DEEP EUTECTIC SOLVENT (DES) AS A NEW SOLVENT FOR LIPASE-CATALYZED SYNTHESIS OF GLYCERYL MONOCAFFEATE VIA TRANSESTERIFICATION

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by

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LIST OF SYMBOLS

[A]	Concentration of substrate A
[B]	Concentration of substrate B
[EC]	Concentration of ethyl caffeate
[Gly]	Concentration of glycerol
A	Substrate A
A	Arrhenius pre-exponential factor
B	Substrate B
BF_4^-	Tetrafluoroborate ion
E	Enzyme
E_a	Activation energy
F	Enzyme intermediate state
k	Rate constant
K_{iA}	Dissociation constant for substrate A
K_{iB}	Dissociation constant for substrate B
K_{iEC}	Dissociation constant for ethyl caffeate
K_{iGly}	Dissociation constant for glycerol
K_m	Michaelis constant
K_{mA}	Michaelis constant for substrate A
K_{mB}	Michaelis constant for substrate B
K_{mEC}	Michaelis constant for ethyl caffeate
K_{mGly}	Michaelis constant for glycerol
P	Product P
PF_6^-	Hexafluorophosphate ion
Q	Product Q
R	Alkyl group
R	Gas constant
v	Initial velocity/rate of reaction
V_{max}	Maximum velocity/rate of reaction

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BOD	Biological oxygen demand
CA	Caffeic acid
CAPE	Caffeic acid phenethyl ester
CCD	Central composite design
CDAGs	Caffeoylated diacylglycerols
ChCl	Choline chloride
CMAGs	Caffeoylated monoacylglycerols
DES	Deep eutectic solvent
DMSO	Dimethylsulfoxide
EC	Ethyl caffeate
ESI-MS	Electrospray ionization mass spectrometry
GDC	Glyceryl dicaffeate
GMC	Glyceryl monocaffeate
HBDs	Hydrogen bond donors
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
*i*CALB	Immobilized *Candida antartica* B
M	Molecular weight
NIOSH	National Institute for Occupational Safety and Health
NMR	Nuclear magnetic resonance
OFAT	One-factor-at-a-time
RSM	Response surface methodology
U	Unit activity
UV	Ultra violet
VOC	Volatile organic compound

PELARUT EUTEKTIK (DES) SEBAGAI PELARUT BARU BAGI SINTESIS GLISERIL MONOKAFEAT MENGGUNAKAN LIPASE SEBAGAI PEMANGKIN MELALUI TRANSESTERIFIKASI

ABSTRAK

Cecair ionik dan pelarut eutektik (DES) telah dikenal pasti sebagai pelarut alternatif yang mesra alam. Walau bagaimanapun, laporan kajian terbaru tentang cecair ionik telah menunjukkan bahawa bahan mentahnya (kation berasaskan imidazolium) memberi kesan toksik yang terhadap alam sekitar. Penggunaan DES sebagai pengganti cecair ionik memberi sinar baru bagi aplikasi tindak balas menggunakan lipase. Tiada sebarang kajian mengenai tindak balas menggunakan lipase bagi menghasilkan gliseril monokafeat (GMC) di dalam DES. Pengubahsuaian asid kafeik (CA) kepada GMC berpotensi untuk meluaskan aplikasi aktiviti biologi CA di dalam sistem berasaskan air dan minyak. Transesterifikasi antara etil kafeat (EC) dan gliserol untuk menghasilkan GMC telah dijalankan dengan menyaring parameter-parameter berikut yang memberi kesan terhadap tindak balas iaitu jumlah enzim daripada 250 U sehingga 1500 U, kandungan air bermula daripada 0 % (v/v) sehingga 40 % (v/v), kelajuan pusingan di antara 100 rpm dan 250 rpm, nisbah molar substrat etil kafeat dan gliserol daripada 1:40 sehingga 1:90, masa tindak balas bermula daripada 0 minit sehingga 240 minit dan suhu di antara 30 ºC hingga 60 ºC. Penukaran etil kafeat sebanyak 88.4 % telah diperolehi pada 1250 U jumlah enzim, 20 % (v/v) kandungan air, 200 rpm kelajuan pusingan, 1:50 nisbah molar substrate etil kafeat dan gliserol, 30 minit masa tindak balas dan 40 ºC suhu. Mekanisma kinetik dan parameter kinetik telah dijalankan untuk menentukan kadar awal tindak

balas untuk pelbagai kepekatan substrat. Hasil menunjukkan bahawa tindak balas menggunakan lipase bagi menghasilkan GMC di dalam DES mematuhi mekanisme Ping Pong Bi-Bi dengan V_{max}, 10.9 mmol min^{-1}; K_{mEC}, 126.5 mmol dan K_{mGly}, 1842.7 mmol. Seterusnya, kajian tentang tenaga pengaktifan telah dijalankan dengan mempelbagaikan suhu daripada 30 ºC sehingga 55 ºC dan menghasilkan nilai sebanyak 50.4 kJ/mol. Akhir sekali, metodologi tindak balas permukaan (RSM) berdasarkan reka bentuk komposit pusat (CCD) telah dijalankan untuk memperoleh nilai optimum dengan penukaran EC diperolehi sebanyak 94.71±0.06 % daripada faktor berikut yang signifikan iaitu: jumlah enzim, 705 U; jumlah air, 20 % (v/v) dan masa tindak balas, 113 minit. Secara kesimpulannya, DES berpotensi sebagai pelarut bagi tindak balas menggunakan lipase.

