APPLICATION OF EUTECTIC SOLVENTS IN CHEMICAL AND ENZYMATIC REACTIONS OF UPSTREAM PROCESSES FOR BIODIESEL PRODUCTION

SHAHIDAH NUSAILAH BINTI RASHID

FACULTY OF ENGINEERING
UNIVERSITY OF MALAYA
KUALA LUMPUR

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SHAHIDAH NUSAILAH BINTI RASHID

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ABSTRACT

This study introduces novel deep eutectic solvents (DESs) that act as catalyst in esterification of palm oil and media for enzymatic hydrolysis to produce palmitic acid. The conventional organic solvent will have to be a substitute in the near future by DES due to the economic viability and environmental concerns. The development of DES-based solvent and catalysis is at an exponential rate. Acidic crude palm oil (ACPO) with 9.2 % of free fatty acid (FFA) generated from industrial palm oil was pre- treated (esterified) before utilizing it as feedstock for biodiesel production. The pre-treatment of ACPO was conducted using (1R)-(-)-camphor-10-sulfonic acid (10-CSA) with choline chloride (ChCl); CSA-ChCl-ES and p-toluenesulfonic acid monohydrate (PTSA) mixed with benzyltrimethylammonium chloride (BAC); BAC-DES as the novel DES-based catalysts to remove the FFA to less than 2% at optimized condition. The optimal reaction conditions for CSA-ChCl-ES were 2.5 wt% of catalyst dosage, 10:1 molar ratio, 60 ^{0}C of reaction temperature and 40 minutes of reaction time. While the reaction conditions for BAC-DES were 2 % catalyst dosage, 10:1 molar ratio of methanol to oil, 30 min of reaction time and reaction temperature of 60 °C gives high conversion and yield of 90 %. In the second part of this study, hydrolysis reaction of DES with lipase enzyme was investigated. The results show that DES is able to activate and stabilize lipases enzyme in hydrolysis reaction. DESs of the aqueous glycerol solution (GLY 85) as the hydrogen bond donor (HBD) with methyltriphenylphosphonium bromide (MTPB) and ChCl as salt was applied as new reagents media for enzymatic hydrolysis. The physicochemical properties such as viscosity, conductivity, density, surface tension, and freezing point were measured to establish DES characteristics. The results showed that DES at a ratio of 1:3 of ChCl-based DES and 1:5 of MTPB-based DES has their eutectic points at 213.4 K and 255.8 K respectively. The enzymatic activity of *porcine pancreas* and *Rhizopus niveus* lipases in DESs were examined. Both lipases were stable

in all ratios of the DES especially in 80% concentration of DES. Overall, phosphonium-based DES showed higher activation towards *porcine pancreas* lipase (7.2 fold) compared to *Rhizopus niveus* lipase (0.9 fold) under the same conditions. To the best of our knowledge, this is the first time that aqueous glycerol was used to prepare DESs and subsequently applied for enzyme-based processes. Overall, these results proved that DES especially ChCl-based DESs could replace conventional solvents as they possess enormous potential; especially in the electrochemical technology given their values are higher than average conductivities. They also demonstrated a stabilizing effect on lipases in which the activity was stimulated in the presence of DESs in aqueous solution system. This feature could assist in transesterification in biodiesel synthesis.

ABSTRAK

Kajian ini memperkenalkan pelarut eutektik baru (DES) yang bertindak sebagai katalis dalam reaksi pengesteran minyak sawit dan media untuk hidrolisis enzimatik untuk menghasilkan asid palmitik. Pelarut organik konvensional akan menjadi pengganti dalam masa terdekat oleh DES disebabkan kebergantungan ekonomi dan kebimbangan alam sekitar. Kajian yang dijalankan dalam bidang pembangunan pelarut dan katalis berdasarkan DES adalah pada kadar yang tinggi. Minyak sawit asid mentah (ACPO) dengan 9.2% asid lemak bebas (FFA) dihasilkan daripada minyak sawit industri telah dirawat terlebih dahulu (esterified) sebelum menggunakannya sebagai bahan mentah untuk pengeluaran biodiesel. Pra-rawatan ACPO telah dijalankan menggunakan (1R) - (-) - asid camphor-10-sulfonat (10-CSA) dengan choline chloride (ChCl); CSA-ChCl-ES dan p-toluenesulfonic monohydrate (PTSA) bercampur dengan benzyltrimethylammonium chloride (BAC); BAC-DES sebagai katalis berasaskan DES baru untuk menghapuskan kadar FFA kepada kurang daripada 2% mengikut parameter yang telah dioptimumkan. Keadaan tindak balas optimum untuk CSA-ChCl-ES ialah 2.5% jumlah dos katalis, nisbah 10: 1 molar, 60 ^{0}C suhu tindak balas dan 40 minit masa tindak balas. Sedangkan untuk BAC-DES adalah 2% dos katalis, nisbah molar 10: 1 methanol kepada minyak, 30 minit masa tindak balas dan suhu reaksi pada takat 60 °C memberikan jumlah minyak terawat yang tinggi iaitu 90%. Dalam bahagian kedua kajian ini, tindak balas hidrolisis DES dengan enzim lipase telah disiasat. Keputusan menunjukkan bahawa DES dapat mengaktifkan dan menstabilkan enzim lipase dalam reaksi hidrolisis. DES bagi larutan cecair gliserol (GLY 85) sebagai penderma bon hidrogen (HBD) dengan metiltriphenylphosphonium bromide (MTPB) dan choline chloride (ChCl) sebagai garam digunakan sebagai media baru untuk hidrolisis enzimatik. Ciri-ciri fizik dan kimia seperti kelikatan, kekonduksian, ketumpatan, ketegangan permukaan, dan titik beku diukur untuk memprofilkan ciri

DES. Hasil kajian menunjukkan bahawa DES pada nisbah 1: 3 dari DES berasakan ChCl dan 1: 5 dari DES berasaskan MTPB mempunyai nilai eutektik masing-masing pada 213.4 K dan 255.8 K. Aktiviti enzim *Porcine pancreas* and *Rhizopus niveus* lipases dalam DES telah dikaji. Kedua-dua lipase menunjukkan kestabilan dalam semua nisbah DES terutamanya dalam kepekatan sebanyak 80%. Secara keseluruhan, DES berasaskan fosfonium menunjukkan pengaktifan yang lebih tinggi terhadap *Porcine pancreas* (7.2 kali ganda) berbanding dengan *Rhizopus niveus* lipase (0.9 kali ganda) di bawah keadaan yang sama. Untuk pengetahuan, ini adalah kali pertama cecair gliserol digunakan untuk sebagai DES dan kemudiannya digunakan untuk proses reaksi berasaskan enzim. Secara keseluruhannya, keputusan ini membuktikan bahawa DES terutamanya DES yang berasaskan ChCl boleh menggantikan pelarut konvensional, kerana ia mempunyai potensi yang sangat besar, terutamanya dalam teknologi elektrokimia berdasarkan hasil kajian yang telah ditunjukkan yang lebih tinggi daripada hasil tanpa DES. Mereka juga menunjukkan kesan penstabilan pada enzim lipase di mana aktiviti itu dirangsang dengan kehadiran DES dalam sistem larutan cecair. Ciri ini boleh membantu dalam transesterifikasi dalam sintesis biodiesel.

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LIST OF SYMBOLS AND ABBREVIATIONS

DES : Deep Eutectic Solvent
SPO : Sludge Palm Oil
CPO : Crude Palm Oil
ACPO : Acidic Crude Palm Oil
LGPO : Low Grade Palm Oil
FAME : Fatty Acid Methyl Ester
ISI : Institute for Scientific Information
FAAE : Fatty Acid Alkyl Ester
POME : Palm Oil Mill Effluent
EFB : Empty Fruit Bunch
SMM : Simultaneous Mode
WCO : Waste Cooking Oil
NaOH : Sodium Hydroxide
KOH : Potassium Hydroxide
BCL : Burkholderia cepacia lipase
ChCl : Choline Chloride
FFA : Free Fatty Acid
10-CSA : (1R)-(-)-camphor-10-sulfonic acid
BAC : benzyltrimethylammonium chloride
MTPB : methyltriphenylphosphonium bromide
HBD : Hydrogen Bond Donor
LPR : Lipase from porcine pancreas
LRN : Lipase from Rhizopus niveus
LCR : Lipase from Candida rugosa
LAB : Amano Lipase PS was utilized from Burkholderia cepacia (BCL)
ICALB : lipase B candida Antartica immobilized on immobead whom recombinant from aspergillus oryzea,

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CHAPTER 1: INTRODUCTION

1.1 **Background**

Energy is one of the vital components for human in their daily life. Energy is also the key factor for the transformation of technologies all around the world. The demand and consumption of energy were never deprived however the sources are. Many innovations were developed in order to maintain and balance both demand and sustainability of energy. However, exploitation of the sources of energy without a proper sustainability management causes deprivation and it also upsets the ecosystem and biodiversity of nature. It causes severe environmental catastrophe such as global warming, extreme climate change, and ozone layer depletion.

Petroleum or fossil fuel is one of the dominant and highly dependent sources of energy nowadays, especially for the transportation sector. It is so vital that even the stability of global economy depended on it (Mohaddes, & Pesaran, 2017). In addition to that, individual, industry and company’s income is spent on procuring fuel products (Hasan, & Rahman, 2017). However, the fluctuating prices of fossil fuel and the concern for the negative impact on the environment, people are now shifting to renewable sources of energy such as biofuel, hydro, solar and wind energy.

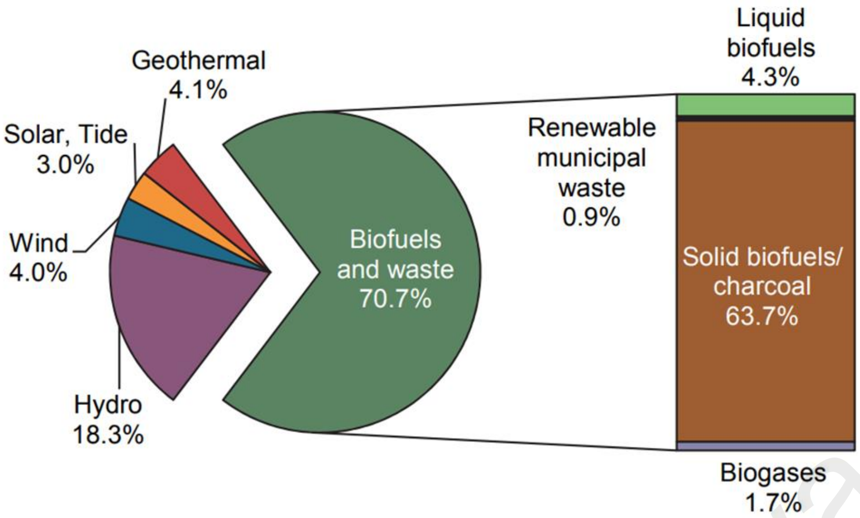


Figure 1.1.1: Product shares in world renewable energy supply for 2015 (IEA, 2017)

As shown in Figure 1, the world largest renewable energy supply comes from biofuels which cover 70.7% of the total supply. Among biofuel, only 4.3 % biodiesel produced by the manufacturer. In fact, during the last 10 years, biodiesel production has elevated from 736 m^3 to 3,419,838 m^3 in South America alone (De Oliveira, & Coelho, 2017). Generation of biodiesel started since 1930s when edible oils were used occasionally especially during emergency time where diesel oil reserve decreases (Ma, & Hanna, 1999). Biodiesel is fuel derived from biomass and compared to fuel from petroleum, it emits lower CO_2 to the atmosphere (Hao et al., 2018). Adopting and blending of biodiesel with diesel fuel will lower the intensity of greenhouse effect and improve air quality (Musa, 2016).To date, another type of source has been used to produce biodiesel such as using non-edible oil, oil from algae, and also waste oil recycle. The competitiveness of biodiesel industry is at the same par with the diesel fuel. The current market for biodiesel is accelerating in exponential phase wherein between 2005 to 2015, it increases at a rate of 23% per annum (Naylor, & Higgins, 2017).

Rise in the awareness to reduce environmental pollution builds the idea of zero waste emission system and it draws the attention from both manufacturer and researcher around the world to utilize and recycle waste as feedstock (Shimada et al., 2002). It is the most efficient method to both preserve the environment as the waste are not dumped away and instead, they are reused to produce biofuel. Waste palm oil or sludge palm oil has been studied extensively and showed great positive impact as feedstock in biodiesel production (Hayyan et al., 2010b) . The optimization for production of biodiesel was also studied such as the dosage of catalyst, the molar ratio of solvent to feedstock, reaction temperature and reaction time. The innovation of this research is on the application of a novel solvent or deep eutectic solvent (DES) as the co-solvent in both chemical and enzymatic esterification for production of biodiesel. DES is able to improve the hygroscopicity of chemical catalyst and durability of enzyme catalyst in esterification of waste oil. These will lead to a comprehensive understanding and a fill in the hole of research on biodiesel production focusing on the development of solvent and catalyst used.

1.2 Problem Statement and Significant of Study

As Malaysia is one of the largest palm oil producers, the palm oil refinery produces a lot of products and by-products. It has high value and significance in the production of biodiesel. For example, sludge palm oil (SPO), crude palm oil (CPO), acidic crude palm oil (ACPO) have already been used as the main raw material for the production of biodiesel (Hayyan et al., 2014b). The author reported that high free fatty acid (FFA) level in CPO or mixed CPO with SPO has been successfully decreased using various types of acidic catalysts. Other components such as the empty fruit bunches and ashes from the refinery process can also be used as catalyst (Ho et al., 2014; Mosarof et al., 2015).

Other than raw material, components such as solvent and catalyst also play an important role in the esterification and transesterification reactions. Enzymatic treatment is more favorable than chemical treatment regardless of the fact that it is more expensive and lowers catalytic activity depending on the type of enzyme used. Enzymatic treatment is non-toxic and has a higher cycle of recyclability that will offer greener and more effective production of biodiesel for the industry.

Concerning the cost and availability of palm oil in Malaysia and Indonesia and also the potential to scale up the production of biodiesel in this region, a study on the significance of DES in biodiesel production especially on esterification reaction is worthy to investigate. This is important to contribute to advancement of information on the optimization and dynamic study of biodiesel production. The use of catalyst and support solvent properties within the production will also be studied. This is important especially to the research community and regional community that are involved in this industry.

A clearer view on the effect of DESs in biodiesel production optimization needs more attention. Synthesis of efficient and favorable DESs may offer great potential for the enhancement of esterification and transesterification reactions. This potential of enhancement will be influenced by various combinations of salt and hydrogen bond donor (HBD) with different chemical and physical characteristics such as hydrogen bond, nature of cation-anion and viscosity.

1.3 Research Philosophy

This research aspires to provide a fundamental knowledge on optimization of biodiesel production focusing on the pretreatment process by applying novel solvent in the system. DES is selected as the most practical supporting solvent for the treatment of acid waste oil prior to biodiesel production for study, owing to the simplicity of its synthesis and derived from low-cost material. Understanding the interactions between the DES with both chemical and enzyme catalyst is vital for designing new catalyst for production of biodiesel. Preliminary studies of enzyme lipase activity and without catalyst were performed before optimization to investigate the effect of each parameter (reaction time, reaction temperature, mixing speed and chain number of alcohol used as solvent). The recyclability and validation steps were also performed for each type of selected catalyst to give an overall view of the effect of each novel catalyst.

1.4 Research Objective

1. Synthesis of different DESs and investigation of the physical properties.
2. Application of DESs in the enhancement of lipase enzyme activity.
3. Investigate the catalytic activity of chemical based DES catalyst for esterification using a different type of salts and hydrogen bond donors.
4. Study the recyclability of DESs after the reaction.

1.5 Research Methodology

The specific stages of the research methodology are listed below.

1. Background study of biodiesel production using chemical and enzymatic pathway.
2. Analysis of performance of lipase enzyme activity using UV-spectrophotometry in the hydrolysis reaction.
3. Study on physical properties of proposed DES.
4. Conversion of high content free fatty acid oil into biodiesel using a DES-based catalyst.
5. Optimization, validity, and recyclability of esterification reaction using a DES-based catalyst.

1.6 **Outline of the Thesis**

This thesis comprises of five chapters, as follows:

Chapter 1 provides an overview of the current state of biodiesel production, the problem statement, research objectives, and finally the research methodology.

Chapter 2 discusses a literature review covering the challenges in the fuel industry, the need for biofuel especially biodiesel, the pathway for production of biodiesel using chemical and enzymatic treatment and finally a current study of DES in biochemistry and application of DES as a support system in chemical and enzyme-based catalyst.

Chapter 3 gives the detailed study of novel eutectic solvent; (1R)-(-)-camphor-10-sulfonic acid (10-CSA) with choline chloride (ChCl) for esterification of crude palm oil mixed with sludge palm oil.

Chapter 4 presents the optimization study of benzyltrimethylammonium chloride (BAC) with the aid of p-toluenesulfonic acid monohydrate (PTSA) as catalyst based DES in esterification of low grade palm oil.

Chapter 5 presents the study which introduces DES as a novel and efficient solvent for enzymatic hydrolysis. The enzymatic activity of lipase from porcine pancreas (LPR) and Rhizopus niveus lipases LRN and physical properties of DES were also investigated.

Chapter 6 presents the optimization study of hydrolysis reaction of ChCl/Glycerol 85% with LPR and LRN.

Chapter 7 provides conclusions and potential improvement for future work.

CHAPTER 2: LITERATURE REVIEW

2.1 Biodiesel as Alternatives Fuel

Biodiesel is a fuel that is made from renewable natural sources such as vegetable oil and animal fats. Scientifically, biodiesel is a monoalkyl ester (methyl, ethyl, or propyl) that consists of long carbon chains from a chemical reaction between lipids and acyl acceptor such as alcohol and dimethyl carbonate (Lee et al., 2016). The most adopted acyl acceptor to produce biodiesel is methanol (Verma et al., 2016). This is due to its low price and short carbon chains. Alternatively, standard specification of blended biodiesel with diesel has already been applied commercially since 1991. The European standard EN 14214 (October 2008) and USA ASTM D6751 (November 2008) are among the widely adopted blended specification of biodiesel at the present (Mahmudul et al., 2017). These standards are applied to manufacture biodiesel without requiring any engine modifications. Biodiesel possesses similar thermal properties to petroleum fuel such as cetane number and calorific value. However, some physical properties such as pour point, viscosity and density vary depending on the feedstock used (Agarwal et al., 2017). Thus, it limits the compatibility of biodiesel to some parts of the diesel engine. For example, it is shown that the viscosity of biodiesel from pure castor oil is considerably greater than the European specification limit for biodiesel (DIN – 14214) and only by blending it with 20% v/v of soybean and cotton the biodiesel becomes compliant with the specification limit (Albuquerque et al., 2009).

Biodiesel is currently one of the topics most intensely explored not only by academics and scientist but also by industrial society and environmentalist. Statistically, the research on this topic exponentially increased in the late 2000s. It is probably due to the scarce source of petroleum-based fuel and necessity to find alternative sources. Moreover, there has been a rise in awareness to reduce and eliminate negative environmental pollutions such as emission of hazardous greenhouse gasses and toxic particulate in air (Muhammad et al., 2015).

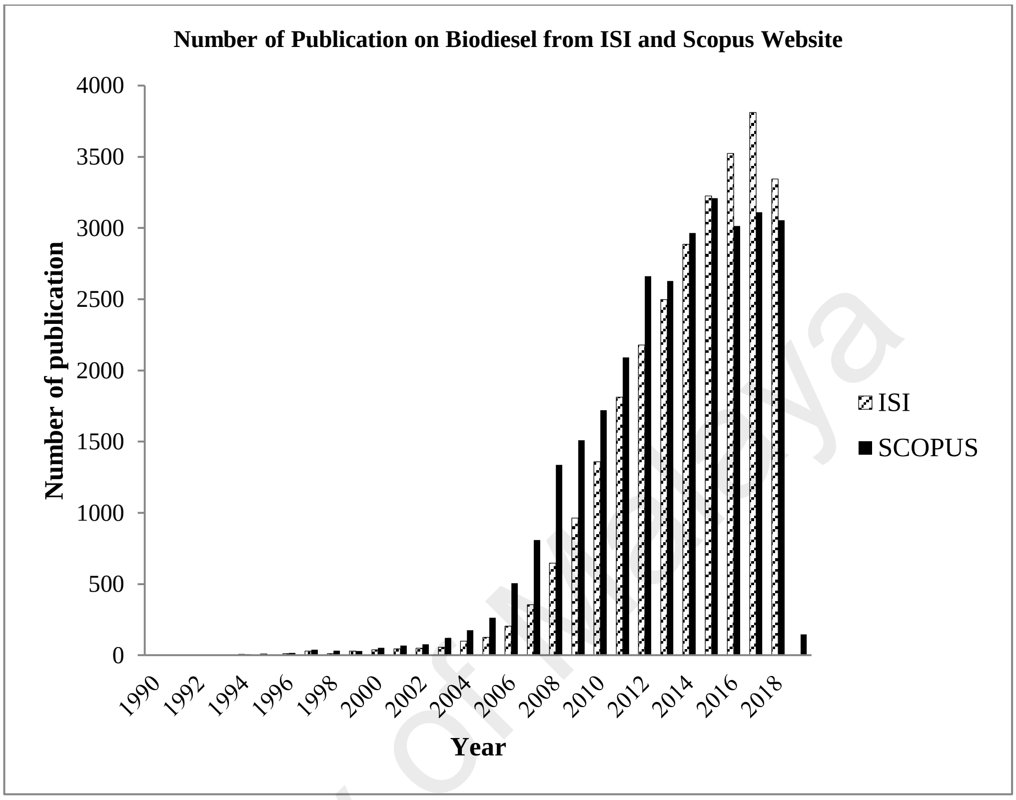


Figure 2.1: Trending of a research article on biodiesel (Adapted from Web of Knowledge and Scopus)

2.2 Economic Overview

Biodiesel has many advantages including job opportunity increase in rural regional community, potential to utilized, lowering dependency on fossil fuel and reduction of the emission of environmental pollutants such as greenhouse gases. The United States national average price of biodiesel (B20), (B99-B100) and diesel between Jun 1st and July 31st, 2017 were $2.49, $3.22 and $2.47 per gallon respectively (U.S. Department of Energy, 2017). The price of biodiesel (B99-B100) was $0.75 higher than the market price of diesel making it unfeasible to be transported in a massive scale. Costs of feedstock and plant capacity for biodiesel were the biggest aspects influencing the economic viability of biodiesel industry (Zhang et al., 2003). In fact, 75% of production cost came from feedstock alone (Mahmudul et al., 2017).

2.3 Renewable and Sustainable Feedstock of Biodiesel

Biodiesel can be produced from various types of feedstock either edible or non-edible oil. List of different types of feedstock with their respective country and yield of crude oil is given in Table 2.1. As mentioned above, choice of feedstock is the most important aspect in the manufacture of biodiesel as it covers 75% of the total production cost. In the early years of the attempt to produce biodiesel, vegetables and seed edible oil were used as feedstock. Soybean oil was the common biodiesel feedstock for South and North America continent, while rapeseed oil was the major oil crops for the production of biodiesel in Europe (Agarwal et al., 2017; Mahmudul et al., 2017). On the other hand, in South East Asia, palm oil has become the main biofuel feedstock because abundant source is available in this region due to massive plantation crops (Khatun et al., 2017).

However, relatively high prices of these oils and competitive issue with demand from the food supply, edible oil as a feedstock was becoming impracticable (Khan et al., 2014). The high price of edible oil makes the turnover of edible oil biodiesel much lower than fuel from petroleum and therefore, it becomes impractical for industry to replace current diesel as it is not economically competitive. Due to these reasons, non-edible oil has been extensively studied to replace edible oils. The most widely adopted non-edible oil used as feedstocks of biodiesel are *Jatropha curcas*, Karanja (*Pongamia Pinnata*) and microalgae oil (Khan et al., 2014; Lau et al., 2016; Patel, & Sankhavara, 2017). Though this non-edible oil was the best to replace the expensive edible oil, it still could not be applied widely and globally as the location and climate conditions influence the oil yield and oil properties (Patel, & Sankhavara, 2017). For example, greater availability of feedstock of jatropha and karanja oil is in the continent of Asian and Africa, yet their feedstock availability is very low in

European and American continent. Different continent relies on different sources of oil crops.

Recently, a new alternative with low cost and sustainable waste feedstocks has been studied. It offers more advantages than plant-based non-edible oil especially towards the environment. Presently, many types of waste oil have successfully synthesized biodiesel. Cooking oil waste with high free fatty acid (FFA) value is able to transform into biodiesel with a cetane number of 57.1 and flash point of 161 (Ullah et al., 2017). In fact, all the physicochemical properties of synthesized biodiesel from this waste cooking oil have met ASTM D-6751 and EN14214 specifications. Another study on the production of biodiesel from waste palm oil has also shown a high yield of 92.7% and 90.7 % of FAME using coconut meal residue and ethanesulfonic acid as catalyst (Hayyan et al., 2011b; Thushari, & Babel, 2018). The beauty of using oil waste as feedstock is that it simultaneously resolves the disposal issue of hardly degradable material which is the waste oil itself. Waste oil requires costly treatment process and long period of degradation. By utilizing and recycling this cheap material as feedstock it will be advantageous for the environment because a lesser amount of waste will be disposed to landfill. It is also a sustainable source of feedstock as the demand for oil such as cooking oil, motor oil or even machinery oil is escalating with time thus the waste produced from them also increases.

Table 2.1: Feedstock of Biodiesel (Clare, 2017; Ito et al., 2012; Lam et al., 2010; Mahmudul et al., 2017; Pourzolfaghar et al., 2016)

Feedstock	Country	Oil Content (%)
Vegetable/ seed edible oil		
Soybean Oil	Brazil, India, Argentina	15-20
Rapeseed Oil	China, India Sweden, France, Germany, Italy, Turkey, UK, Canada	38-46
Palm Oil	Indonesia, Malaysia, Thailand, Iran, Singapore, Ghana, Peru	30-60
Sunflower oil	India, France, Spain, Italy, Turkey,	25-35
Corn oil	US, China, Brazil, Mexico, Russia	48
Rice Bran oil	India, China, Japan	15-23
Coconut oil	Indonesia , Philippines, India	63-65
Olive oil	Italy, Spain, Greece	45-70
Castor oil	Iran, Kenya, Brazil, India	45-50
linseed oil	Spain, China, Belgium, USA, Germany	35-45
Canola	Canada, China, India	40-45
Mahua	India, Bengal	35-40
Animal Fats		
Swine fat	China, USA	-
Tallow	Canada, Australia, New Zealand	-
Fish oil	All Countries	-
Animal Fats	Ireland, Canada, Japan	-
Non- edible /Waste oil		
Sludge palm oil	Indonesia, Malaysia	50
Jatropha curcas	China, Pakistan, Thailand, Iran, Zimbabwe, Mali, Cuba, Peru, Australia, Indonesia	50-60
Pongamia glabra (karanja)	India, Bangladesh, Australia, Philippines	30-40
Moringa oleifera	Cuba	40
Neem oil	Cuba, India, Thailand, Iran	20-30
Microalgae	USA, Europe, India	30-70
Waste cooking oil	New Zealand, Australia, UK, Japan, China, Malaysia, Canada, Taiwan , European, US	-
cotton seed oil	USA, Brazil, Greece, China, Pakistan,	18-25
Grease	Canada	-

Feedstock	Country	Oil Content (%)
Jojoba	Southwestern North America	45-50
Rubber Seed	Sri Lanka, Malaysia, India, Indonesia	40-60

In this study, oil waste from palm oil refineries has been selected as the main feedstock for the production of biodiesel. Malaysia is well known as one of the largest palm oil importers in the world. As the largest palm oil producer, it also contributes to tons of waste every year including palm oil mill effluent (POME), empty fruit bunch (EFB), fruit bunches fiber and shell (Wu et al., 2017). Palm tree originated from West Africa and was brought to Malaysia during British colonization in early 1900 (Yee, & Chandran, 2005). Since then, it continues to grow and become one major economic contributor to Malaysia. Besides, due to suitable tropical climate, location and amount of rainfall every year, other Southeast Asia countries such as Thailand and Indonesia has also increased their palm oil plantation scale. Many parts of the palm tree itself has proven to be useful in the processes of making biodiesel. Using residual oil from POME as feedstock and crude lipase from palm fruit as the catalyst yielded 92.07 ± 1.04% of FAME in enzymatic transesterification reaction (Suwanno et al., 2017).

