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Whole-cell Biotransformation of *Chlorella* Oil Hydrolysates into Medium Chain Fatty Acids

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Abstract A synthetic pathway, which consisted of fatty acid double bond hydratase, alcohol dehydrogenase, and Baeyer-Villiger monooxygenase, was applied to *Chlorella* oil to produce ester fatty acids, which can be hydrolyzed into medium chain fatty acids. Since linoleic acid is a major fatty acid constituent of *Chlorella* oil, a fatty acid double bond hydratase from *Lactobacillus acidophilus* NBRC13951, which is able to convert linoleic acid into 13-hydroxyoctadec-9-enoic acid, was used. Recombinant *Escherichia coli* expressing the fatty acid double bond hydratase from *L. acidophilus* NBRC13951 successfully transformed linoleic acid in *Chlorella* oil hydrolysates into 13-hydroxyoctadec-9-enoic acid with approximately 60% conversion yield. 13-Hydroxyoctadec-9-enoic acid was further converted into ester fatty acids by the recombinant *E. coli* expressing a long chain secondary alcohol dehydrogenase and a Baeyer-Villiger monooxygenase. The resulting ester fatty acids were then hydrolyzed into medium chain fatty acids by a lipase. Overall, industrially relevant medium chain fatty acids were produced from *Chlorella* oil hydrolysates. Thereby, this study may contribute to biosynthesis of medium chain fatty acids from microalgae oils as well as long chain fatty acids.

Keywords : *Chlorella* oil, medium chain fatty acids, linoleic acid, fatty acid double bond hydratase, Baeyer-Villiger monooxygenase

Introduction

Natural oils such as plant oils or microalgal oils are valuable resources for the production of high value-added oleochemicals. Because major components of almost all the vegetable oils or microalgal oils are oleic acid and linoleic acid [1-4], development of techniques producing high value-added chemicals from oleic acid and linoleic acid can be valuable in utilizing natural oils. One of the examples may include biological production of medium chain fatty acids (MCFAs) from natural oils [5,6].

MCFAs including α,ω-dicarboxylic acids and ω

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-hydroxycarboxylic acids can be utilized as chemical products and intermediates such as nylons, polyamides, polyesters, resins, hot-melt adhesives, powder coatings, corrosion inhibitors, lubricants, plasticizers, greases, and perfumes [7]. MCFAs, which can be candidates for various value-added chemicals, could be produced by multi-step enzyme reaction [7,8]. It has been shown that this microbial production of medium chain fatty acids can be applied to various oils including plant oils, yeast-derived oil, and microalgae-originated fatty acid methyl esters [5,6].

Microalgae have been considered one of the most promising sources of triacylglycerol because of its high oil yields and growth in non-arable lands [9]. Like other oils, microalgal oil consists of various fatty acids. For example, *Chlorella*-originated oil consists of average 15.21% of palmitic acid, 18.41% of oleic acid, 14.26% of linoleic acid, and 15.31% of α-linolenic acid [10]. Overall, contents of polyunsaturated fatty acids (PUFA) such as linoleic acid and α-linolenic acid occupy the highest contents compared to saturated fatty acids and monounsaturated fatty acids. Therefore, efficient utilization of PUFA should be accomplished to achieve maximum yield for the production of MCFAs. In addition, because fatty acid content of microalgae can be increased by applying nitrogen depletion, inhibition of carbohydrate synthesis, or radiation of light with specific wavelength [11,12], microalgae have high potential in mass production of natural fatty acids. In multi-step enzyme reaction strategy reported (Fig. 1), the first step of the production of MCFAs from unsaturated fatty acids is the hydration (i.e. generation of hydroxyl group) of double bond. This task can be done by double bond hydratase or 13-lipoxygenase [13,14]. However, depending on the substrate specificity of each oleate hydratase, utilization rate of PUFAs may differ. Therefore, in the case of microalgal oil utilization, development of oleate hydratase having substrate specificity to PUFAs should be achieved. Oh et al. reported the double bond hydratase from *Lactobacillus acidophilus* NBRC13951 having specificity to linoleic acid (i.e. linoleate hydratase (LHTase)) [15]. Identification of such a linoleate hydratase paved a way to the utilization of linoleic acid, which ultimately leads to efficient production of MCFA from microalgal oils. Therefore, in this study, we investigated the production MCFA from *Chlorella* oils using the LHTase from *Lactobacillus acidophilus* NBRC13951 and multi-step enzyme reaction strategy, which had been reported in our previous study [15] (Fig. 1).

