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ARTICLE

One-step transesterification reaction using methanol-stable lipase for omega-3 fatty acid ethyl ester production

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Abstract We investigated transesterification activity of methanol-stable lipase from *Staphylococcus haemolyticus* L62. Lipase L62 transesterified olive oil and menhaden oil using various alcohols including methanol, ethanol, 1-propanol, and 1-butanol. Based on the high stability of lipase L62 toward alcohols, high molar ratios (3:1 or 6:1) of alcohol to oil were employed in the reaction systems. Lipase L62 produced fatty acid esters efficiently by one-step transesterification. Reaction conditions for production of fatty acid methyl esters (FAMEs) and omega-3 fatty acid ethyl esters were further optimized. Lipase L62 produced those esters efficiently when 30–40 % water content was present in the reaction medium. The lipase L62 conversion yield was as high as 92 % for FAME and omega-3 ethyl ester production. These results indicate that lipase L62 is a suitable catalyst for producing an omega-3 ethyl ester concentrate in the pharmaceutical industry as well as for producing biodiesel in the bio-refinery industry.

Keywords Biodiesel · Lipase · Omega-3 ethyl ester · *Staphylococcus haemolyticus* L62 · Transesterification

Introduction

Lipases (triacylglycerolacylhydrolases, E.C. 3.1.1.3) catalyze hydrolysis of carboxylic ester bonds of triacylglycerols. In addition, lipases catalyze esterification, interesterification, and transesterification reactions (Hasan et al. 2006). Lipases have substrate specificity as well as region- and stereo-selectivity (Lin et al. 2010). Thus, lipases are a promising enzyme toolbox for synthesizing pharmaceuticals, biosurfactants, oleochemicals, and cosmetics (Hasan et al. 2006; Sharma et al. 2001).

Various fatty acid esters are produced by lipase-mediated transesterification reactions. Organic solvent-stable lipases are suitable for these reactions. *Candida antarctica* lipase B (CalB) is the most widely used lipase and is an excellent catalyst for various applications (Anderson et al. 1998; Rotticci et al. 2001; Sanchez and Vasudevan 2006; Yoo et al. 2007). However, it is unstable in a high methanol concentration, which makes CalB inadequate for producing fatty acid methyl esters (FAMEs; biodiesel). Thus, many microbial sources have been searched for methanol-stable lipases. The *Staphylococcus haemolyticus* L62 strain, which has been isolated from sewage treatment plants in Korea, produces a typical lipase. Lipase L62 is quite stable in a high concentration of various alcohols and is a suitable enzyme candidate for transesterification (Kim et al. 2013a, b; Oh et al. 1999).

The importance of omega-3 fatty acids (ω3FAs) in human nutrition and disease prevention has been scientifically recognized for some time. ω3FAs include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are found abundantly in marine fish and marine plants such as phytoplankton and algae (Pigott and Tucker 1987). Therefore, increased consumption of marine lipids increases ω3FA dietary intake. In addition, supplementation with ω3FA is recommended as an alternative to natural sources of ω3FA (Shahidi and Wanasundara 1998). The dosage of ω3FA as a dietary supplement should be high enough to achieve the desired biological effects. Marine

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oils can be concentrated as modified triacylglycerols, free fatty acids, or their ethyl esters (Shahidi and Wanasundara 1998). The vast majority of fish oil concentrates sold globally is eicosapentaenoic acid ethyl ester (EPAEE) and docosahexaenoic acid ethyl ester (DHAEE) concentrates. Ethyl ester forms of EPA and DHA have positive actions against atherosclerosis and its complications because EPA and DHA inhibit platelet aggregation and reduce serum triglycerides (von Schacky 2006).

Omega-3 ethyl esters (ω3EEs) can be synthesized via transesterification of fish oil with ethanol (Scheme 1). Fish oils are important sources of EPA and DHA. For example, fish oil from menhaden contains about 25–30 % ω3FA and was used in this study.

The objective of this study was to investigate the transesterification activity of methanol-stable lipase L62. We assessed applying lipase L62 as a suitable catalyst to produce a ω3EE concentrate via a one-step transesterification reaction.

