Choline Chloride-urea Deep Eutectic Mixture Water for the Synthesis of an Amphiphilic Compound of Glyceryl Monocaffeate

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Abstract: The Lipase-catalyzed synthesis of glyceryl monocaaffeate (GMC) in choline chloride-urea of natural deep eutectic solvent (NADES) media is reported to provide amphiphilic character to caffeic acid (CA). The modification of CA into GMC could potentially increase its solubility and widen the application of CA's biological activities in water and oil-based systems. The high conversion was achieved when the reaction was carried out with the addition of more than 20 %v/v water, at a high molar ratio of glycerol and 40°C. It was found that the lipase-catalyzed transesterification of ethyl caffeate (EC) and glycerol in choline chloride-urea of DES media obeyed ping-pong bi-bi mechanism with $V_{\text{max}} = 10.9 \text{ mmol.min}^{-1}$, $K_{\text{mEC}} = 126.5 \text{ mmol}$ and $K_{\text{mGly}} = 1842.7 \text{ mmol}$. 

Key words: eutectic, glycerol, glyceryl monocaaffeate, lipase, enzyme kinetic

1 Introduction

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 as illustrated in Fig. 1. CA are present abundantly in many food sources including coffee, tea, vegetables, and fruits like blueberry, raspberry, blackberry, etc.1 CA is well known for its antioxidant activity. The antioxidant activity is closely associated with the number of hydroxyl groups on the aromatic ring.2 As compared to ferulic acid with methoxy (OCH₃) on its C3 position of the aromatic ring, the efficiency of the antioxidant activity of CA is greater since it contains two hydroxyl groups which contribute to additional resonance stabilization.2-4. Apart from being an antioxidant, CA has also demonstrated anti-microbial, anticancer, anti-inflammatory, and anti-HIV properties3,4. Nonetheless, its lipophilicity or effectiveness in the 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.5 CA also has low solubility in water systems.6 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 esters derivatives which was initially identified in oats.7 The addition of the glycerol moiety 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.8 Glyceryl monocaaffeate (GMC) is a

![Fig. 1 Reaction scheme for the lipase-catalyzed synthesis of GMC via transesterification reaction of EC and glycerol.](image-url)
caffeeic acid ester derivative with solubility in water about 3 times as compared to CA (1.76 mg.mL⁻¹ in the water at 20°C). The enhancement of hydrophilicity and lipophilicity in GMC could provide a promising new type of compound that is water and oil soluble with a plethora of biological activities. GMC could be synthesized via lipase-catalyzed transesterification reaction in an organic solvent at mild reaction conditions. By carried out the reaction in the organic solvent may increase the solubility of the substrate for improving yield, however, this approach denying the green chemistry concept. As alternative deep eutectic solvent or specifically natural deep eutectic solvent (NADES) has emerged as a substitute to organic solvent. Recent success on the enzyme-catalyzed reaction in the NADES has been reported in numerous reports. Besides NADES, propylene carbonate could also be used as a green solvent for lipase catalyzed synthesis where recently proven by Cumming and co-workers during the synthesis of GMC. However, our work was focused on the feasibility of NADES reaction media of choline chloride-urea for the synthesis of GMC via an immobilized lipase-catalyzed reaction of ethyl caffeate (EC) and glycerol. In addition, the possible reaction mechanism was investigated to provide valuable information on the biocatalytic reaction in the used media.

2 Methodology

2.1 Materials and chemicals

The lipase used for the transesterification reaction was originated from Candida Antarctica B immobilized on the acrylic resin (Novozyme 435). CA and urea were purchased from Acros Organic. Choline chloride, glycerol, and 4-nitrophenyl butyrate were purchased from Sigma-Aldrich. Diethyl ether, ethanol, magnesium sulfate anhydrous, acetonitrile, disodium hydrogen orthophosphate, and acetic acid were obtained from Fisher Scientific. Sodium carbonate, sulfuric acid, sodium chloride, sodium hydroxide, and methanol were from Merck-Millipore. Whereas the standard substrate of EC was purchased from Enzo Life Sciences.

