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Ultrasound irradiation accelerates the lipase-catalyzed synthesis of methyl caffeate in an ionic liquid

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ABSTRACT

Methyl caffeate is a natural ingredient with several biological activities, but its preparation is generally limited to chemical synthesis. To set up a simple, high-yield and low-cost synthesis process for obtaining methyl caffeate, a novel synthesis method using the lipase-catalyzed esterification of methanol and caffeic acid in an ionic liquid under ultrasound irradiation was established. A maximum yield of 99.79% was obtained using ultrasound irradiation with an ultrasound frequency of 25 kHz and ultrasound power of 150 W under the following optimal conditions: Novozym 435 as a biocatalyst, [Bmim][Tf_2N] as the reaction medium, lipase concentration of 60 g/L, reaction temperature of 75 °C, and reaction time of 9 h. Using the optimal conditions under ultrasound irradiation, the reaction time was reduced 0.75-fold, and the apparent kinetic parameter (V_m/K_m) was increased 2-fold. The lipase could be reused 11 times without significant loss of activity. The results suggest that ultrasound irradiation can accelerate lipase-catalyzed synthesis of methyl caffeate in an ionic liquid.

1. Introduction

Methyl caffeate (methyl-(E)-3-(3,4-dihydroxyphenyl) prop-2-enoate, MC) is one of the alkyl caffeates extracted from *Polygonum amplexicaule* and the fruit of *Solanum torvum*. Alkyl caffeates have obvious biological functions in antimicrobial, antioxidative, antiviral and antineoplastic activities [1,2]. Among the alkyl caffeates, compared with caffeic acid (CA), MC has molecular polarity comparable to that of CA and has appetite suppressant and larval development inhibitory activities [3]. MC also has strong anti-inflammatory, anticancer, antioxidant, antiviral, detoxicant, anti-clotting, and anti-diabetic effects on diabetic rats induced by streptozotocin [4]. Additionally, MC can be used as an intermediate for the production of natural medicines or food additives such as propyl caffeate (PC) and caffeic acid phenethyl ester (CAPE), which are oxidative dimerization products of MC [5].

Currently, preparation strategies for MC typically focus more on chemical synthesis than on enzymatic synthesis. Several reports have been published on the synthesis of MC from CA and methanol using an acidic catalyst. For example, Shin et al. described the esterification of CA and methanol in the presence of sulfuric acid in 10 h with an MC yield of 71.0% [6]. Then, Wang et al. described a similar process catalyzed by *p*-toluenesulfonic acid (PTSA) for the preparation of MC in 4 h, achieving a relatively higher MC yield of 84.0% [7]. However, acidic catalysts are hazardous, requiring special energy-inefficient processes to dispose of the waste acid. Moreover, chemical methods are associated with problems such as high energy consumption, poor reaction selectivity and high cost of manufacturing. In contrast, the lipase-catalyzed synthesis of caffeates has garnered increasing attention due to its relative advantages. Chen et al. [8] used immobilized lipase as a catalyst to synthesize CAPE from CA and 2-phenylethanol (PE), achieving 93.08% molar conversion of CAPE. The results indicated that enzymatic synthesis could offer several advantages, such as mild reaction conditions and reagents, minimization of side products in the reaction, higher activation energy, and better control over

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the esterification reaction. Thus, there is a need to explore more efficient processes for the lipase-catalyzed synthesis of MC via esterification.

Enzymatic catalysis in ionic liquids (ILs) has been extensively studied for its high substrate conversion and product yield [9], high reaction rates, high activity, high stability, and good enantioselectivity [5]. Recently, the lipase-catalyzed synthesis of caffeates has attracted intense interest, with caffeates having higher solubility than CA in hydrophobic media [10]. The synthesis methods tested include esterification and transesterification, the latter having higher reaction efficiency than the former. As we have reported earlier [11], the use of MC as a substrate during the synthesis of propyl caffeate (PC) significantly improves the yield. Thus, biosynthesis of MC could provide greener avenue for the production of the much needed MC to be used in such process. Moreover, the method of lipase-catalyzed esterification synthesis of MC had yet not been tested in an ionic liquid system. Therefore, it was tested in this study for its effectiveness in obtaining better MC yields.

Recently, ultrasound irradiation has seen numerous applications in organic chemistry as an environmentally benign method [12]. Ultrasound irradiation is a useful tool for enhancing mass transfer in liquid–liquid heterogeneous systems and increasing the mass transfer rate of reagents and the active sites of enzymes [13]. Immobilized enzymes are more resistant than native enzymes to thermal deactivation caused by ultrasound irradiation [14]. Although the application of ultrasound irradiation to enzymatic reactions has not been extensively explored, it has been used to accelerate enzymatic reactions [15], such as esterification of phytosterol and different acyl donors [16], enzymatic esterification of rutin and naringinto catalyzed by Novozym 435 [17], transesterification of glycerol and methyl benzoate in the organic solvent [18], and synthesis of sugar esters in ILs [19]. To date, no known studies have investigated the application of ultrasound irradiation to the synthesis of MC.

Hence, the aim of this study was to explore a new method for the synthesis of MC using lipase-catalyzed esterification of CA and methanol in ILs. The effects of the type of IL, the types of lipase, the concentration of substrate, the concentration of biocatalysts in reaction system, the reaction time and reaction temperature on the esterification yield were investigated under incubator shaking. The experiments were subsequently conducted with incubation under ultrasound irradiation. Nuclear magnetic resonance (NMR) and mass spectrometry were used to identify the product.