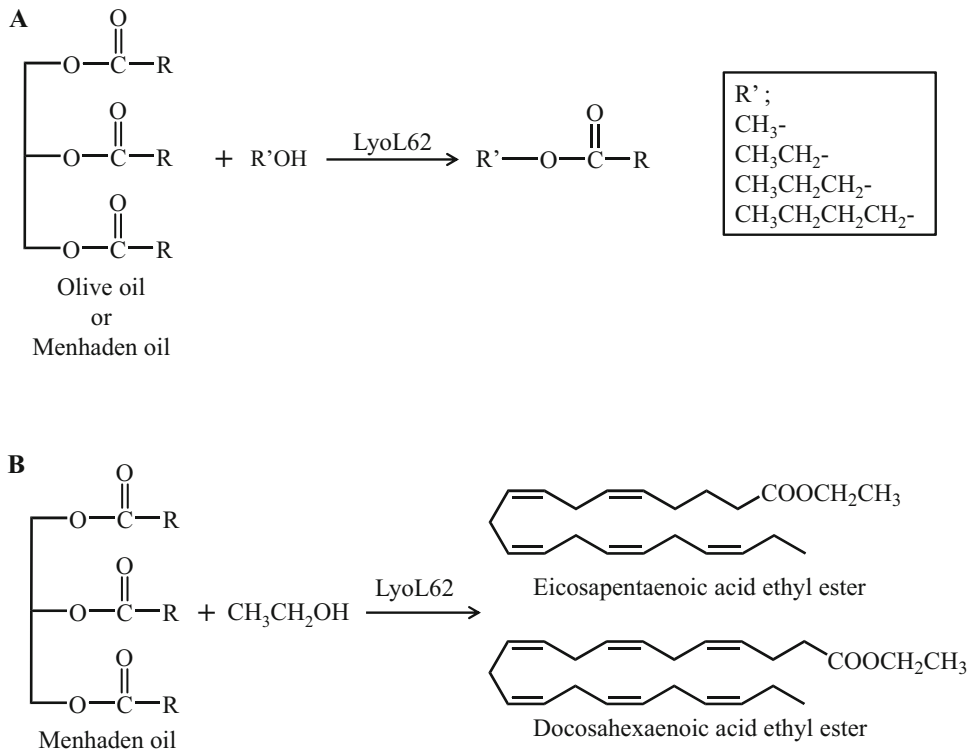
Materials and methods

Materials

CalB adsorbed onto macroporous resin (Novozym 435), menhaden oil, olive oil, and FAMEs (methyl linoleate, methyl oleate, methyl palmitate, and methyl stearate) and fatty acid ethyl esters (FAEE) (ethyl linoleate, ethyl oleate, ethyl palmitate, and ethyl stearate) were purchased as gas chromatography (GC) standards from Sigma-Aldrich Co. (St. Louis, MO, USA). EPAEE and DHAEE were purchased as GC standards from Cayman Chemical Company (Ann Arbor, MI, USA). Hexane for GC analysis was purchased from Avantor Performance Materials (Center Valley, PA, USA). 1-Propanol and 1-butanol were purchased from Junsei Chemical Co. (Chuo-ku, Tokyo, Japan). Analytical grade methanol and ethanol were purchased from Merck Chemical Co. (Darmstadt, Germany).

Production and preparation of lyophilized lipase L62

Recombinant *Escherichia coli* BL21 (DE3) cells containing the lipase L62 gene (Oh et al. 2000) were cultured in 1 L Luria–Bertani broth containing 100 μg/mL ampicillin at 37 °C. When the OD_{600nm} reached 0.5, a final concentration of 1 mM IPTG was added and cultured for an additional 20 h at 20 °C. The cultured cells were harvested via centrifugation (8,000×*g*, 10 min) and resuspended in 20 mL Tris–HCl buffer (50 mM, pH 8.0). The cells were disrupted by sonication, and the soluble fraction was obtained by centrifugation (10,000×*g*, 10 min). The cell-free extract was frozen in liquid nitrogen and lyophilized (Operon Freeze Dryer FDB-5503, Seoul, Korea) for 48 h.



Scheme 1 Schematic representation of lipase-mediated transesterification. **a** Olive oil (or menhaden oil), various alcohols (methanol, ethanol, 1-propanol, and 1-butanol), and LyoL62 are used to produce fatty acid esters. **b** Menhaden oil, ethanol, and LyoL62 are used to produce omega-3 fatty acid esters

Measurement of hydrolytic activity

Hydrolytic activities of lyophilized L62 (LyoL62) toward olive oil and menhaden oil were measured quantitatively by titrating free fatty acids released using the pH stat method (Kim et al. 1998). An oil emulsion containing 1 % oil and 1 % gum arabic was prepared in a Waring blender at maximum speed for 2 min. An appropriate amount of LyoL62 was added, and the release rate of fatty acids was measured with a pH titrator (718 Stat Titrino, Metrohm, Herisau, Switzerland) for 3 min at 30 °C. One unit was defined as the amount of LyoL62 liberating 1 μmol of fatty acid/min.

LyoL62 activities were also measured by hydrolysis of *p*-nitrophenyl caprylate (pNPC) at 35 °C in 50 mM Tris–HCl buffer (pH 8.0) for 3 min. The concentration of the *p*-nitrophenol (pNP) hydrolysis product was measured using a spectrophotometer at 405 nm. One unit of lipases activity was defined as the amount of LyoL62 required to release 1 μmol of pNP under the assay conditions.

