Master's Thesis

Production of Hexanoic Acid from Long Chain Fatty Acid in *Escherichia coli*

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6. 19. 2015

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Abstract

All over the world, the efforts are under way to find the petroleum replacement resources in order to overcome fossil fuel depletion and to reduce greenhouse-gas emissions. In this respect, replacing petrochemicals with renewable alternatives is one of the principal challenges of our day. Hexanoic acid could be widely used as a precursor to biofuels and for the production of chain based chemicals such as adipic acid, hexane, amino-caproic acid and so on. In order to produce specific carbon chain fatty acid, *Escherichia coli* β-oxidation pathway was engineered. First, *fadE* and *fadR* genes were deleted. And then, *E. coli* acyl-CoA dehydrogenase (FadE) was replaced with acyl-CoA oxidase 2 encoded by *POX2* gene from *Yarrowia lipolytica*, which has substrate specificity towards medium to long chain fatty acyl-CoA. Finally the thioesterase Ⅱ (TesB) was overexpressed in the cell. In this study, a maximum of 210mg/L of hexanoic acid was produced by the engineered strain (MGREBA2) from 1g/L of oleic acid in 48hours of biotransformation. Based on these results, it is suggested that various oil as feedstock will be directly applied for the production of short chain fatty acids, especially hexanoic acid.

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Nomenclature

SCFA: Short chain fatty acid

MCFA: Medium chain fatty acid

E. coli: *Escherichia coli*

Y.Lipolytica: *Yarrowia lipolytica*

ACP: Acyl-acyl carrier protein

FAD: Flavin adenine dinucleotide

NAD: Nicotinamide adenine dinucleotide

GC/FID: Gas chromatography/flame ionization detector

OD: Optical density

PCR: Polymerase chain reaction

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

RFP: Red Fluorescent Protein

Aox: Acyl-CoA oxidase

EtAc: Ethyl acetate

HCl: Hydrochloric acid

TMS: Trimethylsilyl

IPTG: Isopropyl β-D-1-thiogalactopyranoside

1. Introduction

All over the world, the efforts are under way to find the petroleum displacement resource in order to overcome fossil fuel depletion and to reduce greenhouse-gas emissions. In economic and environmental sustainability terms, the renewable sources based on fatty acid rich feed stocks such as waste vegetable oil are attractive alternative feedstock for the production of industrial chemicals[2]. Various technology as homogeneous, heterogeneous and enzymatic catalysis for transesterification is being applied in order to directly use biodiesel from waste oil[3]. This waste vegetable oil is attractive feedstock for producing industrial chemicals or biofuel using microorganism because it has sufficient fatty acids as carbon source. However, a few researches have been reported at least for now[3].

1.1 Hexanoic acid

Hexanoic acid (caproic acid) is the carboxylic acid containing six-carbon chain. This short chain fatty acid (SCFA) can be directly applied to perfumes, medicine, food additives, polymer backbone and various industrial materials production[4]. In addition, it could be a precursor for fuel and various chemicals based on six carbon chain such as adipic acid, hexane, hexanol, amino-caproic acid and so on[5]. Most studies of fatty acid production in *Escherichia coli* (*E. coli*) have been focused on production of medium chain fatty acids (MCFAs) including its increase of yield, because the chief ingredients of biodiesel are derived from MCFA[6]. These MCFAs from microorganism could be directly applied for biodiesel production after esterification[6c]. In addition, the short chain fatty acids (SCFAs) containing hexanoic acid have advantage for precursor of polymer because it have shorter chain length than MCFA[7].

1.2 Fatty acids metabolism in *E. coli*

The fatty acid metabolism in *E. coli* is largely divided into two different pathway either synthetic or degradation pathway. The synthetic pathway of fatty acids signify generative processes to acyl-acyl carrier protein (acyl-ACP) from acetyl-CoA through 3ketoacyl-ACP, 3-hydroxy acyl-ACP and enoyl-ACP. Two carbon chain from acetyl-CoA is added to previous acyl-ACP in each cycle. Synthesized acyl-ACP through this pathway is converted to free fatty acid form, which reaction is mediated by thioestrease Ⅰ (TesA). Although native *E. coli* thioesterase Ⅰ is generally located on periplasm[8], cytosolic thioestease Ⅰ without leader sequence has been utilized to increase the fatty acid production[6c,

[9]. In addition, several studies reported that the production of specific carbon chain fatty acid in microorganism is achieved by heterologous expression of plants originated thioesterase[6c, 10]. Owing to dissimilar substrate specificity of each species derived thioesterase, the specific carbon chain length fatty acid can be produced in the pathway of synthetic fatty acid.

