Parameters study of lipase-transesterification reaction of ethyl caffeate and glycerol in deep eutectic solvent (DES)

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Abstract. The toxicity of organic solvent to human and environment has triggered researchers to find an alternative green solvent for the past 20 years. Deep eutectic solvent (DES) is the newly emerging green solvent that is being utilized in biocatalysis. The boiling point of DES is higher than conventional organic solvent thus enables for lipase to perform at its optimum temperature. However, the transesterification reaction in DES is limited due to its high viscosity. Therefore, water was added to DES media to reduce its viscosity. This study reported on the use of choline-based DES for lipase-catalyzed transesterification of ethyl caffeate and glycerol. The transesterification reactions in DES comprised of choline chloride and urea at molar ratio 1:2 resulted in high conversion of ethyl caffeate (over 90%) under the following parameters: enzyme loading (1250 Unit Activity); amount of water (20 vol%) and agitation speed (200 rpm). This work could provide an insight to the potential of DES as a promising alternative solvent for the production of glyceryl caffeate esters due to high conversion, faster reaction time and reduction of dangerous organic solvent consumption.

1. Introduction
The toxicity and harmful effect that the organic solvents possess has boosted researchers to find alternative solvents for synthetic chemical reaction. In addition, one of the green chemistry principles outlined that benign solvent should be used [1]. The most outstanding substitute solvents so far are ionic liquids (ILs) and deep eutectic solvents (DESs). Having non-volatility and thermal stability properties, the latest generation of ionic liquid usually comprised of organic cations such as pyridinium, imidazolium, pyrrolidinium, and phosphonium; and anions like methylsulphate, tetrachloroalumine, methansulfonium and hexafluorophosphate [2]. Despite having great physical properties suitable for synthetic chemical reaction, ILs requires expensive starting material components [3], as well as its common starting material components used, are toxic to the environment according to investigation made by toxicologist, refuting the claim that it is a green solvent [4]. Furthermore, the impurities found in ILs required for purification process of ILs is not economical for further industrial application [5]. The emergence of DESs overcomes the drawback of ILs as it owns physical properties quite similar to ILs. DESs consisting of hydrogen bond acceptor and hydrogen bond donor components to create a liquid with a low melting point than the single components resulting from intermolecular hydrogen bonding between those components [2, 6]. In contrast to ILs, the starting materials of DESs are more environmental-friendly and can be easily prepared from readily available components. The most employed hydrogen bond acceptor, the choline-based quaternary ammonium salt is usually mixed with
amide, urea, carboxylic acids, alcohols, sugars or organic acids to form the DES at a specific molar ratio [6, 7]. DES is utilized in organic synthesis, biocatalysis, nanotechnology, biotransformation, electrochemistry, extraction, analysis and gas adsorption [6, 8]. Currently, lipase-catalyzed reaction for the production of biodiesel is the most widely employed enzymatic reaction that applies DES as the solvent. For instance, in the production of biodiesel from soybean oil [9], rapeseed oil [10] and yellow horn seed oil [11]. DES also has been utilized as a solvent for the production of phenolic compound involving the reaction between methyl p-coumarate and methyl ferulate with 1-octanol [12].

A hydroxycinnamic acid such as caffeic acid (CA, 3,4-dihydroxycinnamic acid) is a phenolic compound that is produced by plant as a secondary metabolite. A plentiful amount of CA is widely distributed in nature for example in fruits, vegetables, oils, and tea. CA exhibits several biological activities like antioxidant [13, 14], anticancer [15] and anti-HIV [16]. Unfortunately, the solubility of CA in a polar and non-polar media demonstrated unsatisfying result thus modification into caffeic acid esters is required to enhance its solubility. The most widely synthesized caffeic acid esters using lipase are alkyl caffeates [17, 18] and caffeic acid phenethyl esters (CAPE) [19]. The first glycerol-based caffeate ester was previously produced in solvent-free system using lipase enzyme [20] and in ionic liquid which functioned both as catalyst and solvent [21]. Glycerol-based caffeate ester has high solubility in water as compared to CA (1.76 mg/ml in water at 20 °C) [20]. At the present time, the production of glycerol-based caffeate esters in DES using lipase has not being reported. The addition of water will improve the performance and activity of lipase in DES media since water is providing hydration to the enzyme. In this work, the lipase-catalyzed transesterification reaction of ethyl caffeate and glycerol to produce glycerol-based caffeate esters using a commercial lipase was carried out. This study aims to investigate the effect of reaction parameters that affect the conversion of ethyl caffeate in DES consists of choline chloride-urea. The outcomes of the investigation are important for better understanding of the potential of DES as a solvent for further application in food, cosmeceutical and pharmaceutical industries.