2.2 Preparation of NADES

The NADES used in the current study is based on a eutectic mixture of choline chloride and urea. The NADES was prepared according to the procedure described by. The choline chloride and urea are hygroscopic, thus it was dried in a vacuum oven at 0.5 bar and 60°C before further use. The dried choline chloride and urea were mixed at a molar ratio of 1.2 in a sealed container. Subsequently, the mixture was heated at 100°C and slowly stirred to form a colourless liquid after approximately two hours. The duration required to form the liquid depends on the total volume, temperature, and homogeneity of the mixture.

2.3 Synthesis of ethyl caffeate

The EC was synthesized through an acid-catalyzed esterification reaction according to the procedure described by. 2.5 g of caffeic acid, 200 mL of ethanol and 2 mL of H₂SO₄ were mixed in a round bottom flask. The reaction has proceeded at 60°C under reflux condition and continuously stirred at 400 rpm for about 8 hr. Beforehand, the molecular sieve was also added to the reaction mixture to absorb the water by-product produced during the reaction. Then, the reaction mixture was cooled and neutralized with 10% v/v of sodium carbonate. The EC produced was extracted using diethyl ether to form a clear yellowish solution. Subsequently, the trace amount of water was removed using anhydrous magnesium sulfate. The liquid mixture was collected and the EC was recovered from diethyl ether by using a rotary evaporator at reduced pressure. The formation of EC was confirmed using the Nuclear magnetic resonance (NMR) analysis.

2.4 Enzyme activity assay

The activity of the enzyme was assayed by using 4-Nitrophenyl butyrate (PNPB) as a standard substrate. The assay mixture was 100 mM sodium phosphate buffer solution with the addition of 0.5% (v/v) Triton X-114. Then, the pH of the resulted assay mixture was adjusted to pH 7.2 at 37°C with a 1 M sodium hydroxide solution. Meanwhile, 50 mM of PNPB was prepared in acetonitrile as the standard substrate solution. During the assay, 10 mg of the immobilized enzyme is added to 1 mL of assay reagent and then incubated in an orbital shaker at 37°C for 30 min. Afterward, 0.01 mL of standard substrate solution is added to allow the reaction to occur for 10 min at 37°C. The reaction solution was filtered by using a syringe filter and poured into a cuvette to remove the immobilized enzyme. The change of colour was quantified by using UV-Vis spectrophotometer at 400 nm and 1 cm light path length. All assays were performed in triplicates. The unit activity was calculated by using the following equation:

\[
\text{Activity (IU)} = \frac{1}{t \cdot V_{\text{Enz}}} \left( \frac{A_{\text{Test}} - A_{\text{Blank}}}{\epsilon \cdot V_{\text{T}}} \right)
\]

Where; \(A\) is the absorbance value (abs), \(V_T\) is the total volume of the assay (mL), \(df\) is the dilution factor, \(t\) is the assay duration (min), \(V_{\text{Enz}}\) is the volume of the enzyme assay solution and \(\epsilon\) is the millimolar extinction coefficient of 4-nitrophenol at 400 nm (1.48 × 10⁻⁵ M⁻¹.cm⁻¹). The unit activity (IU) definition means that one unit will release 1.0 micromole (10⁻⁶ mole) of 4-nitrophenol per minute at pH 7.2 at 37°C using 4-nitrophenyl butyrate as a substrate.

2.5 Lipase-catalyzed transesterification reaction

The substrates of EC and glycerol were dissolved in 5 mL of DES with added water. The resulting mixture was stirred for a few minutes to allow it homogenized. The reaction was carried out in the incubator shaker and started.
when the immobilized enzyme added to the reaction media. A 100 µL of the sample was periodically withdrawn from the reaction media and diluted in 900 µL methanol. Then, the sample was filtered by using 0.2 µm of syringe filter. The dissolved components in the sample was analyzed by using High-performance liquid chromatography (HPLC). All experiments were conducted at least in triplicates. The conversion of the EC was calculated by using the following equation:

\[
\text{Conversion (\%)} = \frac{C_{CE, f} - C_{CE, i}}{C_{CE, i}} \times 100\%
\]

where, \( C_{CE, i} \) and \( C_{CE, f} \) are the initial and final concentrations of EC. Since the conversion was taken as the amount of EC consumed. The conversion should not be taken as an absolute formation of GMC as there are possibilities of the formation of CA due to reverse reaction.

