HALLOYSITE NANOTUBES (HNTs) SUPPORTED CANDIDA RUGOSA LIPASE AS CATALYST FOR MONOOLEIN PRODUCTION

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by

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LIST OF SYMBOLS

%	Percent
±	Plus minus
°C	Celcius
Y	Response variable
α	Alpha
β	Beta
E_a	Activation energy
E_d	Deactivation energy
ΔG	Gibbs Free Energy
ΔH	Enthalpy
ΔS	Entropy

LIST OF ABBREVIATIONS

[A]	Concentration of substrate A
[B]	Concentration of substrate B
µm	Micrometer
A	First substrate
A’	Pre-exponential constant
atm	Standard atmosphere
ANOVA	Analysis of variance
B	Second substrate
CCD	Central Composite Design
CRL	*Candida rugosa* lipase
E	Enzyme
E’	Intermediate enzyme
E’P	Modified enzyme product
EA	Enzyme substrate complex
EB	Dead end complex
EC	Enzyme Commission
E_a	Activation energy
E_d	Deactivation energy
EQ	Enzyme water complex
FTIR	Fourier Transform Infrared Spectroscopy
g	Gram
GC	Gas Chromatography
h	Planck’s constant
K	Rate constant

K	Kelvin
K_B	Boltzman constant
K_d	Inactivation constant
K_i	Inhibition constant
K_m^A	Michaelis constant for substrate A
K_m^B	Michaelis constant for substrate B
K_S^A	Dissociative constant for substrate A
Min	Minute
mL	milliliter
mM	millimolar
mmol	Millimolar
MG	Monoglyceride
MO	Monoolein
NaOH	Sodium hydroxide
NH_2	Amidogen
P	First product
PVA	Polyvinyl Alcohol
Q	Second Product
R^2	Coefficient of determination
RPM	Rotation per minute
RSM	Response Surface Methodology
SEM	Scanning Electron Microscopy
T	Absolute temperature
TEM	Transmission Electron Microscopy
v	Initial velocity
V_{max}	Maximum velocity
XRD	X-Ray Diffraction

LIST OF APPENDICES

HALOISIT NANOTIUB (HNTS) MENYOKONG CANDIDA RUGOSA LIPASE SEBAGAI PEMANGKIN UNTUK PENGHASILAN MONOOLEIN

ABSTRAK

Permintaan biofuel yang semakin meningkat sebagai alternatif kepada bahan api fosil telah menghasilkan sejumlah besar gliserol mentah sebagai produk sampingan. Penggunaan gliserol mentah menjadi produk tambah nilai seperti monoolein menjadi fokus bagi penyelidikan ini adalah untuk menyediakan alternatif bagi memanfaatkan sejumlah besar gliserol mentah yang dihasilkan dari industri biodiesel. Penyekatgerakan lipase ke haloisit nanotiub menggunakan dua pendekatan yang berbeza (secara fizikal dan kimia) telah menggambarkan trend yang sangat berbeza tentang aktiviti pemangkin dan kapasiti pemuatan pemangkin. Penyelidikan ini telah berjaya meneroka beberapa faktor utama (nisbah sokongan terhadap pemangkin, pH, suhu, tekanan) yang dapat mempengaruhi kecekapan sekatgerak pemangkin. Adalah kebiasaannya, aktiviti pemangkin dan jumlah pemuatan enzim untuk teknik imobilisasi fizikal lebih tinggi daripada sekatgerak kimia, kecuali untuk faktor pH yang pemuatan pemangkin lebih tinggi semasa disekatgerak secara kimia, namun, aktiviti pemangkin yang dihasilkan tidak berbeza dengan lipase yang disekatgerak secara fizikal. Dengan menggunakan response surface methodology (RSM), pemangkin tersekatgerak dioptimumkan untuk beberapa parameter (nisbah sokongan kepada pemangkin, pH, suhu, tekanan) dan dicirikan menggunakan analisis HRTEM, XRD, BET dan FTIR. Keadaan optimum yang dicadangkan ialah nisbah lipase hingga HNTs 2.48: 1, pH 6, 38,6^{o}C dan 0.33 atm dengan aktiviti relatif eksperimen 81,92%. Dengan menggunakan pemangkin tersekatgerak yang dioptimumkan, esterifikasi menggunakan asid oleik dan gliserol mentah dilakukan

untuk mengkaji kesan masa, suhu, kadar agitasi, dan nisbah molar bahan pada penghasilan monoolein. Keadaan kerja terbaik untuk pemangkin yang disekatgerak secara fizikal untuk menghasilkan sekitar 88.97% monoolein ialah pada suhu 42^{o}C, dengan nisbah 4:1 untuk gliserol:asid oleik, pengadukan pada 200 rpm selama 16 jam. Kajian kinetik telah menunjukkan bahawa pemangkin yang tersekatgerak secara fizikal mengikuti mekanisme bi-bi yang berurutan dengan perencatan asid oleik. Pemalar kinetik adalah seperti berikut: V_{max} = 2.104 mM/min, K_m^A = 0.131 mM, K_m^B = 15.043 mM, dan K_i = 1.391. Sifat termodinamik untuk tindak balas esterifikasi bermangkin lipase yang tersekatgerak secara fizikal menunjukkan tenaga pengaktifan (E_a) dan tenaga denaturasi (E_d) masing-masing 90.36 kJ/mol dan 111.47 kJ/mol. Dengan menggunakan parameter yang dioptimumkan, sifat termodinamik tindak balas esterifikasi pemangkinan lipase yang tersekatgerak secara fizikal pada 40°C memberikan nilai separuh hayat pemangkin tersekatgerak ($t_{1/2}$) = 97.63 jam, pemalar penyahaktifan (K_d) = 0.0071 jam^{-1}, entalpi (ΔH) = 107.40 kJ/mol, entropi (ΔS) = 0.383 kJ/mol.K, dan tenaga Gibbs (ΔG) = -19.113 kJ/mol. Jumlah monoolein yang dihasilkan menggunakan pemangkin tersekatgerak sangat setanding dengan pemangkin bebas, dengan keupayaan HNT-lipase untuk digunakan semula dan kestabilannya pada suhu yang lebih tinggi. Berdasarkan penemuan yang dikemukakan dalam penyelidikan ini, ia mengesahkan HNT berpotensi menjadi perumah yang bagus untuk enzim dan penggunaan gliserol mentah sebagai bahan permulaan yang baik untuk menghasilkan produk tambah nilai yang lebih baik.

