



Lipase-catalyzed Biodiesel Production in [BMIM][PF₆]

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Lipase-catalyzed transesterification of Tung oil in room temperature ionic liquids reaction system was studied with Liaczyme and Limczyme (immobilized lipase from *Candida antarctica*). Limczyme was more perfect biocatalyzers than Liaczyme. The [BMIM][PF₆] was more perfect reaction medium than the others. The optimal technology parameters of production biodiesel from Tung oil using Limczyme as biocatalyzer was as follows: [BMIM][PF₆] as reaction medium, pH 7.5, reaction temperature 40 °C, water content of 6 %, immobilized lipase Limczyme amount of 5 %, shaker speed of 200 rpm, reaction time of 48 h. As double substrates, the molar ratio of methanol to Tung oil should be maintained at 1.0, while methanol should be added into bioreactor step by step.

Keywords: Transesterification, Tung oil, Immobilization lipase, Ionic liquid, Biodiesel.

INTRODUCTION

Biodiesel (fatty acid methyl esters, FAMES), a renewable, biodegradable, nontoxic and cleaning fuel as a part of substitute for conventional fossil diesel, which is made from plant oils or animal fats. Biodiesel is advantage of solving energy crisis and reducing the emission of greenhouse gas. Until now, the traditional methods for producing biodiesel have been the alkali-catalyzed and acid-catalyzed transesterifications from vegetable oils or animal fats with methanol. In the process of producing biodiesel, the problems as energy-intensive nature, difficulties in the recovery of glycerol and chemical catalysts should be solved.

To overcome drawbacks above, people tried to produce biodiesel with lipase or immobilized lipase as a catalyzer. The lipase-catalyzed transesterification for producing biodiesel was considered as effective and promising means¹. The lipase-catalyzed transesterification to produce FAMES must commonly be in non-aqueous solutions to disperse substance very well. However, organic solution is not friendly to environment and make lipase inactive easily as reaction medium.

Room temperature ionic liquids (RTILs) are organic salts formed by ions. Room temperature ionic liquids have recently become increasingly popular as truly 'green' solvents with its low vapor pressure, non-flammable and thermal stability because of its alternative to volatile, toxic, inflammable, explosive organic solvents. Ionic liquids were recycled by evaporation, extraction, supercritical carbon dioxide extraction, membrane separation and other separation technology². The recent trends

of medium engineering (ionic liquids) and enzyme engineering (non-aqueous enzymology) were organically accorded with the current development trend of green chemistry. Bio-catalysis in ionic liquids focus on regulating the physical and chemical properties of ionic liquids, such as melting point, viscosity, solubility, density, hydrophobicity and polarity. Two-phase or multi-phase reaction systems are to facilitate the separation of the final products³.

Ionic liquids could be used as carriers of lipase for transesterification, the concept was proposed by Klibanov and his colleagues⁴. Since then, Lau *et al.*⁵ studied the ionic fluids ([C4mim][BF₄] or [C4mim][PF₆]) as the reaction medium in lipase-catalyzed alcoholysis reaction. At 50 °C, Lozano *et al.*⁶ studied on the activities and stabilities of *Candida antarctica* lipase B in 4 ionic liquids with different water contents and reported that all of the ionic liquids for the lipase-catalyzed reaction was appropriate. In this paper, it was the first time that we have produced biodiesel with Tung oil from Shaan'xi province in China as raw materials, with ionic liquids as reaction mediums, CAL B as bio-catalyst. The relationships between the reaction variables (time and substrate molar ratio, water content *etc.*) and conversion yield were examined.

EXPERIMENTAL

Lipase from *Candida antarctica* (5000 U g⁻¹), BSA, Non-porous Silica Gel was purchased from Merck A. G (Darmstadt, German), CHD-ZT6, prepared by ourselves; *n*-tridecane (internal standard, Geel, Belgium), polyethylene glycols 2000, methyl palmitate, methyl stearate, methyl oleate, ethyl oleate,

methyl linoleate, methyl linolenate (Accu Standard, U.S.A), 4-toluenesulfonyl chloride, boron(tri)fluoride, acetone, 3-glycidioxypropyl trimethoxysilane, pyridine were obtained from China Chemical Group Co., Xi'an, P.R. China. All other chemicals used were of analytical grade. Tung oil, commercially available from the south of Shaan'xi province in China; Liaczyme, immobilized lipase by adsorption-cross-linking method by ourselves; Limczyme, immobilized lipase by modification-coupling method, [BMIM]Cl, [BMIM][BF₄], [BMIM][PF₆].