\subsection*{2.6 Determination of kinetic mechanism}

The experiment to study the kinetic mechanism of the reaction was carried out at the same reaction media. The reaction temperature was 40°C and agitated at 200 rpm. The unit activity and reaction period were remained constant. The initial rate of reaction was taken from the time-progressive curve of the product. The concentration of EC and glycerol were varied between 0.02 to 0.1 M and 1.0 to 1.8 M, respectively. Lineweaver-Burk's plot was used to determine the kinetic mechanism involved.

\subsection*{2.7 HPLC analysis}

The concentration of the substrate and product presence in the sample withdrawn from the reaction media was quantified using the HPLC coupled with ultraviolet (UV) detector at wavelength 325 nm. The injection volume and the flow rate were set at 0.5 µL and 0.5 mL/min. (R,R)-Whelk-O1 chiral column (Regis Technologies, USA) was used for the detection and separation of substrate and product at an oven temperature of 40°C. The mobile phase used to consist of isocratic mixtures of methanol and deionized water (DI) with an addition of acetic acid as a modifier for better separation (methanol: DI + 0.5%v/v acetic acid, 80:20%v/v). The acquisition of the data was carried out for 15 minutes and no obvious peak appeared after that period.

\subsection*{2.8 Nuclear magnetic resonance (NMR) study}

The study was conducted at the School of Chemical Sciences, Universiti Sains Malaysia by using the proton nuclear magnetic resonance (1H-NMR) to confirm the structure of the EC synthesized. The sample was dissolved in deuterated acetone (acetone-d6) for the analysis.

\subsection*{2.9 Electrospray ionization-mass spectroscopy (ESI-MS) study}

The qualitative identification of GMC produced was carried out by MUPA Laboratory, School of Chemical Sciences, Universiti Sains Malaysia using Liquid Chromatography-Mass Spectrometry (LCMS-TOF Agilent 1290) with electrospray ionization (ESI) method. The total run time was 3 min.

\section*{3 Results and Discussion}

\subsection*{3.1 Chemical characterization and analysis}

The results on the chemical characterization are shown in Fig. 2. The structure of EC synthesized was confirmed using NMR analysis. The 1H-NMR data are similar to those reported by Xiang and co-worker (Fig. 2(a)): 1H-NMR (400 MHz, Acetone-d6) δ 1.27 (3H, t, CH3/H-1, J = 5.6 Hz), 4.18 (2H, q, CH2/H-2, J = 5.6 Hz), 6.28 (1H, d, H-4, J = 12.8 Hz), 6.87 (1H, d, H-8, J = 6.4 Hz), 7.04 (1H, d, H-7, J = 6.8 Hz), 7.16 (1H, d, H-11, J = 1.6 Hz), 7.53 (1H, d, H-4, J = 12.8 Hz), 8.29 (1H, s, Ar-OH). The composition of the reaction mixture was analyzed by using HPLC. The peak at 8.8 and 9.4 min (Fig. 2(b)) corresponds to EC and was proved by comparing the chromatogram of the standard EC obtained from Sigma (Fig. 2(c)). The two peaks that appeared at next to each other at different retention times might be due to the stereoisomers of EC (cis-EC and trans-EC). Whereas the peak at 6.8 min belongs to the products. The GMC and EC present in the reaction mixture were further analysed by using the electrospray ionization method mass spectrometry (ESI-MS). The compound can be identified based on their molecular weight which appeared in the spectroscopy spectrum as shown in Fig. 2(d). The formation of CA because of a reverse reaction is possible as this compound was detected in the ESI-MS spectrograph (at m/z 178.9976, Mw 180 g.mol⁻¹). The peaks of deprotonated ions, [M-H]⁻ at m/z 207.0279 and 253.1276 in the MS spectrum under negative ion mode are characterized as EC (Mw: 208.1 g.mol⁻¹) and GMC (Mw: 254.1 g.mol⁻¹), respectively.