DEEP EUTECTIC SOLVENT (DES) AS A NEW SOLVENT FOR LIPASE-CATALYZED SYNTHESIS OF GLYCERYL MONOCAFFEATE VIA TRANSESTERIFICATION

ABSTRACT

Ionic liquids and deep eutectic solvent (DES) have been identified as greener alternatives solvents. Nevertheless, ionic liquid has been reported to display toxicity to the environment due to its starting material (i.e. imidazolium-based cation). Therefore, the design of DES for ionic liquids substitution has shown a bright future for implementation in lipase-catalyzed reaction. Lipase-catalyzed reaction of glyceryl monocaffeate (GMC) in DES has not yet been reported. The modification of caffeic acid (CA) into GMC could potentially be widen the application of CA's biological activities in water and oil-based system. The transesterification of ethyl caffeate (EC) and glycerol to produce GMC was carried out by screening these parameters that affect the reaction such as enzyme loading between 250 U to 1500 U, water content within 0 % (v/v) and 40 % (v/v), agitation speed ranged from 100 rpm to 250 rpm, substrates molar ratio of ethyl caffeate and glycerol from 1:40 to 1:90, reaction time between 0 to 240 minutes and temperatures ranged from 30 ºC to 60 ºC. Final conversion of ethyl caffeate at 88.4% was obtained at 1250 U of enzyme loading, 20 % (v/v) of water content, 200 rpm of agitation speed, 1:50 substrates molar ratio of ethyl caffeate and glycerol, 30 minutes of reaction time and 40 ºC of temperature. The kinetic mechanism and kinetic parameters were investigated to determine the initial rate of reaction of various substrates concentration. The results demonstrated that lipase-catalyzed synthesis of GMC in DES obeyed Ping Pong Bi Bi mechanism

with V_{max}, 10.9 mmol min^{-1}; K_{mEC}, 126.5 mmol and K_{mGly}, 1842.7 mmol. Next, the study on the activation energy was conducted by varying temperature from 30 ºC to 55 º C and resulted in the value of activation energy was 50.4 kJ/mol. Finally, response surface methodology (RSM) based on face-centered central composite design (CCD) was studied to obtain the following optimum conditions with 94.71±0.06 % final conversion of EC from significant factors: enzyme loading, 705 U; water content, 20 % (v/v) and reaction time, 113 minutes. Therefore, it can be concluded that DES could serve as a potential solvent for lipase-catalyzed reaction.

CHAPTER 1

INTRODUCTION

This chapter gives an overview of the research background introducing the phenolic compound, and followed by the review on caffeic acid and its derivative. Then, the potential of ionic liquid and deep eutectic solvent (DES) to substitute conventional volatile organic solvents is elaborated as well as some issues regarding ionic liquid to the environment. Finally, problem statement, research objectives and scope of study are outlined.

1.1 Phenolic compound as an antioxidant

Phenolic compound, a secondary metabolite found mainly in plants has attained great attention due to its antioxidant property. Phenolic compound is characterized by the presence of one or more hydroxyl group on benzene ring. Antioxidants are compounds that function by hindering and slowing the effects caused by free radicals and oxidizing compounds.

Figure 1.1 shows the global phenolic antioxidant industries which is dominated by food industry and followed by plastic and rubber, pharmaceutical, cosmetic, and fuels and lubricants industries. In food industry, antioxidant is effective for preventing rancidity and deterioration of flavor in food due to oxidation (Ho, 1992). Meanwhile, in plastics and rubber industry, antioxidant acts as an additive that maintains the color, flexibility, tensile strength, cracking in thermoplastic and thermosetting resin (Oxiris, 2018).

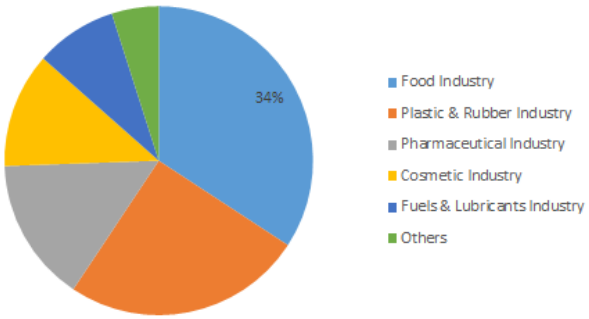


Figure 1.1 Phenolic antioxidant industries (Anand *et al.*, 2017)

1.2 Caffeic acid and its derivative

Caffeic acid (CA), a phenolic acid that is classified as a hydroxycinnamic acid consists of two hydroxyl groups on C3 (*meta*-substitution) and C4 (*para*-substitution) position on the aromatic ring. CA presents abundantly in many food sources including coffee, tea, vegetables and fruits like blueberry, raspberry, blackberry etc. (El-Seedi *et al.*, 2018). Figure 1.2 illustrates the chemical structure of caffeic acid.

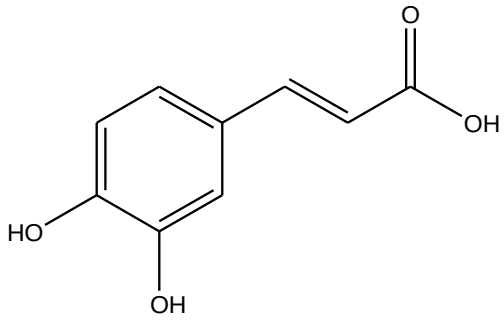


Figure 1.2 Chemical structure of caffeic acid

CA is well known for its antioxidant activity. The antioxidant activity is closely associated to the number of hydroxyl groups on the aromatic ring (El-Seedi *et al.*, 2018). As compared to ferulic acid with methoxy (OCH_3) on its C3 position of the aromatic ring, the efficiency of antioxidant activity of CA is greater due to the fact that it contains two hydroxyl groups which contribute to additional resonance

stabilization (Graf, 1992; Magnani *et al.*, 2014). Apart from being as an antioxidant, CA has also demonstrated antimicrobial, antitumour/anticancer, anti-inflammatory and anti-HIV properties (Al Jitan *et al.*, 2018; Magnani *et al.*, 2014).