2. Materials and methods

2.1. Enzymes and materials

Commercial lipases containing Novozyme 435, Lipozyme TL IM (immobilized lipase from *Thermomyces lanuginosus* (previously *Humicola lanuginosa*), immobilized on a granulated silica carrier), and Lipozyme RM IM (*Rhizomucor miehei*, 275 IUN/g, where IUN represents Interesterification Units Novo; carrier: phenol formaldehyde), were provided by Novo Nordisk A/S (Bagsvaerd, Denmark). Eight ILs, including [Hmim][HSO_4], [Bmim][Tf_2N], [Bmim][PF_6], [Bmin][BF_4], [Emim][TfOH], [Toma]Cl, [Toma][Tf_2N], [Omim][BF_4] were obtained from Shanghai Cheng-Jie Chemical Co. Ltd. (Shanghai, China). The residual chloride content in these ILs was less than 50 ppm. CA was from Nanjing Zelang Pharmaceutical Sci. & Tech. Co. Ltd., China. Methanol and acetonitrile were HPLC-grade (Tedia Co., Fairfield, OH, USA), and other reagents were analytical grade (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China).

2.2. Lipase-catalyzed synthesis of MC in an incubator shaker

The esterification reaction was performed in a 5 mL screw-capped vial at 75 °C for 36 h with a constant stirring speed of 120 rpm. CA (100 mg) was dissolved in methanol (10 mL), then different volumes of CA solution were added to IL, making the total reaction system to be 0.5 mL. The reaction was initiated by adding immobilized enzyme. At regular time intervals (3, 6, 12, 24, 36 and 48 h), 20 μL aliquots were taken from the well-stirred reaction mixtures and diluted using 380 μL methanol in preparation for HPLC analysis. The effects of different reaction temperatures (50–80 °C), different concentrations of substrate (0.25, 0.5, 1, 1.5, 2, 4 and 6 g/L), and concentration of Novozym 435 (20, 40, 60, 80, 100, and 120 g/L) on MC yield were investigated. All experiments were carried out in triplicate.

2.3. Lipase-catalyzed synthesis of MC under ultrasound irradiation

The esterification reaction was performed in a 5 mL screw-capped vial at 75 °C for 9 h under ultrasound irradiation using an ultrasound frequency of 25 kHz and ultrasound power of 150 W. CA (100 mg) was dissolved in methanol (10 mL), and different volumes of CA solution were added to IL, making the total reaction system to be 0.5 mL. The reaction was initiated by adding the immobilized enzyme. At regular time intervals (1, 2, 3, 6, 12, 24, 36 and 48 h), 20 μL aliquots were taken from the well-stirred reaction mixture and diluted using 380 μL methanol in preparation for HPLC analysis. The effects of different ultrasound power (90, 120, 150, 180, 210 W), frequency (15, 20, 25, 30, 35 kHz), operation mode (sweep, pulse), reaction temperatures (50–80 °C), different concentrations of substrate (0.25, 0.5, 1, 2, 4 and 6 g/L), and concentration of Novozym 435 (20, 40, 60, 80, and 100 g/L) on the MC yield were investigated. All experiments were performed in triplicate.

2.4. Complexation extraction process of MC

The complexation extraction process was used to extract MC from enzymatic reaction system using trioctylphosphine oxide (TOPO)–cyclohexane as an extractant [20], in which mass fraction of complex agent TOPO in diluter cyclohexane was 100 g/L. MC was extracted from the mixture to extractant with the ratio of 1:1 (v/v). After cyclohexane was volatilized from extractant, the residue was crystallized from methanol, thus MC could be separated from mixtures to obtain white product with the method of vacuum drying.

2.5. Kinetic study of lipase-catalyzed synthesis of MC

The kinetics of the esterification reaction was investigated by studying the effect of the CA concentration on the initial rate of the reaction. The concentration of CA varied within the range of 1.39–33.32 mM. Initial reaction rates, which expressed as mM CA per hour, were determined from the time course of the reaction by using a second order polynomial curve fitting via regression analysis of the product concentration as well as determining the initial slope of the tangent to the curve.

2.6. Analysis of products by LC–MS and HPLC

As in previous test methods [11], LC-PAD-MS was performed using a Thermo Fisher system. The LC equipment comprised a Finnigan MAT Spectra System P4000 pump, a Finnigan AQA mass spectrometer, and an autosampler with a 50 μL loop. LC separation was performed on a Kromasil C_{18} column (150 mm × 4.6 mm, i.d.; 5 μm, W.R.) and monitored using a UV6000 LP diode array detector. Isocratic elution was used to run the mobile phase, which was

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a mixture of solvent A (methanol) and solvent B (water) at a ratio of 65:35, v/v, respectively. The wavelength range for PAD detection was from 200 to 400 nm. The flow rate was 1.0 mL/min for LC and PAD detection, and the column was maintained at 30 °C. Selected ion monitoring (SIM) was performed under negative ion mode, with capillary voltage set at 1.6 kV, and temperatures of the curved desolvation line (CDL) and heat block both set at 200 °C. Electrospray ionization (ESI) was performed using nitrogen to assist nebulization, with the flow rate set at 1.0 mL/min. The data were processed using Xcalibur 1.2 software. The intense peaks at m/z 179.1 and m/z 193.1 in ESI-MS spectra under negative ion mode correspond to the deprotonated ion $[M-H]^-$ of CA and MC, respectively.

HPLC analyses were performed using a constant flow pump (2PB0540, Beijing Satellite Factory., Beijing, China) with a UV–vis detector (L-7420, Tech comp Co. Ltd., Shanghai, China) and N-2000 workstation (Hangzhou Mingtong S&T Ltd., Hangzhou, China). Separation was achieved on a HC-C_{18} column (250 mm × 4.6 mm, i.d.; 5 μm, W.R. Grace & Co., Deerfield, Illinois, USA) maintained at 30 °C. CA and MC were detected from the mobile phase, which consisted of a solvent mixture of methanol/water (65:35, v/v) at a flow rate of 1.0 mL/min and detection wavelength of 325 nm. All experiments were carried out in triplicate.