\subsection*{3.2 Effect of enzyme loading}

The unit activity of the enzyme was varied between 250 to 1500 IU to investigate its implication on the EC conversion in a pure NADES solvent system. As expected, the conversion of EC was very low (< 22%) due to the nature of pure DES as being highly viscous. Previously, Durand and co-workers reported an even lower conversion of < 2% during esterification of phenolic acid ester and 1-octanol in a pure eutectic mixture of Choline Chloride-Urea media. Besides, the association of EC and glycerol as a part of the NADES hydrogen bond network is also a concern. The formation of hydrogen bonds by the substrates and the NADES may hinder the mechanism of the reaction, especially the formation of a carbocation. Regardless, the EC conversion gradually increases when a
higher unit activity of the enzyme was used as shown in Fig. 3(a). The conversion reaches a maximum level when the unit activity is at 1250 IU. However, it decreases if the unit activity was increased to 1500 IU. Based on the observation, the immobilized enzyme was agglomerated at high loading in the reaction media. This phenomenon is due to the hydrophobic nature of the support made of microporous acrylic resin or may be due to the lower mobility of the immobilized enzyme in the viscous media. This observation is similar to those reported by Sun and co-workers [17]. As a result, it gives a poor distribution of the immobilized enzyme in the media, high steric hindrance, and induces diffusion limitation of the substrate. However, these drawbacks can be solved by reducing the viscosity of the NADES with the addition of water [18]. The mobility and dispersion of the immobilized enzyme should be considered as their hydrodynamic behavior strongly influenced by its chemical characteristic either in aqueous, organic, or DES media.

3.3 Effect of reaction time

The reaction period required to reach maximum conversion is normally overlooked. By taking data too early might result in the wrong conclusion for some cases as the reaction is not completed. In the present work, the data were taken when the reaction has reached a plateau. Several parameters could prolong the time required to reach maximum conversion such as the unit activity of the enzyme (i.e., a specific activity or enzyme loading), temperature, substrate concentration, etc. In the present work, the reaction time was varied between 15 to 240 min as depicted in Fig. 3(b). The enzyme activity, temperature, water content, and EC:Glycerol molar ratio were fixed at 1250 IU, 40°C, 20%v/v, and 1:50, respectively. The conversion rate is relatively fast where the conversion reaches 62% in 15 min. After 60 min the reaction began to reach a plateau at 87% of conversion. In comparison, 72 hr was required for the synthesis of octyl p-coumarate by Novozyme 435 in the same NADES media [16]. This positive result might be due to higher unit activity and EC:Glycerol ratio employed. Fast conversion shall increase productivity, however excess amount of enzyme may not be a cost-effective approach. Hence, it is suggested to conduct cost and yield optimization analysis before actual production. In this study, most of the data were taken after 4 hr to allow the reaction to reach the highest conversion.

3.4 Effect of substrate molar ratio

The transesterification reaction is a reversible process. It is very common to shift the reaction equilibria toward ester formation by using one of the substrates in excess. In the present work, glycerol was used in excess with EC:Glycerol molar ratio varied between 1:40 to 1:90. The EC conversion of more than 95% was achieved as anticipated. Based on Fig. 3(c), the conversion of EC increases from 95 to 98% as the molar ratio of EC to glycerol is increased up to 1:50. Further increase in molar ratio maintains the EC conver-
Choline Chloride-urea Deep Eutectic Mixture for the Synthesis of Glyceryl Monocaffeate