Enzymatic transesterification reaction

The enzymatic transesterification reaction of olive oil into FAMEs was conducted as follows. Methanol and olive oil were mixed at a molar ratio of 6:1. Methanol (0.78 mL), olive oil (3 mL), 1.5 mL Tris–HCl buffer (50 mM, pH 8.0), and 25 glass beads (3 mm, Superior, Marienfeld, Germany) were put in a glass vial. Then, 0.03 g of Novozym 435 or 0.03 g LyoL62 (corresponding to 1,000 U) was added to the reaction mixture, and the conversion reaction was performed for 24 h at 30 °C for LyoL62 and at 50 °C for Novozym 435 in a shaking incubator (230 rpm). One study (Yang et al. 2009) showed that methanol should be added three-stepwise during CalB-mediated transesterification. However, as the purpose of this experiment was to compare performance during one-step transesterification, we added 0.78 mL methanol once at the beginning of the reaction. Biodiesel production was measured qualitatively using thin-layer chromatography (TLC) analysis.

The enzymatic transesterification reaction of menhaden oil into FAEE was conducted as follows. Ethanol and menhaden oil were mixed at a molar ratio of 3:1. Ethanol (0.55 mL), menhaden oil (3 mL), 1.5 mL Tris–HCl buffer (50 mM, pH 8.0), and 25 glass beads were put in a glass vial. Then, 0.03 g of Novozym 435 or 0.03 g of LyoL62 was added to the reaction mixture, and the conversion reaction was performed for 24 h at 30 °C for LyoL62 and at 50 °C for Novozym 435 in a shaking incubator (230 rpm). Production of FAEE* (fatty acid ethyl esters including saturated fatty acids and oleic acid) and ω3EE was measured qualitatively by TLC analysis.

Effect of various alcohols on the transesterification reaction

The effects of various alcohols (methanol, ethanol, 1-propanol, and 1-butanol) on enzymatic transesterification of olive oil and menhaden oil using LyoL62 were assessed using the same transesterification method described above. The molar ratio of methanol and 1-butanol to olive oil (or menhaden oil) was 6:1, whereas the molar ratio of ethanol and 1-propanol to olive oil (or menhaden oil) was 3:1. LyoL62 (corresponding to 1,000 U) was added to the reaction mixture, and the conversion reaction was performed for 24 h at 30 °C in a shaking incubator (230 rpm). The production of various fatty acid esters or omega-3 esters was measured qualitatively using TLC analysis.

Effect of buffer amount on the transesterification reaction

The effects of various Tris–HCl buffer amounts (10–40 %) on enzymatic transesterification of methanol and olive oil (molar ratio 6:1) or ethanol and menhaden oil (molar ratio 3:1) using LyoL62 were evaluated. LyoL62 (corresponding to 1,000 U) was added to the reaction mixture, and the conversion reaction was performed for 24 h at 30 °C in a shaking incubator (230 rpm). The production of FAME, FAEE*, and ω3EE was measured qualitatively using TLC analysis and quantitatively by GC analysis.

Optimization of ω3EE production

FAEE* and ω3EE production via enzymatic transesterification of menhaden oil using LyoL62 was assessed using the same transesterification method described above. Ethanol and menhaden oil were mixed in a molar ratio of 3:1. Ethanol was added at the beginning of the reaction (one-step reaction). A higher amount of LyoL62 (corresponding to 3,000 U) was added to the reaction mixture, and the conversion reaction was performed for 48 h at 30 °C in a shaking incubator (230 rpm). The production of FAEE* and ω3EE was measured qualitatively using TLC analysis and quantitatively by GC analysis.

TLC analysis

The fatty acid esters and omega-3 esters samples were analyzed qualitatively using TLC. At predetermined time intervals, samples (200 μL) were taken from the reaction mixture and mixed with 1 mL hexane for 2 min. After centrifugation (12,000×*g*, 10 min), 0.01 mL of the upper layer was loaded on a TLC silica gel 60 F_{254} plate (Merck). The TLC analysis was performed using hexane:ethyl acetate:acetic acid (90:10:1) as the developing solvent and

methanol:sulfuric acid (1:1) as the coloring reagent. After spraying the coloring reagent over the silica gel plate, the plate was heated at a high temperature and analyzed.