Nonetheless, its lipophilicity or effectiveness in oil system is restricted due to its low solubility in non-polar media and therefore limits it application in oil-based food, pharmaceutical and cosmetic industries (Pang *et al.*, 2013). In fact, CA also has low solubility in water systems (Mota *et al.*, 2008). Thus, the modification into CA derivatives especially caffeic acid esters derivatives would overcome the limitation pose by CA as well as enhance its biological activities.

Glyceryl ester of caffeic acid is one of caffeic acid ester derivatives which was initially identified in oats (Daniels & Martin, 1968). The addition of glycerol backbone to caffeic acid through ester linkage is fascinating due to the formation of amphiphilic compound. This would allow the compound to orient to the oil-water interface (Hall III, 2001). Glyceryl monocaffeate (GMC) is a caffeic acid ester derivative with solubility in water about 3 times as compared to CA with value of 1.76 mg/ml in water at 20 ºC. GMC could be synthesized using enzymatic reaction which is more energy-friendly as it has high selectivity and requires only medium temperature from 30 ºC to 60 ºC to operate (Sun & Hu, 2017b). The enhancement of hydrophilicity and lipophilicity in GMC could provide a promising new type of compound that is water and oil soluble with plethora of biological activities.

1.3 Alternative solvents

According to United States Environmental Protection Agency, green chemistry is the area where chemical products and processes are designed to reduce or eliminate the use or generation of hazardous substances. One of the twelve principles of the green chemistry outlined that benign or less harmful solvents should be employed (Anastas & Williamson, 1998). Green solvents were developed as the result of highly usage of volatile organic compounds (VOCs) in chemical processes.

Organic solvent has been employed in many areas including biocatalysis because of its wide availability in the market. However, these non-aqueous solvents are hazardous to human and environment. According to a survey conducted by National Institute for Occupational Safety and Health (NIOSH), approximately 10 millions of American workers are potentially exposed to organic solvents (Firestone & Gospe, 2009). In addition, polar organic solvents such as acetone, methanol or DMSO contribute to denaturation of the biocatalyst (Kumar *et al.*, 2016). Therefore, the search and development for efficient and greener alternative solvents has boosted interest among the researchers.

Recently, new types of solvents such as ionic liquid and deep eutectic solvent (DES) have emerged to overcome the weakness of organic solvent. Both of these liquids exhibit similar physical properties in terms of low vapor pressure, high viscosity and non-flammability (Smith *et al.*, 2014). These aqueous solvents have been utilized in various research field including enzyme-catalyzed reaction (Durand *et al.*, 2013b), biodiesel synthesis (Troter *et al.*, 2016), metal processing (Jenkin *et al.*, 2016), nanotechnology (Abo-Hamad *et al.*, 2015), natural product (Dai *et al.*,

2013), chemical synthesis (Liu *et al.*, 2015) and sample preparation (Francisco & Jacek, 2014).

1.4 Issues regarding ionic liquids

An ideal green solvent should possess several of these qualities: biodegradability, non-toxic, recyclable, high accessibility and low cost (Kudłak *et al.*, 2015). Having the properties of low vapour pressure, it is thought at first that ionic liquids could be a potential green solvent when comparing them to conventional volatile organic solvents because the risk of air pollution is unlikely to happen (Zhao *et al.*, 2007). However, the 'green label' has been challenged by some aspects namely the toxicity of the cations and its low biodegradability. To make matters worse, ionic liquid have great miscibility and solubility in water (Kudłak *et al.*, 2015). Possible passage of ionic liquid into the environment could occur from accidental spills of effluent from industry or landfill sites leaching (Bubalo *et al.*, 2014).

Furthermore, high stability of ionic liquids poses a great accumulation problem in the environment if they are utilized in large scale applications. Therefore, the ability for ionic liquids to be biodegradable becomes the major concern in order to avoid them to stay persistent in the environment for a long period of time. The evaluation of biodegradability in ionic liquid is conducted by measuring Biological Oxygen Demand (BOD) and showed that most of cations exhibit low biodegradation (Zhao *et al.*, 2007).

Apart from that, ionic liquids require purification as their physico-chemical properties and enzyme activity will be greatly affected with the presence of impurity such as halide ion like chloride ion, Cl^- (Durand *et al.*, 2012). Frequently, MX waste usually NaCl is generated in the preparation of ionic liquid. The removal of Cl^- ions cannot be neglected since it can critically affect the physical properties of the ionic liquid where the viscosity increases (Seddon *et al.*, 2000). The purification of ionic liquids require a laborious process as well as their synthesis consume high cost of starting material thus further industrial application is less practical (Deetlefs & Seddon, 2010).

In brief, the use of ionic liquids as an alternative solvent implies several alarming issues; high toxicity through most of their cations, low biodegradability, presence of impurities, involve tedious time for preparation and high starting material cost. As an alternative, a more environmental-friendly solvent, DES has been prepared to overcome the problems faced by ionic liquids. Although it is being classified as ionic liquid, there are several features that make DES distinguishable from ionic liquids. Further discussion on DES will be emphasized in Chapter 2.

