condition for the FAME synthesis from FCO was selected at MeOH to oil molar ratio of 5.0, 0.71% of enzyme based on oil weight, and 41.3ºC and another condition for WCO at MeOH to oil molar ratio of 5.0, temperature of 38.9ºC, 0.7% of enzyme based on oil weight, both with solvent 1:1 *tert*-butanol based on volume of oil and stirring at 200rpm. The predicted and experimental results agree very well with one another.

5.2 Recommendations

The ME content can be enhanced where future work should consider using a pumping system to circulate the liquid substrate through immobilized lipase in a column. In order to increase the enzyme activity, further improvement in preparation technique may be sought to decrease the size of self-prepared chitosan beads so as to increase the surface area. With the aim to remove the byproduct of glycerol which may block the immobilised enzyme, this may be carried out by washing the enzyme with solvent after every cycle. Future investigation can also strive to enhance the lipase immobilisation technique by better methods to achieve higher ME content. Fourier Transform Infrared Spectrophotometer can be used in order to determine the compound after immobilisation. Furthermore, other parameters can also be investigated such as pH, agitation speed, solvent type, etc. Therefore, the present analysis systems need to be further improved and

experiment works should be continued to explore the full potential of chitosan-immobilised lipase as an efficient and environment friendly catalyst for biodiesel conversion on WCO.

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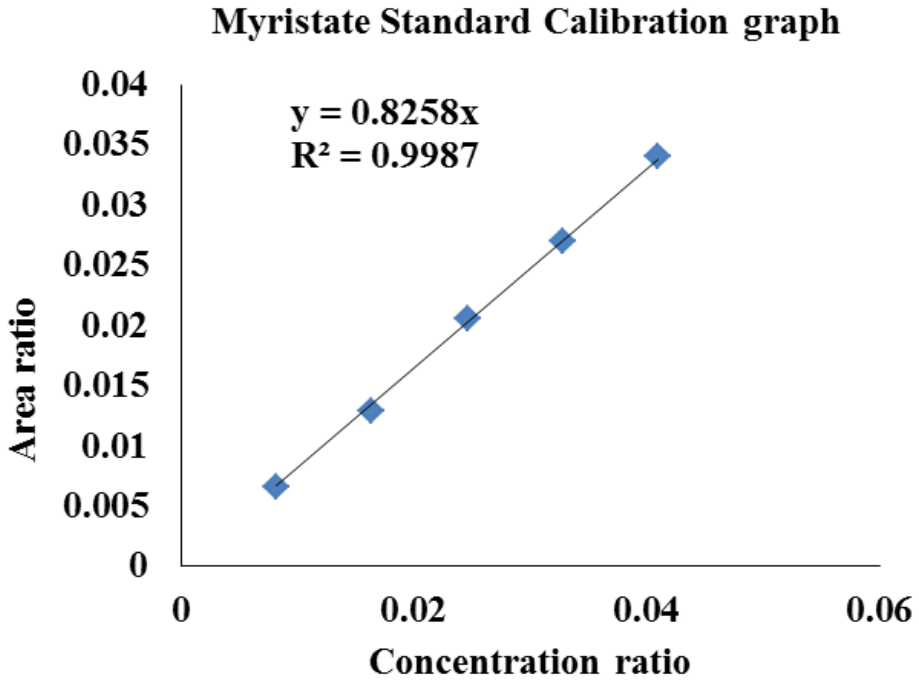
APPENDIX A1


Myristate Standard Calibration graph
y = 0.8258x
R² = 0.9987
Area ratio
0.04
0.035
0.03
0.025
0.02
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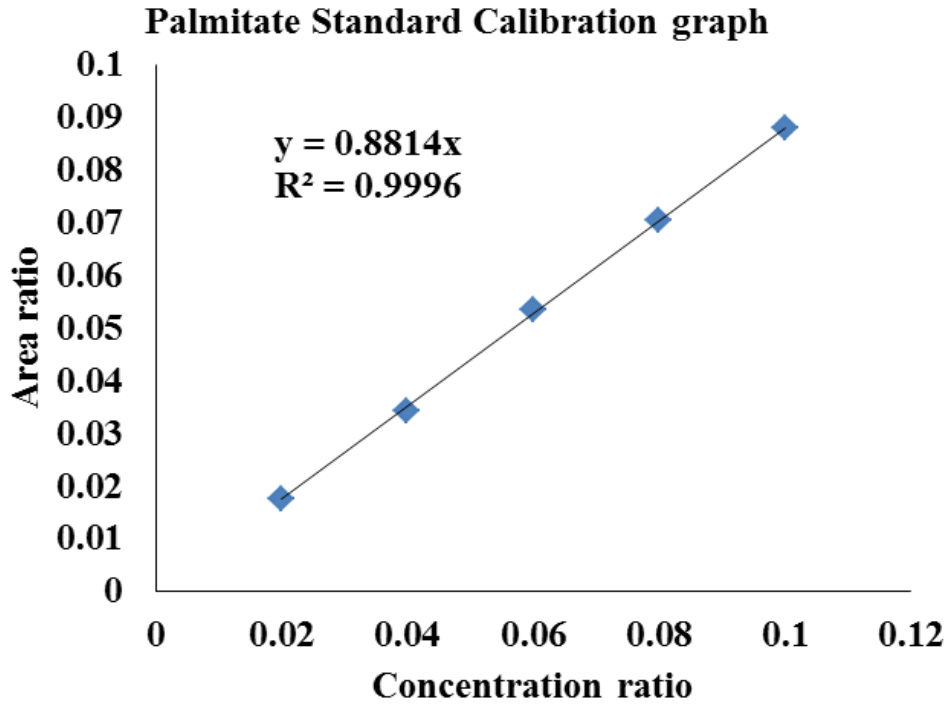
0.01
0.005
0
0
0.02
0.04
0.06
Concentration ratio


Palmitate Standard Calibration graph
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Area ratio
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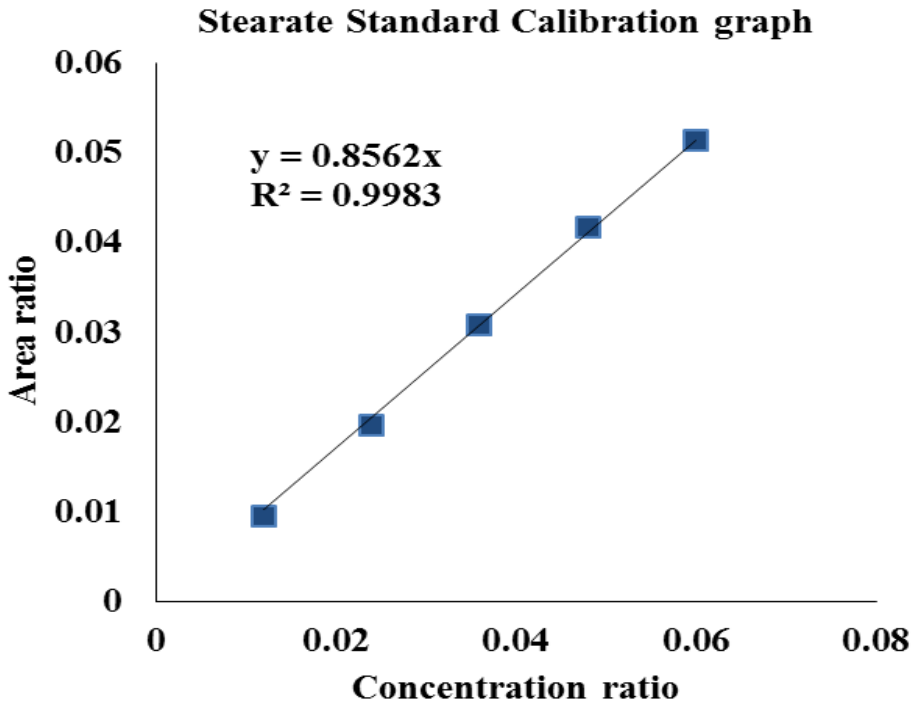
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
0.02
0.04
0.06
0.08
0.1
0.12
Concentration ratio


Stearate Standard Calibration graph
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Area ratio
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0.03
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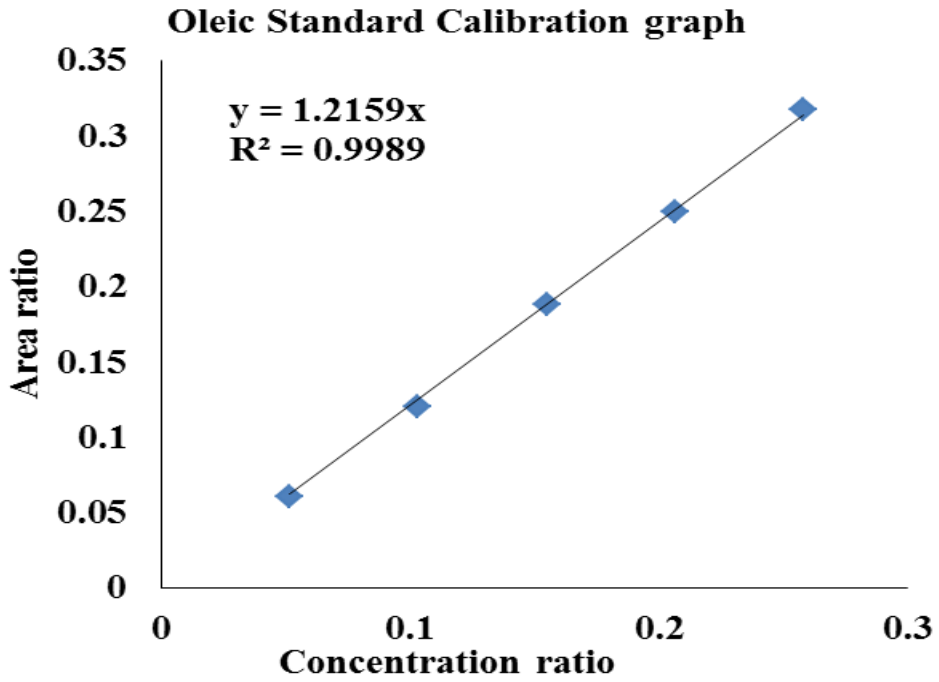
0.01
0
0
0.02
0.04
0.06
0.08
Concentration ratio


Oleic Standard Calibration graph
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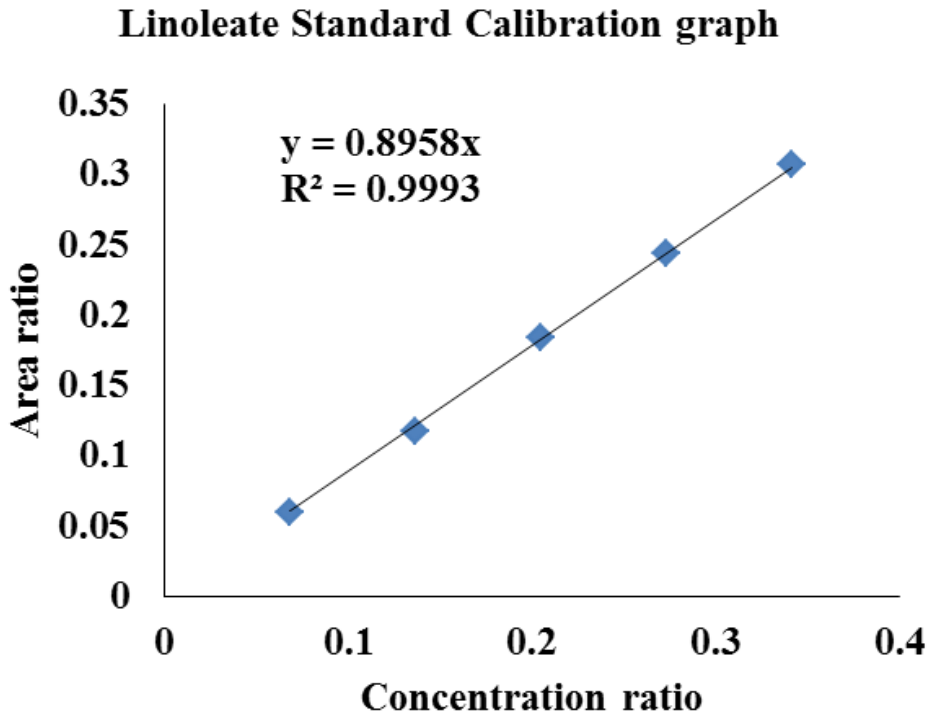
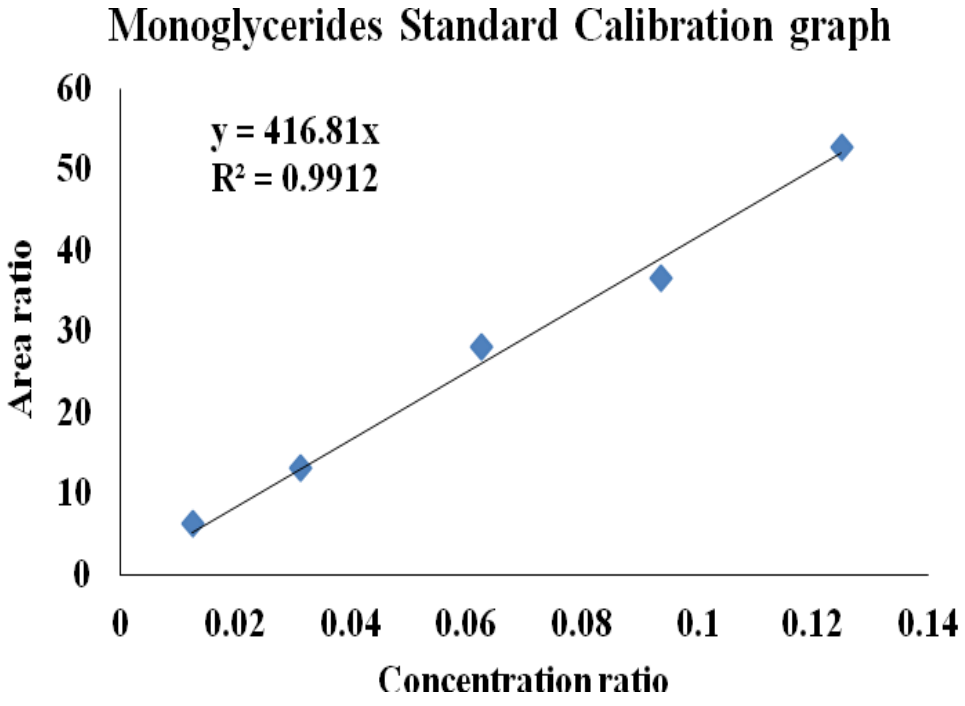
0.25
0.2
0.15
0.1
0.05
0
0
0.1
0.2
0.3
Concentration ratio


Linoleate Standard Calibration graph
y = 0.8958x
R² = 0.9993
Area ratio
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.1
0.2
0.3
0.4
Concentration ratio


Monoglycerides Standard Calibration graph
y = 416.81x
R² = 0.9912
Area ratio
60
50
40
30
20
10
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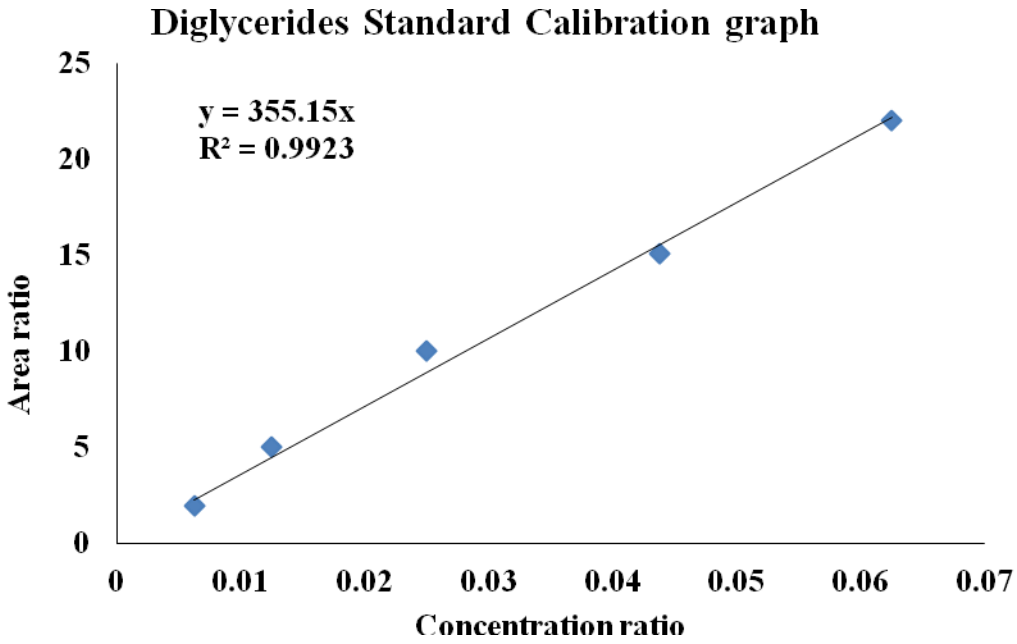
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
Concentration ratio


Diglycerides Standard Calibration graph
y = 355.15x
R² = 0.9923
Area ratio
Concentration ratio
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10
15
20
25
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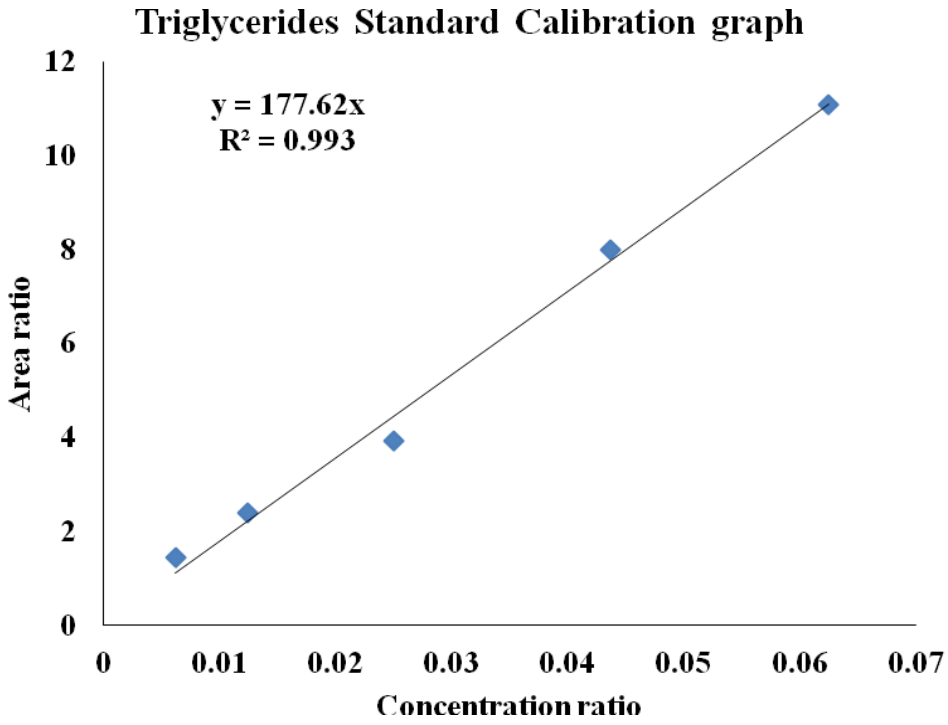
0.01
0.02
0.03
0.04
0.05
0.06
0.07


Triglycerides Standard Calibration graph
y = 177.62x
R² = 0.993
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Concentration ratio
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2
4
6
8
10
12
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07

APPENDIX A2

Calculation for ME mass

ME concentration ratio

$$= \frac{Area\ \ of\ \ ME}{Area\ \ of\ \ IS} \div \quad Slope\ \ from\ \ S\tan dard\ \ Calibration\ \ Graph$$

ME concentration
= ME concentration ratio x Internal Standard concentration

ME mass
= ME concentration x reaction mixture

Total mass of ME
= ME mass x total volume of reactants mixture

ME mass in one gram of oil
= ME total mass divide by mass of oil

Example:
MeOH to oil molar ratio 6:1, 1:1 (v/v) tert-butanol based on the volume of oil, 1% (w/w) of lipase to oil weight, and with stirring 200rpm at 40°C

Palmitate:

Palmitate concentration ratio $= \frac{10103.5}{118308} \div 0.8814$
$= 0.09691$

Palmitate concentration = 0.09691 x 10000µg/mL
= 969.1µg in one ml reaction mixture

Palmitate total mass = 969.1µg/mL x 45.31mL
= 43909.9µg
= 43.91 mg in total volume of reactants

Palmitate mass in one gram of oil = 43.91mg / 16.8g
= 2.6 mg/g

Total ME mass
= (Myristate + Palmitate + Stearate + Oleic + Linoleic) mass

***Same calculation for mass of TG, DG, and MG.**

WCO							ME Conc ratio					Total ME conc (µg/mL)	Total mass (µg)		
No.	°C	Lipase %	MeOH ratio	Time (h)	Reactants volume (mL)	Mass of oil (g)	Myristate	Palmitate	Stearate	Oleic	Linoleic	Total Conc ratio*IS conc	Total Conc * reactants volume	Total mass (mg)	Total mass in 1 g of oil (mg/g)
1	40	1	1	24	41.73	17.06	0	1.427014	0.13338	0.894838	0.120557	25757.89	1074877	1074.877	63.02414
2	40	1	3.5	24	41.82	16.28	0.06665	2.845305	0.299287	1.844843	0.303915	53600.01	2241552	2241.552	137.6875
3	40	1	6	8	45.31	16.74	0.078716	3.541108	0.366056	2.124003	0.348121	64580.04	2926121	2926.121	173.7602
				16			0.087895	3.771546	0.395724	2.41408	0.394634	70638.79	3200644	3200.644	190.062
				24			0.096909	4.228637	0.453607	2.928401	0.514496	82220.51	3725412	3725.412	221.224
				32			0.091084	3.958728	0.411141	2.907558	0.520072	78885.84	3574317	3574.317	212.2516
				40			0.096124	4.638695	0.488555	3.613822	0.654098	94912.94	4300505	4300.505	235.3744
				48			0.096125	4.166576	0.443016	3.340211	0.63349	86794.18	3932644	3932.644	233.53
				56			0.0938	4.226858	0.43498	3.583196	0.703877	90427.11	4097252	4097.252	243.3048
				64			0.093466	4.310082	0.436868	3.704719	0.701301	92464.36	4189560	4189.56	248.7862
				72			0.085439	3.949816	0.391011	3.536448	0.681347	86440.62	3916624	3916.624	232.5786
4	30	1	3.5	24	43.6	16.972	0.06533	2.914141	0.307983	1.886359	0.332046	55058.59	2400554	2400.554	141.4421
5	40	1	3.5	24	43.2	16.28	0.06665	2.845305	0.299287	1.844843	0.303915	53600.01	2315520	2315.52	142.231
6	50	1	3.5	24	43.67	17.002	0.050277	2.269856	0.210021	1.260417	0.187019	39775.9	1737013	1737.013	102.1652
FCO															
1	40	1	1	24	40.94	16.94	0	1.296943	0.118103	0.911227	0.158622	24848.96	1017316	1017.316	60.07182
2	40	1	3.5	24	41.35	16.28	0.069441	2.825946	0.296429	2.02506	0.405031	56219.08	2324659	2324.659	142.7748
3	40	1	6	8	45.31	17.58	0.079321	3.457294	0.361344	2.368488	0.452375	67188.23	2963001	2963.001	168.5822
				16			0.093409	3.62867	0.381737	2.674601	0.532866	73112.84	3224276	3224.276	183.4477
				24			0.095694	3.848365	0.406883	2.919047	0.5947	78646.89	3468328	3468.328	197.3332
				32			0.082665	3.709324	0.341707	2.580485	0.536178	72503.58	3197408	3197.408	181.919
				40			0.102772	3.787198	0.402278	3.082219	0.646157	80206.23	3537095	3537.095	201.2457
				48			0.089533	3.620271	0.390713	3.179503	0.689979	79700	3514770	3514.77	199.9755
				56			0.085549	3.285042	0.347274	2.893303	0.642519	72536.88	3198876	3198.876	182.0025
				64			0.072906	2.925872	0.306232	2.671214	0.596532	65727.55	2898585	2898.585	164.9172
				72			0.111166	4.286857	0.465038	4.164602	0.948379	43253.53	1907481	1907.481	108.5276