J. Oleo Sci.

3.5 Effect of water content

The viscosity of the NADES causes problems in their application for the separation process or reaction media. The viscous solvent exhibits a poor hydrodynamic profile and difficult to operate. Hence, the addition of water has been suggested to reduce its viscosity and make it more convenient to handle. The water content was varied from 0 to 40% v/v during the lipase-catalyzed transesterification of EC and glycerol. As expected, the effect of water addition is very profound to the enzymatic reaction in DES media as depicted in Fig. 3 (d). Without water, the conversion of EC in a NADES media is merely <22%. The EC conversion significantly improves when 5% v/v of water was introduced. This result implies that NADES (choline chloride/urea) was unable to replace the role of water on catalytic action of the enzyme. A complete conversion of the EC is possible when the water content is over 20% v/v. However, the increase in conversion might not be completely due to transesterification nor reduced viscosity, but rather due to the competing hydrolysis reaction of ethyl caffeate to caffeic acid in the presence of an excess amount of water. Hence, we recommend future investigation on the extent of hydrolysis reaction during the transesterification of ethyl caffeate and glycerol within the same reaction system. Even though water is required for enzyme hydration, a small quantity is usually sufficient for transesterification. If the hydrogen bond between the water and choline chloride are unable to inhibit the hydrolysis reaction, dilution with other nonreactive compounds should be proposed.

3.6 Effect of reaction temperature

Chemical synthesis at lower temperatures reduces energy demand and utility costs. However, at lower temperature does not give better conversion even though for biocatalyst such as enzymes. The optimal temperature always exists for any enzyme-catalyzed reactions. Thus, the optimal temperature for lipase-catalyzed transesterification of EC and glycerol was investigated at a temperature between 30 to 60°C. It was found that the conversion of EC to GMC is gradually increased with higher temperatures as shown in Fig. 4 (a). However, this trend is insignificant at a temperature of more than 40°C where the conversion remains close to 90%. This result is consistent with the previous investigation where Novozyme 435 normally shows optimal performance at between 40 to 45°C. The enzyme employed is rather productive at a lower temperature, which gives 72% conversion at 30°C.

3.7 The activation energy of the reaction

During a chemical reaction, sufficient energy is needed to overcome the energy barrier called activation energy ($E_a$) to form the product. In an enzymatic reaction, enzyme increases the rate of reaction by lowering the activation energy.
energy. The activation energy for lipase-catalyzed reaction can be estimated based on Arrhenius's plot; a natural logarithm of the initial rate of reaction $\ln v$ against reciprocal of absolute temperature $1/T$. Based on the negative slope of Arrhenius's plot in Fig. 4(b), the $E_a$ value estimated is 50.4 kJ.mol$^{-1}$ with an $R^2$ value of 0.923. Enzymes with low activation energy are preferable for industrial applications.

Zanin and co-workers reported that normal activation energy for most enzymes is smaller than 104.6 kJ.mol$^{-1}$ [21]. In this study, the value of the activation energy is comparable with the one reported by Sun et al. ($E_a = 44.23$ kJ.mol$^{-1}$) and by Xin et al. ($E_a = 65.04$ kJ.mol$^{-1}$) for lipase-catalyzed transesterification of EC and ethyl ferulate, respectively [17, 22].

### 3.8 Optimization of parameters

Three significant parameters were chosen based on the screening study which are enzyme loading, water content, and reaction time. The effect of the substrate molar ratio was excluded since the reaction is carried out at excess glycerol. Whereas the temperature was fixed at 40°C as the minimal impact was recorded. The optimization was carried out using a central composite design (CCD) available in the Design-Expert software. Based on the analysis, a quadratic model was proposed ($p < 0.05$) to estimate the conversion of the EC with $R^2 = 0.9344$ and the lack of fit was 0.0728 ($p > 0.05$). The final equation expressed in coded factors for conversion of the substrate is represented as Eq. 3:

$$\text{Conversion} = 70.51 + 13.88A + 29.29B + 13.68C + 2.71AB - 0.75AC + 1.81BC - 7.73A^2 - 13.49B^2 - 6.60C^2$$

where; $A$ is water content, $B$ is enzyme loading and $C$ is reaction time.

