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Kinetic study on esterification of palmitic acid catalyzed by glycine-based crosslinked protein coated microcrystalline lipase

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HIGHLIGHTS

- Reaction kinetics on palmitic acid esterification by CL-PCMC-LIP/Gly was studied.
- Effects of reaction parameters on the enzyme catalyzed reaction were studied.
- A maximum FAME yield of over 95% was achieved.
- V_{max} and K_m were determined by Ping-Pong bi-bi model with methanol inhibition.
- High K_i of CL-PCMC-LIP/Gly suggested high tolerance to methanol inhibition.

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ABSTRACT

Enzymatic esterification of fatty acid-rich feedstocks represents an efficient and eco-friendly catalytic process for production of biodiesel. In this study, the reaction kinetics on esterification of palmitic acid with methanol using the heterogeneous glycine-based crosslinked protein coated microcrystalline lipase (CL-PCMC-LIP/Gly) was studied. The maximum FAME yield of over 95% based on a molar basis was achieved at 15% (w/w) enzyme dosage with a 4:1 [MeOH]/[FFA] molar ratio after incubation at 50 °C for 1 h in the presence of *tert*-butanol as a co-solvent. According to the rate equation based on the Ping Pong Bi Bi mechanism model with methanol inhibition, the maximum velocity of the reaction was 4.8×10^{-3} M/min while the Michaelis–Menten's constants for palmitic acid ($K_{m,PA}$) and methanol ($K_{m,MeOH}$) were 2.2 M and 1.4 M, respectively with the inhibition constant of methanol ($K_{i,MeOH}$) of 32.6 M. The turnover number (k_{cat}) of CL-PCMC-LIP/Gly was 2.7 min^{-1} under the optimized experimental condition. The reaction kinetics result provides an important basis for further up-scaling study of the enzymatic esterification process for biodiesel synthesis.

1. Introduction

Biodiesel is an important renewable alternative to petroleum fuel used in diesel engines. It has comparable properties to conventional diesel, but with several environmental advantages such as it is carbon–neutral, which can help reducing emission of carbon monoxide, and its combustion generates lower unburned hydrocarbons, particulate matter and other pollutants [1]. Biodiesel is produced as fatty acid methyl esters by trans-esterification of triacylglycerol (TAG) in vegetable oils and esterification of free fatty acids (FFA) with short chain alcohols in a catalytic reaction using bases, acids, or enzymes as catalysts. The conventionally used alkaline-catalyzed reaction is sensitive to free fatty acids in the crude feedstocks which can cause saponification of FFA, resulting in low product yields and complicate the subsequent separation steps [2]. The use of homogeneous acidic catalysts e.g. sulfuric acid and hydrochloric acid can overcome these drawbacks; however; the acid catalyzed process is slow and has difficulties in catalyst recovery, waste treatment, and corrosion of the equipment, which thus limit its implementation in industry [3].

The lipase-catalyzed biocatalytic process allows for high conversion efficiency on transesterification and esterification of TAG

Abbreviations: FFA, free fatty acid; MeOH, methanol; PA, palmitic acid; *t*-BuOH, *tert*-butanol; FAME, fatty acid methyl ester.

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and FFA in the feedstocks under mild reaction conditions with simple downstream purification steps [4,5]. The use of immobilized lipase for biodiesel synthesis has gained increasing interest as it allows recycling of the biocatalyst and can enhance operational stability of the enzyme. Various forms of immobilized lipases prepared by adsorption [6], covalent bonding [7], entrapment [8,9], and microcrystalline enzymes [10] were studied for synthesis of alkyl esters from different feedstocks. The crosslinked protein coated microcrystalline enzymes (CL-PCMCs) are characterized as an enzyme layer on the surface of a core matrix component. CL-PCMCs can be prepared by rapid dehydration and co-precipitation of the enzyme and a matrix component in an organic solvent with an extra step on enzyme covalent crosslinking by glutaraldehyde to increase the biocatalyst's stability [11]. Recently, zwitterionic glycine has been reported as a potent alternative core matrix for preparation of high performance CL-PCMC lipase from an extensive screening of organic and inorganic solid-state buffer [12]. The glycine-based microcrystalline lipase (CL-PCMC-LIP/Gly) showed high reactivity and stability on transesterification and esterification of various palm oil products, for example, crude palm oil, refined palm oil, palm oil-derived FFA, and palm fatty acid distillate (PFAD) using methanol and ethanol as the nucleophiles, when compared with the microcrystalline lipase prepared on the inert K_2SO_4 used as a conventional core matrix component [12].

Although there has been extensive study of the biocatalytic biodiesel synthesis process using various forms of free and immobilized enzymes, study of the reaction kinetics has been neglected. In this report, the reaction kinetics on esterification of palmitic acid with methanol using CL-PCMC-LIP/Gly were investigated. Effects of agitation speed, enzyme loading, reaction temperature, and molar ratio of methanol to palmitic acid on the reaction were studied and the kinetic parameters were fitted to the data. The work provides an insight on reaction kinetics of the CL-PCMC-lipase catalyzed esterification reaction for production of biodiesel.

