Food Chemistry 220 (2017) 249–256

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Lipase-catalyzed esterification of ferulic acid with lauryl alcohol in ionic liquids and antibacterial properties in vitro against three food-related bacteria

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ARTICLE INFO

Article history:
Received 9 August 2016
Received in revised form 26 September 2016
Accepted 28 September 2016
Available online 4 October 2016

Keywords:
Ionic liquids
Ferulic acid
Lauryl ferulate
Lipase
Biocatalysis
Antimicrobial activity

ABSTRACT

Lauryl ferulate (LF) was synthesized through lipase-catalyzed esterification of ferulic acid (FA) with lauryl alcohol in a novel ionic liquid ([(EO)-3C-im][NTf_2]), and its antibacterial activities was evaluated in vitro against three food-related bacteria. [(EO)-3C-im][NTf_2] was first synthesized through incorporating alkyl ether moiety into the double imidazolium ring. [(EO)-3C-im][NTf_2] containing hexane was found to be the most suitable for this reaction. The effects of various parameters were studied, and the maximum yield of LF (90.1%) was obtained in the optimum reaction conditions, in [(EO)-3C-im][NTf_2]/hexane (V_{ILs}:V_{hexane} = 1:1) system, 0.08 mmol/mL of FA concentration, 50 mg/mL Novozym 435, 60 °C. LF exhibited a stronger antibacterial activity against Gram-negative (25 mm) than Gram-positive (21.5–23.2 mm) bacteria. The lowest MIC value was seen for *E. coli* (1.25 mM), followed by *L. Monocytogenes* (2.5 mM) and *S.aureus* (5 mM). The MBCs for *L. Monocytogenes*, *S.aureus* and *E. coli* were 10, 20 and 5 mM.

1. Introduction

Ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA) is an antioxidant compound as a result of resonance stabilisation of its phenoxy radical by the conjugation of the aromatic nucleus and its extended side chain. This compound is readily isolated from maize waste, and it can be absorbed and easily metabolized in the human body (Li, Li, & Luo, 2005; Ou & Kwok, 2004; Poquet, Clifford, & Williamson, 2008; Zhao, Egashira, & Sanada, 2004). It is widely used in the food and cosmetic industries because of its physiological functions, including antioxidative, antimicrobial, anti-inflammatory, and antithrombotic functions, display anti-cancer activities, protect against coronary disease, lower cholesterol, and increase sperm viability (Ou & Kwok, 2004). However, the poor solubility of FA in either hydrophobic or hydrophilic media limits its application. Incorporation of a hydrophobic moiety (such as aliphatic molecules) into FA offers a viable alternative to ameliorate the solubility of FA in oil-based formulas and emulsions. Moreover, alkyl esters of FA, such as octyl ferulate, have been observed to have higher antioxidant activity than the acid itself and the activity of octyl ferulate was reported to be comparable to BHT (Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002). However, to the best of our knowledge, there are rather few reports describing the antimicrobial activities of FA derivatives.

In recent years, a number of studies reported the enzymatic synthesis of hydrophobic derivatives of FA to increase its oil-solubility (Chigorimbo-Murefu, Riva, & Burton, 2009; Laszlo & Compton, 2006). However, there exists a technical dilemma for creating an efficient nonaqueous enzymatic reaction system: FA have low solubility in most organic solvents (such as *t*-butanol) where the lipase could exhibit decent catalytic behaviors; while in polar solvents (such as DMSO) in which FA have good solubility while the biocatalyst is deactivated or shows low activity. Previous works mainly focused on the enzymatic modification of FA in conventional solvents or solvent-free systems (Compton, Laszlo, & Berhow, 2000; Katsoura et al., 2009; Lee, Widjaja, & Ju, 2006; Yoshida, Kimura, Kadota, Tsuno, & Adachi, 2006). Only a few involved the use of common ionic liquids as media for the enzymatic production of feruloylated acylglycerols (Sun, Yang, Bi, & Xiao, 2009) or oleyl alcohol (Chen

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http://dx.doi.org/10.1016/j.foodchem.2016.09.187
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et al., 2011), cinnamic acid derivatives (Katsoura et al., 2009). Among these research, cases in point of esterification of FA catalyzed with Novozym 435, the optimum productivity of oleyl ferulate in [Hmim][PF_6]/isooctane and various aliphatic alcohol ferulates in [Bmim][PF_6] were better than those in some organic solvents, yet still not satisfying. Many researchers, therefore, have been urging on finding suitable alternative ILs for biocatalytic processes.

Ionic liquids (ILs), which are claimed to be "green" media, carry numerous desirable properties such as wide liquid range, thermal and chemical stability, remarkable solubility with many small molecules, high polarity and conductivity, attractive recyclability, and negligible vapor pressure that are well suited for a myriad of innovative applications (van Rantwijk, Lau, & Sheldon, 2003). The advantages of ILs related to biotransformation include adjustable solubility properties, protective effects or increased stability for enzymes, positive effects on the specificity of enzymes or on the shift of reaction equilibrium, as well as recoverability and recyclability (Gorke, Srienc, & Kazlauskas, 2010; Moniruzzaman, Nakashima, Kamiya, & Goto, 2010; Quijano, Couvert, & Amrane, 2010). In our laboratory, we have been interested in developing new ILs and have an ongoing program to evaluate ILs as novel and stable media for chemical and biological applications (Shi, Fang, Ren, Wu, & Guan, 2008; Shi, Li, & Chu, 2011; Shi et al., 2012). Recently, we have successfully employed ILs containing a specific double imidazolium ring in its cationic moiety as potential PCR enhancing reagents to allow polymerase chain reactions (PCR) amplification of GC-rich DNA (Shi et al., 2012). On the other hand, some reports had disclosed that introduction of alkyl ether moiety in the cationic part of ILs might be a sure approach to design ILs suited for enzymatic reaction (De Diego et al., 2009; Dreyer & Kragl, 2008; Guo, Chen, Murillo, Tan, & Xu, 2006; Zhao, Jones, & Cowins, 2009). According to these observations, we are keen to incorporate alkyl ether moiety into the double imidazolium ring in the cationic part of ILs to ensure the novel ILs more appropriate for lipase-catalyzed reactions (Scheme 1).