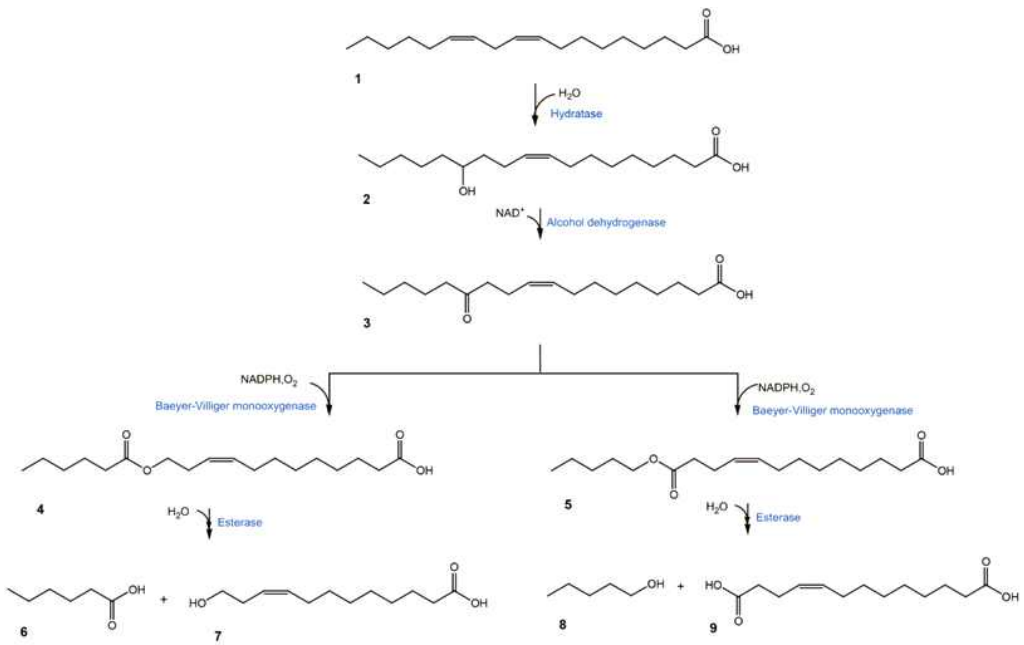

Figure 1. Production scheme of medium chain fatty acids from linoleic acid using multi-step enzyme reactions, which had been reported in our previous studies [15]. (**1**) linoleic acid, (**2**) 13-hydroxyoctadec-9-enoic acid, (**3**) 13-ketooctadec-9-enoic acid, (**4**) (*Z*)-12-(hexanoyloxy)dodec-9-enoic acid, (**5**) (*Z*)-12-((pentyloxy)carbonyl)dodec-9-enoic acid, (**6**) *n*-hexanoic acid, (**7**) 12-hydroxydodec-9-enoic acid, (**8**) *n*-pentanol, (**9**) α,ω-tridec-9-enedioic acid.

Materials and Methods

Microbial strains and culture media

Recombinant *E. coli* strains were grown in a Luria–Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) supplemented with the appropriate antibiotics for seed cultivation. The Riesenberg medium [16], supplemented with 10 g/L glucose and the appropriate antibiotics, was used for the main cultivation and biotransformation. The Riesenberg medium consisted of 4 g/L $(NH_4)_2HPO_4$, 13.5 g/L KH_2PO_4, 1.7 g/L citric acid, 1.4 g/L $MgSO_4$, and 10 mL/L trace metal solution (10 g/L $FeSO_4$, 2.25 g/L $ZnSO_4$, 1.0 g/L $CuSO_4$, 0.5 g/L $MnSO_4$, 0.23 g/L $Na_2B_4O_7$, 2.0 g/L $CaCl_2$, and 0.1 g/L $(NH_4)_6Mo_7O_{24}$). In case of cultivation of *E. coli* ER2566 pAhyd expressing the linoleate 13-hydratase from *Lactobacillus acidophilus* [17], the super optimal broth with catabolite repression (SOC) medium, which contained 3.6 g/L glucose, 20 g/L tryptone, 5 g/L yeast extract, 0.584 g/L NaCl, 0.186 g/L KCl, 0.952 g/L (10 mM) $MgCl_2$, and 2.408 g/L (10 mM) $MgSO_4$, was used as previously reported [17]. Plasmids used in this study are listed in Table 1. Recombinant gene expression was induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and/or 2 g/L rhamnose to the culture broth.

Table 1. Plasmids used in this study.