Most researches on producing fatty acid have been focused on engineering fatty acid synthetic pathway because carbohydrates served as a major carbon source. In this case, *fadD* gene encoding acyl-CoA synthetase or *fadE* gene encoding acyl-CoA dehydrogenase is deleted for inactivation of β-oxidation, because free fatty acids from synthetic pathway can be degraded by β-oxidation pathway[6c, 6d, 11]. In contrast, few studies have been conducted to produce fatty acid by β-oxidation engineering. β-oxidation engineering is material to using fatty acid for carbon source, because it is sole fatty acid metabolism pathway for formation of acetyl-CoA, which is used for energy source on TCA cycle, from fatty acids in *E. coli*[12].

1.3 Beta(β)-oxidation pathway

The initial stage of β-oxidation is mediated by acyl-CoA synthetase (FadD) encoded by *fadD* gene. Prior to catabolism, fatty acids must be converted to an acyl-CoA form. This step is mediated by FadD with energy from ATP [13]. And then this acyl-CoA convert to enoyl-CoA, which oxidation is mediated by acyl-CoA dehydrogenase (FadE) encoded by *fadE* gene. This reaction involves the transfer of two electrons from the substrate to a flavin adenine dinucleotide (FAD) cofactor that must be reoxidized in order for the dehydrogenase to have catalytic function[14]. Unlike other species, *E. coli* has sole acyl-CoA dehydrogenase[15]. Therefore, the strain of deleted *fadE* gene has growth deficiency on sole carbon source of all chain fatty acids, because its substrate specificity is broad chain length. Hence, the β-oxidation engineering is important part for producing specific chain fatty acids, if acyl-CoA dehydrogenase substrate specificity is modulated[16]. The next step is conversion of enoyl-CoA to 3-keto acyl-CoA via 3-hydroxyacyl-CoA mediated by FadB. Finally, the 3-keto acyl-CoA convert to acyl-CoA with cutting out acetyl-CoA and replacing new coenzyme A mediated by FadA (Fig. 1) [17].

In contrast to *E. coli* metabolism of fatty acids, the β-oxidation of mammalian cells occur in both mitochondria and peroxisome separately[18]. Although concrete evidence why they are separated has not yet been identified, a clear contrast is that the mitochondrial fatty acid metabolism involved in energy production while peroxisomal metabolism is not. the mitochondrial β-oxidation is directly connected with electron transport chain thus it can produce ATP for energy [18a]. However, peroxisomal β-oxidation doesn't produce energy directly but form hydrogen peroxide. This difference involves the redox reaction by the co-factor as flavin andenine dinucleotide (FAD) or nicotinamide adenine dinucleotide (NAD).

FAD can be reduced to $FADH_2$ mediated by acyl-CoA dehydrogenase and acyl-CoA oxidase in the mitochondria and peroxisome respectively. This $FADH_2$ reverts to FAD from complex Ⅱ in the electron transport chain in the mitochondria, while its reaction is mediated by the oxygen molecule in the peroxisome. Acyl-CoA oxidases are directly to oxygen to generate hydrogen peroxide in order to revert to FAD from FADH (Fig. 2) [18b] .