Another study by Hayyan's was also done using SPO which oil waste with very high FFA (>23%) treated with a homogenous strong acidic catalyst; sulphuric acid (H_2SO_4) resulted in a reduction of FFA to >2% under esterification reaction for 60 minutes (2011a). Moreover, the palm kernel shell has also been successfully transformed into activated carbon which has high potential to be applied in wastewater treatment. The robust Taguchi method suggested that irradiation time of 17 minute under microwave with power of 800 W of phosphoric acid impregnated palm kernel shell synthesized activated carbon with adsorbing capacity of 1000 mg g^{-1} under

Langmuir model (Kundu et al., 2015). Utilizing palm oil waste as feedstock is significant for Southeast Asia especially Malaysia and Indonesia. As they have the largest plantation crops of palm tree, the availability and sustainability of palm oil waste as the feedstock will not deprived. It is also economical compatible and beneficial for environment compared to edible oil and plant-based non-edible oil.

2.4 Methods to Produce Biodiesel

There are many approaches in order to synthesize biodiesel such as direct use and blending of vegetable oil to diesel, ultrasonic irradiation, pyrolysis (thermal cracking), microemulsions and transesterification (Ma, & Hanna, 1999). In the early years of discovery of biodiesel, vegetable oil was directly used and blended with petrol fuel. However, it has poor low-temperature properties such as polymerization during storage, gum formation due to oxidation, high FFA content and viscosity making the use of vegetables oil impracticable. Though it's not widely adopted, many studies on the production of biodiesel using ultrasonic irradiation method have been successfully done where they use sound wave energy vibrates to improve the contact area between oil interface and alcohol interface by the formation of smaller droplets. For example, intensification of ultrasonic irradiation at 20/28 kHz simultaneous mode (SMM) was able to yield a 96.3% conversion of biodiesel with an acid value of less than 0.4% (Yin et al., 2017).

Another study also shows that ultrasonic irradiation at 40 kHz was able to pre-treat the high free fatty acid of ACPO from 8.7% to 2% which later underwent trans-esterification with 83% of final conversion of FAME (Hayyan et al., 2015b). On the other hand, pyrolysis was meant to synthesize biodiesel at very high temperature through cleavage of chemical bonds in the absence of nitrogen and air (Mishra, & Goswami, 2017). Pyrolysis method is able to reduce operation time and the use of organic solvent. However, the operational cost of pyrolysis is higher when compared to transesterification and ultrasonic irradiation. The synthesized biodiesel from pyrolysed vegetable oils contain standard amounts of copper corrosion values, water content, sediments and sulfur, but they also contain undesirable value of pour point, carbon residual and ash (Atabani et al., 2013). Micro-emulsification has also been adapted to resolve the issue of the high viscosity of vegetable oils in the production of biodiesel. A study reported on using this approach where it involves the formulation of cooking oil waste (COW) with butan-2-ol as the co-surfactant and ethanol as disperse phase resulting in the production of hybrid biofuel which exhibits viscosity equivalent to biodiesel (Bora et al., 2016).

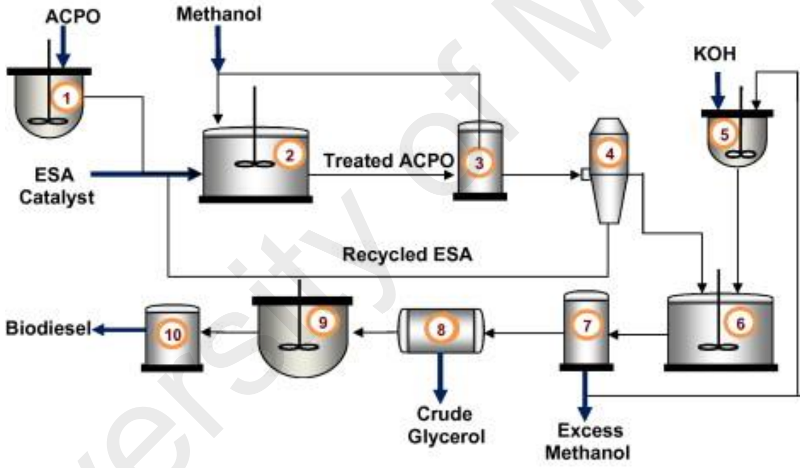


Figure 2.2: Schematic diagram of process for FFA treatment study and production of biodiesel (Hayyan et al., 2011b) Copyright 2017 Elsevier

2.4.1 Transesterification

Among the abovementioned approaches, transesterification reaction is the most highly recognized and adopted method in the industry. This is due to the simple process, yet high yield value of FAME from the reaction as in figure 2.2. Transesterification is the reaction between carboxylic acid normally triglycerides or vegetable oils with short chain alcohol typically methanol or ethanol that gives a product of FAME or FAEE with a byproduct of glycerol. NaOH and KOH are the most widely used base catalyst in transesterification reaction (Srilatha et al., 2012). However, in biodiesel production, the triglycerides or the substrate of the reaction must have an acid content of lower than 2% and a low value of moisture content. As in base catalyzed transesterification, higher acid content and a significant amount of water content which exist in oil will initiate secondary hydrolysis of triglyceride. This lead to formation of soap which hinders its catalytic activity towards transesterification reaction and reduce the quality of biodiesel (Lau et al., 2016)

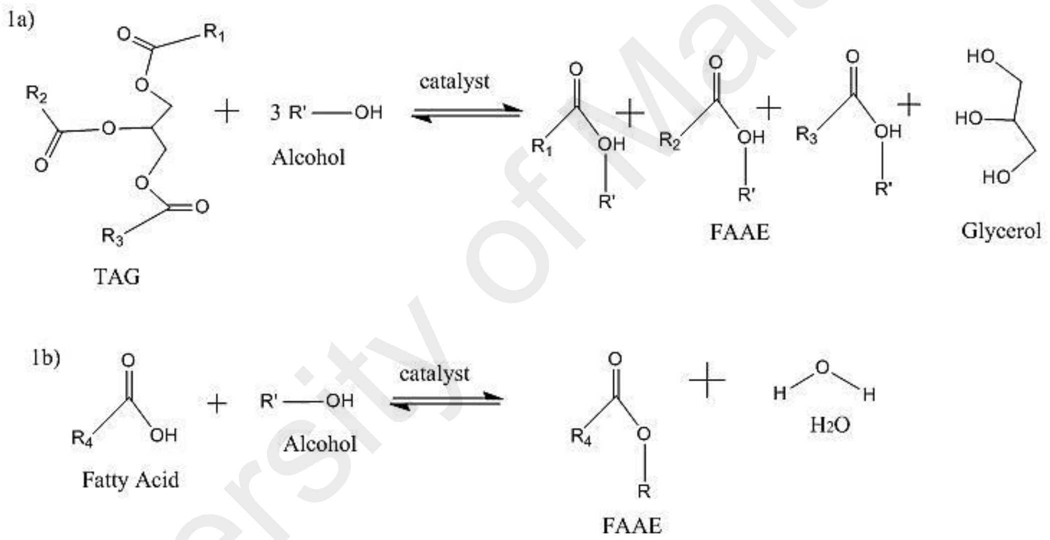


Figure 2.3: An equilibrium reaction to synthesize fatty acid alkyl ester (FAAE) 1a) Transesterification of TAG with 3 moles of alcohol 1b) Esterification of fatty acid with alcohol where R' is alcohol moiety and $R_{1\text{-}4}$ are acyl residues

2.4.2 Pretreatment Process

Due to the presence of high acid content in vegetable oil, a pretreatment process or commonly known as esterification reaction is done to treat the oil. The main objective of esterification is to reduce the acid content or free fatty acid (FFA) in oil. At first, both triglyceride and FFA require a trigger of protonation of the carbonyl groups to drive the reaction (Zhang et al., 2013). Although it can be carried out without a catalyst, the reaction will be very tardy as the rate of the reaction is dependent on the autoprotolysis of the carboxylic acid with the reactant (de Paiva et al., 2015).

Therefore, the selection of catalyst in esterification and transesterification reaction is very crucial in developing a renewable biodiesel from a sustainable source of triglycerides. There are also previous works done with double stage esterification. The advantages of this method are it will move the equilibrium of the reaction towards the product as the esterification is reversible reaction. (Zanuttini et al., 2014). The difference between esterification and transesterification is associated with the molecular characteristics of the materials used; either fatty acid or triacylglyceride. Fatty acid has a more polarized molecule and they are smaller than triacylglyceride which speed up mass transfer into the fatty acid, increasing the reaction rate and assisting the nucleophilic attack of alcohol (Raia et al., 2017). Due to the high FFA content, the esterification process usually uses an acidic catalyst or enzymatic treatment. The activation of the carbonyl group is difficult due to the existence of the long alkyl chain in the triglyceride (Zhang et al., 2013). Acid sites in catalyst make carbonyl carbon more electrophile and also make it easier to eliminate H_2O. Figure 2.4 showed the mechanism of esterification reaction of the fatty acid with alcohol using acid as a catalyst (H^+).

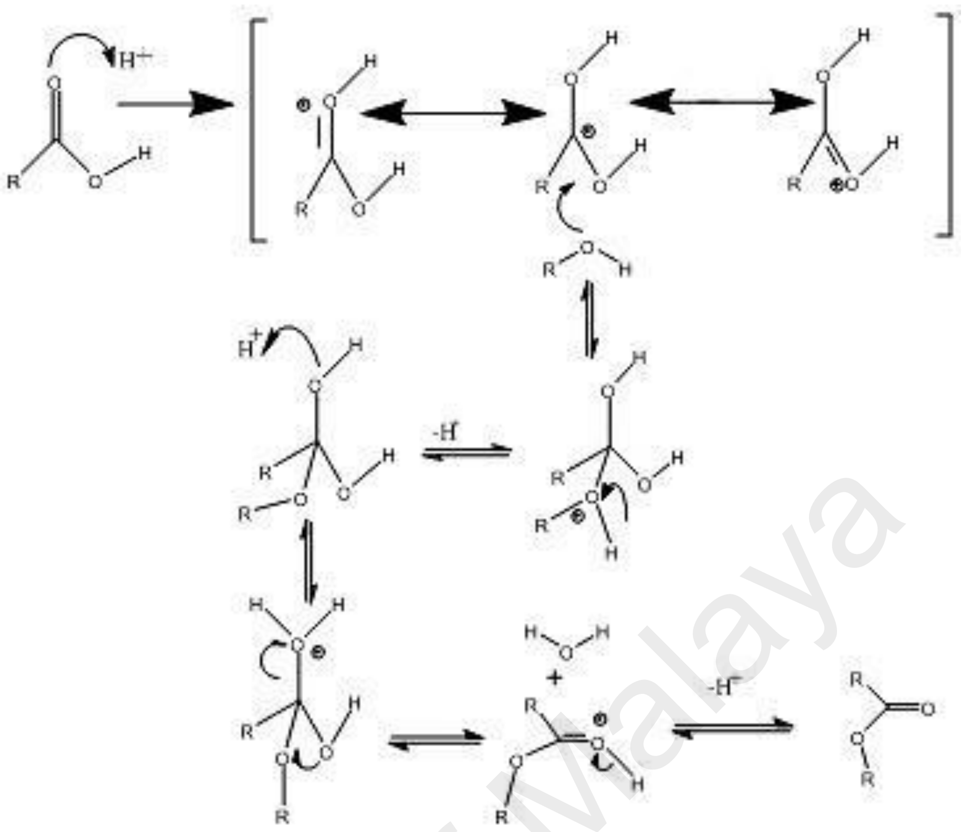


Figure 2.4: Mechanism of pretreatment reaction (acid catalyzed esterification) where it starts with the protonation of carbonyl oxygen, to the 1, 2-addition (of alcohol) whereas the proton from alcohol transfer to OH. Next, 1, 2 – elimination of water to protonated ester and finally ester will be deprotonated

2.4.3 Acid and Lipase Catalyst in Esterification

As mentioned above, esterification was carried out using feedstock to reduce the FFA. This part will exclusively discuss the various acid and lipase catalysts that have been used in the pretreatment of feedstock for the production of biodiesel. Base catalyst is known for negatively impacting in the production of biodiesel from waste feedstock due to the high amount of FFA that makes the feedstock undergo hydrolysis instead of esterification and produces soap instead of the ester.

Table 2.4 and 2.5 review the efficiency of catalyst (based on FFA reduction and yield of treated oil) in the esterification reaction. The tables also highlight the optimized operation condition of each catalyst in different kinds of feedstock.

The total acidic sites in the acid catalyst is quantified based on the total of Bronsted and Lewis acid sites. In acid catalyzed esterification mechanism, Bronsted acid sites act to protonate the nucleophilic attack of the hydroxyl group of the alcohol to activate carbonyl oxygen atom present in the fatty acid. Simultaneously, a reactive complex is formed when Lewis acid adsorbed the carbonyl oxygen atoms of the fatty acids. Water and ester are the product of this reaction (Raia et al., 2017). Reinoso et al. reported the mechanism of Lewis acid zinc carboxylic salts in the esterification of oleic acid with methanol to produce methyl ester and water. The Lewis acid Zn^{2+} attack on the lone pair of carbonyl of the oleic acid triggers the positive character and making it more likely to a nucleophilic attack. The four-center transition state is formed when the hydroxyl group of methanol strikes the carbon atom of the activated carbonyl group. This transition state breaks the water molecule. The catalytic cycle is completed when the Lewis acid catalyst split-up from the carbonyl group and synthesizes the methyl ester (Reinoso et al., 2012).

On the other hand, a mechanism in enzymatic treatment is controlled and happens at the oil-water interface area. Lipase enzyme is responsive and activated when colliding with a substrate that is different in the interface. The conformation of lipase will lid-open to allow substrates with which the oil will be in contact with the active site(s) (Su et al., 2016). Moreover, a multiple sequential hydrolysis and esterification also occur when lipase is used as the catalyst. This produces a lower yield of ester and much longer reaction time needed for enzymatic treatment compared to acid catalyzed esterification.

Fan et al. explained the series of multiple reaction mechanisms of an enzyme-catalyzed reaction. Firstly, diacylglycerol and free fatty acid (FFA) are synthesized from the hydrolyzed triglycerides; then short chain alcohol esterified the fatty acid through catalysis by lipases; later, diacylglycerol is repetitively broken down into monoacylglycerol and FFAs by hydrolysis, and the FFA is esterified repeatedly by the same cycle. Next, monoacylglycerol will be hydrolyzed into glycerol and FFA and the generated FFAs are completely esterified into fatty acid alkyl esters (Fan et al., 2017). Fundamental insight of the mechanism of catalysis in esterification reaction will effectively enhance the design of experiment for the optimization of biodiesel from ACPO. The formula use in this study to calculate the FFA content of the ACPO and treated oil was as equation 2.1;

$$FFA\ content\ (\%) = \frac{25.6 \times 0.0864 \times KOH_N}{Weight\ of\ Oil} \tag{2.1}$$

Where 25.6 is the constant for the equation and 0.0864 is the molecular weight of KOH and KOH_N is the volume of KOH. The efficiency of acidity or FFA reduction of each run was expressed using equation 2.2 adapted from (Cruz et al., 2017).

$$\text{Acidity Reduction (\%)} = \frac{Initial\ FFA - Final\ FFA}{Initial\ FFA} \times 100 \tag{2.2}$$

Table 2.2: Review of acid catalysts in the esterification reaction

CATALYST	TYPE	FEEDSTOCK	OPERATION CONDITION		RESULT	REF.
Sulfuric acid	homogeneous	LGPO	catalyst dosage (CD) : 2% molar ratio methanol to oil(MR): 10:1 speed: N/A reaction time(RT):300min reaction temperature (RTEM): 50◦C equipment: sonoreactor		Initial FFA: 20% Final FFA: 3% Yield :83.72%	(Hayyan et al., 2011a)
		Wet Greasy Sewage Sludge	CD : 0.037 wt.% Mass Ratio methanol to oil: 15.8:1 speed: 4.17 Hz RT: 8 hour RTEM: 60◦C		Initial FFA: 65.4 ± 4.5% Final FFA: 1.7± 0.1% Yield(concentration) :70.4%	(Urrutia et al., 2016)
		Fleshing Oil	CD: 10% MR methanol to oil: 30:1 speed: 300 rpm RT: 60 min RTM: 60◦C		Initial FFA: 12.15% Final FFA: <1%	(Alptekin et al., 2014)
		Animal Fats	**First Stage** CD: 20% MR methanol to oil: 30:1 speed: 300 rpm RT: 60 min RTEM: 60◦C	**Second Stage** CD: 15% MR meth to oil: 40:1 speed: 300 rpm RT: 60 min RTEM: 60◦C	Initial FFA: 26.15%. Final FFA: 0.81% (Two stage Esterification)	
		Oil obtained from waste plum stones	CD: 2% MR methanol to oil: 8.5:1 RT: 60 min RTEM: 45◦C		Initial FFA: 15.8% Final FFA: 0.34% Reduction :98.5% recyclability: 5 consecutive runs	(Kostić et al., 2016)

CATALYST	TYPE	FEEDSTOCK	OPERATION CONDITION		RESULT	REF.
		Butia Yatay coconut oil	**First Stage** CD : 0.75 vol% MR ethanol to oil: 5.2:1 speed: 600 rpm RT:1.5 h RTEM: 70	**Second Stage** CD : 0.75% MR ethanol to oil: 3.4:1 speed: 600 rpm RT: 1.5 h RTEM: 70◦C	Initial FFA: 128 mg KOH g^{-1} Final FFA: 5 mg KOH g^{-1}	(Zanuttini et al., 2014)
methane sulphonic acid	homogeneous	ACPO	CD: 1% MR methanol to oil: 8:1 speed: 300 RPM RT: 30 min RTEM: 60◦C		Initial FFA: 18% Final FFA: 0.7% yield: 96% recyclability : 3 consecutive runs	(Hayyan et al., 2012b)
P-toluenesulfonic acid (PTSA)	homogeneous	SPO	CD : 0.75% MR methanol to oil: 10:1 speed: N/A RT: 60 min RTEM: 60◦C		Initial FFA: 22.33% Final FFA: 2.02% yield: 96%	(Hayyan et al., 2010b)
(1R)-(–)-camphor-10-sulfonic acid (10-CSA)	homogeneous	ACPO mix with SPO	CD : 1.5% MR methanol to oil: 10:1 speed: N/A RT: 30 min RTEM: 60◦C		Initial FFA: 8% Final FFA: <1% yield :87.50% recyclability : 6 consecutive runs	(Hayyan et al., 2014a)
ethanesulfonic acid (ESA)	homogeneous	ACPO	CD : 0.75% MR methanol to oil: 10:1 speed: N/A RT: 30 min RTEM :60◦C		Initial FFA: 8.6% Final FFA: 0.8% yield :96% recyclability : 3 consecutive runs	(Hayyan et al., 2011b)

CATALYST	TYPE	FEEDSTOCK	OPERATION CONDITION	RESULT	REF.
benzenesulfonic acid (BZSA)	heterogeneous	LGCPO	CD : 0.75% MR methanol to oil: 8:1 speed: N/A RT: 30 min RTEM: 60◦C	Initial FFA: 9.3% Final FFA: <1% yield :88.67% recyclability : 3 consecutive runs	(Hayyan et al., 2014c)
Trifluoromethanesulfonic acid (TFMSA)	homogeneous	SPO	CD : 0.75% MR methanol to oil: 10:1 speed: N/A RT: 40 min RTEM: 60◦C	Initial FFA: 16% Final FFA: <2% yield: 84%	(Hayyan et al., 2013c)
propanesulphonic acid (1-PSA)	homogeneous	ACPO mix with SPO	CD : 0.75% MR methanol to oil: 8:1 speed: N/A RT: 30 min RTEM: 60◦C	Initial FFA: 8.5% Final FFA: 0.81% yield :88.67% recyclability : 3 consecutive runs	(Hayyan et al., 2015a)
chromosulfuric acid	homogeneous	LGCPO	CD : 0.75% MR methanol to oil: 10:1 speed: 200 rpm RT: 30 min RTEM: 60◦C	Initial FFA: 7% Final FFA: >1% yield: 85%	(Hayyan et al., 2013f)
5% sulfuric acid solution	homogeneous	high FFA feedstock soybean oil	CD: 10% MR ethanol to oil: 20:1 speed: N/A RT: 60 min RTEM: 60◦C	Initial FFA: 20% Final FFA: >1% yield: 75.12%	(Canakci, & Van Gerpen, 2001)
H3PW12040 (Brønsted acids)	homogeneous	fatty acid	CD : 0.0192 mmol MR methanol to oil: 10.0 mL: 2.50 mmol speed: N/A RT: 4 hour RTEM: 25◦C	Conversion FFA: 92% Yield:94% recyclability : 3 consecutive runs	(Silva et al., 2010)

CATALYST	TYPE	FEEDSTOCK	OPERATION CONDITION	RESULT	REF.
1-butyl-3-methyl-imidazolium hydrogensulfate (BMIMHSO4)	homogeneous	CPO	CD: 4.5 wt.% MR methanol to oil: 12:1 speed: 600 rpm RT: 120 min RTEM: 160◦C	Initial FFA: 3.49% Final FFA: >1% yield: 98.4%	(Elsheikh et al., 2011)
sulphated zirconia (pH 8)	heterogeneous	Jatropha curcas L.	CD: 10 wt.% MR ethanol to oil: 10:1 speed: 800 rpm RT: 8 hour RTEM: 150◦C	Initial FFA: 14.29% Final FFA: N/A yield: 59.4%	(Raia et al., 2017)
sulphated zirconia	heterogeneous	Rapeseed oil + 10 wt% myristic acid	CD : 3 wt.% MR ethanol to oil: 20:1 speed: 600 rpm RT: 6 hour RTEM: 170◦C Pressure: 22 bars	Initial FFA: N/A Final FFA: 2.95% yield: 86%	(Rattanaphra et al., 2012)
chlorosulfonic acid modified zirconia ($HClSO_3$–ZrO_2)	heterogeneous	crude rice bran oil (refined oil with 40 wt% FFA)	CD: 6 wt.% MR methanol to oil: 12:1 RT: 12 hour RTEM: 120◦C	Initial FFA: 40% Final FFA: N/A yield: 92% recyclability : 3 consecutive runs	(Zhang et al., 2013)
1-sulfobutyl-3-methylimidazoliumhydrosulfate([BHSO3 MIM]HSO4)	heterogeneous	oleic acid	CD: 10 wt.% MR ethanol to oil: 4:1 speed: 500 rpm RT: 4 hour RTEM: 130◦C	Initial FFA: 72% Final FFA: N/A yield :94.9% recyclability : 10 consecutive runs	(Li et al., 2014)

CATALYST	TYPE	FEEDSTOCK	OPERATION CONDITION	RESULT	REF.
chlorosulfonic acid modified zirconia	heterogeneous	oleic acid	CD : 3 wt.% MR ethanol to oil: 8:1 speed: 500 rpm RT: 12 hour RTEM: 100◦C	Initial FFA: N/A Final FFA: N/A yield :100% recyclability : 5 consecutive runs	(Zhang et al., 2014)
4-dodecylbenzene sulfonic acid	Heterogeneous	oleic acid	CD: 0.015 moles of SO_3H group/ mol of fatty acid MR methanol to oil: 2:1 speed: 600 rpm RT: 5.5 hour RTEM: 60◦C	yield: 96 %	(Alegría, & Cuellar, 2015)
Amberlyst 15 wet ion exchange	heterogenous	oleic acid	CD: 20% MR methanol to oil: 1:1 speed: 600 rpm RT: 6 hour RTEM: 60◦C	yield: 53 %	(Hykkerud, & Marchetti, 2016)
phosphoric acid Activated montmorillonite STx-1-P0.5M2h	heterogenous	Lauric acid	CD: 12% MR methanol to oil: 12:1 speed: 500 rpm RT: 2 hour RTEM: 160◦C	Initial FFA: 30.21±0.36% Final FFA: 3.42 ± 0.07% yield :96.58 % recyclability : 4 consecutive runs	(Zatta et al., 2013)
nitric acid Activated montmorillonite	heterogenous	Lauric acid	CD: 8% MR methanol to oil: 12:1 speed: 500 rpm RT: 2 hour RTEM: 160◦C Internal Pressure: 12 bar	Initial FFA: 30.21% Final FFA: 6.92±0.79 % yield: 93.08 %	(Zatta et al., 2012)

CATALYST	TYPE	FEEDSTOCK	OPERATION CONDITION	RESULT	REF.
Aminophosphonic acid resin D418	heterogenous	oleic acid	CD : 10.2% MR ethanol to oil: 14:1 RT: 10 hour RTEM: 115◦C	Yield 92.02 ± 0.74%	(Yin et al., 2012)
smectite natural clay-based catalyst (SME_{nat} acid-activated)	Homogeneous	Stearic acid	CD: 200 mg (from 4 mmol of stearic acid) MR methanol to oil: 3:1 RT: 4 hour RTEM: 100◦C	Yield: 99% recyclability: 5 consecutive runs	(Rezende, & Pinto, 2016)
2-Ce/ZrO_2 - TiO_2/SO_2^4 - 600 (600◦C of calcination temperature) (2 wt% of Ce)	heterogenous	Vegetable oil	CD : 5% MR methanol to oil: 6:1 RT: 3 hour RTEM: 65◦C	Initial FFA: 15.2 wt% Final FFA: 0.07% yield :99.53 % recyclability : 6 consecutive runs	(Kaur, & Ali, 2015)
phenolsulfonic acid – formaldehyde resin (PAFR)	heterogenous	Oleic acid	CD : 0.7mol % (5.6mg) MR methanol to oil: 1.0 mmol : 1.2 mol equiv RT: 12 hour RTEM: 60◦C	yield: 93%	(Baek et al., 2016)
Manganese glycerolate (MnGly)	heterogenous	Jatropha oil	CD: 6 wt % MR 95% ethanol to oil: 20:1 RT: 6 hour RTEM: 150 °C	Initial FFA: 4.93 wt% Final FFA: N/A yield :99.7 % recyclability : 3 consecutive runs	(Lau et al., 2016)

CATALYST	TYPE	FEEDSTOCK	OPERATION CONDITION	RESULT	REF.
sulfonated graphitic carbon nitride (Sg-CN)	heterogenous	Oleic acid	CD : 25 mg MR 95% ethanol to oil: 5.0 ml :1.0 g RT: 4 hour RTEM: room temperature	yield :>99% recyclability: 5 consecutive runs	(Baig et al., 2016)

Table 2.3: Review of lipase catalysts in the esterification reaction

CATALYST	FEEDSTOCK	OPERATION CONDITION	RESULT	REF.
lipase from *Rhizomucor*	palm fatty acid	catalyst dosage (CD) : 13U/g	Initial FFA: 97%	(Aguieiras

CATALYST	FEEDSTOCK	OPERATION CONDITION	RESULT	REF.
miehei (free lipase)	distillate (PFAD)	molar ratio methanol to oil(MR): 2:1 speed: N/A reaction time(RT): 24 hour (stepwise) reaction temperature (RTEM): 45◦C	Final FFA: N/A Yield :>90% recyclability : 5 consecutive runs	et al., 2017)
	soybean fatty acid distillate (SFAD)	CD : 13U/g MR methanol to oil: 2:1 speed: 4.17 Hz RT: 6 hour RTEM: 45◦C	Initial FFA: 100% Final FFA: N/A Yield :80% recyclability : 5 consecutive runs	
Novozymes 435 (immobilized lipase)	Wet Greasy Sewage Sludge	CD : 10wt.% Mass Ratio methanol to oil: 40 ml:10g speed: 4.17 Hz RT: 15 hour RTEM: 45◦C	Initial FFA: 65.4 ± 4.5% Final FFA: 10.4± 0.1% Yield(concentration) :64.6%	(Urrutia et al., 2016)
	Saponifiable lipids & microalga as free fatty acid	CD : 0.025:1 w/w (enzyme:FFA) MR methanol to oil: 1.5:1 speed: 200 rpm RT: 4 hour RTEM: 25◦C	Initial FFA: 73.5% Final FFA: N/A Yield :92.6% recyclability : 6 consecutive runs	(Castillo López et al., 2015)
	high-FFA rapeseed oil	CD : 5% Methanol: 4% speed: 1000 rpm RT: 1 hour RTEM: 35◦C	Initial FFA: 20% Final FFA: 0.5% Stability : Yield of 84.8% in 4.4 days	(Nordblad et al., 2016)
	Macauba	CD : 5% wt/wt MR methanol to oil: 2:1 RT: 4 hour RTEM: 30◦C	Initial FFA: 35-43% Final FFA: 1.09 Yield :97.22% Stability : Yield of 82.7% in 600 hours	(Teixeira et al., 2017)

CATALYST	FEEDSTOCK	OPERATION CONDITION	RESULT	REF.
	soapstock oil	CD : 4% MR methanol to oil: 5:1 speed: 150 rpm RT: 10 hour RTEM: 45◦C	Initial FFA: 81.9 mg KOH/g oil Final FFA: N/A Yield :95.2%	(Su, & Wei, 2014)
	Black soldier fly larvae (BSFL)	Solvent: methyl acetate (MA) the reaction time of 12 h molar ratio MA to fat of 14.64:1 enzyme loading of 17.58% the temperature of 39.5 °C	the yield of 96.97% Novozymes 435 could be reused for up to 20 cycles without loss in enzyme activity	(Nguyen et al., 2018)
immobilized Candida sp. 99–125	Waste Cooking Oil	CD : 13.33 % Volume ratio methanol to oil: 5:1 speed: 220 rpm RT: 40 hour RTEM: 40◦C	Initial FFA: 83.9% Final FFA: N/A Yield : 98.9%	(Wang et al., 2014)
Free Bacillus firmus ASU 32 (KP777552)	fungal lipids	CD: 1ml (1.0 g of fungal lipids) Volume ratio methanol to oil: 1:2 speed: 120 rpm RT: 72 hour RTEM: 40◦C	Yield: 71.2%	(Abd-Alla et al., 2015)
Immobilized Bacillus firmus ASU 32 (KP777552)			Yield: 82%	
Lipozyme CALB L	Mamalian Fats	CD : 0.0060 wt/wt. Mass Ratio methanol to oil: 3.25wt/wt RT: 3 hour RTEM: 45◦C	Initial FFA: 8.86% Final FFA: 2.924%	(Mata et al., 2017a)
	Fish oil	CD : 0.0045 wt/wt. Mass Ratio ethanol 99.8* to oil: 4.92wt/wt RT: 3 hour RTEM: 45◦C	Initial FFA :8.86% Final FFA: 1.67% Reduction :76%	(Mata et al., 2017b)
Lipozyme TL 100L		CD : 0.1 wt/wt.	Initial FFA :8.86%	

CATALYST	FEEDSTOCK	OPERATION CONDITION	RESULT	REF.
		Mass Ratio ethanol 96% to oil: 3.24wt/wt RT: 3 hour RTEM: 40◦C	Final FFA: 1.57% Reduction :75%	
Thermomyces lanuginosus	soapstock of vegetable oil refining	CD: 4 wt.% Mass Ratio methanol to oil: 1:1.5 speed: 4.17 Hz RT: 24 hour RTEM: 45◦C	Initial FFA: 65.5% Final FFA: N/A Reduction: 80% recyclability: 5 consecutive runs	(Cruz et al., 2017)
commercial lipase catalyst (Eversa Transform)	crude tall oil	CD: 1 wt.% Mass Ratio methanol to oil: 1:1.5 speed: 4.17 Hz RT:16 hour RTEM: 40◦C	Initial FFA: 53.4% Final FFA: 3% Yield: 96.57%	(Adewale et al., 2017)

2.5 Deep Eutectic Solvent Overview

Recently, exploration of a design solvent has grown enormously. The goal is to offset the hazardous and corrosive conventional solvent with a user-friendly, readily available and inexpensive material (Fox, 2009). In respect to this chemistry research of an alternative and greener solvent, many attempts have been made such as using ionic liquid (IL), supercritical fluids and if possible to avoid using solvent or adapt a "solvent-free" system (Alonso et al., 2016; Zhang et al., 2011).