Plasmids	Description	Reference
pACYC-LHTase	p15A origin, Cm^R, P_{T7}	Oh et al. [15]
pACYC-ADH-FadL	p15A origin, Cm^R, P_{T7}	Cha et al. [18]
pJOE-E6BVMO	pBR322 origin, Amp^R, P_{rhaBAD}	Seo et al. [21]
pETDuet-E6BVMO	pBR322 origin, Kan^R , P_{T7}	Seo et al. [21]
pETDuet-E6BVMO$_{C302L}$	pBR322 origin, Kan^R , P_{T7}	Woo et al. [19]

ADH: alcohol dehydrogenase from *Micrococcus luteus* NCTC2665, FadL: long-chain fatty acid outer membrane transporter from *Escherichia coli* BL21(DE3) (Accession number: CAQ32746), LHTase: linoleate hydratase from *Lactobacillus acidophilus* NBRC13951, BVMO: Baeyer-Villiger monooxygenase from *Pseudomonas putida* KT2440, E6BVMO: hexa-glutamate tagged BVMO, E6BVMO$_{C302L}$: hexa-glutamate tagged BVMO C302L mutant.

Reagents

Linoleic acid, palmitic acid, *n*-hexanoic acid, *n*-pentanol, ethyl acetate, IPTG, Riesenberg medium components, and pyridine were obtained from Sigma (St. Louis, MO, USA). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (TMS) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). 13-Hydroxyoctadec-9-enoic acid (13-HOA) was prepared via hydration of linoleic acid or *Chlorella* oil hydrolysates with a linoleate C12 double bond-hydratase in our laboratory [17]. *Chlorella* oil hydrolysates were obtained from Prof. C.-G. Lee at Inha University.

Hydration of fatty acids into hydroxy fatty acids

Hydration of linoleic acid or *Chlorella* oil hydrolysates was carried out according to our earlier work [17]. Briefly, the recombinant *E. coli* expressing the linoleate 13-hydratase gene was cultivated in the SOC medium at 16°C. After induction of the gene expression, the cell mass was harvested and resuspended into 50 mM citrate/phosphate buffer (pH 6.0) to 20 g dry cells/L. After adding linoleic acid or *Chlorella* oil hydrolysates with 0.25% Tween 40 into the reaction medium, the biotransformation medium was incubated in a shaking incubator (200 rpm and 35°C). One unit (U) of whole cell activity was defined as μmol of hydroxy fatty acid produced per min at 35°C by 1.0 g dry cells.

Biotransformation of hydroxy fatty acids into ester fatty acids

The biotransformation was carried out as previously

reported [6,18-20]. In brief, the biotransformation was initiated at the stationary growth phase, usually 8 h after the induction of gene expression with 0.1 mM IPTG and/or 2 g/L rhamnose. After changing the pH of the culture broth to 8.0 and increasing the temperature to 35°C, hydroxy fatty acids and 0.5 g/L Tween80 were added into the culture broth containing a cell concentration of approximately 3 g dry cells/L. Cultivation and biotransformation was performed in a 100 mL flask (working volume: 10 mL) in a shaking incubator (200 rpm).

Product analysis by gas chromatography-mass spectrometry (GC-MS)

Concentrations of the remaining fatty acids and accumulating carboxylic acids in the medium (e.g., linoleic acid, 13-HOA, 13-keto-octadec-9-enoic acid, ester fatty acids, and carboxylic acids) were determined as described previously [6,18,19]. The reaction medium was mixed with an equal volume of ethyl acetate containing 0.5 g/L methyl palmitate as an internal standard. The organic phase was harvested after vigorous vortexing and then subjected to derivatization with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (TMS). The TMS derivatives were analyzed using a Thermo Ultra Trace GC system connected to an ion trap mass detector (Thermo ITQ1100 GC-ion Trap MS, Thermo Scientific (Indianapolis, IN, USA)). The derivatives were separated on a non-polar capillary column (30 m length, 0.25 μm film thickness, HP-5MS, Agilent Technologies (Palo Alto, CA, USA)). A linear temperature gradient was programmed as 90°C, 5°C/min to 280°C. The injection port temperature was 230°C. Mass spectra were obtained by electron impact ionization at 70 eV. Mass spectra were obtained within 100–600 m/z. Selected ion monitoring was used for the detection and fragmentation analyses of the reaction products.

Results and Discussion

Firstly, we analyzed the composition of *Chlorella* oil hydrolysates. As shown in Table 2, composition of *Chlorella* oil hydrolysates in this study was different from the previous report [10]. The major component was linoleic acid (50.3%), which was more than half of all the fatty acids. This result means that production of MCFAs from *Chlorella* oil hydrolysates would be successful only if linoleic acid is successfully utilized using LHTase. Among the LHTase, we found that LHTase from *Lactobacillus acidophilus* NBRC13951 (LHTase_Laci) has unique activity compared to other LHTases. According to Oh et al., LHTase_Laci has substrate specificity to linoleic acid as well as weak activity to oleic acid [15]. For example, LHTase_Laci produced ~7.5 mM of hydrated product of linoleic acid after 6 hr of reaction whereas it produced less than 2 mM of hydrated product of oleic acid [15]. Therefore, after the evaluation of substrate specificities of known double bond hydratases, we selected the LHTase_Laci as a double bond hydratase of our multi-enzyme synthetic pathway.