External standard method was adopted in calculating of the reaction conversions, and the CA conversion and MC yield of the lipase-catalyzed esterification were calculated as follows:

$$\text{CA conversion}(\%) = \frac{\text{Converted molar amount of CA(mol)}}{\text{Initial molar amount of CA(mol)}} \times 100 \quad (1)$$

$$\text{MC yield}\ (\%) = \frac{\text{Molar amount of MC}\ (\text{mol})}{\text{Initial molar amount of CA}\ (\text{mol})} \times 100 \quad (2)$$

2.7. Structural identification of MC

^{1}H NMR spectra were recorded on a Bruker AVANCE spectrometer 400 (Bruker Biospin Co., Billerica, MA, USA). The samples were dissolved in DMSO-d_6, containing tetramethylsilane (TMS) as an internal standard. Proton spectra were recorded at 400 MHz using a solvent field lock (400 MHz), which was shown as Fig. S1 in Supplementary Materials. The identification results are as follows: MC: ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.60 (1H, s, OH), 9.16 (1H, s, OH), 7.50 (1H, d, J = 15.9 Hz, α-H), 7.07 (1H, d, J = 2.0 Hz, Ph-H), 7.01 (1H, dd, J = 8.1 Hz, Ph-H), 6.77 (1H, d, J = 8.1 Hz, Ph-H), 6.28 (1H, d, J = 15.9 Hz, β-H), 3.69 (3H, s, CH_3). The results were consistent with previous studies [11].

2.8. Statistical analysis

Triplicate experiments were performed for each parameter investigated. The differences in mean values were evaluated using the analysis of variance (ANOVA) method, and standard deviations were calculated to verify the results reliability. Significance was determined at a 95% level of probability.

3. Results and discussion

3.1. Selection of a suitable lipase as a biocatalyst

Fig. 1 shows the catalytic efficiency of three commercially available immobilized lipases (Novozym 435, Lipozyme RM IM and Lipozyme TL IM) as biocatalysts for MC production in 0.5 mL [Bmim][Tf_2N]. The lipase concentration was 100 g/L, and the reaction temperature was 75 °C. The MC yields varied markedly between Novozym 435 and the remaining two lipases; the activities of the three lipases and their highest MC yields were as follows, in descending order: Novozym 435 (93.18%) > Lipozyme TL IM (23.59%) > Lipozyme RM IM (20.63%).

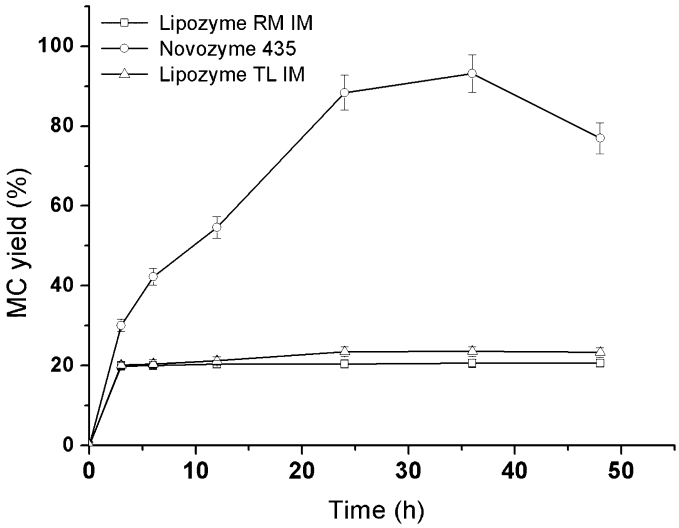

Fig. 1. Effect of different lipases on the MC yield in [Bmim][Tf_2N]. The CA concentration was 0.5 g/L, and the concentration of lipases in TL was 100 g/L. All tests were performed at 75 °C and 120 rpm in an incubator shaker.

Novozym 435 is a lipase of high thermostability, used mainly as a catalyst for the synthesis of esters [21]. It has a maximum enzymatic activity in 70–80 °C. In order to maintain the optimization of productivity, several literatures recommended temperature span between 50 and 60 °C [20,22]. But in this experiment, in order to maintain maximum activity of Novozym 435, the option of the temperature in all process optimizations was according to the highest MC yield. This lipase displayed the highest activity in the esterification of CA with methanol [23]. When compared with Lipozyme RM IM, Lipozyme TL IM is more advantageous for the synthesis of short chain alkyl esters [24]; moreover, considering the great difference in the cost of the two enzymes, Lipozyme TL IM may be considered a better catalyst than Lipozyme RM IM. However, whereas Lipozyme TL IM has commonly been reported to catalyze resolution of chiral alcohol and preparation of biodiesel [25], it has rarely been used to catalyze the synthesis of alkyl caffeates [20]. In conclusion, the two Lipozyme enzymes tested were not clearly adapted to catalyze the synthesis of MC. Therefore it is apparent that Novozym 435 is the most active catalyst for the esterification of CA and methanol. Hence, Novozym 435 was chosen as the most suitable catalyst for the following studies.