1.5 Problem statement

GMC could be catalytically synthesized by using lipase through two synthetic routes i.e. esterification and transesterification reactions. For a lipase-catalyzed esterification reaction, CA and glycerol are utilized as substrates. There are several major drawbacks of using esterification reaction method. First, the presence of *para*-hydroxylated substituent on aromatic ring of CA as well as conjugated double bond on the side chain will inhibit catalytic activity of lipase through resonance

delocalization (Kahveci *et al.*, 2015). Second, production of uncontrollable amount of water as a by-product could potentially shift the reaction towards hydrolysis hence reducing the yield of GMC. Hence, the lipase-catalyzed transesterification reaction in DES using alkyl caffeate (for example ethyl caffeate) with glycerol is more favourable approach for synthesis of GMC. The addition of alkyl chain to CA to become alkyl caffeate stabilizes and activates the carbon centre of carboxylic group through inductive effects thus allowing nucleophilic attack of alcohol (glycerol). In general, this will reduce the inhibition of catalytic activity of lipase and increase the reaction rate. The possible reaction scheme for lipase-catalyzed synthesis of GMC via transesterification is presented in Figure 1.3.

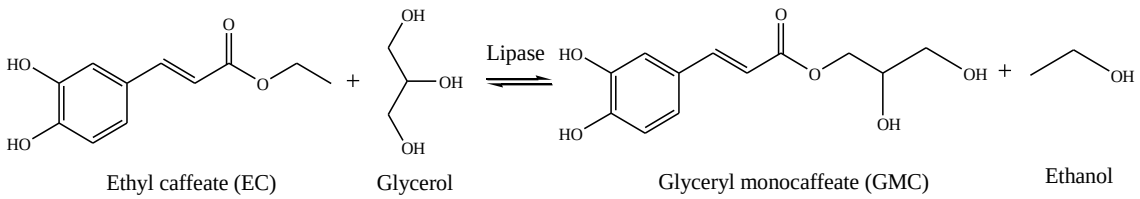


Figure 1.3 Reaction scheme for lipase-catalyzed synthesis of GMC via transesterification reaction of EC and glycerol

The use of ionic liquid to substitute organic solvents in enzyme-catalyzed reaction is now common (Domínguez de María & Maugeri, 2011). This is due to the fact that ionic liquid have the ability to dissolve a wide range of compound especially those with low solubility in non-polar organic solvent. However, as mentioned earlier, due to certain disturbing issues that it poses to the environment, researchers have started to find an alternative solvent to substitute the usage of ionic liquid in lipase-catalyzed reaction. The emergence of DES as a green solvent to replace ionic liquids has given hope to the scientific community since its physicochemical properties is quite similar to ionic liquids. DES is pleasing due to several reasons.

First, it comprised of readily available and non-toxic starting material. Second, the preparation method is not laborious thus causing reduction in cost. Third, it possesses high biodegradability which enable it to be disposed easily (Zhang *et al.*, 2012).

Based on previous study, GMC was synthesized from the lipase-catalyzed transesterification reaction using ethyl caffeate and glycerol as the substrates in solvent-free system. Solvent-free system utilizes excess substrate as the solvent for the reaction. In that study, glycerol was used as the substrate as well as the solvent where there was a production of side product, glyceryl dicaffeate (GDC). However, uncontrollable amount of glycerol lead to the difficulty in controlling GMC production due to the side product, GDC. To date, there is no investigation made on the lipase-catalyzed reaction of GMC in DES. Hence, lipase-catalyzed reaction of GMC in DES could provide valuable information on the behaviour of lipase towards these substrates in DES.

1.6 Research objectives

The main objective of this research project is to focus on the utilization of DES in lipase-catalyzed synthesis of GMC. The following specific objectives are:

i. To study the effect of reaction parameters and effect of optimum condition based on one-factor-at-a-time method (OFAT) for lipase-catalyzed synthesis of GMC in DES.

ii. To determine the kinetic mechanism, kinetic parameters and activation energy for lipase-catalyzed synthesis of GMC in DES.

iii. To determine the interaction effect between reaction parameters and identify the optimum conditions for lipase-catalyzed synthesis of GMC in DES using response surface methodology (RSM).

1.7 Scope of research

In order to achieve the objectives of study, three major scopes are covered in this research includes effect of reaction parameters, determination of kinetic mechanism, kinetic parameters as well as activation energy and the optimization of lipase-catalyzed synthesis of GMC in DES.

1.7.1 Lipase-catalyzed synthesis of GMC in DES

At the beginning of this study, ethyl caffeate (EC) as the main substrate was prepared due to unavailability of commercialized EC in huge amount. The synthesized EC was then confirmed and compared with the standard EC using high pressure liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) before being utilized for further investigation. In the preliminary study, enzyme assay was carried out to determine enzyme unit activity (U) of Novozym 435. After that, lipase-catalyzed transesterification reaction in DES using EC and glycerol was conducted in DES to determine effect of reaction parameters namely effect of enzyme loading, water content, agitation speed, substrates molar ratio, reaction time and temperature based on OFAT method.

1.7.2 Kinetic study and activation energy of lipase-catalyzed synthesis of GMC in DES

Kinetic study was conducted to determine the kinetic mechanism and parameters of lipase-catalyzed synthesis of GMC in DES. In order to obtain the kinetic parameters, initial rate of reaction was determined by varying the substrates concentrations. Then, the kinetic parameters were calculated from the proposed model. Next, activation energy was determined from the initial rate of reaction by varying the temperature.

1.7.3 Optimization of lipase-catalyzed synthesis of GMC in DES

Based on the effect of reaction parameters study, the significant reaction parameters namely enzyme loading, water content and reaction time were studied to determine the optimum conditions with highest conversion of EC. Central composite design (CCD) of response surface methodology (RSM) was employed to monitor the interaction between parameters and finally to obtain the optimum conditions for lipase-catalyzed transesterification reaction of EC and glycerol in DES.