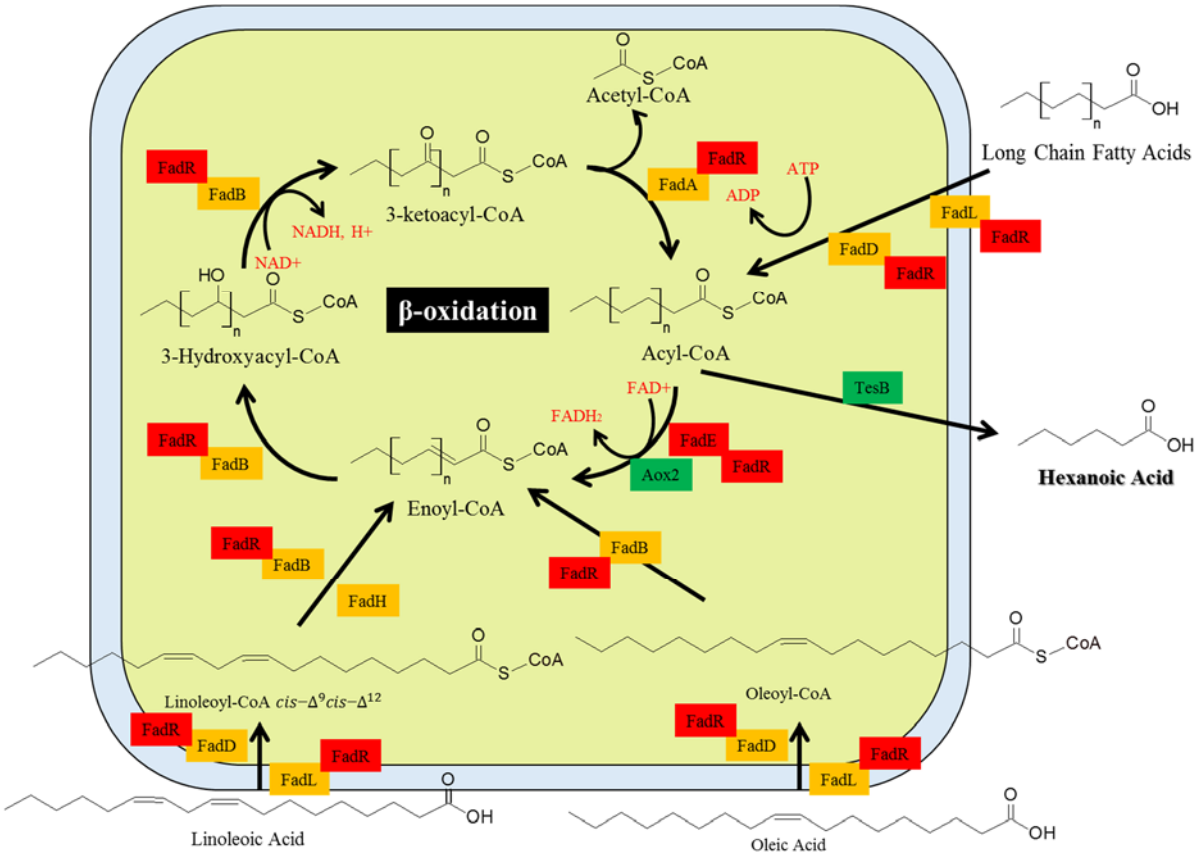

Figure 1. A schema of beta-oxidation engineering to produce hexanoic acid

The red box represents the deleted genes; the green box represents the activation by heterologous- or over-expression. The *fadE* gene, which encodes acyl-CoA dehydrogenase, was deleted. In order to unblock the beta-oxidation pathway, the *fadR* gene, which encodes fatty acid metabolism regulator protein, was also deleted. And the *POX2* gene from *Y. lipolytica* and *tesB* gene, which encodes acyl-CoA oxidase2 and thioesterase Ⅱ respectively, was expressed in pBbE6k plasmid[1].