APPENDIX A3

WCO							Conc ratio			Conc (µg/mL) = Total Conc ratio*IS conc			Total mass (mg)		
No.	**°C**	**Lipase %**	**MeOH ratio**	**Time (h)**	**Reactant volume (mL)**	**Mass of oil (g)**	**MG**	**DG**	**TG**	**MG**	**DG**	**TG**	**MG**	**DG**	**TG**
1	40	1	6	8	45.31	16.84	2.282799	0.32928	4.648475	18262.39	2634.237	37187.8	827.46889	119.3573	1684.979
				16			2.174177	0.247536	0	17393.42	1980.29	0	788.09582	89.72695	0
				24			1.57526	0	0	12602.08	0	0	571.00008	0	0
				32			1.440759	0	0	11526.07	0	0	522.24627	0	0
				40			1.221884	0	0	9775.07	0	0	442.90842	0	0
				48			1.213019	0	0	9704.152	0	0	439.69512	0	0
				56			1.075987	0	0	8607.898	0	0	390.02387	0	0
				64			1.048714	0	0	8389.709	0	0	380.13773	0	0
				72			0.910148	0	0	7281.181	0	0	329.91030	0	0
2	30	1	3.5	24	43.6	16.972	2.141582	0.635582	0	17132.65	5084.658	0	746.9837	221.6911	0
3	40	1	3.5	24	43.2	16.28	2.254978	0.286745	0	18039.82	2293.963	0	779.3203	99.09921	0
4	50	1	3.5	24	43.67	17.002	2.468782	0.823324	4.671521	19750.25	6586.596	37372.17	862.4935	287.6366	635.4016
FCO															
1	40	1	6	8	45.31	17.58	1.459596	0.102185	3.359341	11676.77	817.483	26874.72	514.94543	36.051	1185.175
				16			2.316015	0.193788	0	18528.12	1550.302	0	817.08993	68.36832	0
				24			1.939945	0.142883	0	15519.56	1143.063	0	684.41243	50.4091	0
				32			1.899	0.132	0	15192	1056	0	669.9672	46.5696	0
				40			1.568277	0	0	12546.22	0	0	553.2882	0	0
				48			1.336067	0	0	10688.53	0	0	471.36431	0	0
				56			1.50372	0	0	12029.76	0	0	530.51257	0	0
				64			1.094962	0	0	8759.696	0	0	386.30259	0	0
				72			1	0	0	8000	0	0	352.8	0	0

APPENDIX A4

APPENDIX A5

Calculation for ME content

Example:
MeOH to oil molar ratio 3.5:1, 1:1 (v/v) tert-butanol based on the volume of oil, 1% (w/w) of lipase to oil weight, and with stirring 200rpm at 40°C by FCO

ME content $= \frac{(\sum A) - A_{El}}{A_{El}} \times \frac{C_{El} \times V_{El}}{m} \times 100\%$

$= (\frac{950858.3 - 63916.48}{63916.48}) \times \frac{10 \times 0.178}{4} \times 100\%$

= 21.70%

APPENDIX A6

Calculation for Acid Value

Example: FCO

$$F = \frac{1000W_{KHP}}{204.23V_{eq}}$$

$$AV = \frac{56.1 \times M \times F \times (A - B)}{W}$$

$$= \frac{56.1 \times 0.1 \times 0.084 \times (9.56 - 6.8)}{3.82g}$$

$$= 0.34$$

Calculation for Iodine Value

Example: FCO

$$\mathbf{IV} = \frac{(B - S) \times M \times 12.69}{wt}$$

$$= \frac{(38.1 - 15.2) \times 0.1 \times 12.69}{0.52g}$$

$$= 55.88$$

Calculation for Saponification Value

Example: FCO

$$\mathbf{SV} = \frac{56.1(B - S) \times N}{g}$$

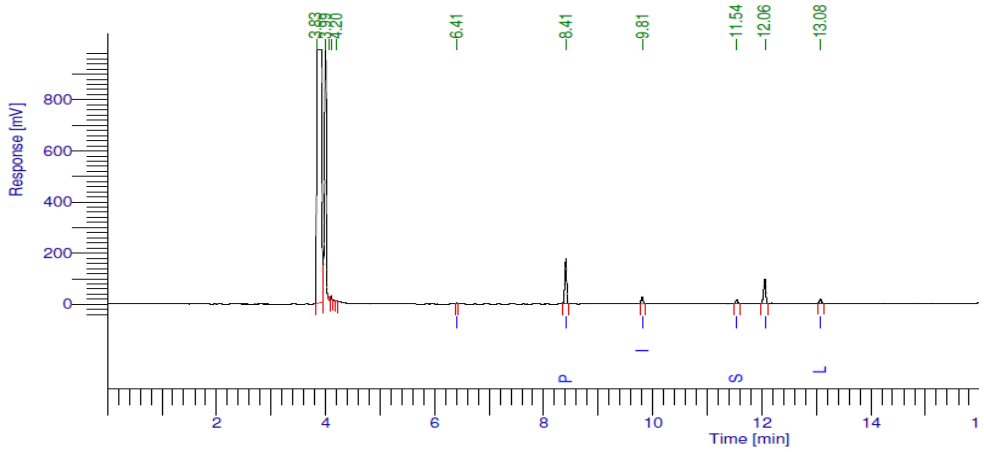
$$= \frac{56.1(46.0 - 0.00) \times 0.5}{6.6}$$

$$= 195.5$$

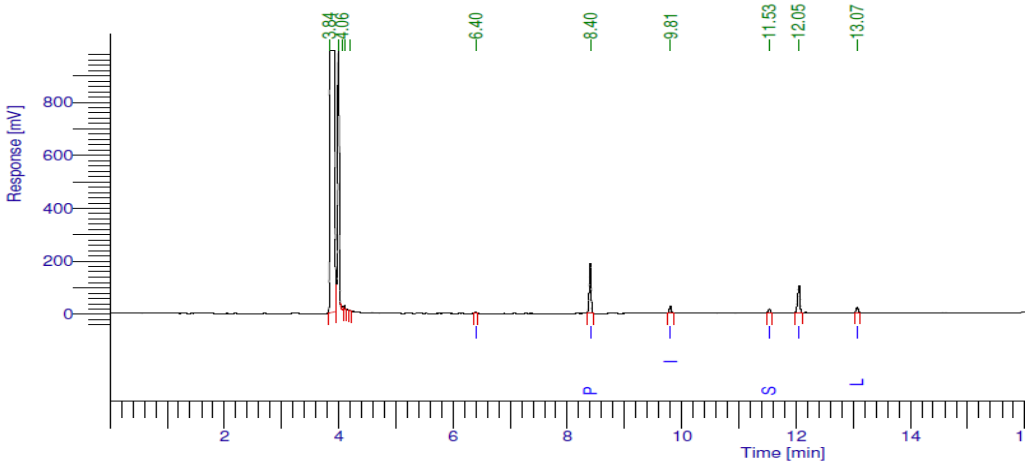
APPENDIX B1

ME content of FCO using chitosan immobilized TL with the condition of MeOH to oil molar ratio 6:1, 1:1 (v/v) tert-butanol based on the volume of oil, 1% (w/w) of lipase to oil weight, and with stirring 200rpm at 40°C based on Figure 4.6.

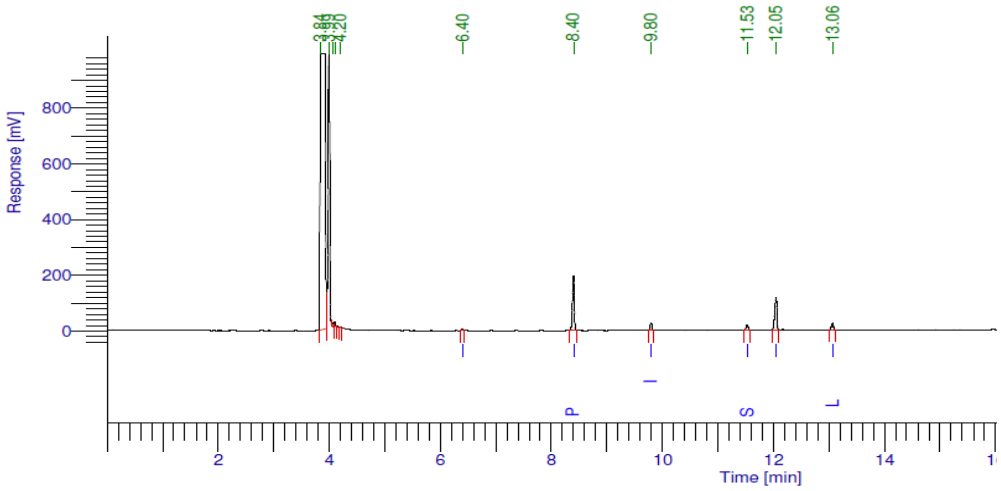
(1) At 8H



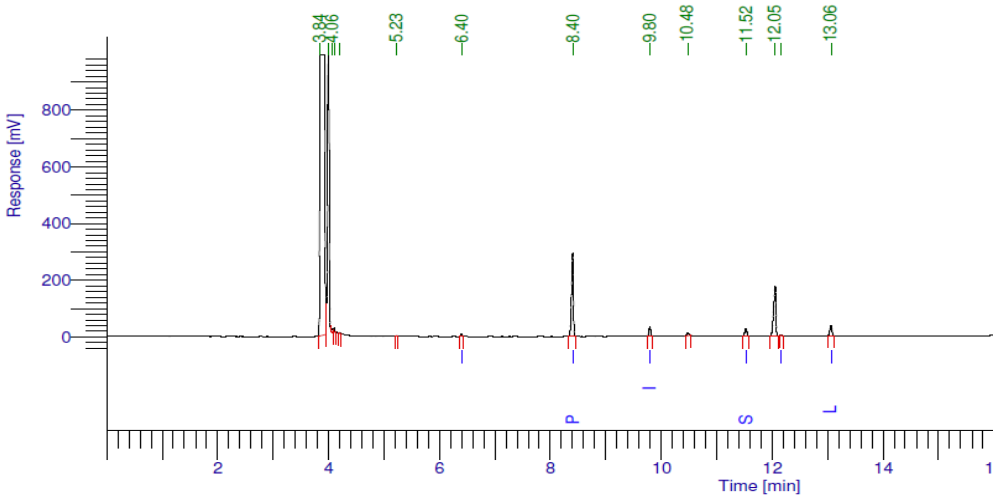
(2) At 16H



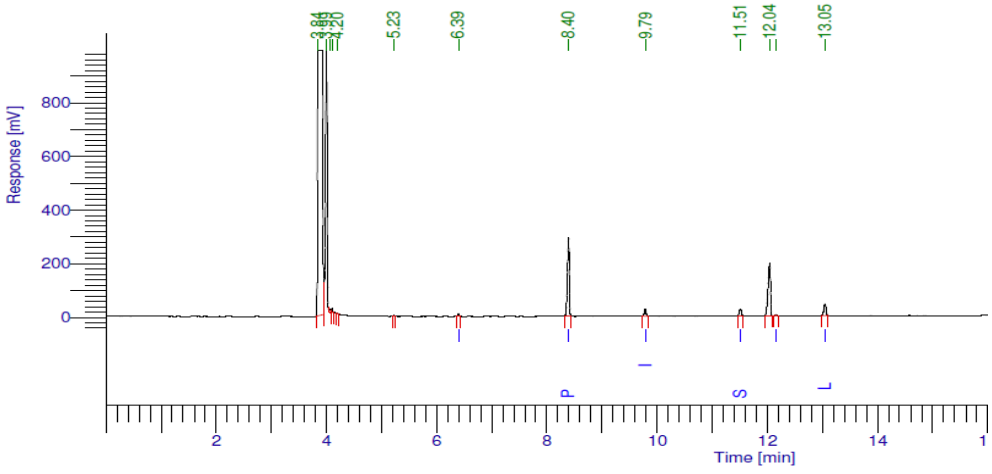
(3) At 24H



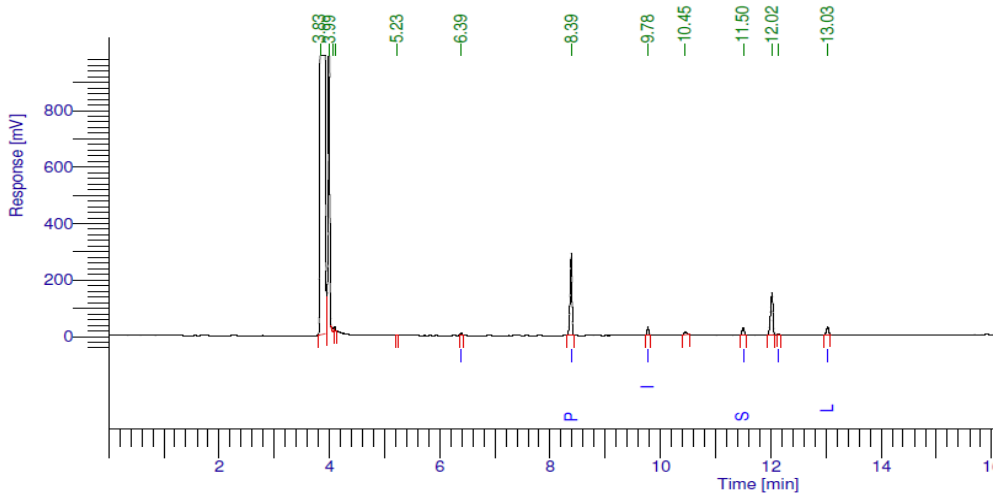
(4) At 32H



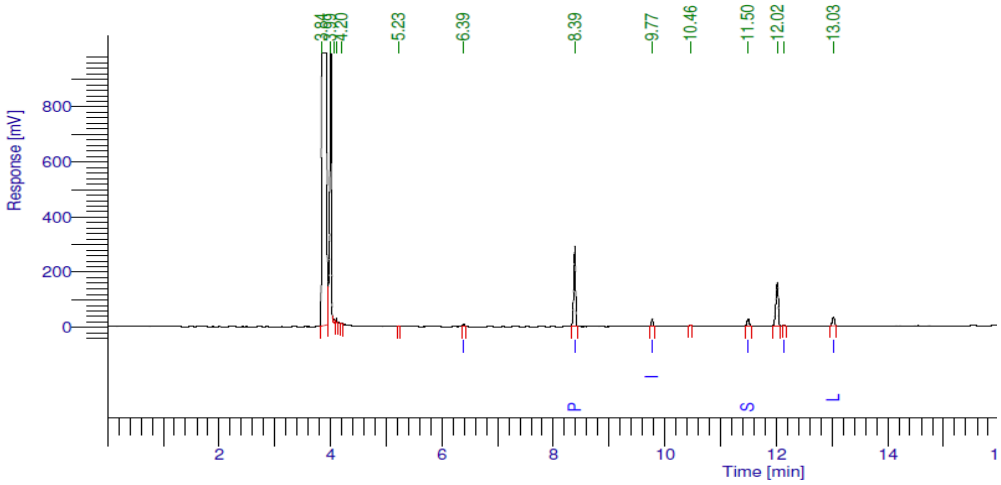
(5) At 48H



(6) At 56H



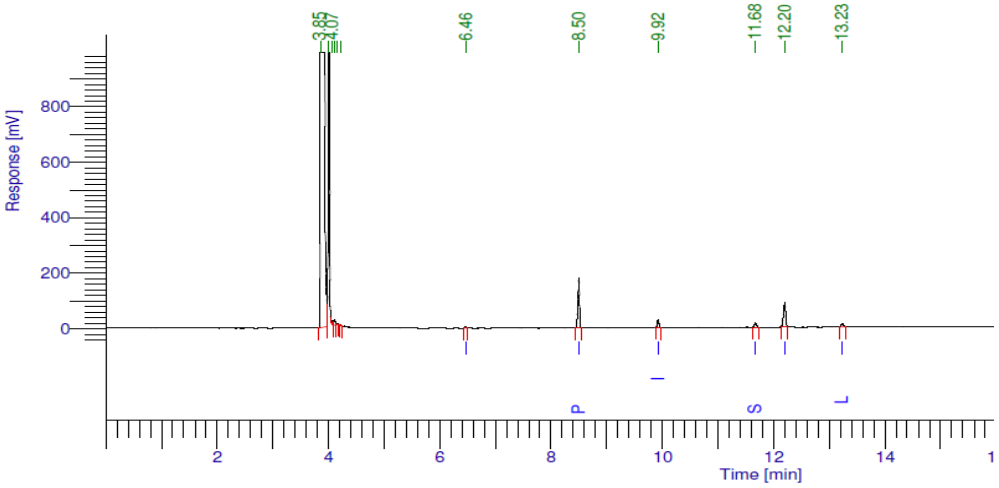
(7) At 72H



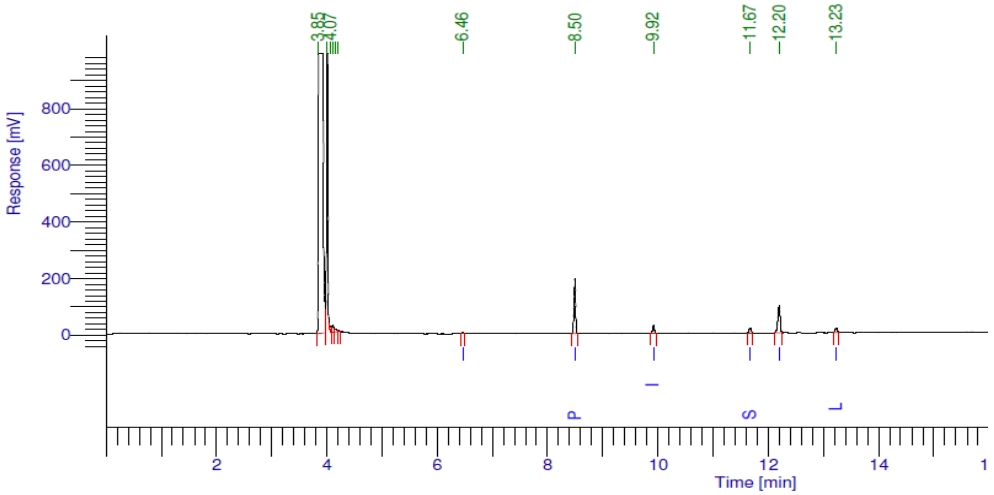
APPENDIX B2

ME content of WCO using chitosan immobilized TL with the condition of MeOH to oil molar ratio 6:1, 1:1 (v/v) tert-butanol based on the volume of oil, 1% (w/w) of lipase to oil weight, and with stirring 200rpm at 40°C based on Figure 4.6.

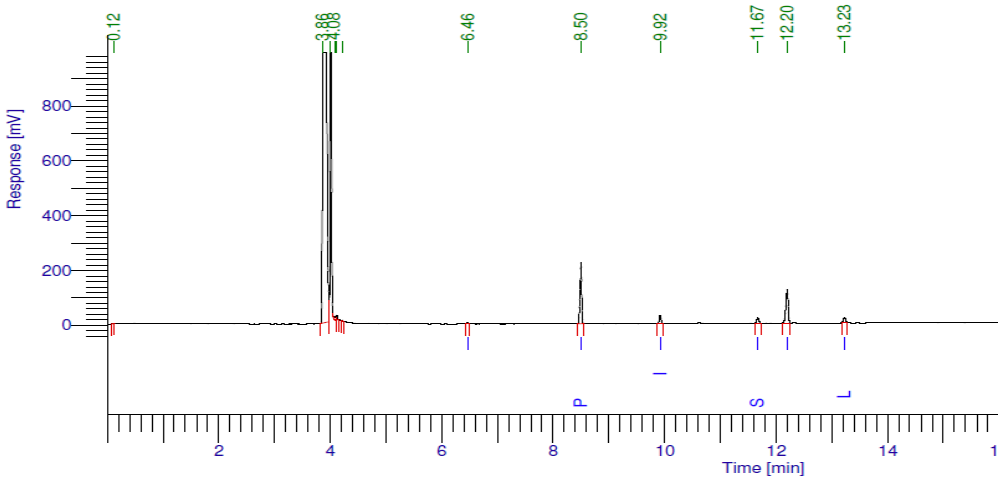
(1) At 8H



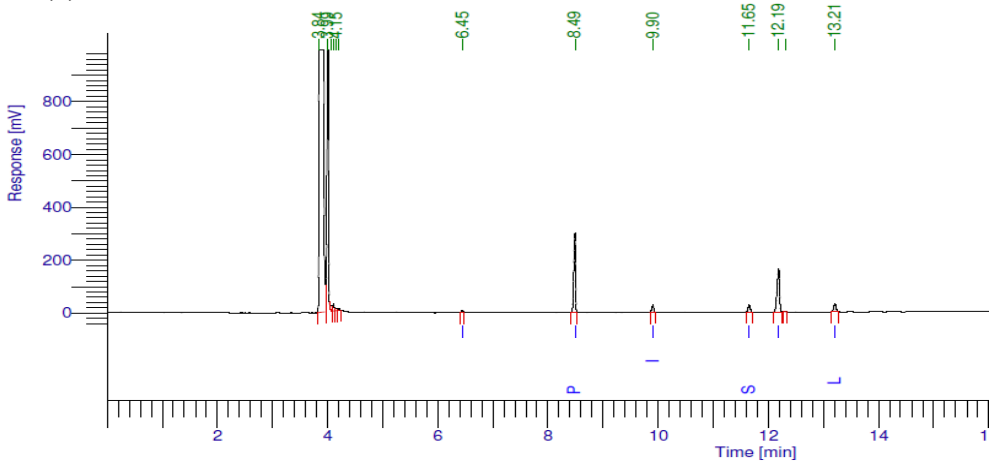
(2) At 16H



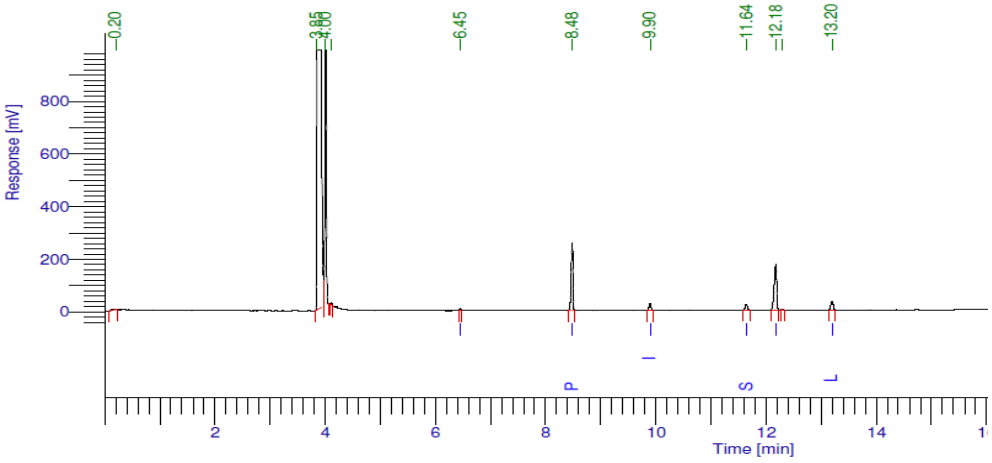
(3) At 24H



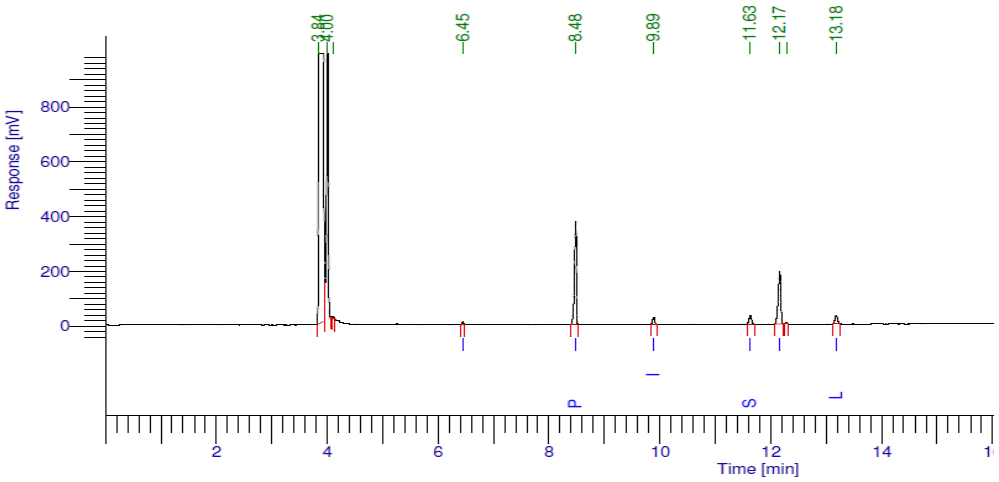
(4) At 32H



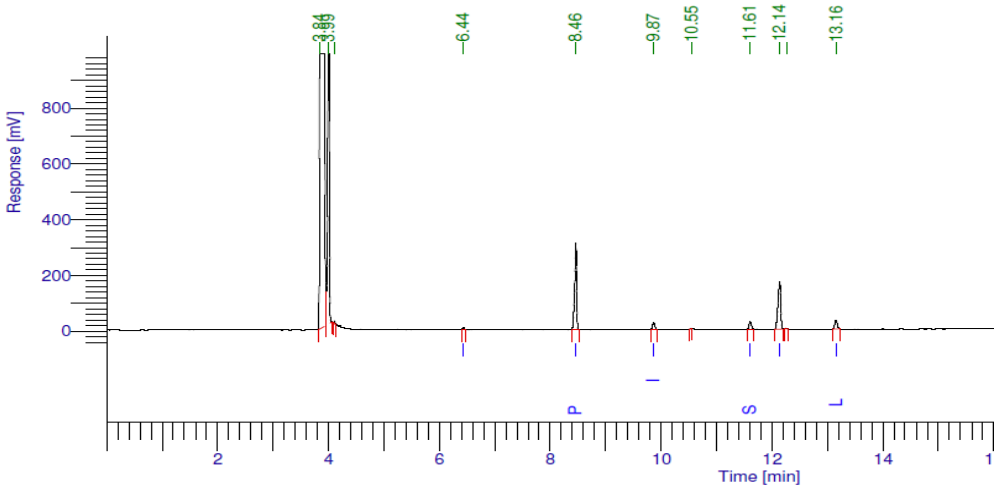
(5) At 48H



(6) At 56H



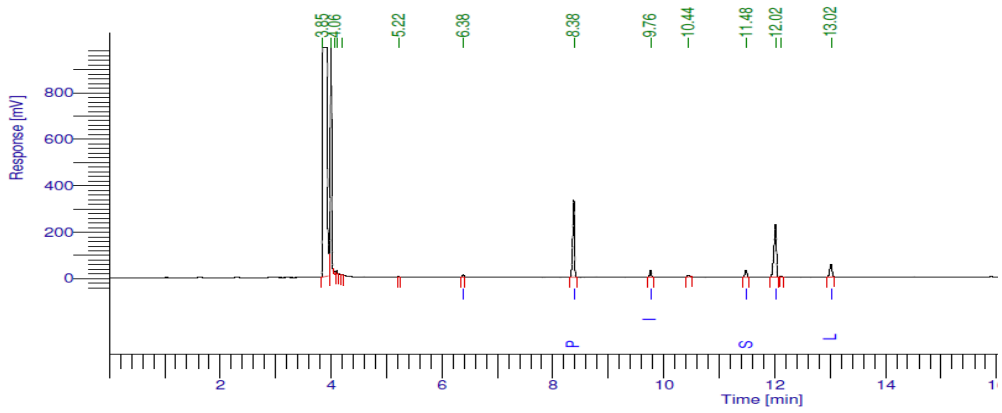
(7) At 72H



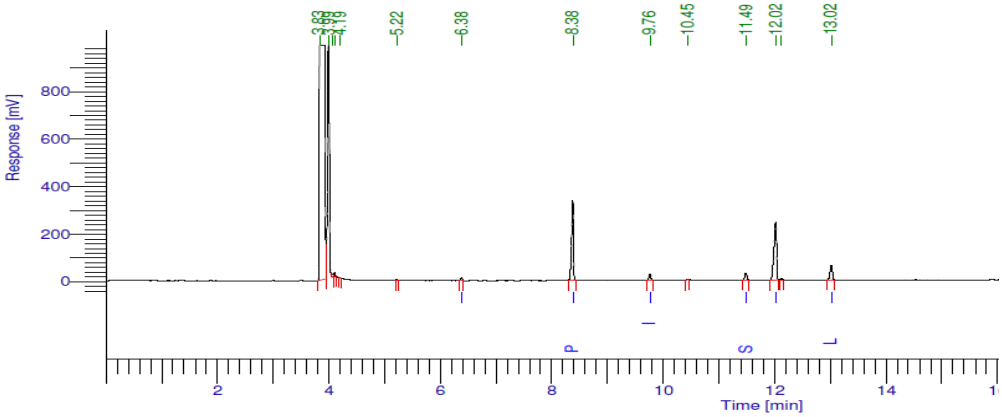
APPENDIX B3

ME content of FCO using commercial TLIM with the condition of MeOH to oil molar ratio 6:1, 1:1 (v/v) tert-butanol based on the volume of oil, 10% (w/w) of lipase to oil weight, and with stirring 200rpm at 40°C based on Figure 4.8.