HALLOYSITE NANOTUBES (HNTS) SUPPORTED CANDIDA RUGOSA LIPASE AS CATALYST FOR MONOOLEIN PRODUCTION

ABSTRACT

A growing demand for biofuels as the alternative to fossil fuel has produced abundance amount of crude glycerol as by-product. Utilization of crude glycerol into a value-added product such as monoolein has been the focus for this present work, as to provide alternative pathway to utilize the mass abandoned of crude glycerol from biodiesel industry. Immobilization of lipase onto the halloysite nanotubes using two different approaches (physical and chemical) have portrayed remarkably different trends on enzyme activity and loading capacity. The present work managed to explore several main factors (ratio of support to enzyme, pH, temperature, pressure) that could affect the efficiency of enzyme immobilization. Most of the time, the enzyme activity and amount of enzyme loading for the physical immobilization technique remained higher than chemical immobilization, except for the different pH treatment. For the latter, lipase loading was higher upon chemical immobilization, yet, the resultant enzyme activity was not that different with the physically immobilized lipase. Using response surface methodology (RSM), the immobilized enzyme was optimized for several parameters (ratio of support to enzyme, pH, temperature, pressure) and characterized using HRTEM, XRD, BET and FTIR analysis. The optimum conditions suggested were 2.48:1 of lipase to HNTs ratio, pH 6, 38.6^{o}C and 0.33 atm with experimental relative activity of 81.92%. By utilizing the optimized immobilized lipase, esterification using oleic acid and crude glycerol was conducted to study the effect of time, temperature, agitation rate, and substrates molar ratio on monoolein production. The best working conditions for physically immobilized lipase was to

produce about 88.97% monoolein at 42°C, with 4:1 ratio of glycerol:oleic acid, agitated at 200 rpm for 16 hours. Kinetic study has revealed that physically immobilized lipase followed an ordered sequential bi-bi mechanism with dead-end inhibition by oleic acid. The kinetic constants were as follows: V_{max} = 2.104 mM/min, K_m^A = 0.131 mM, K_m^B = 15.043 mM, and K_i = 1.391. Properties of thermodynamic for physically immobilized lipase-catalysed esterification reaction revealed an activation energy (E_a) and denaturation energy (E_d) of 90.36 kJ/mol and 111.47 kJ/mol, respectively. Under optimized conditions, the thermodynamic properties of physically immobilized lipase-catalysed esterification reaction at 40°C gave immobilized enzyme half-life ($t_{1/2}$) = 97.63 hours, deactivation constant (K_d) = 0.0071 hour^{-1}, enthalpy (ΔH) = 107.40 kJ/mol, entropy (ΔS) = 0.383 kJ/mol.K, and Gibbs energy (ΔG) = -19.113 kJ/mol. The amount of monoolein produced using immobilized lipase was highly comparable with free lipase, with ability of HNTs-lipase to be reused and was thermally stable at higher temperature. Based on the findings presented in this research, it validated HNT as a promising application potential for enzyme immobilize carrier and crude glycerol as an unseen starting material for better value-added product synthesize.

CHAPTER 1

INTRODUCTION

1.1 Monoglyceride

Partial acyl glycerol or in common term known as monoglycerides (MG) or diglycerides has abundance of applications in various industries. They can be produced by two main routes of esterification of glycerol and fatty acid and inter-esterification of glycerol and fats or oils. Frenchman Berthelot is the first person who synthesized mono and di-glycerides in 1853. However, the usage of monoglyceride only started to burgeon in industrial scale around 1930s following a large-scale demand for the margarine industry (Whitehurst, 2008). Annually, production of partial acylglycerols count up about 75% of the total world production of emulsifier, which is equal to approximately 250,000 metric tons annually (Devi *et al*., 2009). The worldwide food emulsifier market is expected to rise at a growth rate valued at 4–5% compound annual growth rate (CAGR) from 2014 to 2020, comparable to USD 3.2– 4.3 billion. In 2014, the AP owned the largest food emulsifier market share at 34.4%, followed by Europe at 28.6%. The Asia Pacific food emulsifier market is likely to grow at a higher rate of 7–8% CAGR from 2014 to 2020 (Transparency Market Research, 2015).

MG with the non-ionic trait consisting both hydrophilic and hydrophobic regions, are useful as emulsifier, that is widely used in the food industry, cosmetic, pharmaceutical and so etc. This function also makes MG as good lubricant bio surfactant, internally and externally in fiber and textile technology (Chetpattananondh and Tongurai 2008). Among many available MG, we chose monoolein (MO) as our target production for this study. MO or glyceryl monooleate is made up of a hydrocarbon chain which attached to a glycerol backbone by an ester bond. This feature gives its amphiphilic character that has been widely used as an emulsifying

agent for water-in-oil emulsions and micro-emulsions (Kulkarni *et al.,* 2011; Ganem-Quintanar *et al.,* 2010). Monoolein can be synthesized by few routes, such as direct esterification of fatty acids, primarily oleic acid and glycerol, or by the glycerolysis (transesterification) of refined vegetable oils, such as canola oil or sunflower oil. MO is known as one of the vital lipids in the fields of emulsion stabilization, drug delivery, protein crystallization (Nitbani *et al.,* 2020). For this study, direct esterification from oleic acid and glycerol was catalyzed using immobilized lipase-HNTs to produce monoolein.

1.2 Problem Statement

Even though MG are renewable surfactant molecules that have greater emulsifying property than diglycerides, to obtain pure MG in huge quantity is costly and technically complicated as it needs downstream processes to attain the purity. Albeit of being produced in low quantity, with high demands of MG by many industries, they can be highly priced in the market with high profit margin (Keng *et al.*, 2009). Current commercial production routes of MG by direct esterification of glycerol and fatty acid and, transesterification of triglycerides using glycerol are problematic due to employment of high temperatures of 220^{o}C or above and in most circumstances, inorganic catalysts are used. This inorganic catalyst on the hand, known for its risk for toxicity and pollution in the long run. High temperature not only degrading the quality but also will result in uneconomical and laborious purification process to obtain the food grade MG (Sonntag, 1984; Guner *et al*., 1996).

