A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Lipase-catalyzed synthesis of pyridoxine monolaurate as a food emulsifier in solvent-free system

식품 유화제 피리독신 모노로우레이트의 무용매 반응계에서 라이페이스 기반 효소적 합성

February, 2021

Department of Agricultural Biotechnology

Seoul National University

Sungjun Kim

석사학위논문

Lipase-catalyzed synthesis of pyridoxine monolaurate as a food emulsifier in solvent-free system

지도교수 장 판 식

이 논문을 석사학위 논문으로 제출함

2021년 2월

서울대학교 대학원

농 생 명 공 학 부

김 성 준

김성준의 석사 학위논문을 인준함

2021년 2월

위원장 하 남 출 (인)

부위원장 장 판 식 (인)

위원 이 기 원 (인)

Abstract

In recent years, as the demand for natural and eco-friendly products in the food industry increases, many studies have been conducted on natural emulsifiers to replace synthetic emulsifiers. In this study, a new natural emulsifier was synthesized through lipase-catalyzed synthesis in a solvent-free reaction system by using hydrophilic pyridoxine and hydrophobic lauric acid as substrates. Lipase-catalyzed synthesis using Novozym® 435 was analyzed by HPLC, and the synthesis of pyridoxine monolaurate (PML) was identified by LC-ESI-MS analysis and ^{1}H-, ^{13}C-, and ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC) NMR, confirming that the new compound was [5-hydroxy-4-(hydroxymethyl)-6-methylpyridin-3-yl]methyl dodecanoate. Reaction efficiency was compared among in organic solvent monophase system (OS-MPS), solid-liquid biphase system (SL-BPS), and gas-solid-liquid multiphase system (GSL-MPS) with respect to volumetric productivity and conversion yield. In GSL-MPS, the volumetric productivity of 41.24 mmol/L/h was about 3.72-fold higher than that of OS-MPS and 2.08-fold higher than that of SL-BPS. It was determined that GSL-MPS is the most efficient enzyme bioreactor system in lipase-catalyzed synthesis of PML. The operational stability of the enzyme was also evaluated, confirming that there was no significant ($p < 0.05$) decrease in operational stability in GSL-MPS,

but significant ($p < 0.05$) decrease in operational stability in SL-BPS and OS-MPS for 6 times reaction batch. In GSL-MPS, the effect of substrate molar ratio ([pyridoxine] to [lauric acid]) and reaction temperature on reaction efficiency was evaluated, and the reaction temperature of 70°C, the substrate molar ratio of 0.1, and the reaction time of 9 h were established as optimal synthesis conditions. In the conditions, the volumetric productivity of PML was 41.24 mmol/L/h and the conversion yield was 84.56%. In order to evaluate the interfacial activity of PML, the reduction of interfacial tension at the interface between the MCT oil and water with increase in concentration of PML was measured. In the PML concentration range of 0.5~6.0 mM, the interfacial tension decreases with PML concentration increases, identifying PML was the interfacial active compound. The effect of pH and PML concentration on the emulsion properties of oil-in-water emulsion stabilized with PML was determined. Oil-in-water emulsion was prepared with 0.20%(w/w) PML, and Z-average size and zeta potential were measured in the range of pH 2.0~8.0. In the pH range of 2.0~4.0, there was no significant change in Z-average size. Unlike this, Z-average size tended to increase and decrease as pH changes from 4.0 to 6.0 and from 6.0 to 8.0, respectively. There was no significant difference in zeta potential between pH 2.0 and pH 3.0, and in the pH range of 3.0~8.0, the zeta potential tended to decrease significantly

($p < 0.05$) with increase in pH. Z-Average size of the oil-in-water emulsion prepared in the PML concentration range of 0.01~0.20%(w/w) was measured. In the range of 0.01~0.10%(w/w) of PML, Z-average size was significantly decreased ($p < 0.05$) with increasing concentration, and in the range of 0.10~0.20%(w/w), there was no significant change in Z-average size. Finally, PML is expected to be synthesized through lipase-catalyzed synthesis in eco-friendly solvent-free reaction system and used as a natural food emulsifier applied to acidic emulsion foods.

Keywords: pyridoxine monolaurate, small-molecule emulsifier, lipase-catalyzed synthesis, solvent-free reaction system, emulsifying property

Student number: 2019-27667

Contents

List of Tables

List of Figures

Fig. 5. Time courses of lipase-catalyzed synthesis of pyridoxine monolaurate in different enzyme bioreactor systems. All reactions were carried out with Novozym® 435 of 396 mg (26.4 mg/mL) and reaction volume of 15 mL at 70 ℃. Reaction conditions in organic solvent monophase (OS-MPS): pyridoxine, 0.3 mmol; lauric acid, 3.0 mmol; magnetic stirring, 300 rpm; reaction time, 1.5 h. Reaction conditions in solid-liquid biphase system (SL-BPS): pyridoxine, 6.59 mmol; lauric acid, 65.89 mmol; magnetic stirring, 300 rpm; reaction time, 12 h. Reaction conditions in gas-solid-liquid multiphase system (GSL-MPS): pyridoxine, 6.59 mmol; lauric acid, 65.89 mmol; N_2 gas flow, 2.0 L/min; reaction time, 12 h.

Fig. 6. Operational stability of Novozym® 435 over repeated batches of lipase-catalyzed synthesis of pyridoxine monolaurate in different enzyme bioreactor systems. All reactions were carried out with Novozym® 435 of 396 mg (26.4 mg/mL) and reaction volume of 15 mL at 70 ℃. Reaction conditions in organic solvent monophase (OS-MPS): pyridoxine, 0.3 mmol; lauric acid, 3.0 mmol; magnetic stirring, 300 rpm; reaction time, 1.5 h. Reaction conditions in solid-liquid biphase system (SL-BPS): pyridoxine, 6.59 mmol; lauric acid, 65.89 mmol; magnetic stirring, 300 rpm; reaction time, 12 h. Reaction conditions in gas-solid-liquid multiphase system (GSL-MPS): pyridoxine, 6.59 mmol; lauric acid, 65.89 mmol; N_2 gas flow, 2.0 L/min; reaction time, 12 h.

Fig. 7. Effect of substrate molar ratio ([pyridoxine] to [lauric acid]) on lipase-catalyzed synthesis in gas-solid-liquid multiphase system. (a) Time courses of reaction, (b) conversion yield, and (c) volumetric productivity at different substrate molar ratio. Reaction conditions: substrate molar ratio, 0.05~0.30; temperature, 70℃; Novozym® 435, 396 mg (26.4 mg/mL); N_2 gas flow, 2.0 L/min; reaction time, 12 h in 20 mL reactor. (The amount of lauric acid was fixed to 65.89 mmol.)

Fig. 8. Effect of reaction temperature on lipase-catalyzed synthesis in gas-solid-liquid multiphase system. Reactions conditions: pyridoxine, 6.59 mmol; lauric acid, 65.89 mmol; reaction temperature, 50~80℃; Novozym® 435, 396 mg (26.4 mg/mL); gas flow, 2.0 L/min; reaction time, 12 h in 20 mL reactor.

Fig. 9. Influence of pyridoxine monolaurate concentration on the interfacial tension measured at the MCT oil-water interface.

Fig. 10. Influence of pH on (a) Z-average size and (b) zeta potential of 10%(w/w) MCT oil-in-water emulsions stabilized by 0.2%(w/w) pyridoxine monolaurate. All measurements were conducted within 1 h after emulsion preparation.

Fig. 11. Influence of pyridoxine monolaurate concentration on Z-average size of 10%(w/w) MCT oil-in-water emulsions at pH 3.0 stabilized by PML. All measurements were conducted within 1 h after emulsion preparation.

1. Introduction

Emulsifiers reducing interfacial tension at oil-water interface and preventing droplet aggregation are required to stabilize emulsion system (Dickinson, 2003). Synthetic emulsifiers such as polysorbates (Tween) and monoglycerises have been widely used in food industry (Molet-Rodríguez, Salvia-Trujillo, & Martín-Belloso, 2018). Most of the synthetic emulsifiers with small molecular weight are easily adsorbed to the oil-water interface, lowering interfacial tension, and consequently facilitating droplet disruption during emulsion preparation. Therefore, they can stabilize oil droplets even at low concentrations (Dickinson, 2003; Jo & Kwon, 2014; Molet-Rodríguez et al., 2018). Moreover, the small-molecule emulsifiers are more effective to prepare emulsions with smaller size (Qian & McClements, 2011; Raikos, Duthie, & Ranawana, 2017).

Recently, as consumers' demand for more natural and environmentally friendly products has increased, many manufacturers and researchers have been interested in replacing synthetic emulsifiers with natural alternatives (McClements & Gumus, 2016). In response to these demands, lipase-catalyzed synthesis of small-molecule natural emulsifiers in organic solvent

has been studied using various substrates. For example, erythorbyl laurate was synthesized through lipase-catalyzed synthesis as an multifunctional emulsifiers (Park, Lee, Jo, Choi, Lee, & Chang, 2017). In addition, glucose-fatty acid esters with emulsifying properties were produced by lipase-catalyzed synthesis (Liang, Chen, Banwell, Wang, & Lan, 2018; Ren & Lamsal, 2017).

However, the conventional enzymatic synthesis using organic solvents as a reaction medium has several drawbacks. The lower solubility of polar substrate in organic solvents results in decreased productivity due to limited substrate concentration (H. Yu, Lee, Shin, Park, & Chang, 2019). And adverse effect of organic solvent on activity of biocatalyst is also one of the disadvantages in organic solvents reaction system (Inprakhon et al., 2017). Another problem is that the substrate specificity and kinetic parameter of the enzyme change as the molecular structure of the enzyme changes in the organic solvent (Bahamondes, Wilson, Guzmán, & Illanes, 2017; Cui, Stadtmüller, Jiang, Jaeger, Schwaneberg, & Davari, 2020). In addition, there are safety concerns about the production of food ingredients using organic solvents (Foresti & Ferreira, 2005; Santos, Bueno, Molgero, Rós, & de Castro, 2007).

To overcome these limitations, solvent-free system has been studied. Because a simple substrate mixture is used as the reaction medium without organic solvent, much higher concentration of the substrate can be applied in one reaction batch. As a result, more products can be obtained in the same reaction volume (Kim, Youn, & Shin, 2006). When emulsifiers are synthesized in solvent-free system, the conversion yields are very low because of immiscibility of substrates including both of hydrophilic and hydrophobic compounds. In particular, when the substrates belong to different phases, there is an obstacle in that the conversion yield is lowered due to the mass transfer limitation between the two phases (Taha, Nosier, Abdel-Aziz, & El-Naggar, 2020).

In order to overcome mass transfer limitation, various methods such as ultrasonication and microwave have been attempted (Martins, Schein, Friedrich, Fernandez-Lafuente, Ayub, & Rodrigues, 2013; D. Yu et al., 2010). Among them, the gas-solid-liquid multiphase system (GSL-MPS) used to synthesize erythorbyl laurate is an enzyme bioreactor system of improving mass transfer through incorporating gaseous phase (H. Yu et al., 2019).

Pyridoxine was selected as a hydrophilic substrate to synthesize a natural small-molecule emulsifier. Pyridoxine is a nutrient that exists in nature and is

a hydrophilic compound with a high water solubility of 220 g/L. Pyridoxine is absorbed after ingestion and converted into the active form, pyridoxal-5-phosphate (PLP), which is involved in numerous metabolisms, including amino acid metabolism, neurotransmitters biosynthesis, normal level of homocysteine maintenance, and immune function. Lauric acid was chosen as the hydrophobic substrate and the reaction medium, because lauric acid was demonstrated as an appropriate reaction medium in GSL-MPS studied previously (H. Yu et al., 2019). Pyridoxine fatty acid esters were synthesized by lipase-catalyzed esterification of pyridoxine with various fatty acids (Baldessari, Mangone, & Gros, 1998) and the potential of pyridoxine fatty acid esters as emulsifiers was suggested based on the calculated HLB (Baldessari & Mangone, 2002).

In this study, lipase-catalyzed synthesis of pyridoxine mono laurate (PML) was conducted with pyridoxine and lauric acid as substrates. Reaction efficiency and operational stability of lipase-catalyzed synthesis of PML under various enzyme bioreactor systems were compared. Effects of reaction parameters on the reaction efficiency were determined in GSL-MPS. Interfacial and emulsifying properties of PML were evaluated to propose its potential as the natural small-molecule emulsifier.