Herein, we present the results of advanced designs of ILs used as media to enhance the yield for enzymatic esterification of FA with alcohol. Some studies showed that laurate esters of sugars (sucrose, glucose, maltose, galactose, or fructose) inhibited microbial growth (Ferrera et al., 2005; Wagh, Shen, Shen, Miller, & Walsh, 2012). Taken this into consideration, the laurate alcohol was chosen to be substituted on FA. This unique IL could not only help to enhance the solubility of FA at a higher concentration but also create a favorable microenvironment for the enzyme to maximize the bioconversion. The esterification were optimized with respect to the ratios between ILs and the organic solvent, temperature, enzyme concentration, substrate concentration. Furthermore, the biocatalytically produced lauryl ferulate (LF) were assessed for the antibacterial activity against three food-related bacteria by measuring growth inhibition zone diameters, MIC and MBC values.

2. Materials and methods

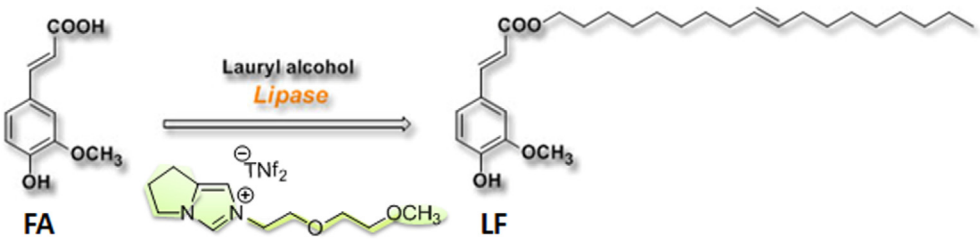
2.1. Materials

Ferulic acid and lauryl alcohol with >98% purity were purchased from Sigma Aldrich (Shanghai, China). Novozym 435 (*Candida antarctica* lipase B immobilized on acrylic resin) was a gift sample from Novo Nordisk (Denmark). Molecular sieves (3 Å and 4 Å) were purchased from Sinopham Chemical Reagent Co., Ltd. (Shanghai, China). Ionic liquids including [(EO)-3C-im][NTf_2] (1-(2-(2-methoxyethoxy)ethyl)-5,6,7,8-tetrahydroimidazo[1,2-a]pyridin-1-ium bis(trifluoromethylsulfonyl)imide) (99%, HPLC) was synthesized and purified in our lab. [Bmim][NTf_2] (1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide), [Bmim][PF_6] (1-butyl-3-methylimidazolium hexafluorophosphate), and [Bmim][BF_4] (1-butyl-3-methylimidazolium tetrafluoroborate) were procured from Shanghai Chengjie Chemical Co. Ltd. (Shanghai, China). All other regents were of analytical grade. All other chemicals of analytical or HPLC grade were purchased from the common commercial sources in China. Lauryl alcohol and other organic solvents were stored over molecular sieves (4 Å), at least 24 h prior to use.

2.2. Synthesis of [(EO)-3C-im][NTf_2]

Methanesulfonyl chloride (36 mmol, in 30 mL dichloromethane) was added into an ice-cooled 50 mL dichloromethane solution of oligo(ethylglycol) methyl ether (22 mmol) and triethylamine (56 mmol). The reaction was carried out under ice bath for 30 min and then washed with 0.5 M citric acid (3 × 20 mL) and 1 M sodium bicarbonate (3 × 20 mL). The organic layer was dried with anhydrous sodium sulfate and removed under vacuum to give colorless liquid methanesulfonylated oligo(ethylene glycol) monomethyl ether. To a round-bottomed flask containing methanesulfonylated oligo(ethylene glycol) methyl ether was added 6,7-dihydro-5H-pyrrolo[1,2-a]imidazole (10 mmol). The mixture was stirred 5 at 75 °C for 24 h. After cooling to room temperature, the reaction mixture was washed with ether (3 × 10 mL) to remove the excess reactants and side products to obtain methanesulfonate salt as a light yellow liquid (65–95% yield). The methanesulfonate salt (2.5 g) was then put into a 150 mL round-bottomed flask, and then bistrifluoromethanesulfonimide lithium salt (1.1 equiv) and water (5 mL) were poured into the bottle. The mixture was allowed to proceed the ion exchange for 12 h at room temperature. The resulting solution was extracted with dichloromethane (3 × 5 mL) and dried with sodium sulfate. Removal of the solvent under reduced pressure to afford the [(EO)-3C-im][NTf_2] as colorless liquid with good isolated yield (75–94%).

[(EO)-3C-im][NTf_2] (1-(2-(2-methoxyethoxy)ethyl)-5,6,7,8-tetrahydroimidazo[1,2-a]pyridin-1-ium bis(trifluoromethylsulfonyl)imide). Colorless liquid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.81 (qn, J = 7.2 Hz, CCH_2C, 2 H), 3.24 (t, J = 7.7 Hz, N = CCH_2, 2 H), 3.33 (s, OCH_3, 3 H), 3.48–3.50 (m, OCH_2, 2 H), 3.57–3.60 (m, OCH_2, 2 H), 3.79 (t, J = 4.5 Hz, 2 H), 4.21 (t, J = 4.8 Hz, NCH_2CO, 2 H), 4.28



Scheme 1. Lipase-catalyzed synthesis of LF in ionic liquid.

