A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Two-step enzymatic synthesis of mixed lauric acid esters with antibacterial and antioxidant activities

항균 및 항산화 활성을 가지는

mixed lauric acid esters의 2단계 효소적 합성

February 2020

Department of Agricultural Biotechnology

Seoul National University

Yerim Byun

석사학위논문

Two-step enzymatic synthesis of mixed lauric acid esters with antibacterial and antioxidant activities

지도교수 장 판 식

이 논문을 석사학위 논문으로 제출함

2020년 2월

서울대학교 대학원

농 생 명 공 학 부

변 예 림

변예림의 석사 학위논문을 인준함

2020년 2월

위원장 최 상 호 (인)

부위원장 장 판 식 (인)

위원 이 기 원 (인)

Abstract

In this study, two-step enzymatic synthesis of multifunctional mixed lauric acid esters (MLE) was conducted in a gas-solid-liquid multiphase system (GSL-MPS) as an alternative to the conventional purification process. Erythorbyl laurate was synthesized in the first reaction, and the second acyl acceptor was added in the second reaction for the conversion of the residual lauric acid into the new products. Glycerol was selected as the second acyl acceptor for the second reaction as it showed the highest esterification reaction efficiency among four candidates including PEG 600 (Polyethylene glycol 600), propylene glycol, glycerol, and lactic acid. The molar ratio of lauric acid to glycerol for the second reaction of the two-step MLE synthesis was determined as 1.0, considering the monolaurin content, which affects the emulsion stability. As a result, MLE composed of 1.33% erythorbyl laurate, 39.39% monolaurin, 44.19% dilaurin, 2.27% trilaurin, 2.84% erythorbic acid, 2.93% lauric acid, and 7.06%(w/w) glycerol was produced from the two-step enzymatic synthesis. The effects of MLE on emulsion properties were evaluated by the measurements of the droplet size and zeta potential of 5.0%(w/w) oil-in-water emulsions prepared with various concentrations of

MLE. Emulsions containing MLE with the concentration below 2.0%(w/w) were stable over 15 days of storage at 25°C, and this might be attributed to the negatively charged and monodispersed oil droplets. Antibacterial activity of MLE against two Gram-positive and two Gram-negative bacteria was investigated in the oil-in-water emulsion. The time-kill curves of MLE showed the concentration-dependent bactericidal effect against Gram-positive bacteria at the concentration ranging from 0.5 to 2.0%(w/w). Lipid oxidation inhibition effect of MLE was evaluated in the oil-in-water emulsion by ferric thiocyanate method. The amounts of lipid hydroperoxide produced during thermally accelerated oxidation decreased with the increasing MLE concentration, indicating that lipid oxidation inhibition effect was dependent on MLE concentration. In conclusion, MLE produced via the two-step synthesis could be used as a multifunctional food additive with antibacterial and antioxidant activities for the oil-in-water emulsion-type food products.

Keywords: two-step enzymatic synthesis, lipase-catalyzed esterification, oil-in-water emulsion, antibacterial activity, antioxidant activity

Student number: 2018-21443

Contents

List of tables

List of figures

1. Introduction

Erythorbyl laurate is an amphiphilic compound produced by lipase-catalyzed esterification of erythorbic acid with lauric acid. As the amphiphilic characteristic gives it surface-active properties and high foaming stability, erythorbyl laurate can be used for the stabilization of the oil-in-water emulsion (Park, Lee, Jo, Choi, Lee, & Chang, 2017). Besides, erythorbyl laurate can act as an antioxidant since it inhibits lipid oxidation in the oil-in-water emulsion during thermally accelerated oxidation or photo-oxidation (Park, Lee, Jo, Choi, Lee, & Chang, 2017). Erythorbyl laurate also exhibits both bacteriostatic and bactericidal effects against Gram-positive foodborne pathogens by the alteration of the cell membrane permeability (Park, Jo, Yu, Park, Choi, Lee, et al., 2018). Therefore, it was expected that multifunctional erythorbyl laurate with emulsifying, antibacterial, and antioxidant activities in a single molecule could control the microbial contamination and lipid oxidation in the oil-in-water emulsion-type foods.

Production of erythorbyl laurate in an organic solvent system has limitations such as safety issues and low production yield caused by the low solubility of the substrates in the organic solvent (Park, Lee, Sung, Lee, & Chang, 2011).

On the other hand, a solvent-free reaction system, an environmentally friendly system, has several advantages derived from the absence of organic solvents, such as greater safety and higher volumetric productivities (Dossat, Combes, & Marty, 2002; Freitas, Perez, Santos, & Castro, 2007). In addition, solvent-free system in the food industry is highly preferred as it is safe (Abed, Wei, Ali, Korma, Mousa, Hassan, et al., 2018). However, it was hard to synthesize erythorbyl laurate in the solvent-free system because erythorbic acid was not soluble in lauric acid and existed as solid-phase at the maximum working temperature of Novozym® 435 (110ºC) (Verdasco-Martín, Villalba, dos Santos, Tobajas, Fernandez-Lafuente, & Otero, 2016), causing mass transfer limitation. Therefore, a gas-solid-liquid multiphase system (GSL-MPS), enhancing the dispersion of the solid-phase by incorporating the gaseous phase, was established in the previous study (Yu, Lee, Shin, Park, & Chang, 2019). Even though the production of erythorbyl laurate in GSL-MPS had numerous advantages, including higher volumetric productivities in a batch reactor, the conversion yield was still at a low level due to the presence of the solid-phase. Therefore, large amounts of the residual substrates were unavoidable after the synthesis of erythorbyl laurate in GSL-MPS.

The purification process is generally accomplished by solvent extraction, recrystallization, silica gel chromatography, molecular distillation, or the

combination of these methods for the removal or recovery of the residual substrates after the synthesis (Enayati, Gong, Goddard, & Abbaspourrad, 2018; Viklund & Hult, 2004; Zhang, Wang, Xie, Zou, Jin, & Wang, 2018). However, solvent extraction, recrystallization, and silica gel chromatography require organic solvents, and molecular distillation consumes large amounts of energy due to high operating temperature. Therefore, the purification process could cause the problems of safety, environmental concerns, and costs. Purification of erythorbyl laurate, for example, is carried out by solvent extraction based on the difference in the solubility between erythorbic acid, lauric acid, and erythorbyl laurate. Lauric acid is removed with *n*-hexane, followed by the removal of erythorbic acid with water in the same manner. Those intricate purification process has become a hurdle that limits the application of erythorbyl laurate in the food industry.

The addition of another substrate for the conversion of the residual lauric acid into the new products was expected to be an alternative to the conventional purification process. Besides, a mixture of erythorbyl laurate and the second reaction products produced by the additional esterification reaction was expected to exhibit the same functionalities as that of erythorbyl laurate, such as antibacterial and antioxidant activities in the oil-in-water emulsion.

In the present study, glycerol, having the highest reaction efficiency for the

conversion of lauric acid among the candidates, was selected as the second acyl acceptor. Erythorbyl laurate was synthesized in the first reaction, and glycerol was added in the second reaction, making the process as the two-step synthesis of the multifunctional mixture. Consequently, successive two-step synthesis produced mixed lauric acid esters (MLE) composed of erythorbyl laurate and glyceryl laurates. The effects of MLE on emulsion properties were evaluated, and antibacterial and antioxidant activities of MLE in the oil-in-water emulsion were investigated.

2. Materials and methods

2.1. Materials

Novozym® 435, immobilized lipase from *Candida antarctica*, was purchased from Novozymes (Bagsvaerd, Denmark) with a catalytic activity of 10,000 PLU/g (Propyl laurate unit, 1 PLU is the amount of enzyme activity which generates 1 μmol of propyl laurate per minute). Lauric acid (≥ 99.0%) and soybean oil were purchased from Daejung Chemicals & Metals Co., LTD. (Siheung, Korea), and Tween 20 was purchased from Ilshinwells (Cheongju, Korea). Erythorbic acid (≥ 98.0%), glycerol (≥ 99.5%), polyethylene glycol 600 (PEG 600), propylene glycol, and lactic acid were purchased from Acros Organics (Geel, Belgium), Fisher Chemical (Loughborough, Leicestershire, UK), Wako Pure Chemical Industries, LTD. (Osaka, Japan), Junsei Chemical Co., LTD. (Tokyo, Japan), and Showa Chemical (Tokyo, Japan), respectively. All other reagents and solvents were analytical or HPLC grade.

2.2. Lipase-catalyzed synthesis in GSL-MPS

All lipase-catalyzed synthesis was carried out in GSL-MPS (Fig. 1). During the reaction, nitrogen gas was distributed through the glass filter (0.5 cm thickness, 27.5 μm pore size) with a flow rate of 2.0 mL/min. Reaction vessels were preheated, and the reaction temperature was kept constant at 60°C by a water circulator. Lauric acid was added into the reaction vessel and melted for 20 min, followed by the addition of an acyl acceptor. The reaction was initiated by adding Novozym® 435. After the reactions were finished, the nitrogen gas generator was replaced with the vacuum pump, and the reactants were filtrated through the glass filter.

The initial conversion rate of lauric acid was obtained from the slope of the linear part of the curve. The conversion of lauric acid was calculated according to the equation below

$$\text{Conversion of lauric acid (\%)} = \frac{C_o - C}{C_o} \times 100$$

where C_o is the initial lauric acid concentration and C is the residual lauric acid concentration after reaction time.