2. Materials and Methods

2.1. Material

Pyridoxine (≥ 98.0%) and lauric acid (dodecanoic acid ≥ 98.0%) were purchased from Sigma-Aldrich (St. Louis, MO). Immobilized lipase from *Candida antarctica* (Novozym® 435) with a catalytic activity of 7,000 PLU/g (PLU refers to millimoles of propyl laurate synthesized per min at 60℃.) was kindly provided by Novozymes (Bagsvaerd, Denmark). High-performance liquid chromatography (HPLC)-grade acetonitrile and water were purchased from J.T. Baker (Phillipsburg, NJ). Guaranteed reagent grade trifluoroacetic acid (≥ 99.9%), extra pure grade *n*-hexane (≥ 95.0%) and *tert*-butanol (≥ 99.0%) were purchase from Samchun Chemicals (Seoul, Republic of Korea). Medium chain triglycerides (MCT) oil was purchase from Korea Medical Foods (Seoul, Republic of Korea).

2.2. Lipase–catalyzed synthesis of pyridoxine monolaurate

For the production of pyridoxine monolaurate (PML) in organic solvent monophase system (OS-MPS), the reaction was performed in acetonitrile at 70℃ with magnetic stirring of 300 rpm. Lauric acid of 601.0 mg (3.0 mmol) and pyridoxine of 50.8 mg (0.3 mmol) were pre-dissolved in acetonitrile of 15 mL and the reaction was initiated by adding the Novozym® 435 of 396 mg

(26.4 mg/mL, 2,772 PLU). For the synthesis of PML in solvent-free system, lauric acid was used as reaction medium and also acyl donor. Lauric acid of 13,200 mg (65.89 mmol) was melted at 70℃ and pyridoxine of 1,114.8 mg (6.59 mmol) was dispersed in lauric acid medium with magnetic stirring of 300 rpm in solid-liquid biphase (SL-BPS) and N_2 gas flow of 2.0 L/min (purity ≥ 99.9%) in GSL-MPS. The reaction in GLS-MPS was constructed by slightly modifying method described in previous study (H. Yu et al., 2019). After pre-incubation for 10 min, the reaction was initiated by adding Novozym® 435 of 396 mg (26.4 mg/mL, 2,772 PLU). Scheme of the reaction is presented in Fig. 1. To evaluate reaction efficiency in each enzyme bioreactor systems, conversion yield and volumetric productivity were determined. The definition of conversion yield (%) was the percentage of the produced concentration of PML (mmol/L) divided by initial concentration of pyridoxine (mmol/L). And volumetric productivity (mmol/L/h) indicated produced concentration of PML (mmol/L) divided by reaction time attaining equilibrium (h).

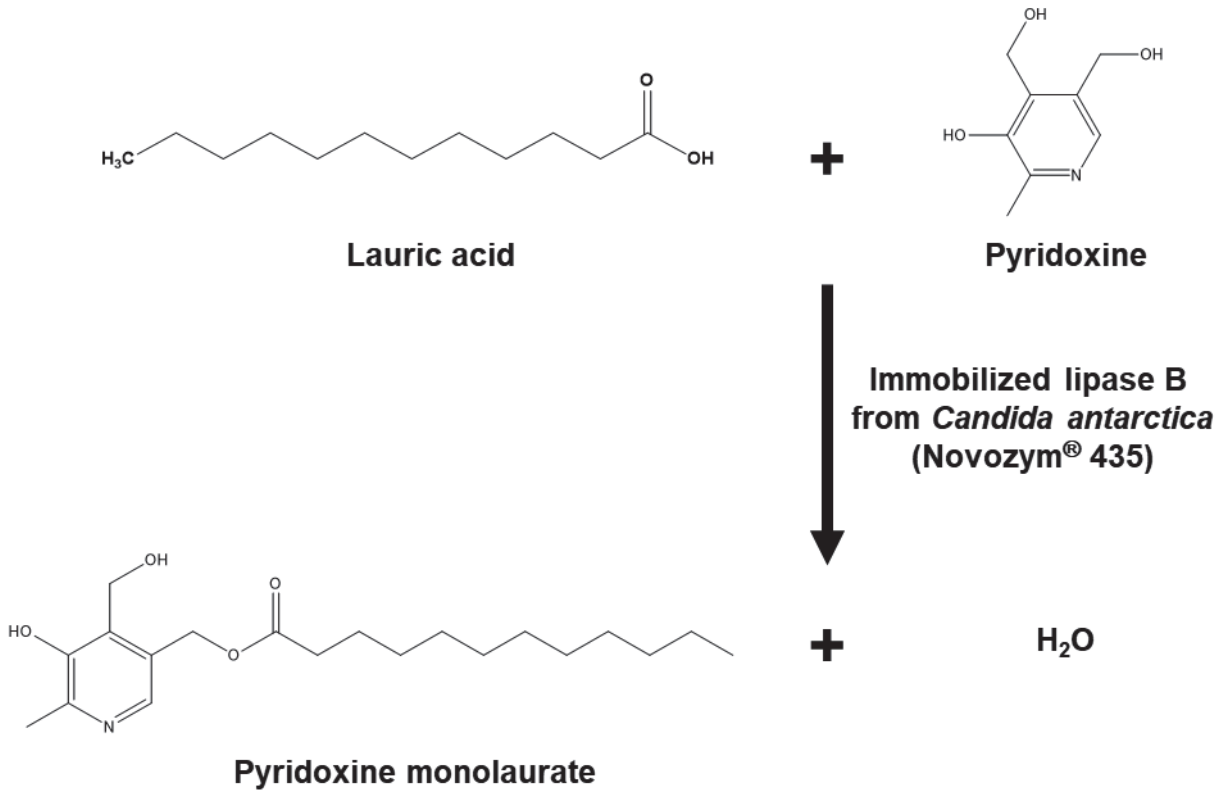


Fig. 1. Scheme of lipase-catalyzed synthesis of pyridoxine monolaurate.

2.3. Quantitative analysis using HPLC

Quantitative analysis of pyridoxine monolaurate were conducted by a HPLC instrument (LC-2002, Jasco, Tokyo, Japan) with an ultraviolet detector (UV-2075, Jasco), a refractive index detector (RI-2031, Jasco) and a silica-based column (5 μm, 4.6 x 150 mm: Luna C18, Phenomenex, Torrance, CA, USA). Acetonitrile/water/trifluoroacetic acid (80:20:0.1, v/v/v) was used as a mobile phase with a flow rate of 1.0 mL/min at 40℃ for 15 min. Pyridoxine and PML were detected by the UV detector at a wavelength of 292 nm while other compounds were detected by the RI detector. Components in the reactants were identified by their retention times and the concentrations were calculated from the standard curves of each compound ($R^2 > 0.992$).

In OS-MPS, reactants of 5 μL were withdrawn, filtered through 0.45-μm syringe filter (PTFE-D, hydrophobic) to separate Novozym® 435, and diluted in mobile phase of 245 μL. In SL-BPS and GSL-MPS, reactants of 50 μL were withdrawn, dissolved in *tert*-butanol of 950 μL, and filtered through 0.45-μm syringe filter (PTFE-D, hydrophobic). The filtered solutions of 5 μL were diluted in mobile phase of 370 μL. After dilution, aliquots of 20 μL were injected into HPLC system for quantitative analysis in all systems.

2.4. Purification and structural analysis

PML was purified from the reactants by solvent-separation. In GSL-MPS, the reactants were filtered through 0.45 μm glass filter to separate the Novozym® 435. The concentrate was washed with *n*-hexane and centrifuged at 13,000 ×g for 30 min; then the supernatant was discarded to remove residual lauric acid. The pellet was washed in distilled water and centrifuged at 13,000 ×g for 50 min; then the supernatant was discarded to remove residual pyridoxine. Both washing processes were repeated 6 times. Purified PML was lyophilized at -76℃ and stored at 4℃ until use.

To identify the purified product, LC electrospray ionization mass spectrometry (LC-ESI-MS) and ^{1}H-, ^{13}C-, and ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC) NMR were conducted. The product was separated using an LC system (Ultimate 3000 RS; Thermo Fisher Scientific, Massachusetts, CA, USA) equipped with a U-VDSpher PurC18-E column (1.8 μm, 50×2.0 mm; VDS optilab, Chromatographie Technik GmbH, Germany). Each peak was analyzed using MS (LTQ; Thermo Fisher Scientific) and compared with the theoretical molecular weight of pyridoxine monolaurate. Product was prepared in dimethyl sulfoxide-d_6 at 10 mg/mL for ^{1}H-, ^{13}C-, and ^{1}H-^{13}C HMBC- NMR analysis. These NMR spectra were recorded using a

Bruker Advance 600 (Bruker, Rheinstetten, Germany). Chemical shift values (δ in ppm) were expressed in ppm relative to tetramethylsiliane (TMS; δ=0).

2.5. Determination of operational stability of Novozym® 435

Operational stability of Novozym® 435 was determined by the following equation:

$$\text{Operational stability (\%)} = \frac{[\text{PML}]\ \text{produced from the } n^{th} \text{ batch (mmol/L)}}{[\text{PML}]\ \text{produced from the } 1^{st} \text{ batch (mmol/L)}} \times 100(\%)$$

Reactions were carried out for 1.5 h in OS-MPS and 12 h in SL-BPS and GSL-MPS under the same reaction conditions described in "2.2. Lipase–catalyzed synthesis of pyridoxine monolaurate." After the reaction, the reactants were withdrawn to separate Novozym® 435 by the same way presented in "2.3. Quantitative analysis using HPLC." After that, the separated Novozym® 435 was directly reused in the next batch of reaction.

2.6. Interfacial tension measurement

The interfacial tension between the aqueous phase (distilled water) and the oil phase (PML dissolve in MCT oil, 0.0~6.0 mM) was determined using droplet shape analyzer (DSA 100, Krüss GmbH, Hamburg, Germany) with pendant drop method (R. Li et al., 2019). A straight-needle with a diameter of

1.275 mm was used to create a pendant drop of water. The water drop was extruded into a cuvette containing oil phase. All samples were measured at 25℃ for 15 min, at 30 s intervals, which was long enough to ensure that the interfacial tension reached a steady value. Digital images were acquired by the instrument's camera and analyzed by the software (ADVANCE). Interfacial tension was calculated using the Young–Laplace equation.

2.7. Emulsion preparation

The oil-in-water emulsion was prepared by adding 1 g of oil phase (PML dissolved in MCT oil, 0.1~2.0%(w/w)) to 9 g of buffer solution (citric acid-sodium citrate, 10 mM, pH 3.0). Initially, coarse emulsions containing relatively large oil droplets was created by homogenizing the simple mixture using a homogenizer (ULTRA-TURRAX IKA T18 basic with S25N-10 G, IKA Works Inc., Wilmington, NC, USA) at 11,000 rpm for 2 min. Subsequently, the coarse emulsions were further homogenized to fine emulsions for 10 min (5 s on and 5 s off) at 30% power by ultrasonicator (Sonomasher, S&T Science, Seoul, Republic of Korea) in ice. The pH of emulsion was adjusted to pH 2.0~8.0 by 5 M NaOH or HCl solution.

2.8. Measurement of droplet size and zeta potential

Droplet size and zeta potential of the emulsions were analyzed at 25℃ using zetasizer Nano ZS90 (Malvern Instruments Ltd., Worcestershire, UK). To prevent multiple scattering, the emulsions were diluted at 1:500 (v/v) with buffer solution adjusted to the same pH to emulsion. Droplet size was expressed as the Z-average size (intensity-weighted mean droplet diameter), and width parameter was presented as the polydispersity index (PdI).

2.9. Statistical analysis

All data were analyzed by analysis of variance (ANOVA) using SPSS software version 26 (SPSS, Inc., Chicago, IL, USA), and presented as means and standard deviations of triplicate experiments. The differences between mean values were compared using Tukey's multiple range test ($p < 0.05$).

3. Results and Discussion

3.1. HPLC analysis of reactants

HPLC chromatograms of reactants from the synthesis in OS-MPS using acetonitrile as areaction medium at various reaction times were presented in Fig.2. The retention time of pyridoxine and lauric acid was 1.320 min and 4.907 min, respectively. As the reaction time increased, the peak area of pyridoxine decreased, and unknown peak was observed at the retention time of 2.017 min. Peak area of the unknown peak increased continuously, indicating that a new compound was produced. It was proposed that the new compound was PML due to its retention time. Because C18 column was used in reverse phase chromatography, the retention time of the unknown peak which was between that of pyridoxine and that of lauric acid indicated that the compound was more hydrophobic than pyridoxine and more hydrophilic than lauric acid. For identification of this compound, structural analysis was conducted.