In relation to that catalyst limitation, there are many factors that have been previously studied and proved to have influence the reactions efficiency such as sources of catalyst, temperature, pH, substrate molar ratio, organic solvents and initial water content, to name a few. The replacement of inorganic catalysts by lipases has been suggested as a better alternative for the industrial production of MG. This biocatalyst is not only capable of operating at mild conditions of temperature but also has its own specificity towards substrate and produce less undesired products which may lead to zero purification of finished product (Cao *et al*., 1996). Lipase from *Candida antartica*, *Candida rugosa* (CRL), *Pseudomonas fluorescens*, *P. cepacia* and *Penicillium cyclopium* are example of lipases utilized by previous studies in MG production (Coteron *et al*., 1998; Diks & Bosley, 2000). For this reason, this study proposes the use of biocatalyst instead of commonly used inorganic catalysts for catalyzing the production of MG with the choice of lipase used is *C. rugosa*.

Despite attempts by industrial fronts to shift towards the utilization of biocatalyst as it is a safer and considered a greener technology, some drawbacks related to low stability, aqueous solubility, difficulties in recovery and recycling and high cost must be overcome. One way is to immobilize the target biocatalyst on the support carrier, which is CRL in this study. Enzyme in its immobilized form has many favorable qualities, including enhanced stability towards extreme environment, convenient handling, ease separation of products, and efficient recovery and reuse (Sheldon, 2007).

For these reasons, natural halloysite nanotubes (HNTs) was chosen in this study as support for the lipase immobilization. HNTs are naturally occurring clay with ultra-hollow tubular structure, inexpensive and naturally abundant in many countries such as Australia, Brazil, China, Japan, Korea, Morocco, New Zealand, United States

and others (Joussein *et al*., 2005; Rawtani and Agrawal, 2012; Joseph *et al*., 2013). Thus, instead of using free form enzyme, this work would be carried out the immobilization of lipase under several parameters via two different approaches; i) vacuum entrapment which is denoted as a physical approach and ii) functionalization of HNT's surface with aminopropyl group using chemical (APTES), namely the chemical approach. Although several works have reported the preparation and application of immobilized lipase CRL on HNTs, there are yet any studies examining the influence of different immobilization approaches on the catalytic activity of the enzyme. The two methods proposed within this study (physical vacuum adsorption and chemically immobilization) would reveal the effect of the surface chemistry and textural characteristics on the lipase adsorption capacity whereby the activity and loading of immobilized lipase was studied. Parameters such as substrate molar ratio, pH, temperature, and pressure were varied to study their effect on lipase loading and activity.

Thus, this study proposed to synthesize MG from free fatty acid and crude glycerol using HNTs as the support to immobilize lipase. In this study, the esterification conditions to enzymatically synthesize MO such as reaction time, agitation rate, temperature and substrate molar ratio were analyzed to assess the optimum operating conditions to produce MG. The findings of this study also offer a great benefit for industrial who would want to utilize inexpensive carrier for the enzyme supports that later can be pursued for manufacturing line of products.

1.3 Objectives

The aim of this study is to produce MO using HNTs immobilized lipase. The objectives are;

- To compare immobilization approaches between physically-adsorption of HNTs-lipase and chemically-functionalization of HNTs-lipase and further optimize the better immobilization method
- To determine the optimum conditions of temperature, agitation rate, reaction time and substrate molar ratio in maximizing MO production using immobilized HNTs-lipase
- To deduce the thermodynamic and kinetic properties of enzymatic esterification of crude glycerol and free fatty acid by immobilized HNTs-lipase

CHAPTER 2

LITERATURE REVIEW

2.1 Enzymes

Enzymes are proteinaceous macromolecules that have the ability to catalyze or accelerate both biochemical and metabolic reactions. Each and every of them share a relatively common ancestor that was defined by structure similarity, thus being categorized into several classes. Experts from biochemistry and enzymology have initiated an organized framework to name and classify enzymes using the information gathered from overall catalyzed reactions (Robinson, 2015; Sarmah *et al*., 2017). Presently, Enzyme Commission (EC) number is a way to systematically identify an enzyme using four-level code that led to enzyme classification based on the chemical transformation of substrate into product. The first level code represents six different classes corresponding to type of chemistry being put through. EC 1 stands for oxidoreductases which catalyze oxidation and/or reduction reaction, EC 2 for transferases that able to transfer a chemical group during the reaction, EC 3 represents hydrolases that carry out hydrolysis of chemical bond, EC 4 is for lyases where they cleave chemical bonds, EC 5 is called isomerases with ability to catalyze structural and geometric changes between isomers, and lastly, EC 6 stands for ligases that help linking two molecules. These EC codes are further divided into lots of subclasses and sub-subclasses which both may represent type of chemical bond formed or cleaved, the transferred chemical group, the reaction center and any cofactor used for catalysis. The last fourth level of EC number is meant to define substrate specificity (Cuesta *et al*., 2015). For instance, lipase is a hydrolase (EC 3), subclass of esterases (EC 3.1) acting on the carboxylic link (EC 3.1.1) in the triacylglycerol molecule (EC 3.1.1.3).

Lipases are a class of hydrolytic enzymes which able to catalyze triacylglycerols into glycerol and fatty acids at the interface of substrate and water. Aside of hydrolytic properties, lipases have the capability to catalyze many reactions, including esterification, aminolysis, alcoholysis, acidolysis, etc (Sarmah *et al*., 2017; Rajendran *et al*., 2009) . Not only widely used at the laboratory scale, lipases are also extensively used commercially for wide ranging applications, including for environmentally friendly substitution, upgrading raw materials, utilization of waste into value added product, bioconversion, and so forth.

The high demand of industrial enzymes as nanoscale biocatalysts is substantially rising due to their vast applications in many areas, including pharmaceutical, environmental, and production of fine chemicals for daily use in food, detergent, paper and textiles industries (Raveendran *et al*., 2018; Mojsov, 2011). Despite the attempt by industrial fronts to shift towards utilizing biocatalyst as it is safe and considered a green technology, it exerts some drawbacks because of its low stability, aqueous solubility, cumbersome in recovery and recycling and high cost (Reis *et al.,* 2019; Sharma and Kanwar., 2014) .