(t, J = 7.3 Hz, NCH_2, 2 H), 7.25 (d, J = 1.7 Hz, imH, 1 H), 7.35 (d, J = 1.7 Hz, imH, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.9, 25.6, 48.2, 49.3, 58.6, 68.7, 70.2, 71.4, 117.4, 119.5 (q, J_{CF} = 320 Hz, CF_3), 126.1, 152.9; FAB-HR-MS m/z $[M]^+$ calcd for $C_{13}H_{19}F_6N_3O_6S_2$ 491.062, found.

2.3. General procedure of the lipase-catalyzed esterification for LF

Lipase-catalyzed esterification of FA with lauryl alcohol in organic solvents or ionic liquids/organic solvent mixtures was conducted in a 30 ml vial, equipped with a tight plastic cap to prevent the escape of organic solvents. For the organic solvent reaction system, 0.05 mmol of FA and 0.40 mmol of lauryl alcohol were dissolved in 10 mL of different organic solvents. The reaction was allowed to stand with agitation at 60 °C in an orbital air shaking bath (200 rpm) with 60 mg of Novozym 435 and 60 mg of molecular sieve (3 Å). In the ILs containing organic solvent system, 0.06 mmol FA and 0.24 mmol lauryl alcohol were mixed with 2 mL of media (1 mL of ILs and 1 mL of hexane). The reaction was incubated at 60 °C in an orbital air shaking bath (200 rpm). 100 mg Novozym 435 and 100 mg molecular sieve (3 Å) were added to the mixture. Samples from the reaction mixture were taken at intervals of 24 h. After centrifugation, the supernatants were analyzed by TLC and high performance liquid chromatography. Examinations on the effects of temperatures (from 45 to 65 °C), substrate concentrations of FA (0.01–0.20 mmol/mL), volume ratios of ILs to hexane (3:1, 1:1, and 1:3) and enzyme concentrations (10–100 mg/mL) on the yield of esterification were performed. In order to chemically characterize the reaction products, the reactions were scaled-up to 10 mL. The biocatalyst was filtered and washed with the reaction solvent (3 × 5 mL) to extract LF. The solvent was then removed under reduced pressure and the crude residue was purified by column chromatography on a column (1.5 × 30 cm) packed with silica gel 60 (230–400 mesh, Merck) using hexane: ethyl acetate 8:2 (v/v) as eluent.

Lauryl Ferulate (*LF*). *Dodecyl-(E)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-enoate*. solid, ^1H NMR: δ 0.88 (t, J = 7.0 Hz, CH_3, 3H); 1.26–1.36 (m, $8CH_2$, 16H); 1.57 (m, CH_2, 2H), 1.70 (m, CH_2, 2H); 3.93 (s, OCH_3, 3H); 4.19 (t, J = 7.0 Hz, OCH_2, 2H); 6.29 (d, J = 16.0 Hz, OH, 1H); 6.91 (d, J = 10.0 Hz, 1H); 7.04 (d, J = 2.0 Hz, 1H); 7.10 (d, J = 2.0 Hz, 1H); 7.11 (dd, J = 10.0; 2.0 Hz, 1H); 7.64 (d, J = 16.0 Hz, 1H). ^{13}C NMR: δ 14.1; 22.7; 25.7; 28.7; 29.2; 29.3; 29.4: 29.5; 29.6; 32.8; 55.9; 64.6; 109.3; 114.7; 115,6; 123.0; 127.0;144.6; 146.8; 147.9; 163.8. HR-ESI-MS: $[M + H]^+$ = 363.2525; $[M + Na]^+$ = 385.2351.

2.4. Analytical Procedure

Reaction were followed by TLC on Silica gel 60F254 aluminum sheets (0.2 mm thickness, Merck) in hexane/ethyl acetate 3:2 (v/v) with UV light (254 nm) for detection. HPLC Analysis was performed by high performance liquid chromatography using a Waters pump (Waters 1525) with a UV detector at 325 nm. A Hypersil reversed-phase C18 column (25 cm × 4.6 mm, 5 μm) was used. A sample (10 μL) taken out from the reaction system was centrifuged, diluted with 990 μL of methanol to an appropriate concentration, and injected at a volume of 20 μL. Elution was conducted with methanol/water (95:5, v/v) at a flow rate of 1 mL min^{-1} and a column temperature at 35 °C. The ^1H NMR spectra were recorded on a Bruker AVANCE 400 MHz spectrometer using $CDCl_3$ as the solvent and TMS as the internal standard. Chemical shifts are reported in parts per million (ppm). The ESI-MS were obtained on Agilent 1946A-MSD. The yield of LF was defined on the basis of the concentration of LF and the initial concentration of FA. Data are expressed as means standard deviation of three replicates.

2.5. Bacterial strains and growing conditions

Three common food-related bacterial strains (two Gram positive and one Gram negative) were selected for the study. The Gram positive bacteria were *Listeria monocytogenes* CICC 21529 and *Staphylococcus aureus* CICC 21600 while the Gram negative bacteria was *Escherichia coli* O157:H7 CICC21530. All strains were obtained from China Center of Industrial Culture Collection, Beijing, China, and were grown and maintained in nutrient broth and on nutrient agar (Hangzhou Microbiological Agents Co. Ltd, China). Strains were maintained on TSA slants at 4 °C. Cells were prepared by 16 h culture in TAB at 37 °C. A 16 h culture was diluted with TSB to achieve an inoculum of 1×10^6 CFU/mL approximately. The number of cells in the suspensions was determined by duplicate plating from ten-fold serial dilution on TSA and counting the colonies after incubation at 37 °C for 24 h.