Table 2. Composition of *Chlorella* oil hydrolysates

Fatty acid	Percentage
C16:0	28.7%
C16:1	1.3%
C16:2	0.7%
C18:0	6.7%
C18:1	4.0%
C18:2	50.3%
C18:3	2.8%
C18:4	0.8%
C20:5	4.7%

Next, we examined the production of 13-HOA usin g LHTase_Laci, *Chlorella* oil hydrolysates and linol eic acid. When 10 mM *Chlorella* oil hydrolysates or 10 mM linoleic acid was used as substrate, *E. coli* ER2566::pACYC-LHTase_Laci produced ~6 m M or ~7 mM of 13-HOA, respectively. Conversions of reactions using 10 mM of *Chlorella* oil hydrolysat es or linoleic acid were ~60% and ~70%, respectivel y. These conversions were similar when 20 mM of

Chlorella oil hydrolysates or 20 mM of linoleic acid were used as substrates. Among the two substrates, i.e. linoleic acid and *Chlorella* oil hydrolysates, linol eic acid was more efficient for biotransformation yiel ding about 70% conversion. This result was promisin g because about 70% of conversion is similar to the value of conversion when oleic acid was converted to 10-hydroxystearic acid [5]. Therefore, we thought that, thanks to the LHTase_Laci, utilization of linolei c acid became as efficient as the utilization of oleic acid. In addition, high conversion of linoleic acid by LHTase_Laci was also confirmed by ~60% conversi on of *Chlorella* oil hydrolysates into 13-HOA. Becau se about 50% of *Chlorella* oil in this study is linoleic acid, high activity of LHTase_Laci to linoleic acid should be helpful to the conversion of *Chlorella* oil hydrolysates.

After the enzymatic reaction of LHTase_Laci, we analyzed the reaction using GC-MS after the extraction of organic materials using ethyl acetate. When linoleic acid was used as substrate, a peak containing 13-HOA was well separated with the 13-HOA purity of 75% (Fig. 3 (a)). When *Chlorella* oil hydrolysates were used as substrate, a peak containing 13-HOA was also well separated (Fig. 3 (b)). However 13-HOA purity was 25%, which was very low compared to the reaction using linoleic acid. Using the reaction medium extract, we further tested the alcohol dehydrogenase (ADH) and Baeyer-Villiger monooxygenase (BVMO) reactions using recombinant *E. coli*. Although it has been constantly overcome, the main problem in synthetic pathway in Fig. 1 was low stability of BVMO. To improve the low stability of BVMO from *Pseudomonas putida* KT2440 (BVMO_Pput), various protein engineering were applied to BVMO_Pput [19,21] as well as the screening of other BVMOs [20]. Because hexa-glutamate tag [21] and C302L mutation [19] were the successful engineering cases, we investigated the activity of hexa-glutamate-tagged BVMO (E6BVMO) and C302L mutant of E6BVMO. In addition, we tested the vector effect for the activity of cells expressing BVMO by incorporating another BVMO-expressing vector. Therefore, recombinant hosts for the production of **(3)** in Fig. 1 were *E. coli* BL21(DE3)::pACYC-ADH-fadL,pJOE-E6BVMO, *E. coli* BL21(DE3)::pACYC-ADH-fadL,pETDuet-E6BVMO, and *E. coli* BL21(DE3)::pACYC-ADH-fadL,pETDuet-E6BVMO$_{C302L}$ (fadL: gene encoding outer membrane long-chain fatty acid transporter). Firstly, we tested the activity of our recombinant hosts using 13-HOA from the reaction using linoleic acid. As shown in Fig. 4, *E. coli* BL21(DE3)::pACYC-ADH-fadL,pJOE-E6BVMO, *E. coli* BL21(DE3)::pACYC-ADH-fadL,pETDuet-E6BVMO, and *E. coli* BL21(DE3)::pACYC-ADH-fadL,pETDuet-E6BVMO$_{C302L}$ produced ester fatty acids (compound **4** and **5** in Fig. 1) with conversion of 62%, 78%, and 79%, respectively. Since the conversion yield of 13-HOA from linoleic acid was 77% (Fig. 2 (a)), the overall yield of ester fatty acids from linoleic acid reached 48 to 61%. Among all three cases, ratio between compound **4** and **5** was 2:1. Enhanced conversion by *E. coli* BL21(DE3)::pACYC-ADH-fadL,pETDuet-E6BVMO and *E. coli* BL21(DE3)::pACYC-ADH-fadL,pETDuet-E6BVMO$_{C302L}$ from BL21(DE3)::pACYC-ADH-fadL,pJOE-E6BVMO seemed to be the higher expression strength of T7 promoter of pETDuet plasmid than rhaBAD promoter of pJOE [22]. Therefore, we thought that the difference in the amount of expressed BVMO caused the difference in the production of ester fatty acids. However, interestingly, the ratio between compound **4** and **5** were same for all the reactions. Because we used same BVMO for all the reactions, ratio between compound **4** and **5** would be the same, even with C302L mutant.