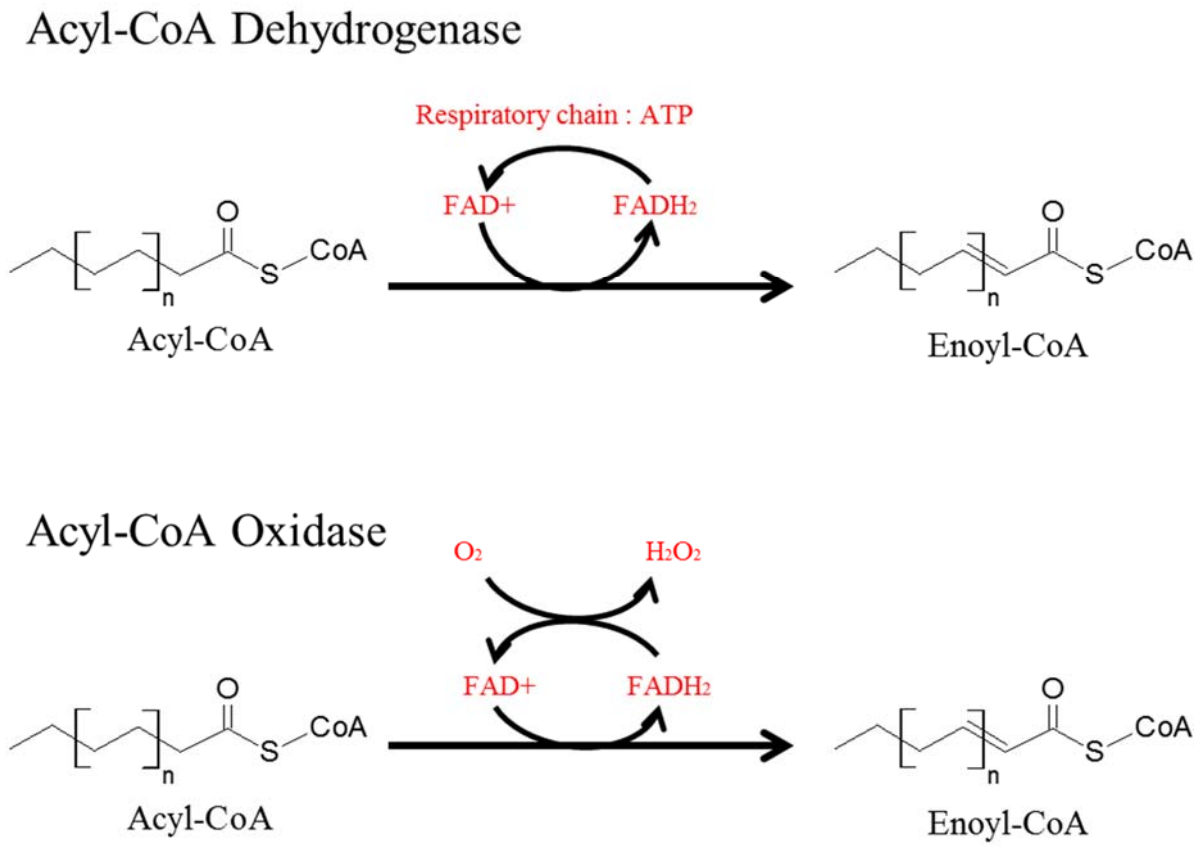

Figure 2. Difference between acyl-CoA dehydrogenase and acyl-CoA oxidase

$FADH_2$, which mediated by acyl-CoA dehydrogenase with conversion to enoyl-CoA form, reverts to FAD from complex Ⅱ in the electron transport chain, Acyl-CoA oxidases are directly to oxygen to generate hydrogen peroxide in order to revert to FAD from FADH.

The yeast is known that the β-oxidation occur only in peroxisome whereas animal cell is taken place both mitochondria and peroxisome [19]. The yeast *saccharomyces cerevisiae* (*S. cerevisiae*) has sole acyl-CoA oxidase as POX1, which has substrate specificity for short to long chain substrate specificity. In contrast to *S. cerevisiae*, *Yarrowia lipolytica* (*Y. lipolytica*) and *Candida* sp. have several acyl-CoA oxidases with different chain length specificities. Therefore, each of acyl-CoA oxidases reacts following each chain length fatty acyl-CoA. The β-oxidation of *Y. lipolytica* is involving by five acyl-CoA oxidase as AOX1 to 5, which is encoded by *POX*1 to 5 genes. From among these AOXs, AOX2 has specificity for long-chain length fatty acyl-CoA[20]. In recent study, MCFA production was conducted in *S. cerevisiae*. The AOX1 deleted *S. cerevisiae* was not able to grow on sole carbon source of oleic acid. And then, although AOX2 from *Y. lipolytica* was expressed in this strain, its growth defect was shown

because of the restriction of the carbon flow toward the TCA cycle. In order to solve this problem, the *Mus musculus*'s *CROT* gene, which encodes carnitine O-octanoyltransferase was introduced to transport the accumulated medium chain acyl-CoAs out of the peroxisome. Therefore, CoA concentration in peroxisome was balanced, then the growth of strain was restored[16].

1.4 Thioesterase specificity in *E. coli*

In *E. coli*, three types of thioesterase are reported; Thioesterase Ⅰ, Ⅱ and III[21]. Even though the accurately physiological role of thioesterase is not identified yet, several studies reported that these enzymes play a vital role in catalyst the hydrolytic cleavage of fatty acyl-CoA thioesters[8, 22]. Thioesterase Ⅰ, encoded by *tesA* gene, cleaves fatty acyl-acyl carrier protein (ACP), whereas ThioesteraseⅡ(TesB), encoded by *tesB* gene, has specificity for acyl-CoA form[23]. ThioesteraseIII, which was identified at comparatively recent, was shown to have the substrate specificity for long chain acyl-CoA derived unsaturated fatty acid[24]. Among these three thioesterase, Thioesterase Ⅱ has the most broad substrate specificity of acyl-CoA chain length. Thioesterase Ⅰ has specificity for C12 to 18 chain length of acyl-CoA ester, while Thioesterase Ⅱ has C6 to C18 chain length specificity as broad chain length[23]. Therefore, in the present study, we selected TesB for hexanoic acid production because it has C6-acyl-CoA specificity.