GC analysis

FAME, FAEE*, and ω3EE were also analyzed quantitatively using GC. The reaction sample prepared for TLC analysis was injected into a GC system (Hewlett-Packard 7890, Palo Alto, CA, USA) and analyzed using an HP-5 column (cross linked 5 % PH ME Siloxane 0.32 mm × 30 m) with a flame ionization detector. Column temperature was increased from 70 to 300 °C at a rate of 10 °C/min and maintained for 3 min at 300 °C. The total quantities (in moles) of fatty acid esters in the reaction mixture were calculated by comparing the retention times and peak areas of the standard FAME, FAEE*, and ω3EE peaks.

Results and discussion

Preparation of LyoL62

Escherichia coli BL21 (DE3) cells containing the lipase L62 gene were cultured, and a cell-free extract was prepared. The cell-free extract was lyophilized, and the resulting lyophilized preparation (LyoL62) was used as the biocatalyst in the following lipase reactions.

Lipase L62 expression was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a lipase activity assay. SDS-PAGE showed that lipase L62 (43 kDa) was expressed in *E. coli*, and that soluble lipase L62 was included in the cell-free extract (Fig. 1a). LyoL62 lipase activity was investigated by both pNPC assay and the pH stat method. As shown in Fig. 1b, LyoL62 lipase had hydrolytic activities toward all substrates tested. The cell-free extract prepared from *E. coli* without the lipase L62 gene had no lipase activity toward any of the substrates (data not shown).

LyoL62 showed relatively high hydrolytic activity toward triglyceride substrates compared to that of the pNPC substrate. It showed the highest hydrolytic activity toward tributyrin (592 U/mg) followed by olive oil (236 U/mg) and menhaden oil (85 U/mg).

Enzymatic transesterification of LyoL62

LyoL62 was used to transesterify olive oil and methanol into FAMEs, and another transesterification reaction was used to transform menhaden oil and ethanol into FAEE* and ω3EE.

The one-step transesterification rate of LyoL62 was compared with CalB (Novozym 435). Figure 2a, b show a comparison of the LyoL62 and CalB transesterification rates to produce FAMEs from olive oil and methanol. The FAME spot increased along with reaction time. Bigger FAME spots were observed when LyoL62 was used as the catalyst compared with CalB during the same reaction time.

The olive oil spot nearly disappeared in a 24 h reaction, indicating that almost all of the olive oil was converted to FAME (Fig. 2a). This result suggests that LyoL62 is a potential catalyst for FAME (biodiesel) production.

A previous study reported that CalB is rapidly inactivated in the presence of high concentration of methanol (Yang et al. 2009). FAME yield is limited by a one-step reaction; therefore, multiple stepwise addition of methanol is required. Many lipases are inactivated during the transesterification reaction when the reaction mixture contains more than 1.5 M methanol (Ranganathan et al. 2008). In contrast, lipase L62

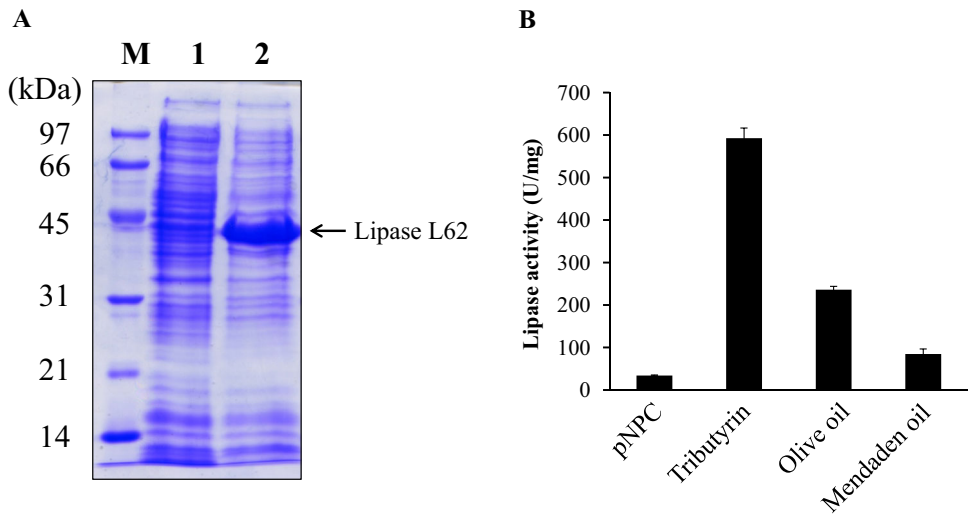


Fig. 1 Sodium dodecyl sulfate-polyacrylamide gel electrophroeis (SDS-PAGE) and LyoL62lipase activity. **a** SDS-PAGE shows 43 kDa-sized lipase L62 band. *Lane M* standard proteins, *lane 1* recombinant *E. coli* cell-free extract (without IPTG induction), *lane 2* recombinant *E. coli* cell-free extract (with IPTG induction). **b** Hydrolytic activity of LyoL62 toward various substrates is shown

