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Kinetic study of esterification of rapeseed oil contained in waste activated bleaching earth using *Candida rugosa* lipase in organic solvent system

Enoch Y. Park *, Masahiro Mori

Laboratory of Biotechnology, Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

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Abstract

The effect of activated bleaching earth (ABE) from the oil processing industry on the production of fatty acid alkyl esters (FAMEs) by the lipase-catalyzed alcoholysis of waste plant oil with methanol in an organic solvent system was investigated. When ABE was added to a reaction mixture, the FAME formation rate improved nine-fold over the mixtures that did not contain ABE. The optimum ratio of ABE to ABE and rapeseed oil was 0.7 for the esterification of the rapeseed oil, where the lipase activity remained high without inactivation. The methanol was adsorbed onto the ABE and the equilibrium concentration (S) in the reaction mixture was represented as a function of ABE (m) and the initial methanol concentrations (S_i) as follows: $S = 0.16m^{-1.76} \cdot S_i^{2.17}$. In the case of the increase in the ABE concentration in the reaction mixture, the initial FAME formation rate increased rapidly until 1.8 mM/min and remained at a high rate regardless of the increase in the initial methanol concentration. The inhibition kinetics are summarized as a function of methanol on the conditions of the sufficient existence of rapeseed oil and lipase as follow: $v = \frac{v_m}{K_s + S + \frac{S^2}{K_i}}$, where v, K_s and K_i denote the initial FAME formation rate, the Michaelis–Menten constant and the methanol inhibition constant, respectively. Subscript 'm' denotes the maximum. The kinetic parameters, v_m, K_s and K_i, were determined to be 2.9 mM/min, 0.09 mM and 0.98 mM, respectively. These results indicate that the presence of ABE relieves the inhibitory effect of methanol on the enzyme because of the adsorption of methanol by ABE.

Keywords: Activated bleaching earth; Fatty acid methyl ester; Recycling of waste vegetable oil; *Candida rugosa* lipase

1. Introduction

The synthesis of methyl esters by chemical transesterification has accomplished high yields in a short reaction time. However, pretreatment of the substrate is required when water is present, and there are drawbacks to the synthesis, including difficulties in the recovery of catalyst and glycerol, the high energy requirements, and the treatment of wastes, all of which are disadvantages in alkali or acid-catalyzed processes. Therefore, the application of lipase to hydrolyze or synthesized esters has attracted great interest because lipase is environmentally friendly. Lara and Park [1] applied waste activated bleaching earth (ABE) to the production of fatty acid methyl ester (FAME) using *Candida rugosa* lipase in an organic solvent system. Activated bleaching earth is one of the most commonly used absorbents due to its high absorption capacity. In an oil refinery process, the upgrading of crude oils from a vegetable origin requires the use of absorbents for the removal of carotene, chlorophyll and other components formed during the refining process (e.g., phosphatides and soaps). Waste ABE contains nearly 40% of its weight as oil [2], a substrate that should be utilized for the synthesis of a wide range of products to be used as bulk chemicals. This waste ABE was applied to produce the FAME using lipase in either a water [2] or organic solvent [1] system. When the extracted vegetable oil from the waste ABE was used for esterification using lipase, the conversion of vegetable oil in the organic solvent systems was only 13% (w/w). However, in the presence of ABE in the organic solvent system, the conversion improved dramatically, to higher than 80% for an 8 h reaction [1]. This means that it is possible for the waste ABE to be used as a substrate for the production of FAME without any pretreatment.

* Corresponding author. Tel.: +81 54 238 4887; fax: +81 54 238 4887.
E-mail address: yspark@agr.shizuoka.ac.jp (E.Y. Park).

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The present paper discusses the effect of ABE on the lipase-catalyzed synthesis of FAMEs in an organic solvent system. This study aims to clarify the function of ABE and the reutilization of waste ABE for the production of FAMEs.