CHAPTER 2

LITERATURE REVIEW

In this chapter, the previous literatures related to this study are reviewed. The literature review begins with previous method of synthesizing GMC as a basis for development of lipase-catalyzed transesterification reaction to produce GMC in DES. Next, the history and general introduction of DES is presented for deep understanding in the potential of DES as a solvent. Then, a brief introduction on choline-based DES comprises of choline chloride and urea is focused for better understanding on the properties and advantages as a solvent in lipase-catalyzed reaction. Finally, the objectives of study are focused from the reviews on the critical reaction parameters, enzyme kinetic study, activation energy and optimization study using RSM.

2.1 Synthesis of glyceryl monocaffeate (GMC)

Being an amphiphilic structure, glyceryl monocaffeate (GMC) is able to facilitate in water and oil medium thus can be widely used in food, pharmaceutical and cosmetic industries. Synthesis of GMC had been previously conducted by Sun and co-workers (2017). Initially, the groups utilized ethyl caffeate (EC) and glycerol as the substrates catalyzed by Novozym 435 for synthesis of GMC (Sun & Hu, 2017b). Later on in the same year, they substitute glycerol with monoacylglycerols such as monooleate and monostearate as the caffeoyl acceptors. In the study, other products beside GMC were also formed such as glyceryl dicaffeate (GDC), caffeoylated monoacylglycerols (CMAGs) and caffeoylated diacylglycerols (CDAGs) (Sun & Hu, 2017a). The same groups further explored way of synthesizing

GMC through the employment of ionic liquid (imidazolium-based ionic liquid) as the catalyst as well as solvent (Sun *et al.*, 2017).

2.2 The discovery of ionic liquid and deep eutectic solvent (DES)

Ionic liquid consists of one type of discrete anion and cation that melt at temperature below 100 °C (Gorke *et al.*, 2010b). In earlier times, ionic liquid namely ethylammonium nitrate (Melting temperature, T_m = 12 °C) was first synthesized in 1914 but possessed limited use (Kudłak *et al.*, 2015). Two decades later, in 1934, pyridinium-based molten salt was created with the ability to dissolve a certain amount of cellulose (Domínguez de María & Maugeri, 2011). The first generation of ionic liquid which raised the interest of many researchers to study extensively about ionic liquid emerged in the 1982 when a clear colourless liquid of dialkylimidazolium chloroaluminate formed (Wilkes *et al.*, 1982). Nevertheless, these generation of ionic liquids that mainly composed of metal halides anions have drawbacks in which they are water-sensitive and air-sensitive (Gorke *et al.*, 2010b). As a consequence, second generation of ionic liquids was developed. This generation of ionic liquids substituted the metal halides anions with weakly coordinating anions such as BF_4^- and PF_6^- (Domínguez de María & Maugeri, 2011). In later work, most of the studies used these liquids with countless publication has been documented. The basic cation core usually used in first and second generation of ionic liquid are cation based on imidazolium, pyridinium, pirolidynium, ammonium and phosphonium (Kudłak *et al.*, 2015).

The toxicity of most of these ionic liquids generations are contributed by their cation. Several cations that usually make up ionic liquids are *N,N'*-dialkylimidazolium, *N*-alkylpyridinium, alkylammonium, alkylphosphonium and alkylsulphonium (Bubalo *et al.*, 2014). The most common cation used in ionic liquid is alkyl chains of imidazolium (e.g. 1-alkyl-3-methylimidazolium). Extensive studies have been made on this type of cation reporting its toxicity on aquatic and soil organisms. Most of the studies proved that longer alkyl chain on the imidazolium-based cation resulted in higher toxicity (Zhao *et al.*, 2007).

As for the third generation of ionic liquid, a more biodegradable and readily available cations (choline) and/or anions (sugar or sugar analogs, amino acid, alkylsulfates or alkylphosphates) are utilized to overcome the weakness of previous generation (Gorke *et al.*, 2010b). DES is included in this third generation of ionic liquids (Domínguez de María & Maugeri, 2011). Figure 2.1 summarized the evolution of ionic liquid discoveries.