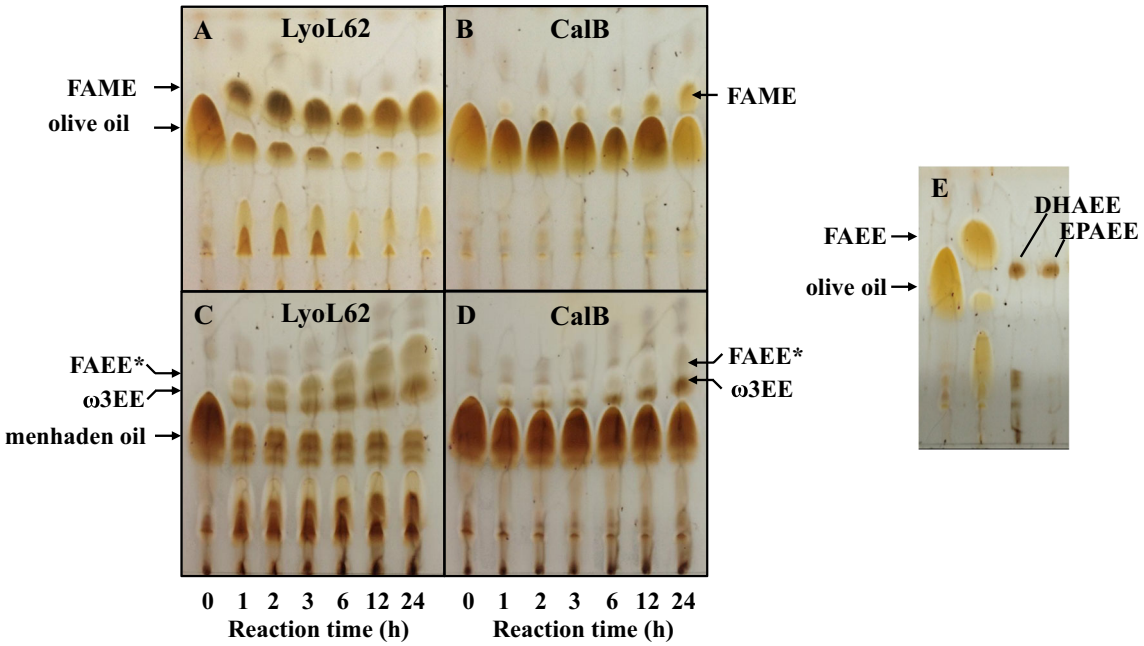


Fig. 2 Transesterification activities of LyoL62 and *Candida antarctica* lipase B (CalB). Transesterification of olive oil and methanol were performed using LyoL62 (**a**) and CalB (**b**). Transesterification of menhaden oil and ethanol was performed using LyoL62 (**c**) and CalB (**d**). **e** The position of fatty acid ethyl esters (FAEE), and eicosapentaenoic acid ethyl esters (EPAEE), and docosahexaenoic acid ethyl esters (DHAEE) are shown on thin-layer chromatography plates

has a high tolerance to methanol and produces biodiesel even when the reaction mixture contains more than 3.5 M methanol (Kim et al. 2013b).

A similar result was observed in the transesterification reaction when menhaden oil and ethanol were used to produce FAEE* and ω3EE (Fig. 2c, d). The FAEE* and ω3EE

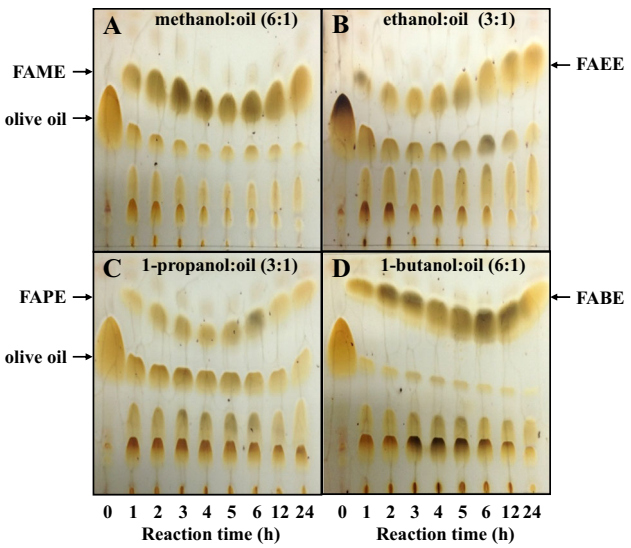


Fig. 3 The effect of various alcohols on transesterification. Transesterification reactions were performed using olive oil and methanol (**a**), ethanol (**b**), 1-propanol (**c**), and 1-butanol (**d**). The production of fatty acid methyl ester (FAME), fatty acid ethyl ester (FAEE), fatty acid propyl ester (FAPE), and fatty acid butyl ester (FABE) was measured qualitatively using thin-layer chromatography