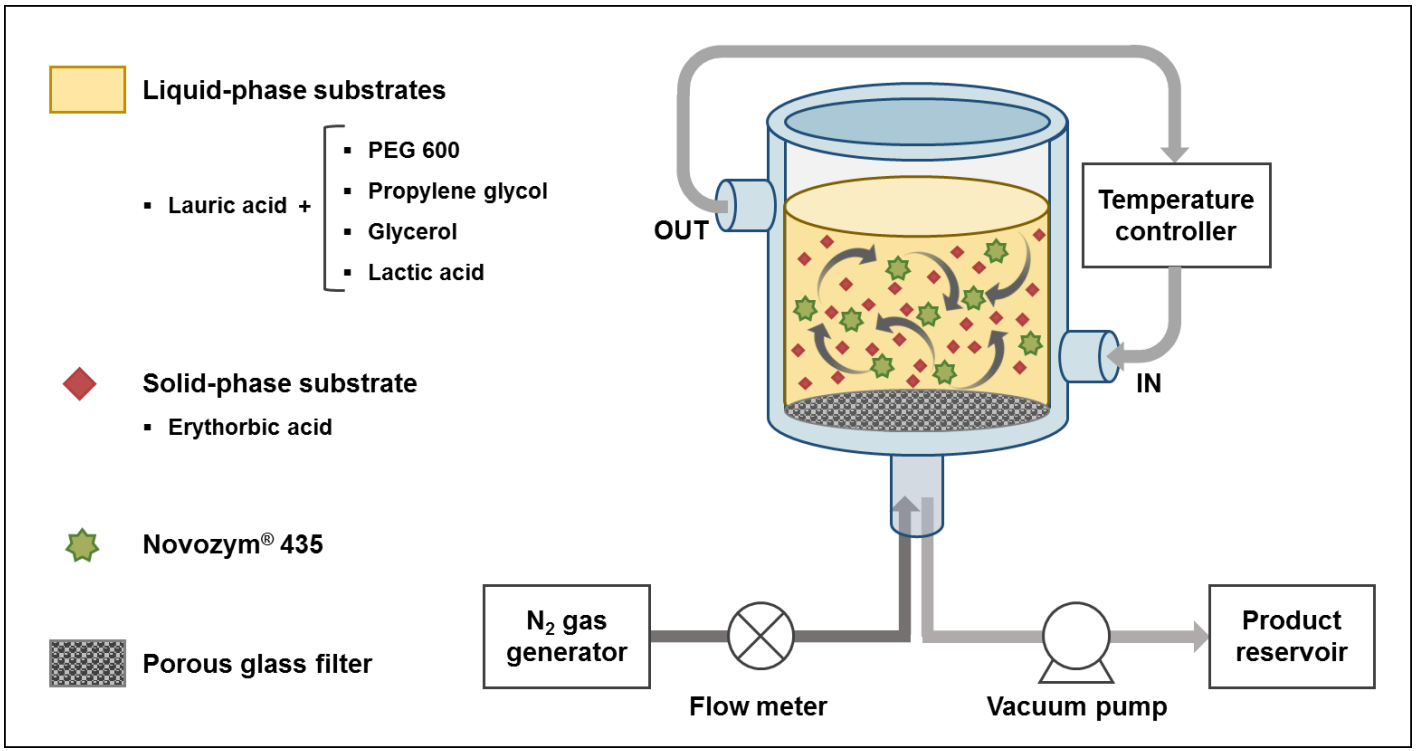


Fig. 1. Illustration of GSL-MPS for the lipase-catalyzed solvent-free synthesis.

2.3. Quantitative analysis using HPLC (High-performance liquid chromatography)

The esterification reaction was monitored by HPLC (LC-2002, Jasco, Tokyo, Japan) equipped with an ultraviolet detector (UV-2075, Jasco) and a refractive index detector (RI-2031, Jasco). Separation of the compounds was carried out on a silica-based column (5.0 µm, 4.6 × 150 mm: Luna C18, Phenomenex, Torrance, CA, USA) and the column temperature was held at 30^{o}C. The mobile phase for the analysis of the reactants of the esterification reaction of lauric acid with PEG 600, propylene glycol, and lactic acid was methanol/water/acetic acid (90:5:5, v/v/v). For the analysis of the reactants of the esterification of lauric acid with glycerol, the mobile phase consisted of acetonitrile/acetone (50:50, v/v), whereas the reactants of the esterification of lauric acid with erythorbic acid was analyzed with the mobile phase composed of acetonitrile/water/acetic acid (90:5:5, v/v/v). All mobile phases passed through the column with a flow rate of 1.0 mL/min. Erythorbic acid and erythorbyl laurate were detected on the UV detector at a wavelength of 265 nm while other compounds were detected on the RI detector. All compounds were identified by their retention time, and the concentrations were calculated from the standard curves of each compound ($R^2 > 0.994$).

2.4. Emulsion preparation

The water phase consisted of Tween 20 (1.0%, w/w) and distilled water (up to 100.0%, w/w) while the oil phase consisted of the soybean oil (5.0%, w/w) and various concentrations of MLE. The concentration of the soybean oil and Tween 20 was designed to mimic a beverage emulsion (Perugini, Cinelli, Cofelice, Ceglie, Lopez, & Cuomo, 2018). Both water and the oil phase were heated up to 70ºC to melt MLE, and the water phase was transferred to the oil phase. Water and the oil phase were mixed using a high-speed blender (Ultra-Turrax IKA T18 basic, IKA Works Inc., Wilmington, NC, USA) at 12,000 rpm for 3 min to form a coarse emulsion. The coarse emulsion was further homogenized for 10 min (1 s on, 4 s off) using an ultrasonicator (Sonomasher, S&T Science, Seoul, Korea) to produce a fine emulsion. The emulsion was immediately cooled down to room temperature (25ºC) with tap water. The emulsions used for time-kill assay were prepared with 20 mM phosphate buffer (pH 7.4) instead of distilled water. Each emulsion was prepared in triplicate. The emulsions prepared without MLE was considered as the control emulsion.

2.5. Measurements of droplet size and zeta potential

Droplet size and zeta potential of the emulsions were analyzed at 25ºC using

Zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK), and each measurement was performed in triplicate. For the measurement of the droplet size, the emulsions were diluted at 1:1,000 (v/v) with distilled water to prevent multiple scattering. The average droplet size was expressed as the intensity-weighted mean droplet diameter (Z-average size), and the width parameter was presented as the polydispersity index. For the measurement of the zeta potential, the emulsions were diluted at 1:100 (v/v) with distilled water.

2.6. Time-kill assay in the oil-in-water emulsion

Two Gram-positive and two Gram-negative bacteria used in this study were *Staphylococcus aureus* ATCC 12692, *Listeria monocytogenes* ATCC 19115, *Escherichia coli* ATCC 35150, and *Salmonella* Typhimurium ATCC 43971. All bacteria were cultured in MHB (Mueller-Hinton broth) at 37°C, 220 rpm for 12-16 h. Cultures were diluted in MHB until the turbidity was reached to 0.5 McFarland standard (1.5×10^8 CFU/mL). McFarland standard suspensions were further diluted to have a cell density of 5.0×10^6 CFU/mL.

Emulsions (3,600 μL) containing 0.0, 0.5, 1.0, and 2.0%(w/w) MLE were mixed with bacterial suspensions (400 μL) and incubated at 37°C, 220 rpm. Aliquots (200 μL) were taken out at 0, 0.5, 1, 2, 4, 6, 8, 10, and 12 h, and serially diluted in 20 mM phosphate buffer (pH 7.4). Diluted samples (50 μL)

were spread on TSA (Tryptic soy agar), and viable cells were counted after incubation at 37ºC for 24 h. All experiments were triplicated.

2.7. Measurements of lipid oxidation in the oil-in-water emulsion

Emulsions (6 mL) were placed in 10 mL screw-capped glass vial and allowed to be oxidized with thermal acceleration at 37ºC. Lipid hydroperoxides were measured as primary lipid oxidation products according to the method by Shantha and Decker (Shantha & Decker, 1994). Every 24 h, emulsion (300 μL) was taken out and mixed with 1,500 μL of isooctane/2-propanol solution (3:1, v/v) and vortexed (10 s, 3 times). The mixed solution was centrifuged at 2,000 × *g* for 2 min (Micro-12, Hanil Scientific Inc., Gimpo, Korea). The upper organic layer (200 μL) was mixed with 2,800 μL of methanol/butanol solution (2:1, v/v), followed by the addition of 15 μL of 3.94 M ammonium thiocyanate and 15 μL of Fe^{2+} solution. The Fe^{2+} solution was prepared freshly from the supernatant of 0.132 M $BaCl_2$ in 0.4 M HCl and 0.144 M $FeSO_4$. The solution was vortexed and held 20 min at room temperature, and the absorbance was measured at 510 nm in a UV-vis spectrophotometer (Optizen POP-BIO, Mecasys Co., Ltd., Daejeon, Korea). Lipid hydroperoxide concentrations were determined using a standard curve prepared from hydrogen peroxide ($R^2 = 0.9978$).

2.8. Statistical analysis

All data were analyzed by analysis of variance (ANOVA) using SPSS software version 25 (SPSS, Inc., Chicago, IL, USA), and presented as means and standard deviations of triplicate experiments. The differences between mean values were compared using Duncan's multiple range test ($p < 0.05$).

3. Results and discussion

3.1. Selection of second acyl acceptor

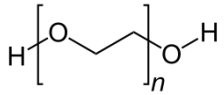
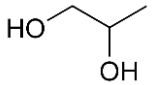
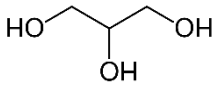
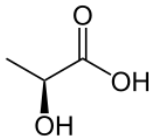
The first criterion for the selection of the second acyl acceptor was the presence of the hydroxyl group because the lipase catalyzes the esterification of the carboxyl group of lauric acid with the hydroxyl group of the acyl acceptor. The second criterion was the melting point lower than the optimum temperature of Novozym® 435 (40 - 60°C) (Lee, Widjaja, & Ju, 2006) because the low conversion caused by the mass transfer limitation could be obtained if the second acyl acceptor exists as a solid-state (Erbeldinger, Ni, & Halling, 1998). Therefore, PEG 600, propylene glycol, glycerol, and lactic acid were chosen as the second acyl acceptor candidates, among the compounds having hydroxyl groups, such as sugar alcohols, monosaccharides, disaccharides, phenolic compounds, hydroxylated organic acids, PEGs, glycerol, and propylene glycol (Table 1).

Lipase-catalyzed esterification of lauric acid with the second acyl acceptor candidates, except for lactic acid, reached equilibrium in 12 h (Fig. 2). On the other hand, esterification of lauric acid with lactic acid did not occur. The esterification reaction efficiency of the second acyl acceptor candidates was

expressed as the initial conversion rate of lauric acid and the conversion of lauric acid at the reaction time of 12 h (Table 2). Both the initial conversion rate of lauric acid and the conversion of lauric acid were the highest in the reaction with glycerol, followed by PEG 600 and propylene glycol.

In CALB (*Candida antarctica* lipase B)-catalyzed esterification, the number of the hydroxyl group and the types of alcohol could affect the reaction efficiency since the primary alcohol is more reactive than the secondary alcohol (Gustini, Noordover, Gehrels, Dietz, & Koning, 2015). Therefore, the reason for the lowest reaction efficiency of lactic acid can be explained by the absence of primary alcohol. Besides, a carboxyl group adjacent to the secondary alcohol might have lowered the nucleophilicity of lactic acid. The higher reaction efficiency of PEG 600 compared to propylene glycol could be attributed to the difference in the number of primary alcohols. PEG 600 has two primary alcohols, while propylene glycol has one primary alcohol and one secondary alcohol. The highest reaction efficiency of glycerol can be explained by the fact that glycerol has two primary alcohols and one secondary alcohol, which makes glycerol have more opportunity for the formation of the ester bonds. Thus, out of four candidates, glycerol showing the highest reaction efficiency was chosen as the second acyl acceptor for the second reaction of the two-step MLE synthesis.