2. Materials and method

2.1. Enzyme and materials
Commercial lipase containing Novozym 435 was purchased from Novozymes (Bagsvaerd, Denmark). Choline chloride (ChCl), glycerol (purity 99.5%) and 4Å molecular sieve were from Sigma-Aldrich, Co. (United States). Urea (U) and caffeic acid (CA) were purchased from Acros Organics (New Jersey, United States). Ethyl alcohol (ethanol), anhydrous sodium sulfate and glacial acetic acid (HPLC grade) were purchased from Fisher Scientific (M) Sdn. Bhd. (Malaysia). Sodium carbonate and methanol (HPLC grade) were from Merck (Germany). Diethyl ether was from Fine Chemicals Corporation (PTY) Ltd (Cape Town, South Africa).

2.2. DES preparation
Hydrogen bond acceptor, choline chloride was dried under vacuum oven at 60 °C as it is hygroscopic. ChCl and U were weighed immediately at molar ratio 1:2 so that only minimum air exposure occurred. The mixture was heated on hot plate with magnetic stirrer (11-100-49SH, Fisher Scientific) at 100 °C with 300 rpm rotation speed until a colourless liquid is obtained (typically 2 hours).

2.3. Synthesis of ethyl caffeate
Ethyl caffeate (EC) synthesis was altered slightly from the method previously described by Pang and Fiuza [17, 18]. Caffeic acid (2.5 g) was refluxed with 200 ml of ethanol and 2.5 ml H2SO4 for approximately 8 h with the addition of molecular sieve to absorb any water produced from the reaction. The rotation speed was set at 400 rpm (MS-DMS, PLT Scientific). The mixture was then neutralized with 10 vol% Na2CO3 solution. The extraction of ethyl caffeate was done utilizing diethyl ether (3x150 ml) and the organic phases collected were then combined and washed with distilled water (3x200 ml) until a clear yellowish solution was acquired. Later, the solution was dried with anhydrous MgSO4 followed by solvent removal under reduced pressure. The residue was filtered and evaporated until
yellowish solid formed.

2.4. Lipase-catalyzed transesterification of ethyl caffeate and glycerol
Enzymatic transesterification of ethyl caffeate and glycerol was carried out according to the procedure previously described by Sun and co-workers [20], with slight modifications. Ethyl caffeate and glycerol (molar ratio 1:50) were dissolved in DES in tight closed jar and the reaction was initiated by adding Novozym 435 at 40ºC in orbital shaker (Infors HT Ecotron). At a certain period of times, the aliquots taken were diluted with methanol, filtered using 0.20 µm syringe filter and injected to HPLC. The aliquots were assessed with HPLC (Shimadzu Corporation, Japan) using a chiral column (Regis Technologies, Inc., Illinois, United States) connected to a UV detector set at a wavelength of 325 nm. The isocratic elution was performed using a mobile phase of methanol: water and 0.5% acetic acid (80:20, v/v). All experiments were conducted in triplicates.