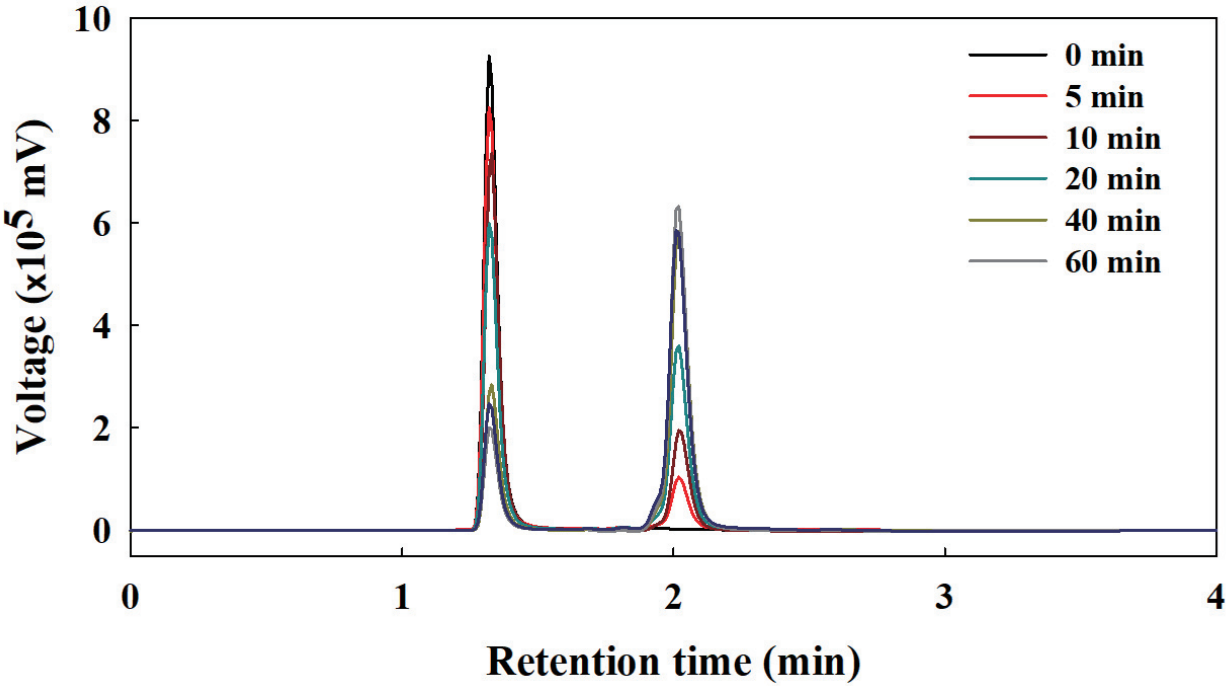


Fig. 2. HPLC chromatograms of reactants from lipase-catalyzed synthesis of pyridoxine monolaurate in acetonitrile at various reaction times.

3.2. Structural analysis of pyridoxine monolaurate

After purification process by solvent separation, high purity of PML (99.26 ± 0.06%, w/w) was determined by HPLC analysis (Data not shown). The purified PML was identified by LC-ESI-MS (Fig. 3). The ESI mass spectrum showed molecular ions at *m/z* 352.3 ($[M+H]^+$). This value corresponded to the molar mass of single-charged ion of the PML (351.5 g/mol) calculated from molecular structure. This result demonstrated that monoester of pyridoxine was synthesized. Because pyridoxine molecule contains three hydroxyl groups able to be esterified with lauric acid, it was necessary to identify which hydroxyl group was participated in esterification by NMR analysis.

Each peak in ^{1}H-NMR and ^{13}C-NMR were annotated as PML based on previous study (Baldessari et al., 1998). In ^{1}H-NMR spectra (Fig. 4b), signals at 5.11 and 4.69 ppm could not be distinguished. According to previous study, signals at 172,62, 149.53, 147.69, 140.04, 132.06, and 127.70 ppm in ^{13}C-NMR (Fig. 4c) were identified as C(14), C(3), C(6), C(2), C(4), and C(5). Therefore, to find the esterification site, exact annotation of signals at 5.11 and 4.69 ppm in ^{1}H-NMR was needed through analyzing ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC) NMR. In ^{1}H-^{13}C HMBC spectra (Fig. 4d),

the long-range connectivities of the signal at 5.11 ppm indicated a distinct column of cross peaks to carbon C(5) (127.79 ppm), C(4) (132.06 ppm), C(6) (140.04 ppm), and C(14) (172.62 ppm). The CH_2 signal at 4.69 ppm showed three cross peaks to carbon C(5) (127.79 ppm), C(4) (132.06 ppm), and C(3) (149.53 ppm). The cross peaks to C(3) indicated that signal at 4.69 ppm annotated to CH_2(8), which indicated that signal at 5.11 ppm annotated to CH_2(9). In the column of cross peaks connected to the CH_2(9) signal, there was long-range connectivity to carbonyl carbon C(14) (172.62 ppm) which indicated the esterification site was hydroxyl group bonded to C(9). As a result, structure of PML was determined as [5-hydroxy-4-(hydroxymethyl)-6-methylpyridin-3-yl]methyl dodecanoate.

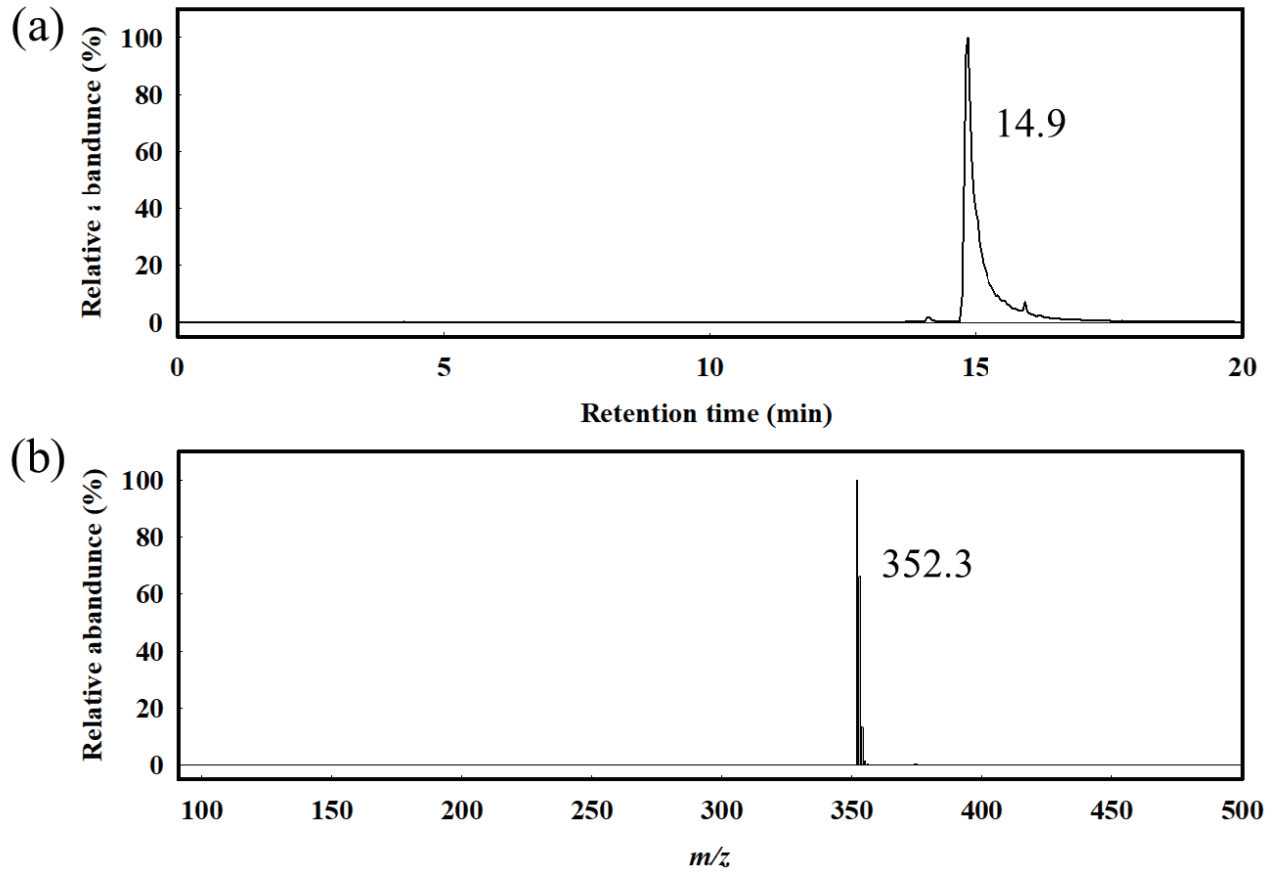
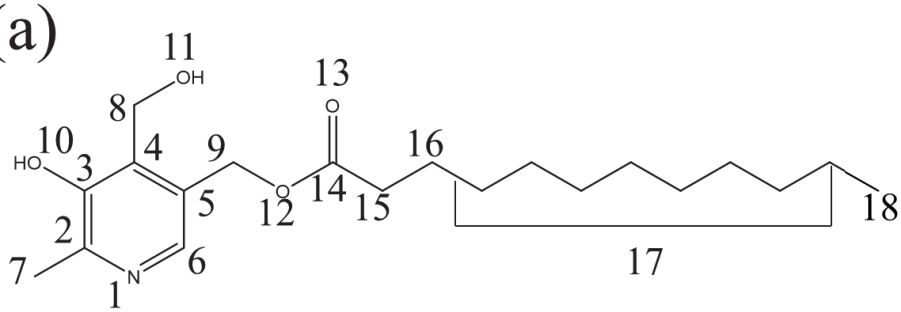
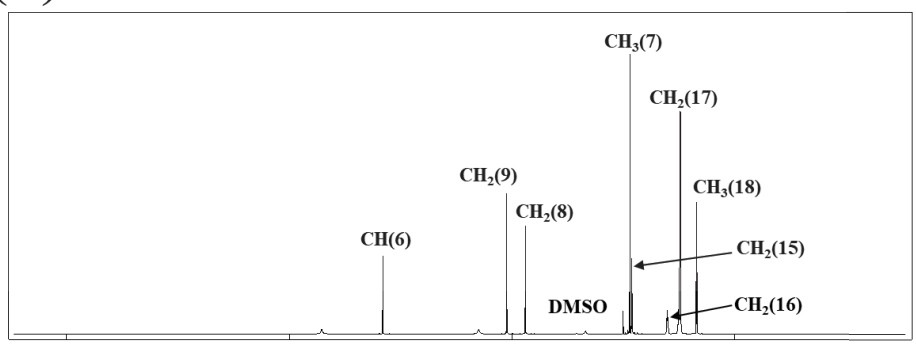


Fig. 3. LC-ESI-MS analysis of pyridoxine monolaurate; (a) HPLC chromatogram and (b) mass spectra of peak with retention time of 14.89~14.92 min.

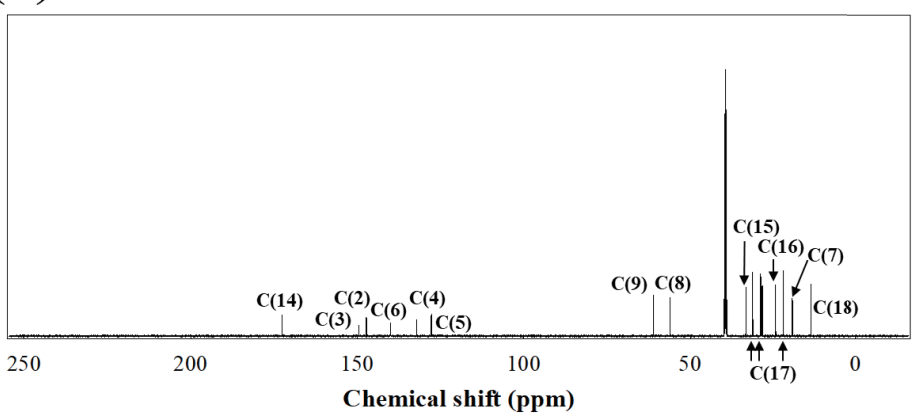
(a)


11
OH
8
13
O
10
HO
3
4
9
16
5
O
12
14
15
18
2
7
N
6
1
17

(b)


CH3(7)
CH2(17)
CH2(9)
CH3(18)
CH2(8)
CH(6)
CH2(15)
DMSO
CH2(16)
15
10
5
0
Chemical shift (ppm)

(c)


C(15)
C(16)
C(7)
C(9)
C(8)
C(14)
C(2)
C(3)
C(6)
C(4)
C(5)
C(18)
250
200
150
100
50
0
C(17)
Chemical shift (ppm)