HH-501 super constant temperature water bath pan, Changzhou Guohua Electric Appliance Co., Ltd.; DS-1 high-speed crushing machine, the Shanghai Biaoben model, factory; BS 224S electronic balance, Sartorius Corporation; HQD98L constant temperature culture shaker, Wuhan Haishengda equipment Co., Ltd.; SP-3420 gas chromatograph, Beijing Rayleigh Analytical Instruments Co., Ltd.; KB-1 capillary column (30 m × 0.25 mm × 0.25 μm), the United States Kromat company; TGL-16C High-speed centrifuge, Changzhou Guohua Electric Appliance Co., Ltd.; LSC-60D Moisture Analyzer, Shenyang Dragon Electronics Co., Ltd..

General procedure

Lipase-catalyzed methanolysis: Before the reaction, ionic liquids in a vacuum drying oven were dried at 50 °C for at least one day in order to remove all the moisture as much as possible. Based on the preceding analysis, the water contents of ionic liquids were not more than 0.10-0.15 % after a day due to the ionic liquid absorbent⁷. Ionic liquids or *tert*-butanol (2.5 mL), Tung oil (2.5 mL) and immobilized lipase Liaczyme, Limczyme (2 wt. % Tung oil based on weight), placed in a 10 mL plastic capped vial, then adding different molar ratios methanol. For the solvent-free system, Tung oil (2.5 mL) and immobilized lipase Liaczyme, Limczyme (2 wt. % based on Tung oil weight), placed in a 10 mL plastic capped vial, then adding different molar ratios methanol. At 40 °C and 150 rpm shaking rate of reciprocal shaker, transesterification has been on for different reaction time.

Detection method

Lipase activity assay: The activity of free and immobilized lipase was determined by monitoring the hydrolysis of olive oil. Olive oil emulsion (200 mL) was prepared by mixing olive oil (50 mL) and gum polyvinyl alcohol solution (150 mL, 4 %, w/v). The assay mixture consisted of emulsion (4 mL), phosphate buffer (5.0 mL, 0.0025 M, pH7.5) and free lipase (1 mL) or immobilized lipases (0.2 g). The hydrolysis of oil was carried out at 4 °C for 15 min in a water bath. The reaction was stopped by the addition of 15 mL of ethanol. The liberated fatty acids in the medium were determined by titration with 50 mM NaOH solution. Assays were performed over a pH range of 4 to 9 and a temperature range 20-60 °C to determine the pH and temperature-dependent activity profiles of the free and immobilized lipases.

1 Lipase unit (U g⁻¹) is defined as the activity required by 1 g of immobilized lipase powder to produce 1 μL of fatty acid per minute from olive oil.

$$A_{\text{rel}} = \frac{A_{\text{im}}}{A_{\text{i}} - A_{\text{res}}} \quad (1)$$

where, A_{rel} is the relative lipase activity, A_{i} is the initial lipase activity; A_{res} is residual lipase activity; A_{im} is the immobilization lipase activity.

GC analysis: Sample (300 μL) taken from the reaction mixture was mixed with 100 μL of water and 100 μL of hexane to extract fatty acid methyl esters (FAMES). Hexane of 90 μL phase which contains FAMES was mixed with 10 μL of *n*-tridecane (internal standard). The methyl ester contents in the reaction mixture were quantified by a SP-3420 gas chromatograph (Beijing Bei Feng Co, Ltd., China) using a KB-1 capillary column (30 m × 0.25 mm × 0.25 μm, Kromat, U.S.A.). The oven temperature was kept at 120 °C for 1 min, raised to 280 °C at 10 °C min⁻¹, maintained at this temperature for 1 min. The temperatures of the injector and detector were set at 290 °C and 300 °C, respectively. Pure nitrogen was used as a carrier gas. The volume of injected sample: 2 μL.