2.6. Agar diffusion assay

The antimicrobial activity of LF was determined by agar diffusion assay method described by Hosseini, Razavi, and Mousavi (2009) with some modification. TSB cultures of bacteria were grown at 37 °C for 12 h. Suspensions (100 μl) of the bacteria, adjusted to final concentration of 10^6 CFU/mL, were added into Petri dishes (95 mm × 15 mm) with 20 mL of sterile TSA media (about 55 °C), and the mixture was mixed immediately and allowed to solidify. Thereafter, holes measuring 7.0 mm of the diameter were dug with sterile hole puncher and 100 μL of the LF with different concentration were added to each hole. The plates were incubated for 24 h at 37 °C and diameter (mm) of the inhibition zones were measured to determine the antimicrobial activity of the LF. The tabulated data are the means ± standard deviations of three replicates.

2.7. Minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC)

MIC and MBC were determined by broth macrodilution assay (Wilson et al., 2005). Serial two fold dilutions of LF were prepared in TSB at concentrations of 40, 20, 10, 5, 2.5 and 1.25 mM. One tube with the same volume of TSB was set as control. Inocula were added into all the tubes to achieve an initial inoculum of approximate 10^6 CFU/ml. All tubes were incubated at 37 °C for 24 h, a 1 ml portion was removed from each tube for colony counting by decimal dilution in 0.85% (w/v) sodium chloride solution, and plated out onto TSA. All experiments were conducted in duplicate. MIC is defined based on the logarithmic difference in population (logDP). The logDP is expressed by the following equation:

$$\log DP = \log(N/N_o) = (\log N) - (\log N_o)$$

N is the population after incubation for 24 h and N_o is the initial population. MIC is defined as the lowest concentration resulting in maintenance or reduction of inoculums viability (log DP $\leqslant$ 0). The minimum bactericidal concentration (MBC) is defined as the concentration where 99.9% or more of the initial inoculums are killed (log DP $\leqslant$ -3).

3. Results and discussion

3.1. Effects of solvent systems on the enzymatic esterification

Most of the biocatalysis and biotransformation are greatly influenced by the reaction media. The physical-chemical properties of the reaction media have essential effect on the activity and even selectivity of enzymes. Lipase differs in their sensitivity or activity

towards different organic solvents and are generally more unstable in polar water miscible-solvents than in water-immiscible solvents. According to some results reported before, some purified lipases could be stable in the presence of organic solvents, such as DMSO, acetone, butanol, n-hexane, isooctane (Chen et al., 2011; Ganske & Bornscheuer, 2005; Hu, Guo, Lue, & Xu, 2009; Katsoura et al., 2009). In this section, some organic solvents, including butanol, n-hexane, cyclohexane, n-octane, isooctane, benzene, toluene, were used to investigate the effect of reaction media on the enzymatic esterification of FA with lauryl alcohol. As showed in Fig. 1(A), the bioconversion yield reached 3.0%, 48.3%, 36.2%, 46.8%, 43.2%, 15.6% and 11.5% for *tert*-butanol, n-hexane, cyclohexane, n-octane, isooctane, toluene and benzene, respectively, after 48 h. Among them, hexane and 1-octane offered relatively higher yield of LF. With the fact that the former is available, cheaper and it's more beneficial for ionic liquids recycling taken into consideration, hexane was chosen as a preferred organic solvent to examine the enzymatic esterification of FA with lauryl alcohol in different ionic liquids containing the organic solvent.

There is no doubt that a drawback of the reaction mediated by ILs is the high viscosity of the system. Adding organic co-solvent to lower the viscosity of ILs may offer another advantage besides increasing the solubility of the substrate that has low solubility in conventional solvents (Chen, Guo, Let, Lue, & Xu, 2008). Fig. 1 (B) depicted the time course of enzymatic synthesis of LF in different ionic liquids containing hexane. Albeit all ILs selected in this section belong to imidazolium-type ILs, the yield of reaction varied