3.2. Selection of a suitable IL for reaction media

Fig. 2 shows the MC yield in each IL with the esterification of CA and methanol. The yields in these reaction media were as follows, in descending order: [Bmim][Tf_2N] (96.14%) > [Bmim][PF_6] (66.64%) > [Toma][Tf_2N] (54.60%) > [Omim][BF_4] (48.93%) > [Toma]Cl (41.69%) > [Bmim][BF_4] (41.01%) > [Emim][TfOH] (39.60%) > [Hmim][HSO_4] (39.54%). This could be due to the report that ILs can increase the substrate solubility [26], and enhance the synthetic activity of lipase because ILs increase the polarity of the system [27]. Therefore, usually, enzymatic media containing ILs possess weakly coordinating anions, such as [Tf_2N] and [PF_6]. However, other ILs possess strongly coordinating anions ([HSO_4], [BF_4] and [TfOH]) and show dramatically lower yields because they are more nucleophilic than [Tf_2N]. In addition, the complex nature of the ILs leads to many possible types of interactions, including hydrogen bonding and

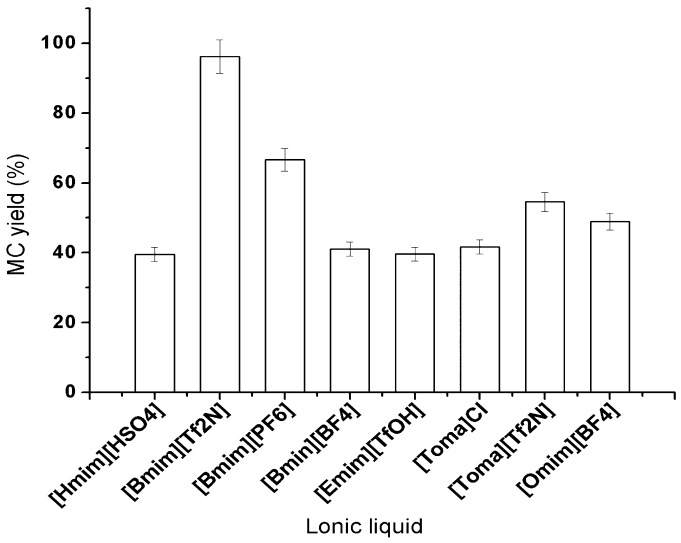

Fig. 2. Effects of different ILs on the MC yield. The CA concentration was 0.5 g/L, and the concentration of lipase was 100 g/L. All tests were performed at 75 °C and 120 rpm for 36 h in an incubator shaker.

hydrogen-bonding basicities [28]. Thus, the interactions among [HSO_4], [BF_4] and [TfOH] and positively charged sites in the structure of the enzyme could cause conformational changes in the enzyme, which then becomes deactivated. These results agreed with previous findings that the anion in an IL plays an essential role in determining lipase activity [5]. Therefore, [Bmim][Tf_2N] was selected as the most suitable medium for the esterification reaction of CA and methanol.

3.3. The effect of temperature

Fig. 3A and B shows the effect of the reaction temperature on the yield of MC catalyzed by Novozym 435 in an incubator shaker and under ultrasound irradiation, respectively. The substrate concentration was 0.5 g/L, the solvent was [Bmin][Tf_2N], and the lipase concentration was 100 g/L. Fig. 3A shows that the MC yield increased with increasing reaction temperature in the range of 50–75 °C within 24 h. However, the MC yield showed a slight decline after 24 h at 70 °C. After 36 h at 75 °C and 80 °C, the MC yield continued to exhibit a declining trend. A maximum MC yield of 93.18% was achieved with 36 h of incubation at 75 °C under ultrasound irradiation. In addition, Fig. 3B shows that an increase in the reaction temperature in the range of 50–80 °C led to increasing MC yields within 9 h. Generally, the MC yield declined slightly after 9 h, except at 70 °C. It is possible that an excessive duration of ultrasound irradiation produces excessive heat, which leads to perturbation of the lipase tertiary structure, ultimately reducing its enzymatic activity to some extent [29,30]. The maximum MC yield of 98.71% was achieved with 9 h of incubation at 75 °C under ultrasound irradiation, the same optimum temperature as which found in the experiments using the incubator shaker.

The temperature usually has an important influence on enzymatic reactions, including esterification and transesterification, because it can influence the enzymatic enantioselectivity [31] and the reaction rate [32]. Increasing the temperature within certain limits could improve the enzyme activity, reduce the ionic liquid viscosity, and increase the mass transfer speed, thus improving the yield of the product. However, when the temperature increases beyond optimal, the yield decreases. It can be inferred that higher temperatures cause partial thermal inactivation of the lipase, which enables the enzyme thermodynamic parameters to be determined but delays reaction times [33]. As a whole, the reaction time was significantly shorted (by three fourths) under ultrasound irradiation compared to the reaction using the incubator shaker. The reason may be that during the enzymatic reaction, ultrasound irradiation can reduce the particle sizes of the substrate and enzyme, consequently increasing the enzyme surface area, which is useful for reducing mass transfer limitations [15]. This concept was further validated in studies using dehydrated enzyme powders to catalyze reactions in an organic solvent [34]. Thus, the optimal temperature and reaction time are 75 °C and 9 h, respectively, for the synthesis of MC under ultrasound irradiation.

3.4. The effect of substrate concentration

Fig. 3C and D shows the effects of different CA concentrations on the MC yield in the incubator shaker and under ultrasound irradiation, respectively. [Bmin][Tf_2N] was chosen as the reaction solvent, and the Novozym 435 concentration in the reaction system was 100 g/L. Fig. 3C shows that when the optimum CA concentration was 0.5 g/L, the MC yield increased to 96.54%. Any further increase or decrease in substrate concentration resulted in a decline of MC yield. Fig. 3D shows that under ultrasound irradiation, the highest MC yield achieved was 96.0%. Meanwhile, any further increase or decrease in substrate concentration led to results similar to those shown in Fig. 3C. Therefore, mass transfer limited the ability of the substrate to reach the active center of the enzyme [35], and the charge viscosity of IL added to the mass transfer limitations, which resulted in low CA conversion. In addition, this inhibition could also be due to the production of generated water which led equilibrium reaction toward to ester hydrolysis, herein, resulting in a lower MC yield.