2.2 Enzyme Immobilization

Consequently, many researchers have developed another route to improve and stabilize the catalytic properties of enzyme, including protein engineering, chemical crosslinking and enzyme immobilization (Patel and Kharat, 2012; Sheldon, 2010). For several decades, enzyme immobilization has been brought to the attention since its first successful invention of invertase entrapment onto the charcoal by Nelson and Griffin in 1916, where they found that the enzymatic activity exhibited by immobilized invertase was the same as free invertase (Nelson and Griffin, 1916). This phenomenal

finding has created the budding interest among the researchers to explore more on the efficiency of immobilization. Enzyme in its immobilized form has many favorable qualities, including enhanced stability towards extreme environment, resistant to microbial attack, convenient handling, ease separation of products, and efficient recovery and reuse (Reis *et al.,* 2019; Homaei *et al.,* 2013; Theron *et al.,* 2008) .

2.2.1 Types of Immobilization

Immobilization of lipase have been explored by varies immobilization methods using several organic and inorganic support, such as, kaolin (Tanaskovic *et al.,* 2017), mesoporous silica (Ali *et al.,* 2016), microporous polymeric matrix (Chen *et al.,* 2019), hydrophobic sol-gel (Meunier and Legge, 2013), agarose, chitin, chitosan, polyvinyl chloride, sepharose, and trisacryl (Zucca *et al.,* 2016; Datta *et al.,* 2013; Bellusci *et al.,* 2012). One study categorized immobilization methods into three groups; support binding (adsorption, ionic or covalent attachment), entrapment and cross-linking (carrier-free) (Sheldon, 2007). While another study proposed that, when the enzymes are either adsorbed, entrapped or encapsulated on or within a solid support matrix, they are called physical immobilization which involves weak attraction forces of hydrogen bonds, van der Waals and hydrophobic interactions between them. Whereas, chemical immobilization takes place with the formation of ionic or covalent bond between the enzyme and support (Herr *et al.,* 2013). Figure 2.1 illustrated the schematic diagram for two different methods of enzyme immobilization that would be used in this study, i) physical vacuum adsorption, and ii) chemically modified HNTs for CRL immobilization.

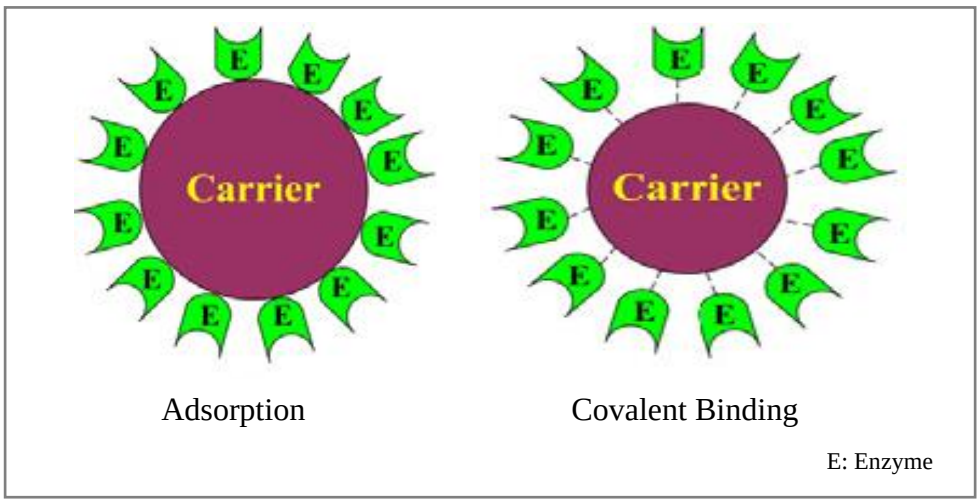

Figure 2.1 Schematic diagram for type of enzyme immobilization used in this study (Zhao *et al.*, 2015)

Adsorption

Adsorption is the simplest yet most economical technique to localize the enzyme on supports. In the absence of chemical, adsorption is performed by direct contact adsorbate (enzyme) with adsorbent (support) at appropriate conditions needed. Despite of possessing only weak attraction forces such as hydrogen bonding and van der Waals forces between enzyme and support, the binding mechanism involved would not cause conformational change which will not affect the enzyme activity. It is not only the cheapest method compared to the others; after deactivation of enzyme, reversibility of adsorption allows the recovery of support for the next cycle by adjusting the parameters. Among the conditions that give impact to the enzyme adsorption are pH, solvent, isoelectric point, ionic strength, and surface morphology of support (Villeneuve *et al*., 2000; Alloue *et al*., 2008). Support with porosity has larger surface area, thus, more capacity for enzyme loading compared to nonporous support. Enzyme are better dispersed on the larger surface area support. Adsorption of enzyme involves two-stage process; at first, the adsorbate (enzyme) will be diffused

into the adsorbent (support) and later, binding at localized adsorption sites. As for the lipase, since binding stage occurs faster than diffusion, the adsorption is dependent on diffusion (Palomo *et al*., 2002; Knezevic *et al.*, 2004). The supports used for lipase adsorption can be either organic or inorganic, for instance; alumina, activated carbon, alginate, bentonite, cellulose, chitosan, dextran, eggshell, kaolinite, and so on (Zivkovic *et al*., 2015; Dong *et al*., 2013; Tahir *et al*., 2012; Rodrigues *et al*., 2008).

Covalent attachment

The method of covalent attachment of CRL was also investigated by this study. Covalent bonds are formed between the residue of amino acid outside the active site of enzyme and the active groups available on supports. Usually, an active agent is used to activate the support's surface, so that the functional groups become strongly electrophilic to react with nucleophilic groups of enzymes. Reactive agent known as spacer arm is attached on the support via chemical reactions and, example of reagents typically used for activating the supports are carbodiimides, glutaraldehyde, 3-aminopropyltriethoxysilane and so on. The thermal stability and rigidity of enzyme being immobilized via this technique is better than adsorption, entrapment and encapsulation because the bonding strength is more intense. Thiol and amine on enzymes are common groups that take part in covalent binding.

For the enzyme, lysine residues are rarely involved in the catalytic active site, instead, they can be exploited for binding purposes, due to its widespread surface exposure and high reactivity on enzyme. Some researchers even claimed that, the crucial aspect to create the most stabilize immobilized enzyme is to aim the immobilization on the region with the densest amount of lysine residues. However, covalent attachment can cause changes in enzyme conformational and its catalytic properties due to harsh utilization of chemicals and sometimes coinciding of same

amino groups at active site that being required in enzyme-support binding (Mohamad *et al*., 2015; Lopez-Gallego *et al*., 2013; Stoytcheva *et al*., 2011).