IL is molten salt which consists of positive and negative ions and has a melting point below 100°C (Abo-Hamad et al., 2016; Zhao et al., 2011). ILs offer a wide range of polarity, and solubility, low flammability, low vapor pressure, high thermal conductivity, high ionic conductivity as well as, high chemical and thermal stability (Zhao et al., 2011). However, the high cost of ionic salt and complexity during synthesizing become the barrier for the industry to use IL in their processing. Moreover, recently the "green solvent" of IL was doubted due to waste formation/ byproduct within the complex synthesis of IL (Domínguez de María, & Maugeri, 2011).

Over the last 10 years, a so-called eutectic solvent systems have emerged and manifest significant attention in many industrial fields such as oil and gas, pharmaceutical and food processing (Abbott et al., 2004a; Alonso et al., 2016). A eutectic mixture is an exclusive composition consisting of two or more chemical elements or components that possess a specific lower melting temperature than their single chemical components (Alonso et al., 2016). Deep eutectic solvents (DESs) refer to a mixture formed from anionic and/or cationic species with hydrogen bond donor (HBD) such as amines, carboxylic acids or alcohols (Abo-Hamad et al., 2015; Alonso et al., 2016; Gouveia et al., 2016). Anion parts of halide salts interact with HBD and position themselves in the interstitial spaces. The mixing disrupt its crystalline pattern by diminishing the interaction with cation parts (Gorke et al., 2010). This consequently triggers a decrease in its melting point than its single components (Taher, & Al-Zuhair, 2016).

As a potential alternative to ILs, DESs is well known to possess similar physicochemical properties (refractive index, surface tension, density, conductivity, chemical inertness, viscosity, etc) to those of ILs. Unlike ILs, the protocol to synthesize DESs is very simple with no formation of by-products and it does not require any additional solvent during the process. (Cvjetko Bubalo et al., 2014). The syntheses of DES also comes from a cheap, natural and readily available feedstock such as choline chloride and glycerol and the resulting DESs were easily biodegrade and are less toxic compared to ILs (Abbott et al., 2007a; Alonso et al., 2016) .

Recently, DES has been applied as a new route in (bio)chemical transformations that attracted widespread interests (Huang et al., 2014b). This includes study on bioactive compounds extraction such as extraction of phenolic compounds using choline chloride (ChCl) /1,2-propanediol(1:1) and ChCl/xylitol (2:1) (García et al., 2016). This also include, studies on enzymatic transesterification for production of biodiesel using chloride/glycerol (1:2) (Zhang et al., 2016; Zhao et al., 2013) and synthesis of specific metabolite bovine serum albumin (BSA), from the enzymatic structures of protein using ChCl/glycerol (1:1) (Xu et al., 2015). In spite of these innovations, the chemistry of DES is still not clear. Industrial communities are still reluctant to fully adopt DESs in their application. Therefore, a broader knowledge on the chemistry of DES is vital. Currently, many aspects of DES have been reviewed. This present work overview and focus on the profiles of DESs in chemical and biochemical transformations especially in enzymatic activity and treatment of free fatty acids.

2.6 What are DES

Deep eutectic solvents or commonly known as DES is a eutectic mixture from a combination of:

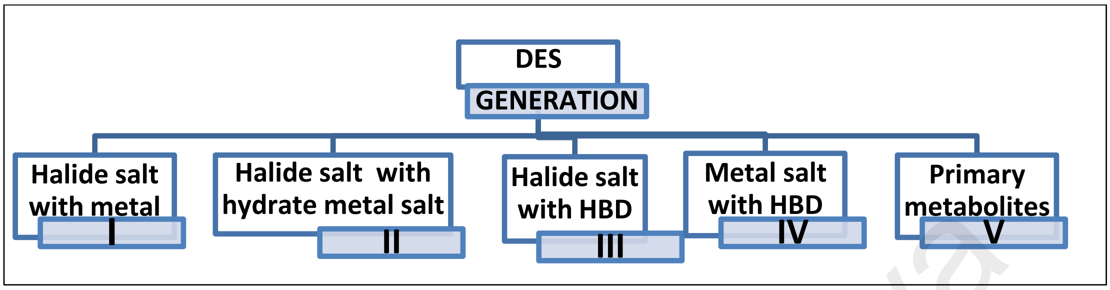


Figure 2.5: Generation of DES (Choi et al., 2011; Juneidi et al., 2015; Mbous et al., 2017; Smith et al., 2014)

The largest intramolecular bond within DES is the hydrogen bond (Mbous et al., 2017). However, van der Waals interaction and electrostatic forces also exist (Zhang et al., 2012b). The hydrogen bonds between halide anion with metal salt/HBD form a large nonsymmetrical ions complexion. This phenomenon causes the DESs to have low lattice energy thus causing them to have a low melting point (eutectic phenomena)(Smith et al., 2014).

The first generation of DES (type I) is focusing on metal electrodeposition and it is limited due to the non-hydrated metal halides which are sensitive to moisture. Some examples of the type I DESS are eutectic mixture of chloroaluminate/imidazolium salt with the following metal halides: AgCl, $CdCl_2$, CuCl, $CuCl_2$, $FeCl_3$ $LaCl_3$, LiCl, $SnCl_2$, YCl_3, and $ZnCl_2$ (Smith et al., 2014).

Type II increases the capability of DES to be adapted in various reactions as hydrate metal salt IS used. Type II DES ChCl /chromium chloride at 1:2 ratio possesses properties identical to imidazolium-based ionic liquids (Abbott et al., 2004b). DES of ChCl/chromium chloride is able to electrodeposit chromium with a crack-free deposit.

The use of hydrate DES in electrodeposition of metals is significant and environmentally friendly as it could replace the use of a bulk amount of strong acids or bases (Jenkin et al., 2016).

Type III has the highest citation in academic research. This is probably due to readily available, less-toxic and low cost of HBD (glycerol, urea, ethylene glycol, etc) (Alonso et al., 2016; Radošević et al., 2015; Zainal-Abidin et al., 2017). Moreover, Type III has simple synthesis procedure and a wide range of combination of Halide salt with Hydrogen Bond Donor (HBD) (AlOmar et al., 2016; Khandelwal et al., 2016).

In contrast, type IV DESs is still in pioneer stage where the research and development of research article on this type of DES still lacking. In 2007, Abbot's group synthesize a metal salt, $ZnCl_2$ with HBD (acetamide, ethylene glycol, urea, and 1,6- hexanediol) and found that type IV DES possesses eutectic characteristic as well as a comparable conductivity and viscosity to ILs. (Abbott et al., 2007a). A new type of DES, natural deep eutectic solvents (NADES) are emerging due to their sustainable and low toxicity profile as they are synthesized from materials of natural origin (Hayyan et al., 2016). By using NMR analysis, it is proven that a primary metabolite, 1,2-propanediol has strong hydrogen bond interaction with the protons on the hydroxyl group of choline chloride (Dai et al., 2013). The author also proved that NADES is able to solubilize macromolecules such as protein and DNA and they are also excellent solvents for many substrates and effective media for reactions as they cover a wide range of polarity and high capacity of solubility (Choi et al., 2011; Dai et al., 2013). NADES offers many advantages such as biocompatibility, sustainability and green solvent making it a potential next-generation solvent in advanced research of biocatalysis, biomedical, electrochemistry applications (Paiva et al., 2014).

Table 2.4: Advantages of DESs

Properties	Evidence /Clarification	Reference
Biodegradable	Choline chloride-based DESs with glucose, glycerol and oxalic acid were classified as ′readily biodegradable′ based on their high levels of mineralization (68–96%).	(Radošević et al., 2015)
	ChCl-based DESs possess higher biodegradability than ChAc-based. While among HBD, acetamide and urea are more biodegradable than ethylene glycol and glycerol.	(Wen et al., 2015)
	The biodegradability for all the tested DESs with levels of biodegradation were as follows: amine-based DESs ≈ sugar-based DESs > alcohol-based DESs > acid−based DESs, thus these DESs are regard as biodegradable green solvents.	(Zhao et al., 2015)
	Cholinium-based DES was found to be readily biodegradable as degradation result of more than 60%.	(Juneidi et al., 2015)
Less-toxic	ChCl which known to be salt of DES is a non-toxic salt that has been endorsed for use as a nutritional additive in all species without a time restraint under Council Directive 70/524/EEC8	(Additives, & Products or Substances used in Animal, 2011)
	Choline chloride-based DESs with glucose, glycerol and oxalic do not inhibit the wheat seed germination and shown a low cytotoxicity to moderate cytotoxicity based on phytotoxicity and cytotoxicity data.	(Radošević et al., 2015)
	The phosphonium-based DESs revealed slight antibacterial activity while ChCl based DESs were totally non-toxic for the tested bacteria (*Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* and *Escherichia coli*)	(Hayyan et al., 2013g; Hayyan et al., 2013h) (Wen et al., 2015)
	Low concentration of DESs, ChCl and choline acetate (ChAc) based DES) (<75mM) inhibit up to 10% of *E. coli* growth. However increasing the concentration (0.75M) of the DESs, all the tested DESs became highly hazardous, inhibit up to 93.8%	

Properties	Evidence /Clarification	Reference
	E. coli growth. Choline chloride DESs (mixed with acids, alcohols, salt, and sugars) were found to be less toxic than their mixture or individual components. The LC50 of synthesized ChCl/$ZnCl_2$ was slightly toxic while DES of ChCl/Malonic acid was practically harmless compared to its former component. This implied that some hydrogen bond donors of DESs are possible to lower the acute toxicity of their former components.	 (Juneidi et al., 2016)
Cheap Material	A cost of ChCl is less than the US $960 per metric ton for 98% purity which the most used cationic salt in DES. The DESs constituent is low priced, for example, US $20 per kg of urea, US $65 per kg of choline chloride, US $35 per kg of glycerol.	(Kim et al., 2016) (Gorke et al., 2008)
Tunable	The DES could be tailored for specific applications as the quaternary ammonium salt and amide strongly influence the fluid properties such as the conductivity and viscosity. Theoretically, from various combinations of anions and cations, the syntheses of a large number of DESs are possible which bring huge opportunity to design greener catalytic condition. Depending on requirements of different processes or reactions, tuning of anions and cations will change the coordination properties/acidity of deep eutectic solvents from the combinations.	(Abbott et al., 2003) (Khandelwal et al., 2016)
High polarity	ChCl/urea/thiourea showed the highest of the combination of polarizability and polarity compared to other DES. It can be concluded that among HBD partners for DES, thiourea had the greatest influence on polarity. ChCl-based DESs with 1,2-ethanediol, urea, malonic acid and glycerol were determined relatively dipolar. The DES dipolarity was influenced by the molecular structure of the H-bond donor in a DES	(Kim et al., 2016) (Zainal-Abidin et al., 2017) (Pandey et al., 2014)
High solvation power	DESs are able to form hydrogen bonds by accepting or donating proton or electrons which allow them for distinctive dissolution properties. Reducing the molar ratio and increasing the temperature during synthesizing are able to increase the solubility of DES. Moreover, the sodium salts solubility was significantly affected by the chemical structure of the DES.	(Zhang et al., 2012b) (Abo-Hamad et al., 2015)
High	Addition of 10% ChCl/urea/glycerol to phosphate	(Kim et al.,

Properties	Evidence /Clarification	Reference
biocompatibility	buffer (pH 7.0) enhanced the activity of lipase up to 55%. It proved that DES is lipase activators.	2016)
	ChCl/triethylene glycol DES successfully extracted rutin from *Sophora japonica* and able to replace the conventional solvents and ILs in the extraction process.	(Zhao et al., 2015)

2.6.1 Physical properties and toxicity profile of DES

One of the subjects that have been addressed by the researcher on DES was their unique physical properties and toxicity. Due to a wide range of material and component to synthesize DES, their physical properties and toxicity are different depending on their former constituent. In this particular subtopic, we are aiming to review the physical properties and toxicity profiles of choline chloride-based DES and glycerol-based DES as these are the two components highly studied on numerous types of reaction (Kim et al., 2016; Paiva et al., 2014; Smith et al., 2014; Zhao et al., 2015).

The eutectic freezing point of benzyltriphenylphosphonium chloride (BTPC)/glycerol (1:16) and ChCl/Gly (1:2) are in the range of -21.99 to -33.47°C respectively (AlOmar et al., 2016). It was also reported that DES of methyltriphenylphosphonium bromide (MTPB)/glycerol at a molar ratio (1:1.75) have a freezing point of -4.03°C (Kareem et al., 2010). It is much lower than the freezing point of its former component which is 17.85°C for glycerol. Other than the freezing point, the viscosity of DESs is an important characteristic because it strongly affects the rate of mass transport within a solution. It depends on the interaction between the salt and HBD and their coordinating ability (Bahadori et al., 2013). The viscosity of (MTPB)/ glycerol (1:1.75) is 16.6 mPa.s at 65°C whereas (BTPC): glycerol (1:16) and ChCl/Gly (1:2) are 2.84782E−07 and 9.48393E−05 281 mPa.s at 25° respectively (AlOmar et al., 2016; Kareem et al., 2010).

Early 2000, DES was considered as green solvent as the synthesis process does not produce any byproduct or side reaction. It also possesses a simple and easy synthesis steps. However, in 2013, the issue on the toxicity of DES was raise as Hayyan's group reported their study on the toxicity of choline chloride-based DES on different type of bacteria including *Bacillus subtilis*, *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* and also on brine shrimp larvae (Hayyan et al., 2013h). The author reported that cytotoxicity of DESs is much greater than their single constituent. Moreover, the synthesized DES does not only have different physical properties but also different chemical properties than their single constituents. Based on the acute toxicity test on *Cyprinus carpio* fish, the LC_{50} ChCl: Glycerol (1:2) is relatively harmless (8000 mg/L) (Juneidi et al., 2015). It is also found that DES of type III (halide salts with HBD) demonstrated significantly lower toxicity than type I (halide salt with metal) and II (halide salt with hydrate metal salt). This was due to the existence of metal component in the DES.

2.7 **Applications of DES**

DES has been extensively applied in many chemical fields as they offer many advantages similar to the common properties of conventional solvent. Table 1 summarized the evidence of common and novel properties of DESs. DES is also favorable to adept in chemical fields because of the easy preparations by low-cost components. These parts explain on the various applications of DES in the chemical and biochemical transformation such as synthesis, separation and analysis.

2.7.1 DES in the Chemical and Biochemical Transformation

In a typical chemical or biochemical process, solvent play a significant major role in almost all stages such as solubilizing agent, media for the substrate, and also in separation and purification of the desired product. The conventional organic solvents used by industry are usually not only hazardous (irritant, explosive, flammable, volatile, etc.) but it also shows high carcinogenicity and toxicity to human beings and environment (Khandelwal et al., 2016).

Green solvents as alternatives have been explored to solve this issue. Water can be considered as the greenest solvent substitute as the resource for water is the most sustainable compared to other alternatives (Castro-Puyana et al., 2017). For examples, pressurized hot water has been applied in the extraction of five flavonoid glycosides from defatted *Camellia oleifera* seeds (Liau et al., 2017). Using subcritical water also has been studied and showed a successful extraction of polyphenols and volatile fraction of coriander (*Coriandrum sativum L.)* extracts (Zeković et al., 2016). However pure and clean water, itself has a limitation as there is competition with the demand of society especially in third class countries where clean water source deprived. Moreover not all biochemical reaction are able to be processed using water as solvents due to its physiochemical properties and high selectivity of the reactant (Zeković et al., 2016).

Other than water, recently ionic liquids (ILs) have also obtained great attention in biochemical transformation due to its attractive physicochemical properties. As mentioned above, ILs possesses tunable physicochemical properties (polarities, solvation, conductivity etc.) by altering the anions and cations (Gorke et al., 2010). Using IL [BMIm][PF6](1- butyl-3-methylimidazolium hexafluorophosphate) as solvents give 93.5% yield of fatty acid methyl ester (FAME) when catalyzed by *Penicillium expansum lipase* whereas the yield is only 68.0% in tert-butanol . IL [BMIm][PF6] is more attractive not just because it gives higher yield, it also does not deactivate the lipase enzyme and can be recycled up to five-run without being degraded. It shows that ILs have superior stabilizing effect as a solvent. However, the high cost of IL and its synthesizing complexity are the main limitations for the industry to use IL in their process. (Juneidi et al., 2017a; Juneidi et al., 2016; Kim et al., 2016; Zainal-Abidin et al., 2017; Zhang et al., 2016)

On the other hand, DESs are derived and synthesized from a low-cost material. For example, glycerol which is the common HBD of DES and also the byproduct of oleochemicals and biodiesel manufacturing was in a surplus state where the production each year exceeded its demand (Ciriminna et al., 2014). This has led to a consistently low price of glycerol each year.

DESs of tetrabutylammonium bromide (TBAB): Sulfolane (1:7) are able to efficiently remove the component of benzene from cyclohexane-benzene mixtures up to 4 extraction cycles without being degraded (Salleh et al., 2017). Though the separation of cyclohexane from benzene is almost impossible due to their close boiling points, TBAB:Sulfolane (1:7) has shown high selectivity and high regeneration in the separation process. In H-NMR analysis, DES of TBAB:Sulfolane (1:7) confirmed that the DES conserved its structure in the presence of benzene, and the solubilization of sulfolane was inhibited by TBAB in the cyclohexane-rich phase.

In similar studies of liquid-liquid extraction on the separation of toluene from n-heptane, DESs of tetrabutylammonium chloride with levulinic acids (1:2) gave high selectivity towards toluene (Gouveia et al., 2016). This study also found that DES with aromatic ring in the HBA triggers high selectivity and distribution coefficients of tie lines for ternary mixture in the phase diagram.

In the investigation of the application of DESs as media for enzymatic reaction, an ammonium salt (ChCl or choline acetate) with common HBD (urea, glycerol, acetamide, ethylene glycol) is successfully activated, stabilizing the structure of horseradish peroxidase (Wu et al., 2014). The author observed that the structure of horseradish peroxidase contained higher α-helix and slightly more relaxed tertiary structure when exposed to DES hence facilitating the horseradish peroxidase activity.

In term of purification of product, DES also plays a major part and shows an effective result at this stage. In the purification of fatty acid butyl ester (FABE) and fatty acid propyl ester (FAPE) and removal of glycerol (a byproduct of biodiesel), ethylene glycol-based DESs were able to remove the glycerol content to 100% (Homan et al., 2017). Based on Figures 2.6 and 2.7, the molar ratio of ChCl/ethylene glycol of 1:2 (DES4), 1:3 (DES 5) and 1:4 (DES 6) all gives a similar result on the removal of glycerol. The purification step also enhances the quality of biodiesel (FABE and FAPE) to the standard of ASTM D6751 and EN14214. This is in agreement with the previous study done by Abbott's group where DES of ChCl/glycerol at 1:1 molar ratio was the best ratio to purify the biodiesel from its byproduct glycerol to up to 99% purification (Abbott et al., 2007b). The ability of the salt to draw glycerol out of the other phase may be dependent on the affinity of Lewis basic mixture.

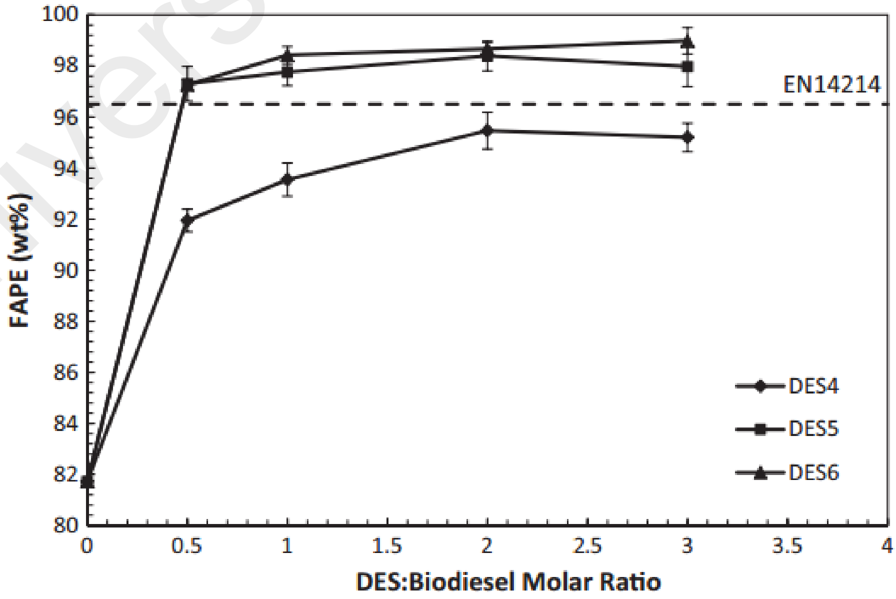


Figure 2.6: FAPE content after purification using ethylene glycol-based DESs. Reprinted with permission from Homan et al. (2017), Copyright 2017 Elsevier.

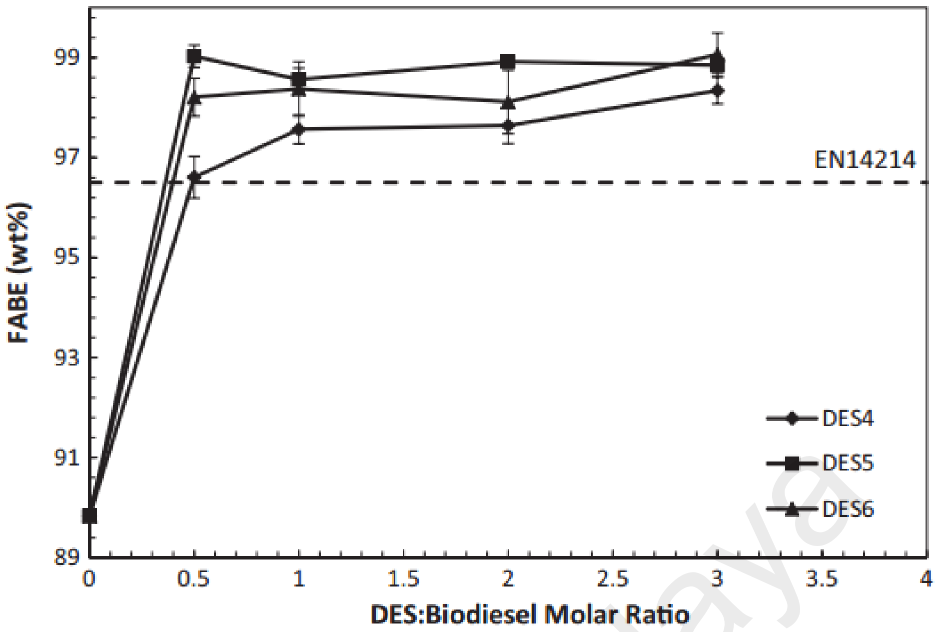


Figure 2.7: FABE content after purification using ethylene glycol-based DESs. Reprinted with permission from (Homan et al., 2017), Copyright 2017 Elsevier.

2.7.2 Application of DES in Lipase Activity

The study on the application of DES in biocatalytic reaction is increasing these recent years. Taking full advantage of DES, researchers in various fields, especially on pharmaceutical and medicine are catching the opportunity to substitute the expensive organic solvent and complexity of their reaction with DESs. *Candida Antarctica* lipase B or Novozymes 435 is one of the most cited lipases studied in recent years (Angajala et al., 2016; Taher, & Al-Zuhair, 2016; Zhang et al., 2012a; Zhang et al., 2016).

Lipases have a strong impact on manufacturing industry such as food, pharmaceutical and oleo-chemical industry. It is an active constituent in numerous biological processes. The catalytic activity of lipase enzyme is a crucial aspect of lipid metabolism as well as organic synthesis. It is able to catalyze all mono-, di-, triglyceride and free fatty acids of oil (Zhang et al., 2012a). Moreover, biotechnology industrial based and research has been accelerated in massive pace due to its cheaper production method, de novo enzyme synthesis and applicable to new fields (Beilen, & Li, 2002). Lipase enzymes are among the major focus in this field.

Due to the wide range of applications such as improved aroma in beverages, emulsifier in food and cosmetics, and hydrolysis in paper product and pharmaceutical, lipases remain as an attractive topic of intense study (Angajala et al., 2016). Lipases (EC 3.1.1.3 triacylglycerol acyl hydrolase) are a group of water-soluble enzymes.

Among the early study reported on the application of DES in the enzymatic reaction are done by Gorke's group. In their study, although the individual component of DES is unstable in aqueous solution, the eutectic mixture of DESs was used as a catalytic activator and as co-solvents for reactions where they boosted the hydrolase-catalyzed reactions up to 20-fold (Gorke et al., 2008). The addition of 25 vol% of ChCl/ Glycerol in the hydrolysis of styrene increased the rate of epoxide hydrolase to up to 92% compared to using buffer which gave only 4.6%. Moreover, addition of 10 vol% of the same DES; ChCl/glycerol in the hydrolysis of p-nitrophenyl acetate increased moderately to three-fold for *Rhizopus oryzae* esterase and pig liver esterase and a 25% increase for *Pseudomonas fluorescens* esterase and lyophilized *Candida Antarctica* lipase B. The activity of Novozyme 435 in DES of ChCl/glycerol or in ChCl/urea was also discovered to be similar and even greater than those by imidazolium-based ILs such as [BMIM]BF_4 or [BMIM]NTf_2. Overall in their study, the authors highlight that DESs are suitable as a solvent candidate for a wide range of reaction. This is due to the reason that hydrogen-bond network in DESs possesses lower reactivity compared to its former component hence lowering the chemical capability (Gorke et al., 2008).