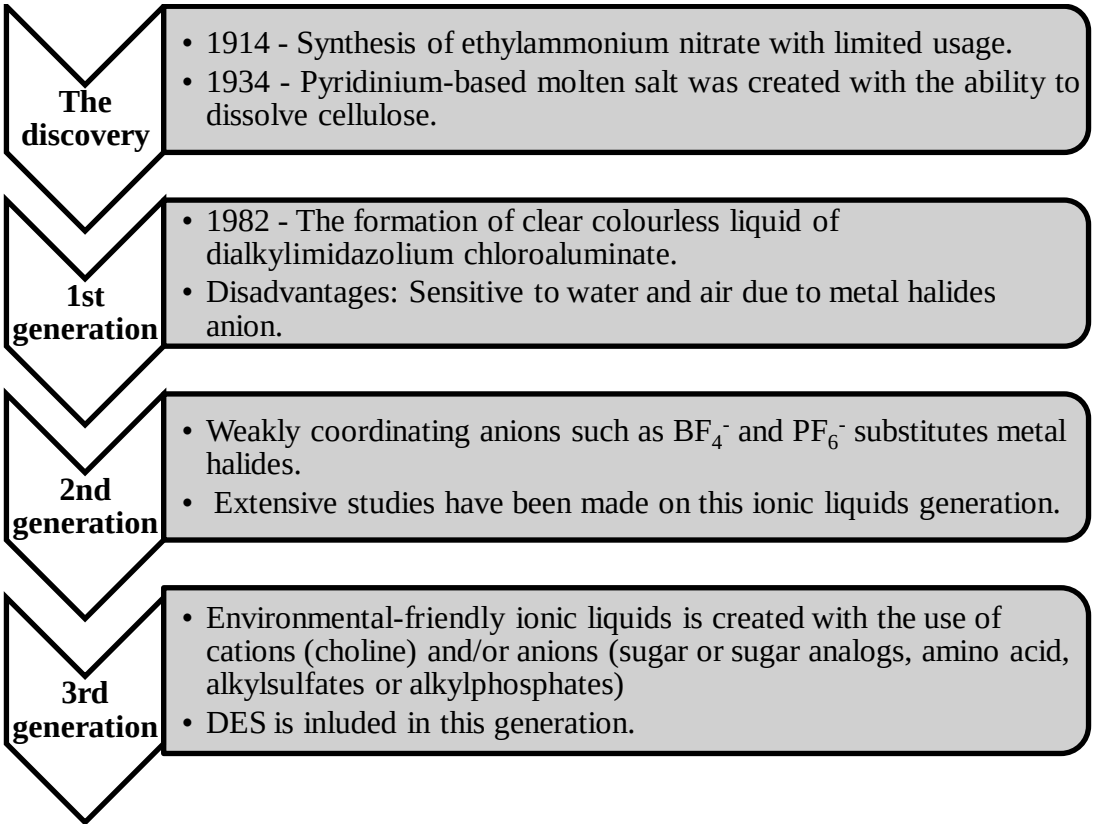
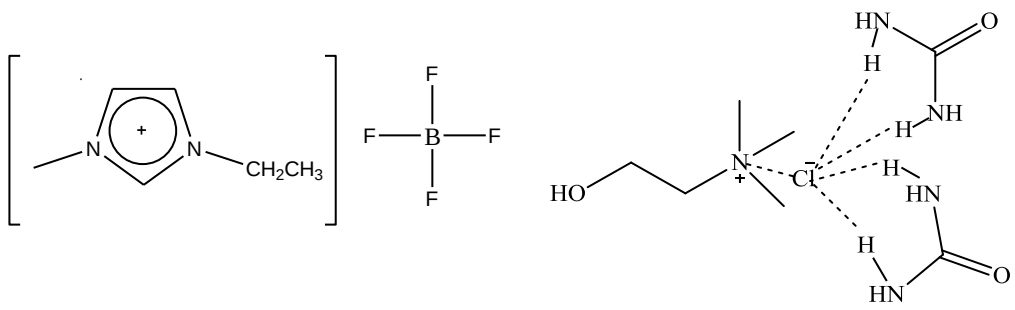


Figure 2.1 Evolution of ionic liquid discoveries

DES is a mixture of two or more components in a certain molar ratio that give a eutectic mixture, the melting point of the liquid is lower than the melting point of the individual components (Abo-Hamad *et al.*, 2015). Unlike ionic liquid which constituted entirely with ions, DES consisted of uncharged molecule of hydrogen bond donor for example urea, glycerol and ethylene glycol. Furthermore, DES differs from ionic liquid in terms of the interaction of molecules within the liquid in which DES formed through hydrogen bonding. On the other hand, ionic liquid formed through ionic bonding (Mbous *et al.*, 2017). Therefore, DES is recognized as different name from ionic liquid. Figure 2.2 (a) and Figure 2.2 (b) illustrate the differences between ionic liquid and DES in term of bonding.



(a) Ionic liquid comprised of 1-ethyl-3-methylimidazolium and tetrafluoroborate (Seddon *et al.*, 2000)

(b) Hydrogen bonding interaction in DES comprised of choline chloride and urea at molar ratio 1:2 (Liu *et al.*, 2015)

Figure 2.2 Chemical components of (a) ionic liquid (b) DES

2.3 Deep eutectic solvent (DES)

2.3.1 General properties

DESs form through intermolecular hydrogen bonds of two or three ingredients in which the mixture of those components resulting in the melting point lower than the melting point of its individual component. For example, DES comprises of choline chloride (T_m = 302 ºC) and urea (T_m = 133 ºC) forms a simple eutectic mixture at molar ratio 1:2 with melting temperature, T_m of 12 ºC (Abbott *et al.*, 2003). Melting point of DESs differs in term of hydrogen bond donors (HBDs), organic salts, anion of choline-based DESs and molar ratio of salt and HBD. There are some DESs that form liquid at room temperature while some are not. Generally, the melting temperatures for all reported DESs are below 150 ºC and those with melting point lower than 50 ºC are much more interesting since most of them appear as a liquid form at room temperature thus making them possible to be applied in

many fields as a solvent (Zhang *et al.*, 2012). As for viscosity, relatively high viscosities are reported for most DES at room temperature (> 100 cP).

2.3.2 Type of DES

The first investigation on DES was reported by Abbott and co-workers involving the eutectic mixtures of choline chloride and urea (2003). Generally, DES is classified into four types as depicted in Table 2.1. Type I DES make up from the combination of organic salts and metal halides (typically metal chloride, MCl_x). Type II DES comprises of organic salts and hydrated metal halides ($MCl_x.zH_2O$). Type III DES consists of organic salts and hydrogen bond donor (urea, glycerol, ethylene glycol, acetamide, etc). Finally, type IV DES composes of metal chloride and hydrogen bond donor.

Table 2.1 Types of DES

Type	General formula	Example	References
I	$Cat^+X^-.zMCl_x$	Choline chloride + $ZnCl_2$	(Abbott *et al.*, 2007; Kudłak *et al.*, 2015; Tang & Row, 2013; Zhang *et al.*, 2012)
II	$Cat^+X^-.zMCl_x.yH_2O$	Choline chloride + $CoCl_2.6H_2O$	
III	$Cat^+X^-.RZ$	Choline chloride + urea/glycerol	
IV	$MCl_x.RZ$	$ZnCl_2$ + urea/ethylene glycol	

$Cat^+ = R_1R_2R_3R_4N^+$ (typically ammonium, phosphonium, sulfonium cation)

Choline-based DES has been emphasized in majority of studies as it has low toxicity, low cost, biodegradable and readily available components. Furthermore, extensive investigations on type III DES has been studied with various hydrogen

bond donors. This class of DES offers tailorable physical properties depending on hydrogen bond donor chosen.