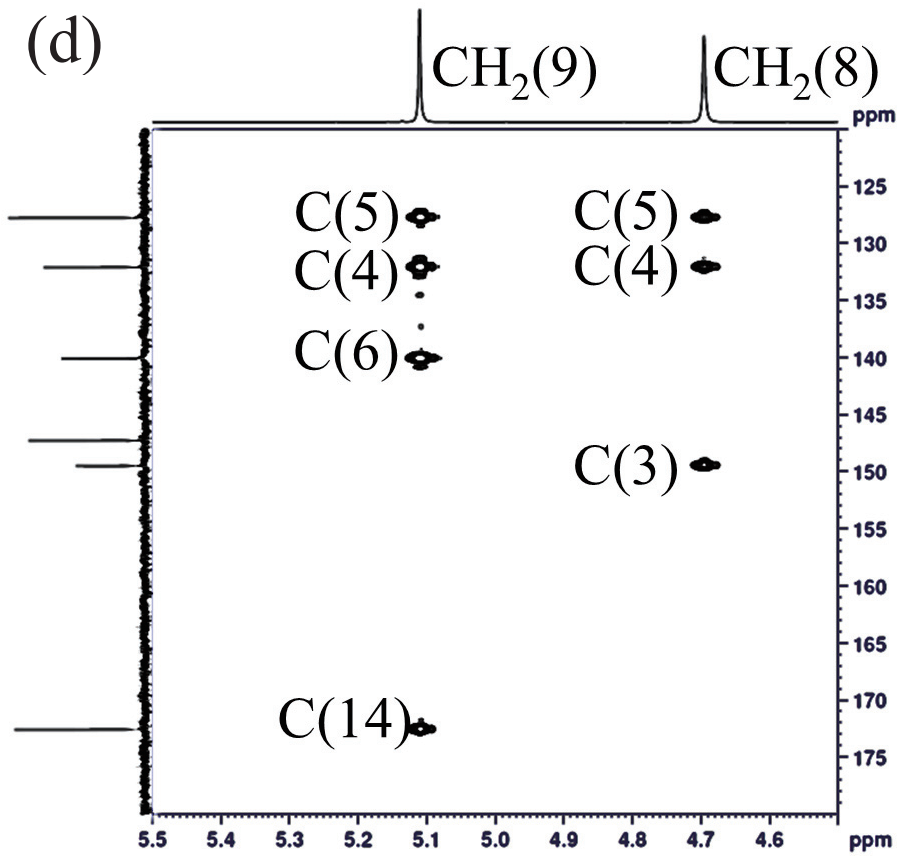


Fig. 4. Structural analysis of pyridoxine monolaurate; (a) Molecular structure of pyridoxine monolauate with atom numbering; (b)^{1}H-NMR and (c) ^{13}C-NMR spectra; (d) Selected regions of ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC) NMR spectra of pyridoxine monolaurate

3.3. Comparison of reaction efficiency in different enzyme bioreactor systems

PML was synthesized in three different kinds of enzyme bioreactor systems (OS-MPS, SL-BPS, and GSL-MPS) and their reaction efficiencies were compared among the systems. Acetonitrile was used as the organic solvent in OS-MPS because it has been reported that the lipase-catalyzed synthesis of pyridoxine esters achieved high yield in acetonitrile solvent (Zhang, Bai, & Sun, 2007). Time courses of lipase-catalyzed synthesis of PML were presented in Fig. 5. Lipase-catalyzed synthesis of PML reached equilibrium at 1.5, 12.0, and 9.0 h in OS-MPS, SL-BPS, and GSL-MPS, respectively. Reaction efficiency was determined by the conversion yield and volumetric productivity (Table 1). These values in the three systems were calculated at equilibrium time. The volumetric productivity of reaction in GSL-MPS (41.24 ± 2.89 mmol/L/h) was significantly higher ($p < 0.05$), followed by SL-BPS (19.86 ± 0.49 mmol/L/h) and OS-MPS (11.10 ± 0.33 mmol/L/h). There was no difference in conversion yield between GSL-MPS (84.56 ± 5.90%) and OS-MPS (79.56 ± 2.33%), but conversion yield in SL-BPS (54.29 ± 1.35%) was significantly lower than other systems ($p < 0.05$).

In OS-MPS, the high conversion yield and reaction rate were caused by

increased mass transfer of reactants in the presence of organic solvent (Mendoza-Ortiz et al., 2020). Despite high conversion yield and reaction rate, low initial pyridoxine concentration (20 mmol/L) due to limited solubility in acetonitrile resulted in the lowest volumetric productivity (Kim et al., 2006). In the solvent-free system, however, pyridoxine could be used without limitation of solubility in organic solvent. Higher volumetric productivity was achieved because higher initial pyridoxine concentration (439 mmol/L), about 22-fold higher than in OS-MPS, was used in SL-BPS and GSL-MPS.

Because pyridoxine is solid phase in lauric acid reaction medium, it is important to increase mixing and mass transfer between liquid and solid in solvent-free systems. When mixing and mass transfer increase, the rate of phase mixing and solid-liquid interface area increase, resulting in enhancing reaction efficiency (Gogate, 2008; Khan, Gawas, & Rathod, 2018). In GSL-MPS, the gaseous phase improves the dispersion of pyridoxine in the lauric acid reaction medium, resulting in significantly higher ($p < 0.05$) volumetric productivity and conversion yield than in SL-BPS through enlargement of solid-liquid interface area (H. Yu et al., 2019). Consequently, the GSL-MPS was suggested to be the most efficient enzyme bioreactor system for mass production of PML.

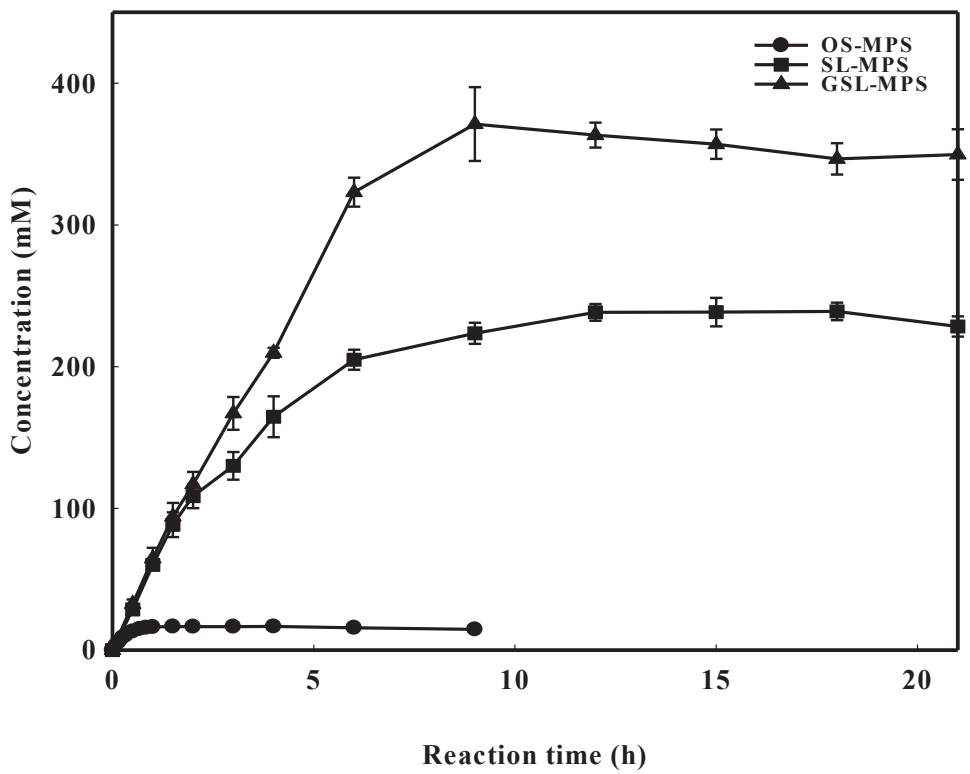


Fig. 5 Time courses of lipase-catalyzed synthesis of pyridoxine onolaurate in different enzyme bioreactor systems. All reactions were carried out with Novozym® 435 of 396 mg (26.4 mg/mL) and reaction volume of 15 mL at 70 ℃. Reaction conditions in organic solvent monophase (OS-MPS): pyridoxine, 0.3 mmol; lauric acid, 3 mmol; magnetic stirring, 300 rpm. Reaction conditions in solid-liquid biphase system (SL-BPS): pyridoxine, 6.59 mmol; lauric acid, 65.89 mmol; magnetic stirring, 300 rpm. Reaction conditions in gas-solid-liquid multiphase system (GSL-MPS): pyridoxine, 6.59 mmol; lauric acid, 65.89 mmol; gas flow, 2.0 L/min.

Table 1. Comparison of conversion yield and volumetric productivity in different enzyme bioreactor systems

Enzyme bioreactor system [1)]	Conversion yield (%)	Volumetric productivity (mmol/L/h)
Organic solvent-monophase system [2)]	79.56 ± 2.33 [a]	11.10 ± 0.33 [a]
Solid-liquid biphase system [3)]	54.29 ± 1.35 [b]	19.86 ± 0.49 [b]
Gas-solid-liquid multiphase system [4)]	84.56 ± 5.90 [a]	41.24 ± 2.89 [c]

Values in the same columns with different letters are significantly different ($p < 0.05$). Conversion yield and volumetric productivity were calculated at equilibrium time attained in each system.

[1)] All reactions were carried out with Novozym® 435 of 396 mg (26.4 mg/mL) and reaction volume of 15 mL at 70 °C.

[2)] Reaction conditions: pyridoxine, 0.3 mmol; lauric acid, 3 mmol; magnetic stirring, 300 rpm.

[3)] Reaction conditions: pyridoxine, 6.59 mmol; lauric acid, 65.89 mmol; magnetic stirring, 300 rpm.

[4)] Reaction conditions: pyridoxine, 6.59 mmol; lauric acid, 65.89 mmol; N_2 gas flow, 2.0 L/min.

3.4. Comparison of operational stability of Novozym® 435 in different enzyme bioreactor systems

The operational stability of Novozym® 435 in specific enzyme bioreactor system is one of the most important factors determining practical applicability of the system (Aguieiras et al., 2016; Martins, Graebin, Lorenzoni, Fernandez-Lafuente, Ayub, & Rodrigues, 2011; Martins et al., 2013; Xing et al., 2019). To find out the most appropriate system to reuse of Novozym® 435, operational stability of it was evaluated in each system and the decreasing tendency of operational stability decrease was observed in all systems (Fig. 6). Unlike other systems, however, there was no significant ($p < 0.05$) difference in operational stability between all batches in GSL-MPS. Operational stability at 6th batch was 82.66 ± 1.81%, 88.15 ± 0.98%, and 94.45 ± 7.62% in OS-MPS, SL-BPS, and GSL-MPS, respectively.

The decreasing tendency of operational stability of Novozym® 435 in all systems was attributed to ① the thermal deactivation of enzyme, ② the effect of organic solvent, and ③ the mechanical stress to Novozym® 435 exerted by magnetic stirring.

Decreases of operational stability in all systems were attributed to thermal deactivation of Novozym® 435 by the energy accumulated during reactions at

high temperature (70°C). It is well known that the enzymes are deactivated at high temperature (Nelson, Lehninger, & Cox, 2008). Enzyme structures are stabilized with disulfide bridge and various non-covalent interactions, such as hydrophobic interaction, hydrogen bonds, and electrostatic interactions. If more energy is accumulated than thermal inactivation energy corresponding minimum energy causing protein unfolding, enzymes are deactivated (Olusesan et al., 2011). Although immobilization enhances thermal stability of enzymes to overcome thermal deactivation, it has been reported that immobilized enzymes are also deactivated by thermal deactivations at higher temperature (Bansode, Hardikar, & Rathod, 2017; Gharat & Rathod, 2013; Pirozzi & Greco Jr, 2004; Zhou, Li, & Zheng, 2019).

The organic solvent also had an effect on activity of Novozym® 435 in OS-MPS. It has been reported that acetonitrile, a hydrophilic organic solvent, leads to dissolution of the supporting material from the immobilized enzyme (Novozym® 435) and liberation of free lipase from the immobilized medium (José et al., 2011). In addition, acetonitrile has an effect on protein denaturation by weakening the hydrophobic interaction and enhancing the peptide-peptide hydrogen bond. Thus, lipase could have been deactivated in acetonitrile (Gekko, Ohmae, Kameyama, & Takagi, 1998). Lauric acid medium did not affect the dissolution of supporting material and deactivation

of enzyme due to its hydrophobic property.

Mechanical stability is one of the important factors to determine the long term application of immobilized enzymes because mechanical stress can cause disintegration of the enzyme(Liese & Hilterhaus, 2013). The mechanical stress caused by the magnetic stirring usually destroys the immobilized lipase and thereby reduces operational stability in OS-MPS and SL-BPS (Hilterhaus, Thum, & Liese, 2008). Consequently, because reaction in GSL-MPS was not affected by organic solvent and mechanical stress, it was suggested that GSL-MPS was most appropriate enzyme bioreactor system for lipase-catalyzed synthesis of PML with respect of enzyme reuse.