2. Materials and methods

2.1. Materials

Lipase from *C. rugosa* was obtained from Meito Sangyo Co. Ltd. (Nagoya, Japan). The methanol and refined rapeseed oil were of analytical grade and were purchased from Wako Pure Chemical Industries (Osaka, Japan). *n*-Hexane was used as the solvent in the synthesis of FAME. The standards for the gas chromatography (GC) of methyl pentadecanoic acid and the methyl fatty acids were from Sigma Chemical Co. (St. Louis, MO).

Mizusawa Chemical Industries Co. (Niigata, Japan) provided the activated bleaching earth used for the oil refining and the waste ABE discarded in the oil refinery process.

2.2. Effect of ABE on the esterification of rapeseed oil contained in waste ABE

The effect of ABE on the esterification of rapeseed oil was investigated using various ratios of ABE to the amount of ABE and rapeseed oil. Rapeseed oil 70 g, *n*-hexane 120 ml, lipase 6 g and an ABE ratio to ABE and rapeseed oil of 0–0.75 were mixed on a 500 ml Erlenmeyer flask capped with rubber stopper and incubated at 37 °C for 30 min with shaking 120 strokes per minute (spm) in a reciprocal shaker (Bio-Shaker, Takasaki Sci. Inst. Co., Kawaguchi, Japan). The reaction was started by the addition of methanol 10.2 g (molar ratio of 4 to rapeseed oil) at the same condition, and carried out for 8 h. A sampling was carried out every 10 min. The initial FAME formation rate was defined as the maximum production rate of FAME calculated by plotting the formed FAME (mM) and reaction time (min).

To investigate the free methanol in the reaction mixture, the order of the addition of methanol, lipase and a solvent was varied in six ways, as shown in Table 1. Two hundreds grams of waste ABE containing 70 g of rapeseed oil was added to a 500 ml Erlenmeyer flask capped with rubber stopper. In Runs 1 and 2, the methanol was added first and mixed well, and then lipase and solvent were added. In Runs 3 and 4, lipase or solvent were added first and mixed well, and then methanol was added and finally solvent or lipase. In Runs 5 and 6, lipase or solvent were added and mixed well, and then finally methanol. The amounts of lipase, methanol and solvent used in this experiment were 2 g, 10.2 g and 120 ml, respectively. The reaction condition was the same as that described above.

2.3. Effect of methanol on the esterification of rapeseed oil contained in waste ABE

The reaction mixture for the investigation of the methanol effect on FAME formation consisted of 114 mM of rapeseed oil, 1.8 g/l of lipase (added as powder), ABE ranging from 0 g/l to 450 g/l, *n*-hexane and methanol. The initial methanol concentration in the reaction mixture was varied from 25.6 mM to 800 mM. The final reaction volume was adjusted to 28 ml with *n*-hexane. The 30 ml capped test tube containing the reaction mixtures were incubated at 37 °C at 120 spm for 30 min with six samplings.

2.4. Methanol adsorption by ABE in n-hexane system

To investigate methanol adsorption by ABE, the initial methanol concentration was varied in the range of 80–920 mM in the presence of ABE ranging from 0 g/l to 500 g/l. The final volume was adjusted to 20 ml with *n*-hexane. The methanol was incubated at 37 °C in a reciprocal shaker at 120 spm for 30 min and was adsorbed by ABE. When the adsorption was at equilibrium state, the mixture was centrifuged and the residual methanol concentration in *n*-hexane measured.