Table 1. Chemical structure and melting point of the second acyl acceptor candidates

Compound	Chemical structure	Melting point (°C)
PEG 600	 n=13	15 - 25
Propylene glycol		-60
Glycerol		18
Lactic acid		17

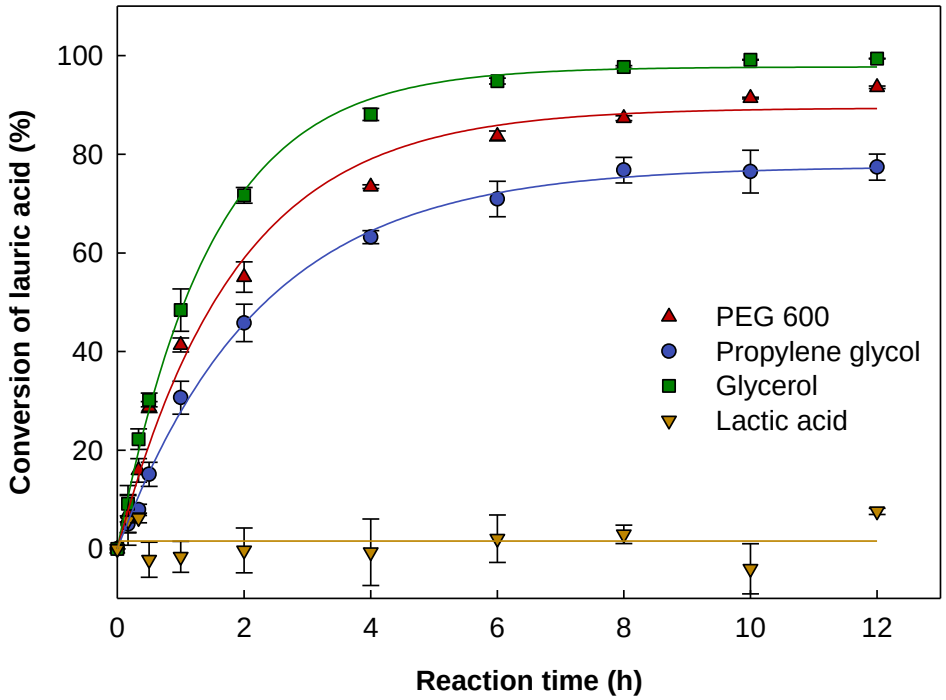


Fig. 2. Time courses of the esterification of lauric acid with the second acyl acceptor candidates (PEG 600, propylene glycol, glycerol, and lactic acid) in GSL-MPS. Reaction conditions: lauric acid, 60.66 mmol; second acyl acceptor candidate, 30.33 mmol; enzyme, 3,000 PLU Novozym® 435; reaction temperature, 60ºC; reaction time, 12 h; N_2 gas flow, 2.0 L/min.

Table 2. Initial conversion rate of lauric acid and conversion of lauric acid obtained from the esterification with the second acyl acceptor candidates

Compound	**Initial conversion rate of lauric acid (mmol/h)**	**Conversion of lauric acid (%)**
PEG 600	29.23 ± 1.50^{b}	93.59 ± 0.20^{b}
Propylene glycol	19.97 ± 1.19^{c}	77.40 ± 2.16^{c}
Glycerol	40.28 ± 2.07^{a}	99.38 ± 0.05^{a}
Lactic acid	N.D.	N.D.

The values with different superscripts in each column are significantly different ($p < 0.05$) by Duncan's multiple range test. N.D.: Not determined.

3.2. Effect of the molar ratio of lauric acid to glycerol

Oil-in-water emulsions are the basis of many food products, such as beverages, salad dressings, ice creams, and mayonnaise (Cheng, Xiong, & Chen, 2010). The emulsions consist of the oil droplets dispersed in the aqueous phase, and the emulsifiers are added to stabilize thermodynamically unstable emulsions (McClements & Jafari, 2018). Monoglycerides have been widely used for the stabilization of the oil-in-water emulsions with small-molecule co-emulsifiers (Chen, Liang, Li, He, Zeng, Gao, et al., 2019; Kantekin-Erdogan, Ketenoglu, & Tekin, 2019; Mao, Calligaris, Barba, & Miao, 2014) due to their amphiphilic characteristics. On the other hand, diglycerides and triglycerides are not suitable for the stabilization of the oil-in-water emulsion as they are highly oil-soluble. During the esterification of lauric acid with glycerol, three types of glycerides including monolaurin, dilaurin, and trilaurin can be synthesized. Among them, the content of monolaurin in MLE was considered as the critical determinant of the oil-in-water emulsion stability.

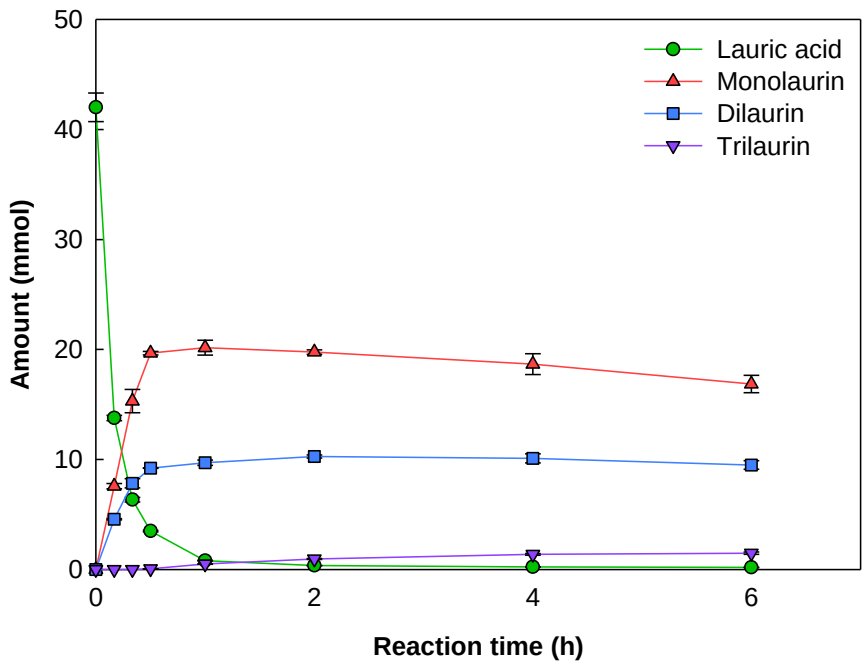
The parameters determining the glyceride composition in the lipase-catalyzed esterification reaction include the substrate molar ratio, the specificity of the lipase, the reaction time, and the water activity (Chand, Adlercreutz, & Mattiasson, 1997). The molar ratio of lauric acid to glycerol was thought to be the most critical factor with reference to the several studies

identified that the molar ratio of fatty acid to glycerol affects the glyceride composition (Rosu, Yasui, Iwasaki, & Yamane, 1999; Tüter, 1998). Therefore, lauric acid esters of glycerol were synthesized at the molar ratio of 0.5, 1.0, 1.5, 2.0, and 3.0 to investigate the effect of lauric acid to glycerol molar ratio on the glyceride composition.

The conversion of lauric acid and the glyceride composition were highly dependent on the molar ratio of lauric acid to glycerol (Fig. 3 and Table 3). The conversion of lauric acid was higher than 99.6% at the substrate molar ratio between 0.5 and 2.0, whereas the molar ratio of 3.0 resulted in the lowest value of 91.94%. This suggests that the decreasing glycerol led to less conversion of lauric acid. The content of monolaurin increased and of trilaurin decreased as the molar ratio decreased from 3.0 to 1.0. On the other hand, no significant difference in the glyceride composition was observed at the substrate molar ratio between 0.5 and 1.0, but the concentration of the products was lowered when the substrate molar ratio was 0.5. Diglyceride content from total glyceride was the lowest at the substrate molar ratio of 3.0, and it increased with the increasing molar ratio from 0.5 to 2.0. The results obtained in this study were in accordance with other studies which revealed that the high substrate molar ratio of fatty acid to glycerol suppresses both monoglycerides production and fatty acid conversion (Freitas, Perez, Santos,

& Castro, 2007; Ghamgui, Miled, Rebaï, Karra-chaâbouni, & Gargouri, 2006; Kantekin-Erdogan, Ketenoglu, & Tekin, 2019; Watanabe, Yamauchi-Sato, Nagao, Yamamoto, Ogita, & Shimada, 2004). Therefore, considering monolaurin content from total glyceride, conversion of lauric acid, and the amount of the residual glycerol, the substrate molar ratio of lauric acid to glycerol for the second reaction of the two-step MLE synthesis was set to 1.0.

A


Lauric acid
Monolaurin
Dilaurin
Trilaurin
Amount (mmol)
Reaction time (h)
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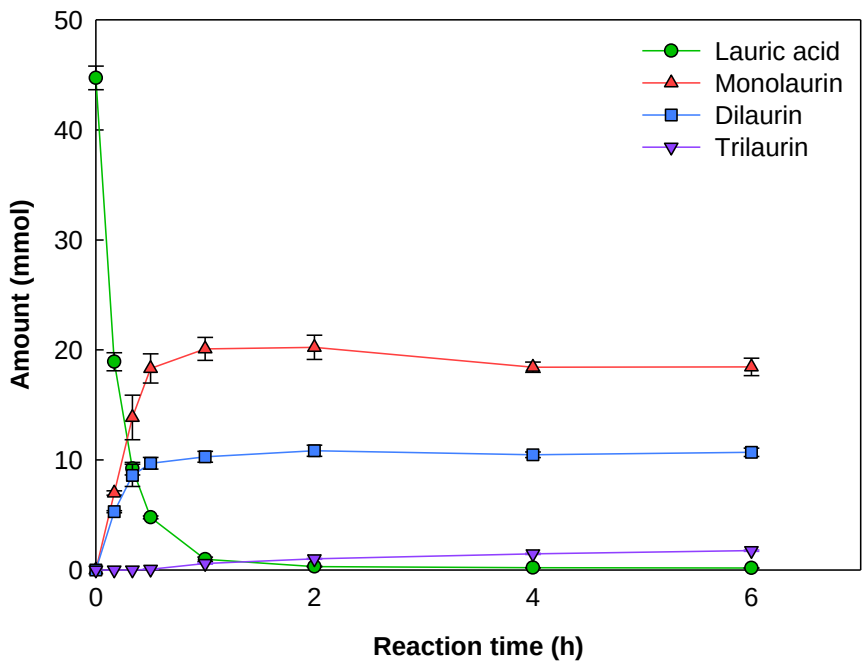
6