This reaction is the first order reaction equation, and the Thiele modulus ϕ was calculated by follows:

$$\eta = \frac{1}{\phi}\left[\frac{1}{\tanh(3\phi)} - \frac{1}{3\phi}\right] \quad (3)$$

$$\eta = \frac{R_s}{R_{s_0}} \quad (4)$$

where η is internal diffusion factor effectively, R_s is actual reaction rate in granular (mol/(s g)), and R_{s_0} is reaction rate when concentration in granular is the same as surface (mol/(s g)).

Therefore, effective diffusion coefficients of both processes were calculated as follows:

$$k_v = \frac{r_{max}}{K_m} \quad (5)$$

$$\phi = \frac{R}{3}\sqrt{\frac{k_{v1}}{D_e}} \quad (6)$$

where coefficient K_m is the reaction kinetic parameter (mM), and r_{max} is reaction rate (mol/m^3 s); R is enzyme radius (cm), k_{v1} is reaction rate constant (s^{-1}), and D_e is the effective diffusion coefficient.

D_e is in proportion to the corresponding porosity and pore size, increased D_e can indicate the enlarging on corresponding porosity and pore size. The micro-porous diffusion coefficient of immobilized enzyme between incubator shaker and ultrasound irradiation could be measured by D_i/D_u (D_i was the diffusion coefficient in incubator shaker; D_u was the diffusion coefficient in ultrasound irradiation), and D_i/D_u was 0.33 in this two conditions, it illustrated that porosity and pore size were increased under the ultrasound process.

The highest MC yield in ultrasound irradiation condition was slightly lower than that an in incubator shaker. When CA concentration was 0.5 g/L, the best reaction time was decreased greatly from 36 h to 9 h. Therefore, some certain errors were inevitable existing. In addition, the results showed that ultrasound irradiation had little impacts on substrate concentration than reaction

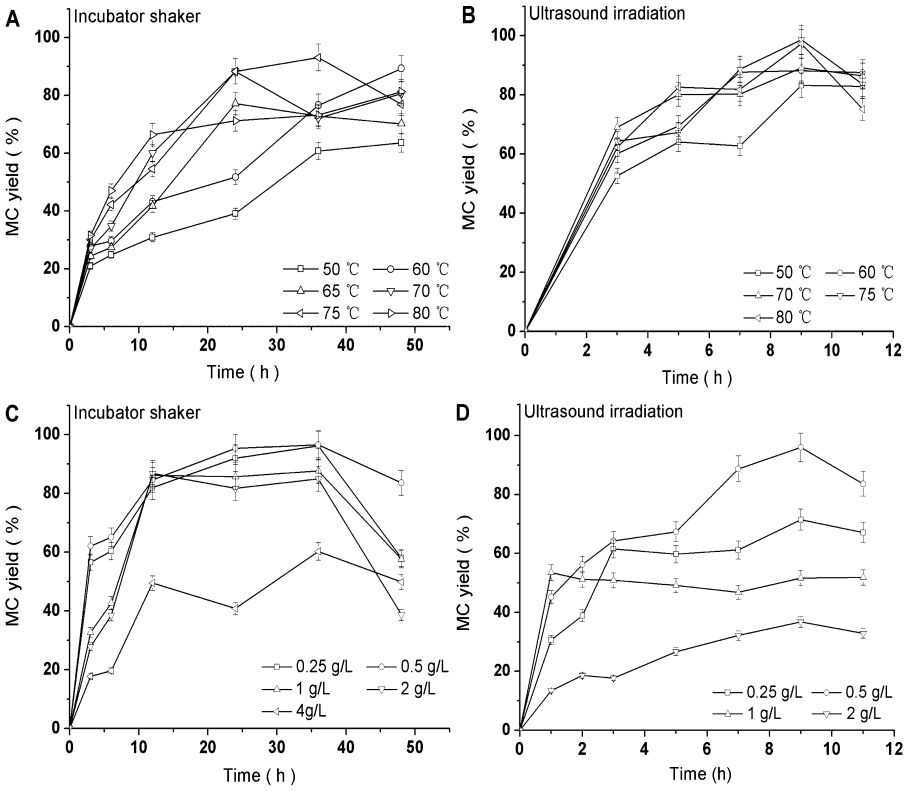

Fig. 3. Effect of temperature (A and B) and substrate concentration (C and D) on the MC yield in [Bmim][Tf_2N]. The lipase used was Novozym 435, CA concentration was 0.5 g/L, and the concentration of lipase was 100 g/L. The reactions analyzed in (A) and (C) were performed at 120 rpm in an incubator shaker; those analyzed (B) and (D) were 10 W under ultrasound irradiation.

time. These results suggest that the interaction between the substrate molecules and the enzyme was maximized at a substrate concentration of 0.5 g/L. Therefore, 0.5 g/L was treated as the optimum CA concentration in [Bmim][Tf_2N], and further experiments on concentration of substrate were carried out at various lipase concentration levels.