3. Results and Discussions
3.1. Effect of lipase loading amount
Novozym 435 with unit activities ranged from 250 U to 1500 U was chosen to monitor the effect of enzyme loading on the lipase-catalyzed transesterification in DES. Final EC conversion increased from 250 U to 1250 U due to the increase in active site for substrates to bind. However, the conversion rate started to decline at 1500 U (see Figure 1). The movement of substrates and enzymes were restricted because of slow mass transfer in the system when more enzymes were added in addition with the high viscosity of DES. Furthermore, enzyme aggregation occurred due to its tendency to attract with each other when in excess resulting in reduction of binding of substrates to the enzyme [17, 22]. Enzyme with unit activity of 1250 U was employed for further investigation.

![Figure 1](image)

**Figure 1.** Effect of lipase loading amount on EC conversion. Reaction conditions: molar ratio of ethyl caffeate to glycerol is 1:50; agitation speed, 150 rpm; temperature, 40 ºC.

3.2. Effect of water content
The high viscosity of DES led to a practical problem as such the transferring process of the solvent was extremely time-consuming [23]. Diluting it with water could reduce the viscosity. The water content in the lipase-catalyzed transesterification in DES was investigated by varying the amount of water from 1 vol% to 40 vol% of the total volume of the mixture. The conversion achieved a plateau at 20 vol% with conversion over 98% within 4 hours (see Figure 2). Water is essential for enzyme to stay hydrated and
maintain its natural conformation in order to fully function. The addition of water was found to increase the conversion and yield for the lipase-catalyzed transesterification of methyl ester with several alkanols in DES as reported by Durand and his co-workers [24]. Since water can cause hydrolysis reaction of ethyl caffeate to caffeic acid thus 20 vol% was chosen for the next parameter investigation to minimize hydrolysis reaction. Moreover, a higher concentration of water in DES could rupture the choline chloride-urea bond and making the components appeared as free forms in the DES liquid, resulting in the disturbance of DES properties [23, 25].

3.3. Effect of agitation speed
Mass transfer limitations could also be overcome by investigating the effect of agitation speed on the lipase-catalyzed transesterification reaction in DES. Figure 3 depicted the effect of agitation speed on the initial rate of lipase-catalyzed transesterification in DES investigated at four different speeds, namely 100, 150, 200 and 250 rpm. The initial rate showed an increment when the agitation speed increased due to the high collision between substrates and enzymes. However, at higher agitation speed than 200 rpm, the initial rate remained steady. The previous study reported that the initial rates were unchanged as the agitation speed was higher than 200 rpm. They deduced that no external diffusion factor when the agitation speed was higher than 200 rpm [26]. EC conversion as illustrated in Figure 4 showed that no significant change can be seen making 200 rpm the chosen agitation speed.

Figure 2. Effect of water content on EC conversion. Reaction conditions: molar ratio of ethyl caffeate to glycerol is 1:50; agitation speed, 150 rpm; temperature, 40 ºC; lipase loading, 1250 U.

Figure 3. Effect of agitation speed on initial rate. Reaction conditions: molar ratio of ethyl caffeate to glycerol is 1:50; temperature, 40 ºC; lipase loading, 1250 U; water content, 20 vol%.
Figure 4. Effect of agitation speed on EC conversion. Reaction conditions: molar ratio of ethyl caffeate to glycerol is 1:50; temperature, 40 ºC; lipase loading, 1250 U; water content, 20 vol%.

4. Conclusions
In the present study, it was found that lipase-catalyzed transesterification of ethyl caffeate and glycerol can be carried out in DES. The overall conversion of more than 90% was achieved using 1250 Unit Activity of Novozym 435 with 20 vol% water and agitation speed of 200 rpm in 1:2 molar ratio of choline chloride: urea. It is suggested that the addition of water has reduced the viscosity of DES and reducing the mass transfer limitation during the reaction. Nevertheless, water should be controlled to minimize the hydrolysis reaction and to prevent total rupture of choline chloride-urea bond in DES. A study on the optimization of the reaction parameters, kinetic and thermodynamic analysis should be carried out before it could be applied to industry. This study provides valuable information on the prospect of DES as a solvent which highly biodegradable and possesses no threat to the environment.

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