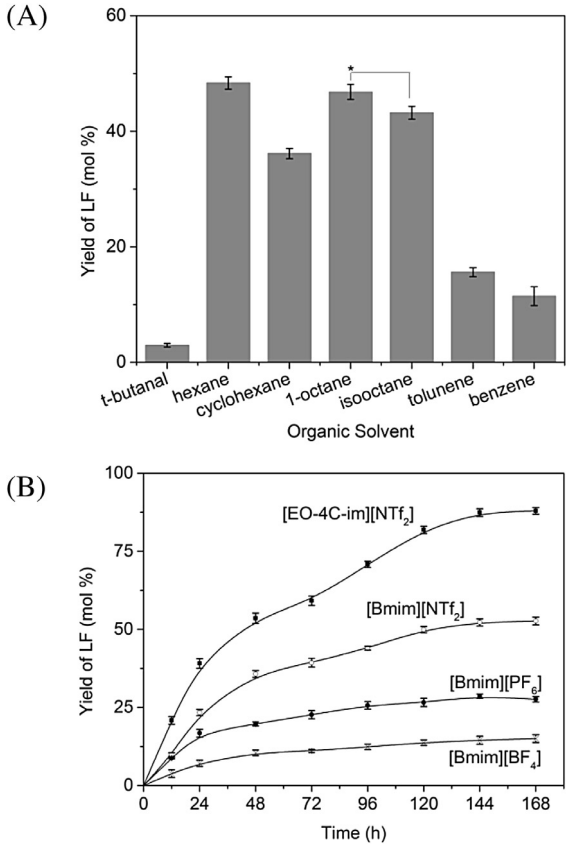


Fig. 1. Solvent dependency of lipase-catalyzed esterification of FA with lauryl alcohol. (A) Organic solvents; (B) ILs/hexane. The error bars represent the standard deviations, and the asterisks indicate significant difference between each other. (A) Reaction conditions: 0.005 mmol/mL FA, 0.04 mmol/mL lauryl alcohol, 10 mL of solvent, 60 mg Novozym 435, 60 mg molecular sieves, 200 rpm, 60 °C, 48 h. (B) 0.03 mmol/mL FA, 0.12 mmol/mL lauryl alcohol, 50 mg/mL Novozym 435, 50 mg/mL molecular sieves, V_{ILs}:V_{hexane} = 1 mL:1 mL, 200 rpm, 60 °C, 168 h.

significantly with the anions and the structure of cationic moiety (namely single or double imidazolium ring). The highest yield of LF (87.9%) was achieved in [(EO)-3C-im][NTf_2]/hexane after 168 h, followed by [Bmim][NTf_2]/hexane (52.7%) at the same reaction conditions. However, remarkably low productivity was yielded in [Bmim][PF_6]/hexane (27.6%) and [Bmim][BF_4]/hexane (15.0%), as can be observed from Fig. 2.

In our previous work, we found the ionic liquid possessing a double imidazolium ring in its cationic moiety could be used to facilitate the polymerase chain reaction (PCR) of DNA by improving the selectivity of polymerase without compromising the natural activity of it (Shi et al., 2012). On the other hand, Zhao et al. (2009) have reported which ILs that have an alkyl ether moiety as a cationic part acted as good solvents for lipase-catalyzed reactions. We envision therefore novel ionic liquids could be synthesized with incorporating both advantages from the structures of double imidazolium ring with alkyl ether moiety to render them more suitable for this scenario. After evaluation of the IL [(EO)-3C-im][NTf_2] as compared with other common ILs in this section, we established that [(EO)-3C-im][NTf_2] acted as a more useful solvent for the esterification of FA with lauryl alcohol.

The nature of ILs (viscosity, polarity, hydrophobicity, nucleophilicity, H-bond basicity, and kosmotropicity/chaotropicity) affect the activity and even selectivity of enzymes. The H-bond basicity, like anion nucleophilicity, can be responsible for the loss of activity in enyzme-incompatible ILs, since high H-bond basicity exacerbates interference with the internal H-bonds of the enzyme. For ILs with these common anions, the order of decreasing H-bond basicity [BF_4^-] > [PF_6^-] > [NTf_2^-] (considering the spreading of negative charge over fluorine atoms) is more consistent with the positive effect on lipase activities. Katsoura et al. (2009) also observed the negative effect of [BF_4^-] containing ILs on the performance of the enzymatic esterification of FA as compare with [PF_6^-] containing ILs when imidazolium-based ionic liquids were employed as reaction media. Hu et al. (2009) investigated the enzymatic esterification with Novozym 435 in ILs-organic solvent mixed systems. For the three chosen ionic liquids anions, namely, [PF_6^-], [BF_4^-], and [NTf_2^-], their thermal stability, viscosity, and strength of H-bonding between anion and water are different. The reaction in an IL possessing the [NTf_2^-] had higher lipase activity than [PF_6^-] and [BF_4^-]. The observation in this work also agreed with their results. [(EO)-3C-im][NTf_2] mediated systems were selected for further parameter optimization.

The error bars represent the standard deviations, and the asterisks indicate significant difference between each other. (A) Reaction conditions: 0.005 mmol/mL FA, 0.04 mmol/mL lauryl alcohol, 10 mL of solvent, 60 mg Novozym 435, 60 mg molecular sieve, 200 rpm, 60 °C, 48 h. (B) 0.03 mmol/mL FA, 0.12 mmol/mL lauryl alcohol, 50 mg/mL Novozym 435, 50 mg/mL molecular sieves, V_{ILs}: V_{hexane} = 1 mL:1 mL, 200 rpm, 60 °C, 168 h.

3.2. Reaction temperatures

Reaction temperature influenced enzymatic reactivity by affecting not only the lipase activity but also the rate of molecular movement. Moreover, the increase of reaction temperature could lower the viscosity of ILs and affect the thermodynamic equilibrium of enzyme-catalyzed reactions. The effect of temperature on the yield for LF synthesis was studied in [(EO)-3C-im][NTf_2]/hexane system, varying the temperature from 45 to 65 °C. We found that increase of the temperature remarkably accelerated the reaction, and a high yield in the esterification of FA with lauryl alcohol catalyzed by Novozym 435 was obtained within 48 h when the reaction was carried out in [(EO)-3C-im][NTf_2]/hexane system at 60 °C (Fig. 2 (A)). This behavior could be explained by the protective effect of IL against enzyme thermal deactivation (Lozano, De Diego, Carrié,