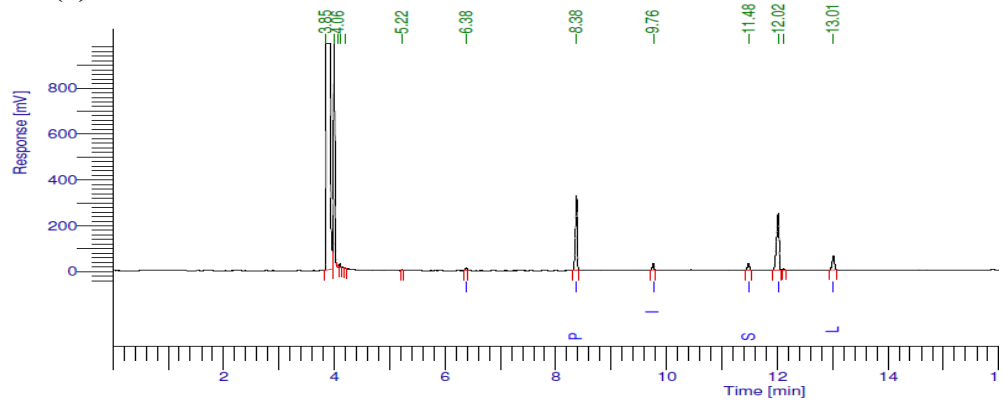
(1) At 8H



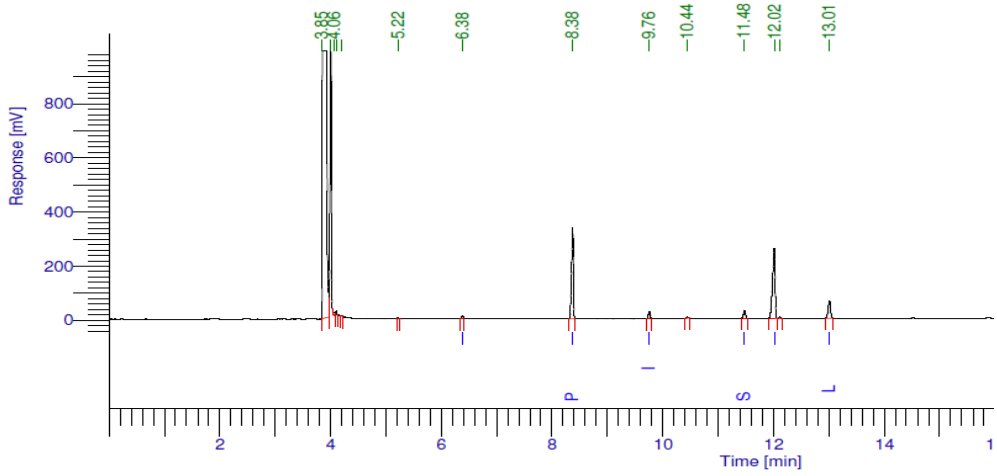
(2) At 16H



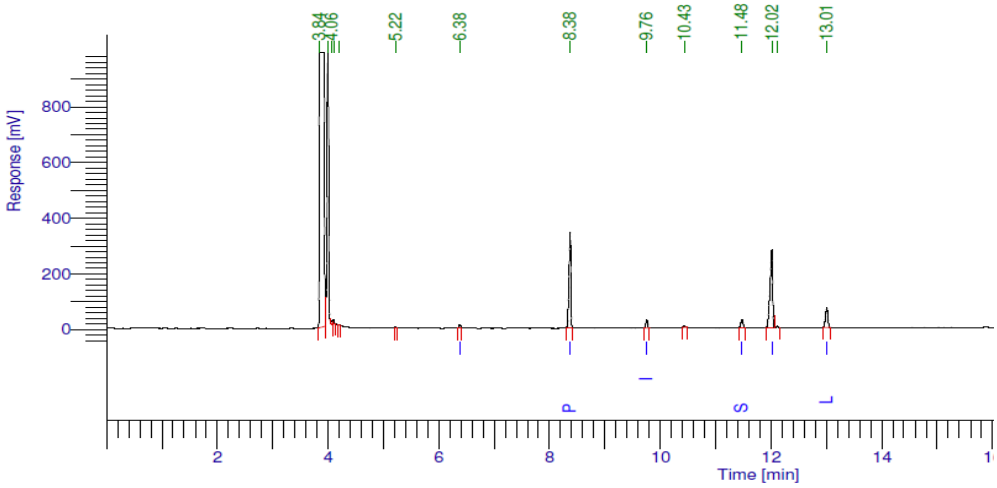
(3) At 24H



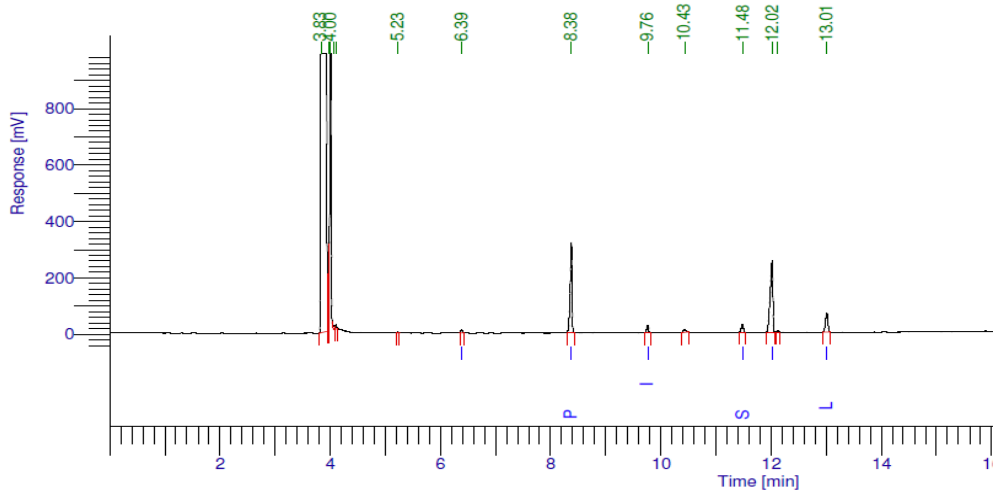
(4) At 32H



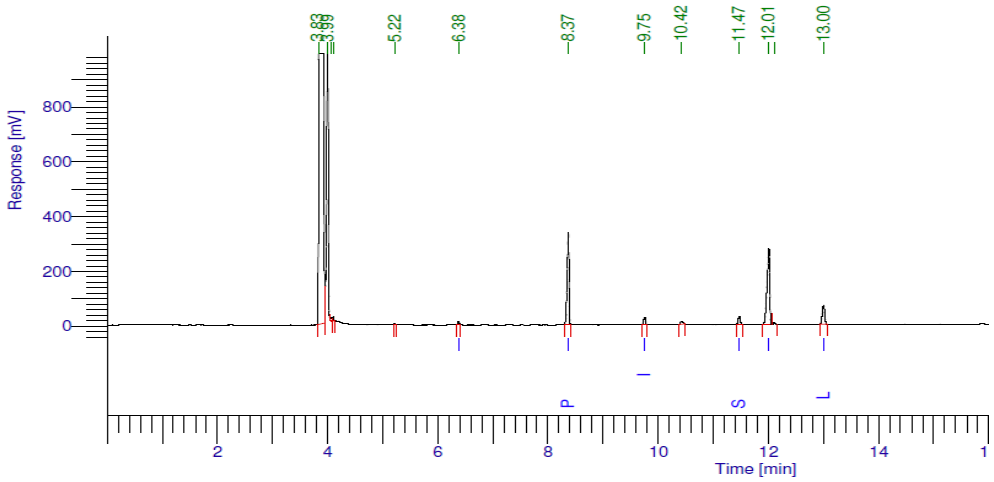
(5) At 40H



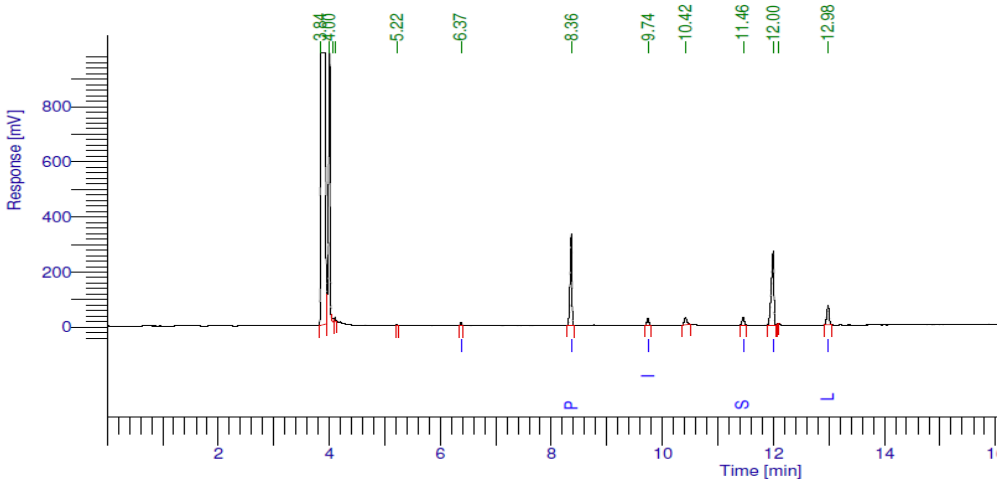
(6) At 48H



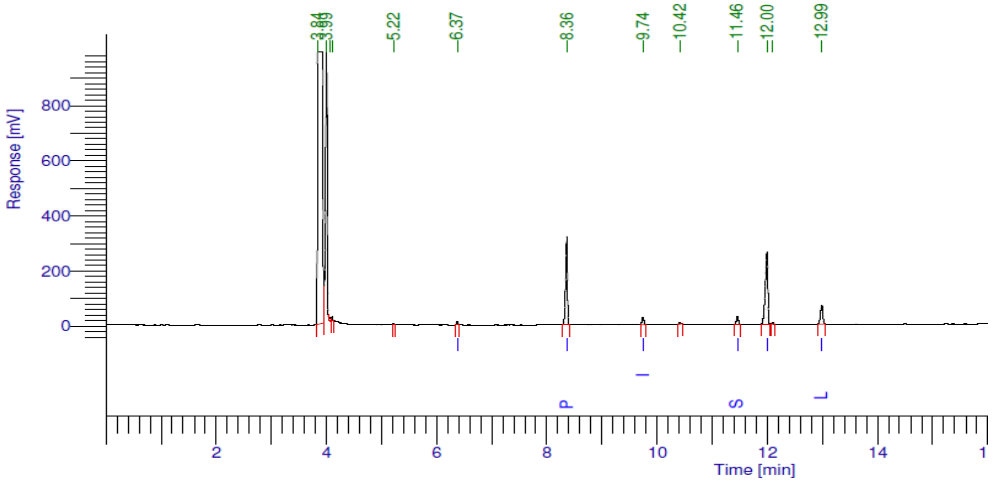
(7) At 56H



(8) At 64H



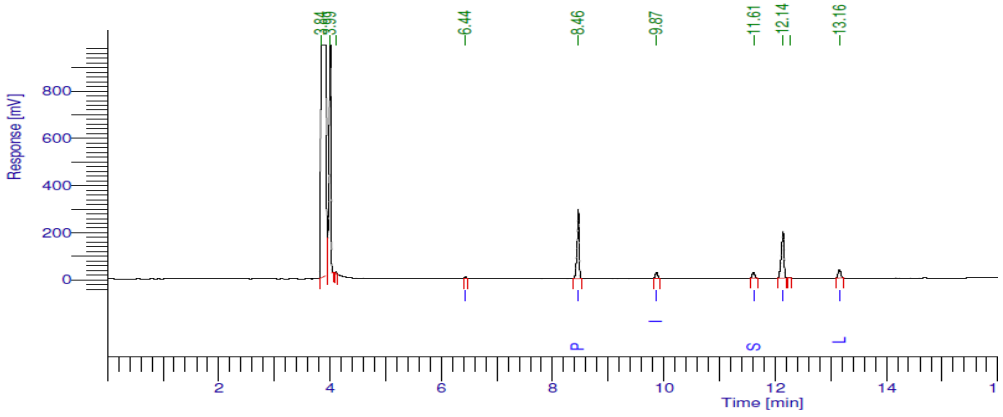
(9) At 72H



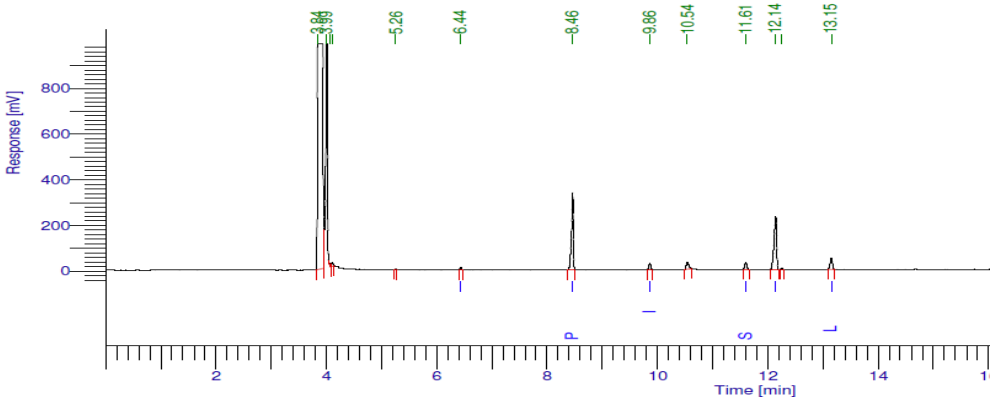
APPENDIX B4

ME content of WCO using commercial TLIM with the condition of MeOH to oil molar ratio 6:1, 1:1 (v/v) tert-butanol based on the volume of oil, 10% (w/w) of lipase to oil weight, and with stirring 200rpm at 40°C based on Figure 4.8.