Recently, based on the study of hydrolysis reaction, ChCl based DES with two different types of hydrogen bond donor result in high lipase enzyme activity and stability (Kim et al., 2016). The chemistry behind the activation and stabilization were investigated using solvatochromic parameters. The authors observed that the solvatochromic parameter E_T^N (the intermolecular charge transfer absorption band of

Reichardt's betaine dye) of DES mixture ranged from 0.814 to 0.857. These results are much higher than short chain alcohols and proctic solvent (secondary amines) such as Nmethylacetamide with 0.657 and methanol with 0.762. The E_T^N value of Reichardt's betaine dye is greatly affected by the hydrogen bond acidity of the solvent (Reichardt, 2005). The increment of E_T^N is parallel with the increment of lipase activity. The relative activities of lipase in ChCl/glycerol (1:2 molar ratio), ChCl/urea (1:2 molar ratio), and ChCl/urea/glycerol (1:1:1) aqueous solutions were 103%, 122%, and 155% respectively. The synergistic effect existed when DES was synthesized with 2 different HBD compared to one type of HBD hence increasing the lipase activity.

Moreover use of ethylene glycol and glycerol as HBD in the synthesis of DES also proves to significantly enhance the thermal stability of lipase in aqueous solutions. The half-life time of lipase in ethylene glycol and glycerol-based DES increases by 5.3–8.9 and 2.9–9.2 times respectively when compared to that of lipase in the buffer. The combination of dipolarity and polarizability (π*) of DES mixture from this study is within the range of 1.112 to 1.245 which is much higher than water (1.09) and ILs. Whereas, the hydrogen bond acidity (α) and basicity (β) were higher than reported organic solvent. Overall from this study, the author concludes that α, β, and π* are closely associated with the thermal stability and storage stability of lipase (Kim et al., 2016).

Previously in 2014, Huang's group also conducted a similar study on hydrolytic reaction of *Penicillium expansum* lipase (PEL) to investigate the significance of DES as an enzyme activator and stabilizer (Huang et al., 2014b). In figure 2.8, the result shows 2.4 times increment of activity when the PEL was mixed with DES of choline acetate (choline acetate /glycerol) (1:2) compared to free PEL hydrolysis reaction. The authors confirmed that the increment of activity and stability of PEL in choline acetate /glycerol (1:2) was not because of the synergistic effect of dissociated components but it was due to the strong hydrogen-bond interactions between the anion and HBD and strong ionic interactions between the cation and the anion. They also studied the toxicity of ChCl and urea and discovered that eutectic mixture was able to weaken or reduce the toxicity of its salt/component (in this study, ChCl). This observation also strongly supports the argument that in an aqueous state, the hydrogen-bonding network of DES is not dissociated into components but remain integrated as a eutectic mixture (Huang et al., 2014b; Wu et al., 2014).

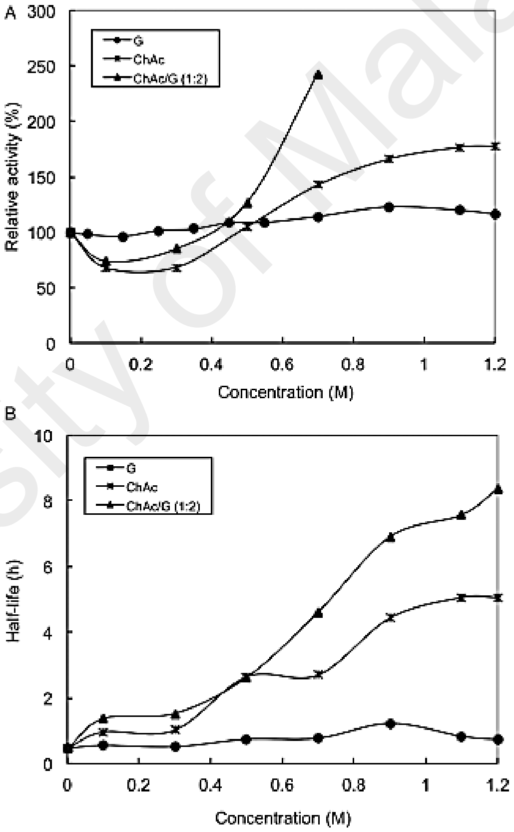


Figure 2.8: Activity (A) and stability (B) of PEL in an aqueous solution containing ChAc, glycerol, and ChAc/G (1:2) at different concentrations. The relative activities (%) presented in (A) refer to the percentages of the initial reaction rates obtained by the enzyme in the presence of different components relative to the one obtained in the buffer solution alone (which is 11.7 µmol L-1 min-1). The half-lives of the enzyme presented in (B) were obtained at 40 °C. Reprinted with permission from (Huang et al., 2014), Copyright 2014 John Wiley and Sons.

2.7.3 Application of DES in Treatment of FFA & Biodiesel Production

Biofuel field and industry are emerging due to an increase in environmental issues such as global warming and greenhouse effect and also depletion of non-renewable sources of energy such as petroleum and natural gases. Based on the report given by OPEC, about 4.1 billion liters of diesel fuel were consumed every day worldwide in the year 2012 and it is forecasted to increase to about 5.7 million liters (5.7ML) per day by 2035 (Hajjari et al., 2017). Researchers and industry are making great progress in finding new technology to find the alternatives to replace non-renewable sources of energy. Biodiesel or scientifically known as fatty acids methyl esters (FAME) is one of the most current topics that is extensively reported in the renewable energy field for the last three decades (Alhassan, & Kumar, 2016).

Another area that earned significant interest in this concern is the catalysis in biodiesel production (Alhassan, & Kumar, 2016). Table 1 summarizes the type of co-solvent and novel catalyst that was studied in these recent years. Various homogenous as well heterogeneous catalysts have been studied extensively in this field such as (1R)-(–)-camphor-10-sulfonic acid and 1- propanesulphonic acid (Hayyan et al., 2014a; Hayyan et al., 2015a) sulfonated graphene (Nongbe et al., 2017), calcium oxide (Ye et al., 2016).

It is DES that is able to replace ILs completely in biodiesel production considering the various advantages offered by DES such as benign and cheap components for its synthesis. Zhou proved that the invention of green solvent DES could substitute the volatile organic solvent in the preparation of biodiesel (Zhao et al., 2013) . In enzymatic transesterification of soybean oil with methanol, DES-based ChCl and glycerol (1:2) was able to activate immobilized *Candida Antarctica* lipase B (iCALB) and showed the highest conversion to 88% triacylglycerol in 24 hours (Zhao et al., 2013). In the experiment, their DES are biodegradable, non-toxic and inexpensive showed high stability toward iCALB. However, the authors found that lower content of glycerol reduced lipase activity. This is due to the high content of anion species in DES that destabilized the lipase enzyme. Previously, in 2011, the authors investigated the activity of iCALB using DES based choline acetate with glycerol (1:1.5 molar ratio) and resulted in 97% conversion of triglyceride from enzymatic transesterification of Miglyol® oil with methanol within 3 hours (Zhao et al., 2011a). They also found out that, DES also improves the physicochemical properties of ILs (hygroscopicity and viscosity) depending on the design of molar ratio between cholinium salts and HBD.

This is in agreement with the previous work of Durran's group where DES of ChCl combined with either urea or glycerol activated iCALB activity in the transesterification of vinyl laurate (Durand et al., 2013). It was also reported by Zhang's group that ChCl with glycerol (ratio 1:2) as a solvent showed higher activity of iCALB for enzymatic transesterification of yellow horn seed oil with 95% conversion yield under microwave irradiation, confirming their possible superiority over organic solvents (Zhang et al., 2016).

Table 2.5: List of a component of DES as co-solvent/catalyst in esterification and transesterification reaction

Halide Salts	**Molar ratio**	**Hydrogen Bond Donor**	**Type of feedstock**	**Biodiesel/ FFA Conversion Yield**	**reference**
choline acetate	1:1.5	Glycerol	Miglyol® oil 812	97%	(Zhao et al., 2011)
Choline Chloride	1:2		soybean oil	88%	(Zhao et al., 2013)
			yellow horn seed oil	95%	(Zhang et al., 2016)
			vinyl laurate	100%	(Durand et al., 2012)
			Millettia	91.9%	(Huang

Halide Salts	**Molar ratio**	**Hydrogen Bond Donor**	**Type of feedstock**	**Biodiesel/ FFA Conversion Yield**	**reference**
			pinnata seed oil		et al., 2014b)
			rapeseed oil	98%	(Gu et al., 2015)
			used cooking oils	97%	(Kleiner et al., 2016)
Choline Chloride	1:3		oleic acid	98%	(Pan et al., 2016)
	1:2	Urea	vinyl laurate	100%	(Durand et al., 2012)
	1:1	Acetic acid	*Chlorella* sp	24%	(Pan et al., 2017)
	1:3	p-toluenesulfonic acid monohydrate	acidic crude palm oil	92%	(Hayyan et al., 2014b)
	1:4		*Pongamia pinnata* seed oil	97.53%	(Alhassan, & Kumar, 2016)
allyl triphenylphosphonium bromide	1:3		low-grade crude palm oil	90.53%	(Hayyan et al., 2013d)
			oleic acid	95%	(Williamson et al., 2017)
N,N-diethylenethanol ammonium chloride	1:3		low-grade crude palm oil	93.67%	(Hayyan et al., 2013b)

2.8 Summary

Biodiesels are any renewable source of oil that can be used as diesel. It is derived from either vegetable oils, algae, animal fats or even waste oil. The main factors affecting the economic viability of biodiesel production are prices, availability of feedstock oils and the plant capacity. The most conventional and commercially used technological approach in biodiesel production is via transesterification of animal fats or vegetable oil with an alcohol in the presence of an acid or alkali catalyst. Edible vegetables oils such as rapeseed oil, soybean oil and palm oil have been used as feedstock in biodiesel production. However, the price of these refined oils is relatively expensive compared to petrol fuel. The cost of feedstock covers 70%-95% of total production cost. In order to overcome this issue, esterification of waste oil was introduced prior to the transesterification. Utilizing waste oil as the alternative cheap feedstock will reduce the overall production cost of biodiesel. Esterification was done as the pretreatment of oil waste which normally has FFA content of more than 1%. This is the limit of content of FFA for transesterification reaction. Cooking oil waste, animal fats waste and sludge palm oil from refinery is the example of oil waste that has been used as the feedstock for esterification reaction. DESs as the co-solvent and catalyst are reported to be effective to esterify and trans esterify from fatty acid and triglycerides. Till date, there is no report on the use of DES in acid and enzymatic esterification. The current work collects and reports the most updated study done on acid/lipase enzyme for the production of biodiesel.

This review comprises the potential of application of DES in chemical and biochemical, especially in biodiesel production. The conventional organic solvent will have to be a substitute in the near future by DES due to economic viability and environmental concerns. The development of DES-based solvent and catalysis is an an exponential rate. As per the reported literature, DES has been applied in various fields and at many stages from the extraction to the purification step. DES has been proven to escalate and activate the free as well as immobilized lipase enzyme activity. This finding is important in biodiesel production as the main drawback of utilizing lipase is due to its high cost. By activating and stable in DES, enzyme lipases could be adapt and regenerated in an enzymatic reaction that eventually will increase the economic viability of biodiesel production. However, to date, there is no study were done on a comparison of eutectic novel solvents/DES in chemical and enzymatic reactions for biodiesel production. Therefore, this suggests that a comprehensive study on the capability of DES enhance chemical type catalyst and activate free lipase enzyme on the biodiesel production especially in the treatment of high FFA during esterification.

CHAPTER 3: SYNTHESIS OF NOVEL EUTECTIC SOLVENT FOR ESTERIFICATION OF LOW GRADE PALM OIL

(Published in Journal of Oil Palm Research 29 (3), 373-379)

3.1 Introduction

In this study, a new eutectic solvent (ES) was synthesized for the first time via mixing (1R)-(-)-camphor-10-sulfonic acid (10-CSA) with choline chloride (ChCl). CSA-ChCl-ES was used as a catalyst in the esterification reaction of acidic crude palm oil mixed with sludge palm oil to reduce the free fatty acid (FFA) content to less than 2%. The optimal reaction conditions are 2.5 wt% of catalyst dosage, 10:1 molar ratio, 60^0C of reaction temperature and 40 minutes of reaction time. This study revealed that the novel eutectic solvent has high catalytic activity and it can be recycled numerous times, therefore this shows that it has high potential in the production of biodiesel.

3.2 Literature Review

The declining of non-renewable source of fossil fuel, unstable and constantly fluctuating prices of petrol fuel in the market and concerns of the threat of global warming have given rise intense attention from around the world to synthesize alternatives fuel from renewable-based feedstock (Salvi, & Panwar, 2012; Silva et al., 2010). To date, the most highly recognized alternative fuels are biodiesel. Biodiesel is defined as mono-alkyl long chain fatty acids esters and obtained from transesterification of fatty acids such as animal fats or vegetables oil (Nieves-Soto et al., 2012).

The crude palm oil (CPO) has been widely used as feedstock for biodiesel production (Elsheikh et al., 2011; Ho et al., 2014). Sludge palm oil (SPO) and acidic crude palm oil (ACPO) were reported as potential industrial raw material for biodiesel production (Hayyan et al., 2014b; Hayyan et al., 2013c). However, high free fatty acid (FFA) content in low grade oils and fats is the main obstacle to produce biodiesel using alkaline catalyst. It reduces the yield of biodiesel and the conversion rate as other reaction occurred, such as saponification (Canakci, & Van Gerpen, 2001). The authors added that the formation of soap diverts the separation process of glycerin from crude biodiesel. Therefore, esterification reaction with acid catalysts is required to convert FFA to fatty acid methyl ester (FAME) before alkaline transesterification reaction (Canakci, & Van Gerpen, 2001; Silva et al., 2010).

The common acid catalyst for esterification reaction in industry is sulfuric acid and p-toluenesulfonic acid monohydrate (PTSA) (Canakci, & Van Gerpen, 2001; Hayyan et al., 2010b; Silva et al., 2010). Recently, (1R)-(–)-camphor-10-sulfonic acid (10-CSA) was introduced as solid organic catalyst for the treatment of FFA and was reported that 10-CSA demonstrated a good catalytic activity in FFA content reduction with high conversion of FAME (Hayyan et al., 2014a). 10-CSA is a weak organic acid and it has promising industrial applications. However, it was also reported that 10-CSA is an organic hygroscopic acid (Hayyan et al., 2014a). Hygroscopicity may reduce its potential to be applied in industrial scale for biodiesel production. Conversion of hygroscopic acids to eutectic solvents has been introduced by Hayyan group. Sulfonic acid based eutectic solvents are relatively more stable compared to conventional sulfonic acids in powder form (Hayyan et al., 2014b).

Eutectic solvents or deep eutectic solvents (DES) have captivated huge interest recently due to their great potential as environmentally excellent and favorable solvents with eutectic characteristic (Alhassan, & Kumar, 2016; Smith et al., 2014). The application of DES in many industrial processes has been reported such as electrochemical energy for storage device (Bahadori et al., 2013), synthesizing of aromatic compound from nucleophilic reagent (Liu et al., 2015) as substrate in enzymatic esterification of selective oleic acid (Zeng et al., 2015) and as reaction medium for synthesizing a novel organic synthesis of oxazoles for pharmaceutical purposes (Singh et al., 2013).

DESs are biodegradable solvents and they have relatively low toxicity compared to the conventional ionic liquids and organic solvents (Hayyan et al., 2014b). DESs are generally prepared using hydrogen bond donors (HBDs) such as sugars, polyols, and carboxylic acids and simply mixed with a hydrogen bond acceptors (HBAs) such as choline chloride (ChCl) (Zhang et al., 2012c). The infusion of HBD with anion of HBA induces a huge depression in the melting point of the new mixture (Durand et al., 2013). Thus the aim of this study is to investigate the functionality of eutectic solvent made from 10-CSA and ChCl for esterification of FFA content.

3.3 Materials and method

3.3.1 Raw material and chemicals

The crude palm oil (CPO) and SPO were collected from local mills at Carey Island, Selangor Darul Ehsan. Methanol, (1R)-(–)-camphor-10-sulfonic acid (10-CSA) and choline chloride (ChCl) were purchased from Merck Sdn Bhd, Malaysia with purity of ≥ 99%.

3.3.2 Methodology

CPO was mixed with SPO to prepare ACPO with 8.1% FFA content. The FFA limit for this study was fixed to be 2%. Jacketed reactor with reflux condenser control by feedback controlled system is used for the treatment of FFA content where the preheated oil (at 70^{o}C for one hour) samples were esterified using methanol with the presence of CSA-ChCl-ES as an acidic catalyst. This method was adopted from a previous study reported by Hayyan et al. 2013.

The DES was prepared by mixing the ChCl with 10-CSA in a mass ratio of 1:1 for 3 hr at 80 °C in the moisture controlled environment (Scheme 1). The CSA-ChCl-ES was sealed and kept in the vial before being used. The CSA-ChCl-ES was mixed with the methanol and then poured in the reactor with the pre-heated oil. CSA-ChCl-ES was used at different dosages and other parameters such as the reaction time, molar ratio, and reaction temperature was optimized.

In the recyclability test, the used catalyst dissolved in methanol was separated from the treated oils after esterification reaction using centrifugation and continuously used for the next batch of a fresh sample of ACPO. The FFA content after esterification reaction was determined according to AOCS official method Ca 5a-4 (AOCS, 1997). The amount of active catalyst used in the reaction was calculated using catalyst consumption (C.C) equation (3.1). C.C is defined as the mass of catalyst consumed per mass of synthesized product (Hayyan et al. 2010).

$$C.C = \frac{Mcat}{Mp} \tag{3.1}$$

Where:
C.C = catalyst consumption.
Mcat = mass of catalyst used to give the synthesized mass of product biodiesel (mg).
Mp = mass of synthesized product biodiesel (g).

3.4 Result and Discussion

3.4.1 Effect of 10-CSA DES at Different Dosages

Figure 3.1 shows the effect of CSA-ChCl-ES at different dosages for FFA reduction. CSA-ChCl-ES has high catalytic activity, decreasing the FFA from 8.1 % to 1.71% and 1.2% using only 2.5 wt% and 3 wt% of eutectic solvent to oil respectively. The reaction time was fixed at 30 minutes for all runs. This can be concluded that the

FFA reduction reduced in a very short period of a time. Figure 1 also shows that FFA was reduced to 1.71 % and the FFA conversion was 90 % when the catalyst dosage is 2.5 %. Using 3.5 % of CSA-ChCl-ES to oil was sufficient to reduce the FFA content to 1 %. It was reported by (Hayyan et al., 2014a) that the best dosage of the catalyst is the minimum dosage that is sufficient to reduce the FFA content rapidly during the course of the esterification reaction, therefore 2.5 % was selected to be the optimum for catalyst dosage. In table 3.1, based on catalyst consumption (CC) of the CSA-ChCl-ES on the conversion rate of FFA, the yield of treated oil was proportional to the amount of catalyst used. At 2.5 % of dosage, 25.91 mg was needed to produce 1 gm of treated oil which is regarded as the low capacity of catalyst in biodiesel production and gives lower cost in pre-treatment process. In a previous study, (Hayyan et al., 2014a) used 10-CSA, and the current study used the 10-CSA as hydrogen bond donor. It was found that the acid will not lose its catalytic activity when it has been converted to DES. CSA-ChCl-ES as an organic catalyst shows slightly low catalytic activity compared to ammonium and phosphonium-based DES (Hayyan et al., 2013a; Hayyan et al., 2013b).

Table 3.1: Effect of CSA-ChCl-ES on the yield of treated ACPO and catalyst consumption

Dosage of Catalyst (%)	Yield (%)	Catalyst Consumption (mg gm^{-1})
0.5	90.0	5.55
1.0	91.5	10.93
1.5	96.0	15.63
2.0	96.2	20.79
2.5	96.5	25.91
3.0	96.6	31.06
3.5	97.0	36.08

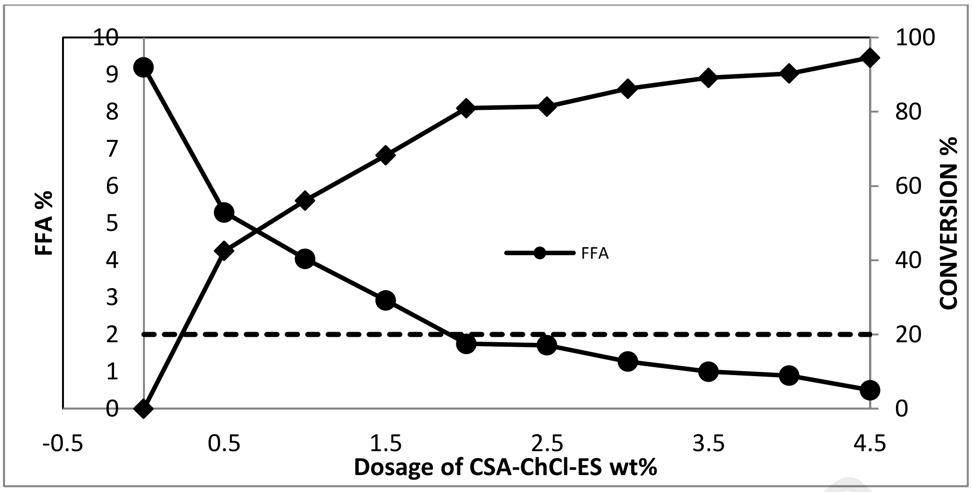


Figure 3.1: Effect of dosage of CSA-ChCl-ES on FFA content reduction at 60 min reaction time, 10:1 molar ratio, 60 °C, and 300 rpm.

3.4.2 Effect of molar ratio

In term of choice of solvent in the esterification, methanol is more favorable compared to another alcohol chain. Methanol has lower viscosity compared to ethanol, offers lower cost, shorter reaction time, less energy required and better performance during the reaction (Guerrero-Romero, & Sierra, 2011). Figure 3.2 shows the effect of methanol loading for esterification of FFA content. Results showed that 8:1 can be used for FFA reduction. However, the FFA level was 2 % for the molar ratio of 8:1 while 10:1 can reduce the FFA content to less than 2 %. Figure 3.2 shows that there no significant improvement using methanol loading more than 10:1. A similar result was also obtained by (Suresh et al., 2017) where there was not much increase of conversion rate after molar ratio 10:1 using 1 % of partially sulfonated polystyrene (PSS). Therefore, 10:1 considered the optimum molar ratio for the esterification reaction. Ma and Hanna mentioned that an excess ratio of methanol is required to maximize the yield of FAME and to drive the reversible reaction towards equilibrium (Ma, & Hanna, 1999). Moreover, 10:1 molar ratio is applicable in the massive plant as the cost of the production of biodiesel is strongly affected by the amount of solvent used (Hayyan et al., 2014b).

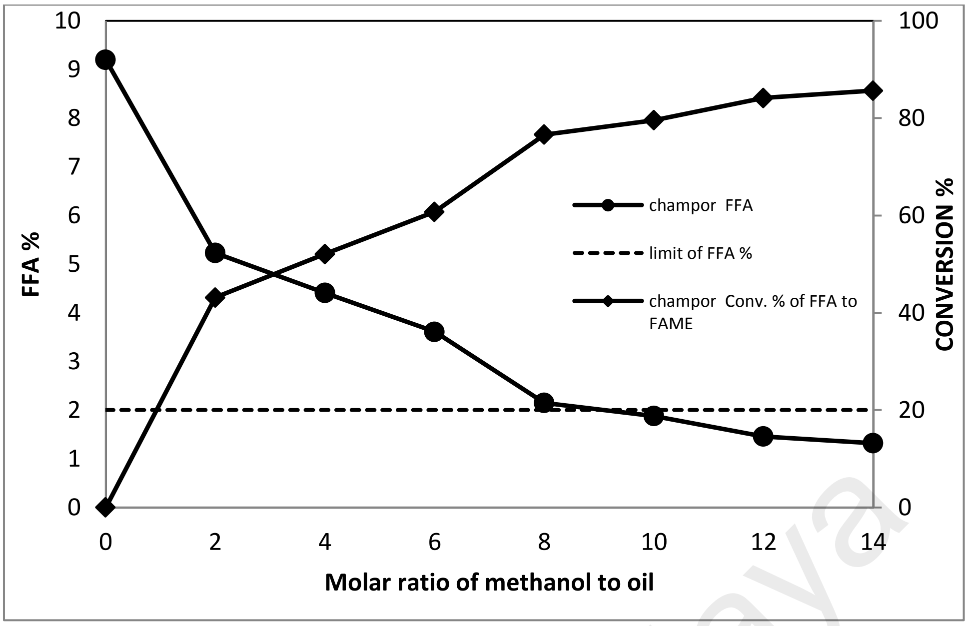


Figure 3.2: Effect of molar ratio on FFA content reduction at 2.5% of dosage of CSA-ChCl-ES, 60 min reaction time, 60 °C, and 300 rpm.

3.4.3 Effect of reaction time

In order to complete the esterification, an adequate reaction time is required. Reaction time for this study was evaluated from 10-120 min as shown in Figure 3.3. The FFA level was reduced simultaneously with increasing reaction time and the reduction of FFA occur very rapidly even at 10 and 20 minutes which 2.16 % and 2.02 % respectively. The rate of the conversion increased with reaction time. Both 30 and 40 were sufficient to reduce the FFA content. However, 40 minutes was chosen as the optimum reaction time. This optimum reaction time is still acceptable in industrial scale and considered as rapid reaction time for the esterification reaction.

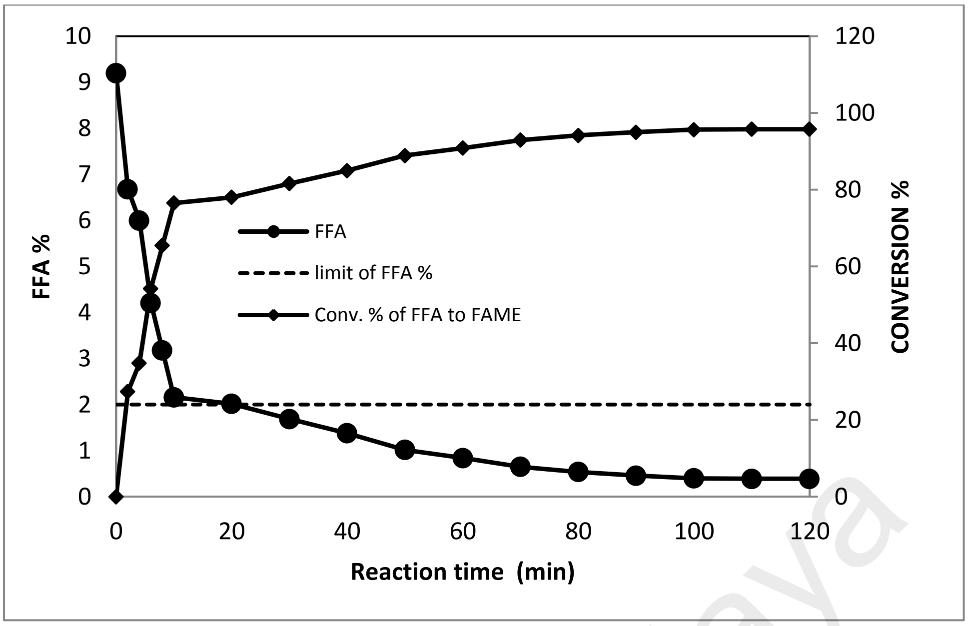


Figure 3.3: Effect of reaction time on FFA content reduction at 2.5% of dosage of CSA-ChCl-ES, 10:1 molar ratio, 60 °C, and 300 rpm.

3.4.4 Effect of reaction the temperature

Thermodynamically, the methanol and triglyceride are unable to react at room temperature and atmospheric pressure. This is due to the low solubility of methanol in oil (Nieves-Soto et al., 2012). Thus, the temperature is a vital parameter to induce esterification reaction. Figure 3.4 illustrates the reaction temperature effect on the reduction of FFA. In order to increase the conversion rate of treated oil, the optimum temperature is required for the system to exceed the activation energy of the reaction (Fan et al., 2017). It can be seen that increasing the reaction temperature will decrease the FFA level. The slightly increased value of FFA content at 80 °C was due to methanol evaporation. In addition, the higher temperature required higher energy consumption (Hayyan et al., 2013a). Reaction temperature at 70 °C can reduce the FFA content from 8.01% to 1.04%. However, in order to save the cost of maintenance and operation and also save energy, a minimum reaction temperature is favorable in esterification reaction. Thus, a temperature of 60 °C was selected as optimized reaction temperature as it sufficient to reduce the FFA level to 1.88% which is lower than the limit of FFA desired in the industry.