To investigate the more real substrate, we performed reaction using organic materials extracted from LHTase reaction using *Chlorella* oil hydrolysates. As shown in Fig. 5, *E. coli* BL21(DE3)::pACYC-ADH-fadL,pJOE-E6BVMO, *E. coli* BL21(DE3)::pACYC-ADH-fadL,pETDuet-E6BVMO, and *E. coli* BL21(DE3)::pACYC-ADH-fadL,pETDuet-E6BVMO$_{C302L}$ produced ester fatty acids with conversion of 51%, 66%, and 62%, and the ratio between compound **4** and **5** of 2:1, 2:1, 1.85:1, respectively. Use of more complex starting material reduced the conversion by 11~17%, whereas the ratio between compound **4** and **5** was similar. These lower conversions may be due

to the competitive substrate inhibition by saturated fatty acids and unsaturated fatty acids included in the *Chlorella* oil hydrolysates such as palmitic acid, stearic acid, and oleic acid. Therefore, to make our synthetic pathway more efficient in complex starting material, competitive substrate inhibition by other fatty acids to LHTase, ADH, and BVMO should be investigated and overcome.

Although we did not investigated the hydrolysis of compound **4** and **5** using lipases, we believe that various lipases such as lipase from *Candida rugosa* or lipase from *Thermomyces lanuginosus* having substrate specificity would surely hydrolyze to produce compound **6**, **7**, **8**, and **9**.

In this study, we investigated the utilization of fatty acids from *Chlorella* oil to produce MCFAs. Successful hydration of linoleic acid proved that employment of LHTase_Laci was successful. We also have shown that 13-HOA could be converted its corresponding ester fatty acids, which may further hydrolyzed to produce various MCFAs. Our study also showed the hurdles (e.g., unreacted fatty acids) in utilization of *Chlorella* oil using synthetic pathway in Fig. 1. To make our synthetic pathway more robust, hurdles such as competitive substrate inhibition should be solved for LHTase, ADH, or BVMO. By incorporating heterologous genes into genome [23], we can construct recombinant *E. coli* cells which does not need expensive antibiotics, and which is more stable than *E. coli* with plasmid-based heterologous expression.