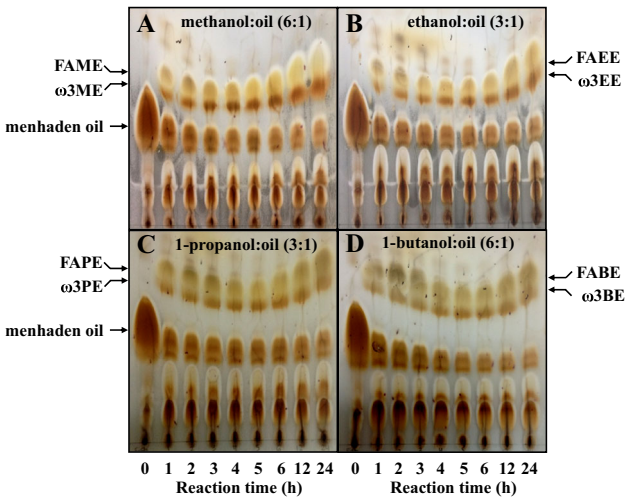


Fig. 4 The effect of various alcohols on transesterification. Transesterification reactions were performed using menhaden oil and methanol (**a**), ethanol (**b**), 1-propanol (**c**), and 1-butanol (**d**). The production of fatty acid methyl ester (FAME), fatty acid ethyl ester (FAEE), fatty acid propyl ester (FAPE), fatty acid butyl ester (FABE), ω-3 methyl ester (ω3ME), ω-3 ethyl ester (ω3EE), ω-3 propyl ester (ω3PE), and ω-3 butyl ester (ω3BE) was measured qualitatively using thin-layer chromatography

spots were compared with standard FAEE and ω3EE spots (Fig. 2e). The FAEE* and ω3EE conversion yields increased along with reaction time, and LyoL62 produced a higher transesterification yield than that of CalB in a 24 h reaction.

Effect of various alcohols on the transesterification reaction

We also observed the effect of various alcohols on the LyoL62 transesterification reaction (Scheme 1). One-step transesterification reactions were performed using olive oil and various alcohols (methanol, ethanol, 1-propanol, and 1-butanol). When methanol and 1-butanol were used, the olive oil spot on the TLC plate nearly disappeared in a 24 h reaction, showing that the transesterification rates were high (Fig. 3a, d).

As shown in Fig. 3b, c, the olive oil spot still remained after a 24 h reaction when ethanol and 1-propanol were used in the transesterification reaction. This result shows that high concentrations of ethanol and 1-propanol have an