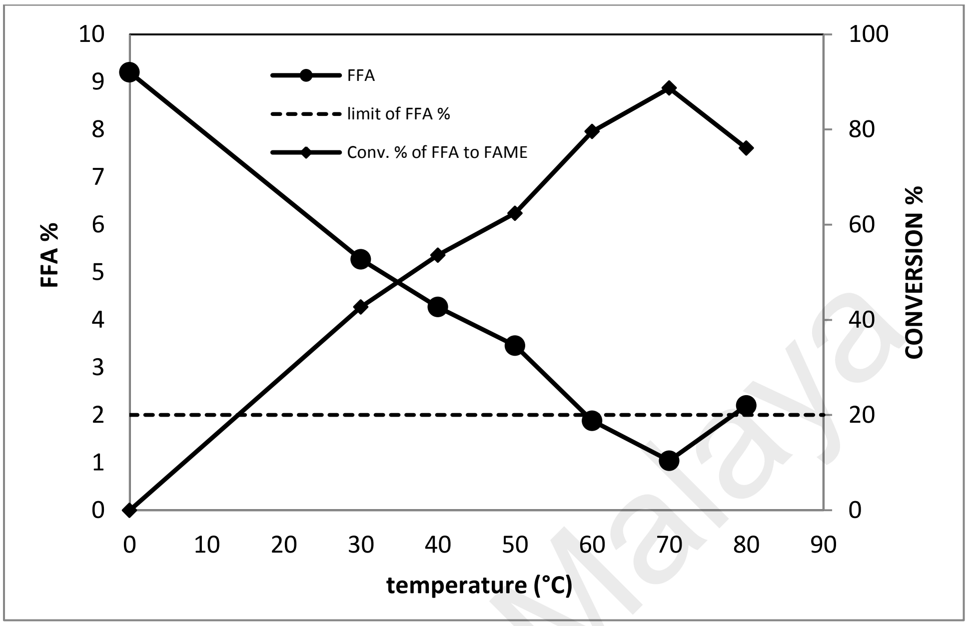


Figure 3.4: Effect of temperature on FFA content reduction at 2.5 % of dosage of CSA-ChCl-ES, 10:1 molar ratio, 40 minutes of reaction time and 300 rpm.

3.4.5 Validation and Recyclability

It was found that the optimum conditions for FFA reduction to less than 2 % were 60 ^{0}C temperatures, 2.5 wt% dosage of CSA-ChCl-ES to oil, the reaction time of 40 min and 10:1 molar ratio. The reusability and recovery of catalyst have a significant impact on the economic gain of industrial processes (Suresh et al., 2017). Figure 3.5 shows that this catalyst can be recycled for five consecutive runs and this gives an indication that this catalyst has promising potential industrial application in biodiesel production.

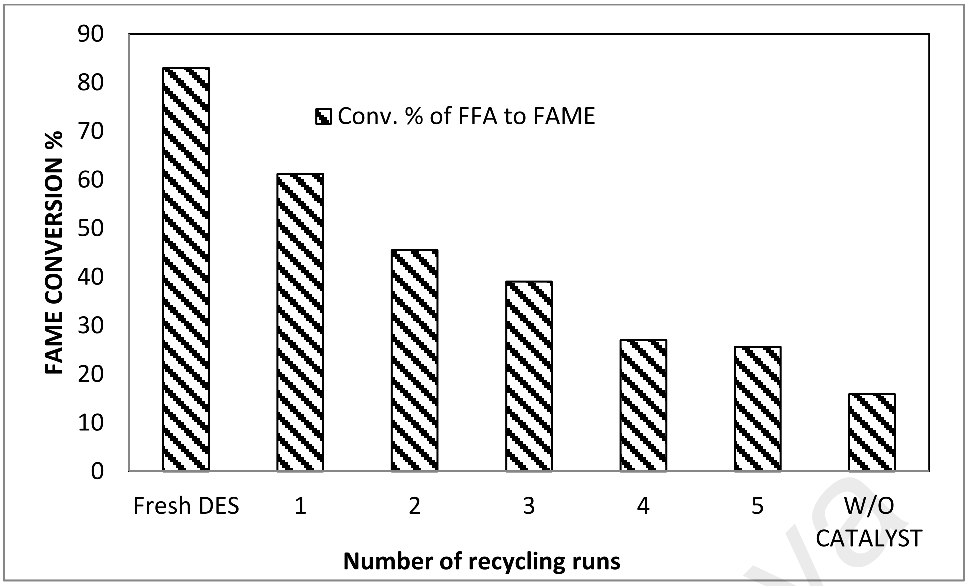


Figure 3.5: Recycling of CSA-ChCl-ES at 2.5 % of dosage of CSA-ChCl-ES, 10:1 molar ratio, and 40 minutes of reaction time, 60 °C and 300 rpm.

3.5 Conclusion

The optimum conditions for this study were 2.5 wt% of catalyst dosage, 10:1 molar ratio, 60^{0}C of reaction temperature and 40 minutes of reaction. The agro-industrial feedstock, ACPO and CPO (palm oil crops) were the most effective raw materials for this product due to its high availability and low price in Malaysia as the world largest palm oil producer. The FFA level of this mixture of ACPO and CPO was successfully reduced from 8.1% to less than 2% which is the limit desired in industrial scale using the selected optimum condition mention above. The reusability of the catalysts is also high. From this experiment, it is proven that CSA-ChCl-ES is capable and favorable to become a novel catalyst for the esterification reaction.

CHAPTER 4: PRODUCTION OF FATTY ACID METHYL ESTER FROM LOW GRADE PALM OIL USING EUTECTIC SOLVENT BASED ON BENZYLTRIMETHYLAMMONIUM CHLORIDE

(Published in IOP Conf Series: Materials Sci and Eng 210 (1), 012012)

4.1 Introduction

In this study, high content of free fatty acid (FFA) from low grade palm oil (LGPO) was treated using benzyltrimethylammonium chloride (BAC) based DES as catalyst. The treatment process of low grade palm oil from refineries was achieved with the aid of p-toluenesulfonic acid monohydrate (PTSA) mixed with salt; BAC. Various effect of reaction conditions such as catalyst dosage (0.5 % to 4.5 %), molar ratio (2:1 to 12:1), reaction time (10 to120 min) and reaction temperature (30 °C to 80 °C) were studied. Results showed that the optimum reaction condition of 2% catalyst dosage, 10:1 molar ratio of methanol to oil, 30 min of reaction time and reaction temperature of 60 °C gives high conversion and yield of 90 %. BAC-based DES catalyst is highly potential to be commercialized and applied in industrial scale due to its high catalytic activity with low cost production.

4.2 Literature Review

Extensive study has been done on the optimization of biodiesel production. The studies were motivated by a demand for renewable source of energy and fluctuating prices of petrol diesel in the current market (Bisen et al., 2010; Jayakumar et al., 2017). A mixture of acidic crude palm oil (ACPO) and sludge palm oil (SPO) showed a tremendous excellent result in producing biodiesel (Hayyan et al., 2013b). These low grade oil mixtures were the waste oil rejected from palm oil refineries due to its high free fatty acid value of over 5%. It is perfect candidates for biodiesel production as it low in cost and available domestically.

A new concept of applying a deep eutectic solvent (DES) with the chemical catalyst in the esterification reaction also has been studied. DES is eutectic mixtures which easily synthesize from combination of either salt: salt, hydrogen bond donor (HBD): HBD, and salt: HBD (AlOmar et al., 2016; Singh et al., 2013). An ammonium based deep eutectic solvent; N,N-diethylenethanol ammonium chloride with p-toluenesulfonic acid monohydrate (PTSA) was applied as a novel recyclable catalyst. Moreover, the esterification reaction was run with the same amount of dosages of using PTSA alone in the previous study; 0.75 % (Hayyan et al., 2013b) . PTSA also was mixed with allyltriphenylphosphonium bromide as a phosphonium-based deep eutectic solvent in other study and also shown a great impact on recyclability study where it can be used up to four runs (Hayyan et al., 2013a).

Another type of hydrogen bond donor was proposed in this study as the novel recyclable catalyst; benzyltrimethylammonium chloride (BAC). This research work presents the catalytic activities of BAC-DES catalyst; BAC mixed with PTSA in esterification of low grade palm oil (LGPO). The optimum condition of the esterification reaction is proposed to be studied such as dosage of catalyst, reaction temperature and time and molar ratio. A multi-unit reactor with reflux condenser using feedback controlled system was used to conduct the experiment.

4.3 Methodology

4.3.1 Materials and Method

Low grade palm oil (LGPO) with FFA content of 12% to 10% was collected from local palm oil refinery of Sime Darby Corp in Carey Island, Selangor Darul Ehsan, and Malaysia. The other chemicals, i.e. methanol, potassium hydroxide, benzyltrimethylammonium chloride; BAC ($C_6H_5CH_2N$ $(CI)(CH_3)_3$) p-toluenesulfonic acid monohydrate; PTSA ($CH_3C_6H_4SO_3H \cdot H_2O$) were purchased from Merck and Sigma Aldrich, were of analytical grades.

4.3.2 Synthesized of DES based catalyst

The BAC-DES catalyst was prepared using method of Hayyan et al. (2013b). The molar ratio of BAC to PTSA was 1:3. The required amount of BAC powder was mixed with PTSA gradually until it becomes clear homogenous solution. The mixing condition is at 60 °C and 350 rpm using hot plate and magnetic stirrer. The mixture was sealed and placed in desiccator with silica gel to prevent absorption of moisture.

4.3.3 Synthesized of FAME from LGPO

LGPO was pre-heat at 60 °C before esterification. The required amount of oil sample was placed in multi-unit reactor with reflux condenser and speed controller using feedback controlled system. The specific dosage of BAC-DES catalyst was mixed with methanol until homogeneous prior addition to multi-unit reactor. Esterification was carried out with different catalyst dosage (0.5% to 4.5%, based on oil weight), molar ratio of methanol to oil (2:1 to 12:1), reaction time (10 to120 min) and various reaction temperature (30 °C to 80 °C). The standard batch was run with 30 g of oil, 12 g of methanol and 0.3 g of catalyst placed in the jacketed vessel. The reaction conditions were at 60 °C, 30 min, and under stirring at 350 rpm. After esterification, the solvent and catalyst was separated using centrifugation. After separated, treated oil was further heated at 75 °C in oven to remove excess methanol. The reusability of BAC based DES catalyst was investigated using selected optimum condition and was run without addition of fresh catalyst. The yield and FFA reduction was evaluated using titration according to AOCS official method Ca 5a-4 (American Oil Chemists' Society, 1997). The transesterification of treated oil was fixed as follow 1 % of KOH solubilize in methanol (10:1 molar ratio of methanol to oil) at 60 °C, 30 min, and 350 rpm stirring speed (Hayyan et al., 2010a).

$$\text{Yield} = \frac{\text{Mb-Ma}}{\text{Mb}} \times 100\% \qquad (4.1)$$

Where Mb and Ma are the mass of oil before and after esterification (g)

4.4 **Result and Discussion**

4.4.1 Effect of BAC-DES catalyst and molar ratio

BAC based DES catalyst was first time studied in esterification of LGPO to produce biodiesel. BAC is an off-white quaternary ammonium compound with a structural resemblance to acetylcholine (Ghanayem, 2000). As mentioned above, the targeted FFA after esterification was to be at ≤ 2%. From Figure 4.1, the reduction of FFA was proportional with increasing dosage of catalyst. The result shows that BAC based DES possessed high catalytic activity despite the presence of foreign compound existed in the mixture; BAC. 1.5% dosage of catalyst is sufficient to lower the FFA content from 9.2% to 1.8% and gives 88.5% yield of conversion to FAME. Though it used higher amount compared to other studies, 2% is still considered acceptable dosage to be applied in esterification at industrial scale. Thus 2% of BAC based DES was selected as the optimum dosage in esterification of LGPO as DES from BAC mixed with PTSA is sufficient to reduce the FFA content to less than 2%. From table 1, the yield of treated oil was found to be increased with increasing of dosage used.

At fixed dosage of BAC-DES catalyst (1.5%), another reaction condition was studied to improve the pre-treatment process of LGPO. Molar ratio was varied from 2:1 to 12: of methanol to oil. Stoichiometrically, during esterification, the three fatty acid chains of triglycerides will release and combine with methanol to produce FAME (Corro et al., 2014). From the Figure 4.22, 6:1 molar ratio was sufficient to reduce the

FFA content to ≤ 2%. No enhancement in the reduction of FFA content after molar ratio 10:1. Thus 10:1 was selected as the optimum molar ratio to esterify LGCPO with the reduction of FFA content from 9.2% to 1.06% yield of 93.3%. BAC based DES was slightly advantage compared to other study done by Corro et al. where 12:1 molar ratio of methanol was used to esterify Jatropha curcas crude oil with the aid of heterogeneous catalyst (Corro et al., 2014).

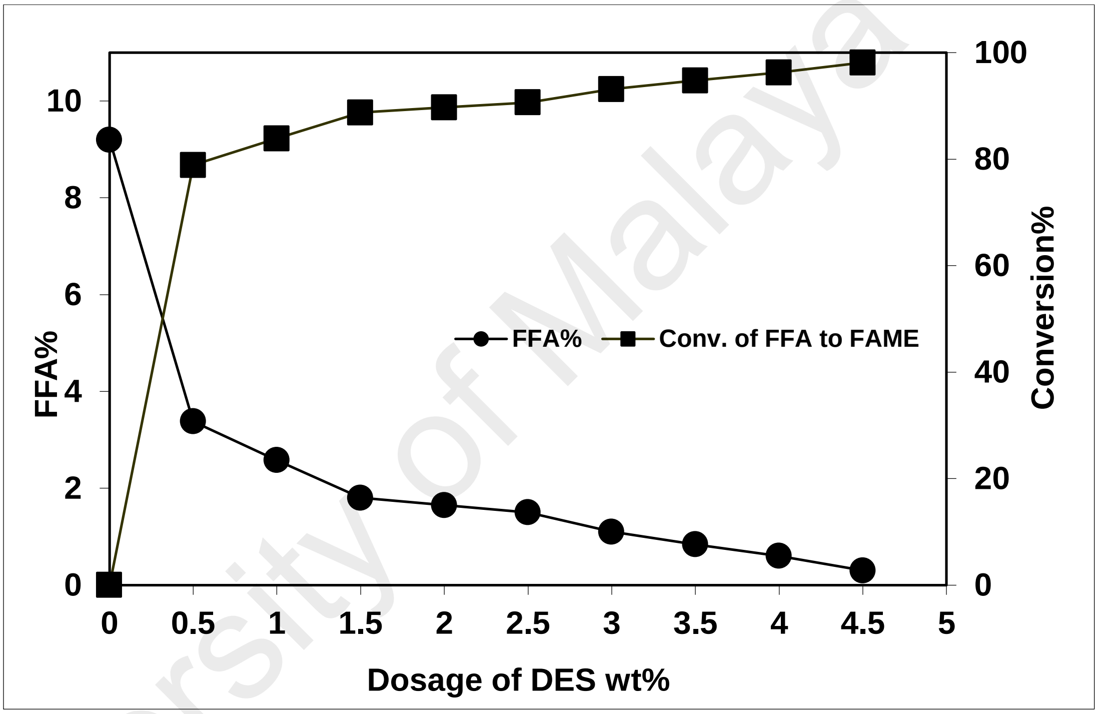


Figure 4.1: Effect of BAC based DES dosage on the yield of treated LGCPO and the correspondence reduction of FFA content at 30 min reaction time, 10:1 molar ratio, 60 °C, and 350 rpm.

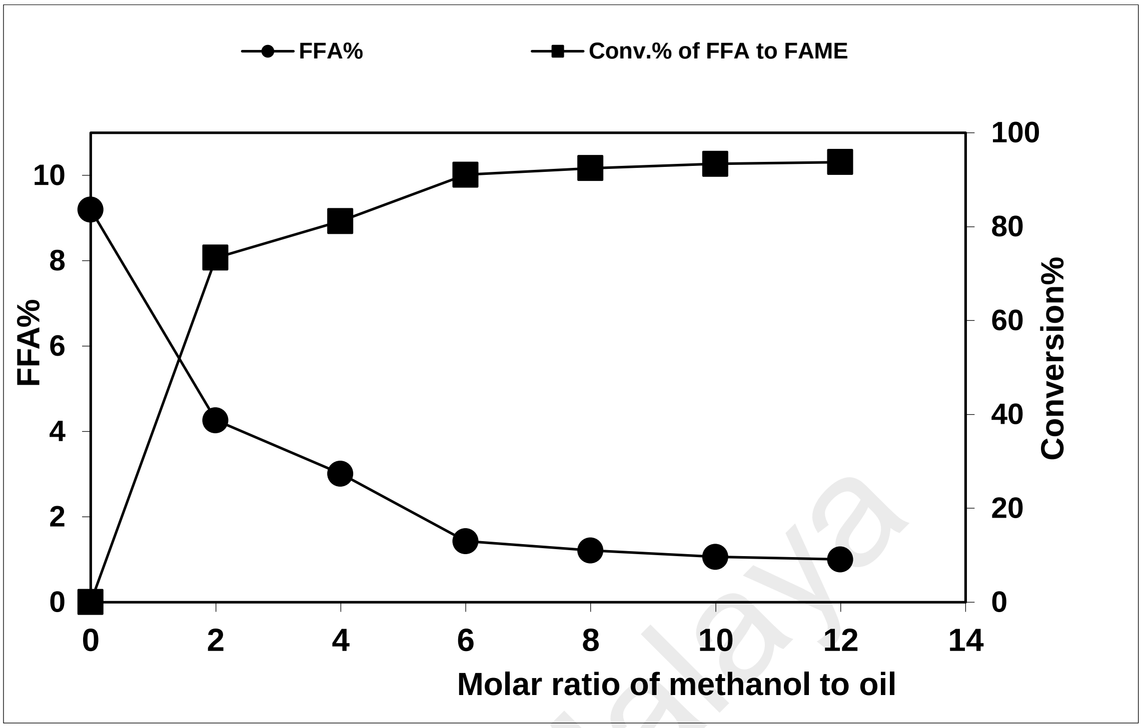


Figure 4.2: Effect of molar ratio on the yield of treated LGPO and the correspondence reduction of FFA content at 30 min reaction time, 60 °C, 1.5% catalyst dosage and 350 rpm.

4.4.2 Effect of reaction time and reaction temperature

As shown in the Figure 4.3, the reduction of FFA to targeted limit was achieved even only with 10 min reaction time. It revealed that BAC based DES possessed strong catalytic activity and was able to react with reactant in short period during esterification. The aim of this study is to investigate the optimum conditions that work best in esterification of LGPO using BAC-DES catalyst. Although prolong the reaction time could lower the FFA content from 9.2% to 0.39%, 30 min was deemed as the optimum reaction time since it reduced the FFA content to 1.61%. A short period of time is preferred to save operating cost of biodiesel production (Lamba et al., 2017). By using PTSA alone in esterification, it needs longer reaction time up to 60 min in order to reduce the FFA content to 2% (Hayyan et al., 2010a).

From the Figure 4.4, reaction temperature of 70 °C showed the highest reduction of the FFA content which from 9.2% to 1%. However, 60 °C was chosen as the optimum reaction temperature to reduce energy consumption and for economic feasibility. At that temperature, it reduced FFA up to 1.3% which lower than industrial limit (Hayyan et al., 2013a). The reaction temperature selected for this study is below the boiling point of the methanol; 64.7°C. As can be seen from Figure 4.4, the FFA reduction was increased at 80°C and reduced the conversion of FAME to 86.25%.Too high reaction temperature may cause the reaction yield to decrease. The amount of loss is believe caused by vaporization of methanol during reaction (Cho et al., 2012).

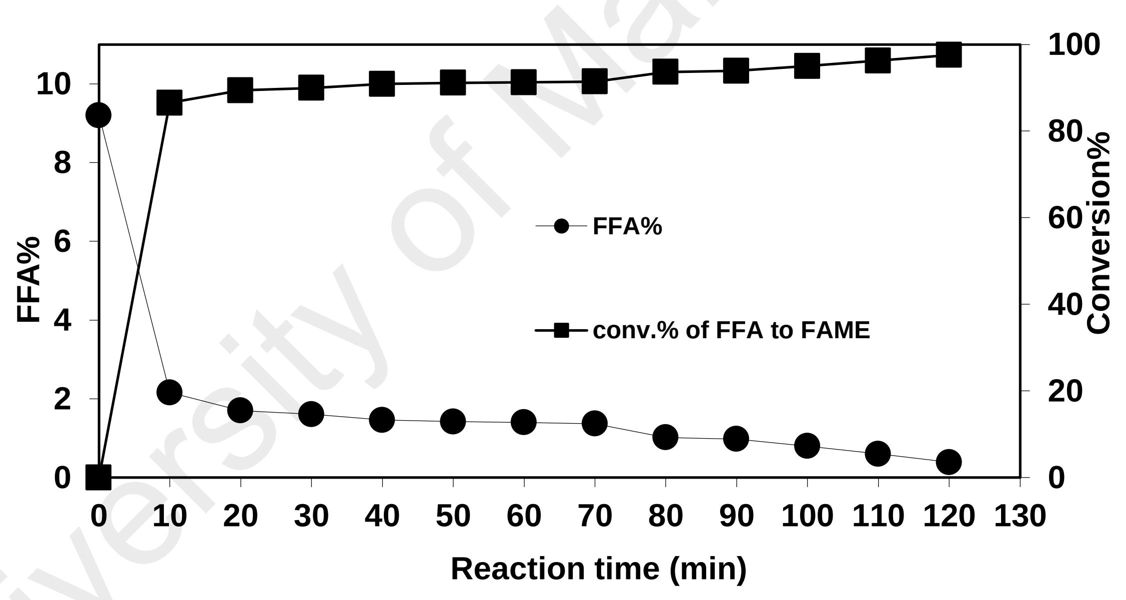


Figure 4.3: Effect of reaction time on the yield of treated LGCPO and the correspondence reduction of FFA content at10:1 molar ratio, 60 °C, 1.5% catalyst dosage and 350 rpm

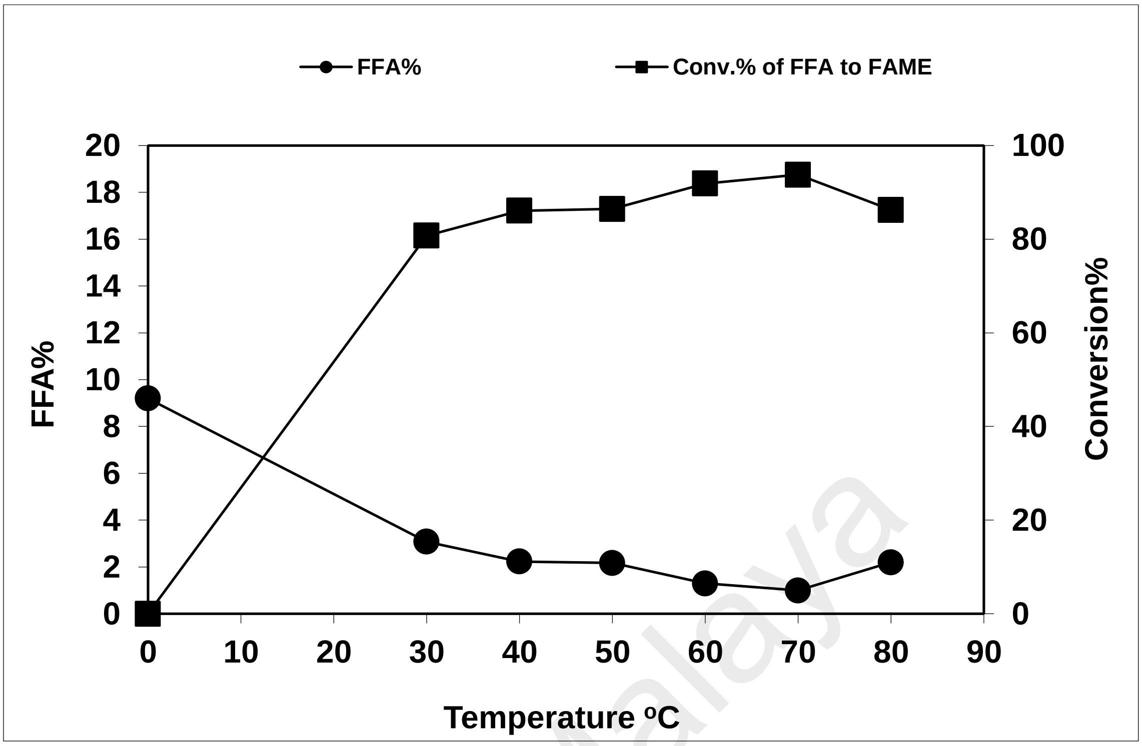


Figure 4.4: Effect of reaction temperature on the yield of treated LGCPO and the correspondence reduction of FFA content at10:1 molar ratio, 30 min reaction time, 1.5% catalyst dosage and 350 rpm

4.4.3 Validity of optimized condition and recyclability

In the present study, 2% of catalyst dosage, 10:1 molar ratio of methanol to oil, 30 min of reaction time, reaction temperature of 60 °C, and 350 rpm was selected as the optimum operating condition for esterification of LGPO, using BAC-based DES catalyst. This is in agreement with the previous study, where infusing catalyst into DES retains the former strong catalytic activity of DES. Study of PTSA alone as catalyst in the esterification of palm oil also gave almost similar optimum operating condition (Hayyan et al., 2010a). However, as mention above, the addition of DES as a catalyst in the esterification demonstrated shorter reaction time compared to its former catalyst from 60 min to 30 min. The optimum condition was able to reduce the FFA content from 9.2% to 1.23%. The 6 consecutive run was conducted without adding fresh catalyst. According to Figure 4.5, the percent of reduction of FFA decrease to 50% in the 2nd run, and constantly showed 50% reduction of FFA.

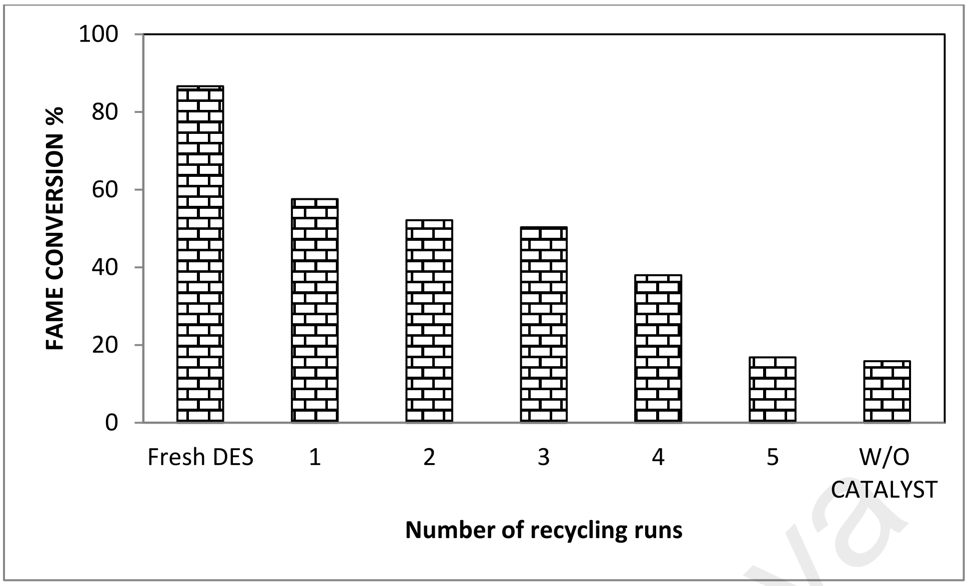


Figure 4.5: Recyclability study using BAC-DES catalyst in optimum reaction condition

4.5 Conclusion

Thus, it can be concluded that application of DES in the biodiesel production may give significant improvement in pre-treatment process. It proved to have high catalytic activity as of homogeneous acid catalyst and also considered cheap compared to ionic liquids. This study exhibited that 2 % catalyst dosage, 10:1 molar ratio of methanol to oil, 30 min of reaction time and reaction temperature of 60 °C was the optimum condition as it gives high conversion and yield of 90 %. BAC-based DES catalyst is highly potential to be commercialized and applied in industrial scale due to its high catalytic activity.

CHAPTER 5: DEEP EUTECTIC SOLVENTS: AN ACTIVATOR FOR LIPASES IN HYDROLYSIS

(Submitted to ACS Sustainable Chemistry & Engineering IF 6.1)

5.1 Introduction

This study introduces deep eutectic solvents (DESs) as a new media for enzymatic hydrolysis. The DESs were synthesized at different specific molar ratios from aqueous glycerol as the hydrogen bond donor (HBD) and salts such as methyltriphenylphosphonium bromide (MTPB) and choline chloride (ChCl). The eutectic point of the ChCl-based DES (ratio of 1:3) and MTPB-based DES (ratio of 1:5) is 213.4 K and 255.8 K, respectively. The aim of this study is to introduce DES as a novel and efficient solvent for enzymatic hydrolysis. The enzymatic activity of lipase from porcine pancreas (PPL) and *Rhizopus niveus* lipases RNL was also investigated, where both lipases were stable at all DES ratios. Overall, PPL showed higher activity compared to the RNL in the phosphonium-based DES. To the best of our knowledge, this is the first attempt using aqueous glycerol to prepare DESs as a medium for enzymatic hydrolysis.