The chosen parameters indeed significantly affect the conversion of EC in the following order; water content ($p < 0.0001$) > enzyme loading ($p = 0.0027$) > reaction time ($p = 0.0029$). However, the interaction between parameters was insignificant to EC conversion with $p$-values more than 0.4 at a 95% confidence level. This means other parameters would not give any significant impact on the relationship between independent and dependent parameters. For instance, the interaction between enzyme loading and the EC conversion is not affected by water content or reaction time. Its behavior remains the same regardless of the amount of water content and the duration of the reaction. A 3D response surface plot of the parameter interaction is shown in Fig. 5 for better illustration of its impact on the EC conversion.
Choline Chloride-urea Deep Eutectic Mixture for the Synthesis of Glyceryl Monocaffeate

J. Oleo Sci.

3.9 Enzyme kinetic analysis

The study on the kinetic mechanism of lipase-catalyzed transesterification of EC and glycerol was carried out to understand the order of substrates binds and product release from the active site of the enzyme. For a reaction between two substrates, there are three possible cases of reaction mechanism namely; random-sequential, ordered-sequential, and ping pong bi-bi mechanisms. For the sequential mechanism, it involves the formation of a ternary enzyme-substrate complex whereas for the ping pong mechanism it involves the formation of the secondary enzyme-substrate complex. To determine the mechanism, a double reciprocal plot (reaction rates and initial substrate concentration) or Lineweaver-Burk’s plot was adopted, and the trend is shown in Fig. 6.

Both figures show linear lines in parallel as the substrate concentration increased which means the biocatalytic reaction in compliance with the ping-pong bi-bi mechanism. This conclusion is in agreement with the most works related to transesterification by Novozyme 435\(^{23-25}\). However, there is no indication of a substrate or product inhibition since the rate of reaction maintained an upward trend as the concentration of both substrates is increased.

Previously, the alcohol substrates and for some cases, the product was reported to be inhibitors. 2-Phenylethanol and \(n\)-butanol are the inhibitors during the lipase-catalyzed synthesis of ethyl-3-phenyl propanoate and caffeic acid phenethyl ester, respectively\(^{23,25}\). Whereas, citronellyl acetate is the inhibitor during lipase-catalyzed transesterification between vinyl acetate and citronellol\(^{24}\). To confirm that the substrates are not the inhibitor, the data were fitted to four models namely; ping pong bi-bi without inhibition (Eq. 4), ping pong bi-bi with inhibition by EC (Eq. 5), ping pong bi-bi with inhibition by glycerol (Eq. 6) and ping pong bi-bi with inhibition by both EC and glycerol (Eq. 7).

\[
V_{\text{max}} = \frac{K_{\text{mEC}}[\text{EC}][\text{Gly}]}{K_{\text{iEC}}[\text{EC}]+[\text{EC}][\text{Gly}]} 
\]

\[
V_{\text{max}} = \frac{K_{\text{mEC}}[\text{EC}][\text{Gly}]}{K_{\text{iEC}}[\text{EC}][\text{Gly}]+[\text{EC}][\text{Gly}]} 
\]

\[
V_{\text{max}} = \frac{K_{\text{mEC}}[\text{EC}][\text{Gly}]}{K_{\text{iEC}}[\text{EC}][\text{Gly}]+K_{\text{iGly}}[\text{Gly}][1+[\text{EC}]/K_{\text{EC}}]}+\text{EC}[\text{Gly}] 
\]

\[
V_{\text{max}} = \frac{K_{\text{mEC}}[\text{EC}][\text{Gly}]}{K_{\text{iEC}}[\text{EC}][\text{Gly}]+K_{\text{iGly}}[\text{Gly}][1+[\text{EC}]/K_{\text{EC}}]+[\text{EC}][\text{Gly}]} 
\]

Subsequently, the values of the kinetic parameters were predicted by using non-linear regression analysis and tabulated in Table 1. The result showed that ping pong bi-bi without inhibition gave a very small sum of squared error (SSE) of 7.4 \(\times 10^{-4}\) indicating a good fit of the model. Meanwhile, the kinetic parameters from other models gave unrealistic and negative value as well as higher SSE values.