2.3.3 Lipase-catalyzed reaction in choline-based DES

Triacylglycerol acylhydrolase, EC 3.1.1.3 or its common name, lipase is a type of hydrolase enzyme which catalyzes reactions such as esterification, transesterification or alcoholysis, interesterification, hydrolysis, acidolysis and aminolysis through acting on carboxylic ester bonds. Lipase is extracted from microorganisms such as bacteria and fungi. Many lipases are commercially available from different suppliers and widely utilized in food, detergent and pharmaceutical industries. In addition, lipase has shown a great potential applications in other industries such as leather, textile, oil and fat, cosmetic and paper industries (Houde *et al.*, 2004).

Novozym 435 is one of the commercially available lipase from *Candida antartica B* immobilized on hydrophobic acrylic resin (*i*CALB) produced by Novozymes. The use of immoblized lipase is economical over free lipase as it is easy to be recovered and recycled thus it can be used for continuous operations until the catalytic activity is lost entirely (Stepankova *et al.*, 2013). Novozym 435 is stable over broad pH range (Novozymes, 2016). Therefore, the utilization of this kind of lipase is suitable for weakly basic DES comprised of choline chloride and urea. In addition, previous studies reported that Novozym 435 was active in all type of choline-based DES studied including DES composed of choline chloride and urea (Gorke *et al.*, 2008; Gorke *et al.*, 2010a). Besides, most of researchers in the previous

studies employed Novozym 435 as a source of lipase-catalyzed reactions. Thus, Novozym 435 is preferable in this study

Lipase-catalyzed reactions are commonly carried out in organic solvents. However, toxicity own by organic solvents as well as it causes the inactivation of lipase has embarked the use of alternative solvents such as ionic liquid and DES. From the aforementioned statement, most of starting materials of ionic liquids possessed toxicity effect to the environment especially aquatic species possibly through accidental spillage (Kudłak *et al.*, 2015). The emergence of DES as a solvent in lipase-catalyzed reactions creates a prospective future. A number of publications have been produced on the utilization of DES in lipase-catalyzed reactions for the past few decades especially type III choline-based DES. The previous study of lipase-catalyzed reaction in type III choline-based DES is summarized in Table 2.2.

Table 2.2 Lipase-catalyzed reaction in choline-based DES

DES	Lipase	Reaction	Substrates	Remarks	References
ChCl:U (1:2)	Novozym 435	Transesterification	Ethyl valerate and 1-butanol	Conversion = 93%	(Gorke *et al.*, 2008)
ChCl:U (1:2)	Novozym 435	Transesterification	Ethyl valerate and 2-butanol	Conversion = 74%	(Gorke *et al.*, 2010a)
ChCl:U (1:2) ChCl:Gly (1:2)	Novozym 435	Transesterification	Vinyl laurate and alcohols (butanol, octanol and octadecanol)	Conversion = 100% (for all alcohols) Selectivity = >99% (for all alcohols)	(Durand *et al.*, 2012)
ChCl:U:H_2O (1:2:3)	Novozym 435	Transesterification	Methyl *p*-coumarate and 1-octanol	Conversion = 100% Yield = 83% Hydrolysis = 10%	(Durand *et al.*, 2013a)
			Methyl ferulate and 1-octanol	Conversion = 90% Yield = ≥90% Hydrolysis = <2%	
ChCl:U: H_2O (1:2:1.5)	Novozym 435	Transesterification	Methyl *p*-coumarate and 1-alkanol (1-butanol, 1-octanol, 1-dodecanol, 1-hexadecanol)	Conversion = >70% (1-butanol), >90% (1-octanol, 1-dodecanol, 1-hexadecanol)	(Durand *et al.*, 2014)
ChCl:U: H_2O (1:2:2)			Methyl ferulate and 1-alkanol (1-butanol, 1-octanol, 1-dodecanol, 1-hexadecanol)	Conversion = >80% (1-butanol), >90% (1-octanol, 1-dodecanol, 1-hexadecanol)	

Table 2.2 Continued

ChCl:Gly (1:2)	Novozym 435	Transesterification	Propyl gallate and methanol	Conversion = 17.4% Yield = 60.4% Hydrolysis = 2%	(Ülger & Takaç, 2017)
ChCl:U: H_2O (1:2:1) ChCl:EG:H_2O (1:2:1.5)	Novozym 435	Esterification	Acetic anhydride and 1-butanol	Yield = 20% Yield = >70%	(Bubalo *et al.*, 2015)
ChCl:U (1:2) ChCl:Gly (1:2)	Lipozyme CALB L	Esterification	Oleic acid and decanol	Yield = 97.8% Yield = 97.7%	(Kleiner & Schörken, 2015)
ChCl:U (1:2) ChCl:Gly (1:2)	Lipozyme CALB L	Transesterification	Rapeseed oil and ethanol Used cooking oil and ethanol	Yield = 97.6% Yield = >80%	(Kleiner *et al.*, 2016)
ChCl:Gly (1:2 with 3% w/w H_2O)	Novozym 435	Transesterification	Waste cooking oil and methanol	Yield = 50%	(Merza *et al.*, 2018)
ChAc: Gly (1:1.5)	Novozym 435	Transesterification	Miglyol® oil 812 and methanol	Conversion = 97%	(Zhao *et al.*, 2011)
ChCl:Gly (1:2)	Novozym 435	Transesterification	Soybean oil and methanol	Conversion = 88%	(Zhao *et al.*, 2013)
ChCl:EG (1:2 with 4% v/v H_2O)	*Burkholderia cepacia* lipase (BCL)	Hydrolysis	*p*-nitrophenyl palmitate	Lipase activity in initial reaction rate = >9 U/mg	(Juneidi *et al.*, 2017)