RESULTS AND DISCUSSION

Screening of ionic liquids: In free solvent reaction system, *tert*-butanol reaction system, 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) reaction system, 1-butyl-3-methyl-tetrafluoroborate ([BMIM][BF₄]) reaction system, 1-butyl-3-methyl-hexafluorophosphate ([BMIM][PF₆]) reaction system, Tung oil and methanol (batches times to add methanol, methanol oil molar ratio: 3:1) as raw materials had been used to produce biodiesel catalyzed using immobilized lipase Liaczyme and Limczyme for 24 h.

The experimental results were shown in Fig.1 that as biocatalyzer Limczyme were more effective than Liaczyme. In *tert*-butanol reaction system, it had good performance as reported⁸, Limczyme catalytic FAMES yield reached 70.9 %, while in hydrophobic ionic liquid [BMIM][PF₆] system Limczyme catalytic FAMES yield reached 82.1 %, which was the highest than in the others. [BMIM][PF₆] reaction system was our choice in the following experiments, the catalyzed behave of the right Limczyme for production biodiesel was investigated.

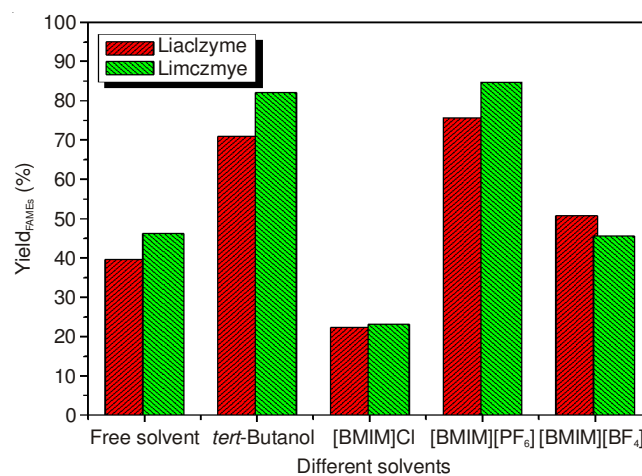


Fig. 1. Comparison of the yields of FAMES from Tung oil in five different medium systems after production for 24 h

The difference of FAMES yields in the 1-butyl-3-methyl chloride salt reaction system and 1-butyl-3-methyl hexafluorophosphate reaction system was not obviously, which may be

related to the types of ionic liquids selected but maybe nothing to do with the catalytic activity and stability of immobilized lipase. In the [BMIM]Cl reaction system, it may be due to water absorption characteristics of chloride ions that the system of water was excessive and lead to inactivation of immobilized lipase (Liaczyme and Limzyme). In hydrophilic ionic liquid reaction system, [BMIM][BF₄] easily combined with the substrate Tung oil. Because of the lack of necessary water, it was difficult that protons passed between lipase amino and substrate, affected the formation of product transition states and resulting in low FAMES yield (45.6 %).

Effect of temperature on Limzyme catalysis in [BMIM][PF₆] system: Under the condition of pH 7.5, we produced biodiesel (FAMES) in [BMIM][PF₆] system at different reaction temperatures and took a sample every 4 h to investigate the FAMES yield each time. The results were shown in Fig. 2, FAMES yields were also improved and the catalytic activity of Limzyme was reduced with reaction temperature increased. At the same reaction time, the conversion rate has also been increasing as the temperature increased, especially in the initial reaction time (16 reaction hours ago) was particularly prominent, after that it was split occurred. 40 °C was the optimal reaction temperature for Limzyme in [BMIM][PF₆] system.

The loss of lipase activity didn't happen in [BMIM][PF₆] as in conventional solvents, which mainly due to the lipase's protective effect making the Limzyme heat-inactivation slow down and becoming stable⁹. On the other hand, with temperature increased, water molecules (a nucleophile receptors) in [BMIM][PF₆] system was relatively less than before. With regard to the effect temperature on the ionic liquids catalytic activity of lipase, Lou *et al.*¹⁰ studied that the process on Novozym 435 catalyzed hydrolysis D, L-phenyl glycine methyl ester to produce D-phenyl glycine in [BMIM][PF₆] system. In the range of 20 to 50 °C, the initial rate could rise rapidly with temperature increased. At 30 and 35 °C, from the beginning the conversion rate was still smaller than the others. The reaction rate was smaller because it was trouble that the viscosity of ionic liquids for mass transfer at lower temperature. At 45 °C and 50 °C, the viscosity reduction is conducive to mass transfer, but higher temperatures were challenge for thermal stability of lipase.