2.2.2 Immobilization Carrier

Since the immobilization methods involve the interaction between the support and the enzyme, the selection of supports is vital to avoid denaturation or non-functional immobilized enzyme. For industrial uses, the utilized supports should demonstrate proper porosity depending on the size of the target enzyme, suitable hardness and density (Zhongjie *et al.,* 2013). However, this study preferable the use of inorganic supports compared to organic ones, for their high stability, resistance against microbial attacks and harsh organic solvents as well as easy reusability and disposal. Examples of inorganic supports are glass beads, zeolites, hydrotalcites, porous molecular sieves, and alumina (Jesionowski *et al.,* 2014; Datta *et al.,* 2013; Yagiz *et al.,* 2007).

Table 2.1 listed different source of lipases immobilized on different type of supports via several immobilization techniques. Most importantly, enzymes immobilized on such supports have been used with good success to produce MG, using three different siliceous mesoporous catalyst of MCM-41 (mobile crystalline material), HMS (hexagonal mesoporous silica) and silica gel that were organofunctionalized using propyl sulfonic acid groups (SO_3H) (Hermida *et al*.,2010). Previous study by Chaari, Neji and Frikha (2017) used acidic clay montmorillonite to catalyze the fatty acid esterification and they noticed the conversion of oleic acid increased proportionally with molar ratio of substrates and achieved the highest conversion of 93.5%.

However, some of these supports might also have microporous surfaces which can limit the access of molecules larger than 2 nm, which make the poor candidates as support to immobilize enzyme. Since most of the sizes of enzymes are larger than 2 nm, this could impede the success of immobilization from occurring (Tully *et al.,* 2016; Zhai *et al.*, 2010). So far, an inorganic support suitable for enzyme immobilize is the HNTs. This is because HNTs have large surface area and pore diameter, valuable features that indicate potential nanocarrier that allows immobilization of various molecules, including anticorrosion agents, drugs, and proteins (Holesova *et al.,* 2018; Kurczewska *et al., 2017;* Tully *et al.,* 2016;).

Halloysite Nanotubes (HNTs)

HNTs are tubular clay material that is chemically two-layered aluminosilicate, composing equal ratio of alumina octahedron sheet and one silica tetrahedron sheet and share the same chemical formula with kaolin ($Al_2Si_2O_5(OH)_4$) except with the monolayer water molecules in between the unit layers. HNTs are cheap and widely available and the length ranges between 400 nm to over 1000 nm, with an outer diameter of less than 100 nm and the lumen with a diameter between 15-20 nm. (Tully *et al.,* 2016; Falcon. *Et al.,* 2015; Rawtani, 2012). The availability of silicon dioxide on the outer surface cause HNTs to exhibit a negatively charged external surface. Whereas, the availability of aluminum hydroxide on the internal surface causes the lumen to be positively charged. When the surrounding medium is at a moderate pH (between 3 to 10), the external surface and lumen would retain opposing charges, thus allowing immobilization to occur for selective charged molecules (Lvov and Abdullayev, 2013; Shchukin *et al.,* 2005). The immobilization of enzymes inside the lumen will form weakly interactions of hydrogen bonding or van der Waals between them. Functionalization of HNTs is necessary using organosilane (3-aminopropyl

triethoxysilane (APTES)) in order to introduce aminopropyl groups on the surface of HNTs, thus, enable the enzyme to immobilize on the HNT's. These groups are expected to have a strong affinity towards specific functional groups of enzymes (Tan *et al.,* 2013).

Table 2.1 Different source of lipases immobilized on different type of supports via several immobilization techniques

Immobilization		Source of lipase	Reference
Technique	Support		
A	Activated carbon	*Candida antartica*	Rodrigues et al. (2008)
A	Chitosan	*Candida rugosa*	Nasratun et al. (2009)
A	Dextran	*Rhizopus arrhizuz*	Tahir et al., 2012
A	Hydrotalcite	*Thermomyces lanuginosus*	Yagiz et al. (2007)
A	Polymethacrylates, Polystyrene	*Candida rugosa, Pseudomonas cepacia*	Palocci et al. (2007)
A	Resin	*Candida antartica*	Wang et al. (2006)
A	Textile membrane	*Candida sp. 99-125*	Lu et al. (2007)
A	Zeolite	*Rhizomucor miehei*	Vasconcellos et al. (2012)
COV	Carbon nanotubes	*Yarrowia lipolytica*	Feng, Sun, & Ji, (2012); Li, Feng, & Pan, (2013).
COV	Magnetic nanostructures	*Candida rugosa*	Lei et al. (2009); Yong et al. (2008); Lee et al. (2009)
COV	Olive pomace	*Thermomyces lanuginosus*	Yucel (2011)
COV	Resins	*Pseudomonas fluorescens, Thermomyces lanuginosus*	Mendes et al. (2011)
COV	Silica	*Rhizopus oryzae, Candida rugosa*	Lee et al. (2011)

A : Adsorption COV : Covalent attachment

2.3 Monoglyceride Production

2.3.1 Lipase as A Biocatalyst

Lipase (triacylglycerol acyl-hydrolase, E.C. 3.1.1.3) has known as one of the most useful biocatalyst in many industry, with wide-ranging of substrate it can work with, good stability in organic solvents, able to activate itself without the need of cofactor and many more (Sandoval, 2012). In this study, the lipase from CRL is the enzyme used for immobilization on two different methods. The catalytic properties of CRL such as substrate specificity, allows the CRL to control the products produced and increase the yield by reducing by-products (Benjamin and Pandey, 1998).

Figure 2.2 showed 3D structure of CRL in its open form. Generally, lipase is made up of a polypeptide chain folded into two domains, the N terminal and C terminal. The N-terminal domain is characterized by both alpha and beta hydrolase fold that holds the active site with a hydrophobic tunnel from serine residues to its surface which gives lipase an ability to hold a long fatty acid chain. While the non-catalytic domain called C terminal, carries beta sheet sandwich that interacts with colipase, thus enable lipase to anchor itself to water-lipid interface (Akoh and Min, 1998; Ollis *et al.,* 1992). In the present of hydrophobic interface is present, lipase would shift from closed lid structure to open ring structure initiated the enzyme activation. The conformation changes of lid opening, would expose the active site binding for the lipid. Then, the substrate able to move into the hydrophobic tunnel enclosing with the active site and this phenomenon is known as interfacial activation (Thomas *et al.*, 2005; Sussman *et al.*, 1991).