2.5. Analytical methods

The products from the enzymatic reaction mixtures were centrifuged at $2146 \times g$ for 1 min, the supernatant containing the *n*-hexane layer was transferred to a heat block kept at 70 °C, and the *n*-hexane was evaporated by blowing off nitrogen gas. For quantitative determinations, 0.025 g of fatty acid methyl ester was redissolved in 1.5 g of chloroform and 2 μl aliquot was injected onto a gas chromatograph (GC-14B, Shimadzu, Kyoto) coupled with a glass column (3 mm × 2 mm) packed with 5% Advans DS on 80/100 meshes Chromosorb W (Shimadzu, Kyoto) and a flame ionized detector. The detector and injection port temperatures were 240 °C, and an isothermal column temperature of 190 °C was maintained. Nitrogen gas as a carrier flowed at a rate of 50 ml/min, and the pressures of

Table 1
Esterification of waste ABE contained rapeseed oil in a different addition order

Run no.	Addition order	Initial FAME formation rate (mM/min)[a]	Correlation coefficient
1	Waste ABE, MEOH, enzyme, solvent	1.2	0.98
2	Waste ABE, MEOH, solvent, enzyme	1.2	0.99
3	Waste ABE, enzyme, MEOH, solvent	0.7	0.97
4	Waste ABE, solvent, MEOH, enzyme	1.1	0.99
5	Waste ABE, solvent, enzyme, MEOH	0.4	0.96
6	Waste ABE, enzyme, solvent, MEOH	0.5	0.97

MEOH denotes methanol.

[a] FAME synthesis was carried out for 12 h of reaction. The FAME was measured every 3 h of reaction.

the used hydrogen gas and air were 0.6 kg/cm^2 and 0.5 kg/cm^2, respectively. All GC measurements were performed in triplicate and the concentration determined from the standards of methyl fatty acids and by utilizing methyl pentadecanoate as an internal standard.

To measure the methanol concentration at equilibrium state, the mixture of methanol and ABE was centrifuged at 3354 × *g* for 1 min, and 2 ml of its supernatant containing *n*-hexane and methanol was sampled. The methanol in the *n*-hexane layer was extracted with 2 ml of distilled water, and the samples dissolved in water were injected onto a gas chromatograph (GC-14B, Shimadzu, Kyoto) coupled with a glass column (3 mm × 2 mm) packed with Gaskuropack 54 on 60/80 meshes (GL Science, Kyoto) and a flame ionized detector. The detector and injection port temperatures were 240 °C and 240 °C, respectively, and an isothermal column temperature of 110 °C was maintained. Nitrogen gas as a carrier flowed at a rate of 50 ml/min, and the pressures of used hydrogen gas and air were 0.6 kg/cm^2 and 0.5 kg/cm^2, respectively.

Enzyme activity (IU/ml), defined as the amount of enzyme that liberated 1 μmol of free thiol groups per minute, was measured using a Lipase Kit S (Dainippon Pharmaceutical, Osaka).

3. Results

3.1. Effect of ABE on the esterification of rapeseed oil

When the ABE ratio was lower than 0.3, no FAME was detected. However, the initial FAME formation rate increased rapidly with the increase in the ABE ratio from 0.5 to 0.7 (Fig. 1). Higher than 0.7, the initial FAME formation rate decreased rapidly, which might be due to incomplete mixing of the viscous reactant. The optimum ABE ratio was 0.7 where the initial FAME formation rate was the highest.