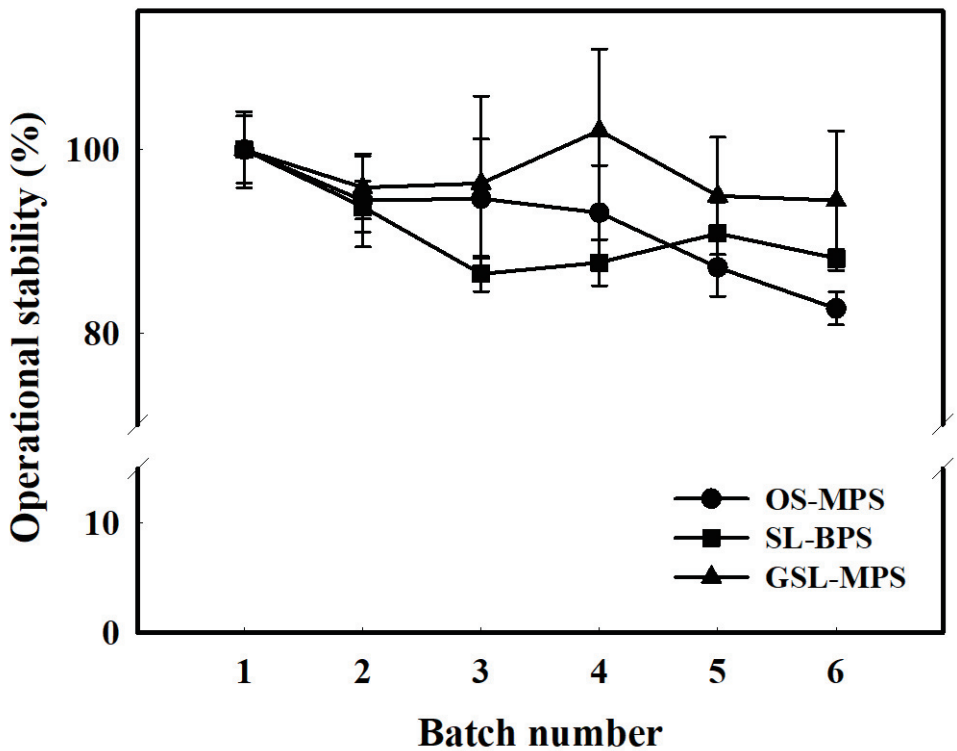


Fig. 6. Operational stability of Novozym® 435 over repeated batches of lipase-catalyzed synthesis of pyridoxine monolaurate in different enzyme bioreactor systems. All reactions were carried out with Novozym® 435 of 396 mg (26.4 mg/mL) and reaction volume of 15 mL at 70 °C. Reaction conditions in organic solvent monophase (OS-MPS): pyridoxine, 0.3 mmol; lauric acid, 3.0 mmol; magnetic stirring, 300 rpm; reaction time, 1.5 h. Reaction conditions in solid-liquid biphase system (SL-BPS): pyridoxine, 6.59 mmol; lauric acid, 65.89 mmol; magnetic stirring, 300 rpm; reaction time, 12 h. Reaction conditions in gas-solid-liquid multiphase system (GSL-MPS): pyridoxine, 6.59 mmol; lauric acid, 65.89 mmol; N_2 gas flow, 2.0 L/min; reaction time, 12 h.

3.5. Effects of reaction variables on reaction efficiency in gas-solid-liquid multiphase system

3.5.1. Substrate molar ratio

The effect of substrate molar ratio ([pyridoxine] to [lauric acid]) on the reaction efficiency in GSL-MPS was investigated in the range of 0.05~0.30 (Fig. 7a). Volumetric productivities at substrate molar ratio of 0.10 (41.24 ± 2.89 mmol/L/h), 0.20 (39.00 ± 1.80 mmol/L/h) and 0.30 (36.53 ± 2.39 mmol/L/h) were determined as the same value ($p < 0.05$). Volumetric productivities at substrate molar ratio of 0.15 (34.07 ± 1.37 mmol/L/h) and 0.05 (20.87 ± 0.95 mmol/L/h) were significantly ($p < 0.05$) lower than that at 0.10 (Fig. 7b). For substrate molar ratio from 0.10 to 0.30, the conversion yields decreased significantly ($p < 0.05$) with increasing the substrate molar ratio (Fig. 7c). At the substrate molar ratio of 0.05 (85.51 ± 3.91%) and 0.10 (84.56 ± 5.90%), the conversion yields were not significantly ($p < 0.05$) different, but it decreased in the order of 0.15 (46.54 ± 1.87%), 0.20 (39.95 ± 1.85%), and 0.30 (24.95 ± 1.63%). Therefore, substrate molar ratio 0.10 was the optimum condition with the highest volumetric productivity and conversion yield.

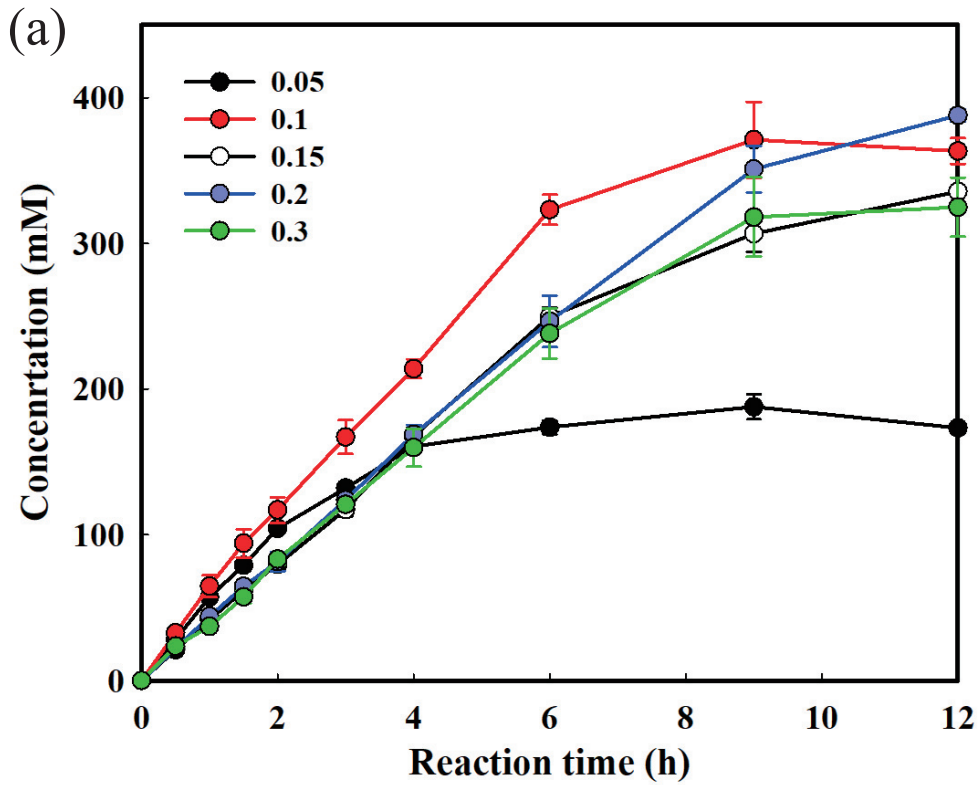
The difference of reaction efficiency was attributed to ① the effect of

ratio of [acyl donor] to [acyl acceptor] on conversion yield and ② the effect of pyridoxine concentration on viscosity of reaction medium. The conversion yield generally increases with ratio of [acyl donor] to [acyl acceptor] in lipase-catalyzed esterification (Ren et al., 2017; Song & Wei, 2002; Zhang, Bai, Dong, & Sun, 2007). Moreover, in the multiphase system with insoluble solid, the increase in apparent viscosity was observed with increasing solid concentration (Kantarci, Borak, & Ulgen, 2005). It was also reported that viscosity of reactants increased with concentration of solid substrate in lauric acid medium (H. Yu et al., 2019). Likewise, increase in viscosity of reaction medium was observed as high pyridoxine concentrations was used. High viscosity also could limit both the mass transfer of substrates to the active site of the lipase and liberation of products from the enzyme-substrate complex, resulting in low reaction efficiency (Kim et al., 2006; Sun, Yu, Curran, & Liu, 2012). Consequently, it is estimated that the ratio of [acyl donor] to [acyl acceptor] and viscosity of reaction medium together affected reaction efficiency.

3.5.2. Reaction temperature

The effect of reaction temperature on the reaction efficiency in GSL-MPS was investigated in the range of 50~80℃ (Fig. 7). It was observed that the initial velocity of reactions increased with increasing reaction temperature. At reaction time of 6 h, corresponding to equilibrium time of the reaction at 80℃, volumetric productivity of reaction at 70℃ (53.85 ± 1.70 mmol/L/h) and 80℃ (57.40 ± 3.43 mmol/L/h) was not significantly different ($p < 0.05$). At reaction time 9 h, corresponding to equilibrium time of the reaction at 70℃, the volumetric productivity of reaction at 70℃ (41.24 ± 2.89 mmol/L/h) and 80℃ (38.61 ± 1.07 mmol/L/h) were not significantly different ($p < 0.05$), too. As a result, 70℃ is the optimum reaction temperature, because the lower reaction temperature, the less energy consumption and the less enzyme deactivation occurs. Because an unknown peak with low intensity was detected by UV detector at retention time of 13.0~14.5 min, it was estimated that pyridoxine dilaurate was synthesized from PML at 80℃ after 9 h. (Data not shown). Since the enzymatic reaction temperature affects the activity and stability of the enzyme, temperature is an important factor to determine the reaction efficiency. In general, the enzyme activity increases as the temperature increases, but the enzyme stability decreases at a certain temperature or higher. The reason for the increase in conversion yield as the temperature increases is that the

viscosity of the reaction medium decreases at high temperature and the diffusion rate of the substrate increases. Accordingly, the number of productive collisions between the enzyme and the substrate increases at high temperatures. In addition, the turnover number of enzymes increases as the temperature increases. However, at high temperature, the active conformation of enzyme may be disrupted, which leads to loss of activity. As a result, as the temperature increase, both an increase in enzyme activity and in enzyme deformation rate had effect on reaction efficiency (Kuperkar, Lade, Prakash, & Rathod, 2014; Paiva, Balcao, & Malcata, 2000).


(a)
0.05
0.1
0.15
0.2
0.3
Concenrtation (mM)
0
100
200
300
400
0
2
4
6
8
10
12
Reaction time (h)

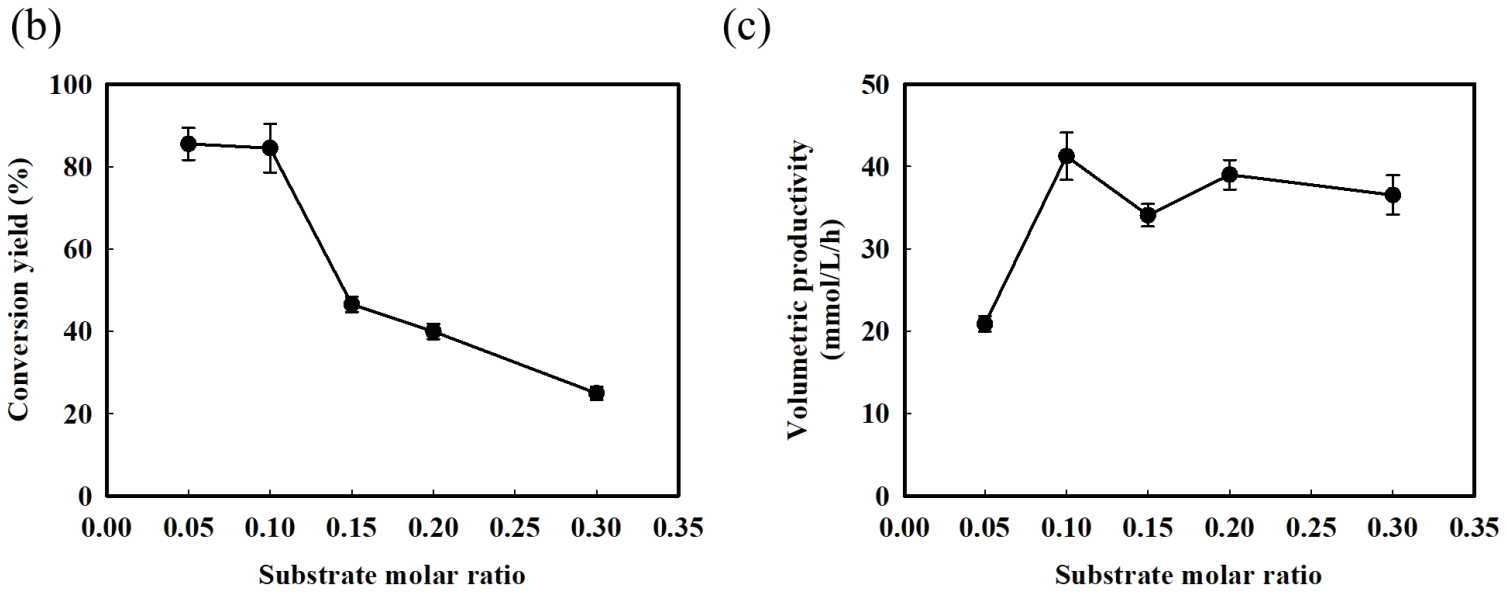


Fig. 7. Effect of substrate molar ratio ([pyridoxine] to [lauric acid]) on lipase-catalyzed synthesis in gas-solid-liquid multiphase system. (a) Time courses of reaction, (b) conversion yield, and (c) volumetric productivity at different substrate molar ratio. Reaction conditions: substrate molar ratio, 0.05~0.30; temperature, 70°C; Novozym® 435, 396 mg (26.4 mg/mL); N_2 gas flow, 2.0 L/min; reaction time, 12h in 20 mL reactor. (The amount of lauric acid was fixed to 65.89 mmol.)