2. Materials and methods

2.1. Materials

Palmitic acid and fatty acid ester standards were obtained from Sigma–Aldrich. Liquid *Thermomyces lanuginosus* lipase (Lipolase 100T) was purchased from Novozyme (Bagsvaerd, Denmark). Glycine was obtained from Sigma–Aldrich. Chemicals and reagents were analytical grade and obtained from major companies (Sigma–Aldrich, Merck, and Fluka). All reagents were dehydrated with 3 Å molecular sieves (Fluka, Buchs, Switzerland) before use.

2.2. Preparation of CL-PCMC-LIP/Gly

CL-PCMC-LIP/Gly was prepared based on the method reported by Raita et al. [12]. The *T. lanuginosus* lipase (500 mL) was clarified by centrifugation (12,000*g*, 10 min) and pre-concentrated (10×, to 50 mL) using ultrafiltration on a Minimate tangential flow filtration system equipped with a Minimate TFF capsule and 10 kDa MWCO membrane (Pall, Easthills, NY). Initially, 1.5 volumes of a saturated solution of glycine were added to 1 volume of the concentrated lipase solution (1000 mg/mL). This combined mixture was then added drop-wise to a stirring vial (150 rpm) containing 20 volumes of acetone. The precipitate was obtained by centrifugation at 2200*g* for 5 min and then washed thrice with 0.5 volumes of acetone. The enzyme precipitate (i.e. PCMCs) was resuspended in 2.5 volumes of acetone, followed by the addition of 0.05 volumes of glutaraldehyde (25% v/v in water). The PCMCs were incubated at 4 °C with stirring at 300 rpm for 1 h and then washed thrice with acetone. The air dried precipitate was used as the biocatalyst. Protein content was determined at the PCMC stage with Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) based on Bradford's method using bovine serum albumin as the standard.

2.3. Lipase catalyzed esterification

For the standard optimized reaction, 250 mg of palmitic acid and methanol were reacted in a molar ratio of 4:1 ([MeOH]/[FFA]) in the presence of *tert*-butanol at a 1:1 M ratio ([*t*-BuOH]/[FFA]). The CL-PCMC-LIP/Gly was added at 15% (w/w based on FFA) in the reaction and incubated at 50 °C on a vertical rotator at 40 rpm. The amount of esters formed was then determined by gas chromatograph according to the method described in Raita et al. [10].

Initial rates of esterification catalyzed by CL-PCMC-LIP/Gly were determined at various concentrations of palmitic acid (0.74–2.24 mol/L) and methanol (2.0–8.96 mol/L) made up to a total volume of 0.65 mL with *tert*-butanol. The reaction was performed under the optimized conditions for esterification in the presence of 15% enzyme loading at 50 °C with shaking at 40 rpm.

2.4. Analysis of FAME yield

FAME yields were analyzed by gas chromatograph equipped with a flame ionization detector (Shimadzu 2010, Kyoto, Japan) and a polyethylene glycol capillary column (Carbowax 20 M, 30 m × 0.32 mm, Agilent Technologies, Santa Clara, CA). The column oven temperature was 200 °C, with injector and detector temperatures at 250 and 260 °C, respectively. Helium was used as the carrier gas at a constant pressure of 64.1 kPa with linear velocity at 25 cm/s. The amount of FAME was determined based on standard curves using the corresponding esters. The product yield (%) is the amount of FAME converted from fatty acid on a molar basis.

2.5. Reaction kinetic analysis

The reaction kinetics on esterification were assumed to follow the Ping Pong Bi Bi mechanism [13,14]. The Ping Pong Bi Bi mechanism with alcohol inhibition is depicted following Cleland's notation in Fig. 1. In this reaction mechanism, the free fatty acid (A) first binds to the free enzyme (E) and forms a non-covalent enzyme-acid complex (EA), which releases the first product, water (P) and acylated enzyme (E*). Next, the second substrate, alcohol (B) reacts with E* to give the complex E*B, which is transformed to the complex EQ, and gives the ester (Q) as the final product and the free enzyme (E). At the same time, B can also form a complex [E_iB] by binding with free enzyme [E]. The reaction rate on enzymatic esterification of palmitic acid with methanol was fitted to Eq. (1):

$$v = \frac{v_m[\mathrm{PA}][\mathrm{MeOH}]}{K_{m,\mathrm{MeOH}}[\mathrm{PA}] + K_{m,\mathrm{PA}}[\mathrm{MeOH}]\left(1 + \frac{[\mathrm{MeOH}]}{K_{i,\mathrm{MeOH}}}\right) + [\mathrm{PA}][\mathrm{MeOH}]} \quad (1)$$

where v is the reaction rate; v_m is the maximum reaction rate; $K_{m,\mathrm{PA}}$ and $K_{m,\mathrm{MeOH}}$ are the Michaelis–Menten's constants for palmitic acid

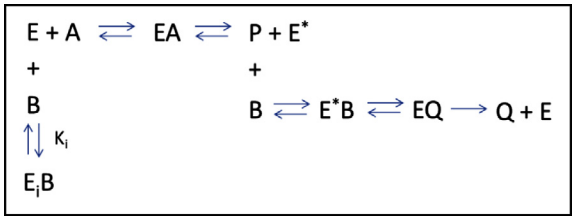

Fig. 1. Schematic representation of the Ping Pong Bi Bi mechanism with competitive inhibition by alcohol.