B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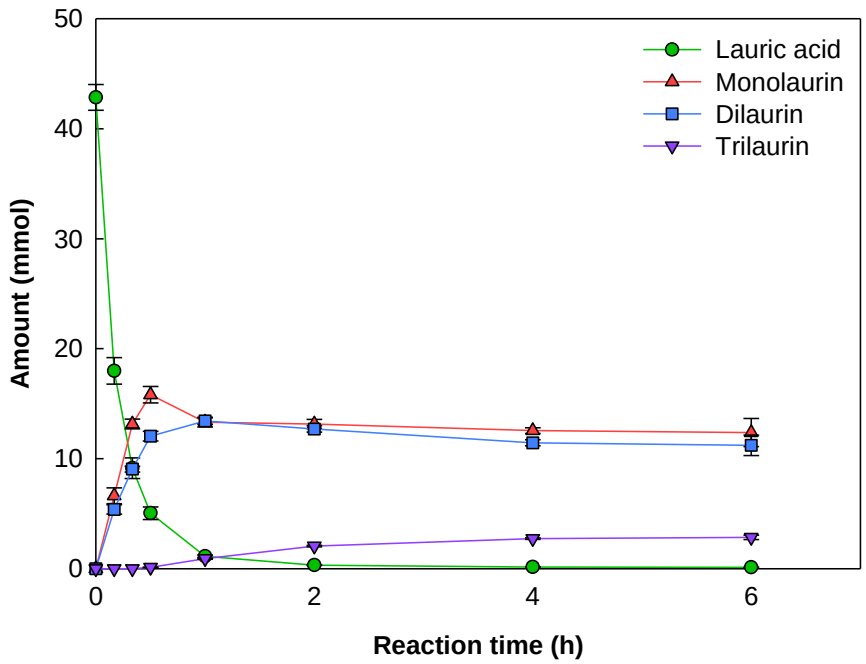
Lauric acid
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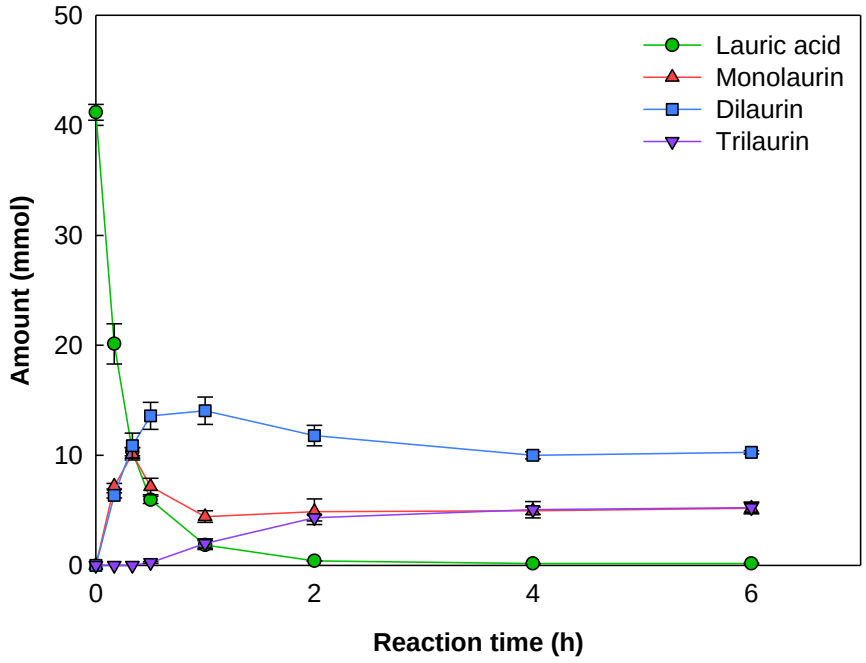
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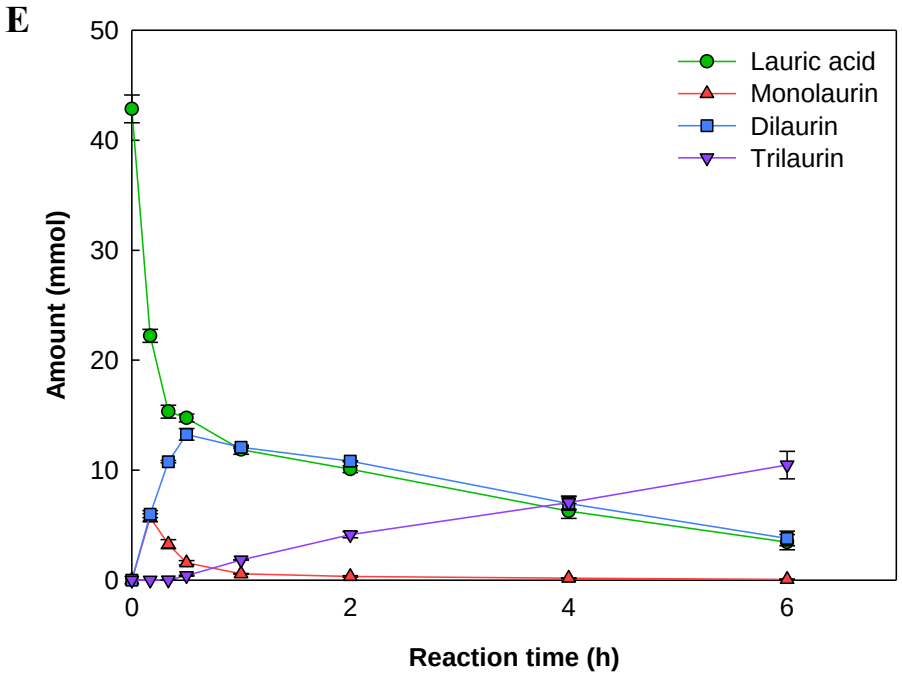


Fig. 3. Time courses of the esterification of lauric acid with glycerol at the substrate molar ratio (lauric acid to glycerol) of (A) 0.5, (B) 1.0, (C) 1.5, (D) 2.0, and (E) 3.0 in GSL-MPS. Reaction conditions: lauric acid, 43.33 mmol; enzyme, 12,000 PLU Novozym® 435; reaction temperature, 60°C; reaction time, 6 h; N_2 gas flow, 2.0 L/min.

Table 3. Effect of the molar ratio of lauric acid to glycerol on the conversion of lauric acid and the glyceride composition

Substrate molar ratio (Lauric acid / glycerol)	Conversion of lauric acid (%)	Glyceride composition (mol %)		
		Monolaurin	Dilaurin	Trilaurin
0.5	99.56 ± 0.03^{a}	60.56 ± 0.07^{a}	34.13 ± 0.17^{c}	5.32 ± 0.12^{d}
1.0	99.60 ± 0.02^{a}	59.73 ± 0.19^{a}	34.60 ± 0.16^{c}	5.68 ± 0.05^{d}
1.5	99.72 ± 0.03^{a}	46.78 ± 0.63^{b}	42.44 ± 0.38^{b}	10.79 ± 0.25^{c}
2.0	99.61 ± 0.12^{a}	25.03 ± 2.38^{c}	49.62 ± 0.05^{a}	25.35 ± 2.38^{b}
3.0	91.94 ± 1.30^{b}	0.46 ± 0.23^{d}	26.65 ± 5.85^{d}	72.89 ± 5.64^{a}

The values with different superscripts in each column are significantly different ($p < 0.05$) by Duncan's multiple range test.

3.3. Two-step synthesis of MLE

MLE was synthesized by the two-step esterification reaction (Fig. 4). In the first reaction, erythorbyl laurate was synthesized for 72 h with the production yield of 15.82 ± 0.25 mg/mL (Fig. 5A), in accordance with the previous study (Yu, Lee, Shin, Park, & Chang, 2019). In the second reaction, monolaurin, dilaurin, and trilaurin were synthesized for 6 h by the addition of glycerol (Fig. 5B). The time course of the second reaction was the same regardless of whether the first reaction is performed of not. The composition of MLE obtained by the two-step synthesis was analyzed (Table 4) and used for further experiments.

Transesterification is the process of converting the alkoxy group of an ester into another alcohol (Demirbas, 2008). The hydrolysis of the products and the formation of the new products can take place depending on the reaction equilibrium in the transesterification reaction. In the two-step MLE synthesis, the transesterification reaction between erythorbyl laurate and glycerol or between glycerides and erythorbic acid is possible. However, the amount of erythorbyl laurate synthesized in the first reaction did not change during the second reaction, indicating that the transesterification did not occur after the addition of glycerol as the second acyl acceptor.