3.5. The effect of lipase concentration

Fig. 4A and B shows the effect of varying lipase concentration on MC yield in the incubator shaker and under ultrasound irradiation, respectively. Varying Novozym 435 concentrations (20, 40, 60, 80, 100 and 120 g/L) and CA concentrations (0.15, 0.25, 0.5, 1, 1.5 and 2 g/L) were used under the two different incubation conditions. Fig. 4A shows that with the incubator shaker, the highest MC yield (96.42%) occurred when the CA concentration was 0.5 g/L and Novozym 435 concentration was 60 g/L. Excessive amounts of enzyme lead to a marginal decrease in yield. When the substrate concentration was 0.15 g/L and Novozym 435 concentration was 20 g/L, the MC yield reached 88.10%. Further increases in enzyme concentration led to a marginal decrease in MC yield. The most likely reason was that the excess enzyme particles have the potential to attract each other and form enzyme aggregates, which may reduce the accessibility of substrate to enzyme particles. When the lipase concentration exceeded 20 g/L, the probability of collisions between substrate and enzyme diminished [36].

As shown in Fig. 4B, under ultrasound irradiation with ultrasound frequency of 25 kHz and ultrasound power of 150 W, the highest MC yield (96.44%) was obtained when the substrate concentration was 0.5 g/L and Novozym 435 concentration was 60 g/L,

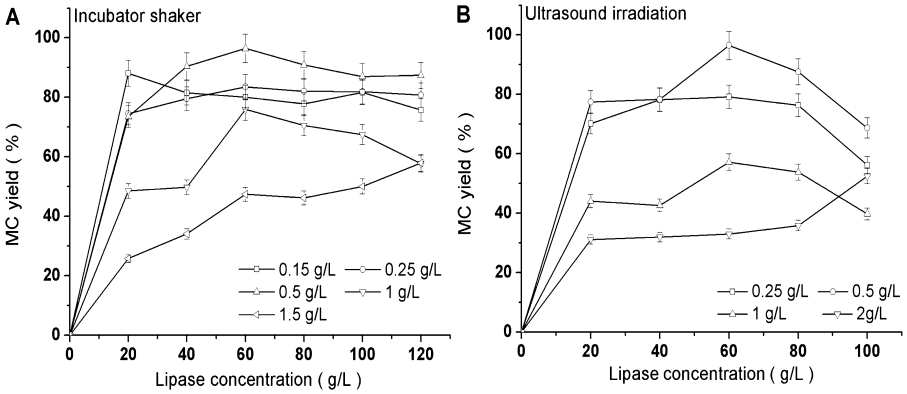

Fig. 4. Effect of lipase concentrations on the MC yield at different CA concentrations in [Bmim][Tf_2N]. The lipase was Novozym 435. The reactions analyzed in (A) were performed at 120 rpm for 36 h in an incubator shaker and those analyzed in (B) were performed at 150 W for 9 h under ultrasound irradiation.

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Table 1
Comparative results for the lipase-catalyzed synthesis of MC (in incubator shaker and ultrasound irradiation) at 75 °C in [Bmim][Tf_2N].

Methods	Substrate concentration (g/L)	Lipase concentration (g/L)	Reaction time (h)	MC yield (%)
Incubator shaker[a]	0.5	60	36	96.54
Ultrasound irradiation[b]	0.5	60	9	99.79

[a] The experiment was conducted 120 rpm in an incubator shaker.
[b] The experiment was conducted with ultrasound power set to 150 W and ultrasound frequency of 25 kHz under ultrasound irradiation.

conditions similar to those for the reactions performed with the incubator shaker. The obvious difference between the results shown Fig. 4B and A for the two different incubation conditions was the effect of the two conditions on the MC yield. Performing the reaction under ultrasound irradiation can obtain a higher MC yield than using the incubator shaker. One possible reason for the improved yield is that ultrasound irradiation may aid the formation of micro bubbles in the solution, which can result in drastic changes to local conditions, such as enhanced mixing that could contribute to the achievement of higher reaction rate. Thus, it is the first time to report a study concerning the biosynthesis of MC from CA and methanol using lipase-catalyzed esterification under ultrasound irradiation.

Table 1 shows the comparative results of the lipase-catalyzed synthesis of MC in an incubator shaker or under ultrasound irradiation at 75 °C in [Bmim][Tf_2N]. The product yield is known to be related to the reaction time: generally, the longer the reaction time, the more products obtained [37]. However, the yield can suffer from instability and oxidation at high temperatures. Therefore, it is important to reduce the reaction time. In this reaction, the reaction time was shortened to 9 h under ultrasound irradiation, whereas it required 36 h in an incubator shaker. In other words, the reaction time under ultrasound irradiation was less than half that of the reaction performed using the incubator shaker. Moreover, the maximum yield reached 99.79% under ultrasound irradiation, which was higher than that of the reaction performed using the incubator shaker. Therefore, the reaction under ultrasound irradiation was more favorable than in the incubator shaker for the lipase-catalyzed synthesis of MC. To further verify these results, the kinetics of lipase-catalyzed esterification was next determined by monitoring the changes in reaction rate with different substrate concentrations. After the optimum reaction conditions were determined, further ultrasound parameters (ultrasound power, frequency, and operation mode) were explored in the following experiments.

3.6. The effect of ultrasound power and frequency

Using pulse mode of ultrasound operation [38], Fig. 5A and B shows the effects of different power (90, 120, 150, 180 and 210 W) and ultrasound frequency (15, 20, 25, 30 and 35 kHz) on MC yield at different reaction time when the CA concentration was 0.5 g/L, reaction temperature was 75 °C, and Novozym 435 concentration was 60 g/L. Different powers were optimized by controlling ultrasound frequency of 25 kHz, which was chosen as an optimum parameter on esterification reaction catalyzed by Novozym 435 [39]. The results indicated that the MC yield increased with the increase of ultrasound power, and reached to the highest value when ultrasound power was 150 W. Continued increasing power led to decrease of MC yield, which was possibly because excessive powerful of ultrasound could result in deactivation of the lipase. It clearly proved that a suitable sonication (power of 150 W and frequency of 25 kHz) has a significant effect on the enzymatic activity [17].