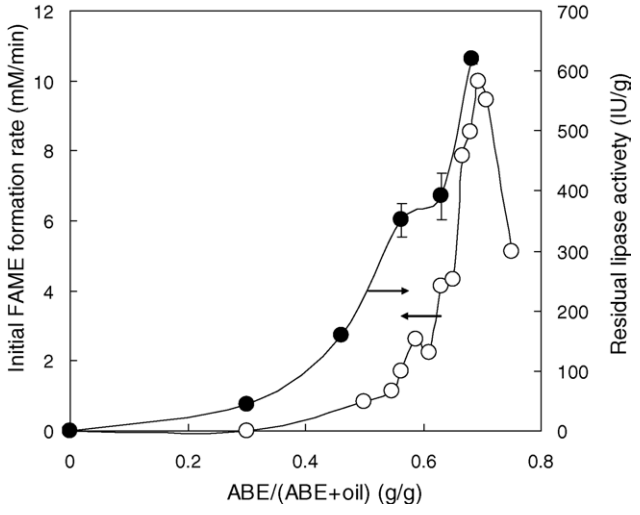

Fig. 1. Effect of ABE ratio on the initial FAME formation rate (open circle) and residual lipase activity (closed circle). Rapeseed oil 70 g, *n*-hexane 120 ml, lipase 6 g and the ABE ratio to ABE and the rapeseed oil of 0–0.75 were mixed on a 500 ml Erlenmeyer flask capped with rubber stopper and incubated at 37 °C for 30 min with shaking at 120 strokes per minute (spm). The reaction was started by the addition of methanol 10.2 g at the same condition and carried out for 8 h.

To find out why the FAME formation rate was affected by the ABE ratio, the lipase activity was measured after the reaction. The residual lipase activity increased with the increase in the ABE ratio (Fig. 1). The ABE maintained the enzyme activity at a high level without the inactivation of lipase; the activity was proportional to the ABE ratio. However, when methanol was not added in the reactant, the enzyme activity remained without inactivation regardless of the presence of ABE in the reaction (data not shown). This result indicates that methanol is the main inhibitor deactivating the lipase in the esterification of rapeseed oil.

3.2. Effect of the addition order of reactant on esterification of rapeseed oil contained in waste ABE

The waste ABE, methanol, enzyme and solvent were added in six different orders, as shown in Table 1. In the case of the first addition of methanol, the initial FAME formation rate was 2.5–3 times as high as that of the third addition of methanol (Table 1). The addition of lipase and solvent did not show a significant influence on the FAME formation rate. This result surmises that the decrease in the FAME formation rate in the third addition of methanol may be caused by the inactivation of lipase due to the presence of methanol in the reaction mixture.

3.3. Adsorption of methanol onto ABE

The adsorption of methanol onto ABE was investigated with various initial concentrations of methanol in the presence of ABE. Specific methanol adsorption by ABE was proportional to the initial methanol concentration (Fig. 2A). The specific methanol adsorption by ABE (r_s) is represented by the following model:

$$r_s = \frac{-\Delta S}{m} = f(m) \cdot S_i \quad (1)$$

where ΔS and m denote the amount of the adsorbed methanol on the ABE and ABE concentration, respectively. Subscript 'i' denotes the initial concentration. The function $f(m)$ was determined from the correlation between r_s and S_i as shown in Fig. 2A, representing a function of the ABE concentration as follows (see Fig. 2B):

$$f(m) = 0.34m^{-0.81} \quad (2)$$

where the correlation coefficient was 0.99.

Eqs. (1) and (2) were combined and yielded the following equation:

$$r_s = \frac{-\Delta S}{m} = f(m) \cdot S_i = 0.34m^{-0.81} \cdot S_i \quad (3)$$

Eq. (3) represents the adsorption of methanol as a function of ABE and the initial methanol concentration.

When the adsorption of methanol on ABE reaches an equilibrium, the methanol concentration in the liquid phase was measured and plotted against the adsorbed methanol in ABE, as