1.5 Modification of β-oxidation in *E. coli*

In this study, we focused on AOX2 derived from *Y. lipolytica* and Thioesterase Ⅱ (TesB) and a novel β-oxidation pathway was constructed in *E. coli*. In order to produce hexanoic acid in *E. coli*, *fadE* gene was deleted for inactivation of acyl-CoA dehydrogenase in wild type strain primarily. In addition, *fadR* gene, which encodes fatty acid metabolism regulator protein (FadR), was also deleted because FadR acts as a repressor of *fad* genes concerned β-oxidation cycle in the presence of long chain fatty acids[25]. Therefore basis in this *fadR* and *fadE* double knockout strain, *POX2* gene from *Y. lipolytica* was the heterologous expressed as Aox2 has substrate specificity towards medium to long chain fatty acids. Therefore it could produce specific chain length acyl-CoA by discontinuing the β-oxidation cycle. In order to increase the product concentration, *tesB* gene was overexpressed. The modification of β-oxidation pathway is described in Figure 1. Through those processes, we constructed novel β-oxidation in *E. coli*. The formation of hexanoic acid was confirmed in engineered *E. coli*.

2. Materials and Methods

2.1 *E.coli* strains and growth condition

Deletion of the *fadE* and *fadR* genes from MG1655 strain was performed using a one-step inactivation method[26]. MG1655Δ*fadE*Δ*fadR*, a β-oxidation disrupted strain derived from *E.coli* strain MG1655, was used for the expression of the *Y.Lipolytica POX2* gene. All *E. coli* strains used in this study are listed in Table 1. In addition, the used primer of deletion for *fadE* and *fadR* is listed in Table 2. The initial culture of strain from colony was performed with 5ml Luria-Bertani (LB) broth (BD, USA) at 37 ℃, which contain appropriate antibiotic as 50 μg/mL of kanamycin or 30 μg/mL chloramphenicol. M9 minimal medium was composed of 1X M9 salts (Bioshop, Canada), 1mM $MgSO_4$, 1M $CaCl_2$, 0.1% carbon source.

2.2 Construction of plasmids and strains

Cloning was conducted through restriction enzyme digestion and ligation method using pBbE6k and pBbB6c as BglBrick vector[1, 27]. In order to amplify target genes as *POX2* from *Y.Lipolytica*, polymerase chain reaction (PCR) (Swift Max Pro, Esco, Singapore) was performed on the condition (95 ℃ - 2 min, (95 ℃ - 40 sec, 55 ℃ - 40 sec, 72 ℃ - 45 sec) × 30 cycle, 72 ℃ -5 sec) The PCR product were digested with *Nde Ⅰ* and *Not Ⅰ* and then ligated into the same restriction site of pBbE6k vector which has been digested with *Nde Ⅰ* and *Not Ⅰ*. Based on the plasmid of pBbE6k-Aox2, the *tesB* gene, which is amplied from MG1655 strain, was inserted in front of *POX2* gene through restriction site of *EcoR Ⅰ* and *Bgl Ⅱ*. This PCR product was digested *EcoR Ⅰ* and *BamH Ⅰ* because *tesB* gene has Bgl *Ⅱ* sequence. *tesA* and *tesB* genes was inserted to pBbB6c vector respectively. The used cloning primer is listed in Table 2.

Based on *fadE* and *fadR* knockout strain, each cloned plasmid was transformed using electroporation method. Basic vector, which is pBbE6k-RFP as Bglbrick vector, was transformed into the *fadE* and *fadR* knockout strain for control cell (Table 1).