ChCl = choline chloride, U = urea, Gly = glycerol, EG = ethylene glycol, ChAc = choline acetate

2.3.4 DES composed of choline chloride and urea

In this study, DES comprised of choline chloride and urea at molar ratio 1:2 was utilized as a solvent for lipase-catalyzed reaction. This type of DES is one of the pioneers in the study of solvent for ionic liquid substitution (Abbott *et al.*, 2003). In fact, majority of studies in DES had incorporated this type of DES as the subject of their studies (Gutiérrez *et al.*, 2009; Smith *et al.*, 2014; Yadav & Pandey, 2014). According to Durand and co-workers (2013b), the pH values of DES is dependent on the individual component of the DES. Therefore, DES composed of choline chloride and urea is considered to be weakly basic. The physicochemical properties of DES composed of choline chloride and urea are tabulated in Table 2.3.

Table 2.3 Physicochemical properties of DES composed of ChCl:Urea (Abo-Hamad *et al.*, 2015)

Choline chloride and urea (ChCl:Urea)	
Molar ratio	1:2
Melting temperature, T_m	12 ºC
Density	1.25 g/cm^3
Viscosity	750 cP (at 25 ºC)
Surface tension	52 mN/m
Conductivity	0.75 mS/cm (at 25 ºC)

2.4 Critical factors influencing lipase-catalyzed reaction

For most lipase-catalyzed reactions, the yields of products were greatly influenced by enzyme concentration, substrate concentration and temperature (Stergiou *et al.*, 2013). Based on previous research, the study on the effect of reaction parameters has been conducted for most of lipase-catalyzed reaction in organic solvent and ionic liquid. However, to date, limited studies are available for lipase-catalyzed reaction in DES. Table 2.4 summarizes the previous study on the effect of reaction parameters in lipase-catalyzed reactions.

Table 2.4 Reaction parameters studied in previous lipase-catalyzed reactions

Substrates and reaction	Lipase	Solvent	Reaction parameter and optimum value	Reference
Propyl gallate (PG) & methanol (Transesterification)	Novozym 435	DES	Water content= 10 % w/w Enzyme concentration= 40 g/l Substrate molar ratio= 1:6 (PG:alcohol) Temperature= 50 ºC Agitation speed= 200 rpm	(Ülger & Takaç, 2017)
Caffeic acid (CA) & phenethyl alcohol (Esterification)	Novozym 435	Ionic liquid	Temperature= 70 ºC Reaction time= 36 to 60 h Enzyme concentration= 1:15 (mass ratio CA:enzyme) Substrate molar ratio = 1:30 (CA:alcohol)	(Ha *et al.*, 2013)
Methyl caffeate (MC)/Ethyl caffeate (EC) & 1-propanol (Transesterification)	Novozym 435	Ionic liquid	Enzyme concentration= 1:20 (mass ratio MC:enzyme), 1:15 (mass ratio EC:enzyme) Temperature= 60 ºC Substrate molar ratio= 1:5 (MC:alcohol), 1:10 (EC:alcohol)	(Pang *et al.*, 2013)
CA & phenethyl alcohol (Esterification)	Novozym 435	Ionic liquid with co-solvent DMSO	DMSO content= 2% v/v Enzyme concentration= 1:18 (mass ratio CA:enzyme) Substrate molar ratio= 1:30 (CA:alcohol) Temperature= 80 ºC CA concentration= 65 mmol/l	(Gu *et al.*, 2014)
MC & 2-cyclohexylethanol/ 3-	Novozym 435	Ionic liquid	Substrate concentration= 50 mM MC, 400 mM	(Kurata *et al.*, 2010)

Table 2.4 Continued

cyclohexyl-1-propanol (Transesterification)			3-3-cyclohexyl-1-propanol Temperature= 80 ºC	
CA & phenethyl alcohol (Esterification)	Novozym 435	Isooctane	Solvent= Isooctane Temperature= 70 ºC Substrate molar ratio= 1:92 (CA:alcohol) Enzyme concentration= 1:15 (mass ratio CA:enzyme) Agitation speed= 50 rpm	(Widjaja *et al.*, 2008)
EC & glycerol (Transesterification)	Novozym 435	Solvent-free system	Reaction system pressure= 90 kPa Temperature= 70 ºC Substrate molar ratio= 1:50 Enzyme concentration= 20% w/w	(Sun & Hu, 2017b)
1) Ferulic acid (FA) & ethanol 2) *p*-methoxycinnamic acid & 2-ethyl hexanol (Esterification)	Novozym 435	Isooctane	Solvent system= isooctane Temperature= 1) 75 ºC and 2) 80 ºC Water content= 1 mg Enzyme concentration= 1:2 (mass ratio acid:enzyme) Substrate molar ratio= 1:5 (FA:alcohol)	(Lee *et al.*, 2006)
Vinyl acetate (VA) & geraniol (Transesterification)	*Pseudomonas cepacia* lipase (PCL) immobilized on biodegradable polymer film	Toluene	Solvent= toluene Agitation speed= 140 rpm Enzyme concentration= 50 mg Temperature= 55 ºC Substrate molar ratio= 4:1 (VA:alcohol)	(Badgujar & Bhanage, 2014)