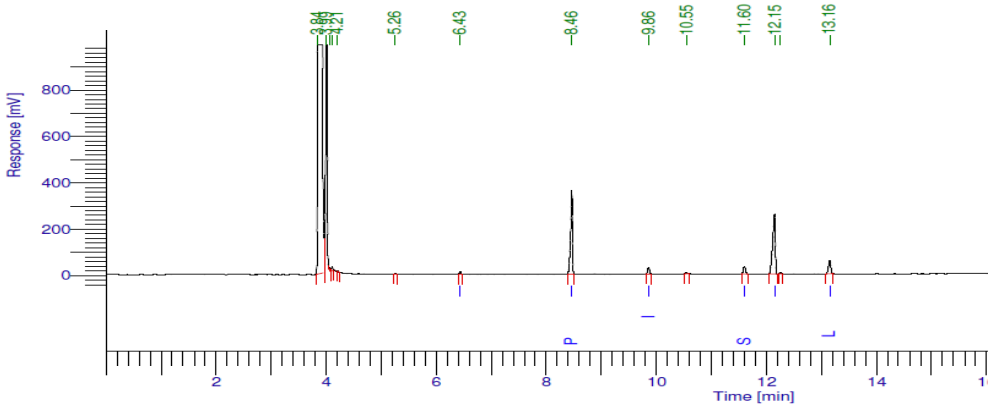
(1) At 8H



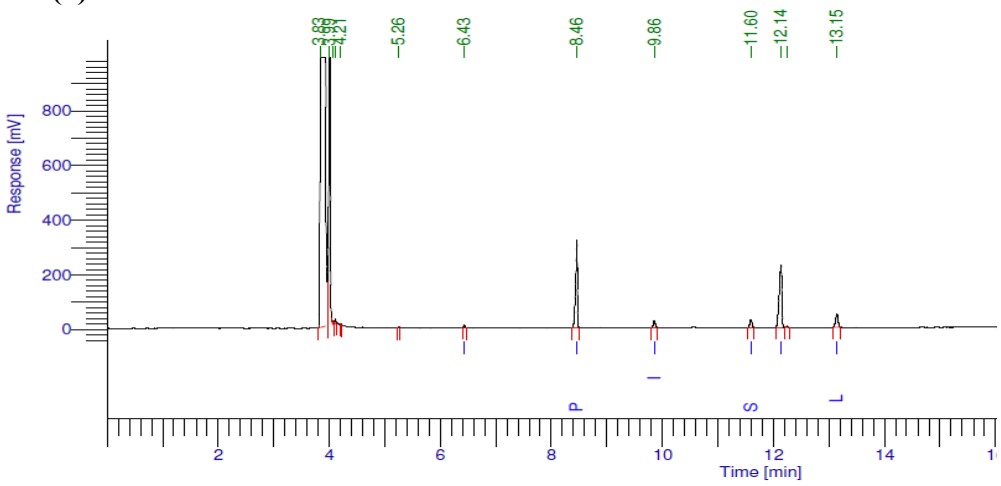
(2) At 16H



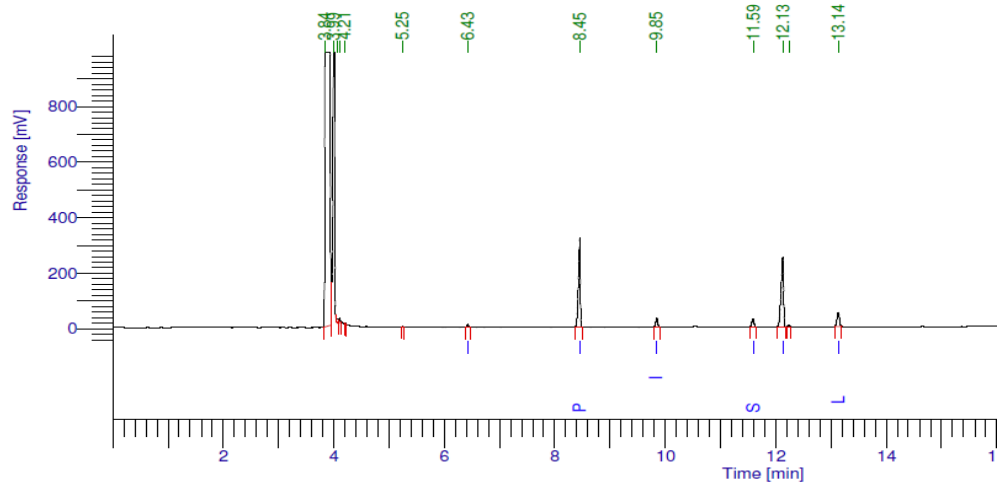
(3) At 24H



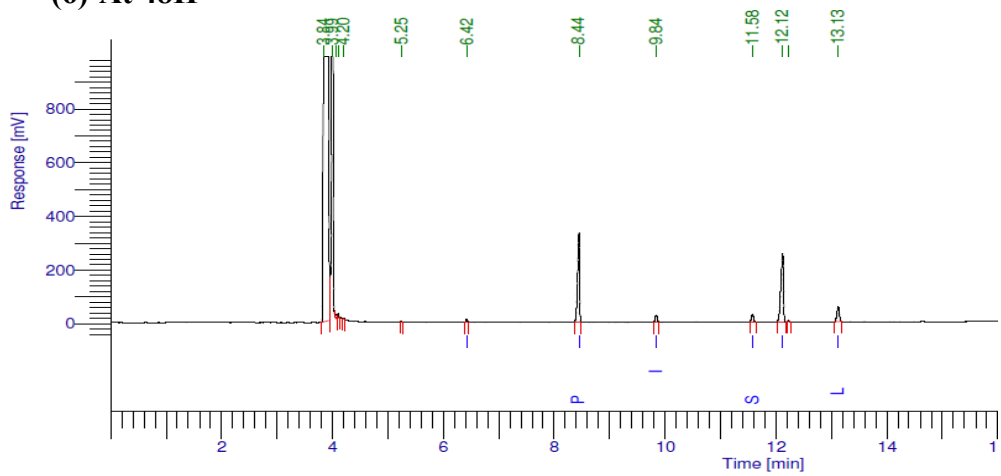
(4) At32H



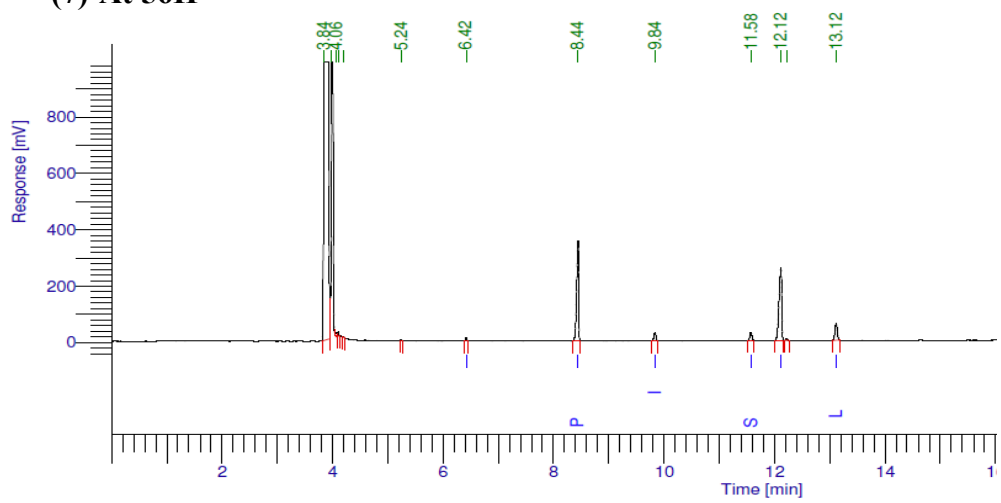
(5) At 40H



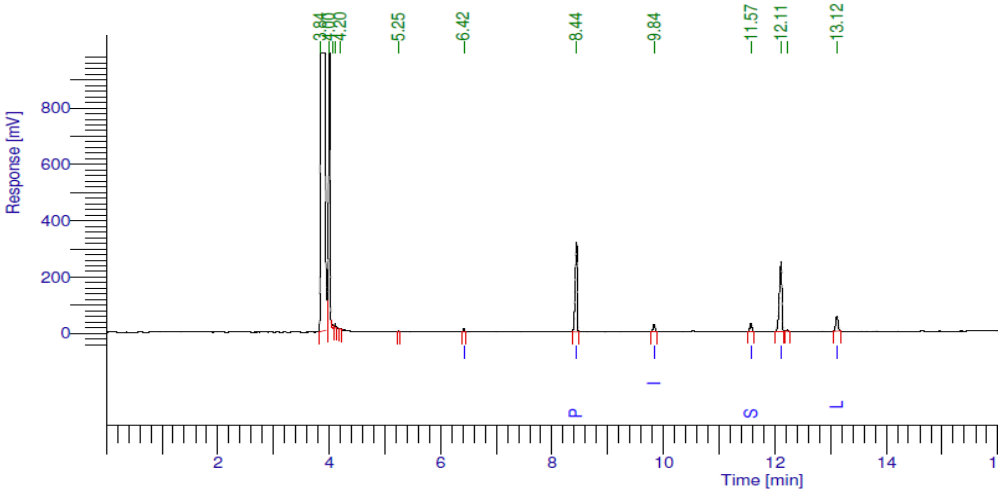
(6) At 48H



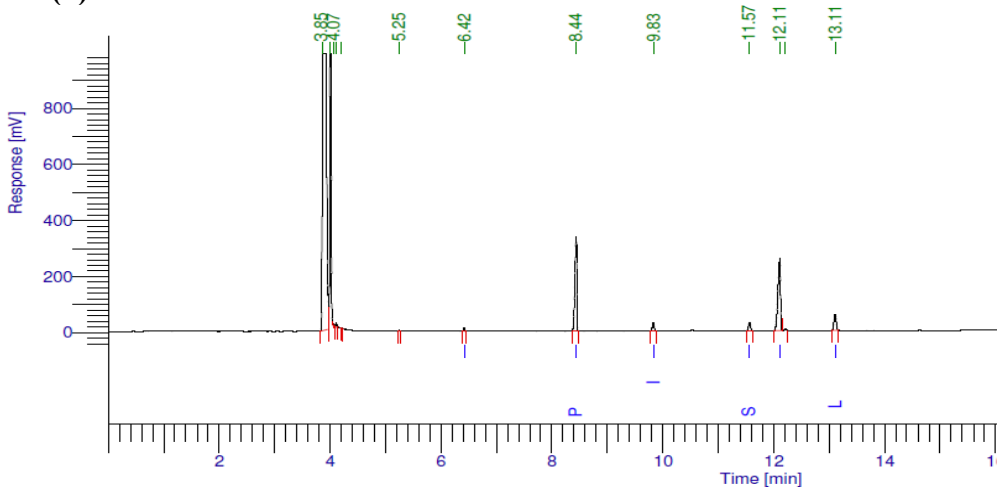
(7) At 56H



(8) At 64H



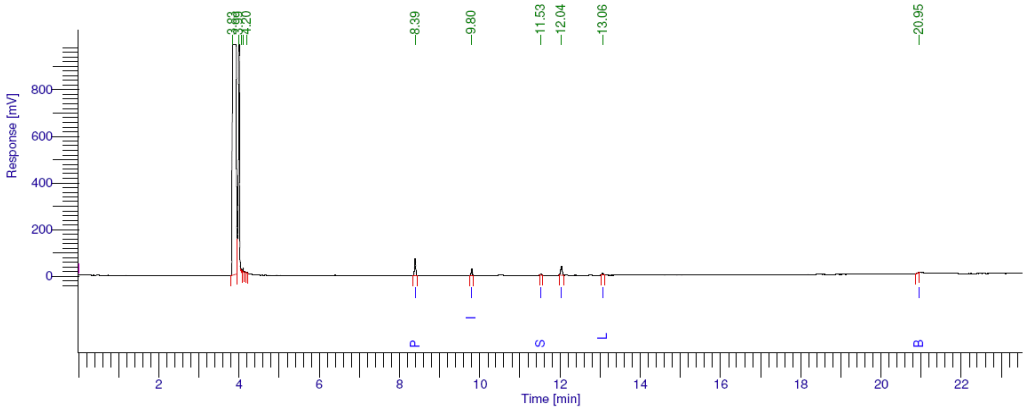
(9) At 72H



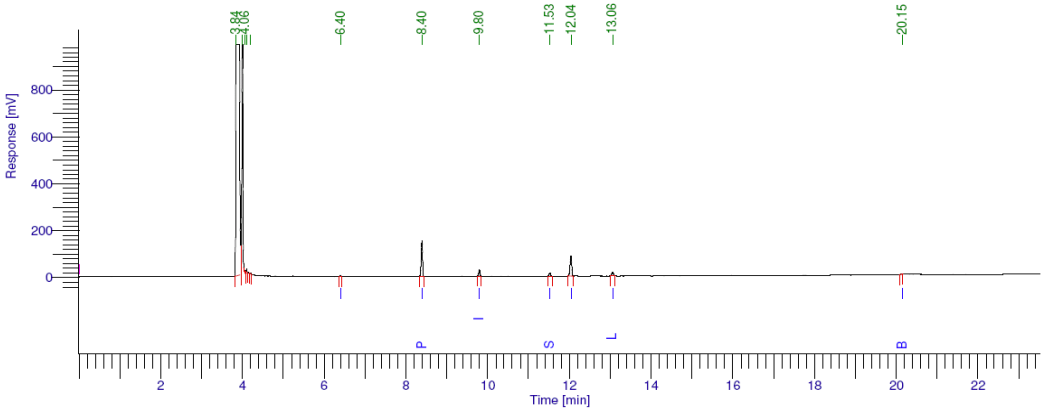
APPENDIX B5

ME content of FCO using chitosan immobilized TL with the condition of 1:1 (v/v) tert-butanol based on the volume of oil, 1% (w/w) of lipase to oil weight, and with stirring 200rpm at 40°C based on Figure 4.9.

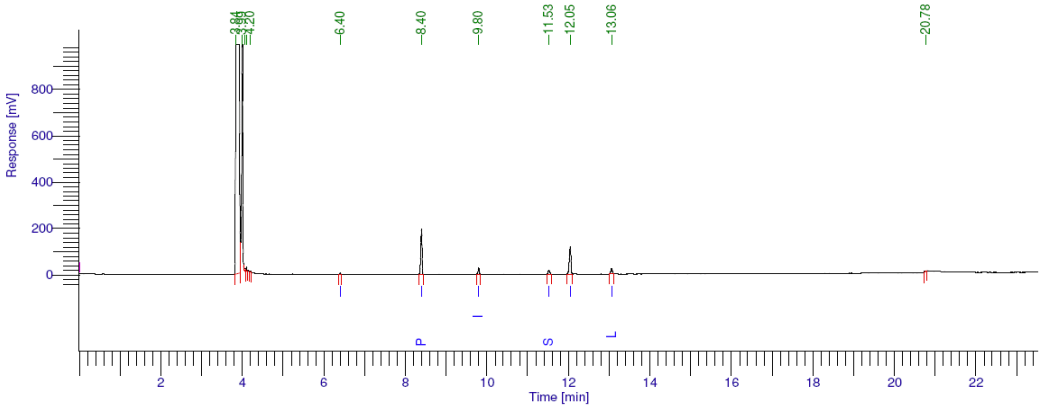
(1) In the condition of MeOH ratio to oil at 1:1, 1:1 *tert*-butanol to oil volume, 1% (w/w) dosage of enzyme to oil weight at 40°C at 24 h.



(2) In the condition of MeOH ratio to oil at 3.5:1, 1:1 *tert*-butanol to oil volume, 1% (w/w) dosage of enzyme to oil weight at 40°C at 24 h



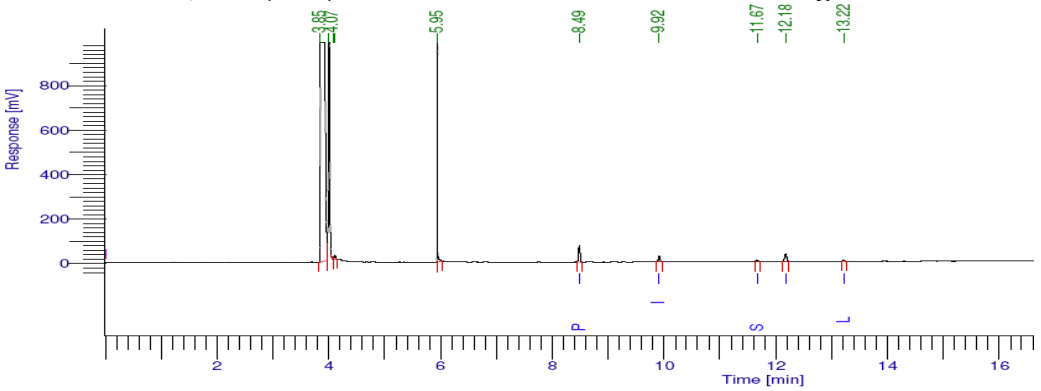
(3) In the condition of MeOH ratio to oil at 6:1, 1:1 *tert*-butanol to oil volume, 1% (w/w) dosage of enzyme to oil weight at 40°C at 24 h



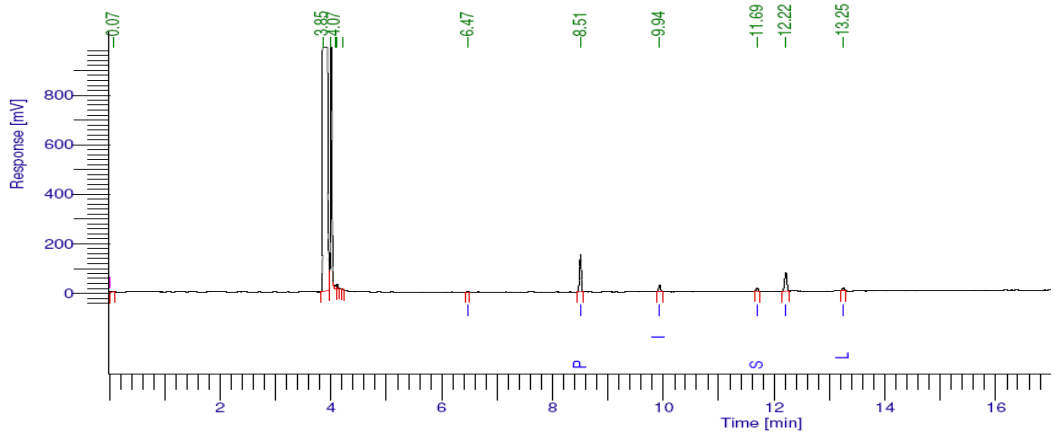
APPENDIX B6

ME content of WCO using chitosan immobilized TL with the condition of 1:1 (v/v) tert-butanol based on the volume of oil, 1% (w/w) of lipase to oil weight, and with stirring 200rpm at 40°C based on Figure 4.10.

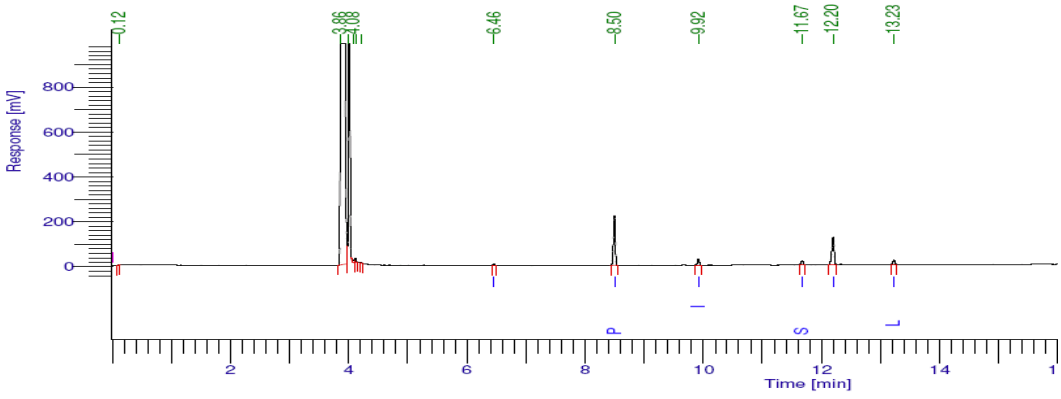
(1) In the condition of MeOH ratio to oil at 1:1, 1:1 *tert*-butanol to oil volume, 1% (w/w) chitosan immobilised TL to oil weight



(2) In the condition of MeOH ratio to oil at 3.5:1, 1:1 *tert*-butanol to oil volume, 1% (w/w) chitosan immobilised TL to oil weight



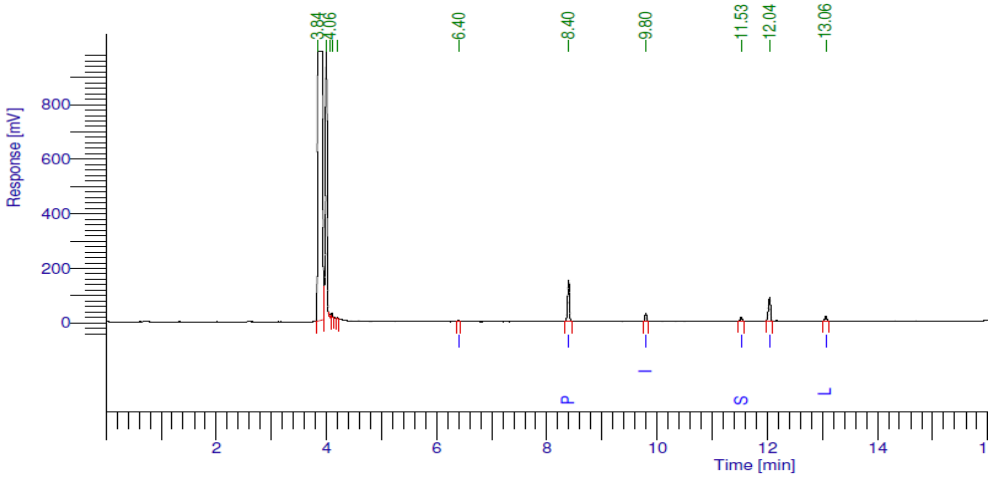
(3) In the condition of MeOH ratio to oil at 6:1, 1:1 *tert*-butanol to oil volume, 1% (w/w) chitosan immobilised TL to oil weight



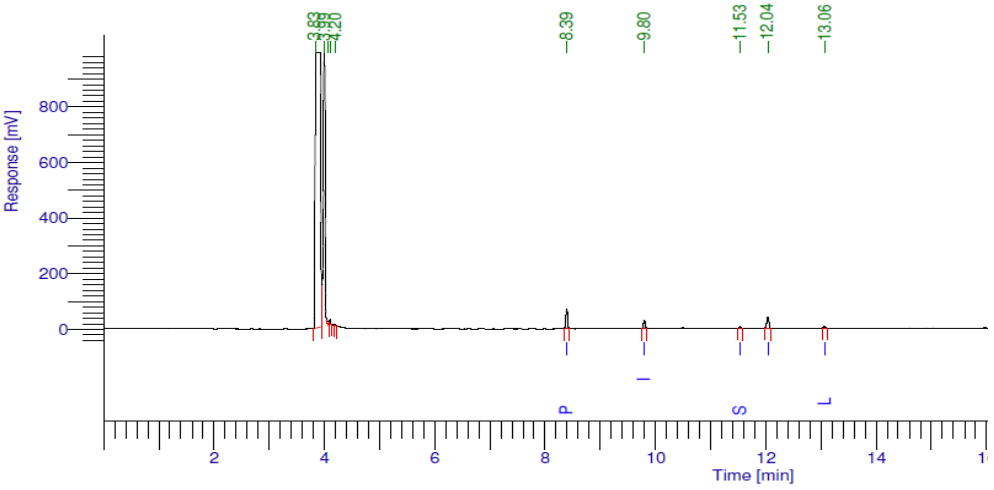
APPENDIX B7

Effect of MeOH/Oil molar ratio on transesterification reaction with 1% (w/w) chitosan immobilised lipase or 10% (w/w) TLIM, 1:1 tert-butanol to oil, and with stirring 200rpm at 40ºC for 24 h °based on Figure 4.11.

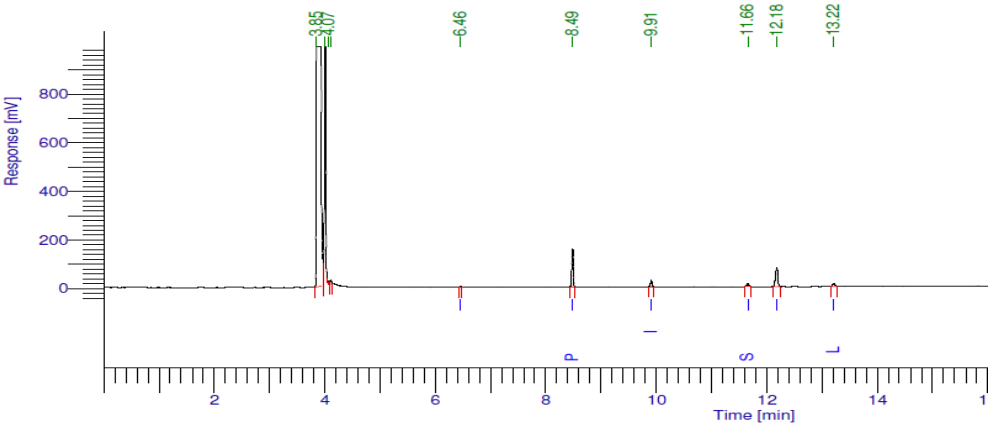
(1) In the condition of MeOH ratio to oil at 1:1, 1:1 *tert*-butanol to FCO volume, 1% (w/w) chitosan immobilised TL to oil weight



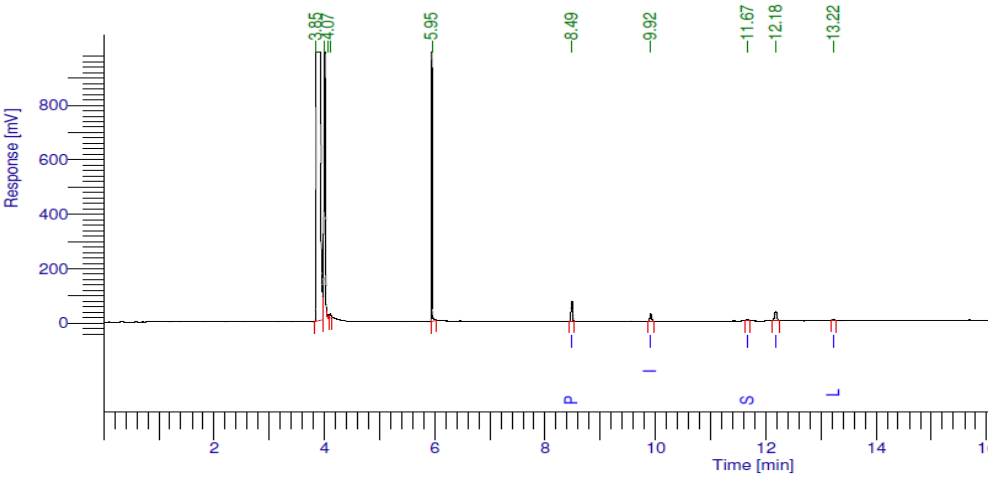
(2) In the condition of MeOH ratio to oil at 3.5:1, 1:1 *tert*-butanol to FCO volume, 1% (w/w) chitosan immobilised TL to oil weight



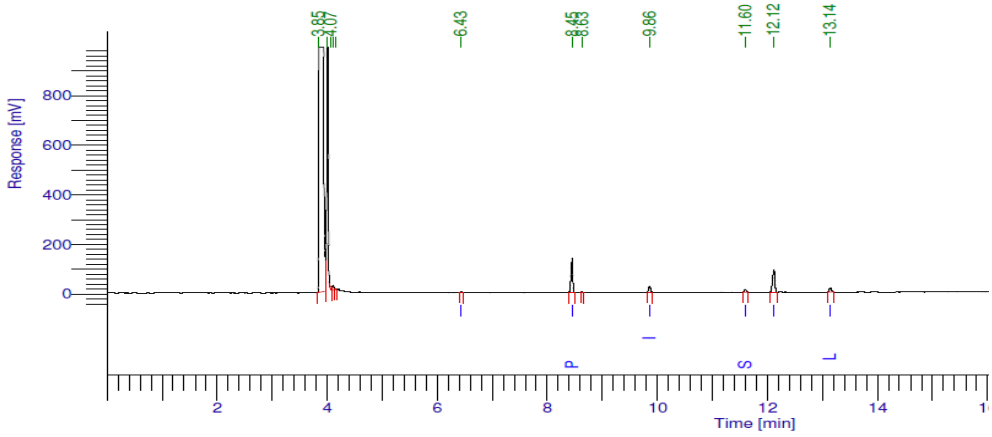
(3) In the condition of MeOH ratio to oil at 1:1, 1:1 *tert*-butanol to WCO volume, 1% (w/w) chitosan immobilised TL to oil weight



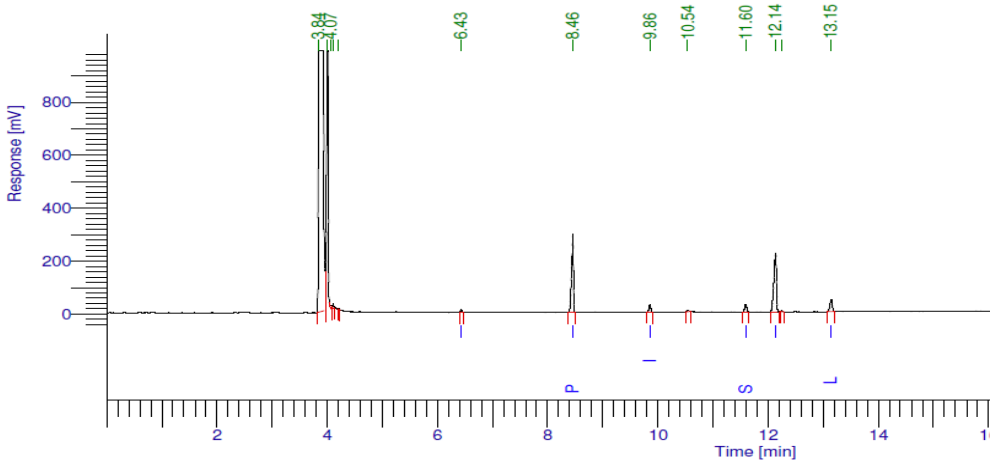
(4) In the condition of MeOH ratio to oil at 3.5:1, 1:1 *tert*-butanol to WCO volume, 1% (w/w) chitosan immobilised TL to oil weight



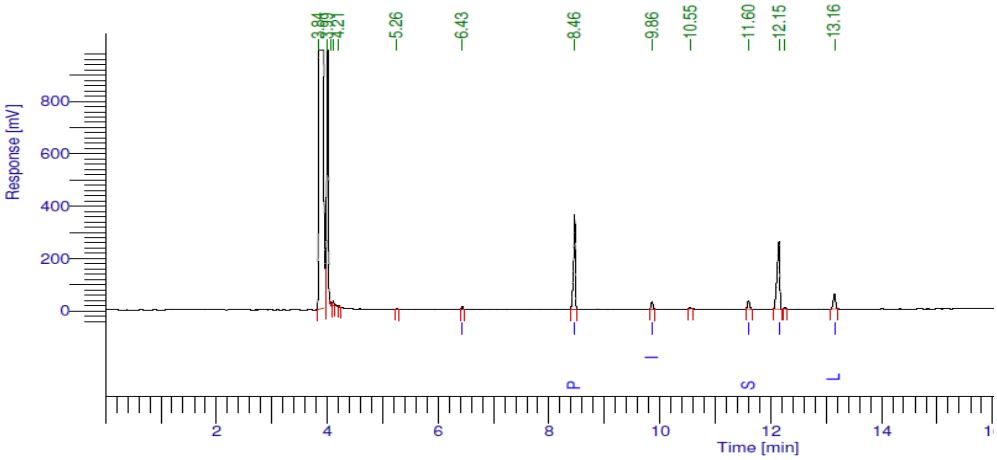
(5) In the condition of MeOH ratio to oil at 1:1, 1:1 *tert*-butanol to WCO volume, 10% (w/w) dosage of TLIM to oil weight