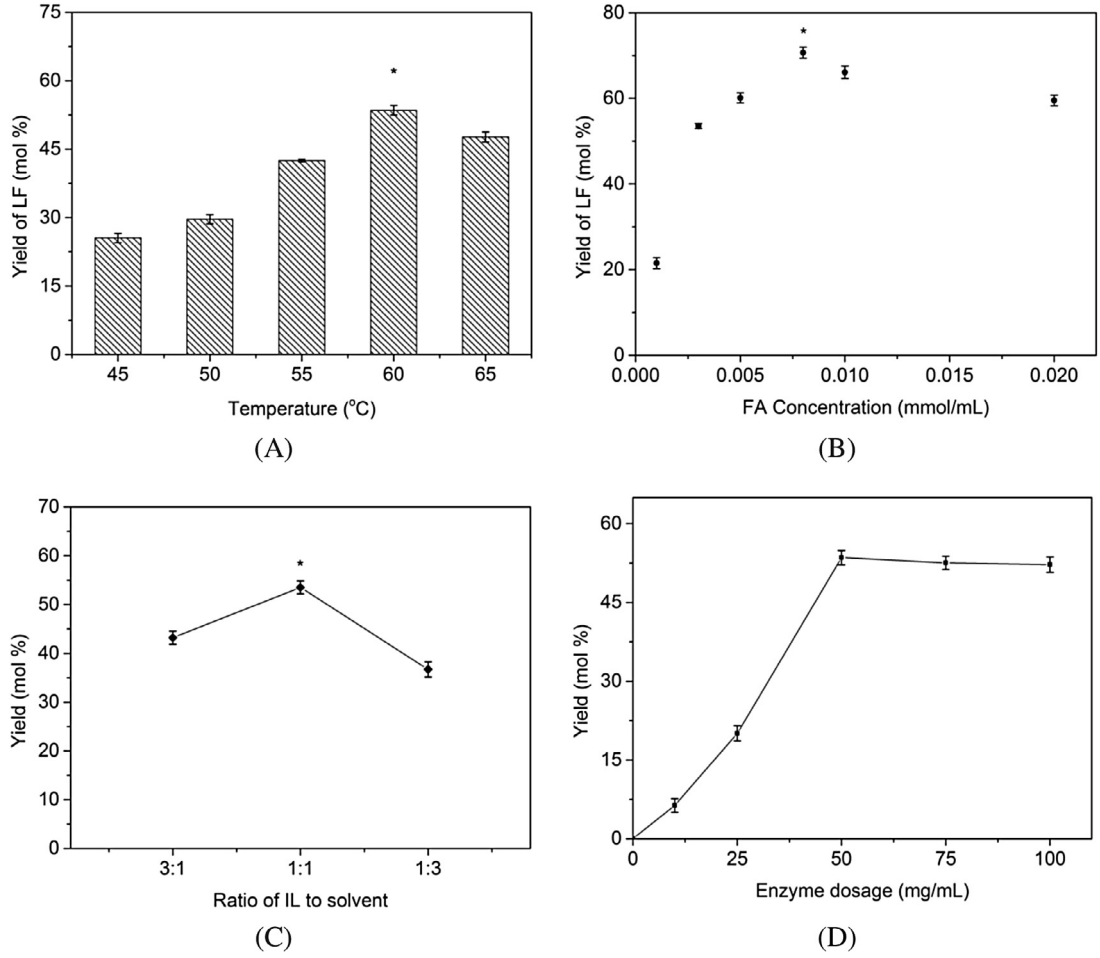


Fig. 2. Effect of various parameters on the yield of lipase-catalyzed esterification of FA with lauryl alcohol. (A) Reaction temperature; (B) substrate concentrations; (C) [(EO)-3C-im][NTf_2]/hexane ratios; (D) enzyme dosage. The error bars represent the standard deviations, and the asterisks indicate significant difference between each other. (A) The reactions were performed at 45, 50, 55, 60 or 65 °C with 0.03 m mol/mL FA, 0.12 mmol/mL lauryl alcohol, 50 mg/mL Novozym 435, 50 mg/mL molecular sieves, V_{ILs}: V_{hexane} = 1 mL:1 mL, 200 rpm, 48 h. (B) FA concentration, 0.001, 0.003, 0.005, 0.008, 0.01 and 0.02 mmol/mL, 60 °C; (C) 0.03 mol/L FA, 0.12 mol/L lauryl alcohol, volume ratio of ILs to Hexane: 3:1, 1:1, and 1:3. (D) Novozym 435, 10, 25, 50, 75, or 100 mg/mL.

Vaultier, & Iborra, 2003; Persson & Bornscheuer, 2003). When the temperature is below 50 °C, low solubility of FA and high viscosity of ILs would inhibit this reaction. Moreover, the volatility of organic solvent and the thermodeactivation effect on lipase would result in the undesired declination of the volumetric productivity as the temperature was over 60 °C.

The reaction time to reach equilibrium depends largely on mass transfer, temperature (kinetics) parameters, temperature-dependent solubility, and so forth. The similar patterns in terms of reaction productivity at different temperatures might be the consequence from all these important variables. The result in this work was in accordance with the previous study reported in other IL-mediated system (Ganske & Bornscheuer, 2005; Katsoura, Polydera, Katapodis, Kolisis, & Stamatis, 2007). In enzymatic production of diglycerids, with increasing temperatures would bring about an improvement in both triacylglycerol conversion and diacylglycerol yield, since the decrease in the viscosity of the IL-hexane- systems was decreased by the increased temperatures. Lou, Zong, Wu Liu, and Wang (2006) and Ganske and Bornscheuer (2005) described a similar effect of temperature on the activity of Novozym 435 for the hydrolysis of D,L-phenylglycine methyl ester to D-phenylglycine in the [Bmim][BF_4]-phosphate buffer and CAL-B for the synthesis of glucose fatty acid esters in the [Bmim][BF_4]/t-BuOH system.