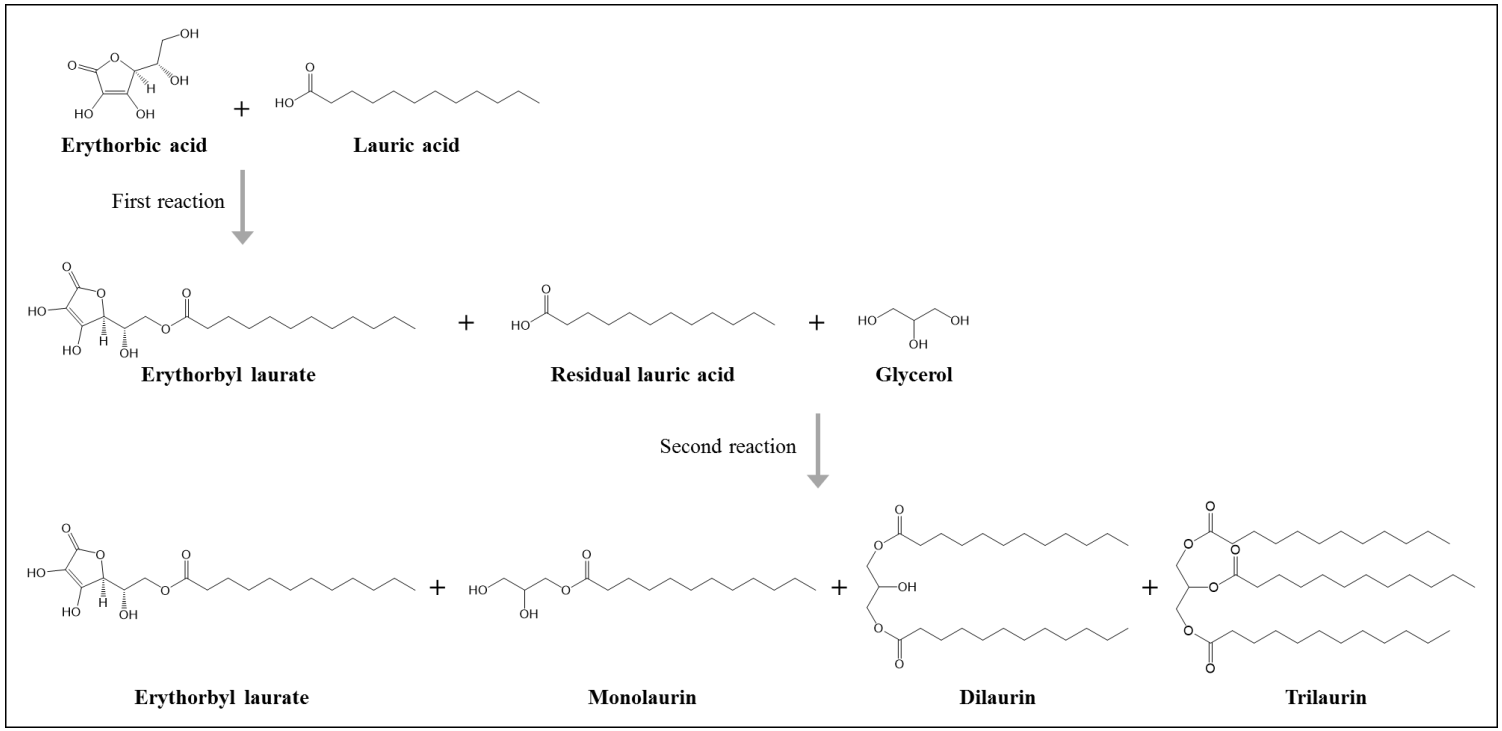


Fig. 4. Reaction scheme for the two-step synthesis of MLE.

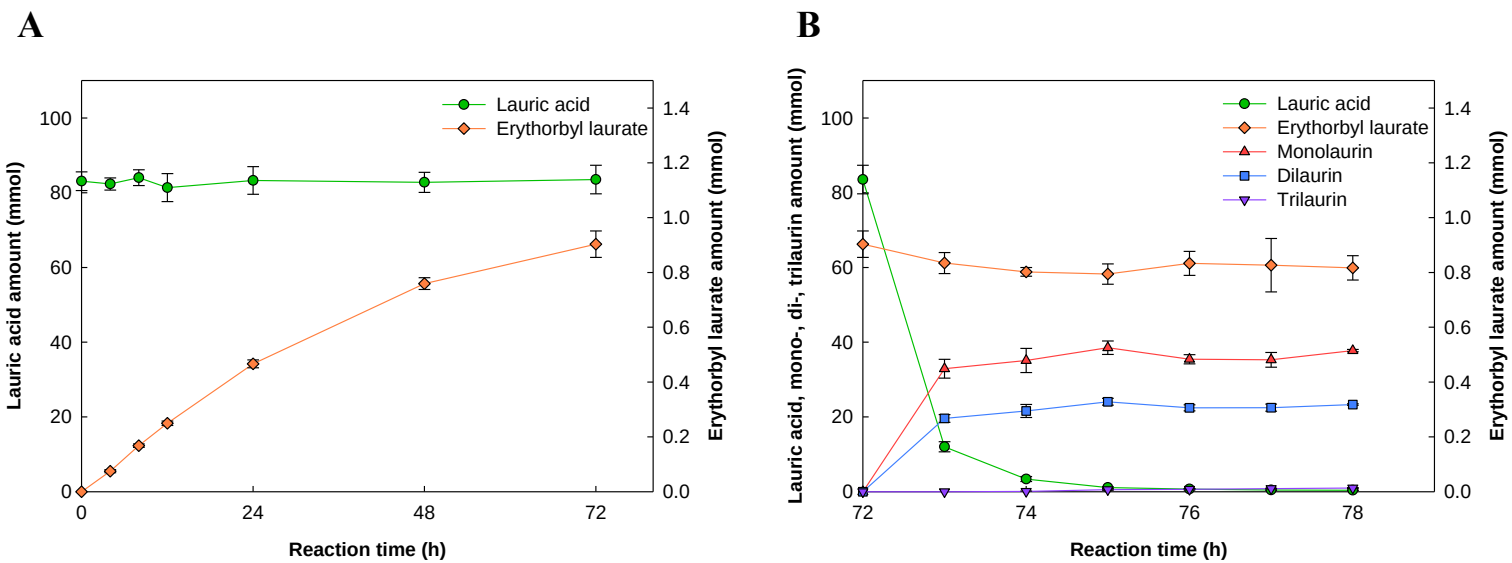


Fig. 5. Time courses of the two-step MLE synthesis in GSL-MPS. Reaction conditions of (A) the first reaction: erythorbic acid, 43.33 mmol; lauric acid, 86.66 mmol; enzyme, 24,000 PLU Novozym® 435; reaction temperature, 60°C; reaction time, 72 h; N_2 gas flow, 2.0 L/min. After 72 h of the first reaction, 84.70 mmol glycerol was added, and the second reaction was performed for 6 h. Other reaction conditions of the second reaction were the same as the first reaction.

Table 4. Composition of MLE

Compound	Concentration (%, w/w)
Erythorbyl laurate	1.33
Monolaurin	39.39
Dilaurin	44.19
Trilaurin	2.27
Erythorbic acid	2.84
Lauric acid	2.93
Glycerol	7.06
Total	100

Concentration is expressed in the average weight percentage of the triplicate.

3.4. Emulsion properties

Initial emulsion stability, initial emulsion properties, and storage stability were investigated to evaluate the effects of MLE on emulsion properties. The initial emulsion stability was evaluated by visual observation of the phase separation of the emulsions containing 0.0 – 5.0%(w/w) MLE after 24 h of storage at 25°C. The emulsions containing MLE with the concentration above 2.0%(w/w) were destabilized within the first 24 h, and the separation of a distinct lipid layer at the top and a relatively transparent aqueous layer at the bottom was observed more clearly as MLE concentration increased from 3.0 to 5.0%(w/w) (Fig. 6). On the other hand, the control emulsion (0.0% MLE) and the emulsions containing MLE with the concentration between 1.0 and 2.0%(w/w) were stable over 24 h of storage at 25°C.

Droplet size and zeta potential of the emulsions containing MLE with the concentration below 2.0%(w/w) were measured to evaluate the initial emulsion properties. The droplet size decreased as MLE concentration increased from 0.0 to 1.0%(w/w), and increased as MLE concentration increased from 1.0 to 2.0%(w/w). The average polydispersity index of all emulsions containing MLE with the concentration below 2.0%(w/w) was lower than 0.2 (Fig. 7), indicating that the emulsions had a narrow size distribution (C. C. Loi, G. T. Eyres, & E. J. Birch, 2019).

The droplet size of the oil-in-water emulsion generally decreases with increasing surfactant to oil ratio (Kundu, Agrawal, Mateen, & Mishra, 2013) and increases with increasing oil concentration (Ni, Hu, Sun, Zhao, & Xia, 2017). The decreasing droplet size with increasing MLE concentration from 0.0 to 1.0%(w/w) might be due to the increasing erythorbyl laurate and monolaurin, which are amphiphilic compounds. They can locate at the oil-water interface, so they might have attributed to split the oil droplets into small pieces to minimize the surface tension and maximize the interfacial area (Saberi, Fang, & McClements, 2013). On the other hand, the increasing droplet size with increasing MLE concentration from 1.0 to 2.0%(w/w) might be caused by the increasing content of oil-soluble dilaurin and trilaurin rather than the effect of erythorbyl laurate and monolaurin (Chia Chun Loi, Graham T. Eyres, & E. John Birch, 2019).

Zeta potential gives information on emulsion stability based on the surface charge of the droplet (Masum, Chandrapala, Adhikari, Huppertz, & Zisu, 2019). The increase in absolute zeta potential indicates the increase in repulsive forces between the oil droplets (Jiang, Liu, Wang, Yang, Chen, Zhong, et al., 2019), and the emulsions having zeta potential more negative than -30 mV are considered stable (Li & Lu, 2016; Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2015). The zeta potential decreased with

the increasing MLE concentration (Fig. 8), and all emulsions containing MLE with the concentration from 0.5 to 2.0%(w/w) had zeta potential below -30 mV, indicating they were stable. The decreasing zeta potential with the increasing MLE concentration might be due to the increase of lauric acid, the free fatty acid having a negative charge on the carboxyl group (Hur, Joo, Lim, Decker, & McClements, 2011).

Storage stability was evaluated by the changes of the droplet size (Fig. 9). At all concentrations of MLE, no significant change in the droplet size was observed during 15 days of storage at 25ºC. Polydispersity index of the emulsions also did not change or slightly changed during the storage time (Table 5). These results suggest that the negatively charged and monodispersed oil droplets contributed to the storage stability of the emulsions containing MLE with the concentrations below 2.0%(w/w). In conclusion, MLE can be added to the oil-in-water emulsion-type foods up to 2.0%(w/w), and the emulsions could remain stable.

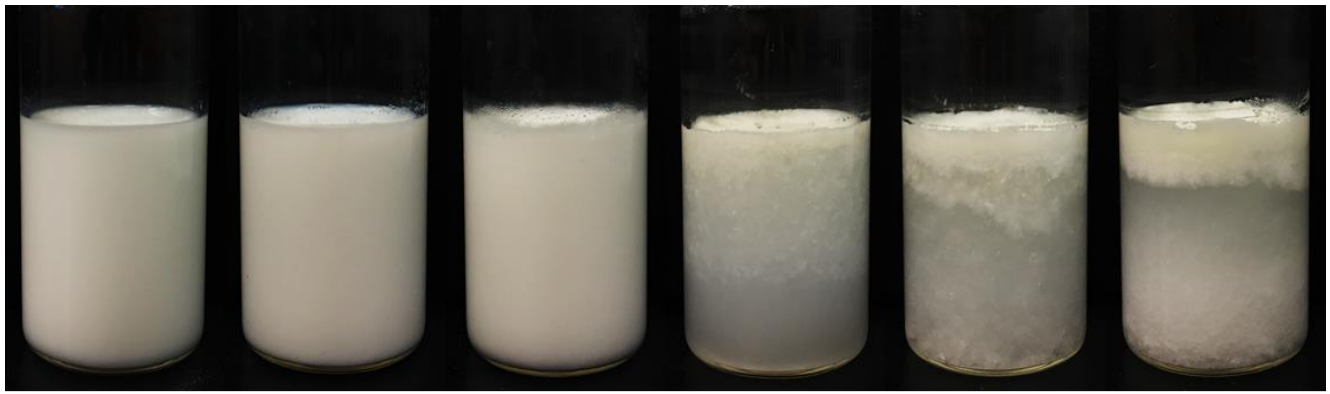


Fig. 6. Photographs of Tween 20-stabilized 5.0%(w/w) oil-in-water emulsion containing 0.0 - 5.0%(w/w) MLE after storage at 25^{o}C for 24 h.