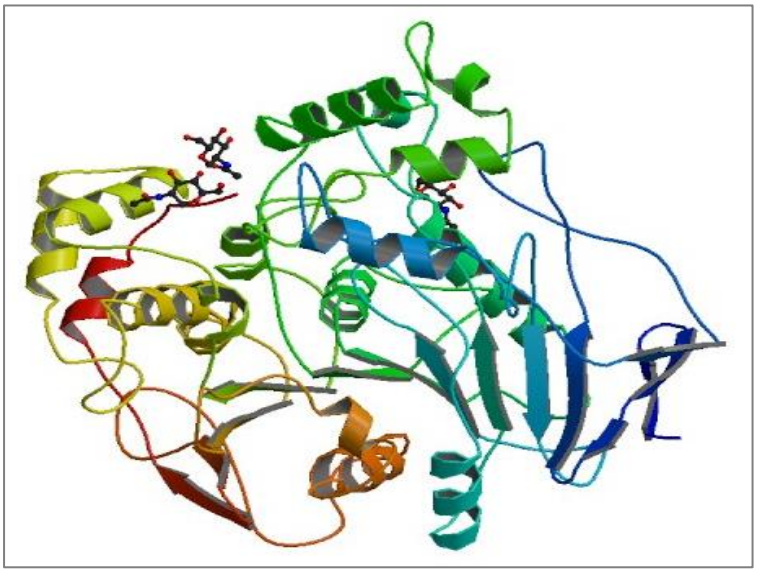

Figure 2.2 An open ring structure of *Candida rugosa* colored by ligand interaction (1CRL from RCSB Protein Data Bank;DOI: 10.2210/pdb1CRL/pdb) (Grochulski *et al.,* 1993)

As shown in Figure 2.3, reaction that being catalyzed by lipase will first formed acyl-enzyme intermediate and alcohol, that originated from the nucleophilic attack of active site serine hydroxyl group (E-OH) on carbonyl carbon of ester bond. This acyl-enzyme intermediate is later hydrolyzed by water or other nucleophiles such as alcohol, to form second product, carboxylic acid. A large number of nucleophiles are capable of deacylating acyl-enzyme intermediates, which lead to the counterproductive reversion of esterification (Toledo *et al.,* 2017).

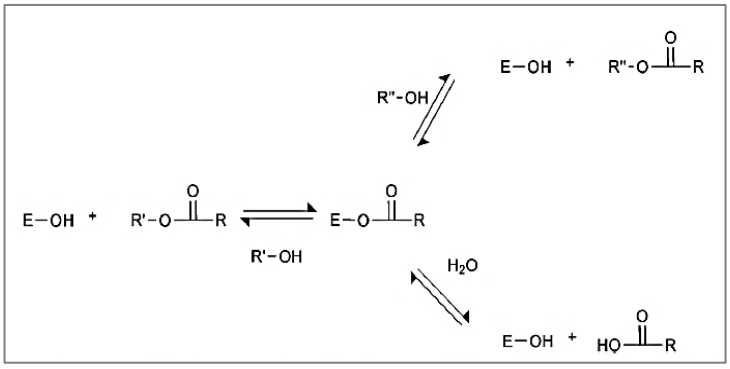

Figure 2.3 Mechanism of lipase-catalyzed reaction (Adlercreutz, 2013)

Utilization of biocatalyst in manufacturing process is known to be non-toxic, hence ecologically safe to perform biochemical reactions under mild reaction conditions but with high selectivity. Moreover, biocatalyst in its free form would not be practical for industrial setting because of its activity and stability tends to deteriorate abruptly over improper temperature and pH applied. Pristine enzyme is not cheap to be repurchased several times during a single manufacturing line of monoglyceride. In view of the drawbacks of free lipase i.e CRL, the study believes a practical cost-effective MO production process can be realized by making CRL reusable via immobilization onto the HNTs.

2.3.2 Monoglyceride Synthesis by Lipases

The use of lipase to produce MG comes in two forms; it can be free lipase or immobilized lipase on a wide range of supports. Lipases are the class of enzymes that have the highest degree of hydrophobicity with 28-30% of hydrophobic amino acid residues. These hydrophobic residues are usually close to the active site which make them prone to activation after being adsorbed on hydrophobic supports like HNTs (Prlainovic *et al.,* 2013; Boncel *et al.,* 2013). There are very few studies that utilizing lipase in its free form to catalyze MG production. One of the earliest studies that succeed in producing high yield of MG at low temperatures using free lipase was by McNeill and team in 1991. In their work, several fats and oils were subjected for the enzymatic glycerolysis using *Pseudomonas fluorescens* under lower temperature. Almost all substrates used in the study could produce more than 65% of MG and olive oil showed the most excellent yield of 90% MG at only 10^{o}C of reaction temperature, while, palm stearin came in second with 86% of MG at 30^{o}C. Due to the low reaction temperature applied, longer time was observed for the olive oil to obtain such high yield of MG, which took about 4 days (McNeill, Shimizu, & Yamane, 1991).

Following the success of lipase being immobilized on different supports for broad range of application, many researchers have further investigated the effectiveness of lipase immobilization towards MG production. Table 2.2 summarizes the previous studies that managed to synthesize MG using immobilized lipases. Several different methods of enzymatic immobilization have been explored to synthesis MG and adsorption technique is observed as the most employed method to localize the enzyme. Adsorption method for enzyme immobilization enables support to be recovered after the enzyme are deactivated. Furthermore, recovery of the absorbent can potentially reduce the manufacturing cost as the support is reusable for the next round of enzyme immobilization (Sheldon, 2007).