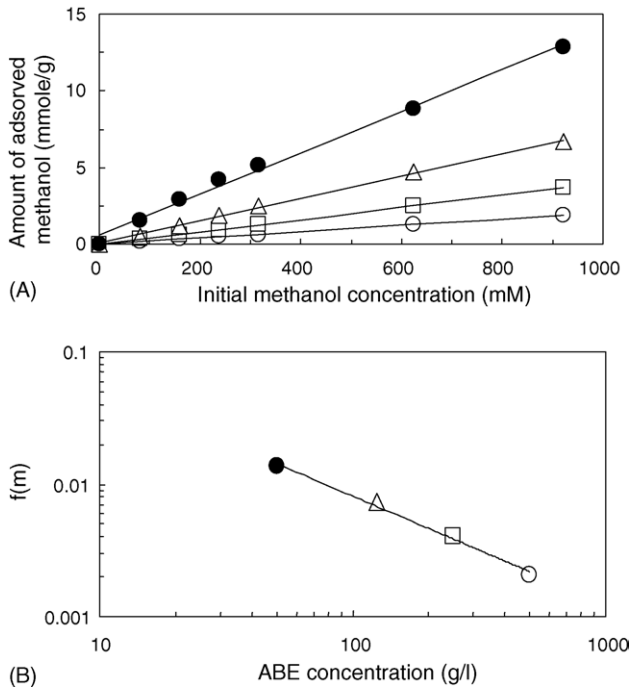

Fig. 2. Methanol adsorption onto ABE (A) and a proportional constant $f(m)$ (B). The initial methanol concentration was varied in the range of 80–920 mM in the presence of various ABE concentrations. The final volume was filled up 20 ml with *n*-hexane. The methanol was incubated at 37 °C in a reciprocal shaker at 120 spm for 30 min and was adsorbed onto ABE. The ABE concentrations were: 50 g/l (closed circle), 125 g/l (open triangle), 250 g/l (open square) and 500 g/l (open circle), respectively.

shown in Fig. 3. The specific methanol adsorption is a function of the methanol concentration in the equilibrium, as follows:

$$r_s = \frac{-\Delta S}{m} = 0.78 \cdot S^{0.46} \quad (4)$$

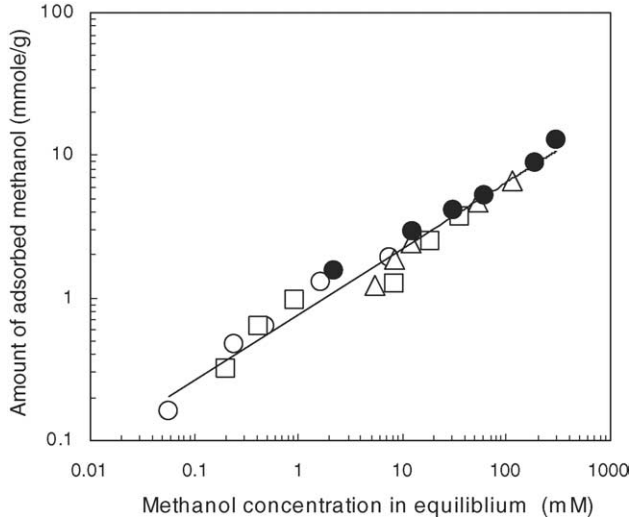

Fig. 3. Methanol adsorption kinetics onto ABE. The symbols are the same as those in Fig. 2. The adsorbed methanol concentration was calculated from the residual methanol concentration in the supernatant of the reaction mixture in the experiments in Fig. 2.

where S denotes an equilibrium methanol concentration. The correlation coefficient between Eq. (4) and the experimental data was 0.97.

From Eqs. (3) and (4), the equilibrium methanol concentration is represented as a function of the initial methanol and ABE concentrations as follows:

$$S = 0.16m^{-1.76}S_i^{2.17} \quad (5)$$

Eq. (5) represents that the methanol concentration in the equilibrium is a function of ABE and the initial methanol concentrations.

3.4. Effect of initial methanol concentration on FAME formation rate

The methanol concentration in the reaction mixture was varied. The effect of this variation on the esterification of rapeseed oil in the presence of ABE and the FAME formation rate were investigated, as shown in Fig. 4. In the absence of ABE in the reaction, the maximum initial FAME formation rate was 0.2 mM/min at the initial methanol concentration of 57.1 mM. With the increase in the ABE concentration in the reaction mixture the FAME formation increased rapidly until 1.8 mM/min. However, when the ABE concentration was higher than 260 g/l, the peak of FAME formation rates shifted from left to right and broadened widely. These results indicate that the presence of ABE relieves the inhibitory effect of methanol on the enzyme, because of the adsorption of methanol by ABE.