3.3. Substrate concentrations

Fig. 2(B) showed the productivity of the LF with different FA concentrations, monitored over 48 h reaction time period. In the [(EO)-3C-im][NTf_2]/hexane reaction system at the volume ratio 1:1, the highest yield of 70.67 % was obtained with a FA concentration of 0.008 mmol/mL after 48 h. However, further increasing the FA concentration from 0.008 to 0.01 mmol/mL led to a slight decrease in the yield of LF from 70.67% to 66.10%. These results suggested that solubility limitation of the substrate might be a key factor. For a specific system only the solubilized substrate is available for reaction. There is a capacity limitation where maximum yield could be reached. Thus, a further increase of substrate load higher than the solubility limitation may not increase the valid substrate concentration for reaction, instead increase viscosity of system and restrain mass transfer, and thus might result in a decrease of reaction productivity. Therefore, 0.008 mmol/mL of FA concentration was treated as optimum option in [(EO)-3C-im][NTf_2]/hexane reaction system. Katsoura et al. (2007) showed the similar effect of substrate concentration on the enzymatic acylation of naringin with vinyl butyrate in [Bmim][BF_4]. They found increasing the substrate concentration of naringin up to a specific value led to an increase in the conversion of naringin, while a further increase of naringin concentration had a negative effect

on the conversion yield. Lv, Pan, and Li (2007) showed the effect of substrate concentration on the biosynthesis of ascorbyl benzoate in organic media. Only at low substrate concentration, the conversion rate increases with rising of substrate concentration.

3.4. Optimization of the ILs/hexane volume ratios

The effects of different [(EO)-3C-im][NTf_2]/hexane ratios on the lipase-catalyzed esterification were investigated with varied ratios at 1:3, 1:1, and 3:1. Fig. 2(C) shows the reaction time course, where increasing the [(EO)-3C-im][NTf_2]/hexane ratios up to a specific value led to an increase in the yield of LF, while a further increase of it had a negative effect on the yield of LF. The maximum yield of LF of 53.5% was obtained with an [(EO)-3C-im][NTf_2]/hexane volume ratio of 1:1. The decrease of yield of LF with higher IL content is more likely due to the high viscosity of the system. On the other hand, decrease of yield LF with lower IL content may attribute to compromise of the favorable microenvironment in ILs for the enzyme or the deleterious effect on enzyme activity from organic solvent. These results rather support our initial envisage that a binary system of IL/organic solvent could reach a promising effect between the contribution from IL for higher substrate solubility and the favorable microenvironment of ILs for the enzyme, as well as the contribution from the organic solvent for buffering the negative effect on mass transfer from the viscosity of IL. The result was also similar to a previous study on enzymatic synthesis of esculin ester, where the esterification rate and esculin conversion increased as the acetone ratios increased in IL-acetone mixtures (Hu et al., 2009).

3.5. Enzyme concentrations

The effect of Novozym 435 dosage on the yield of LF is shown in Fig. 2(D) The yield of LF was enhanced by increasing lipase dosage. The highest yield for the [(EO)-3C-im][NTf_2]/hexane system was 53.53% with 50 mg/mL (100 mg) lipase used. However, a further increase in the enzyme concentration to 100 mg/mL (200 mg) resulted in decreasing yield of LF. In this case, an enzyme concentration of 50 mg/mL was employed. The presence of the higher amount of enzyme provides more active sites for acyl-enzyme complex formation and also increases the probability of enzyme-substrate collision and subsequent reaction (Soo, Salleh, Basri, Rahman, & Kamaruddin, 2004). The negative effect of further increase in enzyme concentration may be due to mass transfer limitation and poor dispersion of enzyme particles as experimentally observed. Zhang, Bai, and Sun (2007) and Li et al. (2006) observed the similar results. The conversion of pyridoxine also increased with the increase of enzyme loading; when the enzyme loading was raised to 10 mg/mL acetonitrile, a further increase in the enzyme concentration was not available for further enhancing the conversion (Zhang et al., 2007).

The error bars represent the standard deviations, and the asterisks indicate significant difference between each other. (A) The reactions were performed at 45, 50, 55, 60 or 65 °C with 0.03 m mol/mL FA, 0.12 mmol/mL lauryl alcohol, 50 mg/mL Novozym 435, 50 mg/mL molecular sieves, V_{ILs}:V_{hexane} = 1 mL:1 mL, 200 rpm, 48 h. (B) FA concentration, 0.001, 0.003, 0.005, 0.008, 0.01 and 0.02 mmol/mL, 60 °C; (C) 0.03 mol/L FA, 0.12 mol/L lauryl alcohol, Volume ratio of ILs to Hexane: 3:1, 1:1, and 1:3. (D) Novozym 435, 10, 25, 50, 75, or 100 mg/mL.

The time course of Novozym 435-catalyzed esterification of FA with lauryl alcohol under the optimum conditions was exhibited in Fig. 3. The yield of LF in [(EO)-3C-im][NTf_2]/hexane increased rapidly in the first 6 days and reached equilibrium at 8 days with yield up to 90.1%.