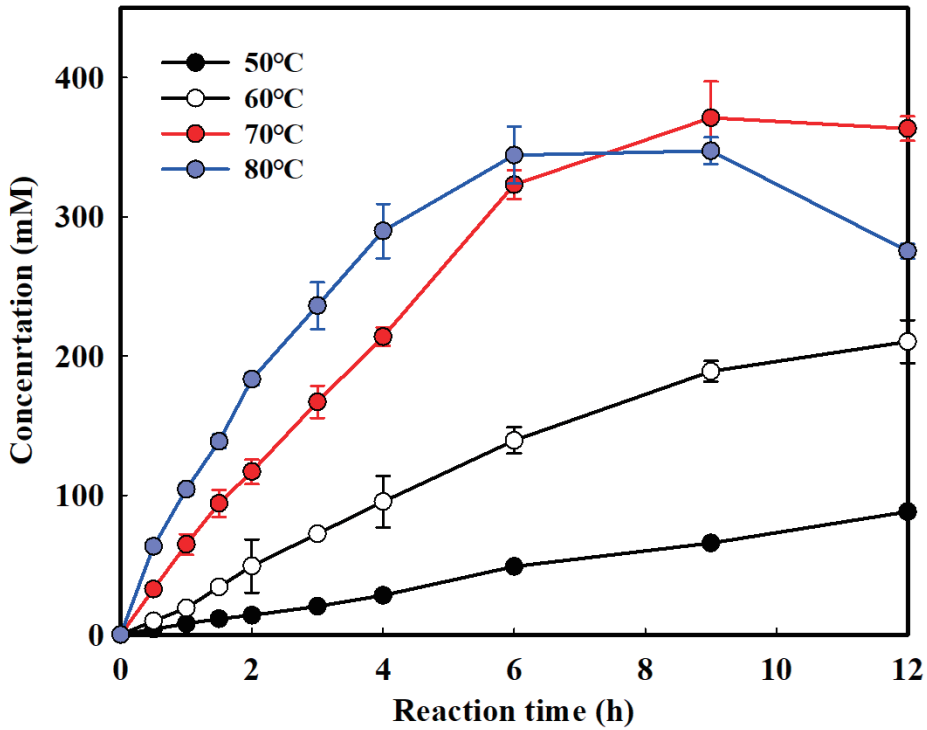


Fig. 8. Effect of reaction temperature on lipase-catalyzed synthesis in gas-solid-liquid multiphase system. Reactions conditions: pyridoxine, 6.59 mmol; lauric acid, 65.89 mmol; reaction temperature, 50~80°C; Novozym® 435, 396 mg (26.4 mg/mL); gas flow, 2.0 L/min; reaction time, 12 h in 20 mL reactor.

3.6. Interfacial characteristics of pyridoxine monolaurate

The interfacial characteristics of emulsifiers play an important role in determining their ability to form and stabilize emulsions (Rodríguez Patino, Rodríguez Niño, & Sánchez, 1999). The interfacial characteristics of the PML were determined by measuring their equilibrium interfacial tension versus emulsifier concentration profiles. When emulsifier was absent in the oil phase, the interfacial tension was measured to be 24.1 mN/m, which is close to the value reported previously (Chung, Sher, Rousset, Decker, & McClements, 2017; R. Li et al., 2019). The interfacial tension decreased with increasing PML concentration (Fig. 9), which indicated that the PML was interfacial active compound and had ability to adsorb to the oil-water interface and lower interfacial tension (Bai, Huan, Li, & McClements, 2017). It was estimated that the higher PML concentration, the more PML was adsorbed to the oil-water interface, resulting in more decrease of the interfacial tension. Because a lower interfacial tension means that less energy is needed to break up the oil droplets, the energy needed to prepare emulsion is lowered too (Santana, Perrechil, & Cunha, 2013). Therefore, it was expected that PML could be used as an emulsifier, as commercial emulsifiers exhibit the same interfacial tension reduction pattern with increasing concentration (Zhu, Wen, Yi, Cao, Liu, & McClements, 2019).

While the ability of compounds to reduce interfacial tension is useful for measuring their ability as interfacial active compounds, it is not necessarily directly related to their ability to prepare and stabilize emulsions. Measurement of interfacial tension is conducted under the static condition, which is different to highly dynamic condition that takes place under complex fluid conditions during preparation of emulsion. Therefore, it was necessary to evaluate the emulsifying properties of PML through emulsion preparation (Bai et al., 2017; Walstra, 1993).

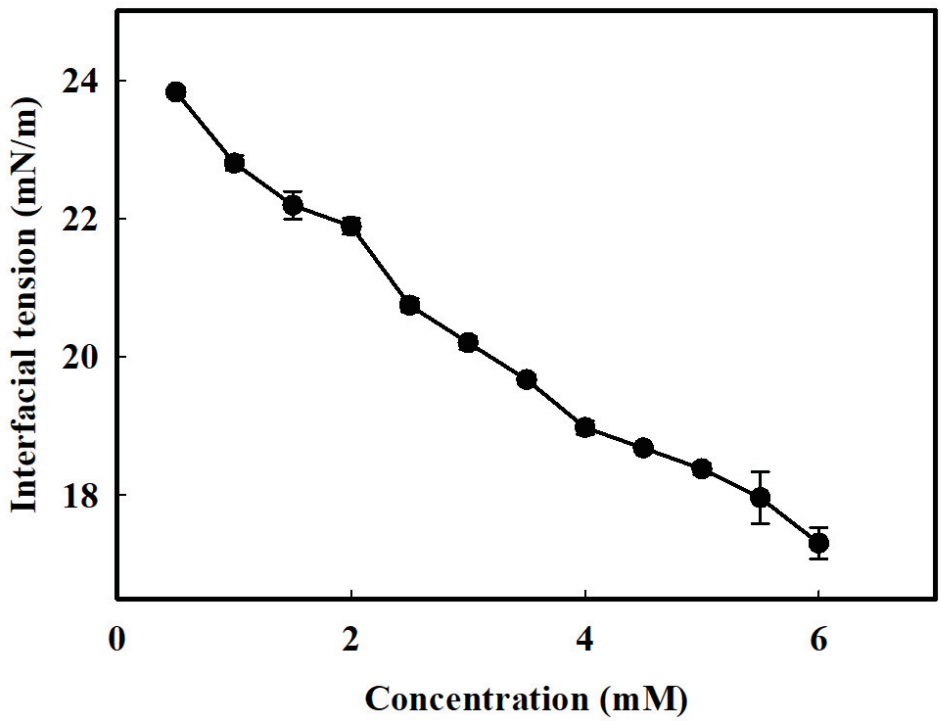


Fig. 9. Influence of pyridoxine monolaurate concentration on the interfacial tension measured at the MCT oil-water interface.

3.7. Emulsifying properties of pyridoxine monolaurate

The effects of pH and PML concentration on emulsifying properties of PML were evaluated. First, the effect of pH on Z-average size and zeta potential was examined. In the case of small-molecule emulsifiers with ionizable sites, the emulsifying properties could be changed by pH (Ozturk, Argin, Ozilgen, & McClements, 2014; Yang, Leser, Sher, & McClements, 2013). For this reason, theoretical pKa values of PML were obtained using ChemAxon software (de Souza, Dottein, Giacobbo, Siqueira Rodrigues, de Pinho, & Bernardes, 2018). It was estimated that PML had three ionizable site, N(1), OH(10), and OH(11) and pKa values of each sites were 5.58, 9.39, and 14.84, respectively,

Z-average size of emulsions stabilized by PML was measured at various pH within 1 h after emulsion preparation (Fig. 10a). At pH 2.0, 3.0, and 4.0, Z-average size of emulsions was not significantly ($p < 0.05$) different. Z-average size of the emulsion increased with pH in the range of pH 4.0 to 6.0 and decreased with pH in the range of pH 6.0 to 8.0. As the pH approached pKa (5.58), the emulsion which had been stabilized with PML was aggregated rapidly.

Zeta potential of emulsions stabilized by PML at various pHs was also

measured to evaluate changes in droplet charge by pH (Fig. 10b). Zeta potential of emulsions decreased from 57.0 ± 4.1 at pH 2.0 to -75.3 ± 1.1 mV at pH 8.0 with increasing pH. The observed zeta potential value at pH 5.0 (-3.0 ± 0.7), near the pKa value, was due to the loss of charge of PML, which was relaated to the rapid aggregation and increase in size of emulsions. At pH 6.0, zeta potential value was -20.3 ± 0.6, which might be attributed to preferential adsorption of OH^- ions from the water phase by the oil droplets or presence of negatively charged free fatty acids in the oil phase (Guan, Chen, & Zhong, 2019; Hur, Decker, & McClements, 2009). It was reported several times that negative zeta potential values were measured in emulsion stabilized by nonionic emulsifiers (Hsu & Nacu, 2003; Mun, Decker, & McClements, 2006). As the pH approached the other pKa values (9.39), it is estimated that PML became negatively charged, leading to decrease of Z-average size of emulsions.

As a result, the degree of ionization of PML located in oil-water interface was very important factor for its emulsifying properties and the main mechanism by which PML stabilized the emulsion was electrostatic repulsion between charged PML in oil-water interface. Accordingly, the PML was suggested to be used as an emulsifier in acid foods at pH ranging reom 2.0~4.0.

Influence of emulsifier concentration on the Z-average sizes of the emulsion stabilized by PML was determined with emulsions prepared at pH 3.0 (Fig. 11). At concentrations of 0.01~0.10%(w/w) of PML, Z-average size decreased with increasing concentration of PML. There was no significant ($p < 0.05$) difference among 0.10, 0.15, 0.20%(w/w) of PML. It has been reported in many studies that droplet sizes decreased with increasing emulsifier concentrations. The decreases in droplet sizes are mainly due to the fact that sufficient emulsifiers can cover the droplet surfaces and emulsifiers could adsorb faster onto droplet surfaces and then better inhibit droplet recoalescence during homogenization (Bai & McClements, 2016). At higher emulsifier concentrations, the oil-water interface of emulsion was saturated by emulsifier, resulting in droplet size unchanged (Tcholakova, Denkov, & Danner, 2004).

In the comparison of emulsions stabilized by same concentration (0.20%(w/w)) of other emulsifiers, Z-average sizes of emulsion stabilized by lecithin, Tween 20, and saponin were 344.1 ± 3.6 nm, 452.0 ± 12.9 nm, and 664.97 ± 18.4 nm, respectively. These results showed that PML was more effective than others in the production of emulsions with small size (Z. Li, Dai, Wang, Mao, & Gao, 2018). The differences in the effectiveness of the emulsifiers may be attributed to many factors, such as the speeds at which

emulsifiers were adsorbed onto the drop interfaces, their ability to reduce interfacial tension and to influence interfacial rheology, and each emulsifier's effectiveness at generating repulsive forces between droplets (Jafari, Assadpoor, Bhandari, & He, 2008; Schubert & Engel, 2004). In addition to these factors, it was inferred that the number of molecules of PML to cover the drop interfaces was higher due to the lower molecular weight of PML, compared to the ather emulsifiers, resulting in effective stabilization.