Fig. 4 was replotted by an equilibrium methanol concentration in the esterification reaction, as shown in Fig. 5. The initial methanol concentration was converted to an equilibrium methanol concentration according to Eq. (5). Except for the

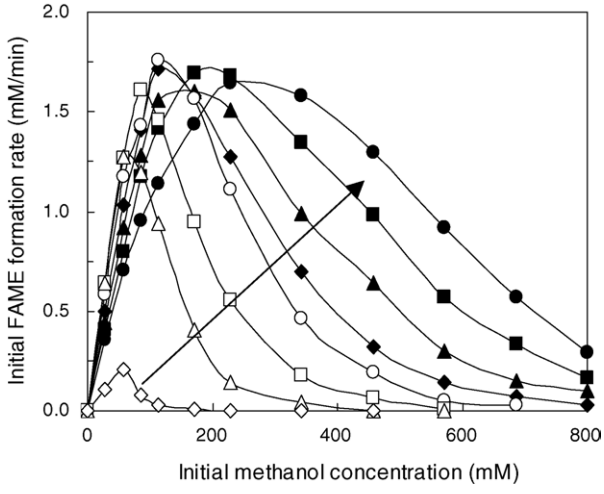

Fig. 4. The effect of the initial methanol concentration in the presence of various ABE concentrations. A reaction mixture consisting of 114 mM of rapeseed oil, 1.8 g/l of lipase (added as powder), ABE ranging from 0 g/l to 450 g/l, *n*-hexane and methanol. The methanol concentration in the reaction mixture was varied from 25.6 mM to 800 mM. The working volume was 28 ml. The ABE concentrations were: 0 g/l (open rhombus), 60 g/l (open triangle), 130 g/l (open square), 190 g/l (open circle), 260 g/l (closed rhombus), 320 g/l (closed triangle), 380 g/l (closed square) and 450 g/l (closed circle), respectively. An arrow indicates the increase of the ABE concentration in the reaction mixture.

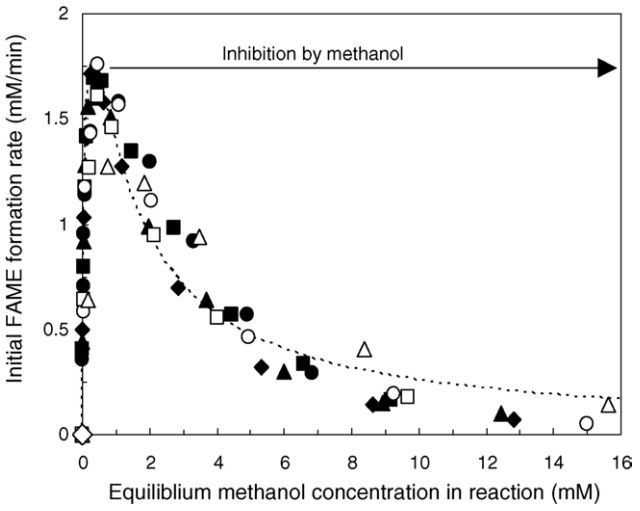

Fig. 5. The generalized FAME formation rate in the esterification of rapeseed oil containing ABE against the methanol concentration in equilibrium. The symbols are the same as those used in Fig. 4. An arrow indicates the inhibitory region of methanol.

ABE concentration of 60 g/l, the FAME formation rates were converged to 1.8 mM/min, which was the maximum FAME formation rate, and decreased significantly with the increase in the equilibrium methanol concentration. When the equilibrium methanol concentration was higher than 0.3 mM, the esterification of rapeseed oil was decreased due to the inhibition of methanol in the esterification reaction.

3.5. General FAME formation model in the presence of ABE in the reaction of esterification of rapeseed oil

The esterification model for FAME formation in the presence of ABE on the condition of a sufficient existence of rapeseed oil and lipase is supposed as follows:

$$v = \frac{v_m S}{K_s + S + \frac{S^2}{K_i}} \quad (6)$$

where v, K_s and K_i denote the initial FAME formation rate, the Michaelis–Menten constant and the methanol inhibition constant, respectively. Subscript 'm' denotes the maximum. The kinetic parameters, v_m, K_s and K_i were determined, from the experimental data in Fig. 5, to be 2.9 mM/min, 0.09 mM and 0.98 mM, respectively. The maximum FAME formation was determined from Eq. (6) to be 1.8 mM/min at 0.29 mM of methanol concentration in equilibrium.