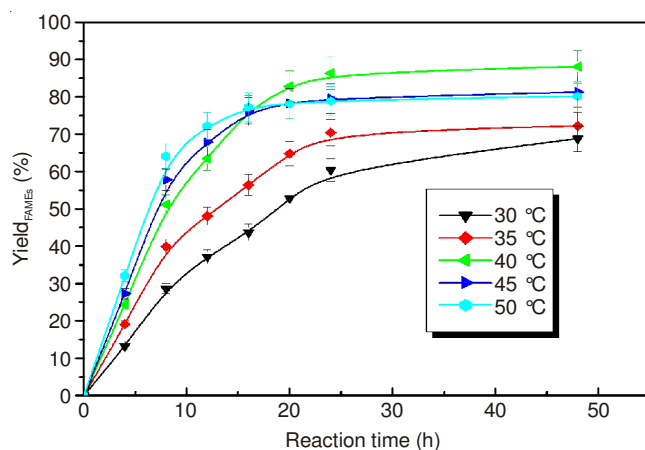


Fig. 2. Effect of temperature in [BMIM][PF₆] reaction system on the yield of FAMES

Effect of water content on Limzyme catalysis in [BMIM][PF₆] system: The water activity in ionic liquids could be adjusted by its pre-balance in saturated salt solution. Eekstein *et al.*¹¹ had quantitative controlled the water activity of reaction medium by pre-balancing with salt solution and found that under low water activity and high-temperature conditions, lipase could show more catalytic selectivity in [BMIM][TfzN] system than in ether solvents. As we know, in reaction system lipase activity could be directly affected by changing water activity with hydration salt. Kuhl and Halling¹² firstly reported that water activity could be controlled by using salt pair. Something else be added into ionic liquids system so that the reaction system tended to be more complex, which was a negative factor for practical application, so we might control water content with pure water (Buffer) in ionic liquid reaction system.

Based on the mention above, we still had [BMIM][PF₆] as non-aqueous medium and Limzyme as lipase catalyst with different quality (based on Tung oil weight) of water (P salt buffer, pH 7.5) and tested for 24 h. The results were shown in Fig. 3 that the relative activity of Limzyme increased from 51 to 83 % with water content increased from 1 to 5 %. When the water content in [BMIM][PF₆] system was in 5 to 8 % range, activity of Limzyme fluctuated on the basic of 80 %. When the amount of water was optimum in [BMIM][PF₆] system, the dynamics rigid and thermodynamic stability of protein structure would reach a balance and show optimal lipase activity. In the [BMIM][PF₆] reaction system, the optimum necessary water content was 6 %.

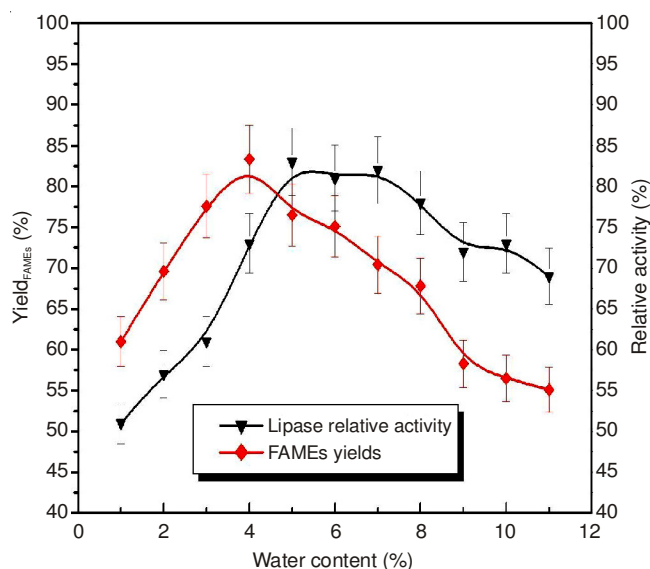


Fig. 3. Effect of water content in [BMIM][PF₆] system on the yield of FAMES

The slight differences of water content would lead to a large change in enzymatic activity because lipase requires a small amount of water to maintain three-dimensional conformation state and keep its activity. At the same time, water plays a vital role on protein structures and functions to determine enzymatic activity and stability. The number of water molecules around lipase molecule was small when water content was less than 1 % and was higher when water content was