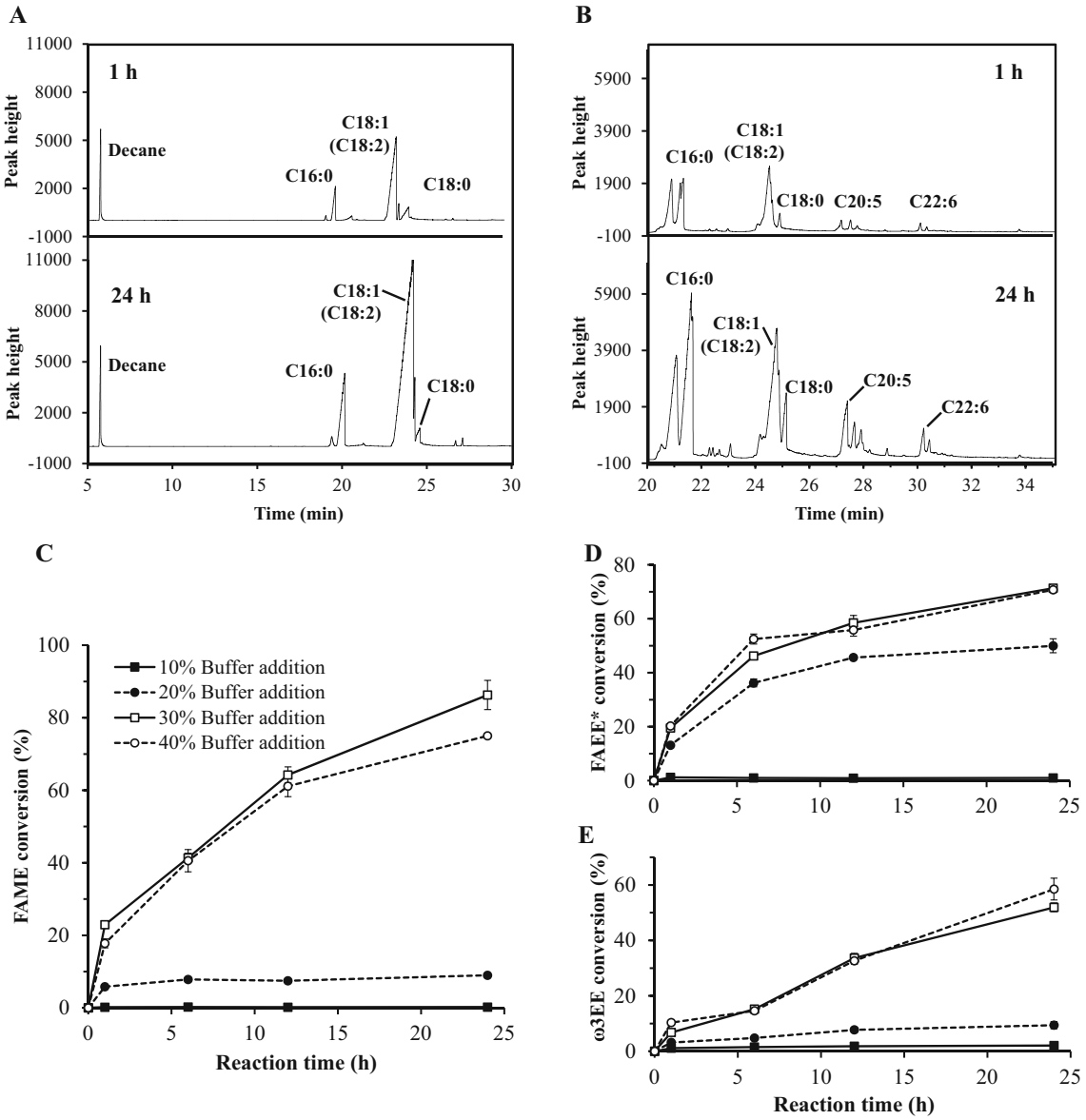


Fig. 5 The effects of buffer content on transesterification. **a**, **c** Transesterification reactions were performed using olive oil and methanol with various amounts of Tris–HCl buffer. **a** Gas chromatography (GC) shows fatty acid methyl ester (FAME) peaks after 1 h and 24 h reactions using 30 % buffer. **c** FAME conversions using various amounts of buffer are shown with the time course. **b**, **d**, **e** Transesterification reactions were performed using menhaden oil and ethanol with various amounts of Tris–HCl buffer. **b** GC shows fatty acid ethyl ester (FAEE) and omega-3 ethyl esters (ω3EEs) peaks after 1 h and 24 h reactions using 30 % buffer. **d**, **e** FAEE* (fatty acid ethyl esters including saturated fatty acids and oleic acid) and ω3EE conversions using various amounts of buffer are shown with the time course

adverse effect on enzyme activity. However, as fatty acid alkyl ester spots were observed on all TLC plates, LyoL62 was confirmed to produce FAMEs, FAEEs, fatty acid propyl esters, and fatty acid butyl esters via the transesterification reaction.

The one-step LyoL62 transesterification reaction using menhaden oil and various alcohols was also performed and analyzed by TLC. As shown in Fig. 4, LyoL62 converted menhaden oil into fatty acid alkyl esters in 24 h. FAEE and omega-3 ester spots increased along with reaction time. Therefore, LyoL62 could perform one-step transesterification reactions efficiently using various alcohols and oil sources.

Effect of various buffer amounts on the transesterification reaction

The effect of various buffer amounts on the LyoL62 transesterification reaction was observed. The transesterification reactions of olive oil–methanol and menhaden oil–ethanol with various amounts of 50 mM Tris–HCl buffer, pH 8.0, were analyzed quantitatively.

As clearly seen in Fig. 5a, a significant increase in FAMEs (C16:0, C18:0, C18:1, and C18:2) was observed at 24 h compared to that at 1 h, indicating that LyoL62 can perform the transesterification reaction from olive oil and methanol when 30–40 % water content is present in the reaction medium. FAME conversion yield was measured quantitatively using GC (Fig. 5c). The highest FAME conversion yield (86 %) was achieved when there was 30 % water content in the reaction medium. This was followed by a 75 % conversion yield when 40 % water content was used in the reaction medium. In contrast, the FAME conversion yield was very low when 10–20 % water content was present in the reaction medium (0.2–8.9 % conversion yield). These results indicate that LyoL62 produces FAME efficiently via the transesterification reaction when there is sufficient water in the reaction medium.

Similar results were obtained from the transesterification reaction using menhaden oil and ethanol. A significant increase in FAEE* (C16:0, C18:0, C18:1, and C18:2) and ω3EE standards (C20:5 and C22:6) at 24 h compared to that at 1 h in the reaction. This result indicates that LyoL62 transesterifies menhaden oil and ethanol when 30 % water content is present in the reaction medium (Fig. 5b).