4. Discussion

ABE has been used in adsorbing the dark color of crude oil, which is caused by chromophoric chloroplast-related materials that undergo different degrees of polymerization. During crude oil refining, ABE adsorbs 40% vegetable oil by weight and is disposed as waste material (waste ABE). This ABE showed a large adsorption capacity (290 m^2/g) because of its activated, porous, three-layer structure of silica–alumina–silica. This conferred a large adsorption capacity of methanol, 13 Mm per 1 g of ABE (from Fig. 2), while in the case of the vegetable oil, 0.5 mM per 1 g of ABE. The adsorption capacity of methanol is three times higher than that of vegetable oil, according to our experiments.

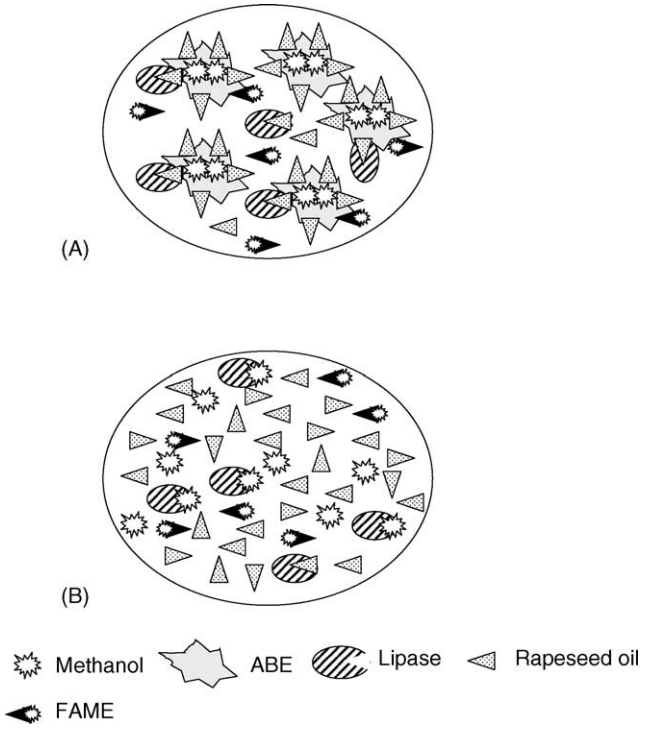

Fig. 6. Schematic representation of FAME formation with ABE (A) and without it (B). When ABE was present, the ABE adsorbed the rapeseed oil and methanol in the *n*-hexane phase and made a complex. However, because lipase cannot be dissolved in the *n*-hexane phase, it existed in a solid phase. The lipase reacted with both the dissolved rapeseed oil in the *n*-hexane phase and the rapeseed oil in the rapeseed oil–methanol–ABE complex. Since the methanol concentration in the *n*-hexane phase was very low, the possibility of a direct collision between the lipase and methanol was low. When ABE was not present, except for lipase, all reactants existed in the *n*-hexane phase. The possibility of a direct collision between the lipase and methanol would be high, which might cause inactivation of the lipase.

The esterification reaction in *n*-hexane was carried out with and without ABE. When ABE was added the FAME formation rate improved remarkably, nine-fold of that when ABE was not added. What caused the improvement in the FAME formation rate in the presence of ABE? When ABE was present in the reactants, the rapeseed oil and methanol were absorbed onto the ABE (Fig. 6A). The rest of the rapeseed oil was dissolved in the solvent, but there was very little free methanol in the solvent due to the low solubility of methanol in an organic solvent. However, the lipase acts a catalyst in the solid phase of an organic solvent, and converts the dissolved and adsorbed rapeseed oil to the FAME (Fig. 6A). The esterification reaction is assumed to be carried out on the surface of the ABE after a co-immobilization of rapeseed oil and methanol, as illustrated in Fig. 6A. The organic solvent may be effective in extracting the triglycerides embedded in waste ABE and facilitating the generation of an active conformation of lipase, which contributed to the marked improvement in FAME production in this study.