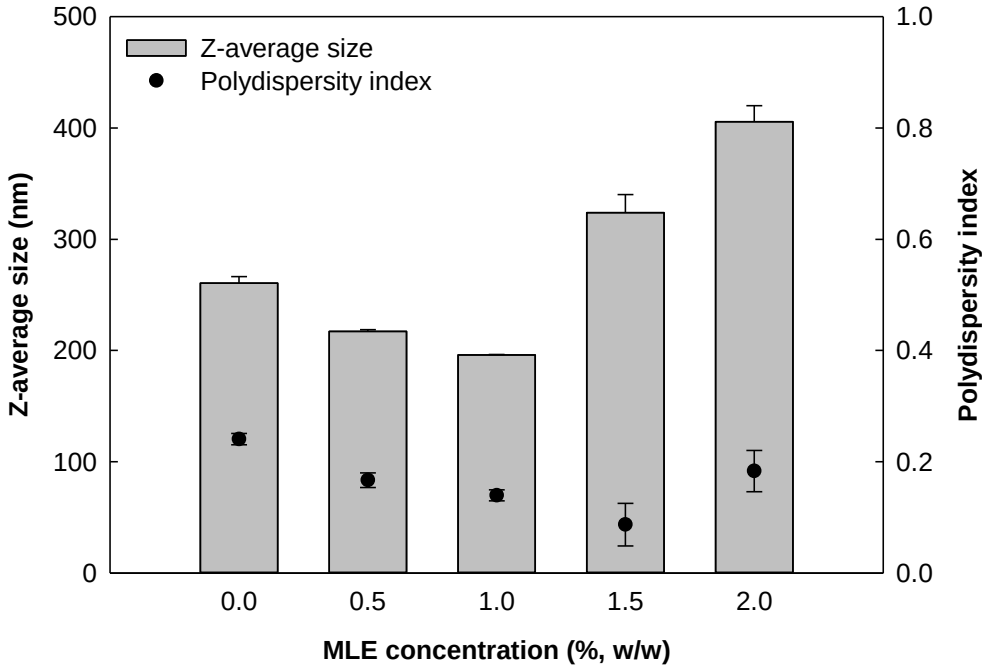


Fig. 7. Effect of MLE concentration (0.0 – 2.0%, w/w) on the droplet size and the polydispersity index of Tween 20-stabilized 5.0%(w/w) oil-in-water emulsion.

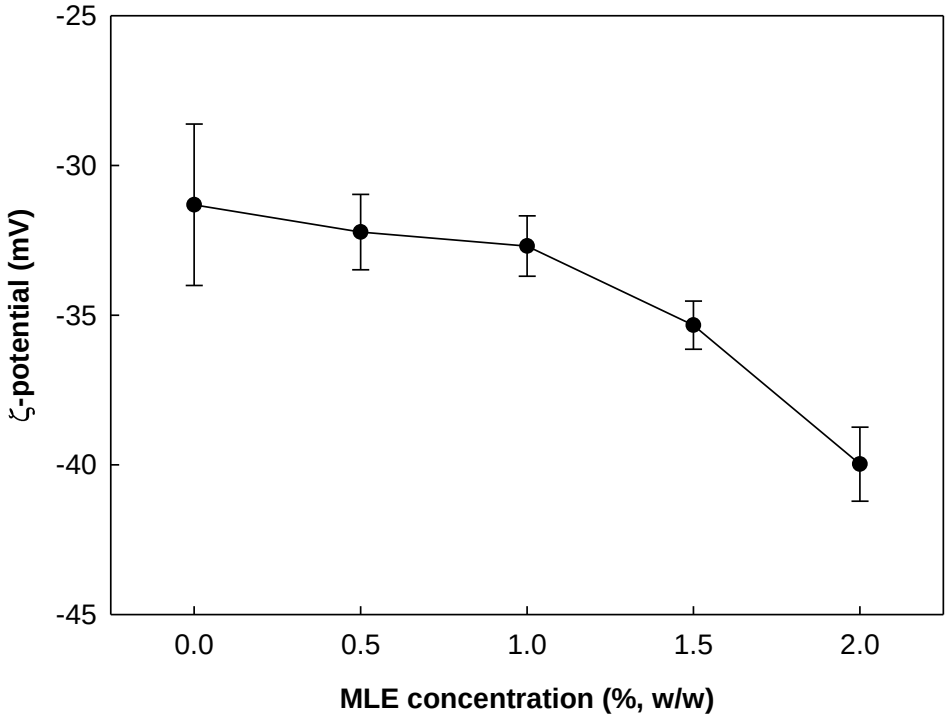


Fig. 8. Effect of MLE concentration (0.0 – 2.0%, w/w) on the zeta potential of Tween 20-stabilized 5.0%(w/w) oil-in-water emulsion.

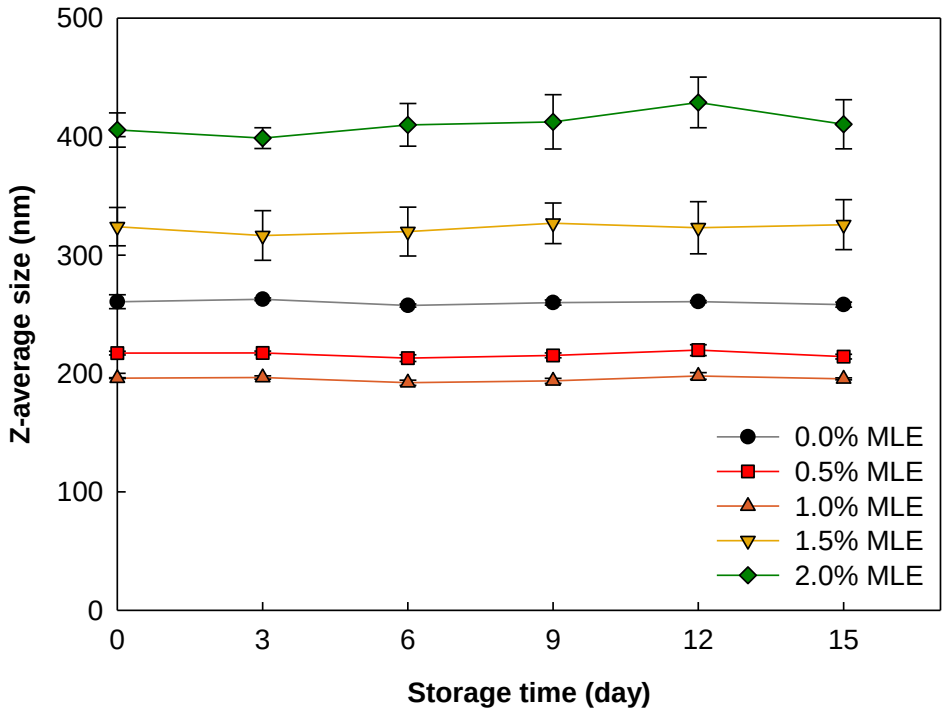


Fig. 9. Droplet size changes of Tween 20-stabilized 5.0%(w/w) oil-in-water emulsion containing MLE (0.0 – 2.0%, w/w) during 15 days of storage at 25°C.

Table 5. Polydispersity index changes of Tween 20-stabilized 5.0%(w/w) oil-in-water emulsion containing MLE (0.0 – 2.0%, w/w) during 15 days of storage at 25°C

MLE concentration (%, w/w)	Polydispersity index					
	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
0.0	0.241 ± 0.008^{a}	0.243 ± 0.009^{a}	0.240 ± 0.018^{a}	0.247 ± 0.007^{a}	0.238 ± 0.004^{a}	0.250 ± 0.016^{a}
0.5	0.167 ± 0.011^{a}	0.181 ± 0.003^{a}	0.184 ± 0.018^{a}	0.167 ± 0.012^{a}	0.169 ± 0.011^{a}	0.175 ± 0.008^{a}
1.0	0.140 ± 0.008^{ab}	0.147 ± 0.007^{ab}	0.159 ± 0.008^{b}	0.126 ± 0.013^{a}	0.147 ± 0.000^{ab}	0.139 ± 0.015^{ab}
1.5	0.087 ± 0.031^{a}	0.135 ± 0.037^{a}	0.136 ± 0.020^{a}	0.090 ± 0.006^{a}	0.110 ± 0.002^{a}	0.124 ± 0.034^{a}
2.0	0.183 ± 0.030^{a}	0.223 ± 0.003^{ab}	0.269 ± 0.015^{c}	0.241 ± 0.014^{bc}	0.218 ± 0.015^{ab}	0.250 ± 0.019^{bc}

The values with different superscripts in each row are significantly different ($p < 0.05$) by Duncan's multiple range test.

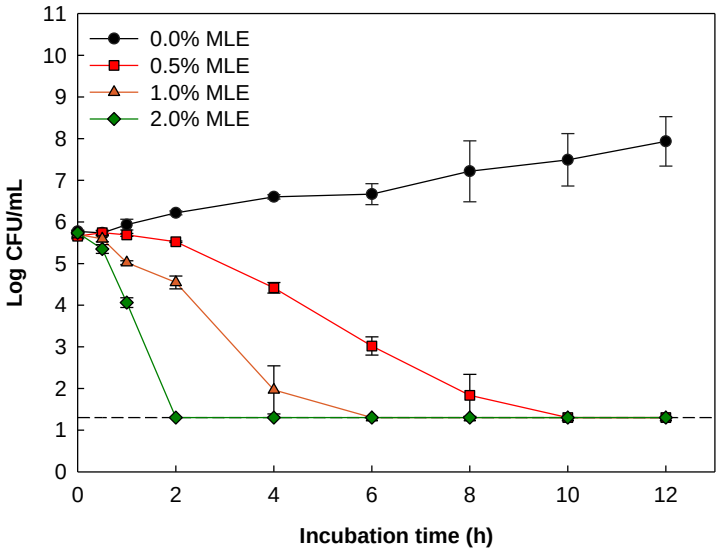
3.5. Antibacterial properties

Antibacterial activity of MLE against two Gram-positive and two Gram-negative bacteria was evaluated in the oil-in-water emulsion by time-kill assay. MLE with the concentration of 2.0%(w/w) did not result in bacteriostatic or bactericidal effect against Gram-negative bacteria, such as *E. coli* ATCC 35150 and *S.* Typhimurium ATCC 43971 (Fig. 10C and D). On the other hand, MLE at all concentrations exhibited bactericidal effect against Gram-positive bacteria, including *S. aureus* ATCC 12692 and *L. monocytogenes* ATCC 19115, and the concentration-dependent effect was observed (Fig. 10A and B).