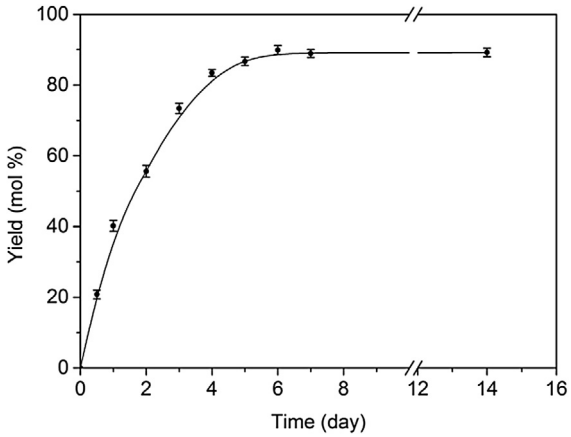


Fig. 3. Time course of lipase-catalyzed esterification of FA and lauryl alcohol in the optimized reaction conditions. Reaction conditions: 1 mL of Hexane/1 mL of [(EO)-3C-im][NTf_2], 0.08 mmol/mL FA, 0.12 mmol/mL lauryl alcohol, 50 mg/mL Novozym 435, and 50 mg/mL molecular sieves for 14 days at 60 °C, 200 rpm.

Reaction conditions: 1 mL of Hexane/1 mL of [(EO)-3C-im][NTf_2], 0.08 mmol/mL FA, 0.12 mmol/mL lauryl alcohol, 50 mg/mL Novozym 435, and 50 mg/mL molecular sieves for 14 days at 60 °C, 200 rpm.

3.6. Antibacterial activity

Initial screening of the antibacterial activities of the investigated LF against three food-related bacteria (including *L. monocytogene*, *S. aureus* and *E. coli*) was conducted by using an agar diffusion assay, which was determined by the presence or absence of inhibition zones. As shown in Fig. S1 (supporting information) and Table 1, LF displayed a variable degree of antibacterial activities against different tested strains. LF showed the strong antibacterial activity against all the tested bacteria, *L. monocytogenes*, *S. aureus* and *E. coli*, with the inhibition zone diameters of 21.5 ± 0.2, 23.2 ± 0.5 and 25.1 ± 0.3 mm, respectively where the concentration of LF is 5-fold MIC. The MICs and MBCs of LF against the tested bacteria are shown in Table 1. The MIC for Gram-negative bacteria *E. coli* was 1.25 mM, while MICs for Gram-positive *L. Monocytogenes* and *S. aureus* were 2.5 and 5 mM, which were higher than that of Gram-negative bacteria. The minimum bactericidal concentrations (MBCs) of LF on bacteria were defined as the lowest concentration of LF that prevent the growth of bacteria after sub-culture on agar media. The MBCs for Gram-positive bacteria *L. Monocytogenes*, *S. aureus* and Gram-negative bacteria *E. coli* were 10, 20 and 5 mM, respectively. Noticeably, the results suggested that the antibacterial activity of LF against Gram-negative bacteria was stronger than that of Gram-positive bacteria. Some sugar monoesters containing laurate, such as lactose monolaurate (LML) and sucrose monolaurate (SML) have been shown to inhibit microbial growth. In contrast, Gram-positive bacteria were more susceptible

Table 1
Antibacterial activity of LF against three tested bacteria.

	Bacteria		
	L.monocytogenes (G^+)	*Staphylococcus aureus*(G^+)	*Escherichia coli*(G^-)
Inhibition zone diameter (mm)[a]	21.5	23.2	25.1
MIC (mM)	2.5	5	1.25
MBC (mM)	10	20	5

[a] The concentration of LF is 5-fold MIC.

than Gram-negative bacteria to both esters. No change in growth of Gram-negative bacteria (*E. coli H7:O157*) was observed in either SML or LML as compared to that of the controls, while both of them were effective to against *L. monocytogenes* EGDe with a MICs of each monoester of 0.2 mM and a MBC of LML of 9.5 mM (Wagh et al., 2012). In this case, it is probable that the aliphatic chain substituted on FA skeleton electronically increase the hydrophobic and lipophilicity, which consequently promote the combination of the LF with the membrane of bacteria and possibly interfere with the bacterial redox systems and enhance the activity against bacterial strains (Sofrata et al., 2011). The exact antimicrobial mechanism of FA derivatives still remain enigmatic and, without doubt, more work is needed.

4. Conclusion

In conclusion, this work examined lipase-catalyzed esterification of FA with lauryl alcohol in [(EO)-3C-im][NTf_2]/hexane binary system, which is found to be capable of obtaining high yield of the LF. Substrate concentration, enzyme content, [(EO)-3C-im][NTf_2]/hexane volume ratio, and temperature are found to be crucial parameters governing the yield of LF and the reaction conditions have been optimized. The optimum yield of LF of 90.1% for [(EO)-3C-im][NTf_2]/hexane system was obtained under its optimized conditions. Biosynthesized LF showed a stronger antibacterial activity against Gram-negative (*E. coli*) than Gram-positive bacteria (*L. Monocytogenes* and *S. aureus*). The lowest MIC value was seen for *E. coli* (1.25 mM), followed by *L. Monocytogenes* (2.5 mM) and *S. aureus* (5 mM). The MBCs for Gram-positive bacteria *L. Monocytogenes*, *S. aureus* and Gram-negative bacteria *E. coli* were 10, 20 and 5 mM, respectively. To our knowledge, this is the first report on novel functionalized ionic liquid enhancement in lipase-catalyzed esterification of FA. More broadly, we expect this IL to find use in facilitating other biotransformation, particularly for nonaqueous systems that were previously shown to be unsuccessful or unsatisfactory in selectivity, bioconversion, even reaction rate. We are currently investigating the general applicability of this IL amplification of a broad range of biotransformation and will report our results in due course.

Acknowledgements

This work was supported by National Natural Science Foundation of China (21106131) and (31101344), Zhejiang Provincial Natural Science Foundation of China (No. Y4100762), as well as Food Science and Engineering the most important discipline of Zhejiang province (JYTsp20142101).

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.foodchem.2016.09.187.

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