5.2 Literature Review

The development of green and sustainable eco-efficient processes is a rising demand due to environmental concerns. Recently, new developments on novel DESs have captivated the research and industrial community. Since its discovery on 2003 by the Abbott's group, there have been numerous studies on DESs and its applications in different fields such as pharmaceutical, manufacturing and chemical industries (AlOmar *et al*., 2016), for example in the synthesis of zeolite analogs (Cooper *et al*., 2004), functionalization of graphene (Hayyan et al., 2015c) and separation of aromatics from aliphatic mixtures (Hizaddin et al., 2015).

The most popular class of the DESs is derived from choline chloride and glycerol mixtures. This is due to the cost-effectiveness and availability of the precursor compounds. Thus research has been focused on the preparation, physical properties and applications of DESs as a medium for different types of reactions. For example, the choline chloride-based DESs are superior alternatives than conventional solvents for the extraction of carrageenan (Das et al., 2016). Furthermore, choline chloride-based DESs have been used as the solvent for the electrodeposition of zinc at copper electrode (Abbott et al., 2011a).

On the other hand, glycerol has been used in many industrial applications, especially as freezing point suppressants and viscosity modifiers in food and pharmaceutical applications, due to their nontoxic nature, biodegradablity and non-flammable character (Abbott et al., 2011b). The use of glycerol as the hydrogen bond donor in the synthesis of most DESs is due to the availability of glycerol as a chemical feedstock in the industry. The main problem associated with glycerol is the high viscosity and poor solubility, which makes it unsuitable for a wide range of applications in the industry. To solve these problems, aqueous solutions of glycerol is used as the solvent and the hydrogen bond donor instead of pure glycerol. This is supported by different reports which stated that water mixtures of DES instead of neat DESs could provide substantial and improved physicochemical properties for many types of industrial applications (Yadav et al., 2014). Thus, an aqueous mixture of glycerol (85% of glycerol and 15% of water) was selected as the hydrogen bond donor in this study. The use of aqueous glycerol implies an inherent presence of water in the preparation of the DESs. From previous work, the presence of water alleviates the high viscosities of DES. Moreover, the presence of water does not affect the vapor pressure of the system; in fact, the vapor pressure decreases according to the Duhring lines. A composition of 80% glycerol-20% water prepared at 343K gave a vapor pressure of 229.59 Mm which is higher than the 80% glycerol-20% water-salt mixture that gave a value of 209.31 Mm (Carr et al., 1925). A less viscous medium allows a quick and facile preparation of the solvent and in the enzymatic context, it enhances the dissolution of substrates with different polarities (AlOmar *et al*., 2016) Furthermore, glycerol is a biodegradable, non-toxic and recyclable alcoholic polyol compound.

DESs are easily prepared by mixing two or three components of salts such as ammonium or phosphonium salts with a hydrogen bond donor (HBD) such as glycerol, ethylene glycol or urea (Hayyan et al., 2013e). The eutectic properties of the prepared DESs are exhibited by the stablity and strong hydrogen bonding between the ionic salts formed after the mixing. This gives a new physicochemical characteristic of the solution which possesses a lower melting point and freezing point compared to the precursor compounds (Juneidi *et al*., 2015). Compared to ionic liquids (ILs), DESs offer similar physicochemical characteristics which is useful in industrial applications such as tunable freezing point, viscosity, good thermal stability and enhanced solvation properties (Hayyan et al., 2013h). On the other hand, DESs offer other advantages especially from an economic viewpoint which makes them a widely used solvent than ILs, as the precursor materials are cheaper and have simple preparation (AlOmar *et al*., 2016). Moreover, the storage of DES requires low maintenance and no further purification steps. Thus, for large scale applications, DESs have brighter prospect than ILs, as the precursor materials and preparations of DES require lower production cost compared to ILs. In addition, DESs could be formulated for a specific application due to their tunable physicochemical properties which broaden the academic research and industrial application of DESs (Abbott et al., 2011a; Yadav et al., 2014). However, the toxicity of DESs is still uncertain and unpredictable, even though most of the HBD and salts are non-toxic, the newly synthesized DESs have shown different toxicological behavior compared to the precursor compounds (Hayyan et al., 2013h).

The toxicity of e DES is still unpredictable and unknown; hence more studies are required on the toxicology of DES. For commercial applications and industrial processes, a comprehensive knowledge of the physicochemical properties of DES is very essential for the industrial community and academic researchers. Thus it is important to establish the theoretical models to understand the relationship between the DESs and its applications. The physicochemical property is one of the main aspects to be understood in this regard, in addition to the design of the theoretical models of the reaction, based on DESs as the raw material or reaction media (Yadav *et al*., 2014).

DES has been introduced in both upstream and downstream processing. The ability to be tuned to the desired physicochemical property has motivated researchers to prepare DES as a reaction media for biocatalyst (Juneidi et al., 2017a). A chiral (1,2)-trans-2-methylstyrene oxide hydrolyzes into epoxide with concentrations until 1.5-fold in choline chloride: urea medium compared to phosphate buffer solution (Lindberg et al., 2010). This study paves a route towards higher product purity by steering the regioselectivity of the enzyme using DES solvents. However, lower efficiencies or the inhibition of enzymes were also reported when DES was used in enzymatic hydrolysis although it showed higher stability with celluloses (Wahlström et al., 2016). In this study, lipase enzymes from porcine pancreas (PPL) and from *Rhizopus Niveus* (RNL) were selected for further investigations. Lipases are environmentally benign catalyst used in the synthesis of commercial esters, in addition to alcoholysis, inter-esterification, esterification, hydrolysis, aminolysis and acidolysis reactions (Rajendran et al., 2009). Although it is widely used in different types of industries, the appropriate kinetic models and mechanisms of catalyzed reactions have yet to be reported especially in the hydrolysis reaction involving PPL in DESs medium. Thus it is important to provide a clear insight on the rate of product formation in DESs medium and its effect on the system conditions.

To date, no study has been performed on the characterization of physicochemical properties of aqueous glycerol-based DESs although there are few studies on the effect of DES on the stability and hydrolytic activity of lipase in these DESs. Therefore, this study investigates the overall physicochemical properties of aqueous glycerol-based DESs using MTPB and ChCl as the precursor salts with different molar ratios. The physicochemical properties include surface tension, conductivity, density, viscosity and pH as a function of temperature. The freezing point from DSC and FTIR analyses were determined for the aqueous glycerol-based DESs and are compared with the precursor salts. The hydrolytic activity of lipases in different molar ratios in aqueous glycerol-based DESs was also investigated to demonstrate its feasibility in enzymatic reactions. The enzymatic activity in the DESs was investigated of their compatibility with biological molecules (enzymes). The kinetic parameters of the selected lipases in DESs media were also investigated to understand its behavior in such media.

5.3 **Methodology**

5.3.1 Materials and Method

Methyltriphenyl phosphonium bromide (MTPB), glycerol 85% (GLY85) Choline Chloride (ChCl), Lipase from porcine pancreas (PPL) and from Rhizopus niveus (RNL) were purchased from Sigma Aldrich with a purity of 99.9%.

5.3.2 DES Preparation

The DESs were prepared according to the molar ratios in Table 5.1. The MTPB and ChCl salts were kept in a vacuum dryer at 353.15 K for 24 hours before mixing due to their hygroscopic nature. The salts and GLY85 were weighed accurately in a 250 ml conical flask according to the molar ratios of the salt to HBD. The DESs were formed by heating at 353.15 K at 350 rpm for at least 1.5 h until the eutectic mixtures are homogeneous and clear without precipitation. All samples were prepared at atmospheric pressure under controlled moisture environment. After the synthesis, all samples were kept in sealed vials in the vacuum dryer for future use.

Table 5.1: Abbreviations and compositions of prepared DESs

Salt	HBD	Molar Ratio	Abbreviation	Appearance at room temperature
ChCl	Gly 85%	1:1	ChCl-DES 1	Colorless Liquid
		1:2	ChCl-DES 2	
		1:3	ChCl-DES 3	
		1:4	ChCl-DES 4	
MTPB		1:3	MTPB-DES 3	
		1:4	MTPB-DES 4	
		1:5	MTPB-DES 5	
		1:6	MTPB-DES 6	
		1:7	MTPB-DES 7	

5.3.3 Analysis

The samples were kept sealed for further analysis to prevent structural changes and moisture imbalance that may affect the DES properties. In this study, the physical properties were investigated in a temperature range of 298.15 K – 353.15 K.

5.3.4 Surface Tension

The surface tension was determined by Surface Tension meter 21 (Fisher Scientific, with AT100 QPS 100VA Transformer). About 1/8 height of ring was soaked into a 40-ml of each sample. The data were recorded at a temperatures range from 298.15 – 363.15 K.

5.3.5 Conductivity

The conductivity was measured using conductivity meter (Mettler TOLEDO), with 631d Water Bath Circulating Controller. At first, 40 ml of the sample was poured into a 100 ml jacketed vessel. The temperature of the circulating water bath was set from 298.15 – 363.15 K. The conductivity of the samples were recorded by a conductivity meter and probe.

5.3.6 Viscosities and Density

The viscosity was measured using the spindle of Brookfield Viscometer (V6.5 LV External Mode) where the temperature probe was attached at the bottom of the vessel. Triplicate values of viscosity were recorded at a temperature range from 298.15 – 353.15 K. The density was determined using a density meter (DM40 Mettler TOLEDO) in the range of 298.15 – 363.15 K for 1 h. A 6 ml sample was injected into the density meter using a 10 ml syringe. The sample was drained from the meter before washing the system with toluene and acetone.

5.3.7 Freezing Points

The freezing points were determined using Differential Scanning Calorimetry (DSC), (STARe System Mettler TOLEDO V9.10). The sample was weighed about less than 10 mg and sealed in a small aluminum crucible with a lid, using nitrogen gas with a heating rate of 1 K/minute and – a temperature range of 293.15 to 203.15 K.

5.3.8 Stability of lipase from porcine pancreas and Rhizopus niveus in DESs

In this section, both PPL and RNL were used in the hydrolysis of pNPP to pNP, in the understudied DESs. The required weight of the enzyme was dissolved in 0.05M phosphate buffer solution (pH 7.0) to yield a final concentration of 1.5 mg/ml. The prepared enzymes were then incubated at 313 K, 350 rpm for 30 min using a thermomixer (Eppendorf Comfort) followed by a pNPP assay at 313 K for 15 minutes. The reaction was halted using 300 μL acetone and ethanol mixture at a volume ratio of 1:1. The absorption spectra of pNP were determined using UV spectrophotometry at a wavelength of 410 nm. The effect of DESs-MTPB 4 and ChCl 4 was also examined in various concentrations (10, 20, 40, 60, 80 to 100%). The enzymatic activity was expresssed in U.mg^{-1} and the residual activity was calculated based on the control reading. The significance of ChCl-DES and MTPB-DES were analyzed using one-way ANOVA, Tukey's test at 95% confidence in Minitab v. 17.1.0, after selecting the closest fitting DESs for lipases activity.

5.3.9 Kinetic Parameters

The Michaelis constant (Km) and the maximum rate (Vmax) were determined by the addition of PPL into ChCl-DES 4 and MTPB-DES 4 (after the selection of the most active lipase with the most stabilizing DESs) with different substrate concentration (0.0189205 to 0.0756819 mM) for 5 min under optimum pH and temperature. The reaction rate of PPL was measured, while the Km and Vmax were calculated from a Lineweaver-Burk plot accordingly. The parameter kcat is a constant that describes the turnover rate of an enzyme-substrate complex to the product and enzyme. It is also the rate of reaction of the catalyst with a particular substrate. The ratio kcat/Km measures the catalytic efficiency of the enzyme. The turnover number (kcat) and the catalytic efficiency (kcat/Km) can be determined when the total amount of enzyme used is known.

5.4 Result and Discussion

The physicochemical properties of a solvent are crucial factors which determine its applications. The physicochemical characteristics of DES differ from one another depending on the type of HBD and HBA precursors used in their preparations. For this reason, we compared two types of DESs: ChCl-glycerol and phosphonium-based DESs, for their physicochemical properties and their ability to stabilize the lipases in the hydrolytic reaction.

5.4.1 Surface Tension

The surface tension of the sample provides important information related to the molecular interactions in the mixtures (Shahbaz et al., 2012). In this study, the surface tension of the eutectic solvent and HBD were measured at a temperature range of 298.15 – 363.15 K. Based on Fig 5.1 and Table 5.2, it can be observed that all DESs have lower surface tension than the precursor compound GLY 85, at room temperature. At room temperature, all ChCl-based DESs had higher surface tension values compared to the MTPB based-DESs. However, at higher temperatures, there is further decrease in the surface tension values of the ChCl-based DES compared to the MTPB-based DES. This implies that the MTPB-based DES is more stable and has higher surface energy at a higher temperature. This is attributed to the changes in the molecular interactions at higher temperatures. In other words, when the temperature increases, the kinetic energy of the molecules also increases which decreases the intermolecular attractions (Widom, 1967). Eventually, the cohesive force of surface tension also decreases. Our results are similar to the previous results where the value of ChCl+ glycerol (ratio 0.5: 1.5) was 57.24 mN m^{-1} at room temperature, even though a lower concentration of aqueous glycerol (85% compared to 99% glycerol) and a different equipment model was used to gather the experimental data (Shahbaz *et al*., 2012). This proved the consistency and the reproducibility of the results. The experimental data obtained in this study is almost similar and consistent compared to the surface tension of 80% glycerol which was 67 mN m^{-1} at 25 °C (Takamura et al., 2012).

Table 5.2: Physical properties of selected DESs measured at 25 °C

	DES Code	Viscosity (relative) (3–5) percent of measured value (180 Shear Stress (mPa.s)	Density (±0.0001 g cm^{-3})	Surface tension (±0.1 mN m^{-1})	Electrical conductivity (± 0.01 µS. cm^{-1})
	GLY 85%	101.00	1.2195	62.9	0.000078
1	ChCl-DES 1	159.67	1.1553	68.0	4.11
2	ChCl-DES	94.63	1.1711	64.2	5.05

	2				
3	ChCl-DES 3	88.67	1.1812	64.0	4.20
4	ChCl-DES 4	82.00	1.1826	62.0	4.28
5	MTPB-DES 3	589.33	1.2808	42.7	0.599
6	MTPB-DES 4	332.00	1.2708	49.5	0.634
7	MTPB-DES 5	271.67	1.2639	53.2	0.787
8	MTPB-DES 6	252.31	1.2496	55.7	0.814
9	MTPB-DES 7	236.14	1.2457	58.4	0.856

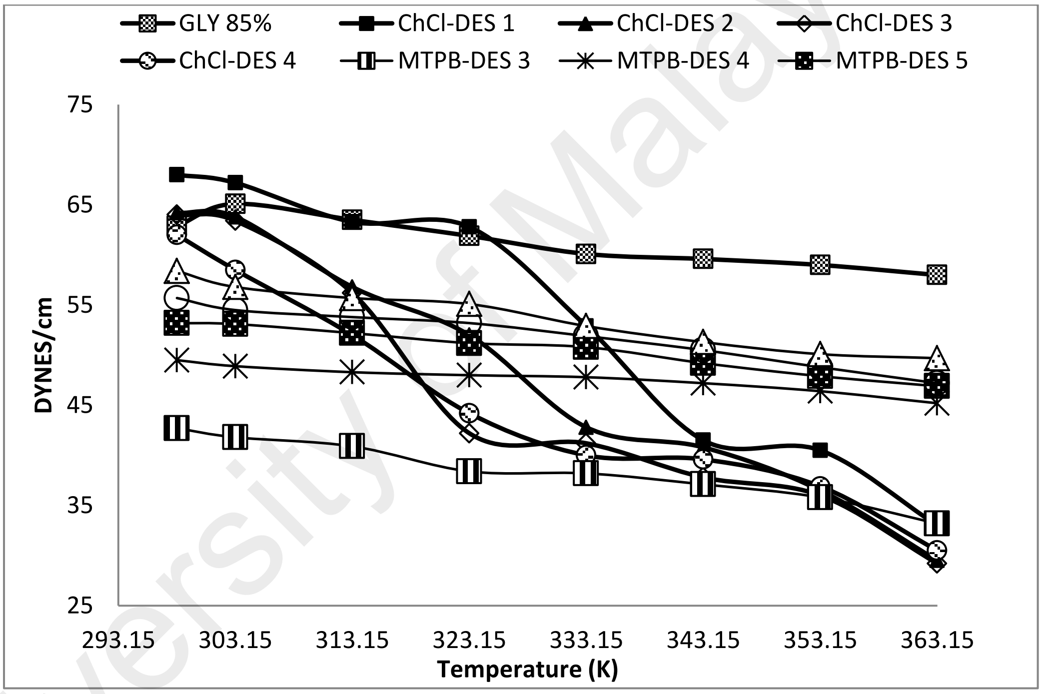


Figure 5.1: Surface tension of DESs as a function of temperature

5.4.2 Conductivity

The electrical conductivity of DES is an important parameter especially when it is used in industrial applications. The conductivity values are an indication of the ionic or electron transport ability of a material (Bagh et al., 2013). Thus it provides useful information on the ability of the DESs to conduct electricity. The conductive behavior of the ChCl-based DESs is higher than the MTPB-based DESs. From the Fig 5.2, it shows that there is an exponential increase in the conductivity at higher temperatures of the prepared DESs, where ChCl-DES 2 showed the highest electrical conductivity. This is due to the increase of charge carriers in the solution as ChCl-DES 2 has higher salt concentrations (Bagh et al., 2013). The conductivity of ChCl-based DESs decreases in the order of ChCl-DES 2 > ChCl-DES 4 > ChCl-DES 3 > ChCl-DES 1 and followed by MTPB-based DESs of MTPB-DES 7 and closely followed by MTPB-DES 6 > MTPB-DES 5 > MTPB-DES 4 > MTPB-DES 3> GLY85. The results in Fig 5.2 indicate that the GLY85 has the lowest conductivity at room temperature (0.000078 mScm^{-1}) and 0.000211 mScm^{-1} at 353.15 K. All the prepared DESs showed similar trends in the conductivity measurements (Fig. 5.2).

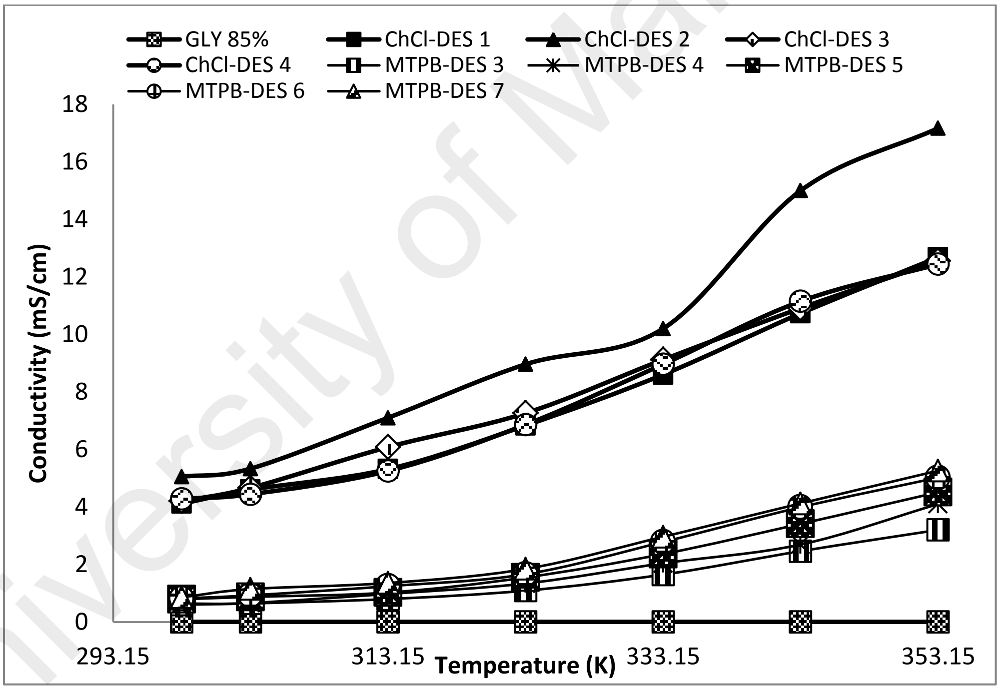


Figure 5.2: Variation of the conductivity of MTPB, ChCl based DES, and HBD at different temperatures

5.4.3 Density

Density is an important physical parameter which describes the condition of an operation, mass transfer and chemical design processes in the industry. The change of density of DES and HBD with temperature were recorded in the range of 298.15-363.15 K. Figure 5.3 shows that the density ranges of HBD and GLY85 are between the

MTPB-DES and ChCl-DES, i.e. 1.2195 g.cm^{-3} to 1.1768 g.cm^{-3} from room temperature to 363.25 K, respectively. The upper limit of density decreases in the reverse order of the molar ratio from MTPB-DES3 to MTPB-DES 7. The density values at room temperature from MTPB-DES3 to MTPB-DES 7 are 1.2808, 1.2708, 1.2639, 1.2496 and 1.2457 g.cm^{-3}, respectively. However, the density values of ChCl-DES are in parallel order, with values of 1.1553, 1.1711, 1.1812 and 1.1826 g.cm^{-3}, from ChCl-DES 1 to ChCl-DES 4, respectively, at room temperature.

Generally, the density decreases as the temperature increases. This is due to the weakening of the attraction forces between the liquid molecules. When the temperature increases the molecules are released to occupy larger volumes of space, hence the density decreases (Syaima et al., 2015). Furthermore, the density values of ChCl-DES 1 and HBD of GLY85 are close to the density of ChCl: HBD (molar ratio 1:1) and pure glycerol, as reported by Al-Omar (AlOmar et al., 2016).

The densities of aqueous mixtures of different types of DESs have been measured by other groups. For instance, Yadav et al. (2014) recently reported the densities and viscosities of an aqueous glycerol based DES composed of choline chloride and glycerol (molar ratio 1:2). However, their results are different from our data as they used a hydrogen bond donor to prepare the DES. Nevertheless, they reported a similar trend of the decrease in the DES density at elevated temperatures (Yadav et al., 2014). Therefore, it can be concluded that most DES densities are dependent on the temperature.

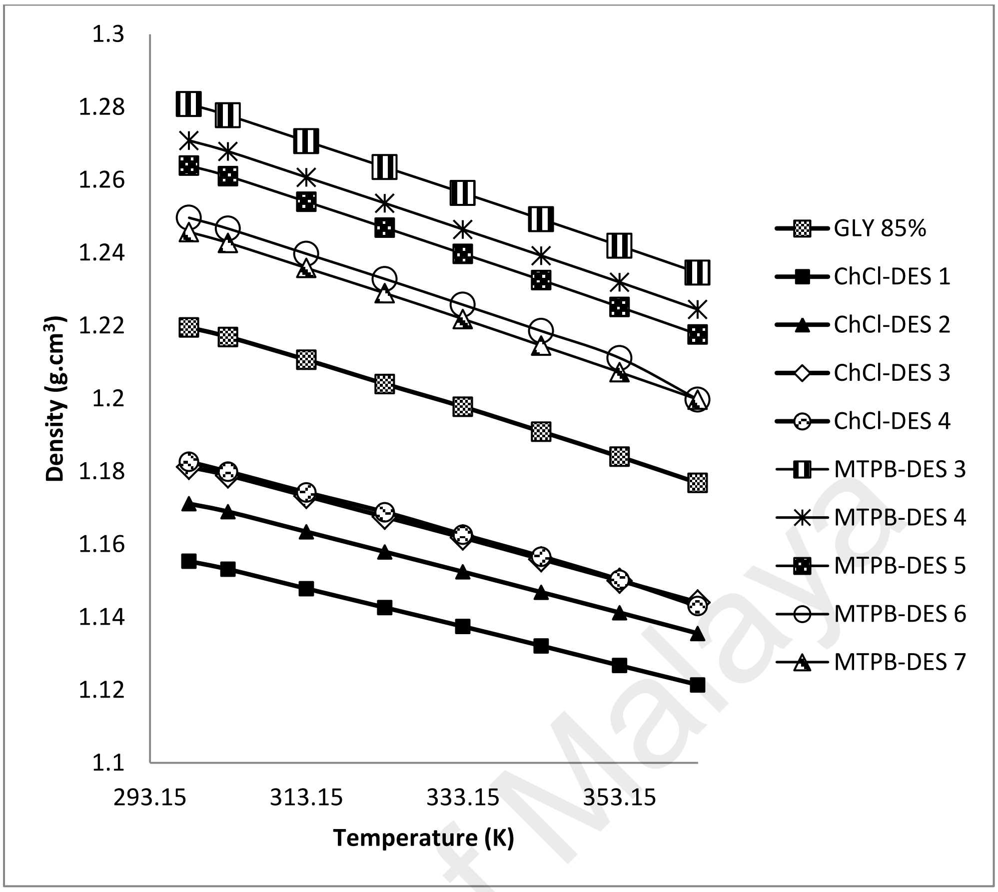


Figure 5.3: Variation of DESs density with temperature

5.4.4 Viscosity

The viscosity is an important parameter which must be reported, as it is the main factor in the design of equipment and fluid flow calculations in a chemical process (AlOmar et al., 2016). The high viscosity of DESs is unfavorable for certain industrial applications and chemical reactions such as liquid-liquid extraction (Hayyan et al., 2013e).

In this work, the temperature range for the measurement of viscosity is between 298.15 and 353.15 K, as presented in Fig. 5.4. At higher temperature, both salt based DESs and GLY85 are easily liquified and are less than 100 mPa.s. In this study, the ChCl-DES 4 had the lowest viscosity at room temperature (82 mPa.s) at a shear stress of 180 cP, while the MTPB-DES 3 had the highest viscosity followed by MTPB DES 4 to7. The viscosity of ChCl-DES 1 to 4 are 90.97, 84.37, and 76.77 mPa.s, respectively, which are close to the viscosity of GLY85(101 mPa.s). It is understood that the mobility of free species in the DESs decreases with the abundance of hydrogen bonding between the MTPB and GLY 85 in MTPB-DES3 (ratio 1:3) (AlOmar et al., 2016)

Even though Yadav's group have reported the viscosity profile of a glycerol based DES as HBD, their results cannot be compared to the current study, as the viscosity was measured as a function of mole fraction of water instead of temperature (Yadav et al., 2014).

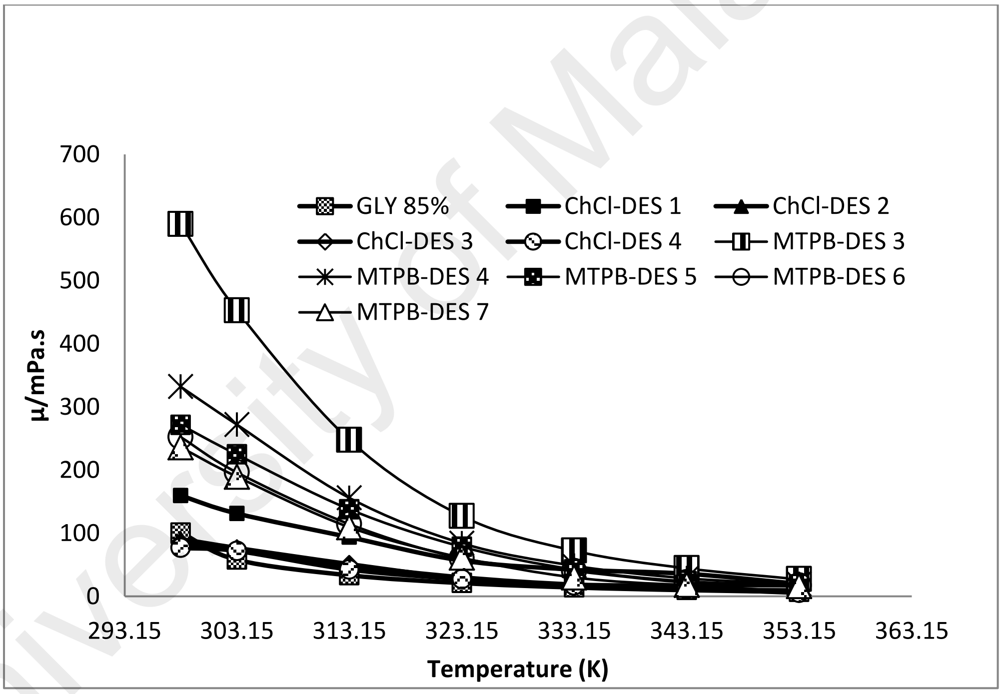


Figure 5.4: Variation of viscosity of DESs with temperature

5.4.5 Freezing point

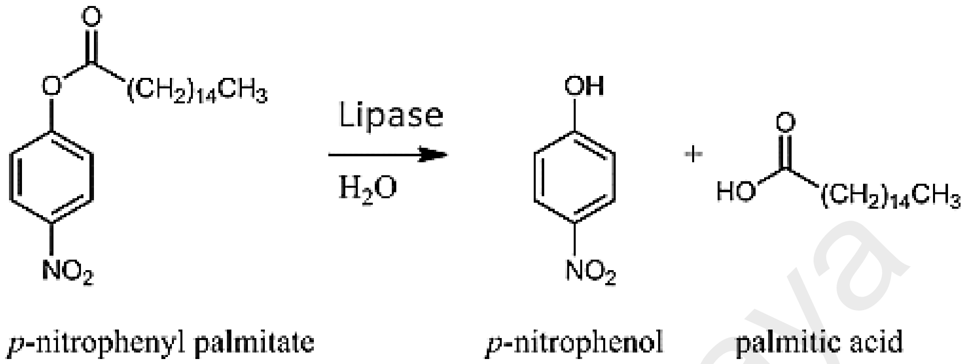
Generally, low freezing point solvents are more favorable in various fields as it offers lower reaction temperature and reaction time in the design of a chemical process. In this study, the freezing point of ChCl based DESs increases in the order of ChCl-DES 3 > ChCl-DES 1 > ChCl-DES 4 > ChCl-DES 2> GLY85. Compared to the ChCl-based DES, the DES-MTPB possesses a higher range of freezing points which decreases in the order of MTPB-DES 5 > MTPB-DES 7 > MTPB-DES 4 > MTPB-DES 6 > MTPB-DES 3 as presented in Table 5.3. It was found that the ChCl-DES 2 with a molar ratio of 1:2 of salt/HBD has close agreement with a previous study by AlOmar (2016). On the other hand, the eutectic point of MTPB-DES is at a ratio of 1:5 (MTPB-DES 5). The eutectic freezing point of ChCl-DES 3 with a molar ratio of 1:3 (ChCl: GLY85) had the lowest freezing point compared to the other mixtures. This suggests the suitability of ChCl-DES 3 as an alternative solvent in industrial applications.