more than 8 %. As a direct result, lipase carboxyl was deprotonated and its amino group was protonated and indirectly making the lower activity of Limczyme. The presence of water surrounding lipase micro-environment could effectively reduce the interaction among polar amino acids of lipase molecules to prevent an incorrect conformation. Experiments also proved that a small amount of necessary water may be effective in shielding the electrostatic interaction between ionic liquids and a certain point on the surface of lipase-protein because of the high dielectric of water and the molecular conformation of lipase was unchanged in [BMIM][PF₆] system as soon as in aqueous system, so Limczyme showed catalytic activity. It is presumed that lipase must be maintained a certain amount of water content to ensure that lipase catalysis in [BMIM][PF₆] as soon as in strong polar organic solvents. Similarly, the molecular sieve could be used to control the water content by being added to the reaction medium to control water content.

Effect of the alcohol-oil molar ratio: For lipase-catalyzed preparation biodiesel, a suitable alcohol-oil molar ratio was extremely critical. This section was based on the optimal reaction conditions, namely, [BMIM][PF₆] as a reaction medium, pH = 7.5, reaction temperature 40 °C, water content 10 %, immobilized lipase Limczyme dosage 5 %, shaker speed 200 rpm.

The appropriate alcohol-oil ratio not only make lipase inactive due to excessive methanol concentration but also could guarantee an appropriate transesterification reaction rate and guide for application production of biodiesel. The results as shown in Fig. 4, when the alcohol-oil ratio was in this range of 0 to 2.0, the reaction rate increased with the alcohol-oil ratio increased and the maximum rate reached $1.95 \times 10^{-4} \text{ mol m}^{-3} \text{ min}^{-1}$. When the alcohol-oil ratio were 1.0, 2.0, respectively, the relative activity of Limezyme accordingly were 91.6 %, 79 % and declined 2.55 % 15.96 % which compared to the relative activity of 94 % with alcohol-oil ratio being 0.8, reaction rate increase 5.8 %, 12.6 % which compared to reaction rate $1.73 \times 10^{-4} \text{ mol m}^{-3} \text{ min}^{-1}$ with alcohol-oil ratio being 0.8, respectively. When alcohol-oil ratio was 1, the data

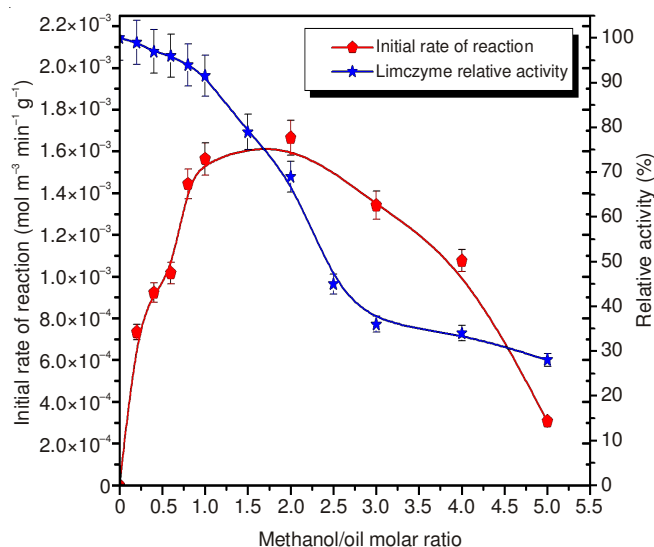


Fig. 4. Effect of methanol-oil ratio on initial rate of enzymatic transesterification reaction

scope of increased reaction rate was smaller than the data scope of declined relative activity of Limczyme. Taken together, the methanol-Tung oil molar ratio being 1 was more appropriate.

