

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

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SYNTHESIS OF GLYCERYL MONOCAFFEATE
VIA TRANSESTERIFICATION**

by

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**Thesis submitted in fulfilment of the
requirements for the degree of
Master of Science**

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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>i</i> CALB	Immobilized <i>Candida antartica</i> 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 °C hingga 60 °C. 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 °C 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 °C sehingga 55 °C 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 °C to 60 °C. 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 °C 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 °C to 55 ° 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).

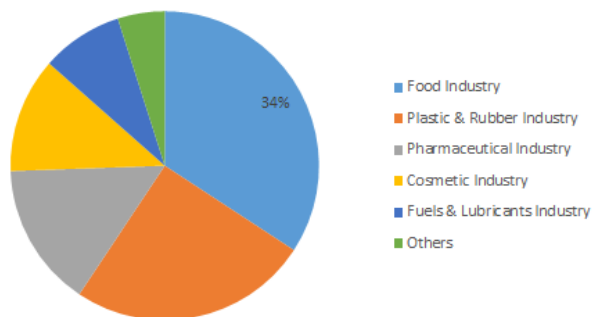


Figure 1.1 Phenolic antioxidant industries (Anand *et al.*, 2017)

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.

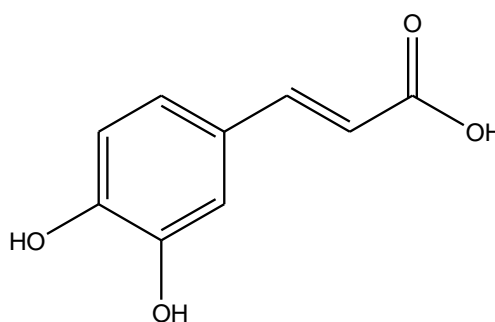


Figure 1.2 Chemical structure of caffeic acid

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 (OCH₃) 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 its 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 posed 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 °C. GMC could be synthesized using enzymatic reaction which is more energy-friendly as it has high selectivity and requires only medium temperature from 30 °C to 60 °C 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 (Kudlak *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 (Kudlak *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.

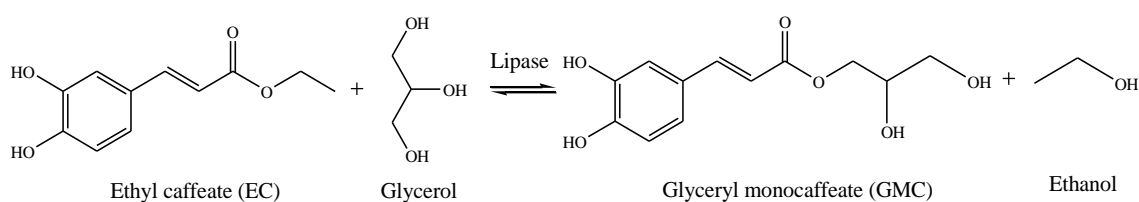


Figure 1.3 Reaction scheme for lipase-catalyzed synthesis of GMC via transesterification reaction of EC and glycerol

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.