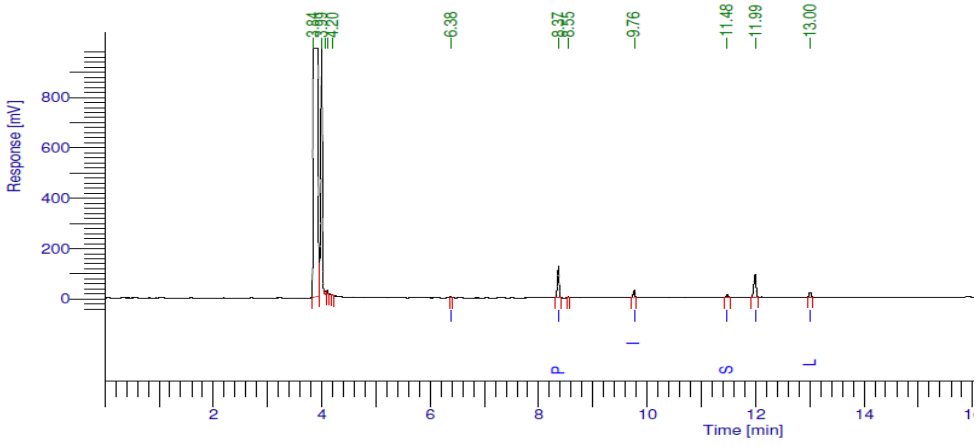
(6) In the condition of MeOH ratio to oil at 3.5:1, 1:1 *tert*-butanol to WCO volume, 1% (w/w) dosage of TLIM enzyme to oil weight



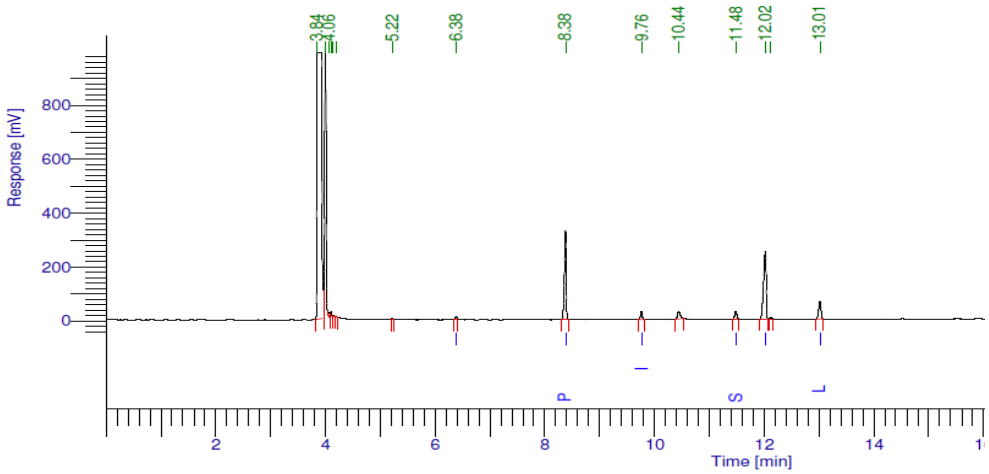
(7) In the condition of MeOH ratio to oil at 6:1, 1:1 *tert*-butanol to WCO volume, 10% (w/w) dosage of TLIM to oil weight



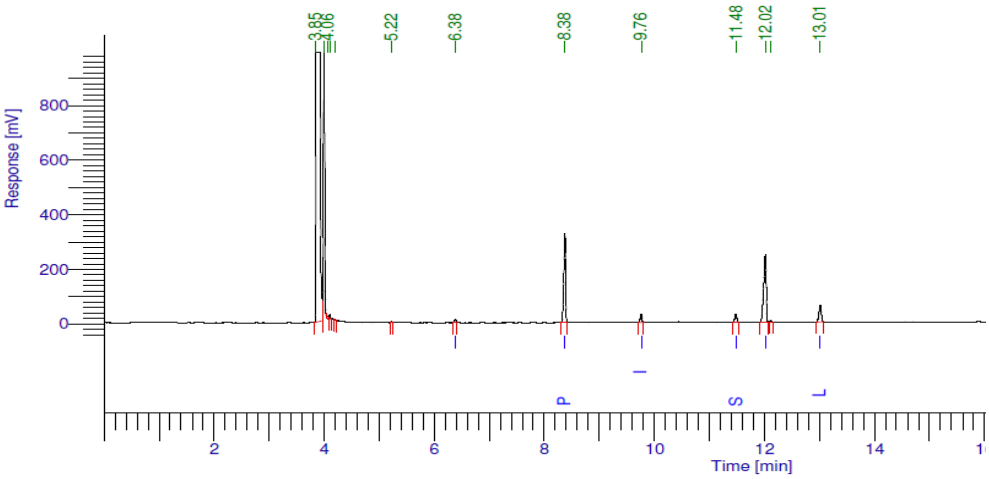
(8) In the condition of MeOH ratio to oil at 1:1, 1:1 *tert*-butanol to FCO volume, 10% (w/w) dosage of TLIM to oil weight



(9) In the condition of MeOH ratio to oil at 3.5:1, 1:1 *tert*-butanol to FCO volume, 10% (w/w) dosage of TLIM to oil weight



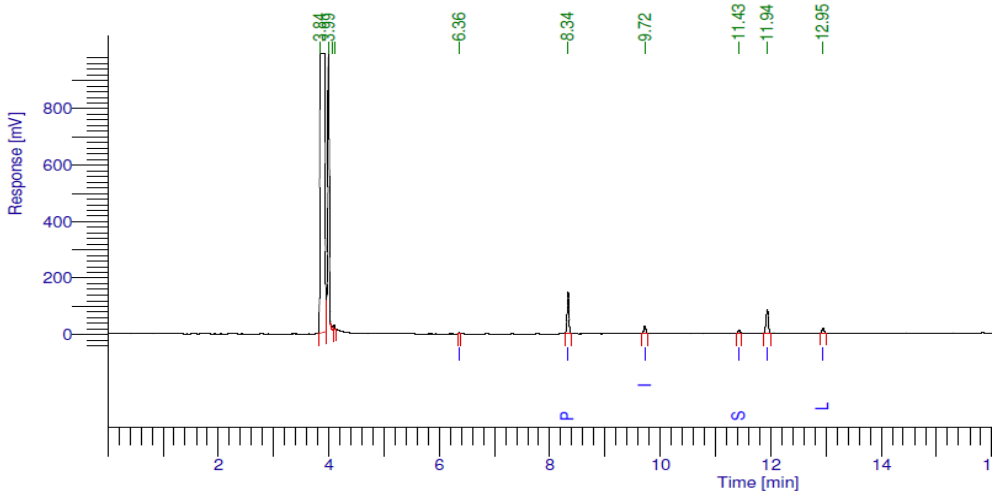
(10) In the condition of MeOH ratio to oil at 6:1, 1:1 *tert*-butanol to FCO volume, 10% (w/w) dosage of TLIM to oil weight



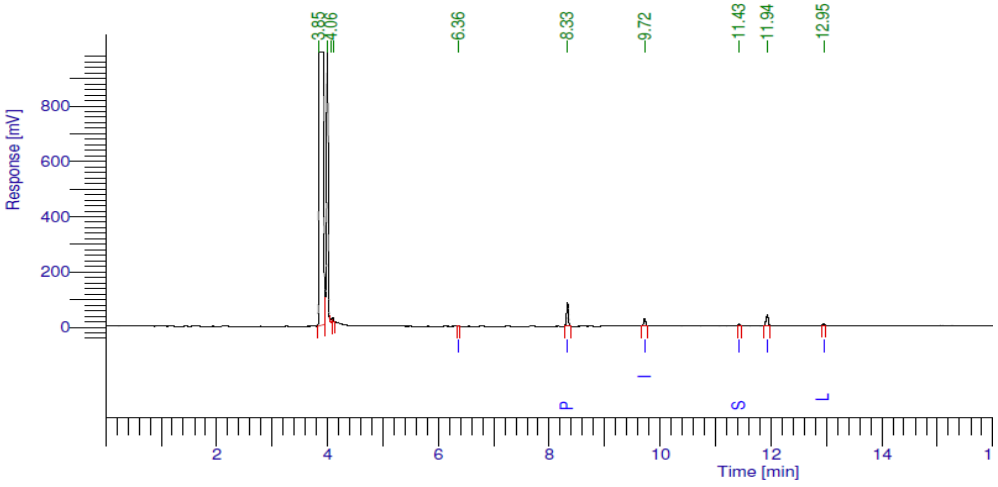
APPENDIX B8

Effect of temperature to transesterification in FCO and WCO with the condition of MeOH ratio of 3.5 to oil, 1:1 *tert*-butanol to oil, 1% (w/w) dosage of enzyme to oil with stirring at 200rpm for 24 h based on Figure 4.12

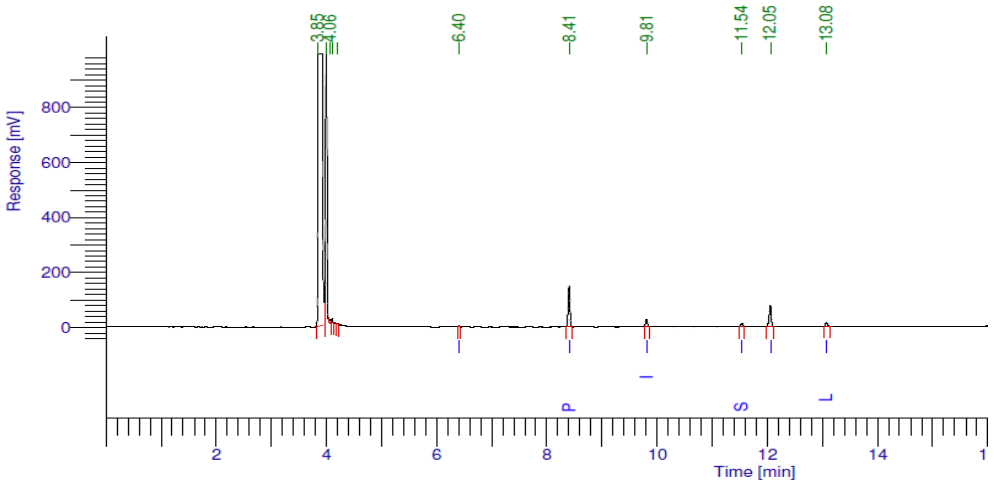
(1) FCO At 30ºC



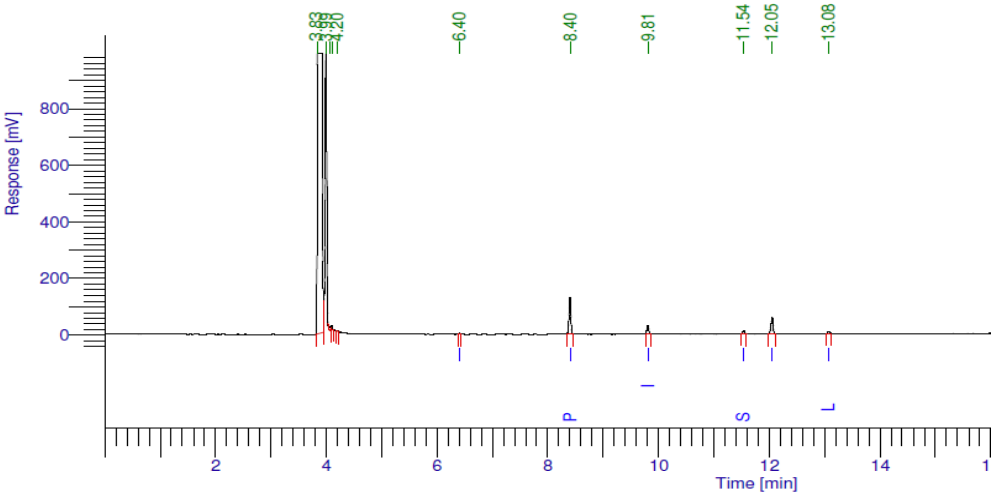
(2) FCO At 50ºC



(3) WCO At 30ºC



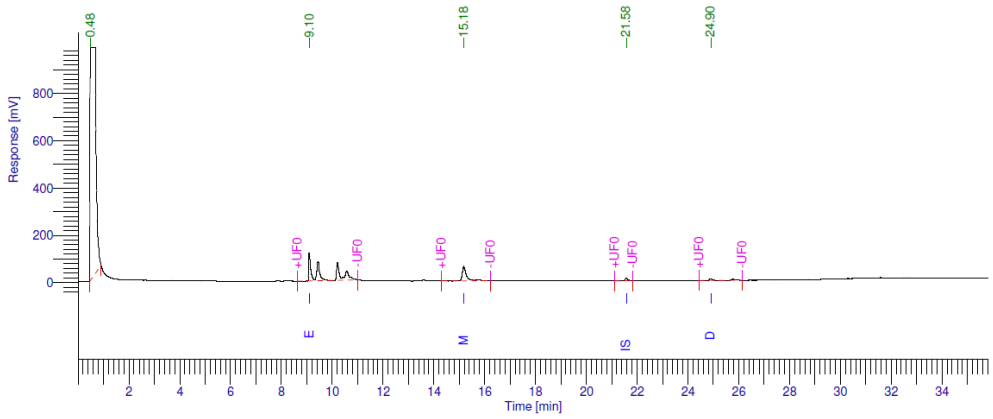
(4) WCO At 50ºC



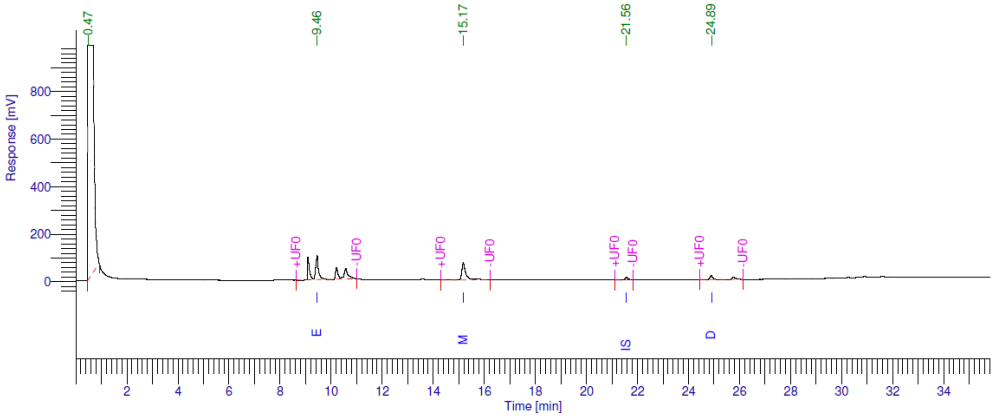
APPENDIX B9

Product composition including TG, DG, MG, glycerols and ME for the reaction mixture of WCO in the condition of MeOH ratio to oil at 3.5:1, 1:1 *tert*-butanol to oil volume, 1% (w/w) chitosan immobilised TL to oil weight at 40°C at 24 h based on Figure 4.13.

(1) WCO At 30°C



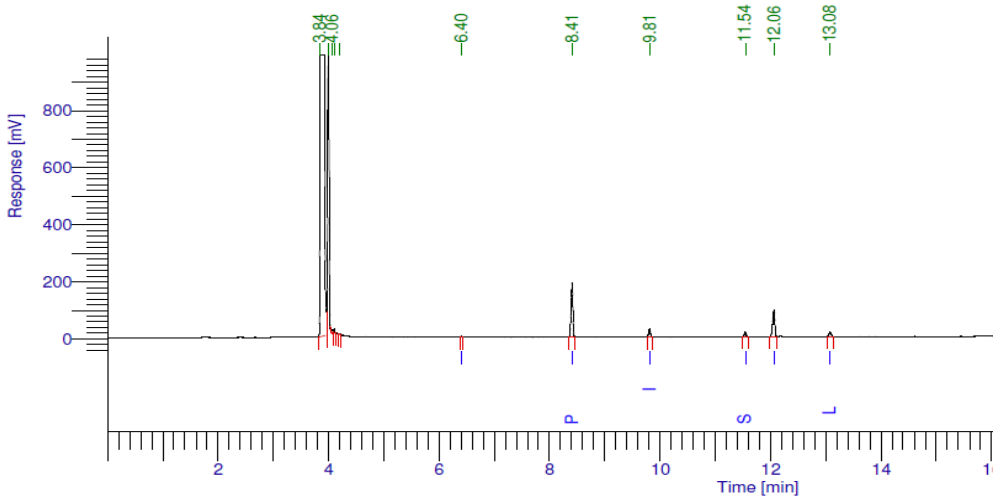
(2) WCO At 50°C



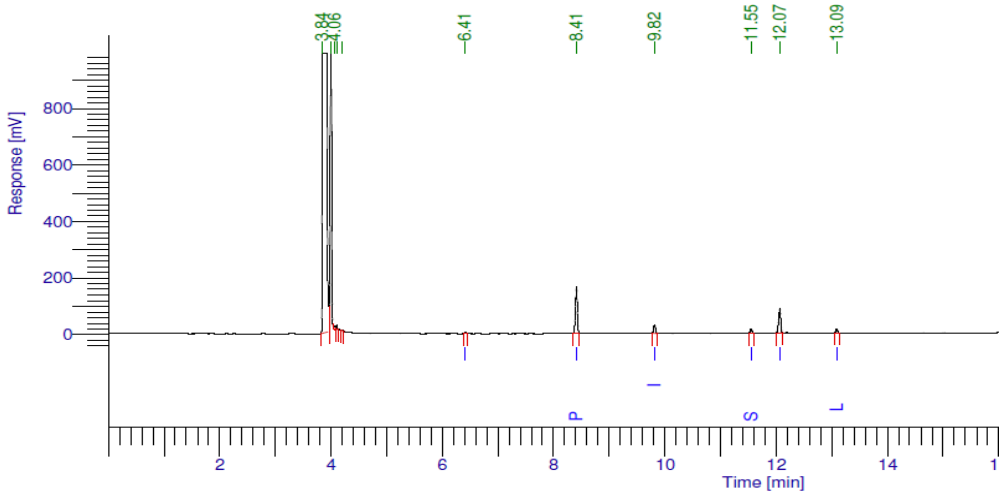
APPENDIX B10

ME content of biodiesel FCO and WCO with different dosage of enzyme at 40°C, MeOH ratio of 3.5 to oil, 1:1 *tert*-butanol to oil with stirring at 200rpm for 24 h based on Figure 4.14

(1) WCO at 0.5% of enzyme



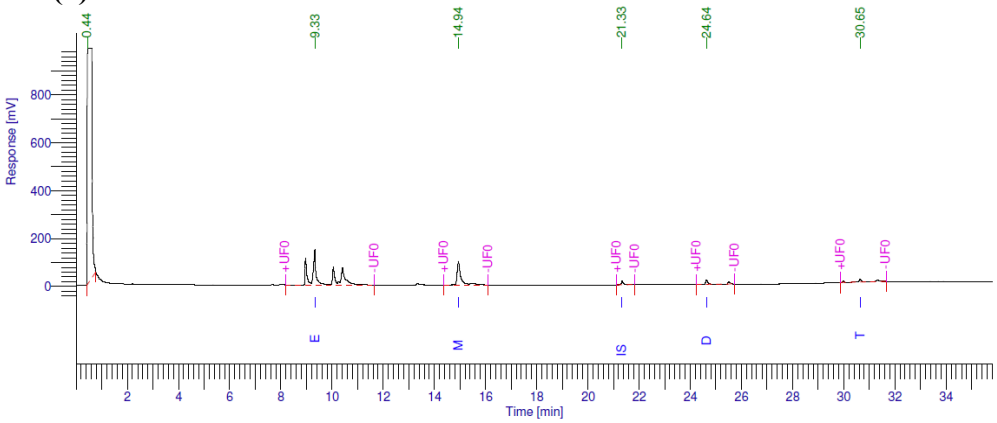
(2) WCO at 1.5% of enzyme



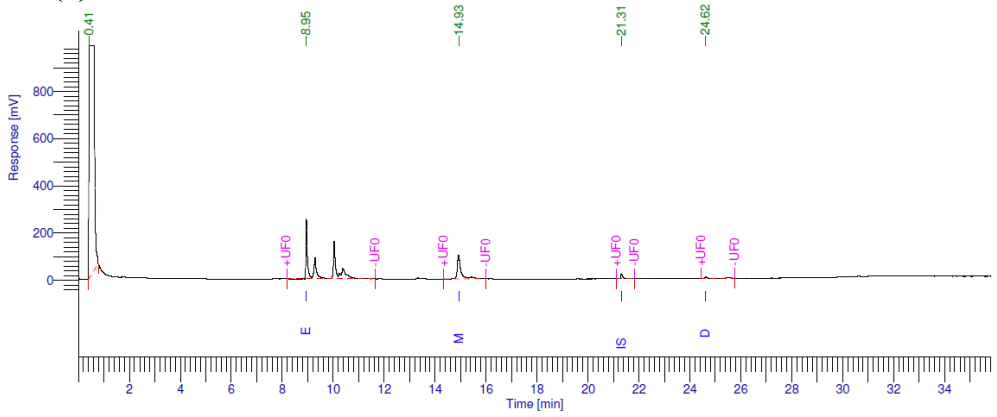
APPENDIX B11

Product composition including TG, DG, MG, glycerols and ME for the reaction mixture of FCO at 72 h in the condition of MeOH ratio to oil at 6:1, 1:1 *tert*-butanol to oil volume, 1% (w/w) dosage of enzyme to oil weight at 40°C based on Figure 4.15.