3.7. Kinetic constants analysis

The enzymatic kinetics in batch reaction systems were determined using the Michaelis–Menten model [40], and the experimental data were fitted to the Lineweaver–Burk equation (Eq. (7)):

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\frac{1}{[S]} \quad (7)$$

where $[S]$ is the initial substrate concentration, V_{max} is the maximum velocity, and K_m is the Michaelis constant. The value $1/V$ can be plotted against $1/[S]$.

The esterification of CA and methanol was accorded with ordered mechanism, namely, two substrates combined with enzyme one after another, it means CA was first coupled on enzyme, later it turns to methanol. In the end, the products were broke away from the enzyme. CA concentrations ranged in control, but the concentration of substrate [S] of methanol far exceeded the enzyme concentration $[E]$ ($[S] \gg [E]$), such that all enzyme could be assumed to form into enzyme–substrate complexes (ES), and the diminishing $[S]$ could be ignored. Under these conditions, the intermediate ES complex concentration remains stable after its initial formation; in other words, the rates of ES formation and its disappearance remain equal, thus achieving a steady state. Furthermore, when all the enzyme complexes with substrate, $[E] = [ES]$, in which case, the

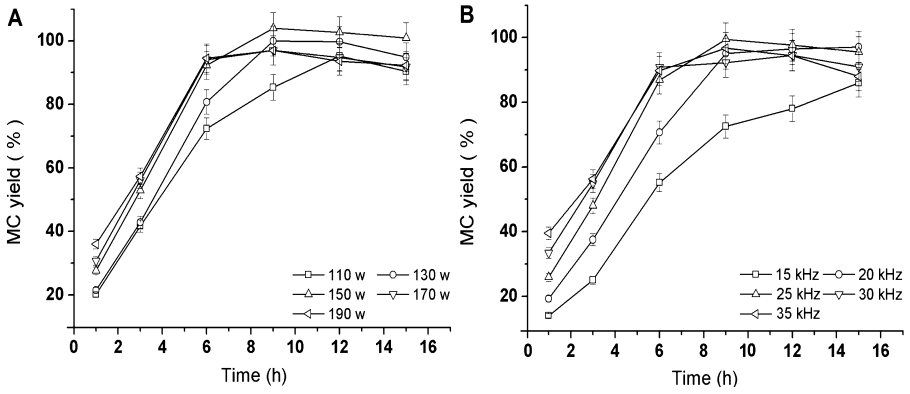

Fig. 5. Effect of ultrasound power (A) and frequency (B) on the MC yield at different reaction time in [Bmim][Tf_2N]. Reaction conditions: temperature – 75 °C, concentration of lipase – 60 g/L, CA concentration – 0.5 g/L. All tests were performed under ultrasound irradiation using pulse mode.

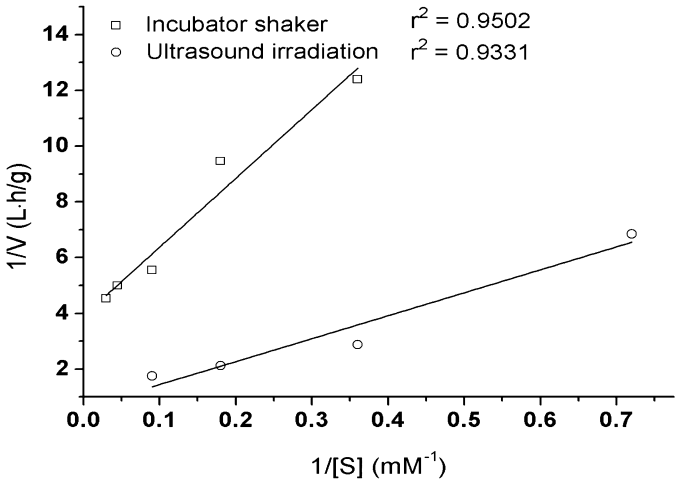

Fig. 6. Kinetic plots of the lipase-catalyzed synthesis of MC in [Bmim][Tf_2N] at different CA concentrations (1.39–33.32 mM). Reaction conditions: temperature – 75 °C, concentration of lipase – 60 g/L, CA concentration – 0.5 g/L. All tests were performed at 120 rpm for 3 h in an incubator shaker and 150 W for 0.5 h under ultrasound irradiation.

parameters of the kinetics of the enzyme could only be described and presented by considering the substrate of CA.

Fig. 6 illustrates Eq. (2) double reciprocal plot obtained for the esterification reaction by studying the effect of the concentration of CA on the initial rate of the reaction. The kinetic parameter values and the corresponding correlation coefficient (r^2) for the lipase-catalyzed synthesis of MC are summarized. It can be observed that r^2 was 0.9502 for the reaction in the incubator shaker, and it was 0.9331 for the reaction under ultrasound irradiation. The kinetic parameters in this study were calculated. The intercept of K_m/V_{max} and slope of $1/V_{max}$ were fit-plotted in Fig. 6. The linear fitting agrees well with the experimental data. The initial rate increased with increasing CA concentration, and the reaction mechanism exhibited Michaelis–Menten kinetics at the low substrate concentration. This result indicates a strong affinity between substrate and enzyme binding sites in this reaction.