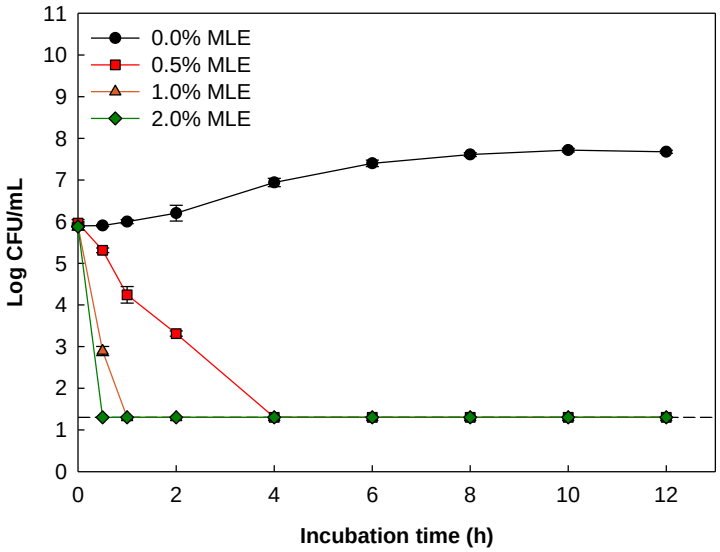
The antibacterial activities of 0.007%(w/w) erythorbyl laurate and 0.493%(w/w) MLE excluding erythorbyl laurate against two Gram-positive bacteria were evaluated to verify the effects of erythorbyl laurate and other compounds obtained from the second reaction on the antibacterial activity of MLE (Fig. 11). The concentration of erythorbyl laurate and MLE excluding erythorbyl laurate was determined to correspond to 0.5%(w/w) MLE. The results suggest that the antibacterial activity of MLE against Gram-positive bacteria might be due to the presence of erythorbyl laurate and monolaurin because they are known to have antibacterial activity against Gram-positive bacteria (Lieberman, Enig, & Preuss, 2006; Park, et al., 2018). Consequently,

MLE could control the contamination of Gram-positive bacteria in the oil-in-water emulsion-type foods.

A


0.0% MLE
0.5% MLE
1.0% MLE
2.0% MLE
Log CFU/mL
Incubation time (h)

B


0.0% MLE
0.5% MLE
1.0% MLE
2.0% MLE
Log CFU/mL
Incubation time (h)

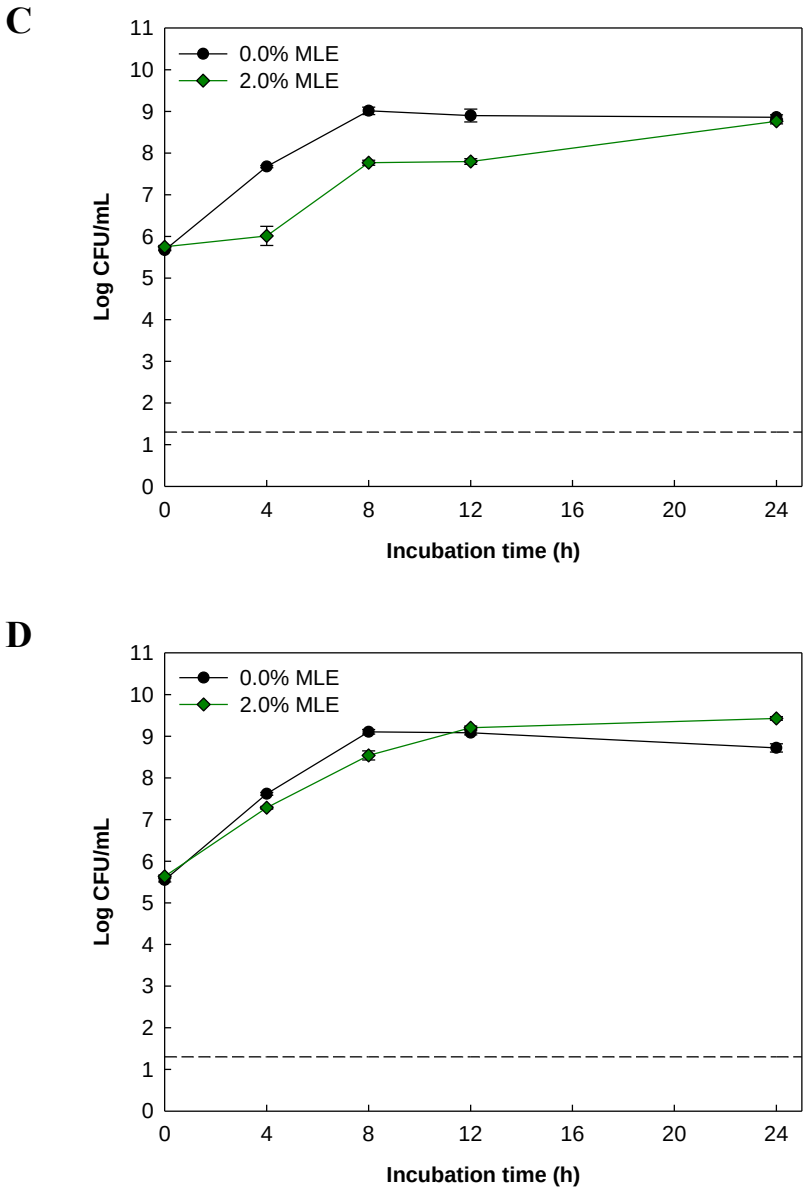


Fig. 10. Time-kill curves of MLE against (A) *Staphylococcus aureus* ATCC 12692, (B) *Listeria monocytogenes* ATCC 19115, (C) *Escherichia coli* ATCC 35150, and (D) *Salmonella* Typhimurium ATCC 43971. Dashed line indicates the limit of detection (20 CFU/mL).

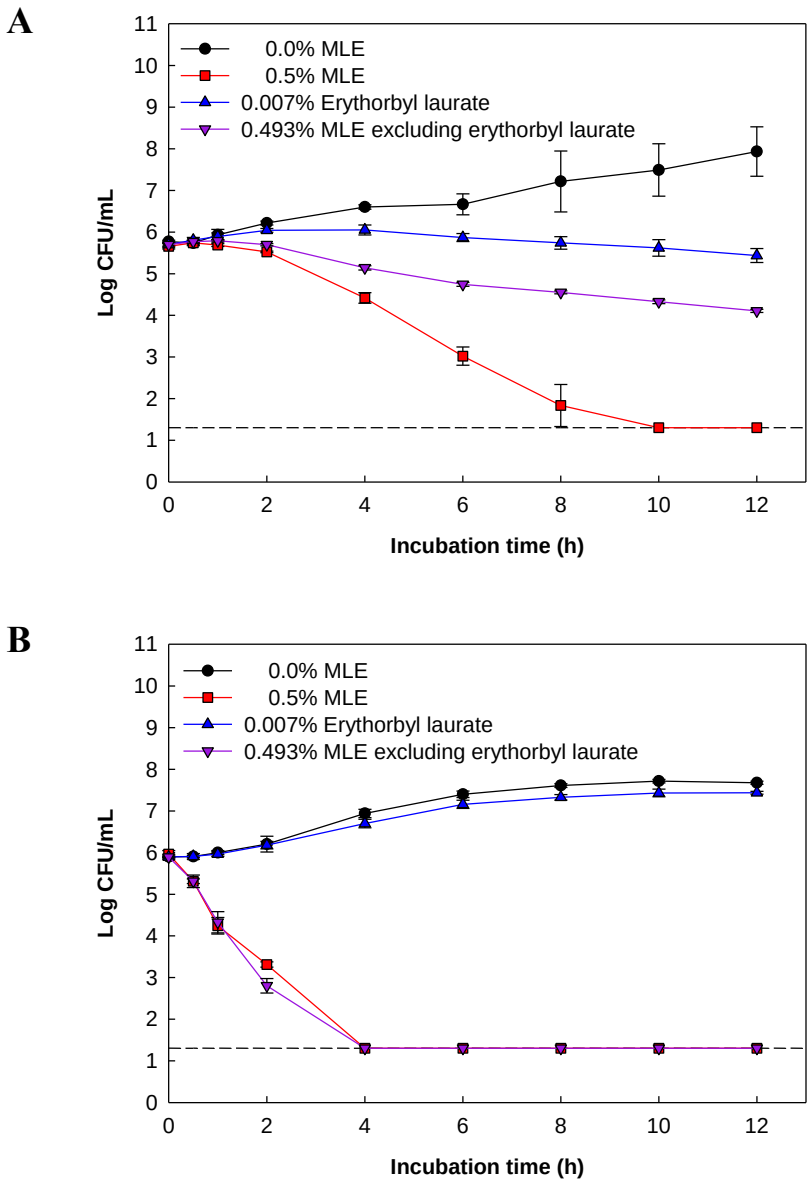


Fig. 11. Time-kill curves of erythorbyl laurate and MLE excluding erythorbyl laurate against (A) *Staphylococcus aureus* ATCC 12692 and (B) *Listeria monocytogenes* ATCC 19115. Dashed line indicates the limit of detection (20 CFU/mL).

3.6. Antioxidative properties

Lipid oxidation inhibition effect of MLE in the oil-in-water emulsion was evaluated using the ferric thiocyanate method during thermally accelerated oxidation. The formation of lipid hydroperoxide in the oil-in-water emulsion was affected by the concentration of MLE (Fig. 12). For the control emulsion, lipid hydroperoxides were produced without a lag phase, whereas the emulsions prepared with 0.5, 1.0, and 2.0%(w/w) MLE had 1, 2, and 3 days of lag phases, respectively. After 9 days of storage at 37°C, lipid hydroperoxide concentrations were 50.0, 84.3, and 98.3% lower than that of the control emulsion in the presence of 0.5, 1.0, and 2.0%(w/w) MLE.