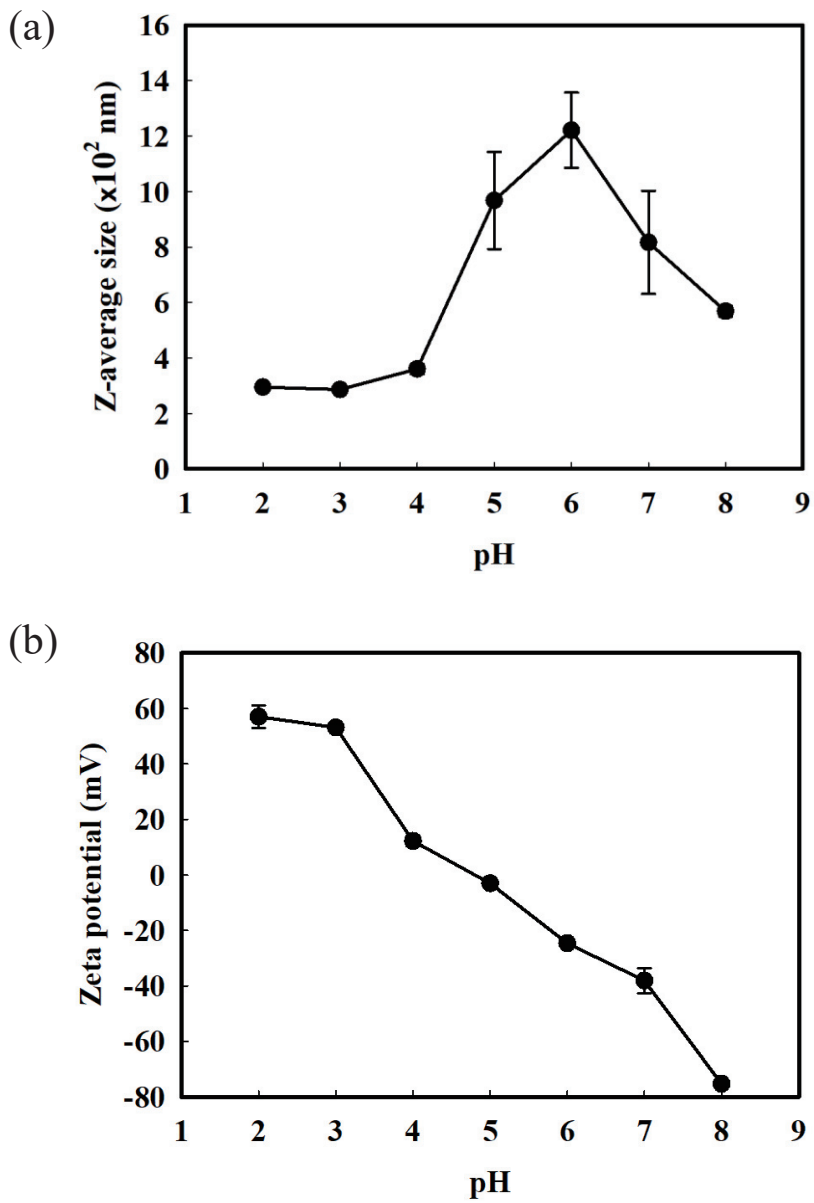


Fig. 10. Influence of pH on (a) Z-average size and (b) zeta potential of 10%(w/w) MCT oil-in-water emulsions stabilized by 0.2%(w/w) pyridoxine monolaurate. All measurements were conducted within 1 i after emulsion preparation.

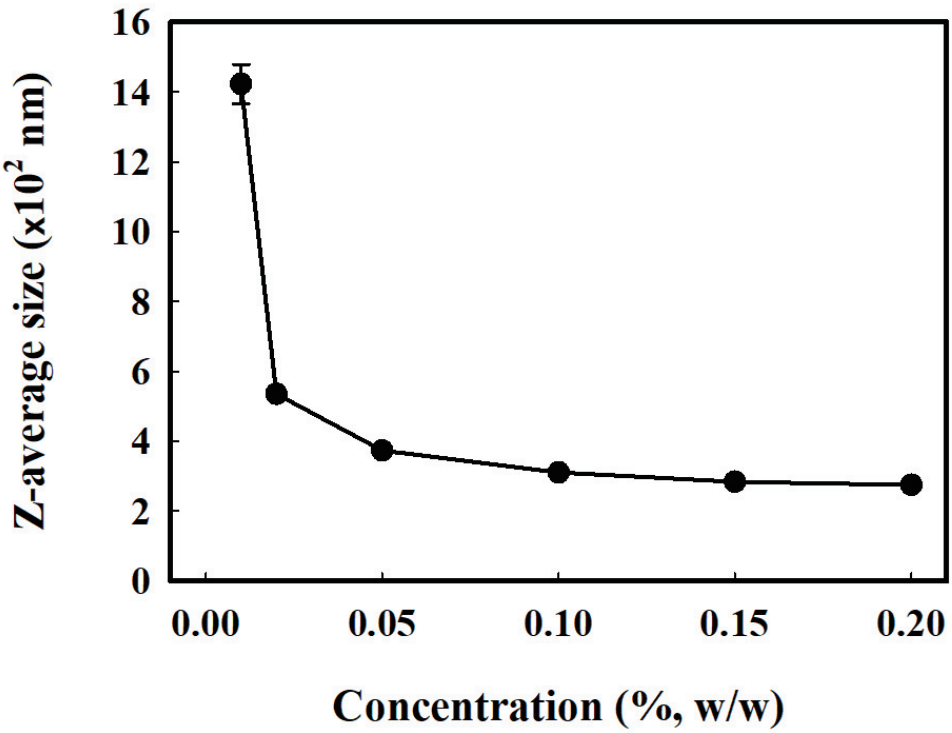


Fig. 11. Influence of pyridoxine monolaurate concentration on Z-average size of 10%(w/w) MCT oil-in-water emulsions at pH 3.0 stabilized by PML. All measurements were conducted within 1 hour after emulsion preparation.

4. Conclusion

In this study, pyridoxine monolaurate was produced by the lipase-catalyzed synthesis in GSL-MPS. Structural analysis of pyridoxine monolaurate was performed by LC-ESI-MS and ^{1}H-, ^{13}C-, and ^{1}H-^{13}C HMBC NMR. Among the enzyme bioreactor systems, the highest conversion yield and volumetric productivity were obtained in GSL-MPS. Moreover, the highest operational stability of Novozym® 435 was found in GSL-MPS. The results indicated that GSL-MPS was the best system for lipase-catalyzed synthesis of pyridoxine monolaurate with respect to reaction efficiency and lipase reusability. The reaction efficiency (conversion yield and volumetric productivity) was the highest when the substrate molar ratio ([pyridoxine] to [lauric acid]) was at 0.10 and the reaction temperature was at 70℃. PML reduced interfacial tension at oil-water interface with concentration-dependent manner. Increase of PML concentration reduced the droplet size in O/W emulsion. The emulsion made of PML showed high emulsion stability at acidic conditions (pH 3.0). These results suggested that PML could be used as a natural emulsifier for acidic emulsion foods.

5. References

Aguieiras, E. C., Ribeiro, D. S., Couteiro, P. P., Bastos, C. M., de Queiroz, D. S., Parreira, J. M., & Langone, M. A. (2016). Investigation of the reuse of immobilized lipases in biodiesel synthesis: influence of different solvents in lipase activity. *Applied Biochemistry and Biotechnology, 179*(3), 485-496.

Bahamondes, C., Wilson, L., Guzmán, F., & Illanes, A. (2017). Mathematical determination of kinetic parameters for assessing the effect of the organic solvent on the selectivity of peptide synthesis with immobilized α-chymotrypsin. *Journal of Bioscience and Bioengineering, 124*(6), 618-622.

Bai, L., Huan, S., Li, Z., & McClements, D. J. (2017). Comparison of emulsifying properties of food-grade polysaccharides in oil-in-water emulsions: Gum arabic, beet pectin, and corn fiber gum. *Food Hydrocolloids, 66*, 144-153.

Bai, L., & McClements, D. J. (2016). Formation and stabilization of nanoemulsions using biosurfactants: Rhamnolipids. *Journal of Colloid and Interface Science, 479*, 71-79.

Baldessari, A., & Mangone, C. P. (2002). Enzyme-catalyzed preparation of

novel fatty acid derivatives of pyridoxine with surfactant activity. *Biocatalysis and Biotransformation, 20*(4), 275-279.

Baldessari, A., Mangone, C. P., & Gros, E. G. (1998). Lipase-catalyzed acylation and deacylation reactions of pyridoxine, a member of vitamin-B_6 group. *Helvetica Chimica Acta, 81*(12), 2407-2413.

Bansode, S. R., Hardikar, M. A., & Rathod, V. K. (2017). Evaluation of reaction parameters and kinetic modelling for Novozym 435 catalyzed synthesis of isoamyl butyrate. *Journal of Chemical Technology and Biotechnology, 92*(6), 1306-1314.

Chung, C., Sher, A., Rousset, P., Decker, E. A., & McClements, D. J. (2017). Formulation of food emulsions using natural emulsifiers: Utilization of quillaja saponin and soy lecithin to fabricate liquid coffee whiteners. *Journal of Food Engineering, 209*, 1-11.

Cui, H., Stadtmüller, T. H., Jiang, Q., Jaeger, K. E., Schwaneberg, U., & Davari, M. D. (2020). How to engineer organic solvent resistant enzymes: Insights from combined molecular dynamics and directed evolution study. *ChemCatChem, 12*(16), 4073-4083.

de Souza, D. I., Dottein, E. M., Giacobbo, A., Siqueira Rodrigues, M. A., de Pinho, M. N., & Bernardes, A. M. (2018). Nanofiltration for the removal of norfloxacin from pharmaceutical effluent. *Journal of*

Environmental Chemical Engineering, 6(5), 6147-6153.

Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids, 17*(1), 25-39.

Foresti, M., & Ferreira, M. (2005). Frequent analytical/experimental problems in lipase-mediated synthesis in solvent-free systems and how to avoid them. *Analytical and Bioanalytical Chemistry, 381*(7), 1408-1425.

Gekko, K., Ohmae, E., Kameyama, K., & Takagi, T. (1998). Acetonitrile-protein interactions: amino acid solubility and preferential solvation. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology, 1387*(1-2), 195-205.

Gharat, N., & Rathod, V. K. (2013). Enzyme catalyzed transesterification of waste cooking oil with dimethyl carbonate. *Journal of Molecular Catalysis B: Enzymatic, 88*, 36-40.

Gogate, P. R. (2008). Cavitational reactors for process intensification of chemical processing applications: a critical review. *Chemical Engineering and Processing: Process Intensification, 47*(4), 515-527.

Guan, Y., Chen, H., & Zhong, Q. (2019). Nanoencapsulation of caffeic acid phenethyl ester in sucrose fatty acid esters to improve activities against cancer cells. *Journal of Food Engineering, 246*, 125-133.

Hilterhaus, L., Thum, O., & Liese, A. (2008). Reactor concept for lipase-

catalyzed solvent-free conversion of highly viscous reactants forming two-phase systems. *Organic Process Research & Development, 12*(4), 618-625.

Hsu, J.-P., & Nacu, A. (2003). Behavior of soybean oil-in-water emulsion stabilized by nonionic surfactant. *Journal of Colloid and Interface Science, 259*(2), 374-381.

Hur, S. J., Decker, E. A., & McClements, D. J. (2009). Influence of initial emulsifier type on microstructural changes occurring in emulsified lipids during *in vitro* digestion. *Food Chemistry, 114*(1), 253-262.

Inprakhon, P., Wongthongdee, N., Amornsakchai, T., Pongtharankul, T., Sunintaboon, P., Wiemann, L. O., Durand, A., & Sieber, V. (2017). Lipase-catalyzed synthesis of sucrose monoester: Increased productivity by combining enzyme pretreatment and non-aqueous biphasic medium. *Journal of Biotechnology, 259*, 182-190.

Jafari, S. M., Assadpoor, E., Bhandari, B., & He, Y. (2008). Nano-particle encapsulation of fish oil by spray drying. *Food Research International, 41*(2), 172-183.

Jo, Y.-J., & Kwon, Y.-J. (2014). Characterization of β-carotene nanoemulsions prepared by microfluidization technique. *Food Science and Biotechnology, 23*(1), 107-113.

José, C., Bonetto, R. D., Gambaro, L. A., Torres, M. d. P. G., Foresti, M. L., Ferreira, M. L., & Briand, L. E. (2011). Investigation of the causes of deactivation–degradation of the commercial biocatalyst Novozym® 435 in ethanol and ethanol–aqueous media. *Journal of Molecular Catalysis. B, Enzymatic, 71*(3), 95-107.

Kantarci, N., Borak, F., & Ulgen, K. O. (2005). Bubble column reactors. *Process Biochemistry, 40*(7), 2263-2283.

Khan, N. R., Gawas, S. D., & Rathod, V. K. (2018). Enzyme-catalysed production of *n*-butyl palmitate using ultrasound-assisted esterification of palmitic acid in a solvent-free system. *Bioprocess and Biosystems Engineering, 41*(11), 1621-1634.

Kim, H. J., Youn, S. H., & Shin, C. S. (2006). Lipase-catalyzed synthesis of sorbitol–fatty acid esters at extremely high substrate concentrations. *Journal of Biotechnology, 123*(2), 174-184.

Kuperkar, V. V., Lade, V. G., Prakash, A., & Rathod, V. K. (2014). Synthesis of isobutyl propionate using immobilized lipase in a solvent free system: optimization and kinetic studies. *Journal of Molecular Catalysis B: Enzymatic, 99*, 143-149.

Li, R., Peng, S., Zhang, R., Dai, T., Fu, G., Wan, Y., Liu, C., & McClements, D. J. (2019). Formation and characterization of oil-in-water emulsions

stabilized by polyphenol-polysaccharide complexes: Tannic acid and β-glucan. *Food Research International, 123*, 266-275.

Li, Z., Dai, L., Wang, D., Mao, L., & Gao, Y. (2018). Stabilization and rheology of concentrated emulsions using the natural emulsifiers quillaja saponins and rhamnolipids. *Journal of Agricultural and Food Chemistry, 66*(15), 3922-3929.