Table 2 shows the kinetic parameter values comparing the lipase-catalyzed synthesis of MC incubated in the incubator shaker and under ultrasound irradiation. From these parameters, the minimum values of the apparent K_m (K_m (app)) and V_{max} were 6.32 mM and 0.26 g/L h, respectively, in the incubator shaker. The results indicate that the affinity of the Novozym 435 toward CA is greater than it is toward PE. A K_m (app) of 13.15 mM and V_{max} of 1.60 g/L h were obtained in the ultrasound irradiation process, increases of 1.08-fold and 5.15-fold, respectively, compared to those from the

Table 2
Kinetic parameters for the lipase-catalyzed synthesis of MC (in an incubator shaker and ultrasound irradiation) at 75 °C in [Bmim][Tf_2N].

Parameter[a]	Value	
	Incubator shaker	Ultrasound irradiation
K_m (app) (mM)	6.32	13.15
V_{max} (g/L h)	0.26	1.60
V_m/K_m (g/mmol h)	0.04	0.12

[a] The kinetic assay was performed with a CA concentration that varied within the range of 1.39–33.32 mM.

incubator shaker. One possible reason for the increase of K_m (app) is that ultrasound irradiation led to a little disruption of the enzyme. Ultrasound irradiation has impact of reducing enzyme activity and even inactivating the enzyme [41]. However, the initial reaction rate improved greatly under ultrasound irradiation compared to the incubator shaker, potentially due to the dispersion effect of ultrasound irradiation, which could have increased the frequency of collisions between enzyme and substrate [42]. The V_m/K_m of 0.12 g/mmol h calculated for the reaction performed under ultrasound irradiation was greater than that for the reaction performed in the incubator shaker (0.04 g/mmol h). Therefore, the process of esterification under ultrasound irradiation was demonstrated to be a more effective method for the synthesis of MC compared to that without ultrasound irradiation.

3.8. Study of lipase reusability

To investigate the reusability of the Novozym 435 for synthesis of MC under ultrasound irradiation, selected reaction conditions were used to test its stability. After each batch, the lipase was filtered by a filter funnel, and then washed the lipase using a little [Bmim][Tf_2N], thus the lipase was recovered. A total of 16 batch cycles were conducted. Fig. 7A shows the reusability of Novozym 435. The activity of lipase was stable during the first 11 batches in [Bmim][Tf_2N], with MC yields more than 82.0%; the highest MC yield was 99.79%. Moreover, Novozym 435 could be reused 15 times while maintaining MC yield more than 50% under ultrasound irradiation, matching the levels of activity and stability reported previously [11]. Immobilized enzymes were more stable compared to soluble enzymes and resisted the deactivating effect of ultrasound [43]. The protein concentration in the reaction medium was measured according to previously explained [44], which increased rarely after each reaction batch (Fig. 7B) in spite of the increase of porosity and pore size in ultrasound irradiation. The results indicated that Novozym 435 was not deactivated or denatured under ultrasound irradiation [45], and the given lipase loading was still

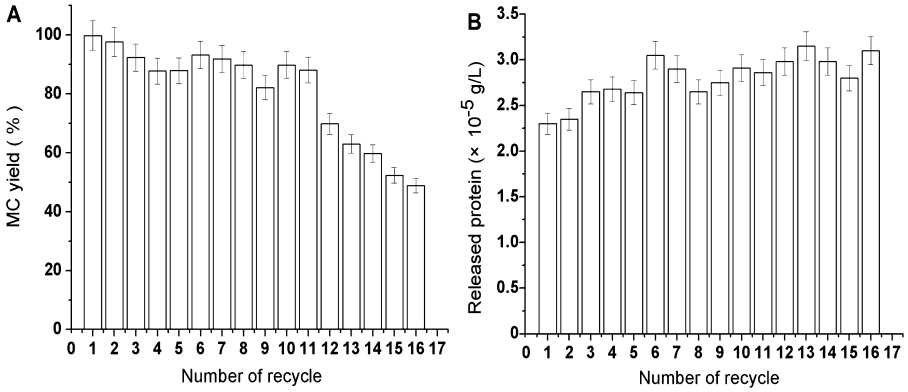

Fig. 7. Reusability (A) and protein leakage (B) of Novozym 435 used in the synthesis of MC in [Bmim][Tf_2N]. Reaction conditions: temperature – 75 °C, concentration of lipase – 60 g/L, CA concentration – 0.5 g/L. All tests were performed at 150 W for 9 h under ultrasound irradiation.

meet the economic feasibility [46]. In addition, by calculating, the overall PC yield (82.74%) [8] could be increased to 98.29%. The results indicated that the developed lipase-catalyzed esterification method of MC under ultrasound irradiation could greatly improve the overall yield of product using lipase-catalyzed transesterification of an intermediate MC and an alcohol in ionic liquid.

4. Conclusions

A novel synthesis method for MC was established using lipase-catalyzed esterification of CA and methanol in an IL using incubation shaking or under ultrasound irradiation, and the effects of various parameters on the yield of MC were investigated. Novozym 435 was found to be most active for catalysis in [Bmim][Tf_2N]; the optimum Novozym 435 concentration was 60 g/L; optimum CA concentration was 0.5 g/L at a temperature of 75 °C. In comparison to incubator shaking, under ultrasound irradiation when ultrasound frequency of 25 kHz and ultrasound power of 150 W, the reaction time reduced from 36 h to 9 h, the kinetic parameter (V_m/K_m) increased from 0.04 g/mmol h to 0.12 g/mmol h, and the maximum MC yield increased from 96.54% to 99.79%. The enzyme could be reused 11 times while maintaining MC yields greater than 82% under ultrasound irradiation. These results indicate that ultrasound irradiation is an efficient way to enhance lipase-catalyzed synthesis of MC in an IL.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2014.11.006.

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