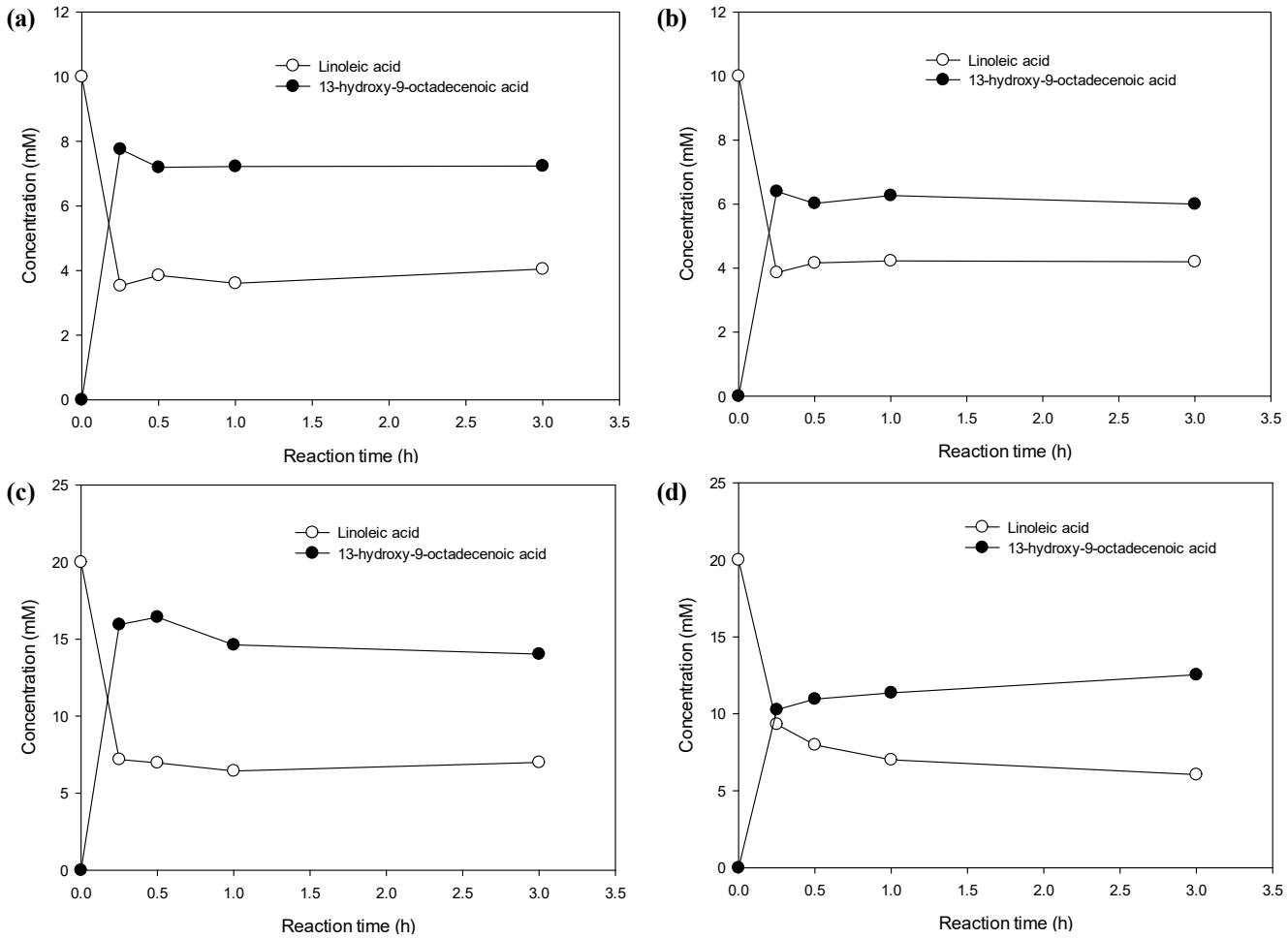

Figure 2. Conversion of *Chlorella* oil hydrolysates or linoleic acid using *E. coli* ER2566::pACYC-LHTase_Laci. (a) Biotransform ation of 10 mM linoleic acid, (b) biotransformation of 10 mM *Chlorella* oil hydrolysates, (c) biotransformation of 20 mM linoleic acid, and (d) biotransformation of 20 mM *Chlorella* oil hydrolysates.

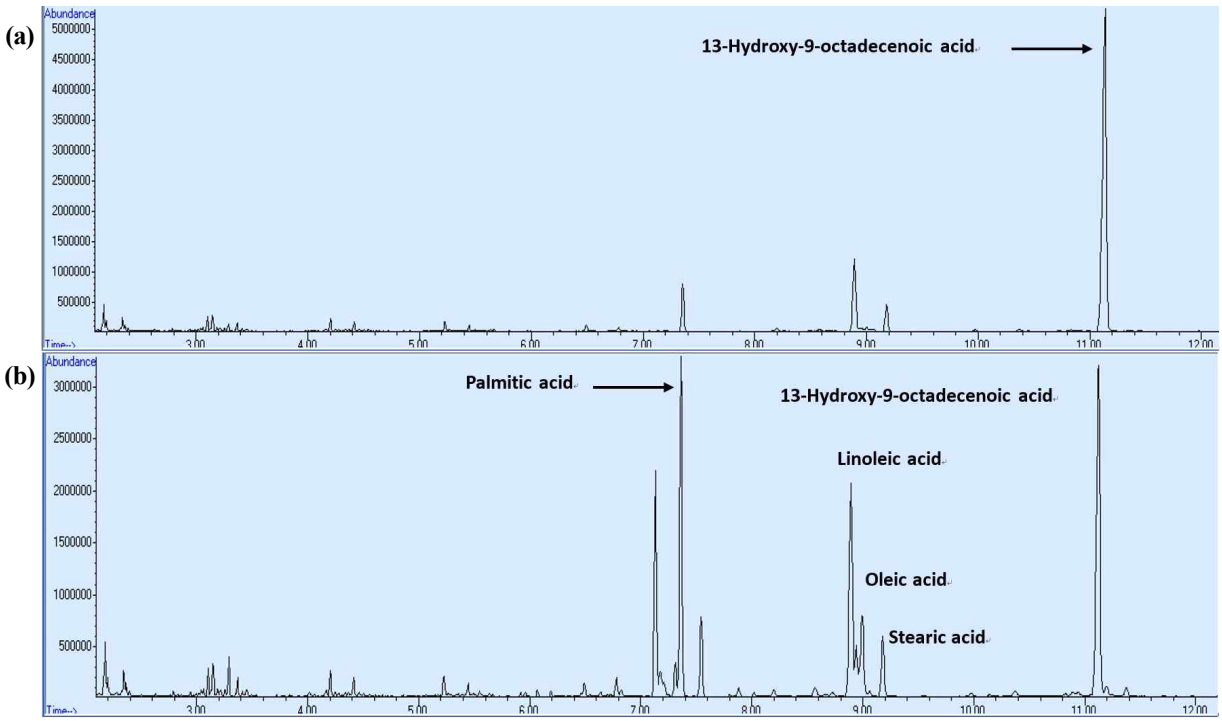

Figure 3. GC-MS spectrum of organic materials in reaction medium extracted using ethyl acetate. (a) GC-MS spectrum of organic materials in reaction medium using linoleic acid, (b) GC-MS spectrum of organic materials in reaction medium using *Chlorella* oil hydrolysates.