Liang, M.-Y., Chen, Y., Banwell, M. G., Wang, Y., & Lan, P. (2018). Enzymatic preparation of a homologous series of long-chain 6-O-acylglucose esters and their evaluation as emulsifiers. *Journal of Agricultural and Food Chemistry, 66*(15), 3949-3956.

Liese, A., & Hilterhaus, L. (2013). Evaluation of immobilized enzymes for industrial applications. *Chemical Society Reviews, 42*(15), 6236-6249.

Martins, A. B., Graebin, N. G., Lorenzoni, A. S., Fernandez-Lafuente, R., Ayub, M. A., & Rodrigues, R. C. (2011). Rapid and high yields of synthesis of butyl acetate catalyzed by Novozym 435: reaction optimization by response surface methodology. *Process Biochemistry, 46*(12), 2311-2316.

Martins, A. B., Schein, M. F., Friedrich, J. L., Fernandez-Lafuente, R., Ayub, M. A., & Rodrigues, R. C. (2013). Ultrasound-assisted butyl acetate synthesis catalyzed by Novozym 435: enhanced activity and

operational stability. *Ultrasonics Sonochemistry, 20*(5), 1155-1160.

McClements, D. J., & Gumus, C. E. (2016). Natural emulsifiers—Biosurfactants, phospholipids, biopolymers, and colloidal particles: Molecular and physicochemical basis of functional performance. *Advances in Colloid and interface Science, 234*, 3-26.

Mendoza-Ortiz, P. A., Gama, R. S., Gómez, O. C., Luiz, J. H., Fernandez-Lafuente, R., Cren, E. C., & Mendes, A. A. (2020). Sustainable enzymatic synthesis of a solketal ester—process optimization and evaluation of its antimicrobial activity. *Catalysts, 10*(2), 218.

Molet-Rodríguez, A., Salvia-Trujillo, L., & Martín-Belloso, O. (2018). Beverage emulsions: key aspects of their formulation and physicochemical stability. *Beverages, 4*(3), 70.

Mun, S., Decker, E. A., & McClements, D. J. (2006). Effect of molecular weight and degree of deacetylation of chitosan on the formation of oil-in-water emulsions stabilized by surfactant–chitosan membranes. *Journal of Colloid and Interface Science, 296*(2), 581-590.

Nelson, D. L., Lehninger, A. L., & Cox, M. M. (2008). *Lehninger principles of biochemistry*: Macmillan.

Olusesan, A. T., Azura, L. K., Forghani, B., Bakar, F. A., Mohamed, A. K. S., Radu, S., Manap, M. Y. A., & Saari, N. (2011). Purification,

characterization and thermal inactivation kinetics of a non-regioselective thermostable lipase from a genotypically identified extremophilic *Bacillus subtilis* NS 8. *New Biotechnology, 28*(6), 738-745.

Ozturk, B., Argin, S., Ozilgen, M., & McClements, D. J. (2014). Formation and stabilization of nanoemulsion-based vitamin E delivery systems using natural surfactants: Quillaja saponin and lecithin. *Journal of Food Engineering, 142*, 57-63.

Paiva, A. L., Balcao, V. M., & Malcata, F. X. (2000). Kinetics and mechanisms of reactions catalyzed by immobilized lipases. *Enzyme and Microbial Technology, 27*(3-5), 187-204.

Park, K.-M., Lee, M. J., Jo, S.-K., Choi, S. J., Lee, J., & Chang, P.-S. (2017). Erythorbyl laurate as a potential food additive with multi-functionalities: interfacial characteristics and antioxidant activity. *Food Chemistry, 215*, 101-107.

Pirozzi, D., & Greco Jr, G. (2004). Activity and stability of lipases in the synthesis of butyl lactate. *Enzyme and Microbial Technology, 34*(2), 94-100.

Qian, C., & McClements, D. J. (2011). Formation of nanoemulsions stabilized by model food-grade emulsifiers using high-pressure homogenization:

factors affecting particle size. *Food Hydrocolloids, 25*(5), 1000-1008.

Raikos, V., Duthie, G., & Ranawana, V. (2017). Comparing the efficiency of different food-grade emulsifiers to form and stabilise orange oil-in-water beverage emulsions: influence of emulsifier concentration and storage time. *International Journal of Food Science & Technology, 52*(2), 348-358.

Ren, K., & Lamsal, B. P. (2017). Synthesis of some glucose-fatty acid esters by lipase from *Candida antarctica* and their emulsion functions. *Food Chemistry, 214*, 556-563.

Rodríguez Patino, J. M., Rodríguez Niño, M. R., & Sánchez, C. C. (1999). Adsorption of whey protein isolate at the oil− water interface as a function of processing conditions: A rheokinetic study. *Journal of Agricultural and Food Chemistry, 47*(6), 2241-2248.

Santana, R., Perrechil, F., & Cunha, R. (2013). High-and low-energy emulsifications for food applications: a focus on process parameters. *Food Engineering Reviews, 5*(2), 107-122.

Santos, J. C., Bueno, T., Molgero, P. C., Rós, D., & de Castro, H. F. (2007). Lipase-catalyzed synthesis of butyl esters by direct esterification in solvent-free system. *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean*

Technology, 82(10), 956-961.

Schubert, H., & Engel, R. (2004). Product and formulation engineering of emulsions. *Chemical Engineering Research and Design, 82*(9), 1137-1143.

Song, Q.-X., & Wei, D.-Z. (2002). Study of vitamin C ester synthesis by immobilized lipase from *Candida* sp. *Journal of Molecular Catalysis B: Enzymatic, 18*(4-6), 261-266.

Sun, J., Yu, B., Curran, P., & Liu, S.-Q. (2012). Lipase-catalysed transesterification of coconut oil with fusel alcohols in a solvent-free system. *Food Chemistry, 134*(1), 89-94.

Taha, M., Nosier, S., Abdel-Aziz, M., & El-Naggar, M. (2020). Solid-liquid mass transfer in a bubble column reactor with tube bank internals. *Experimental Heat Transfer*, 1-13.

Tcholakova, S., Denkov, N. D., & Danner, T. (2004). Role of surfactant type and concentration for the mean drop size during emulsification in turbulent flow. *Langmuir, 20*(18), 7444-7458.

Walstra, P. (1993). Principles of emulsion formation. *Chemical Engineering Science, 48*(2), 333-349.

Xing, X., Jia, J.-Q., Zhang, J.-F., Zhou, Z.-W., Li, J., Wang, N., & Yu, X.-Q. (2019). CALB immobilized onto magnetic nanoparticles for efficient

kinetic resolution of racemic secondary alcohols: long-term stability and reusability. *Molecules, 24*(3), 490.

Yang, Y., Leser, M. E., Sher, A. A., & McClements, D. J. (2013). Formation and stability of emulsions using a natural small molecule surfactant: Quillaja saponin (Q-Naturale®). *Food Hydrocolloids, 30*(2), 589-596.

Yu, D., Tian, L., Ma, D., Wu, H., Wang, Z., Wang, L., & Fang, X. (2010). Microwave-assisted fatty acid methyl ester production from soybean oil by Novozym 435. *Green Chemistry, 12*(5), 844-850.

Yu, H., Lee, M.-W., Shin, H., Park, K.-M., & Chang, P.-S. (2019). Lipase-catalyzed solvent-free synthesis of erythorbyl laurate in a gas-solid-liquid multiphase system. *Food Chemistry, 271*, 445-449.

Zhang, D.-H., Bai, S., Dong, X.-Y., & Sun, Y. (2007). Optimization of lipase-catalyzed regioselective acylation of pyridoxine (vitamin B_6). *Journal of Agricultural and Food Chemistry, 55*(11), 4526-4531.

Zhang, D.-H., Bai, S., & Sun, Y. (2007). Lipase-catalyzed regioselective synthesis of monoester of pyridoxine (vitamin B_6) in acetonitrile. *Food Chemistry, 102*(4), 1012-1019.

Zhou, X., Li, H., & Zheng, L. (2019). Directly covalent immobilization of *Candida antarctica* lipase B on oxidized aspen powder by introducing poly-lysines: An economical approach to improve enzyme

performance. *International Journal of Biological Macromolecules, 133*, 226-234.

Zhu, Z., Wen, Y., Yi, J., Cao, Y., Liu, F., & McClements, D. J. (2019). Comparison of natural and synthetic surfactants at forming and stabilizing nanoemulsions: Tea saponin, quillaja saponin, and Tween 80. *Journal of Colloid and Interface Science, 536*, 80-87.

국문 초록

최근 식품 산업에서 천연 및 친환경 제품의 수요가 증가함에 따라 기존에 사용하던 합성 유화제들을 대체하는 천연 유화제에 대한 연구가 많이 이루어졌다. 본 논문에서는 친수성 피리독신(pyridoxine)과 소수성 로우르산(lauric acid)을 기질로 하는 무용매(solvent-free) 반응계에서 라이페이스 촉매 합성 반응(lipase-catalyzed synthesis)을 통해 신규 천연 유화제를 합성했다. 고정화 효소인 Novozym® 435를 이용한 라이페이스 촉매 합성 반응을 HPLC로 분석하여 피리독신 모노로우레이트(pyridoxine monolaurate, PML)로 추정되는 물질의 합성을 확인했고 LC-ESI-MS 분석과 ^{1}H-, ^{13}C-, 그리고 ^{1}H-^{13}C 이핵 다중 결합 상관관계 NMR을 통해서 해당 생성물이 [5-hydroxy-4-(hydroxymethyl)-6-methylpyridin-3-yl]methyl dodecanoate임을 규명하였다. 유기 용매 단일상 반응계, 고체-액체 이중상 반응계, 그리고 기체-고체-액체 다중상 반응계(gas-solid-liquid multiphase system, GSL-MPS)에서 반응 효율성을 체적 생산성(volumetric productivity)과 전환 수율(conversion yield) 측면에서 비교하였다. GSL-MPS에서 41.24 mmol/L/h로 유기 용매 단일상 반응계에서보다 약 3.72 배,

고체-액체 이중상 반응계에서보다 약 2.08 배 높은 체적 생산성을 보였으므로 세 가지 반응계 중 GSL-MPS가 PML의 합성에 있어서 가장 효율적인 반응계임을 확인하였다. 효소의 안정성 또한 평가하여 GSL-MPS에서는 6 번의 반복 합성에 의한 유의적인 안정성 감소가 없음을 확인했고 SL-BPS와 OS-MPS에서는 유의적인 안정성 감소가 확인되었다. ($p < 0.05$) GSL-MPS에서 기질 몰 비율과 반응 온도 변화에 따른 반응 효율성을 평가하여 70℃의 반응 온도, 0.10의 기질 몰 비율([피리독신]/[로우르산]), 그리고 9 시간의 반응 시간을 최적 생산 조건으로 확립하였다. 해당 조건에서 PML의 체적 생산성은 41.24 mmol/L/h이고 전환 수율은 84.56%였다. PML의 계면 특성을 평가하기 위하여 PML의 농도에 따른 중쇄 지방(MCT oil)과 물의 계면에서의 계면장력을 측정하였다. 0.5~6.0 mM 범위에서 PML의 농도가 증가함에 따라 계면 장력이 감소함을 통해 PML의 계면 활성을 확인했다. PML로 안정화시킨 수중 유적형 에멀션의 에멀션 특성에 pH와 PML 농도가 미치는 영향을 분석했다. 0.2%(w/w) PML을 첨가한 10.0%(w/w) 수중 유적형 에멀션을 제조하고 pH 2.0~8.0 범위에서 입자 크기와 제타 전위를 측정했다. pH 2.0~4.0 범위에서는 유의적인 입자 크기 변화가 없었지

만 pH 4.0~6.0 범위와 pH 6.0~8.0 범위에서는 pH가 증가할수록 각각 입자 크기가 증가, 감소하는 경향성을 보였다. pH 2.0과 pH 3.0에서는 유의적인 제타 전위 차이가 관찰되지 않았고 pH 3.0~8.0 범위에서는 pH가 증가할수록 유의적으로 제타 전위가 감소하는 경향성을 보였다. PML의 농도 0.01~0.20%(w/w) 범위에서 제조한 10%(w/w) 수중 유적형 에멀션의 입자 크기를 측정했다. PML의 농도가 0.01~0.10%(w/w) 범위에서 증가함에 따라 입자 크기가 유의적으로 감소했고 0.10~0.20%(w/w) 범위에서는 유의적인 입자 크기 변화 없었다. 결론적으로, PML은 친환경적인 무용매 반응계에서 라이페이스 기반 효소적 합성으로 높은 반응 효율성으로 생산이 가능하고 산성 유화 식품에 적용할 수 있는 천연 유화제로 활용될 수 있다.

주제어: 피리독신 모노로우레이트, 저분자 유화제, 라이페이스-촉매 합성, 무용매 반응계, 유화 특성

학번: 2019-27667