Table 2.2 Previous studies on MG production via lipase immobilization

Substrate	Lipase	Immobilization		MG Yield (%)	Reusability	Reference
		Technique	Support			
Lauric acid + Glycerin	*Rhizomucor miehei*	A	Acrylic resin	53.67	Six cycles	Mustafa, Karmali, & Abdelmoez (2016)
Docosahexaenoice acid (DHA) ethyl ester + Glycerol	*Rhizomucor miehei, Candida antartica, Alcaligenes sp.*	A	Sepabeads-C_{18} resin	>90	10 cycles	Moreno-Perez et al. (2016)
Coconut oil + Glycerol	*Candida antartica, Thermomyces lanuginose, Rhizomucor miehei*	A	Acrylic resin	60 – 97.6	-	Zha et al. (2014)
Palmitic acid + Glycerol	*Candida antartica, Thermomyces lanuginose, Rhizomucor miehei*	A	Acrylic resin	50	-	Tai & Brunner (2011)
Olive oil + Glycerol	*Candida rugosa, Candida antartica, Burkholderia cepacia*	A	Acrylic resin & Accurel	40 - 61	-	Esmelindro et al. (2008)
Oleic acid + Glycerol	*Staphylococcus simulans*	A	$CaCO_3$	71.68	-	Ghamgui et al. (2006)
Fatty acids + Glycerol	*Penicillium camembertii*	COV	Epoxy SiO2-PVA	32 - 59	-	Freitas et al. (2010)

A : Adsorption COV : Covalent attachment

One study has claimed that the commercial immobilized enzyme (Lipozyme RM IM) was able to retain high enzyme catalytic activity in solvent-less medium even after being used for six reaction cycles. Under optimum conditions of temperature (60^oC), enzyme concentration (4%) and glycerin to fatty acid molar ratio (4:1), they managed to synthesize about 53.67% of monoglyceride from 93.23% of total fatty acid conversion. The high conversion of fatty acid was impressively achieved by the team in only 1 hour of reaction time (Mustafa, Karmali, & Abdelmoez (2016). Even though the commercial immobilized lipase is deemed as expensive, perhaps with its capability to be reused several times under high retention of enzyme activity will counter effect its price. The reusability of immobilized enzyme was stacked up by another researcher into 10 reaction cycles using Sepabed C18 resin as support for three different lipases; *Rhizomucor miehei*, *Candida antartica*, *Alcaligenes sp*. (Moreno-Perez et al., 2016).

This study succeeded in producing monoglyceride incorporated with docosahexaenoice acid (DHA), which is omega-3 fatty acid where its application is undoubtedly broad in nutritional foods/supplements, pharmaceuticals and cosmetics. The same immobilization technique (adsorption) was used but Moreno-Perez and team managed to synthesize high yield of monoglyceride (>90%) at a lower temperature of 37^oC. While at higher temperature ($\geq 50^oC$), product inversion occurred, where the reaction became more favorable towards triglyceride formation. Interestingly, the reaction time required to achieve such high yield of MG was not more 4 hours and the MG produced was stable and remained unmodified for 24 hours without proper storage.

Prior to those studies, Zha and fellow researchers (2014) also succeeded to yield MG more than half of the total fatty acid converted. They compared three lipases (*C. antartica*, *Thermomyces lanuginosa*, *Rhizomucor miehei*) in the glycerolysis of

coconut oil and *C. antartica* managed to produce the highest monoglyceride of all lipases used. The study has worked on two-steps molecular distillation after MG were obtained under its optimal conditions. The optimum parameter of temperature (50^{o}C), enzyme loading (8%), glycerol to coconut oil ratio (4:1) and reaction time (4 hours) happened to only produce about 29.3%. Later, when the MG were placed into this two-step of molecular distillation, the total recoveries obtained was more than 60%. This make the final product of MG to be 97.6%, with 2.0% of diglyceride and triglyceride, nil. The first step of distillation designed to completely omitted the free fatty acids resulted from the glycerolysis, while in the second step, more MG could recover as they claimed that the temperature used (120^{o}C) was in the right range for the coconut oil. When they attempted to increase the temperature, recovery yield of MG decreased slowly with the simultaneous increase of diglycerides and triglycerides that were boiled off and drained into the distillate too (Zha *et al.*, 2014). Despite of lower enzyme loading (<10%), works that have been mentioned previously were still able to synthesize more than 50% of MG. Compared to the study conducted by Tai and Brunner (2011), their optimum enzyme loading was 25% but only bring about 50% MG. The palmitic acid conversion was hardly observed to reach 80% and they claimed that the presence of water has made the reaction selectivity favors diglycerides, while too much of water will shifted the esterification towards hydrolysis.

With the use of biocatalyst, the input cost spends on the heat energy will be minimized as well because to get the enzyme to work at its optimum, the temperature can never be too high, otherwise the enzyme will resort to denaturation. Different lipase has different selectivity level towards monoglyceride, and previous study demonstrated that the same source of substrates produced different composition of end products when catalyzed with several different lipases. The dominant composition

might still be MG but with slightly changed in percentage against the other constituents (diglyceride, triglyceride, free fatty acid) (McNeill, 1991 & Fregolente *et al.*, 2008).

The afore mentioned previous studies, despite some showing low MG yield, the final yield of their works still fulfill the standard recognized by World Health Organization (WHO) which stipulates that food grade MG must comprise of; a) at least 70% of MG + diglycerides, (b) a minimum of 30% of MG, and (c) to have contents of both glycerol and triglyceride below 10% (Arcos and Otero, 1996). Thus, this study aimed to run the esterification at its optimal reaction conditions in order to maximize the MO yield, hence can broaden the use of MO produced into possibly food applications.

2.4 Scenario of Crude Glycerol

Glycerol is an alcohol with three hydroxyl groups, a hygroscopic, viscous liquid which is non-toxic and well known as reactive molecule that able to undergo various reactions to form aldehyde, amine, ester, and ether. Unlike purified and commercial grade glycerol, crude glycerol tends to retain slight impurities such as ash, soaps, fatty acids, glycerides, and solvents, where most of the time, do not consumed the major part of glycerol. Rising concerns over depletion of fossil fuel reserves in the near future has urged industries and researchers to opt for alternative energy sources and this biofuel has enticed many for its renewable and environmentally friendly fuel. An upsurge in biofuel production has eventually generated a vast amount of byproduct, which is crude glycerol (Yang *et al*., 2012). This crude glycerol is produced profusely from saponification and hydrolysis reaction in oleochemicals, and transesterification reaction in biodiesel production. For every 100 lbs of biodiesel produced,

approximately 10 lbs of crude glycerol are produced (Anuar and Abdullah, 2016; Tan *et al.,* 2013; Ayoub and Abdullah, 2012; Yang *et al*., 2012).

Figure 2.4 depicts the transesterification process that results in the formation of crude glycerol. Initially, methanol reacts with triglycerides of vegetable oil or animal tallow to form diglycerides and methyl ester. Methanol later reacts with diglycerides produced from the first step, to form MG and methyl ester. The MG consequently reacts with methanol to form glycerol along with methyl ester. Zhou and co-workers monitored the input of triglycerides in relation to the produced biodiesel and its byproduct, crude glycerol. They discovered that crude glycerol was produced along with biodiesel at a ratio of 1:10. This meant that, every 45.3 kg of biodiesel produced, 4.53 kg of crude glycerol was created (Zhou *et al.,* 2008).