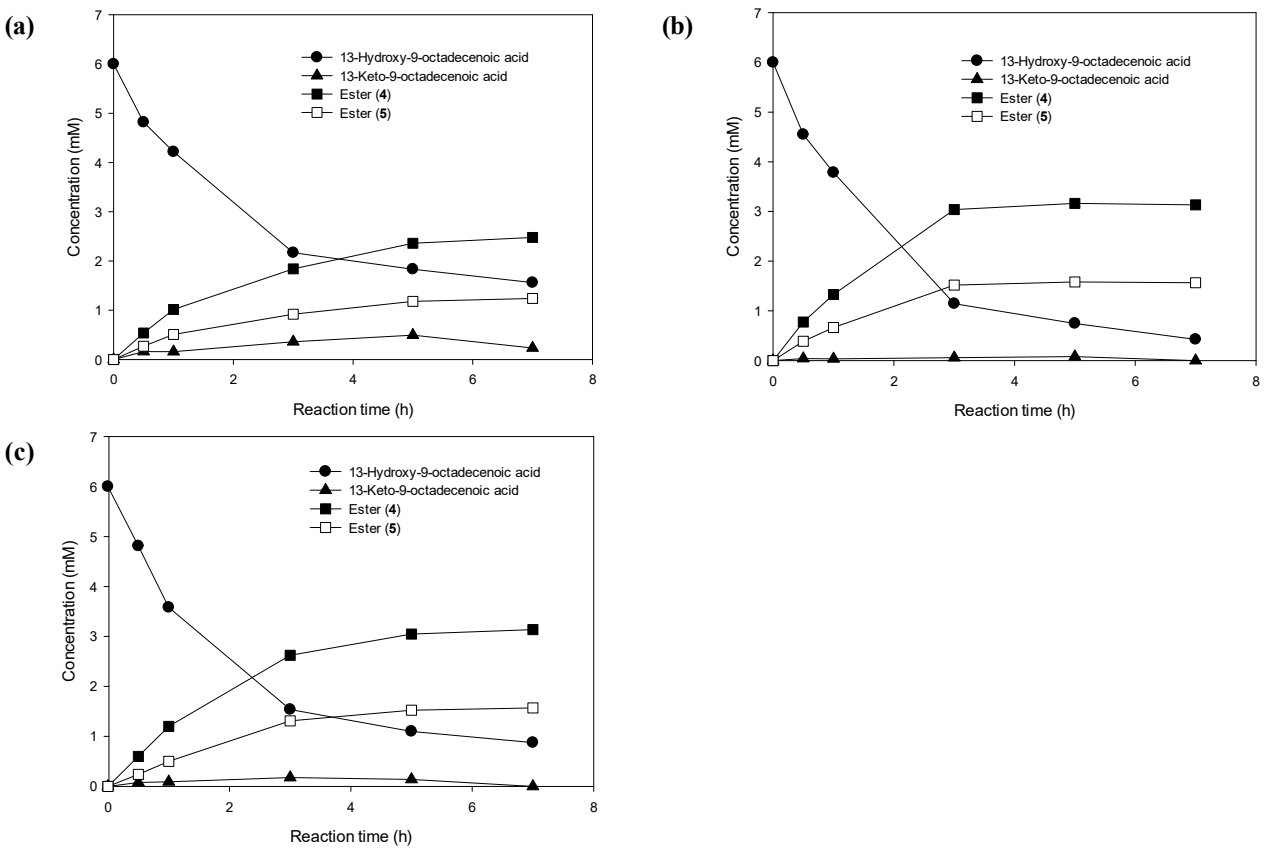

Figure 4. Alcohol hydrogenase (ADH) and Baeyer-Villiger monooxygenase (BVMO) reaction using 13-HOA from the LHTase reaction using linoleic acid. (a) *E. coli* BL21(DE3)::pACYC-ADH-fadL,pJOE-E6BVMO, (b) *E. coli* BL21(DE3)::pACYC-ADH-fadL,pETDuet-E6BVMO, (c) *E. coli* BL21(DE3)::pACYC-ADH-fadL,pETDuet-E6BVMO$_{C302L}$. Ester **(4)** and Ester **(5)** means compounds **4** and **5** in Fig. 1, respectively.