Table 5.3: Freezing Point of DESs based on DSC analysis

Entry		Peak (K)
Components	GLY85%	263
	ChCl	575
	MTPB	504
ChCl-DES	ChCl-DES 1	225.11
	ChCl-DES 2	240.14
	ChCl-DES 3	213.4
	ChCl-DES 4	233.63
MTPB-DES	MTPB -DES 4	258.84
	MTPB -DES 5	255.83
	MTPB -DES 6	259.27
	MTPB -DES 7	257.87

5.4.6 Stability of Porcine Pancreas and Rhizopus niveus lipases in DESs

The hydrolysis of pNPP was selected as a standard reaction to demonstrate the effect of MTPB and ChCl-based DESs on the lipase activity (Scheme 1).



Scheme 1. The general scheme of *p*NPP hydrolysis by lipase.

It can be observed from Fig. 5.5, that both lipases [Porcine pancreas lipase (PPL) and *Rhizopus niveus* lipase (RNL)] are stable in the ChCl-DES. These results confirm the influence of the DES ratio on the lipase activity, as it stimulates the activity of PPL compared to the control buffer solution.

The activity of PPL increases in the following order: ChCl-DES 4(7.2 fold)> ChCl-DES 1(5.5 fold) > ChCl-DES 3(4.9 fold) > ChCl-DES 2 (4.3 fold). While for the RNL, the activity increases in the following order: ChCl-DES 2 (2.6 fold) > ChCl-DES 1 (2.1 fold) > ChCl-DES 3 (1.7 fold) > ChCl-DES 4 (1.6 fold). The PPL activity increased until seven times compared to RNL (two time increase) in the ChCl-based DES.

From the analysis, it is shown that the lipase activity is greatly enhanced in the ChCl-DES 4, thus this medium was selected for further analysis. The results in Fig. 5.6 shows a lower lipase activity in the MTPB-DES, compared to the ChCl-DES. However, similar to Fig. 5.5, the lipase activity especially PPL is greatly enhanced in MTPB at the same molar ratio of ChCl-DES 4 (1:4) compared to the control (buffer solution). While the RNL shows a slightly higher lipase activity in MTPB- DES 4 compared to the control. Therefore, the MTPB- DES 4 was selected for further analysis.

These results show a similar trend with the line graph in the physical properties result in Fig.5.3 and 5.4. The ChCl-DES with lower values of viscosity and density, as well as higher values of conductivity provides a higher stability of the lipase, compared to the MTPB-DES. It was also reported by many researchers that higher viscosity and density of ILs and DESs inhibit the enzyme activity and protein stability.

As seen from Fig. 5.7, the presence of the buffer in the DESs resulted in a lower activity of the enzyme. In addition, the presence of excess aqueous buffer solution in the mixture deactivated the enzyme even further. This observation is supported by the lower activity in the control compared to the DESs. This observation is due to the water content of the mixture. The results showed that a certain amount of water is needed to enhance the hydrolysis. A lower DES concentration (in buffer) contributed to a lower activation of the PPL in the MTPB- DES 4, and only a slight enhancement in the ChCl-DES 4. It is suggested that the non-aqueous DES stimulated the enzyme’s activity. In this case, the mixing of both types of DESs with the buffer to produce an aqueous solution had a negative impact on the enzyme. Durand et al. showed that the presence of only 5 % water dramatically improved the lipase activity and change the selectivity of the proteases (Durand et al., 2013). This is in contrast with our findings, where very low conversions (<2%) were observed in the MTPB-DES 4 (100%), whereas quantitative conversions were achieved in the DES–water binary mixtures. In addition, the composition of MTPB based DES negatively affected the stability of the enzyme due to the charge delocalization with the presence of hydrogen bonding. This inhibits the lipase enzyme since charge delocalization easily destabilizes and denatures the enzyme compared to localized charges (Hayyan et al., 2013h).

This study shows that the PPL instead of the RNL, is highly activated in the ChCl-DES 4. Moreover, a mixture of 60% DES and 40% phosphate buffer gave the highest conversion of pNPP.

The ChCl based DES showed greater stabilization and activation effects on the lipase compared to the MTPB-based DES. This is in close agreement with several studies which demonstrated higher enzymatic activity and stability in the DES, such as *Candida antarctica lipase* B (CALB) and *C. antarctica lipase* A (CALA) (Gorke et al., 2008). Another study found that the ChCl: Glycerol (1:2) and choline acetate / glycerol (1:1.5) with lower viscosities are biocompatible with the immobilized CALB (Zhao et al., 2013). The choline-based DES is low cost, has lower toxicity and is biocompatible with lipase (Huang et al., 2014a). The same group also showed that DESs based on choline salts and glycerol have merits as green solvents in a lipase-catalyzed transesterification and biodiesel preparation (Huang et al., 2014a).

Huang et al. also used cholinium-salt-based DESs as the co-solvents with Penicillium expansum lipase (PEL) in concentrated aqueous solution of 2.0 mol L-1 (Huang et al., 2014a). The lipase showed high acidic stability in aqueous solutions of 10 % Gly-DES mixtures (Kim et al., 2016). They found that the increase in the water content from 2% to 20% in ChCl: Urea increases the residual lipase activity by 10 times (Durand et al., 2013). They proposed the formation of supramolecular complexes involving strong hydrogen bonding between the DES and water molecules (Huang et al., 2014a; Kim et al., 2016). Therefore, the introduction of water in a DES mixture could promote the following: (i) a lower viscosity and enhanced mass transport; (ii) a significant increase in the lipase activity and reaction rate; (iii) a higher stability of the enzymatic activity.

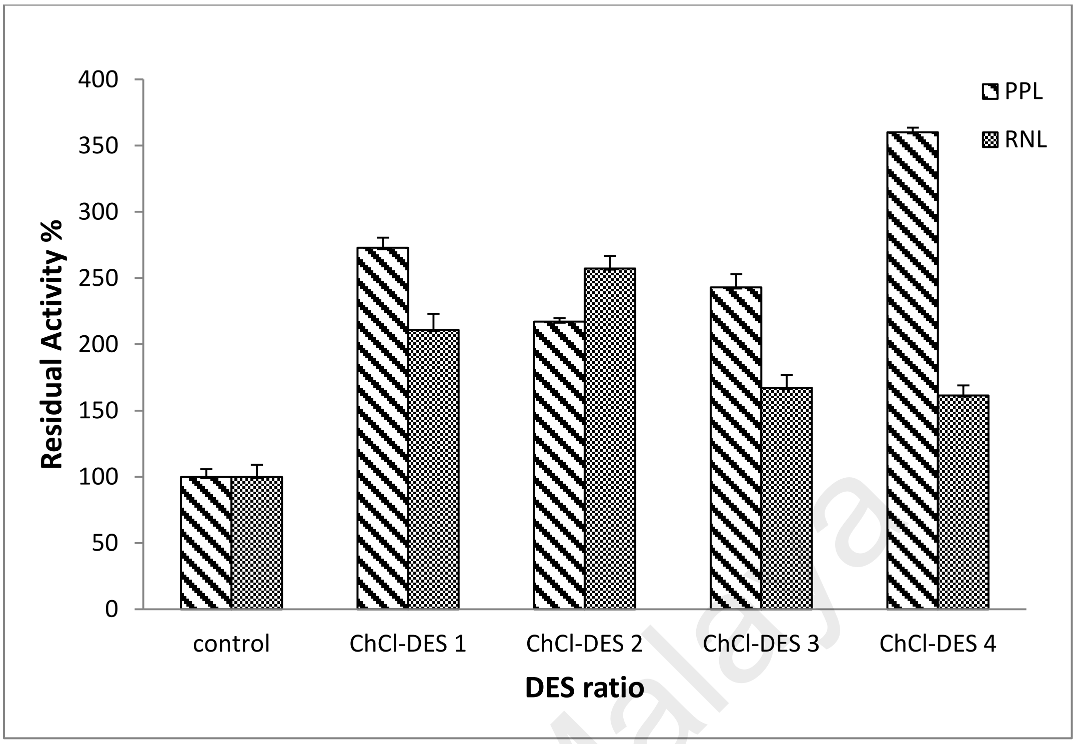


Figure 5.5: Activity of PPL and RNL in ChCl: GLY85 %

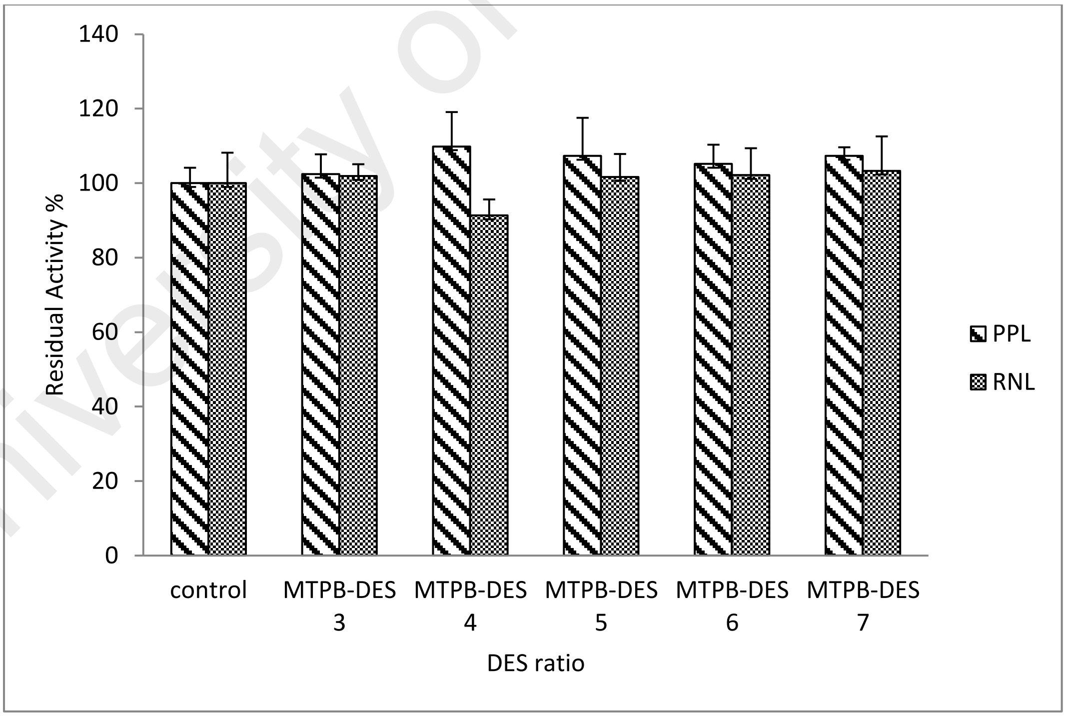


Figure 5.6: Activity of PPL and RNL in MTPB : GLY85 %

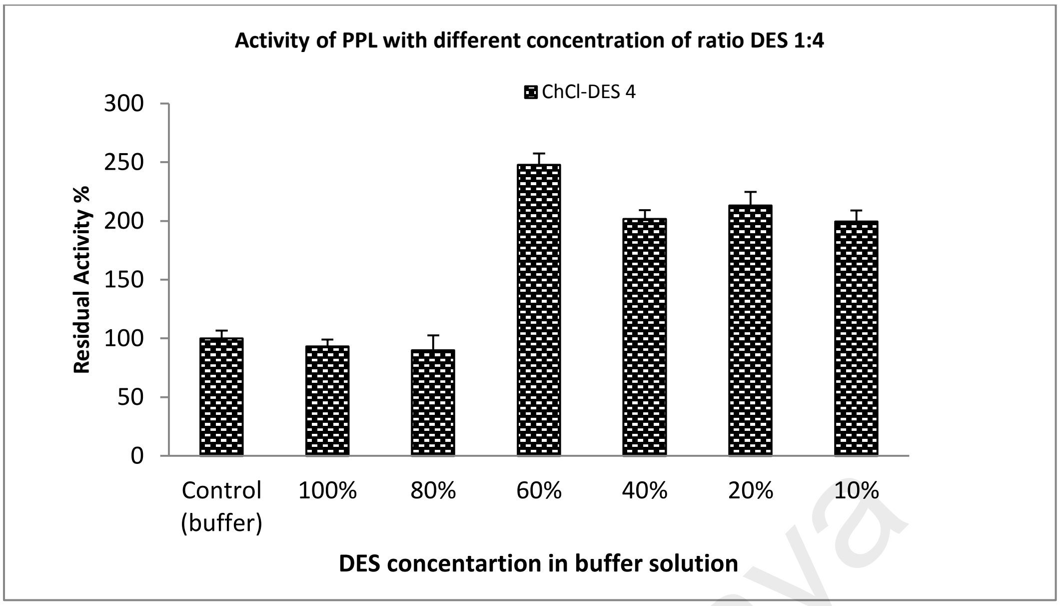


Figure 5.7: Activity of PPL with different concentrations of ratio DES 1:4. Mean values that share the same letter are not significantly different from each other (Tukey's Test)

5.4.7 Kinetic Study of MTPB-DES 4 AND ChCl-DES 4 on PPL

In order to obtain a satisfactory level of significance of the lipase stability in the DESs, the Tukey's test method was performed on the obtained data to categorize the activities based on the mean values. According to the Tukey's test method, the mean values that do not share a letter are significantly different, using a 95% confidence level. In other words, each concentration was categorized in a separate group (A, B, C) as presented in Fig. 5.7. As obtained from the One-Way ANOVA analysis, the results showed the significance of the DES concentration on the activity of the PPL, where it seems to stimulate the activity of the PPL compared to the control. All DES concentrations except for the 100% and 80% of ChCl-DES 4, showed a clear enhancement on the activity of PPL, therefore are placed in group [A and B], separately. From the analysis, it was shown that the lipase activity was mostly enhanced in 60% ChCl-DES 4 compared to the other concentrations. Thus the ChCl-DES 4 was placed in one group [A], thus this group was selected for the kinetic study. In this context, the mixing of DES with the buffer had negative effects on the enzymatic activity at a certain level. It can be observed that an excess of the buffer solution in the mixture resulted in a lower activation of the enzyme. It was reported that the lipase activity in ChCl; urea:-glycerol was 155% higher than in the buffer solution. In general, the glycerol-containing DES mixtures are very useful in enhancing the activity and stability of the lipase (Kim et al., 2016). However this result contradicts with another study by Juneidi et al where the ChCl: Ethylene glycol with 40 % (v/v) (DES ratio to buffer) hydrolyzed the pNPP in the presence of lipase, with an increase of 230% of its activity compared to the free DES system (Juneidi et al., 2017b). This could be due to the presence of different types of HBD, GLY 85 was used in this study compared to ethylene glycol in the other result.

The Lineweaver–Burk plot for the enzyme-catalyzed reaction follows the Michaelis–Menten model, which was used to evaluate the parameters from the experimental results. The kinetic studies showed that the variation of DES was directly influenced by the Michaelis–Menten parameters of the PPL (Table 5.4).

Table 5.4: Kinetic parameters of lipases in pNPP substrate solution using different reaction media

Medium	**MTPB-DES4**	**ChCl-DES 4**	**Phosphate Buffer pH 7.0**
K_m (mM)	0.076±0.01	0.0045±0.001	1.05±0.52
V_{max} (mM min^{-1})	0.046±0.01	0.0180±0.01	3.38±1.21
k_{cat} (min^{-1})	0.229±0.05	0.088±0.005	5.07±0.08
k_{cat}/K_m (min mM^{-1})	3.030±1.05	19.62±2.50	4.83±1.05

Through the Lineweaver-Burk plots, the kinetic parameters, Michaelis-Menten constant Km and the initial maximum reaction velocity Vmax were calculated. The MTPB-DES 4 shows a higher value of Km compared to the ChCl-DES 4, but a lower value of Vmax .The small value of Km represents a higher affinity to the substrate (pNPP). In other words, the ChCl-DES 4 shows better enhancement of the PPL compared to the MTPB-DES 4 and the control. The Vmax of ChCl-DES 4 was the largest among all DES. This indicates the suitability of the ChCl-based DES for the hydrolytic reaction, as it increases the reaction rate.

As reported by Juniedi et al. (2017), a lower Km value but a higher Vmax value is an indication of an enhanced enzymatic activity (Juneidi et al., 2017b). Furthermore, the Kcat/Km of the ChCl-DES 4 was 6 times higher (19.62 min mM^{-1}) compared to the MTPB-DES 4 and is around 4 times higher compared to the control. The highest turnover number (Kcat) was recorded for the control followed by ChCl-based DES.

The strong hydrogen bonding and ionic interaction between the ChCl ion and the HBD in aqueous GLY85 increases the turnover number of the reaction in a saturated substrate concentration of PPL. This is also supported by a higher conductivity profile of the ChCl-DES compared to the MTPB-DES as in Fig. 5.2.

Lindberg et al. (2010) reported that the turnover numbers are not affected by the DESs as the co-solvent, even when the Km increases with the concentration. However, these results cannot be generalized to other enzymes or reactions, as different types of lipases show different activities in different types of DESs. We assumed that the Km value refers to the competitive inhibition, where it might be possible that the DES blocks the active site of the enzyme and prevents more substrate molecules from binding. However, in the current study, the Km value was decreased in the presence of DES, especially in the ChCl-DES 4. This proves that there is no inhibition effect in the lipase-DES system, instead the reaction has been enhanced by the DES activation. The activation can be illustrated based on the allosteric activation of the enzyme (Fig. 5.8). We predict that the DES binds to a site on the enzyme (other than the active site) and causes conformational changes, which might lead to the activation of the lipase, when the lipase is hydrated by water molecules. However, simulation and modelling of such activation should be conducted to help confirm this theory.

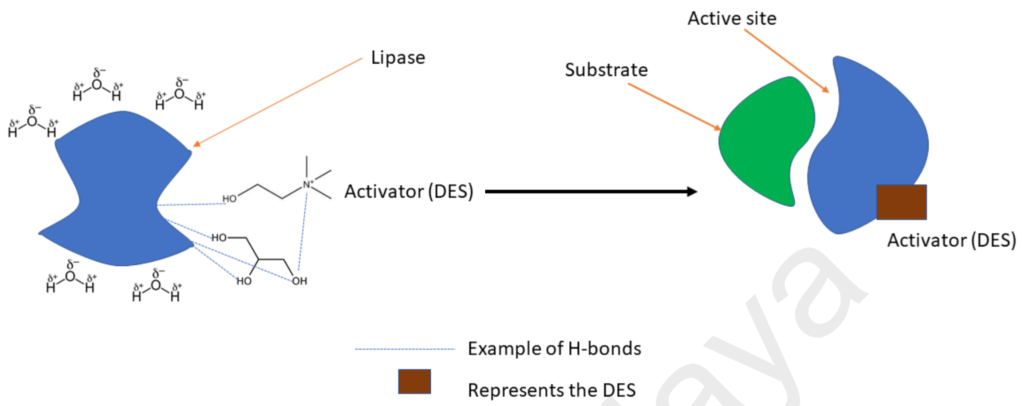


Figure 5.8: Predicted illustration of the activation of lipase by ChCl-Gly DES

The study revealed that the thermodynamic equilibrium of the hydrolysis reaction between the ChCl and MTPB DES is catalyzed by the PPL and is significantly affected by the solvent properties such as freezing point, viscosity and conductivity.

5.5 Conclusion

ChCl and MTPB-based DESs were prepared at molar ratios of 1:1 to 1:4 and 1:3 to 1:7, respectively. The physicochemical properties such as freezing point, density, conductivity, surface tension and viscosity were measured to ascertain the DES characteristics. The molar ratio between ChCl, MTPB (as salt) and GLY85 (as HBD) played an important role in the physicochemical behavior of the DES. The temperature also influenced the DESs properties as changes in the physicochemical properties were observed when the temperature was increased from 298K to 363K. Overall, these results proved that the DES, especially the ChCl-based DESs, could be a viable substitute for conventional solvents, as they possess enormous potential especially in electrochemical applications due to the higher ionic conductivities. They also demonstrated a stabilizing effect on the lipases, where the enzymatic activity was increased in the presence of DESs in aqueous solutions. MTPB-DES 3 showed the highest viscosity and density at room temperature, and this implies that MTPB based DES possess stronger intermolecular forces. These physicochemical features of DES are great advantages in the transesterification process in biodiesel synthesis.

CHAPTER 6: IDENTIFICATION OF LIPASES ACTIVITY TOWARDS DEEP EUTECTIC SOLVENT: A COMPARATIVE STUDY OF LIPASES

(Submitted to journal of molecular liquids IF 4.5)

6.1 Introduction

A new liquid-molten support system in lipase was evaluated in the study of the hydrolysis of lipases using a different type of deep eutectic solvents (DESs). DESs based lipases were prepared using five different synthesized DES namely (ChCl/glucose), (ChCl/polyethylene glycol/ethylene glycol), (alanine/glycerol) (ChCl/Glycerol 85%) and (MTPB/Glycerol 85%) for the hydrolysis of 4 different lipases from porcine pancreas (LPR), from Candida Rugosa (LCR), from Rhizopus Niveus (LRN) and Amano Lipase PS from Burkholderia Cepacia (LAB). One substrate was studied to compare the capability of alcoholic solvent (aqueous methanol) as a dummy prior to the application of lipase in an esterification reaction to synthesized biodiesel. One commercial lipase which lipase B candida Antartica immobilized on immobead whom recombinant from aspergillus oryzea (ICALB) was also used to study the comparison and effect of the commercial immobilized enzyme in hydrolysis. Result clearly showed that the activity of all free lipase was enhanced after immobilized with DESs except for Candida rugosa. ChCl/Glycerol 85% and MTPB/Glycerol 85% are the most promising support system for lipases as the relative activity was elevated to more than 100 % compare to its free lipase in the buffer. The optimum parameters of hydrolysis of ChCl/Glycerol 85% with LPR and LRN were 80% of concentration of DES, at pH 8 and of 48 hour of incubation time. The result shows that DESs are able to stabilize and activated free lipase.

6.2 **Literature Review**

Biochemical industries are in an exponential phase of development over the past few years. The scientific breakthrough of biochemistry and new technology-driven biocatalysts in biochemical industries attracted both market and researcher to explore this industry. Development on biocatalysts enhanced yields of product with lower impact on the environment. One of the breakthroughs in the biotechnology industry was on the enzymology. Enzymes are naturally existing protein constituents in living organisms that regulate and catalyze chemical routes. The history of the employment of enzyme in food processing during ancient civilizations was well documented. Breakthrough and rapid development in the field of biochemistry, enhanced techniques in fermentation and comprehensive purification processes have led to the various types of enzyme production. Only 200 enzymes are in commercialization among the 4000 known enzymes and mostly initiated from microbes (Bornscheuer, 2013).

Lipase enzyme has been used extensively in pharmaceutical industries and is one of the most promising catalysts for the enzymatic transesterification for production of biodiesel (Angajala et al., 2016; Fan et al., 2017). Lipase has great potential in bioprocesses application due to their stability and availability in aqueous as well as in organic media. (Sharma, & Kanwar, 2014). Lipases or scientific name of triacylglycerol acyl hydrolases, (EC. 3.1.1.3) belong to the family of serine hydrolases. This group of enzymes is primarily used for catalysis the formation of ester bonds and amide and performs enantioselective hydrolytic reactions (Angajala et al., 2016). As shown in Figure 5.1, naturally lipases catalyze the hydrolysis of ester bonds at the interface between the aqueous phases with the insoluble substrate phase where the enzyme maintains soluble. Under non-aqueous conditions, they catalyze the reverse reaction such as inter-esterification, esterification, and transesterification converting fatty acids and glycerol into glycerides. The interaction mechanism for the lipases lies in the interface where the helical oligopeptide unit lid shield the catalytic site within the structure of the protein will open in the presence of hydrophobic interface to activate the active site (Angajala et al., 2016; Babaki et al., 2015). The opened active site which composed a traid containing acyl-enzyme complexes, aspartate, serine, and histidine provide free access to the substrate.

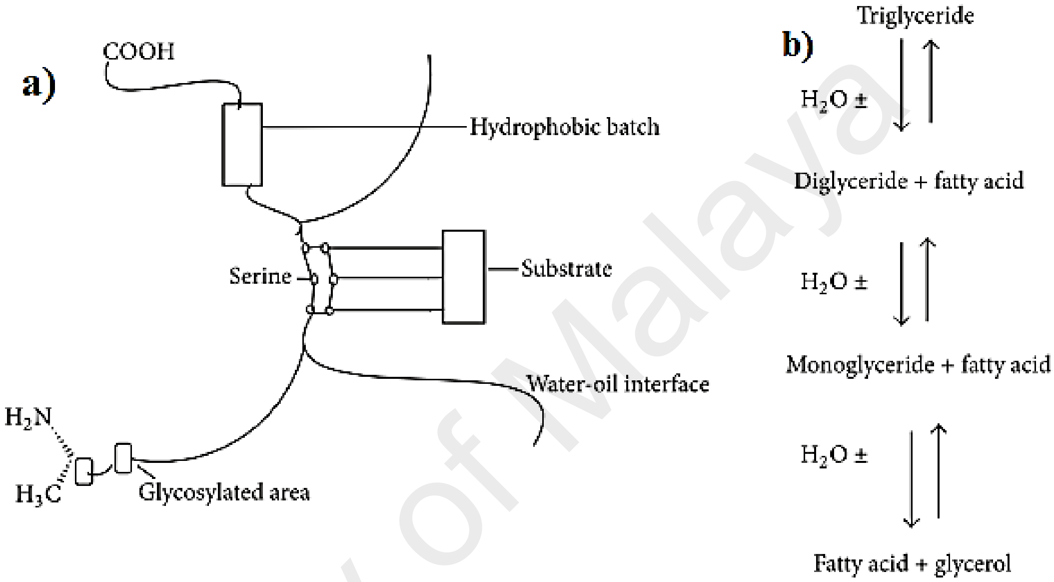


Figure 6.1: a) natural condition for lipases interaction b) Lipases reaction in non-aqueous condition (Sharma, & Kanwar, 2014)

In esterification and transesterification specifically, lipase enzyme holds many advantages if compared to chemical pathways. The enzymatic reaction does not generate any waste whereas alkaline catalysts generate fatty acid alkaline salts (soaps) which need to be washed with water resulting alkaline water that is polluting to the environment (Shimada et al., 2002). It is also environmentally friendly as it from natural sources and certainly non-toxic. However, lipases enzyme were not practical to be adapted to industrial scale due to its high cost compared to chemical and inorganic catalyst (Tacias-Pascacio et al., 2017). There have been many studies that address this issue, and many innovations were accomplished in order to make lipase enzyme more industrial-friendly. One of the major innovation addresses by the researcher was by immobilization of enzyme to endure the hurdle condition during the reaction and durable so it can be recycled many times as enzymes were expensive. Lipase B from Candida Antarctica (CALB) and lipase from Rhizomucor miehei (RML) were covalently immobilized onto epoxy-functionalized silica and were able to produce 94% after 11 cycles and 93% after 14 cycles of fatty acid methyl ester (FAME) respectively (Babaki et al., 2017). Also, Burkholderia cepacia lipase (BCL) immobilized with superparamagnetic multi-walled carbon nanotubes linking polyamidoamine (PAMAM) dendrimers were able to retain 90% of its original activity even after 20 repeated cycles (Fan et al., 2017). The magnetic property of BCL and reasonable cost of immobilization CALB and RML onto epoxy silica makes the production of biodiesel using enzymatic pathways feasible to be up-scale.