Table 1. *E. coli* strains and plasmids used in this study

Strains	**Genotypes**	**Ref.**
MG1655	F- λ- ilvG- rfb-50 rph-1	Blattner, et al. 1997[28]
MGE	MG1655 *ΔfadE::FRT* with pBbE6k-RFP	This study
MGEA2	MG1655 *ΔfadE::FRT* with pBbE6k-Aox2	This study
MGEBA2	MG1655 *ΔfadE::FRT* with pBbE6k-TesB/Aox2	This study
MGECA2	MG1655 *ΔfadE::FRT* with pBbE6k-Aox2 pBbB6c-ChfatB2	This study
MGR	MG1655 *ΔfadR::FRT* with pBbE6k-RFP	This study
MGRA2	MG1655 *ΔfadR::FRT* with pBbE6k-Aox2	This study
MGRBA2	MG1655 *ΔfadR::FRT* with pBbE6k-TesB/Aox2	This study
MGRCA2	MG1655 *ΔfadR::FRT* with pBbE6k-Aox2 pBbB6c-ChfatB2	This study
MGRE	MG1655 *ΔfadE::FRT ΔfadR::FRT* with pBbE6k-RFP	This study
MGREA2	MG1655 *ΔfadE::FRT ΔfadR::FRT* with pBbE6k-Aox2	This study
MGREBA2	MG1655 *ΔfadE::FRT ΔfadR::FRT* with pBbE6k-TesB/Aox2	This study
MGRECA2	MG1655 *ΔfadE::FRT ΔfadR::FRT* with pBbE6k-Aox2 pBbB6c-ChfatB2	This study
Plasmids	**Description**	**Ref.**
pBbE6k-RFP	ColE1 ori, P_{LacO1} Promoter, Km resistant	Lee, et al. 2011[1]
pBbB6c-GFP	BBR1 ori, P_{LacO1} Promoter, Cm resistant	Lee, et al. 2011[1]
pBbE6k-Aox2	pBbE6k harbouring *POX2* gene derived *Y.Lipolytica*	This study
pBbE6k-TesB/Aox2	pBbE6k harbouring *tesB* gene with *POX2* gene	This study
pBbB6c-ChfatB2	pBbB6c harbouring *fatB2* gene derived *Cuphea hookeriana*	This study

Table 2. List of used primer in this study

Primer	Sequence	Ref.
FadE_del_F	CCATATCATCACAAGTGGTCAGACCTCCTACAAGTAAGGGG CTTTTCGTTGTGTAGGCTGGAGCTGCTTC	Baba, et al. 2006[29]
FadE_del_R	TTACGCGGCTTCAACTTTCCGCACTTTCTCCGGCAACTTTAC CGGCTTCGATTCCGGGGATCCGTCGACC	Baba, et al. 2006[29]
FadR_del_F	TCTGGTATGATGAGTCCAACTTTGTTTTGCTGTGTTATGGAA ATCTCACTGTGTAGGCTGGAGCTGCTTC	Baba, et al. 2006[29]
FadR_del_R	AACAACAAAAAACCCCTCGTTTGAGGGGTTTGCTCTTTAAA CGGAAGGGAATTCCGGGGATCCGTCGACC	Baba, et al. 2006[29]
Aox2-F	CCGCATATGAACCCCAACAACACTGGCACC	This study
Aox2-R	CCGTAGCGGCCGCCTATTCCTCATCAAGCTCGCAAAT	This study
TesB-F	ATCGAGAATTCTTTAAGAAGGAGATATACATATGAGTCAGG CGCTAAAAAA	This study
TesB-R	TCGGGATCCTTAATTGTGATTACGCATCA	This study
TesA-F	AAAGAATTCAAAAGATCTTTTAAGAAGGAGATATACATATG GCGGACACGTTATTGAT	This study
TesA-R	TTACTCGAGTTATGAGTCATGATTTACTA	This study
ChfatB2-F	AAAGAATTCAAAAGATCTTTTAAGAAGGAGATATACATATG GTCGCAGCAGCAGCCT	This study
ChfatB2-R	TTACTCGAGTCATTACGACACGCTATTGCCGTTTG	This study

2.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The expression of genes was identified by SDS-PAGE analysis. Overnight culture medium as LB broth at 37 ℃ was inoculated to 50ml fresh LB broth by 1/100 diluted ratio with suitable antibiotics. After 0.5 mM IPTG induction was conducted at OD 0.7, this culture medium was incubated at 30 ℃ during 16 hours. Whole cell was harvested by centrifugation at 3500 rpm during 10min. And then the harvested cell was resuspended in lysis buffer (50 Mm Tris-HCl pH 8.0) and sonicated using Vitra Cell Sonicator (SONICS, USA). After separation of supernatant and pellet by centrifugation at 13000 rpm of centrifugation, and mixed with loading buffer. And then this total protein was denaturated at 80 ℃ for 10 min. Prepared protein was separated on a 10 % SDS-PAGE (Bio-Rad, USA).