In this study methanol should be added into the [BMIM][PF₆] reaction system by continuous adding mode for lipase-catalyzed transesterification production biodiesel with Tung oil. Under alcohol-oil ratio controlled with 1.0 condition, the biodiesel yield should be very closely to 100 % by optimizing the reaction conditions. Every time, the addition amount of methanol should not be on average and alcohol-oil ratio should be 1.0 as the only measure. We would named the phenomenon that FAMES yield was infinitely close to 100 % with the times of adding methanol increased to an infinite number in the [BMIM][PF₆] reaction system.

Reuse of Limczyme: In the process of enzymatic production biodiesel with immobilized lipase, the reused times of immobilized lipase was particularly important for application production biodiesel. After repeated use of 20 times, the residual relative activity of Liaenzyme (adsorption-Cross-linked lipase) was 62.1 %, the residual relative activity of Limczyme (modified-coupled lipase) was 74.4 %. The process of Limczyme reuse shown in Fig. 5, after several reactions the relative activity of Limczyme decreased serious because of lipase molecules breaking off from Limczyme in the response and recovery process, then little change for its activity. After 10 reaction times, the relative activity of Limczyme was 83.2 % much lower than the 92.7 % yield in *t*-butanol reaction system. It could only be attributed to [BMIM][PF₆] that ionic liquid molecules which adhered on Limczyme molecules because of its viscosity might not be completely cleaned from Limczyme and affect Limczyme activity for the enzymatic production biodiesel.

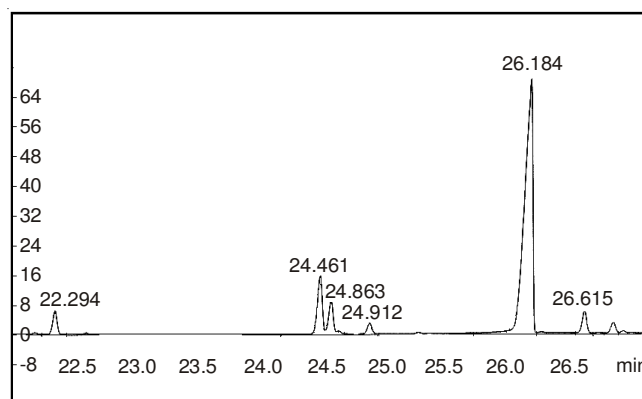


Fig. 5. Gas chromatogram of FAMES in biodiesel from Tung oil

From the thermodynamic point of view, Limczyme should be almost completely inactivated after 10 reaction times at 40 °C for 480 h. However, Limczyme was not completely inactivated and its activity maintained above 83.2 %. The stable structure of [BMIM][PF₆] and its strong polar molecules had maintained the spatial "rigid" molecule structure of lipase, so that the active sites of lipase molecules were not damaged and be able to achieve the desired results. The process of Limczyme-catalyzed production biodiesel with Tung oil as raw material was very effective and feasible in the laboratory

scale and could guide in its application for production of biodiesel.

Conclusion

The results derived from this study revealed that the conditions of Limczyme-catalyzed Tung oil production biodiesel were optimized to determine the process parameters and discuss the enzymatic production biodiesel mechanism. There are some advantages for ionic liquids with non-volatile, non-combustible, electrical conductivity, high heat capacity and small vapor pressure, stabilities and good solubility for many inorganic and organic compounds. Optimizing reaction conditions, obtained the optimal technology parameters of preparation biodiesel from Tung oil using Limczyme as biocatalyzer by transesterification reaction: [BMIM][PF₆] as reaction medium, pH 7.5, reaction temperature 40 °C, water content of 6 %, immobilized lipase Limczyme amount of 5 %, shaker speed of 200 rpm, reaction time of 48 h. As double substrates, the molar ratio of methanol to Tung oil should be maintained at 1.0, used to add methanol approach step by step. The process of Limczyme-catalyzed production biodiesel with Tung oil as raw material was very effective and feasible in the laboratory scale and could guide in its application for production of biodiesel.

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