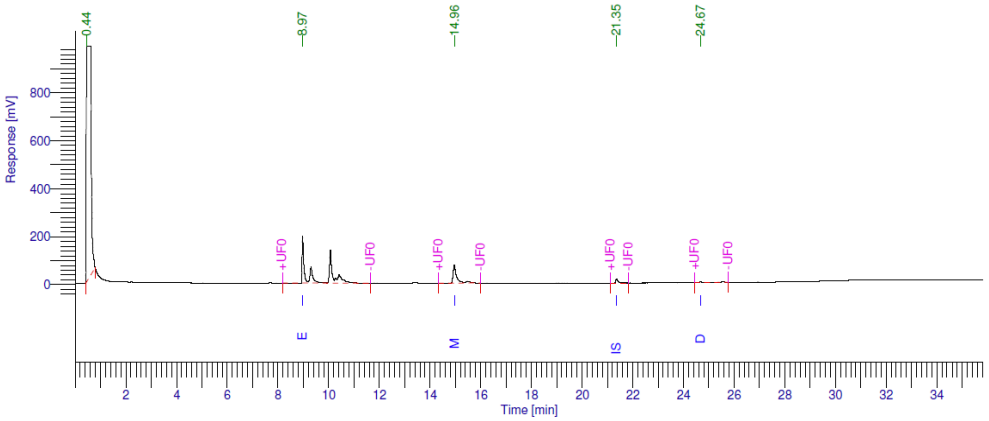
(1) At 8H



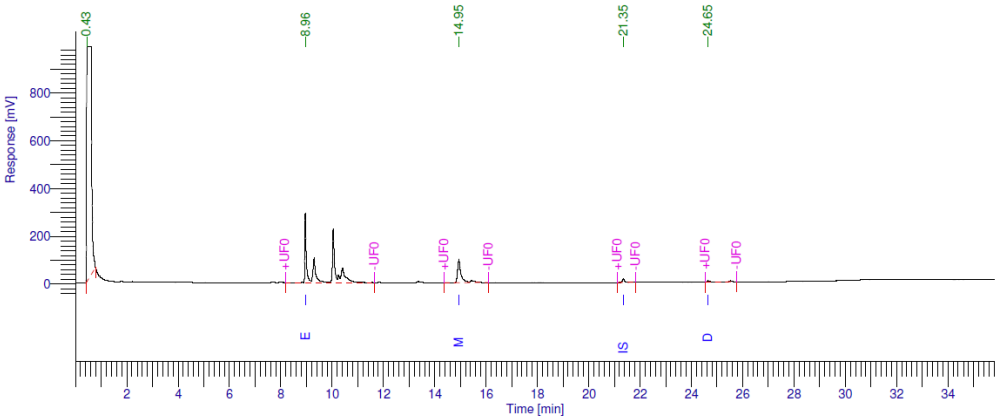
(2) At 16H



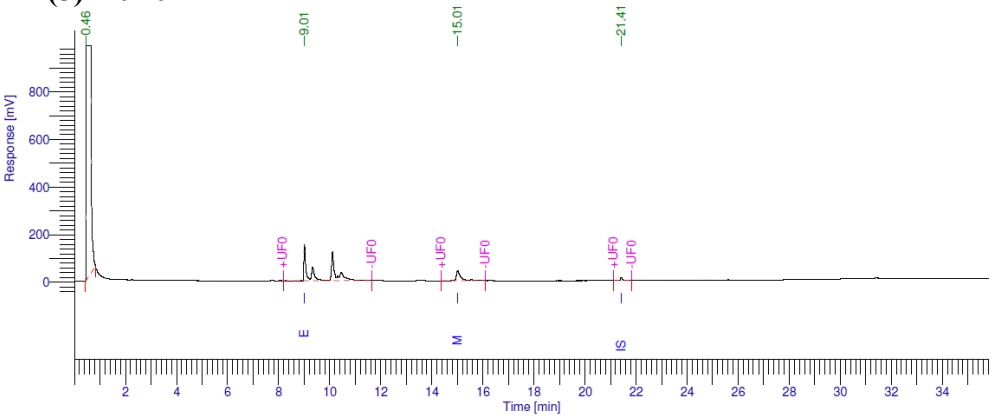
(3) At 24H



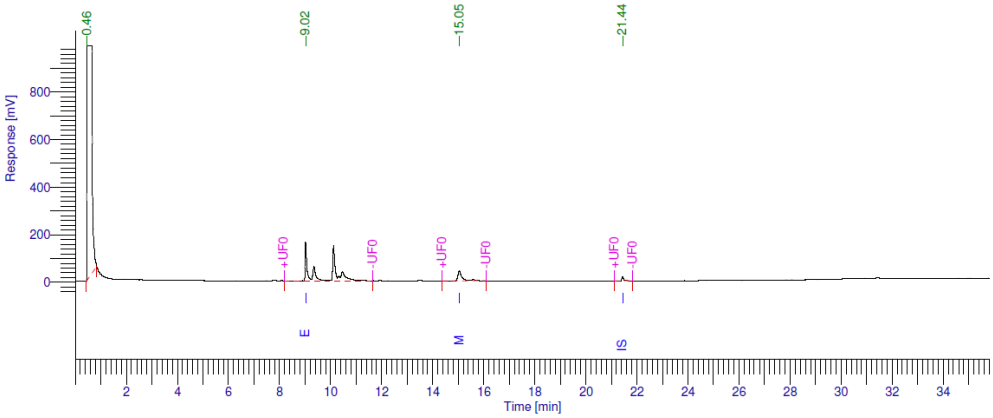
(4) At 32H



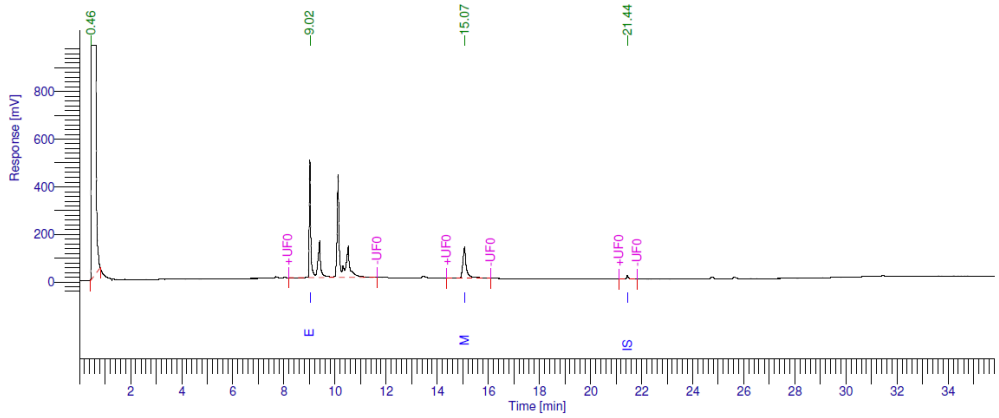
(5) At 40H



(6) At 48H

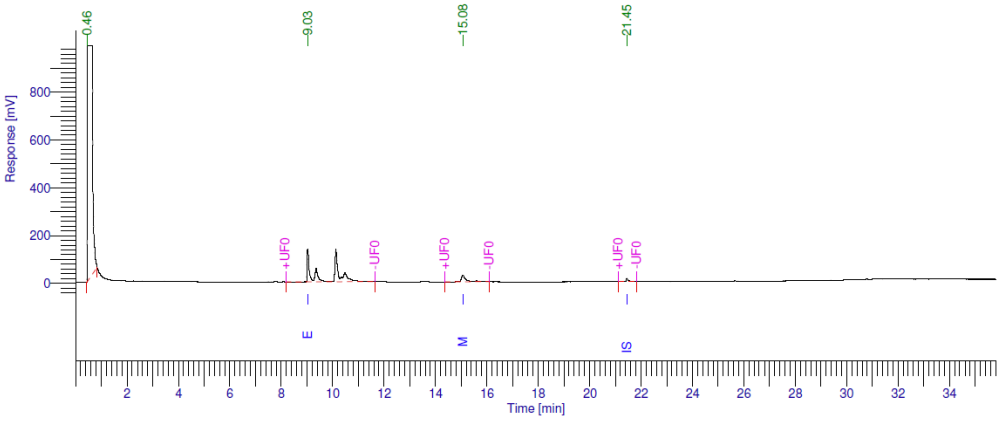


(7) At 56H

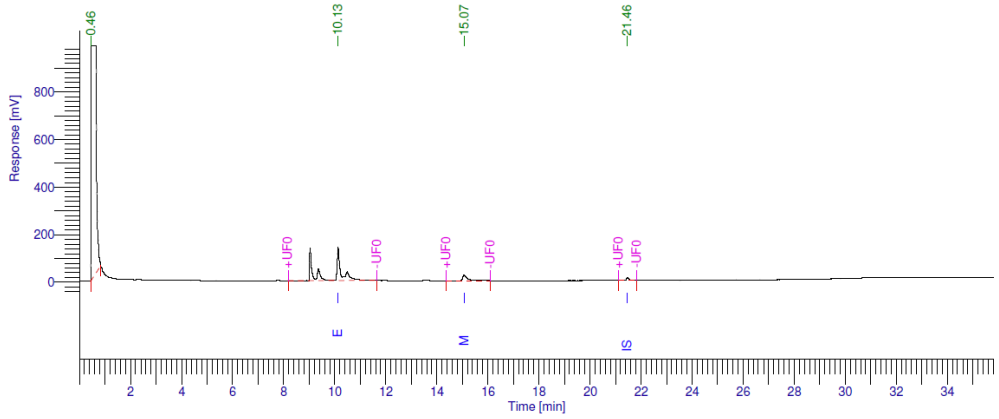


\

(8) At 64H



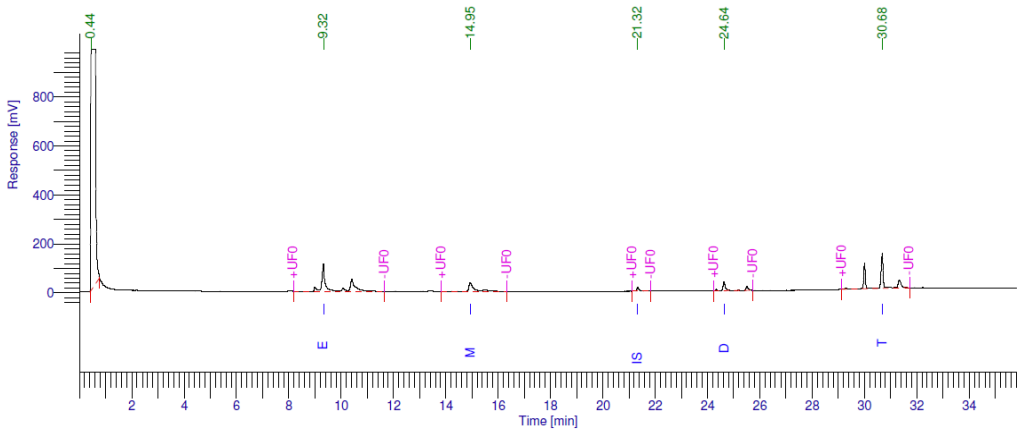
(9) At 72H



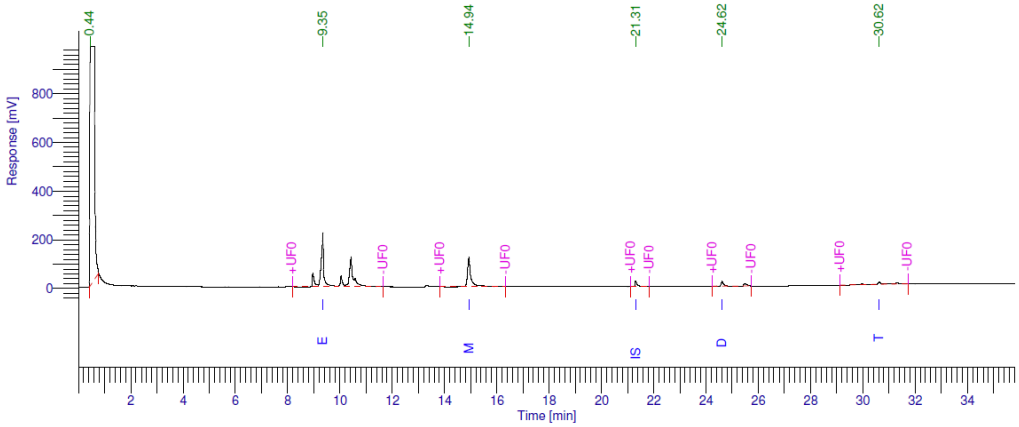
APPENDIX B12

Product composition including triglycerides, diglycerides, monoglycerides, glycerols and ME for the reaction mixture of WCO at 72 h in the condition of MeOH ratio to oil at 6:1, 1:1 *tert*-butanol to oil volume, 1% (w/w) dosage of enzyme to oil weight at 40ºC based on Figure 4.16.

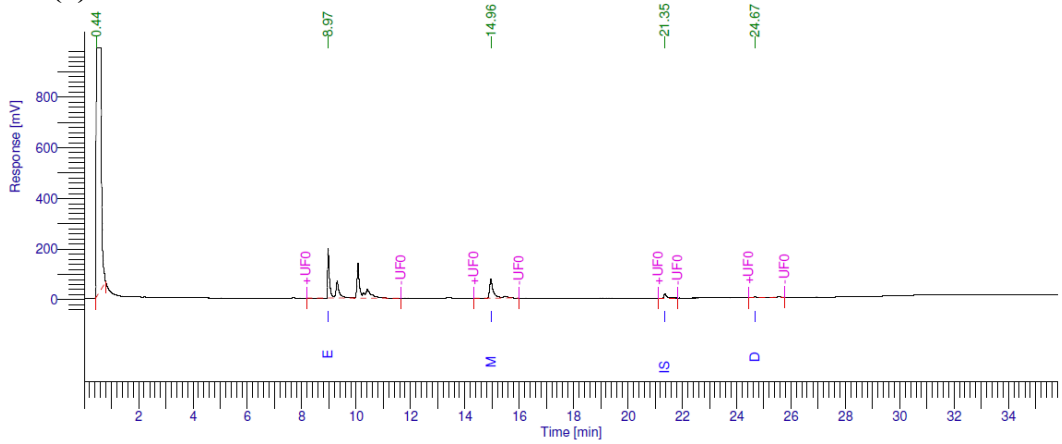
(1) At 8H



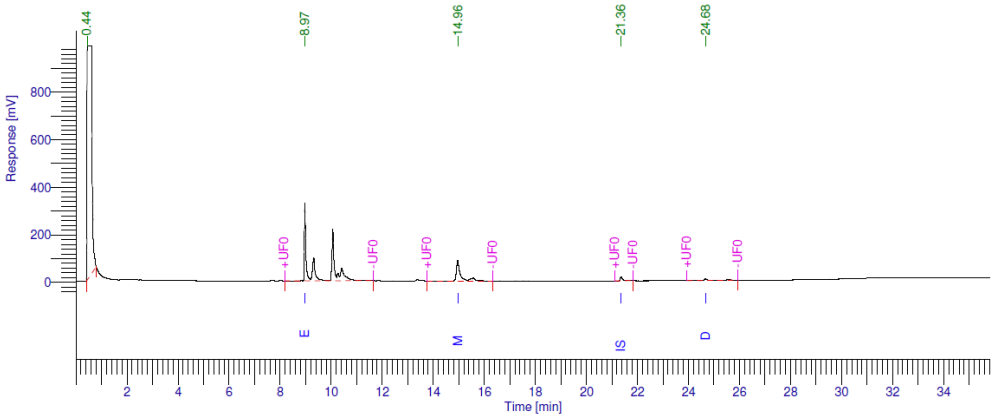
(2) At 16H



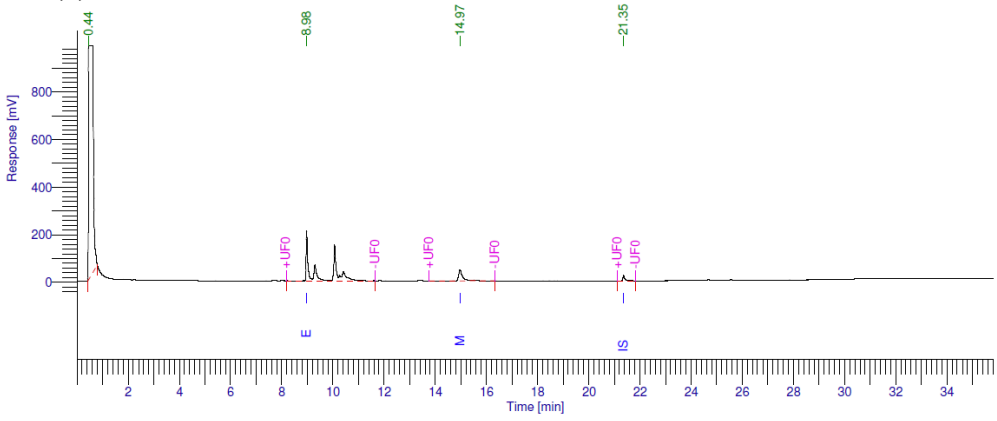
(3) At 24H



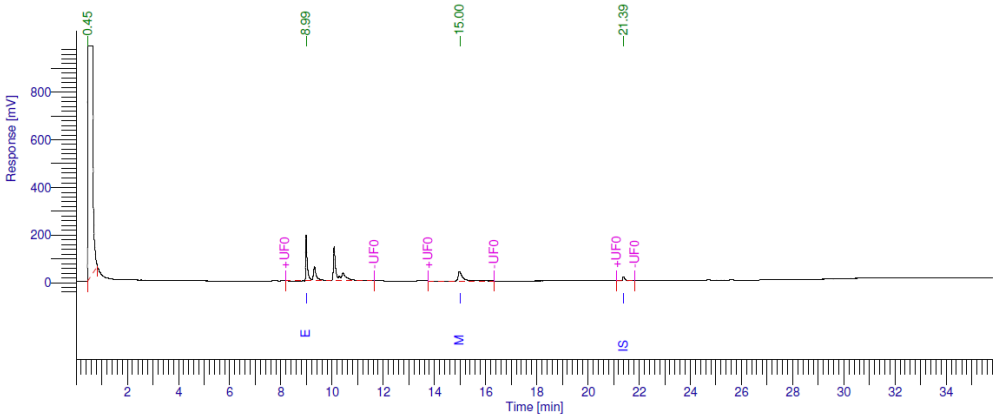
(4) At 32H



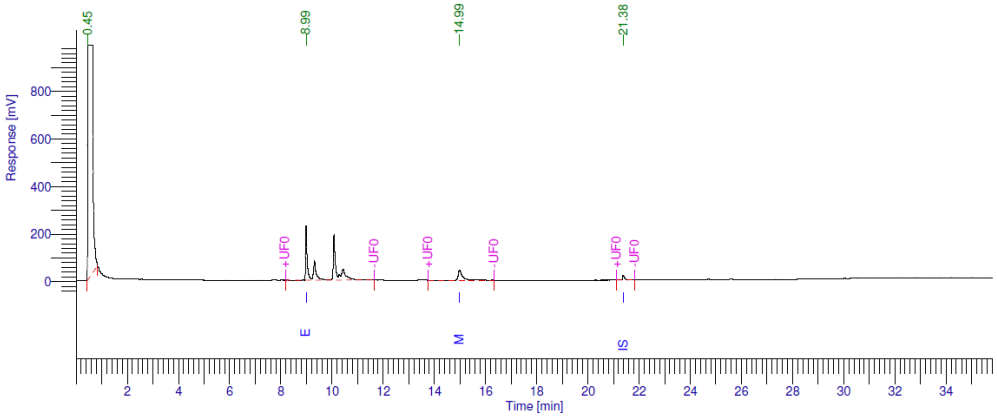
(5) At 40H



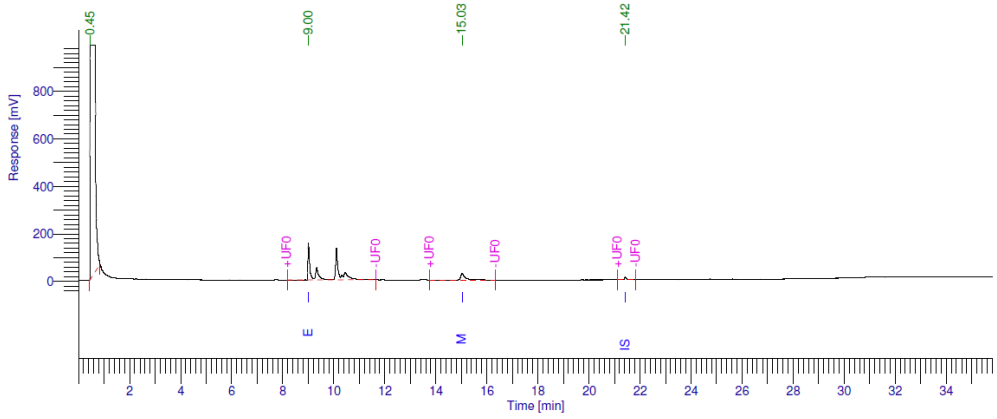
(6) At 48H



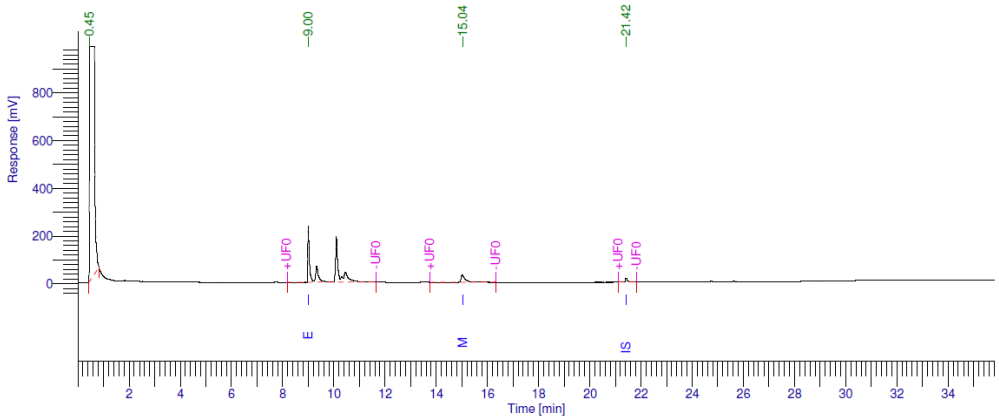
(7) At 56H



(8) At 64H



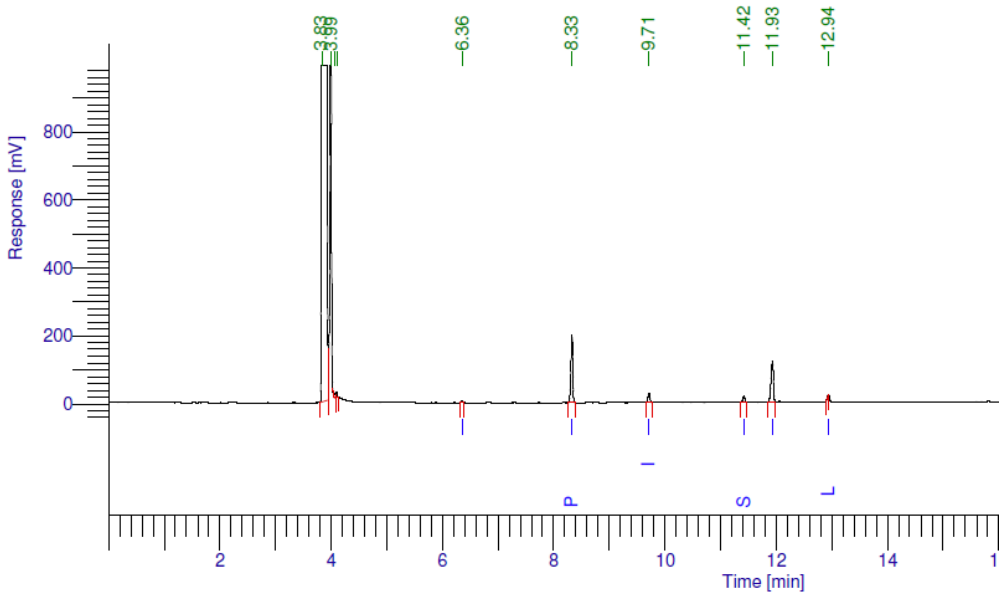
(9) At 72H



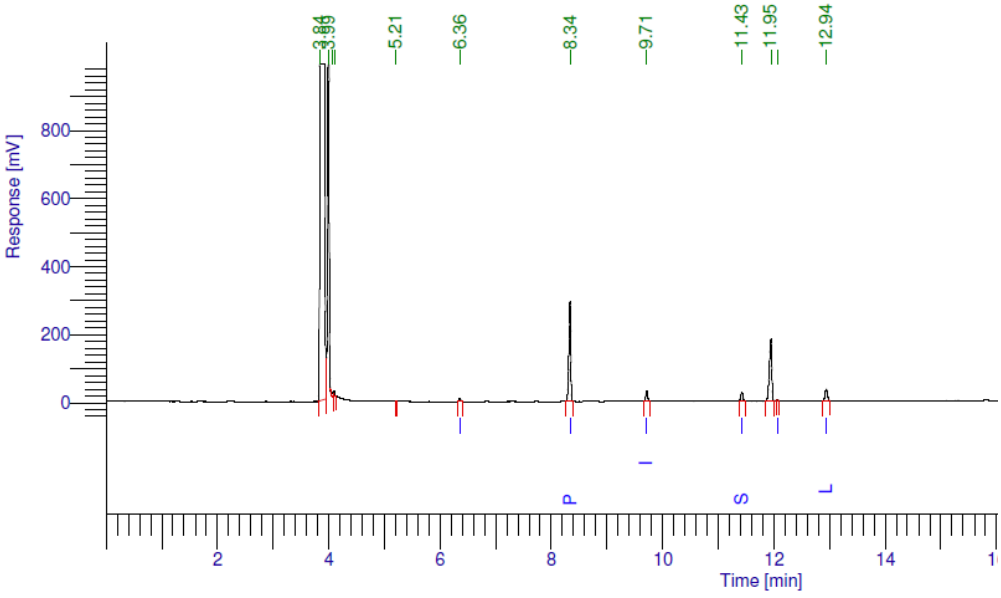
APPENDIX B13

Operational stability of chitosan immobilised lipase during twelve batch reactions in the transesterification of WCO, 0.7% (w/w) of the enzyme amount to oil weight, MeOH/oil molar ratio at 5:1, 200rpm stirring at 46°C based on Figure 4.17.