The ABE may also play an important role in the activation of lipase, probably caused by the interaction of its functional groups with those involved in the conformational change of the enzyme. The interfacial activation of the enzyme may be enhanced by its adhesion to the surface of ABE, leading to a more active conformation of the enzyme (lid opening) and to a better dispersion of the enzyme molecules.

However, when ABE is absent, the lipase exists as a solid phase, but rapeseed oil and methanol exist in a liquid phase. The affinity of methanol to lipase in the reaction mixture is higher than that of organic solvent, which causes an easy inactivation of lipase. This phenomenon is shown in the results (Fig. 4). From the methanol inhibition kinetics, the methanol inhibition constant was 0.98 mM in an organic solvent.

The methanol inhibition is generally observed during lipase esterification and transesterification reactions [3,4]. Methanol which is not only substrate, but also inhibitor of lipase, binds to lipase and makes the lipase–methanol complex, catalytically inactive. To avoid the inhibitory effect of alcohol on the ethanolysis of sunflower oil, Selmi and Thomas [5] added silica gel to the reaction and obtained a higher conversion yield of sunflower oil from ethanolysis than that obtained in the standard condition. Watanabe et al. [6] reported that the inhibition of methanol was due to the interference of the interaction of the lipase molecule with methanol and that three-step esterification successfully converted 94% soybean oil to methyl esters. Xu et al. [7] proposed a new method for FAME production using methyl acetate instead of methanol as the acyl accepter. They showed that the use of methyl acetate could greatly enhance the stability of the immobilized lipase. However, it is not profitable to use silica gel as an additional raw material and very expensive acyl accepter in FAME production to produce biodiesel fuel.

We previously reported on the application of waste ABE from the oil refining process as potential substrates for the synthesis of useful compounds [1]. A large amount of waste ABE needs to be discarded, however, and its release to the environment in landfills is limited by strict environmental regulations. However, we have already proposed a very efficient way to convert waste ABE to biodiesel [1,2]. Moreover, the ABE was a good enhancer of the FAME production in lipase-catalyzed esterification. When ABE was present in the mixture, the glycerol concentration in the reaction mixture was not detectable with a glycerol analysis kit. This indicates that the glycerol produced during alcoholysis is adsorbed in the waste ABE, which may favor the reaction towards product formation.

From a process point of view, a FAME manufacturing process using waste ABE would be much simpler than the process using conventional chemical hydrolysis, by, for example, using sodium hydroxide. This is because when using waste ABE, a FAME is obtained directly following filtration after esterification, while in conventional chemical hydrolysis, additional separation processes, for example, dehydration and the separation of glycerol in the FAME, are required. After filtration, oil-free waste ABE containing FAME, solvent, lipase and glycerol remains. In this step, FAME could be recovered from the oil-free waste ABE by extraction using *n*-hexane. The organic solvent may be recovered and recycled into the process. To avoid the tedious and harmful recycling process, we proposed that an alternative solvent, diesel from petroleum, be used as a solvent for the production of FAMEs from waste ABE [8]. The final byproduct, FAME-free waste ABE containing glycerol and lipase remains, may be reused for the esterification of waste vegetable oil, so long as the lipase is active. Since the lipase is mixed with FAME-free waste ABE, it is impossible to separate the lipase from the final byproduct. The waste edible oils from kitchens or restaurants are a good waste resource for the reuse of FAME-free waste ABE containing lipase. We are considering conducting a study on the reutilization of the lipase contained in the final FAME-free waste ABE.

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