We also measured FAEE* and ω3EE conversion yields when 10–40 % water content was present in the reaction medium (Fig. 5d, e). We observed low conversion (<2 %) of FAEE* and ω3EE when 10 % water content was in the reaction medium at 24 h. When 20 % water content was added to the reaction medium, FAEE* conversion achieved 48 %, although the ω3EE conversion was only 9.4 %. FAEE* conversion yield was similar (70 %) when 30–40 % water content was added to the reaction medium. The highest ω3EE conversion yield (58 %) was achieved when there was 40 % water content in the reaction medium followed by 52 % conversion yield when 30 % water content was in the reaction medium. These results suggest that LyoL62 also requires sufficient water content to produce FAEE* and ω3EE efficiently via the transesterification reaction.

In general, ester bonds are hydrolyzed in aqueous systems but ester synthesis is carried out in a non-aqueous system. However, as shown in Fig. 5c–e, a small amount (<10 %) of water produced a very low conversion yield. These unexpected results can be explained as follows. When water content in the reaction system is low, the methanol concentration in the water phase becomes inversely higher. As a result, lipase L62 dissolved in the water phase might be inactivated by the excessively high methanol concentration (Kim et al. 2013a).

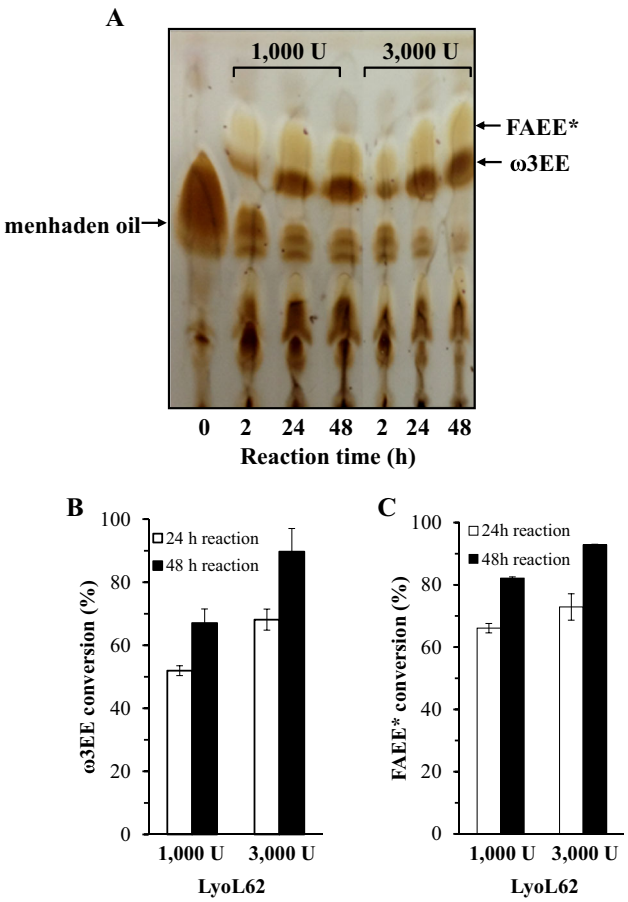


Fig. 6 Effects of enzyme amount and reaction time on transesterification. **a** FAEE* (fatty acid ethyl esters including saturated fatty acids and oleic acid) and ω3EE production from menhaden oil and ethanol was analyzed using thin-layer chromatography. **b** Omega-3 ethyl esters (ω3EEs) conversion was measured by gas chromatography (GC). **c** FAEE* conversion was measured by GC

Optimization of ω3EE production

As shown in Fig. 5e, the ω3EE conversion yield was 50–60 %. Therefore, we tried to optimize ω3EE production yield by increasing the reaction time and the amount of LyoL62. ω3EE conversion yield was 52 % at 24 h and reached 67 % at 48 h when 1,000 U LyoL62 was used (Fig. 6a, b). Conversion yield increased significantly when a greater amount of LyoL62 was used as the catalyst (3,000 U). The conversion yield reached 68 % at 24 h and exceeded 90 % conversion at 48 h. In addition, FAEE* conversion yield also increased when 3,000 U LyoL62 was used (Fig. 6c). Conversion yield reached 72 % at 24 h and exceeded 92 % at 48 h.

Taken together, alcohol-stable lipase L62 can be used as a potential biocatalyst to produce ω3EE, FAEE*, and FAME (biodiesel) by one-step transesterification. A high molar ratio (3:1, 6:1) of alcohol to oil was used in this study. This LyoL62-mediated reaction system can produce many valuable fatty acid esters in the pharmaceutical and bio refinery industries.

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