The maximum rate of reaction (\(V_{\text{max}}\)) is estimated at 10.9 mM.min\(^{-1}\). Whereas the Michaelis constant for EC, \(K_{\text{mEC}}\) was 126.5 mM while the Michaelis constant for glycerol, \(K_{\text{mGlycerol}}\) was 1842.7 mM. The \(K_{\text{m}}\) value can be interpreted as the affinity of the enzyme towards the substrate. Low \(K_{\text{m}}\) value means a higher affinity of the enzyme towards the substrate. It can be observed that \(K_{\text{mEC}}\) is lower than \(K_{\text{mGlycerol}}\) which means that lipase tends to bind with EC rather than glycerol. This is because lipase tends to form the enzyme-substrate complex first with acyl donor as mentioned earlier\(^{26}\).

From the Lineweaver-Burk plot and regression analysis, it can be concluded that the reaction mechanism obeyed ping pong bi-bi without substrates inhibition. It is proposed that EC binds to the lipase enzyme to form an enzyme-EC complex. Enzyme-EC complex then isomerizes into enzyme-acyl-ethanol complex and further releases ethanol as the first product. Then, glycerol combines with an intermediate enzyme (enzyme-acyl) to form an enzyme-acyl-glycerol complex, which again undergoes isomerization to enzyme-GMC complex. Finally, the second product, GMC is released and the enzyme retains its original conformation. The proposed reaction scheme was illustrated in Fig. 7.

![Fig. 6 Lineweaver-Burk’s plot of lipase-catalyzed transesterification of EC and glycerol. (a) At a fixed concentration of EC and (b) at a fixed concentration of glycerol.](image-url)
Table 1  Kinetic parameters based on four ping pong bi-bi models.

| Kinetic parameters | Without inhibition (Proposed) | With inhibition by EC | With inhibition by glycerol | With inhibition by EC & glycerol |
|--------------------|--------------------------------|-----------------------|----------------------------|--------------------------------|
| $V_{max}$ (mM.min$^{-1}$) | 10.9                           | $1.4 \times 10^6$    | 100.4                      | 847.83                         |
| $K_{mEC}$ (mM)       | 126.5                          | $-2324.1$            | $-25.8$                    | $-0.1$                         |
| $K_{mGlycerol}$ (mM) | 1842.7                         | $1.0 \times 10^5$   | $7.1 \times 10^4$         | 5673                           |
| $K_{iEC}$ (mM)       | NA                             | 0.003                | NA                         | 0.08                           |
| $K_{iGlycerol}$ (mM) | NA                             | NA                   | 154.14                     | 0.19                           |
| SSE                 | $7.4 \times 10^4$              | $9.6 \times 10^3$   | $2.7 \times 10^3$         | $2.7 \times 10^3$              |

Fig. 7  Proposed reaction scheme for Ping Pong Bi-Bi mechanism for synthesis of GMC.

4 Conclusion

Based on our study, we concluded that it is difficult to work with pure NADES (Choline chloride-Urea) media for lipase-catalyzed transesterification of EC and glycerol. They are viscous and limit the potential of lipase as a catalyst. However, when water is added they are comparable to aqueous and organic media. Like other media, lipase-catalyzed transesterification of EC is influenced by enzyme activity, water content, substrate molar ratio, temperature, and as well as reaction time. An EC conversion of over 90% is possible with optimized reaction conditions. Besides, the lipase-catalyzed synthesis of GMC follows the ping-pong bi-bi mechanism and there is no indication of substrate inhibition observed within the studied range. This positive result gives an alternative to aqueous media for green synthesis of GMC from EC. However, the formation of CA because of reverse reaction is a concerning issue with the present approach.

Conflicts of Interest

There are no conflicts of interest to declare.

Author Contributions

F.N. Gonawan: Designed research; Analyzed data; Wrote manuscript. P.N.M.A. Bakar: Designed research; Performed research; Wrote manuscript. A.H. Kamaruddin: Funding acquisition; Project administration; Supervised research; Wrote and review manuscript.

Acknowledgment

The authors would like to acknowledge the Malaysian Ministry of Higher Education for funding the current study through the Fundamental Research Gant Scheme (Acc. No: 203.PJKIMIA.6071342). The research facilities provided by Universiti Sains Malaysia are also duly acknowledged.

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Choline Chloride-urea Deep Eutectic Mixture for the Synthesis of Glyceryl Monocaffeate

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