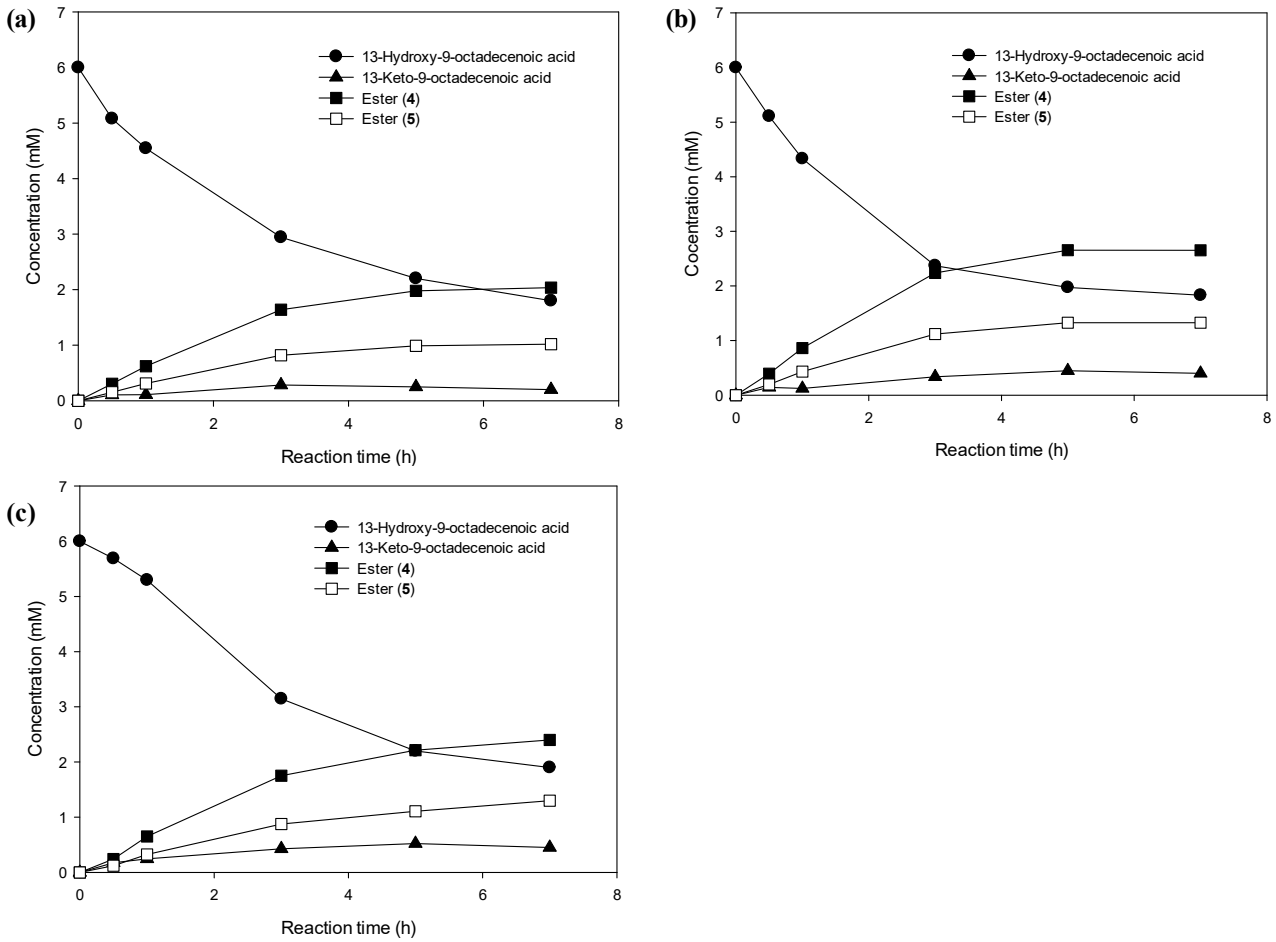

Figure 5. ADH and BVMO reaction using 13-HOA from the LHTase reaction using *Chlorella* oil hydrolysates. (a) *E. coli* BL21(DE3)::pACYC-ADH-fadL,pJOE-E6BVMO, (b) *E. coli* BL21(DE3)::pACYC-ADH-fadL,pETDuet-E6BVMO, (c) *E. coli* BL21(DE3)::pACYC-ADH-fadL,pETDuet-E6BVMO$_{C302L}$. Ester **(4)** and Ester **(5)** mean compound **4** and **5** in Fig. 1, respectively.

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