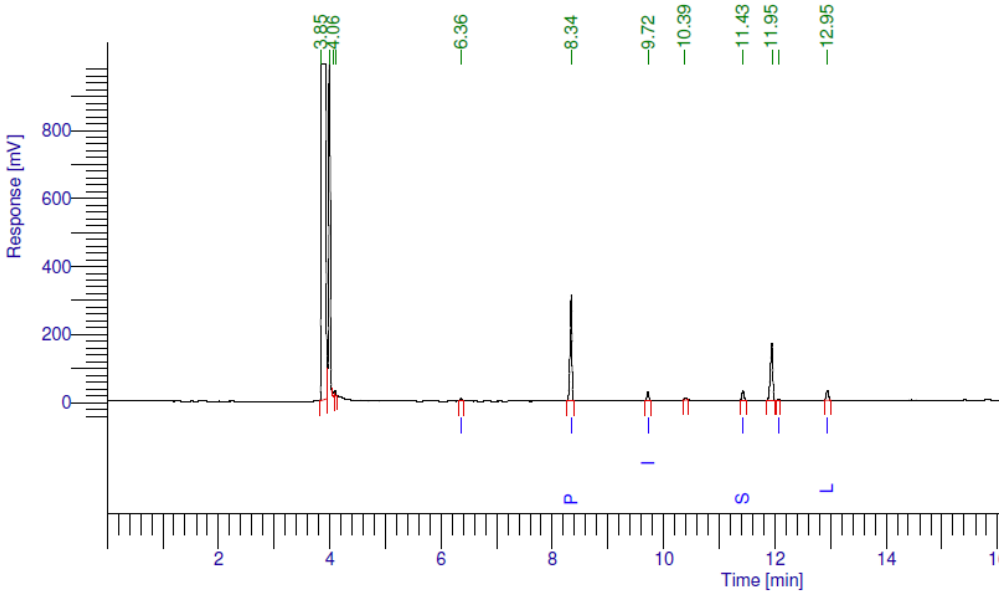
(1) Cycle 1



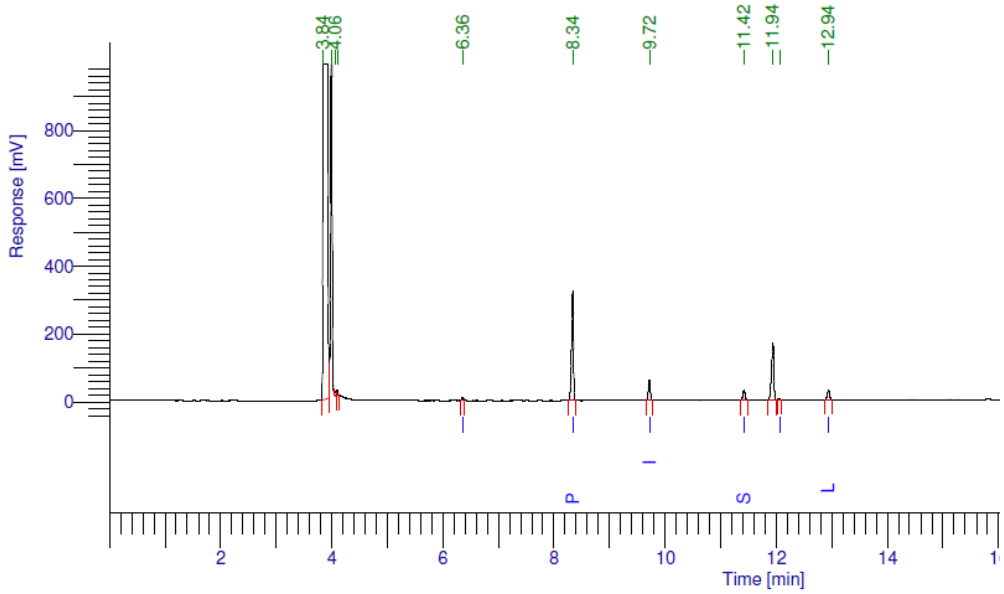
(2) Cycle 2



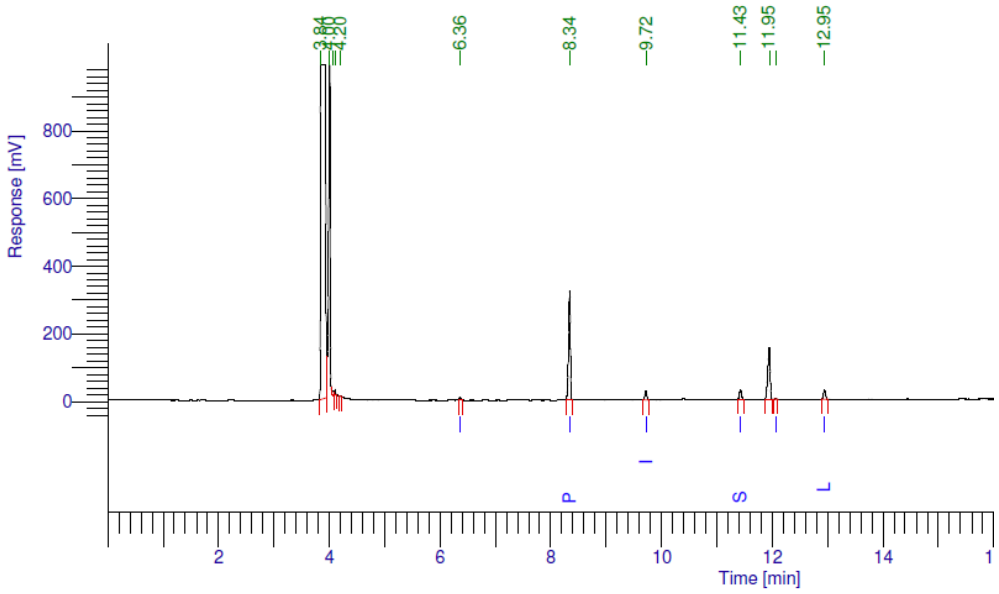
(3) Cycle 3



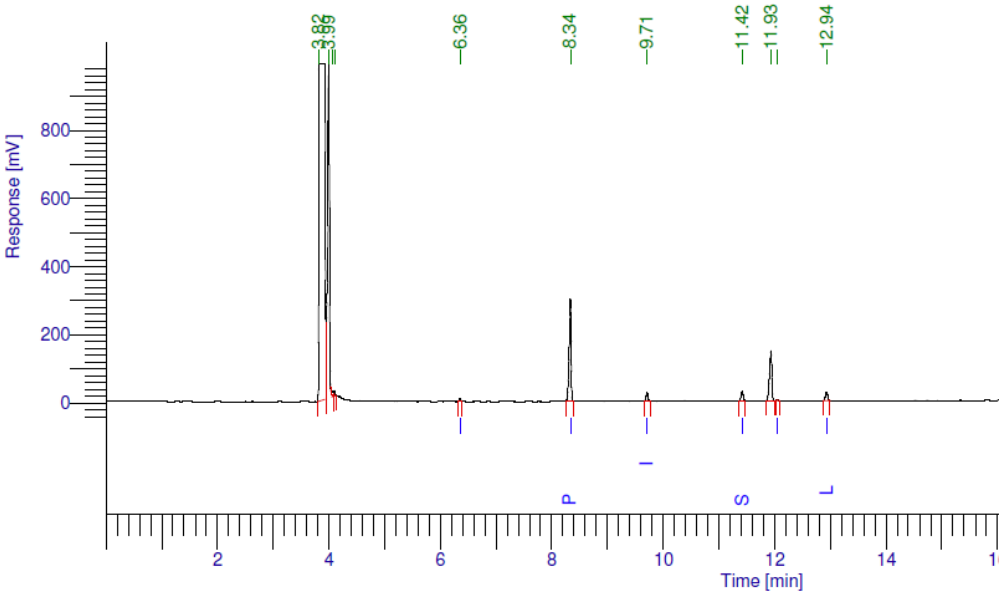
(4) Cycle 4



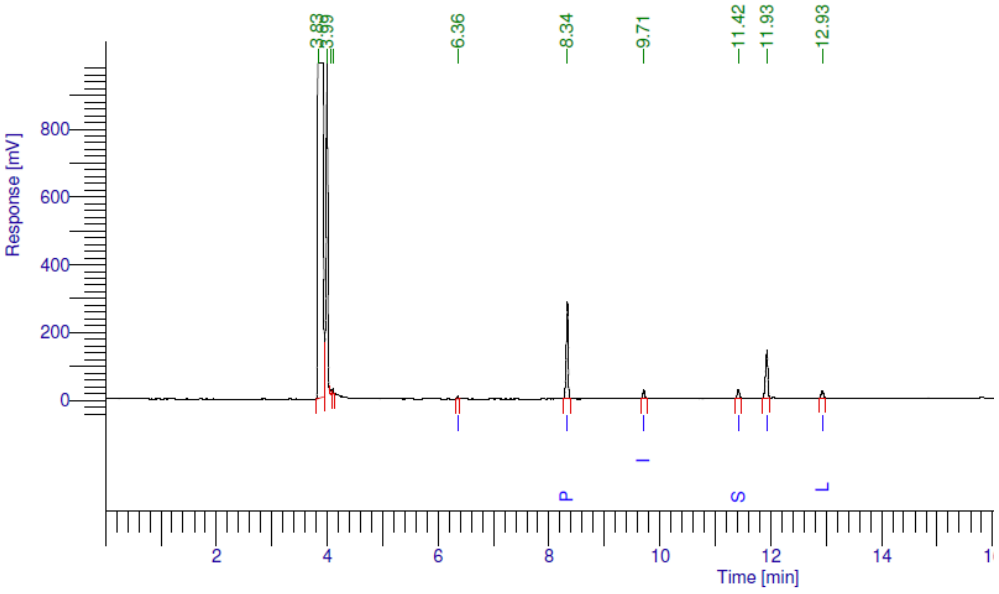
(5) Cycle 5



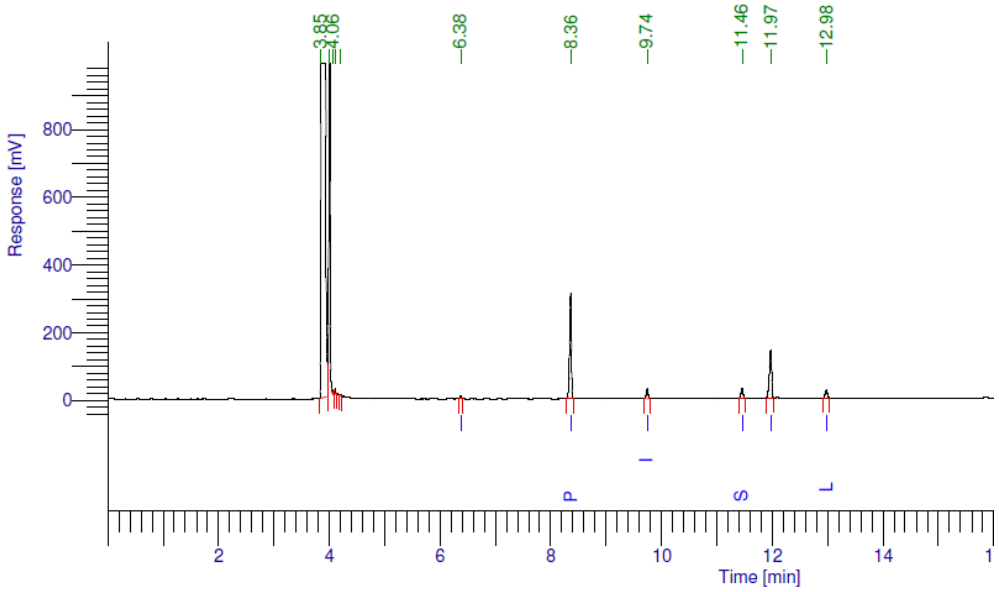
(6) Cycle 6



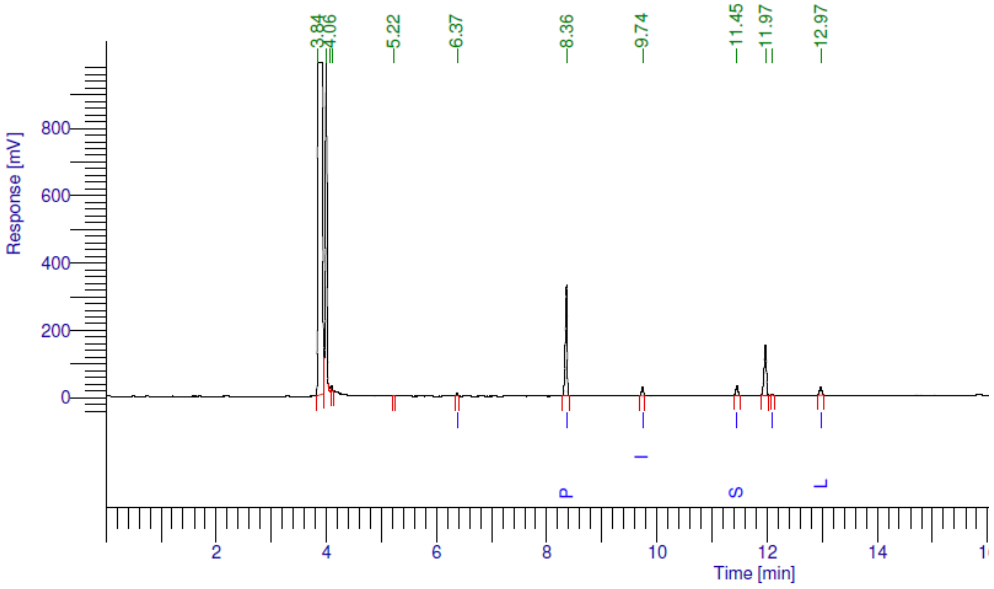
(7) Cycle 7



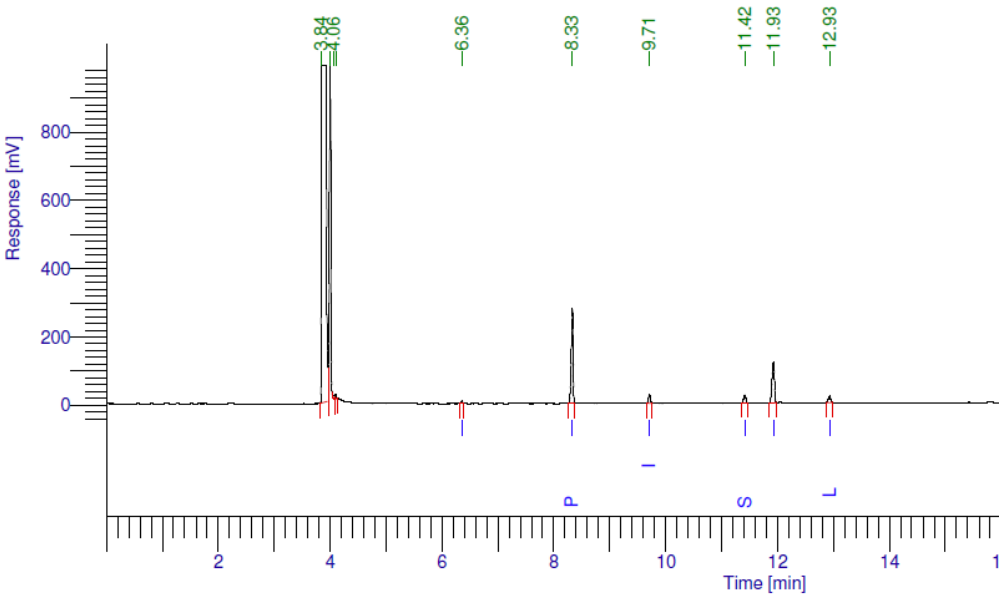
(8) Cycle 8



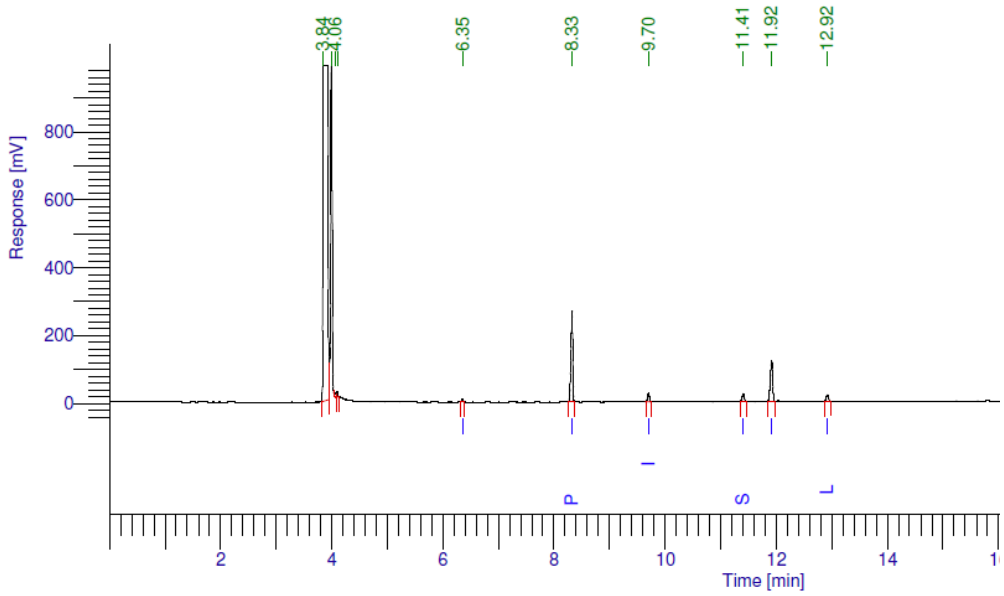
(9) Cycle 9



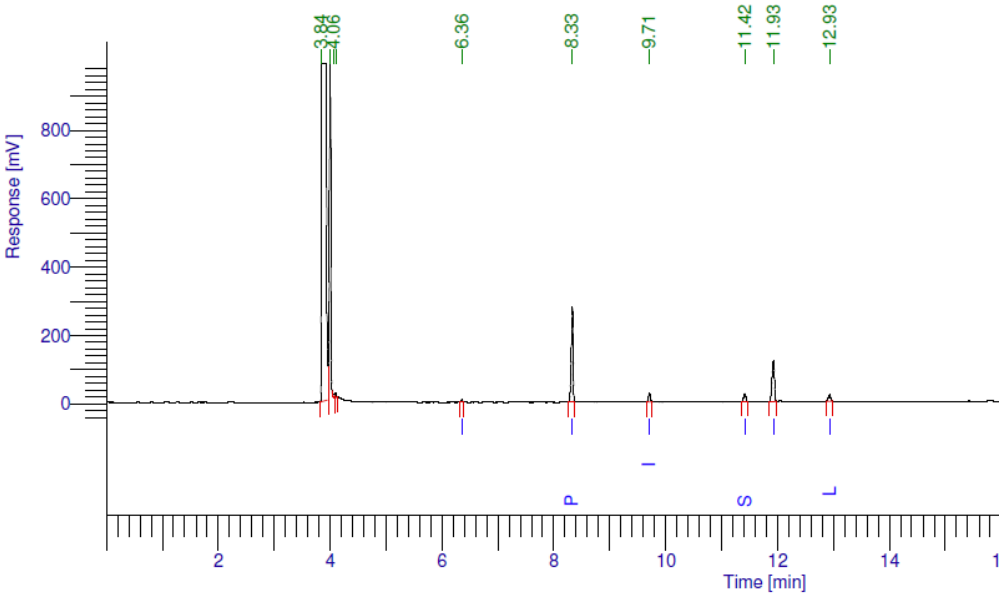
(10) Cycle 10



(11) Cycle 11

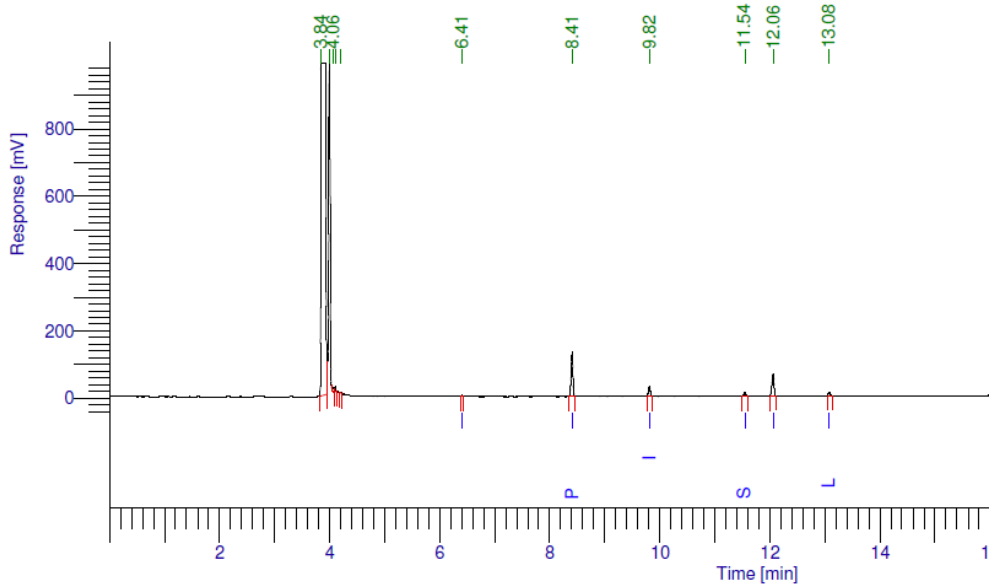


(12) Cycle 12

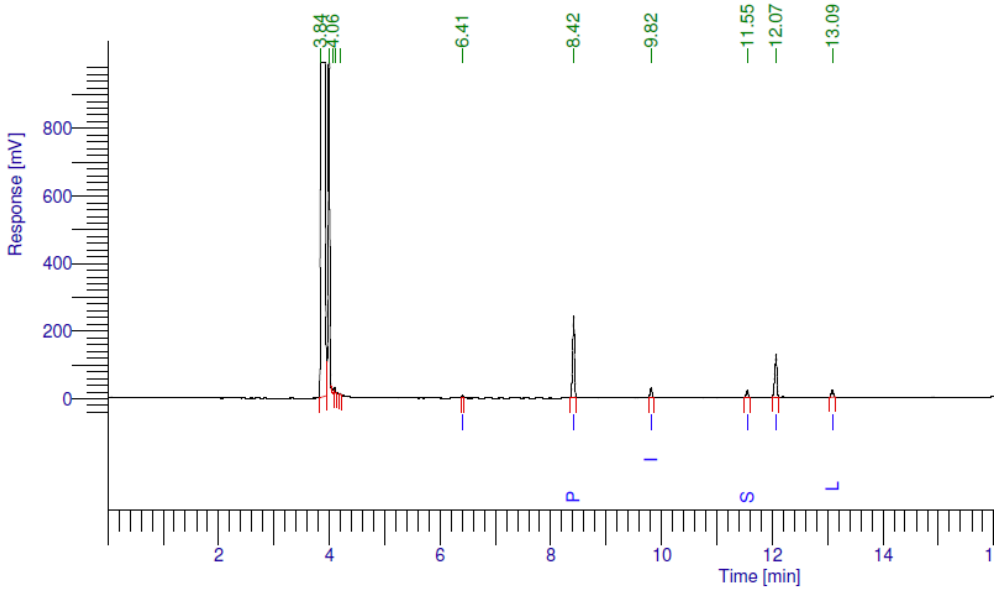


APPENDIX B14

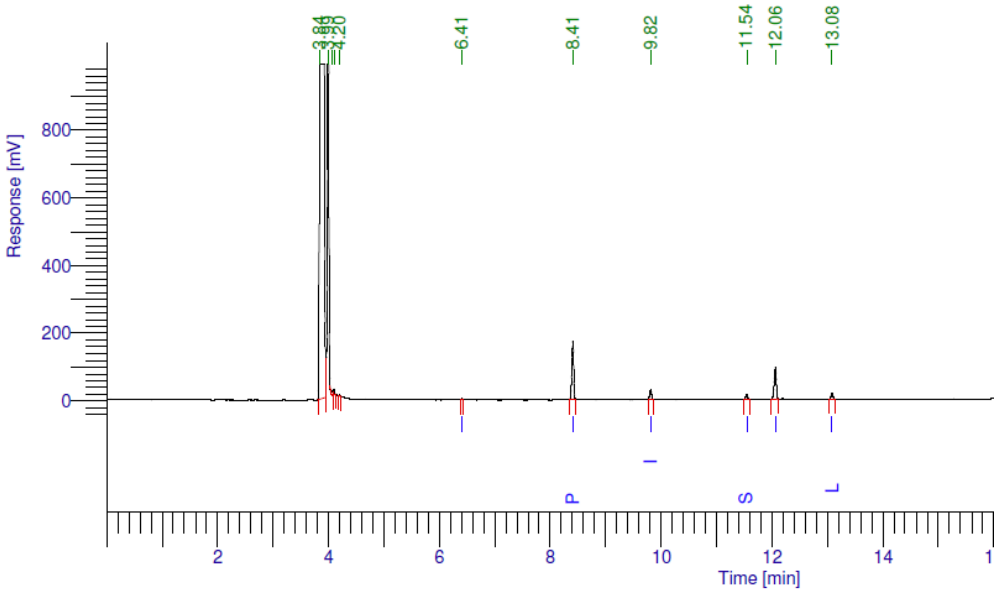
ME content of biodiesel WCO with 0.7% (w/w) dosage of chitosan immobilised enzyme at 34°C, MeOH ratio of 2 to oil, 1:1 *tert*-butanol to oil with stirring at 200rpm for 24 h.



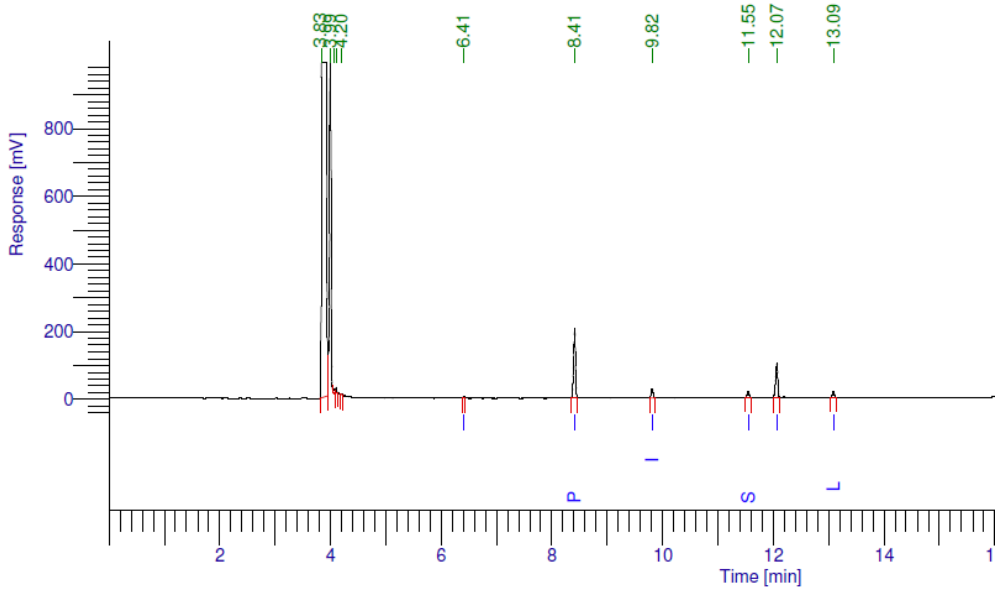
ME content of biodiesel WCO with 0.7% (w/w) dosage of chitosan immobilised enzyme at 34°C, MeOH ratio of 5 to oil, 1:1 *tert*-butanol to oil with stirring at 200rpm for 24 h.