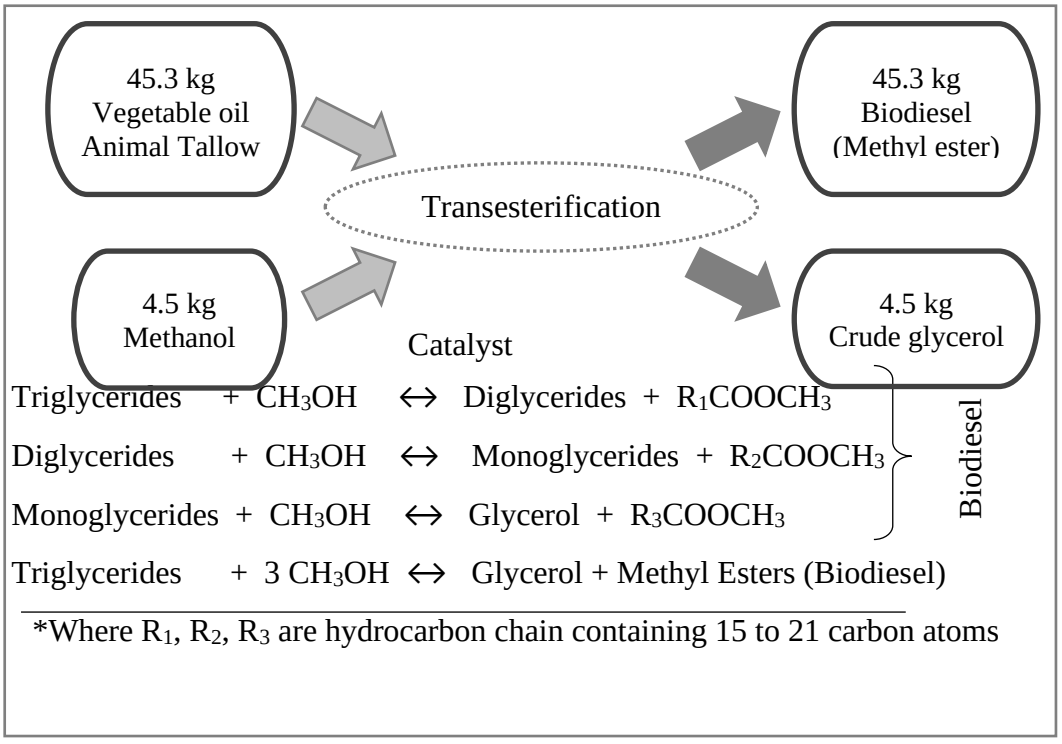

Figure 2.4 Crude glycerol formation during transesterification of biodiesel production (Zhou *et al.,* 2008)

With the rising demand of biodiesel, it is estimated that worldwide glycerol production will reach about 4.2 million tons by 2020. This is far larger than the demand for glycerol which is only predicted to be less than 3.5 million tons in the same year (Viana *et al.,* 2014). The impacts of this large surplus of crude glycerol on the social environment and difficulties faced by industries to dispose enormous quantities of crude glycerol have given impetus for many to turn it into value-added products, instead of the norm of dumping into landfills (Lu *et al.,* 2008).

2.4.1 Value-Added Utilization of Crude Glycerol

It is not common to proceed chemical reactions with crude glycerol instead of pure glycerol as the starting material. Nonetheless, there were few success of using crude glycerol as fermentation feedstock for various production, such as butanol, fumaric acid, humectant (1,3-propanediol), and omega-3 polyunsaturated fatty acid (docosahexaenoic acid (DHA)) and also been used to produce monoglycerides (MG) (Binhayeeding, *et al.,* 2017; Zhou *et al.,* 2014; Powalowska and Bialas, 2014; Kao *et al.,* 2013; Echeverri *et al.,* 2011; Chetpattananondh and Tongurai, 2008; Pyle, 2008).

There is also a recent work, where the food surfactants from monoglyceride and its ester derivatives were obtained by using byproduct glycerol generated from biodiesel manufacturing. The crude glycerol was refined using phosphoric acid prior to the oleic acid esterification. Direct esterification catalyzed by 1.5% para-toluenesulfonic acid (*p*-TsOH) (weight % with respect to oleic acid) achieved about 29% of glycerol monooleate under the optimal reaction conditions of 140^{o}C using 1:2 molar ratio of oleic acid to glycerol for a duration of 90 minutes. The obtained monooleate was further used to synthesize other ester derivatives, such as lactylated glycerol monooleate and acetylated glycerol monooleate, from which lactic acid and acetic anhydride were the starting materials, respectively. Another monooleate

derivative, diacetyl tartaric ester monoglyceride (DATEM) was also produced using the starting substrate of glycerol monooleate with tartaric acid and using acetic anhydride as acetylating agent. The reaction yielded about 49% of DATEM after purification step using column chromatography (Hue *et al.,* 2019). This finding revealed that crude glycerol is a promising substrate for production of MG and the other three ester derivatives of MG.

Crude glycerol from the transesterification process can acidified with sulphuric acid and then gravitationally separate the crude into three distinct phases with glycerol-rich at the middle layer and free fatty acids and inorganic salt in between (Binhayeeding, *et al.,* 2017). The purified crude glycerol was then proceeded to be use for glycerides production catalyzed by two different enzymes, *Candida* sp. Lipase and Pacific white shrimps hepatopancreas. More than 50% of MG, diglyceride and triglyceride were obtained under the optimal glycerolysis conditions using 6:1 mole ratio of glycerol to palm oil, 100 mg of lipase at 40^{o}C for 24 hours.

Another study by Echeverri and co-workers attempted MG production by a glycerolysis reaction between crude glycerol and soybean oil. The crude glycerol was the product of the transesterification of soybean oil and methanol using sodium methoxide and sodium hydroxide as the catalysts. The crude glycerol was utilized directly without the purification process except for the removal of methanol. About 42% of MG was obtained and they claimed that using lower amount of catalyst enhanced the production of monoglycerides. Higher concentrations of catalyst only reduced yield of MG as the reaction would be more favorable to produce diglycerides (Echeverri *et al.,* 2011).

In 2008, Chetpattananondh and Tongurai succeeded in producing MG with palm stearin and followed by two-step processes that include removal of residual