Lipid oxidation inhibition effects of 0.013%(w/w) erythorbyl laurate and 0.987%(w/w) MLE excluding erythorbyl laurate were investigated to examine the antioxidant activity of erythorbyl laurate and other compounds produced by the two-step synthesis. The emulsions containing erythorbyl laurate and MLE excluding erythorbyl laurate consisted of a composition equal to the emulsions with 1.0%(w/w) MLE. Both erythorbyl laurate and MLE excluding erythorbyl laurate inhibited the formation of lipid hydroperoxides (Fig. 13). The amounts of lipid hydroperoxide produced by the oil-in-water emulsions containing 0.013%(w/w) erythorbyl laurate and 0.987%(w/w) MLE excluding

erythorbyl laurate were 75.7 and 65.8% lower than that of the control emulsion after 9 days of storage at 37°C. This result can be explained that the lipid oxidation inhibition effect of MLE derived from erythorbyl laurate and erythorbic acid in MLE excluding erythorbyl laurate. The formation of lipid hydroperoxide was more effectively inhibited by erythorbyl laurate than MLE excluding erythorbyl laurate. This can be explained by the fact that erythorbyl laurate locates at the oil-water interface, where the lipid oxidation usually occurs (Osborn & Akoh, 2004), while erythorbic acid is dispersed in the aqueous phase (Park, Lee, Jo, Choi, Lee, & Chang, 2017). In conclusion, MLE can act as antioxidant inhibiting lipid oxidation in the oil-in-water emulsion-type foods.

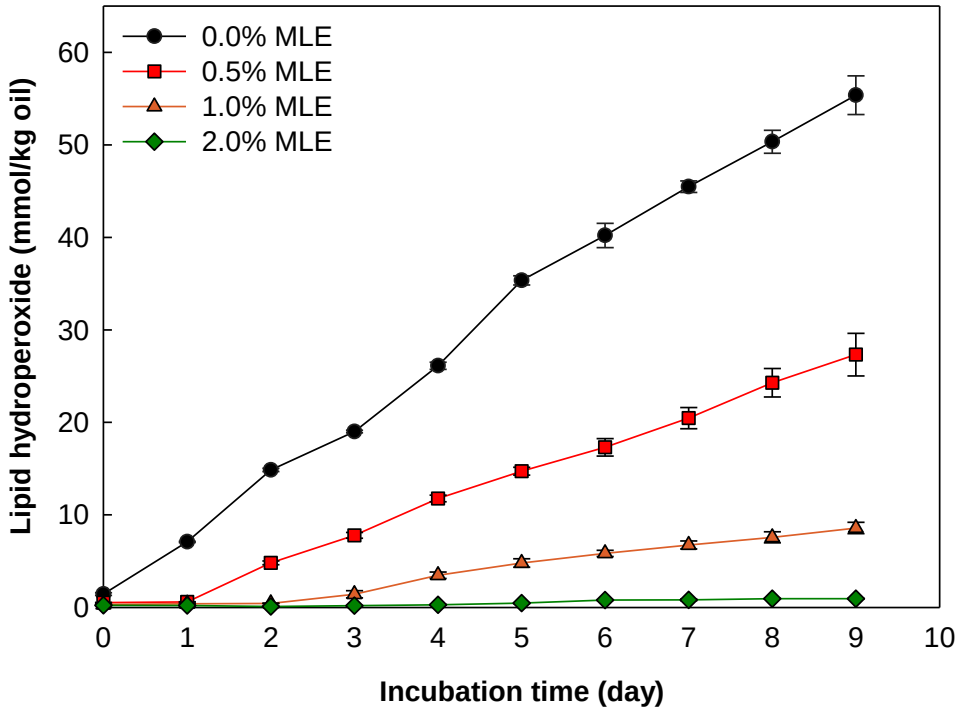


Fig. 12. Effect of MLE on the formation of lipid hydroperoxides during thermally accelerated oxidation.

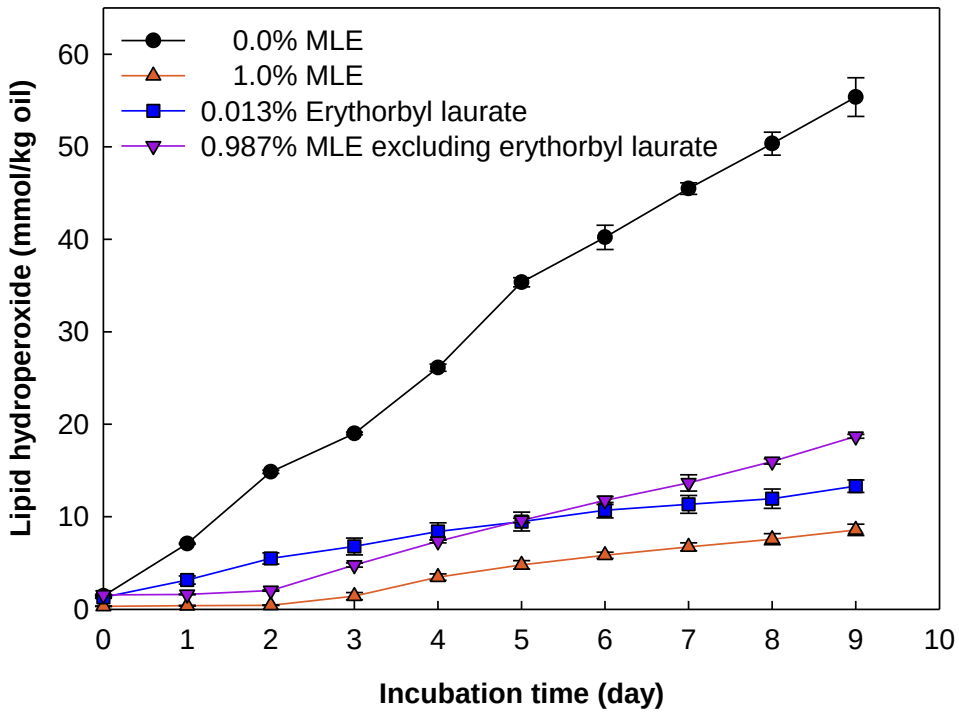


Fig. 13. Effect of erythorbyl laurate and MLE excluding erythorbyl laurate on the formation of lipid hydroperoxides during thermally accelerated oxidation.

4. Conclusions

In this study, multifunctional MLE with antibacterial and antioxidant activities was produced by the two-step enzymatic synthesis as an alternative to the conventional purification process. In the two-step MLE synthesis, erythorbyl laurate was synthesized in the first reaction, and lauric acid esters of glycerol were synthesized in the second reaction. As a result, MLE composed of erythorbyl laurate, monolaurin, dilaurin, and trilaurin was produced. The addition of MLE into the oil-in-water emulsion with the concentration below 2.0%(w/w) did not affect the emulsion stability. Besides, MLE exhibited the bactericidal effect against Gram-positive bacteria and inhibited lipid oxidation in the oil-in-water emulsion. The results obtained in this study suggest the feasibility of a practical application of MLE to the oil-in-water emulsion-type foods.

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국문초록

효소적 합성 후 기질이 잔존하는 경우, 용매 추출, 재결정화, 분자 증류 등의 정제 과정을 통해 잔존 기질이 제거되거나 회수된다. 그러나 이러한 과정은 번거로울 뿐 아니라, 과량의 유기용매와 고에너지를 필요로 한다는 단점을 가진다. 본 연구에서는 복잡한 정제 과정 대신 간단하게 잔존 기질을 활용하는 방법으로 라이페이스-촉매에 의한 다기능성 혼합물의 2단계 효소적 합성에 관한 연구가 진행되었다. 1차 반응에서는 erythorbyl laurate가 합성되었고, 2차 반응에서는 잔존 lauric acid와 에스터 결합 형성이 가능한 2차 아실 수용체가 첨가되어 새로운 에스터 화합물이 추가적으로 합성되었다. 2차 아실 수용체의 선정을 위해 lauric acid와 4가지 후보 물질인 PEG 600, propylene glycol, glycerol, lactic acid의 에스터화 반응 효율을 비교하였다. 반응 효율의 지표인 초기 lauric acid 전환 속도 및 lauric acid 전환율 모두 glycerol과의 반응에서 가장 높게 나타나 glycerol이 2차 아실 수용체로 선정되었다. 2차 반응의 기질 몰 비율 결정을 위해 lauric acid와 glycerol의 몰 비율에 따른 에스터화 반응

을 6 시간 동안 진행한 후 생성된 glyceride의 조성을 비교하였다. Monolaurin, dilaurin, trilaurin으로 구성된 glyceride 중에서 양친매적 구조에 의해 에멀젼 안정성에 가장 큰 영향을 미치는 monolaurin 함량을 고려하여 lauric acid와 glycerol의 기질 몰 비율을 1.0으로 결정하였다. 결과적으로, 2단계 효소적 합성을 통해 1.33% erythorbyl laurate, 39.39% monolaurin, 44.19% dilaurin, 2.27% trilaurin, 2.84% erythorbic acid, 2.93% lauric acid, 7.06%(w/w) glycerol로 구성된 다기능성 혼합물, 즉 mixed lauric acid esters (MLE)가 생산되었다. Erythorbyl laurate가 수중 유적형 에멀젼에 적합한 다기능성 식품 첨가제인 것을 고려하여, MLE가 수중 유적형 에멀젼 특성에 미치는 영향과 에멀젼에서 MLE의 항균력 및 항산화력을 평가하였다. 에멀젼 특성은 MLE를 농도별로 첨가하여 제조된 5.0%(w/w) 수중 유적형 에멀젼의 입자 크기와 제타 전위 측정을 통해 평가되었다. 그 결과 2.0%(w/w) 이하의 MLE가 첨가된 에멀젼은 25°C에서 15일 동안 안정하게 유지되었다. Time-kill assay를 통한 항균력 평가 결과 MLE를 0.5%(w/w) 이상의 농도로 처리하였을 때 그람 양성균인 *S. aureus* ATCC 12692와 *L. monocytogenes* ATCC 19115에 대해 농도 의존적 살

균 효과를 보였고, 열 가속 산화 조건 하에서 MLE의 지질 산화 지연 효과 평가 결과 MLE 농도가 증가함에 따라 지질 과산화물의 생성이 감소하였다. 따라서 2단계 효소적 합성을 통해 생산된 항균력과 항산화력을 가지는 MLE는 수중 유적형 에멀젼 유형의 식품에 다기능성 식품 첨가제로 활용 가능할 것으로 기대된다.

주제어: 2단계 효소적 합성, 라이페이스-촉매 에스터화 반응, 수중 유적형 에멀젼, 항균력, 항산화력

학번: 2018-21443