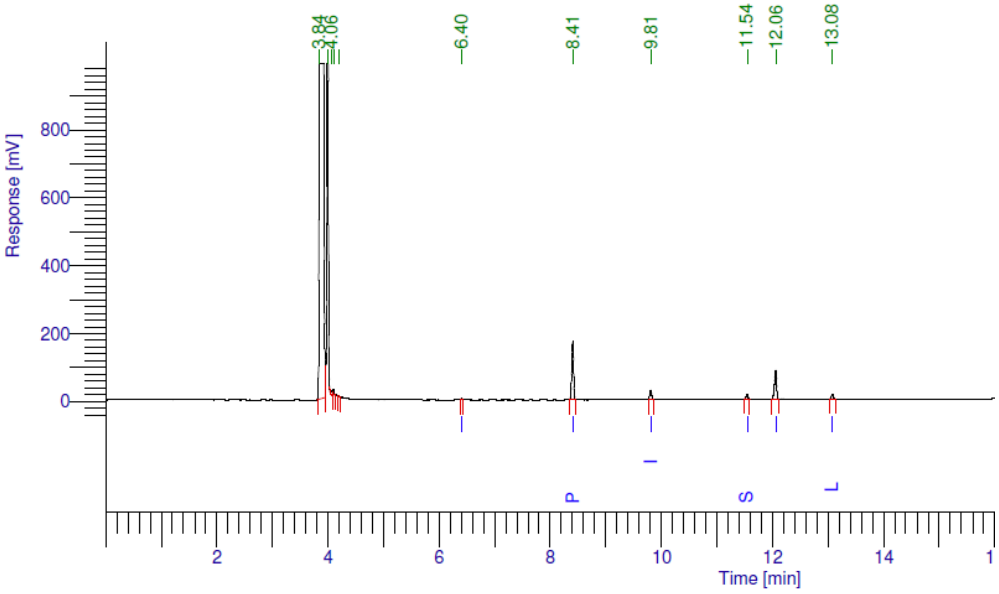
ME content of biodiesel WCO with 1.3% (w/w) dosage of chitosan immobilised enzyme at 34°C, MeOH ratio of 5 to oil, 1:1 *tert*-butanol to oil with stirring at 200rpm for 24 h.



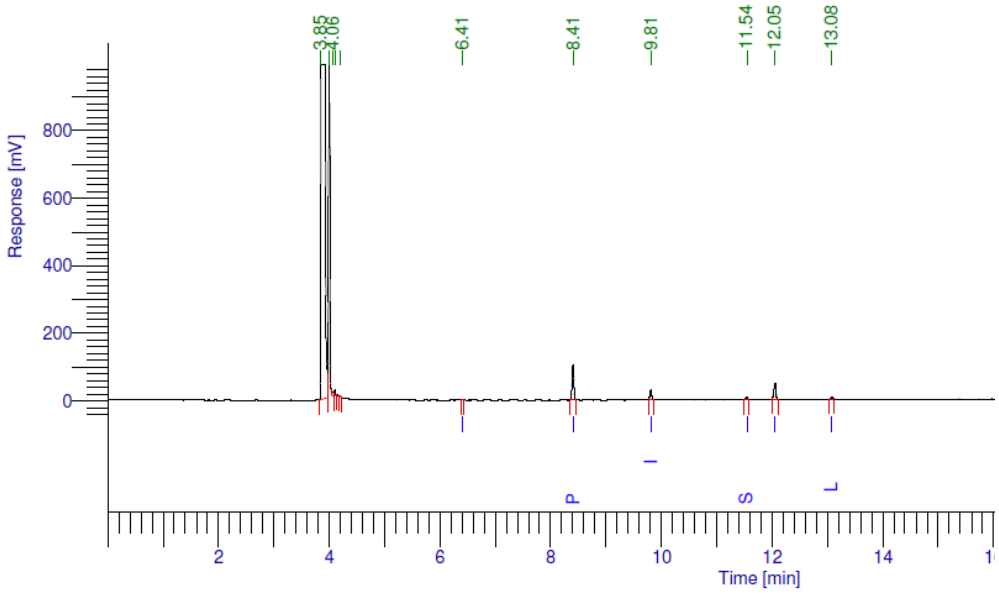
ME content of biodiesel WCO with 0.7% (w/w) dosage of chitosan immobilised enzyme at 46°C, MeOH ratio of 5 to oil, 1:1 *tert*-butanol to oil with stirring at 200 rpm for 24 h.



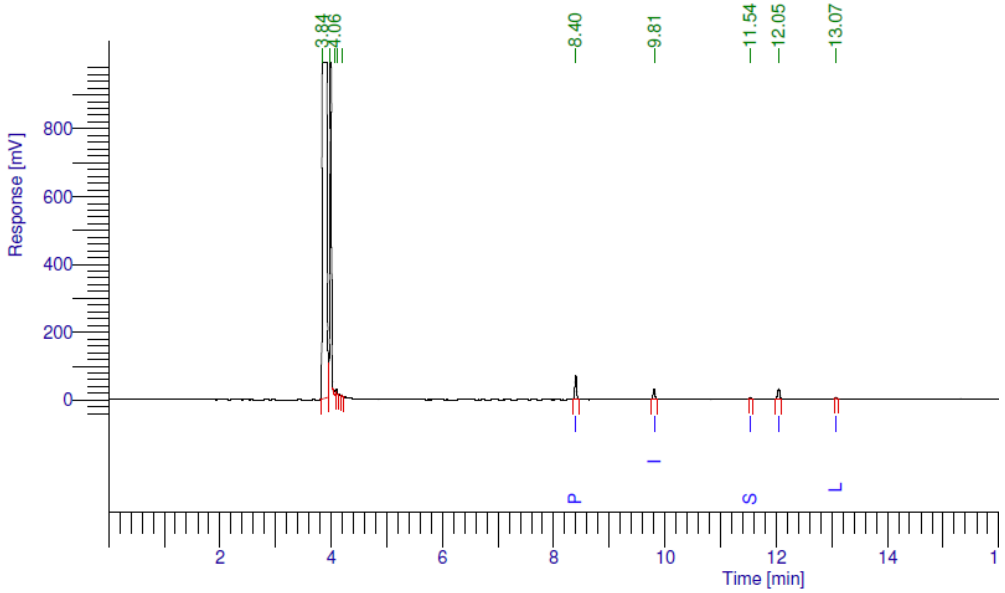
ME content of biodiesel WCO with 1.3% (w/w) dosage of chitosan immobilised enzyme at 46°C, MeOH ratio of 5 to oil, 1:1 *tert*-butanol to oil with stirring at 200rpm for 24 h.



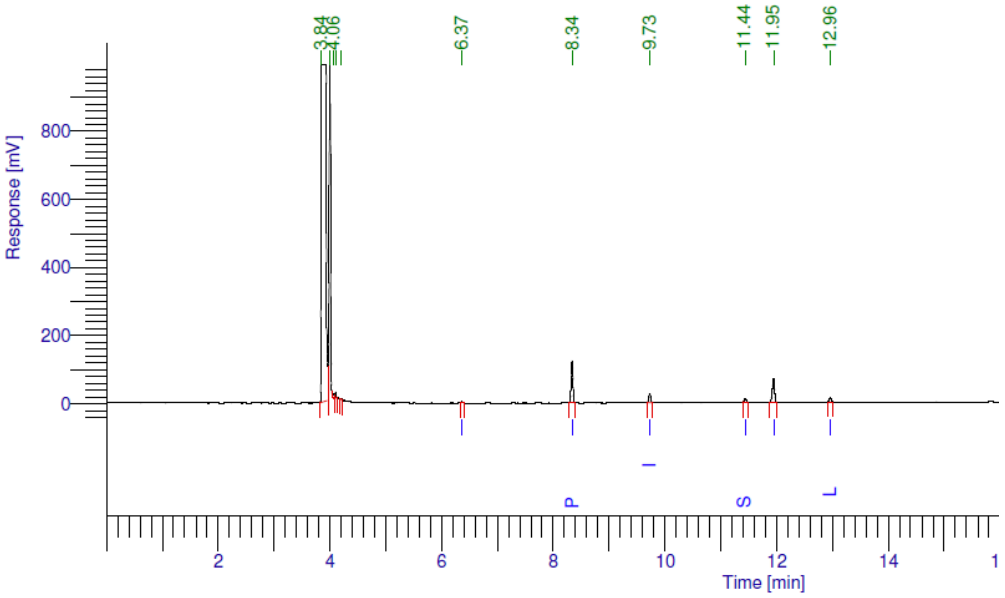
ME content of biodiesel WCO with 0.7% (w/w) dosage of chitosan immobilised enzyme at 46°C, MeOH ratio of 2 to oil, 1:1 *tert*-butanol to oil with stirring at 200rpm for 24 h.



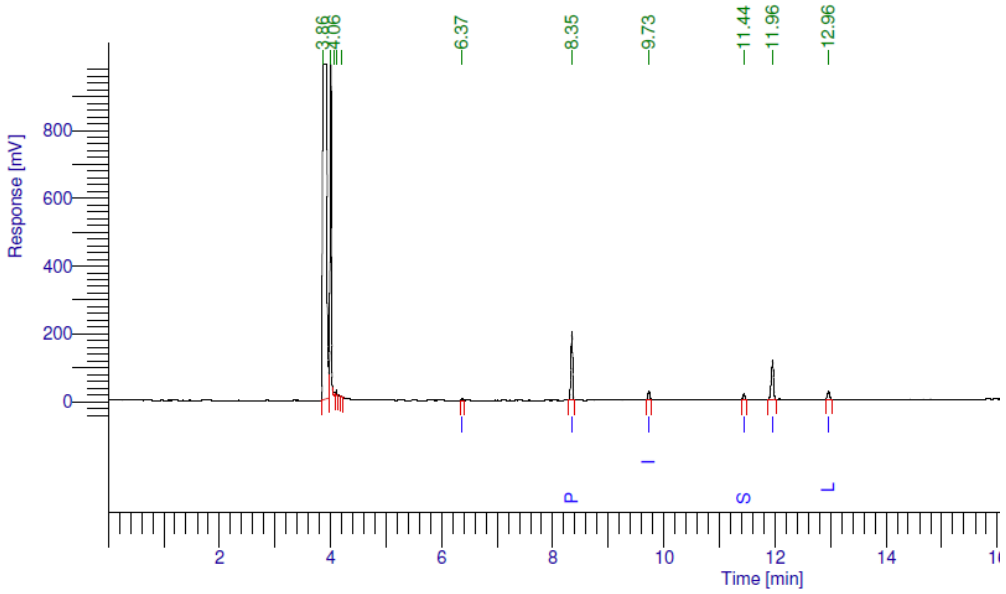
ME content of biodiesel WCO with 1.3% (w/w) dosage of chitosan immobilised enzyme at 46°C, MeOH ratio of 2 to oil, 1:1 *tert*-butanol to oil with stirring at 200rpm for 24 h.



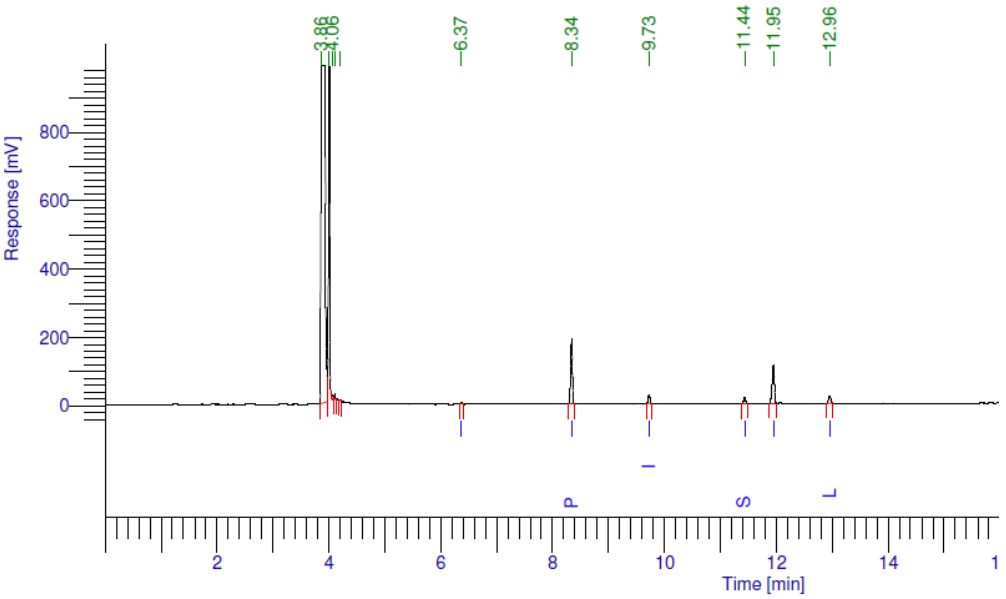
ME content of biodiesel FCO with 0.7% (w/w) dosage of chitosan immobilised enzyme at 34°C, MeOH ratio of 2 to oil, 1:1 *tert*-butanol to oil with stirring at 200 rpm for 24 h.



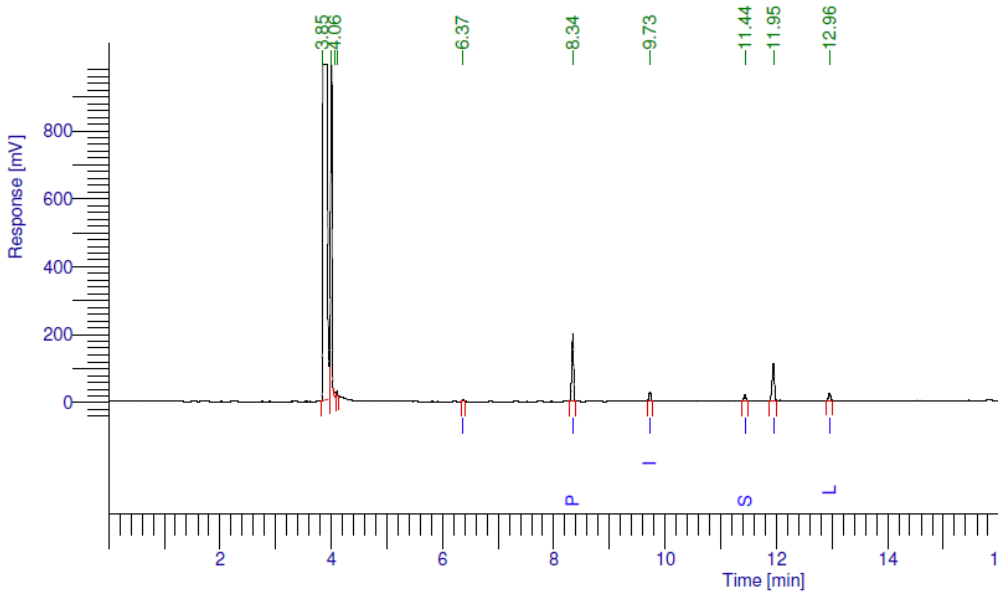
ME content of biodiesel FCO with 0.7% (w/w) dosage of chitosan immobilised enzyme at 34°C, MeOH ratio of 5 to oil, 1:1 *tert*-butanol to oil with stirring at 200rpm for 24 h.



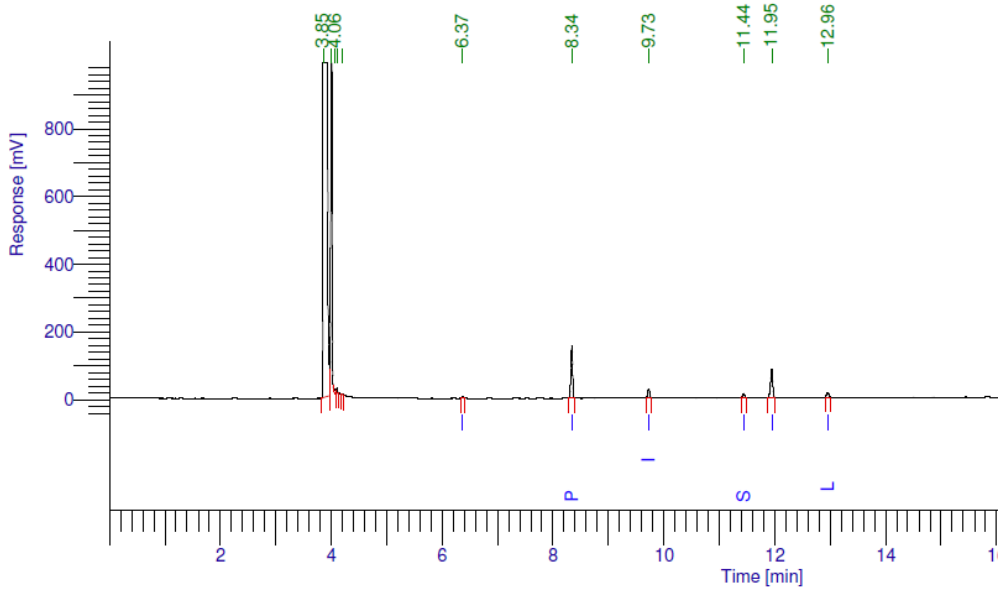
ME content of biodiesel FCO with 1.3% (w/w) dosage of chitosan immobilised enzyme at 34°C, MeOH ratio of 5 to oil, 1:1 *tert*-butanol to oil with stirring at 200rpm for 24 h.



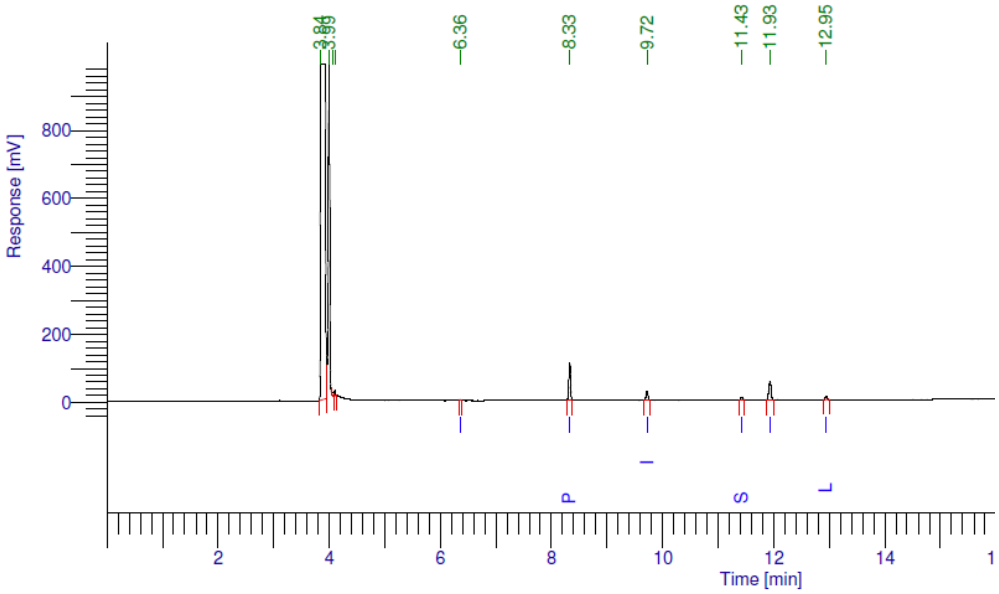
ME content of biodiesel FCO with 0.7% (w/w) dosage of chitosan immobilised enzyme at 46°C, MeOH ratio of 5 to oil, 1:1 *tert*-butanol to oil with stirring at 200rpm for 24 h.



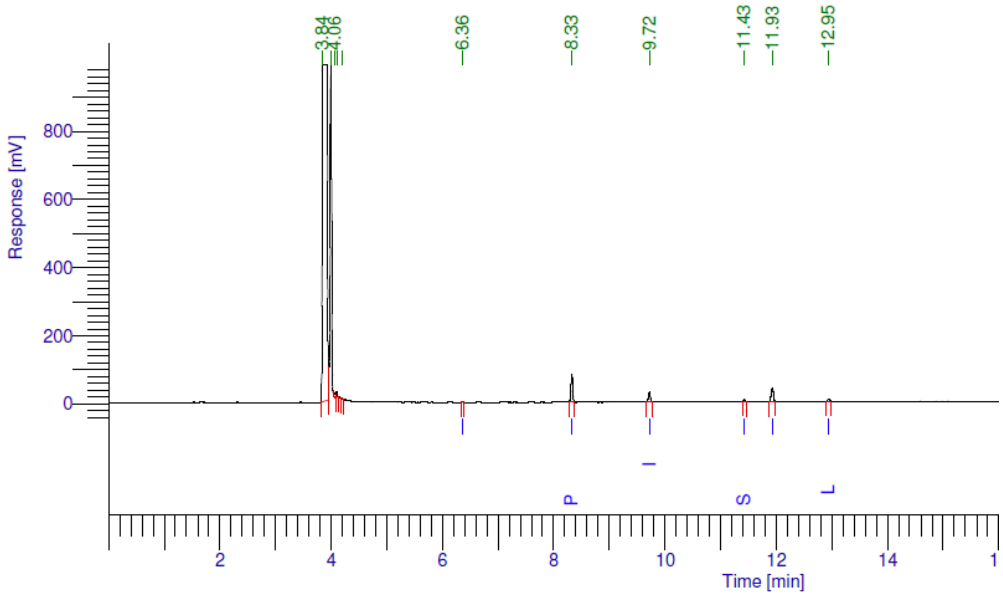
ME content of biodiesel FCO with 1.3% (w/w) dosage of chitosan immobilised enzyme at 46°C, MeOH ratio of 5 to oil, 1:1 *tert*-butanol to oil with stirring at 200 rpm for 24 h.



ME content of biodiesel FCO with 0.7% (w/w) dosage of chitosan immobilised enzyme at 46°C, MeOH ratio of 2 to oil, 1:1 *tert*-butanol to oil with stirring at 200rpm for 24 h.



ME content of biodiesel FCO with 1.3% (w/w) dosage of chitosan immobilised enzyme at 46°C, MeOH ratio of 2 to oil, 1:1 *tert*-butanol to oil with stirring at 200rpm for 24 h.



CHEMRAWN XIX
19th IUPAC International Conference on Chemical Research Applied to World Needs 2011

ENZYMATIC REACTION IN BIODIESEL CONVERSION USING CHITOSAN-IMMOBILIZED LIPASE *THERMOMYCES LANUGINOSUS* ON WASTE COOKING OIL

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Fatty acid methyl ester (FAME), commonly called biodiesel, is a potential alternative fuel to supplement future energy demand. It can be produced from animal or vegetable oils and has non-toxic and environmental friendly properties. Coupled with an increasing need for safe disposal of waste cooking oil (WCO), biodiesel conversion by way of WCO appears to offer an attractive solution to solve both the problems of energy shortage and environment pollution.

A lipase from Thermomyces Lanuginosus (TL) is a suitable biocatalyst for transesterification of oils to produce FAME and has been immobilized on chitosan beads using binary immobilization method in the present study. In order to determine the optimum conditions for biodiesel conversion, a series of experiments conducted with reactants in a conical flask put in an incubator shaker were carried out using methanol to oil (v/v) molar ratio of 1:1 to 6:1, temperature of 30 to 50°C, weight of chitosan-immobilized TL lipase to oil (w/w) of 0.5 to 10%. A three-step methanol addition was used in the presence of solvent tert-butanol. Characterization of the esterified WCO was carried out according to the American Standard Test Method to determine the acid value and iodine value and FAME content was determined by gas chromatography. The reusability of the chitosan-immobilized TL was also investigated.

The present study reveals that highest FAME content was achieved at a mild temperature of 40°C, methanol to oil ratio of 6:1 and immobilized TL lipase to oil ratio of 4% for reaction cycle of 24 hours. If considering the cost of the lipase, lower lipase to oil ratio of 0.5 to 1.5% was preferred though producing slightly lower FAME content. By comparison, transesterification reaction using the present chitosan-immobilized lipase achieved a FAME content of up to 35.5% from the third cycle onwards while commercialized Lipozyme TLIM under the same conditions produced 34.8% content. The amount of bound protein was found to be 29.94 mg g^{-1} and enzyme activity of the self-prepared lipase still managed to retain 76% after 12 cycles. Acid value for esterified WCO was 0.79 mg KOH/g of oil while iodine value was 25.66 g I_2/100g, showing that the transesterification was performed well.