A new type of support system has been studied recently. Rather than used solid support system, the new eutectic solvent was introduced and applied in the enzymatic reaction. The main advantages of this new eutectic solvent are it produced from cheap materials and simple synthesis techniques. This unique solvent or commonly known as deep eutectic solvent (DES) have been applied in the various field of application and reaction. From the synthesis and extraction to the purification and separation process, many researchers successfully substitute the organic, volatile solvent with this benign, biodegradable and low-toxic solvent (Hayyan et al., 2012a). In biodiesel production, DES has been used in both chemical and enzymatic pathways. The hygroscopic p-toluenesulfonic acid monohydrate have been mixed with Choline chloride (ChCl) at 1:3 molar ratio and able to esterified acidic crude palm oil which then used as feedstock for production of biodiesel with a yield of 92 wt% FAME and 0.07% FFA content (Hayyan et al., 2014b). Pseudozyma Antarctica lipase B dissolved in ChCl: glycerol with 1:2

molar ratio give a final ethyl ester content of 95.2 wt.% and 0.8% fatty acids from transesterification of from refined rapeseed oil (Kleiner et al., 2016).

The stability of lipase in DES is one of the major concerns when run an experiment. Gorke reported that DES ChCl/U is more stable than its individual component 5mol L-1 ChCl or 10 mol L-1 urea which was about 20–35-fold of activity of CALB (Gorke et al., 2008).

However, the capability of various types of lipases on esterification reaction has not yet been studied. Therefore, this study presents several types of lipase activity and stability with different type DES. The study was focused on five different types of lipases which are Lipase from porcine pancreas (LPR), *Candida rugosa* (LCR), *Rhizopus niveus* (LRN) , Amano Lipase PS (LAB), and ICALB on the hydrolysis reaction with DES of (ChCl/glucose), (ChCl/polyethylene glycol/ethylene glycol), (alanine/glycerol), (ChCl/Glycerol 85%) and (MTPB/Glycerol 85%). The profile of each enzyme and its interaction with DES in terms of their activity and stability will be used as a preliminary result and predicted hypothesis before esterification reaction of acidic crude palm oil in the next study of enzymatic reaction to produce biodiesel using lipases immobilized with DESs.

6.3 **Methodology**

6.3.1 Materials

Lipase from porcine pancreas, *Candida rugosa*, *Rhizopus niveus,* Amano Lipase PS was utilized from Burkholderia cepacia (BCL), lipase *B candida Antartica* immobilized on immobead whom recombinant from *aspergillus oryzea*, p-Nitrophenyl palmitate, p-nitrophenol, Sodium deoxycholate (purity ≥97%) was purchased from Sigma-Aldrich

6.3.2 Synthesis of DES

DESs were synthesized according to the molar ratio in Table 6.1 where at this molar ratio the DESs were stable; however, DES D was an aqueous methanol which prepared by mixing methanol with distilled water with specific volume ratio (Table 6.1). A fixed amount of hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) were mixed gradually in the isolated fume hood until the synthesized DES soluble with a clear solution. The operating conditions for synthesizing was as followed; 300 rpm at 70 °C for 3 hours. After synthesized the DES were stored in the moisture controlled area before used (Hayyan et al., 2012a)

Table 6.1: Composition and molar ratio of DESs

DES	Composition	Molar ratio
A	ChCl : Glucose	2:1
B	ChCl : Poly ethylene glycol: Ethylene glycol	1:4:0.5
C	Alanine : Glycerol	1:7
D	Methanol : water	83:17 % vol ratio
E	ChCl: Glycerol 85%	1:4
F	MTPB: Glycerol 85%	1:5

6.3.3 Enzyme activity assay

The activity of lipase enzyme in DESs was determined by measuring the intensity in the absorbance at 410 nm produced by the release of p-nitrophenol during the hydrolysis of pNPP (dissolved in isopropanol) in 0.05M phosphate buffer (pH 7.0) using Thermo Scientific Multiskan GO by Thermo Fisher Scientific. The assay contains 0.07g of gum Arabic, 0.14g of sodium deoxycholate and 0.35 mL TritonX100. The assay was run at a temperature of 40°C for 15 minutes after hydrolysis, and the reaction was stopped using 300µL of (acetone/ethanol) at 1:1 v/v%. The hydrolysis reaction for screening of lipases under 7 different types of DESs started with 0.416mg/ml of the lipase suspension (5 mg enzyme in 12 ml phosphate buffer) added to 2 ml of the reaction mixture (volume ratio of 1:1 DES to phosphate buffer) in thermomixer at 40^0C, 350 rpm for 12 hours. The assay was followed by measuring the change in absorbance over 2 min. One unit of enzyme activity (U) was defined as the amount of lipase that consumed 1µ mol of pNP per minute under the assay conditions. The experiment was done in triplicate and the standard deviation was less than 7.

$$Relative\ activity = \frac{Relative\ activity}{initial\ \ activity} \times 100\% \qquad \text{Eq (6.1)}$$

The effect of concentration, pH and stability were all investigated by incubating LPR and LRN in DES E mixture for an hour in varied conditions (temperature, pH, and buffer content). DES concentration corresponded to 10, 20, 40, 60, 80 and 100% DES/ buffer, and incubation time were 6, 12, 24 and 48 hours. As reported in the product specifications, the optimum pH was at 7.0-8.0. The buffer used in this study was 0.05 M sodium phosphate buffer. To change the range for pH 5 - 6 and 8-10; 2 M HCl and 1 M of NaOH were used as acid and base. After hydrolysis for one hour, the lipase assay was executed as previously described.

6.4 Result and Discussion

Figure 6.2 shows the overall residual activity of all lipases with DESs. The free lipase enzymes are from LPR, LCR, LRN and LAB. The immobilized lipase enzyme was ICALB for the comparison of the immobilized enzymes. The hydrolysis was performed with all lipases using five different types of DESs. The objective of this experiment was to investigate the stability and the activity of lipase after immobilization on the DES. This study also presents the finest combination of lipases with DES (most stable and highest activity). Based on the graph of relative activity, all enzymes are stable and exhibited higher activities in hydrolysis when mixed with DESs except for candida rugosa where the activity was lower than free lipase.

The highest relative activity based on 12 hydrolyzes with buffer was achieved by the immobilized lipase ICALB which recorded 106% and secondly by LAB at 63%. When buffer was used as a control, it shows that DES E (ChCl: Glycerol 85%) and F (MTPB: Glycerol 85%) enhanced the activities of both LPR and LRN lipases at least two times compared to their free forms. A slight increment of activity can be observed from ICALB and LRN after incubation with DES. The increase in the activity was probably due to the change in the secondary structure of the lipase during mixing that opened up the “lid” of the active site of lipase to some extent for the substrate(s). The lipase activity increases when the interaction of active site providing easier access to the substrate (Fan et al., 2017). It can be concluded that free lipase of LPR, LRN, and LAB are compatible and stable when mixed with DES. Both DES E and F are the most suitable DESs support system as they do not only stabilize the DES but they also enhanced their activities.

A dilution of methanol was included in this study as a preliminary study for the application of lipases as a catalyst in esterification reaction where methanol was used as a solvent. It has been reported that methanol could irreversibly inactivate the lipase during the esterification when interacting with insoluble methanol (Shimada et al., 2002). However, it has been reported that the deactivation was for the specific enzyme. For example, Novozym 435 was deactivated at a concentration higher than 30% in stepwise addition and 20% in single step esterification (Nordblad et al., 2016). In transesterification, the inhibition of the activity of enzyme differs for different lipases and also it depends on the molar ratio of methanol-to-oil itself (Taher, & Al-Zuhair, 2017). Moreover, the degree of the deactivation has been found to be inversely proportional to the number of carbon chain in the linear lower alcohol (Modi et al., 2007).

Thus this study was executed to investigate the compatibility of tested lipases during hydrolysis with methanol. From Figure 6.2, it can be seen that diluted methanol enhanced the activity of free lipase of LPR, LRN, and LAB. This shows that these lipases are stable in methanol and have a high possibility of not being denatured during esterification reaction. This is important as lipase enzymes are costly, and the preliminary result could reduce the use of lipase during optimization of esterification. However, diluted methanol deactivated LCR and ICALB.

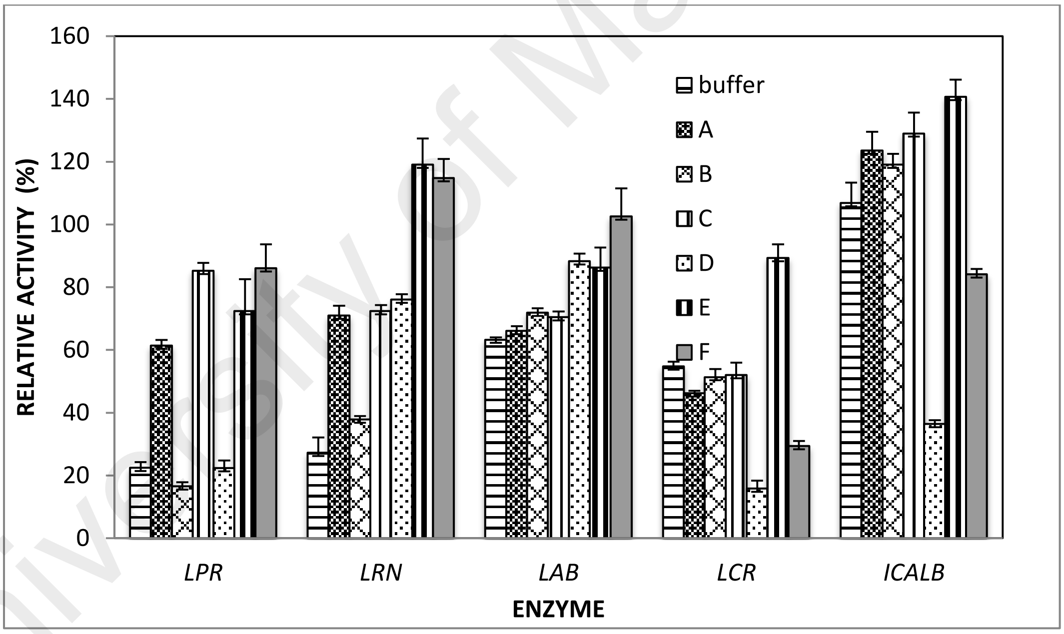


Figure 6.2: Relative activity of lipase after 12 hours of hydrolysis with 7 different type of DES.(R^2= 0.9953)

Apart from the analysis of activity, statistical analysis using one way ANOVA was applied in this study to investigate the compatibility as well as the significance of the activity differences. Based on the graph, the highest intersections are on DES E and F which showed that greater interaction and significance of them on all lipases compared to buffer that are parallel to each other. This interaction supported the observation in Figure 6.3 where DES E and F presented high activation for lipase.

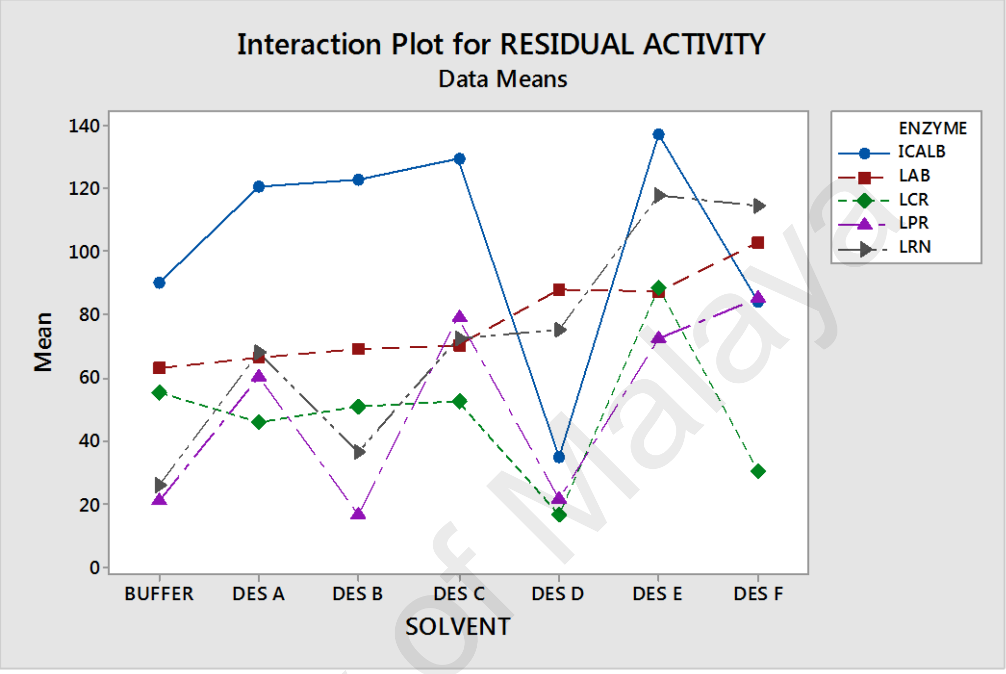


Figure 6.3: Interaction between lipases with DESs using ANOVA analysis

The optimization was done after screening of lipases with 5 different types of DESs. LPR and LRN were selected to be optimized with DES E due to high value of residual activity showed during screening process. DES E showed the highest activity among DESs for all lipases which can be a positive and promising model study of DES in the optimization of hydrolysis of lipase.

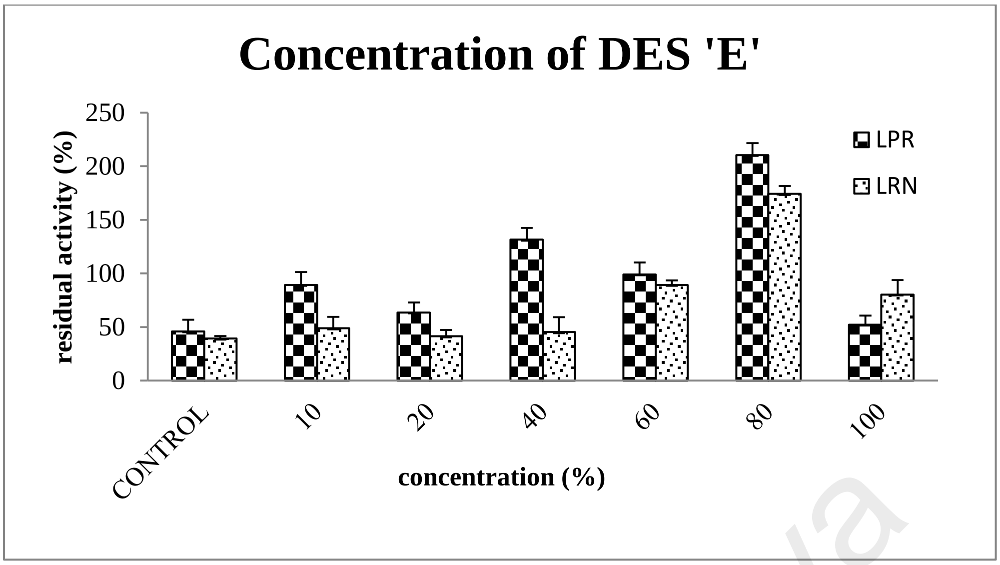


Figure 6.4: Residual activity of LPR and LRN at different concentration of DES “ E ”

Based on figure 6.4, DES E significantly enhanced the activity of both LPR and LRN up to four folds at concentration of 80 % (80 % volume of DES E and 20 % volume of buffer) which more stable than in aqueous solution. Increase the concentration of DES E to 100% showed slight inhibition to both lipases. Compare to control (no mixture of DES), LPR mixed with DES E were activated; as observed from figure 4; 10, 20, 40 and 60 % are enhanced up to 89.44, 63.71, 131.62 and 99.25 % respectively. With the LRN, similar trend was observed with reduced activation effect as the maximum was identified at concentration of 60 and 80 % which are 89.43 and 174.29 % respectively. Thus, 80% of concentration of DES E was selected as the optimum concentration for the optimization.

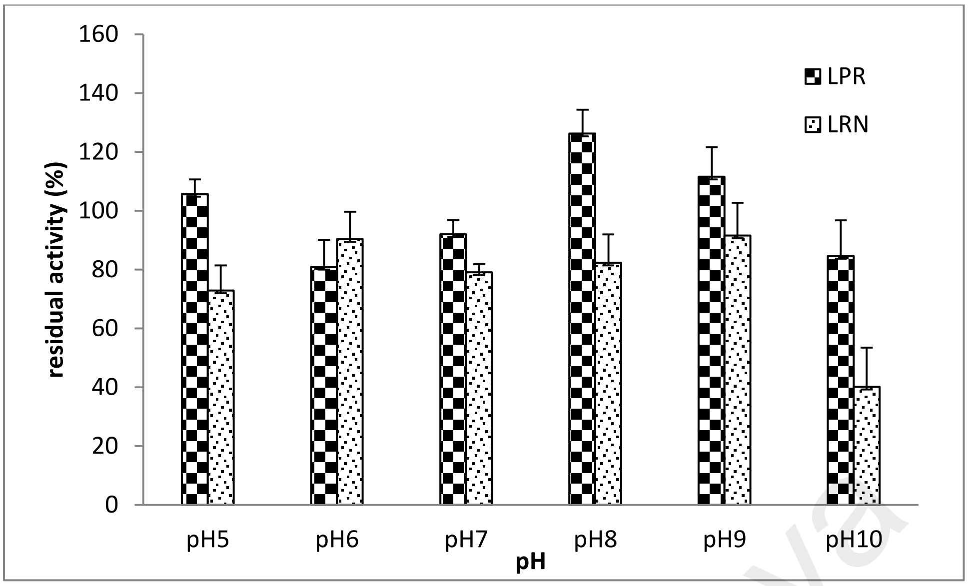


Figure 6.5: Activity of LPR and LRN at different pH values in the presence of 80 % of DES 'E'

From figure 6.5, when mixing 80 % DES E with buffer, it can be observed that there is no significant effect of pH on LPR. The strong HBD clarifies this stability within DES. However, in case of LRN, pH 10 degraded the activity of LRN to 40%. The optimum pH for LPR is at pH 8 where the residual activity is at highest of 126.26 %. Even though the highest residual activity of LRN is at pH 9 which is 91.56%, we have selected pH 8 as the optimum pH for both lipases for the optimization as the residual activity value for RNL is only 3% different. The present result of figure 5.5 agreed with the reported data as lipase were active at about pH 7 to 8.

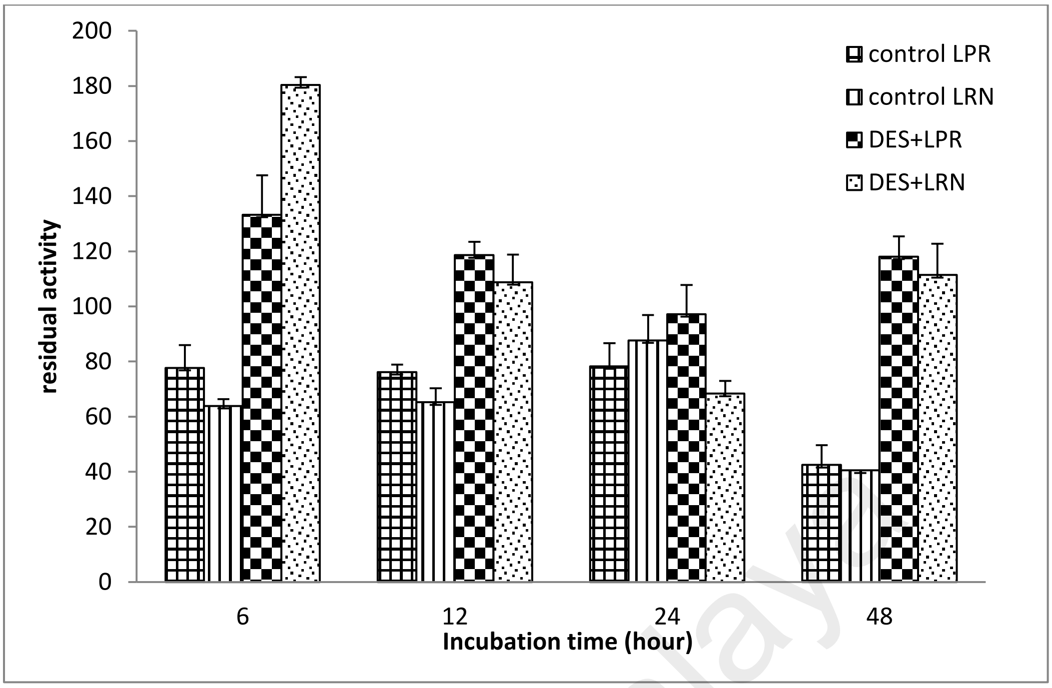


Figure 6.6: Relative activity of LPR and LRN at different incubation time at 80 % concentration of DES E and pH 8

The figure 6.6 indicated the stability of LPR and LRN in DES E, mixture of 80% of DES E constantly stabilize and the enzyme up to 48 hour compare to its control that degraded after 48 hour of incubation. The residual activity of LPR and LRN at 48 hour remains to 118.13 and 111.44 % respectively. The stability and activity could be due to the strong polarity due to the multiple hydroxyl groups based DES and to the nature of DES (Elgharbawy et al., 2018). Mixing DES with free lipase is perhaps will open a new door to handle the fragile enzyme as a simple protocol of ‘immobilization’ is possible to stabilize and activate the free lipase.

6.5 Conclusion

The hydrolysis of five different lipases was studied to investigate the effect of new support system or medium for lipase enzyme. Five different combinations of HBD with HBA were used to DESs. ICALB showed the highest value of the relative activity and enhancement after incubating with DESs. The free lipase of the porcine pancreas, Rhizopus niveus and amano lipase showed significantly enhancement after incubation with ChCl/Glycerol 85 % and MTPB/Glycerol 85 %. Rhizopus niveus and amano lipase also show enhancement when incubate in aqueous methanol (D). This finding could strongly support the hypothesis that both of these lipases with support system from DES are able to esterify the oil for biodiesel production as it stable during hydrolysis with aqueous methanol. The optimization of lipase from porcine pancreas and Rhizopus niveus with ChCl/Glycerol 85 % can be used to assess ideas of applying natural based DES to improve the hydrolysis of free lipases. Targeting lower cost and natural component as a solvent/co solvent for enzymatic protocol is high priority since the cost of enzyme itself is expensive.

CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

7.1 Overall Conclusions

The use of low grade pam oil as feedstocks is a new and promising route for exploitation and development of the renewable fuel industry in Malaysia. DES is a green solvent that is widely used as a co-solvent in various chemical and biological reactions. This study generates an advancement of information on the optimization and dynamic study of upstream processes of biodiesel production. The various types of catalyst and support solvent properties within the production of biodiesel have also been reported in the literature review (Chapter Two).

Therefore, in this study, several types of DESs were synthesized and applied in upstream processes for biodiesel production. CSA-ChCl-ES and BAC-PTSA-DES were utilized as chemical catalysts in the batch esterification reaction for the pre-treatment processes of acidic crude palm oil. On the other hand, ammonium - and phosphonium - based DESs were prepared and used in the enzymatic hydrolysis of pNPP.

7.1.1 Chemical Reaction - Esterification

- Selection of catalysts plays an important role in the esterification reaction. In this study, two types of ammonium DESs were prepared. Two different types of organic acids were used as hydrogen bond donors, i.e. (1R)-(-)-camphor-10-sulfonic acid (10-CSA) and PTSA.
- DES BAC: PTSA (1:3) exhibited high catalytic activity compared to DES 10-CSA: ChCl (1:1). This is probably due to the lower ratio synthesized and weak acidic property possess by the 10-CSA that may have lower catalytic effect compared to the acidic PTSA.

- In addition, BAC- DES required higher dosage of catalyst and slightly higher reaction time compared to CSA-ChCl-ES. Hence, this infers that BAC- DES was a better catalyst than CSA-ChCl-ES in the esterification of ACPO.
- The role of salts in both DESs during esterification reaction was found to be inert, i.e. demonstrated insignificant effect on the activity. Both CSA-ChCl-ES and BAC-DES can be used as the homogenous catalyst in the conversion of acidic oil (ACPO) to biodiesel.
- The recyclability of the catalyst after reaction is one of the most important factors on the viability of biodiesel production. Based on Table 6.1, BAC-DES has higher number of recycling runs compared to CSA-ChCl-ES and therefore it is recommended for biodiesel industry.

7.1.2 Physical Properties of DES

- Aqueous glycerol was used as HBD in the preparation of DESs with two types of salts which were ammonium (ChCl) and phosphonium (MTPB) at specific molar ratio. All the data for the physical properties (surface tension, conductivity, density, viscosity) were reported and with temperatures between 298 K to 363 K;
    - The conductivity values were from 4.11 to 12.68 mScm^{-1} for ChCl-DES and 0.59 to 5.26 mScm^{-1} for MTPB-DES.
    - The surface tension results exhibited values in the range of 29.2 to 68.0 Dynes.cm^{-1} for ChCl-DES and 33.2 to 58.4 Dynes.cm^{-1} for MTPB-DES.
    - The density results showed values in the range of 1.1214 to 1.1826 g cm^{-3} for ChCl-DES and 1.1997 to 1.2808 g cm^{-3} for MTPB-DES.
    - The viscosity values were in the range of 5.68 to 159.67 mPa.s for ChCl-DES and 15.95 to 589.33 mPa.s for MTPB-DES.

- MTPB-DES 3 showed the highest viscosity and density at room temperature, and this implies that phosphonium based DES possess stronger intermolecular forces.
- In general, the physical properties of phosphonium based DES has higher density and viscosity.
- The freezing point of the prepared DES was much lower than their individual component. The eutectic points of DESs for ammonium and phosphonium are at ratio 1:3 and 1:5, respectively.

7.1.3 Enzymatic Reaction-Hydrolysis

In this study, DES showed an exceptional activation and stabilization of the lipase enzyme. Based on the enzyme activity result, the structure of DES seems to have shielding properties rather than only enzyme activation. The prominent enhancement of the enzyme activity in DES may be associated with the hydrogen bonding ability between the salt and HBD, which improves the enzyme affinity during hydrolysis process.

Under a stirring speed of 350 rpm and temperature of 313 K for 30 min with phosphate buffer of pH 7.0, ChCl-DES 4 was able to activate lipase PPL up to 7.2 fold in comparison to the control. Meanwhile, MTPB-DES 4 showed activity of 0.9 fold compared to their control under the same conditions.

In term of kinetic study of PPL, ChCl-DES 4 had Kcat/Km value of 7482.256 min mM-1, which was 46 times higher, compared to the MTPB-DES 4. It was also approximately 1500 times higher compared to the control. Phosphonium-based DES (ChCl-DES 4) showed higher activation towards porcine pancreas lipase compared to Rhizopus niveus lipase.

Several different type of DES were also synthesis and screened with different lipase in hydrolysis reaction. It showed that all DESs are able to activate and stabilizes the lipases in hydrolysis reaction except for candida rugosa which degraded during hydrolysis reaction. After optimization, ChCl/Glycerol 85% with LPR and LRN work best in condition of 80% of concentration of DES, at pH 8 and of 48 hour of incubation time.

7.2 Recommendations

The present work showed positive results for treating acidic oil. However, each component of DES need further study in order to understand the preference of the compound towards the treatment of FFA.

Moreover, in order to study the effect of catalyst to its highest limit, it is recommended to apply different modes of reactor; such as continuous flow reactor to investigate the highest potential and strength of DES-based catalyst. In the batch reaction, most of the catalysts were denatured or degraded during the separation and purification processes. By using continuous flow, it will reduce the risk of catalyst degradation or denaturation after the reaction.

In the enzymatic treatment, free lipases need not endure harsh conditions, such as acidic oil and high reaction temperature. One of the solutions is by inducing the DES lipase enzyme with different media for immobilization, such as CNT and graphene.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

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S. N. Rashid, A. Hayyan, and M. A. Hashim, "Production of Fatty Acid Methyl Ester from Low Grade Palm Oil Using Eutectic Solvent Based on Benzyltrimethylammonium Chloride," IOP Conf. Ser. Mater. Sci. Eng., vol. 210, no. 1, pp. 0–6, 2017.