2.4 Growth study

Overnight culture medium as LB broth on 37 ℃ was separately inoculated to 50 ml fresh LB broth and M9 medium with 0.1 % oleic acid, 0.5 % TWEEN80 and 100 mM potassium phosphate buffer by 1/100 diluted ratio with suitable antibiotics. After 0.1 mM IPTG induction was conducted at OD 0.7, this culture medium was incubated at 30 ℃ for 48 hours. The absorbance for growth of cell was checked by Libra S22 Spectrophotometer (Biochrom, UK) at 600 nm.

2.5 Biotransformation in LB broth

After preparation of initial culture, each engineered strain and control cell was inoculated to 50 ml fresh LB broth in 250 ml flask. After 0.1 mM IPTG induction at OD 0.7, this culture medium was incubated during 4 hour on 30 ℃. Shortly before stationary phase, 0.1 % oleic acid with 0.5 % TWEEN80 was injected to culture medium. This biotransformation medium was conducted at 30 ℃ with a rotation of 200 rpm for 72 hours. One milliliter samples were collected at different time points (0, 6, 12, 24, 48 and 72 hour after oleic acid injection) for GC/FID analysis.

2.6 Whole cell biotransformation in M9 broth

After preparation of initial culture, each engineered strain and control cell was inoculated to 50 ml fresh LB broth in 250 ml flask. When an OD_{600} of 0.7 was approached, 0.1 mM IPTG was added for induction of protein expression. This culture medium was incubated at 30 ℃, 200 rpm for 20 hours. Whole cell was harvested on 3500 rpm at 4 ℃ for 10 min, and then its pellet was washed by 100 mM

potassium phosphate buffer (pH 7.0). After washing steps, this cells were injected to transformation medium which is M9 medium containing 0.1 % oleic acid, 0.5 % TWEEN80, 100 mM potassium phosphate buffer and suitable antibiotic as 50 μg/mL of kanamycin or 30 μg/mL chloramphenicol. Biotransformation was conducted at 30 ℃ with rotation of 200 rpm for 72 hours. 1 ml samples were collected at different time points (0, 6, 12, 24, 48 and 72 hour after oleic acid injection) for GC/FID analysis.

2.7 Analysis of biotransformation products

One milliliter sample was centrifuged at 13000 rpm, 500 μL supernatant was transferred to a 2 ml Eppendorf tube. After 50 μL pure hydrochloric acid (HCl) was added for acidification, 500 μL ethyl acetate (EtAc), which can dissolve fatty acids, was added with internal standard and vortex well for 30 sec. this solution was performed spin down at 13000 rpm during 2 min, and recovered EtAc as top layer was transferred to a new 2 ml Eppendorf tube. This extraction was repeated one more with another added 500 μL EtAc. Collected solution was mixed with vortexing for 10 sec, and 500 μL was transferred to a labeled glass gas-chromatography (GC) vial. For methylation, 50 μL of methanol:HCl (ratio 9:1) was mixed to the extracted EtAc in vial. And then 50 μL of Trimethylsilyl(TMS)-diazomethane solution in 2.0 M hexanes was injected. This vial was taken to GC analysis.

GC analysis was performed on Agilent 7890A GC-FID through DB-WAX column (30 m × 0.320 mm × 0.50 μm, Agilent, USA). The oven temperature was that initial temperature hold 120 ℃ for 2 min, ramp 1 run 150 ℃ for 4 min and ramp 2 run 220 ℃ for 15 min. Fatty acid methyl esters mix (GLC-20, GLC-70, GLC-80, GLC-90, SUPELCO, USA) and hexanoic acid (Sigma-Aldrich, USA) were used as standards.

3. Results

3.1 Expression of acyl-CoA oxidase2 and thioesterases

In order to observe expression of protein, SDS-PAGE was performed on 10 % acrylamide gel. Acyl-CoA oxidase2 and thioesteraseⅡ (TesB) was expressed in pBbE6k vector, and ChfatB2 was expressed in pBbB6c vector. The expression of MGREA2, MGREBA2 and MGRECA2 strain was performed with control as MGRE strain. The SDS-PAGE results are shown in Figure 3. The size of Aox2 is 78.7 kDa, 'TesA is 20.5 kDa, TesB is 32.0 kDa and ChfatB2 is 45.7 kDa.

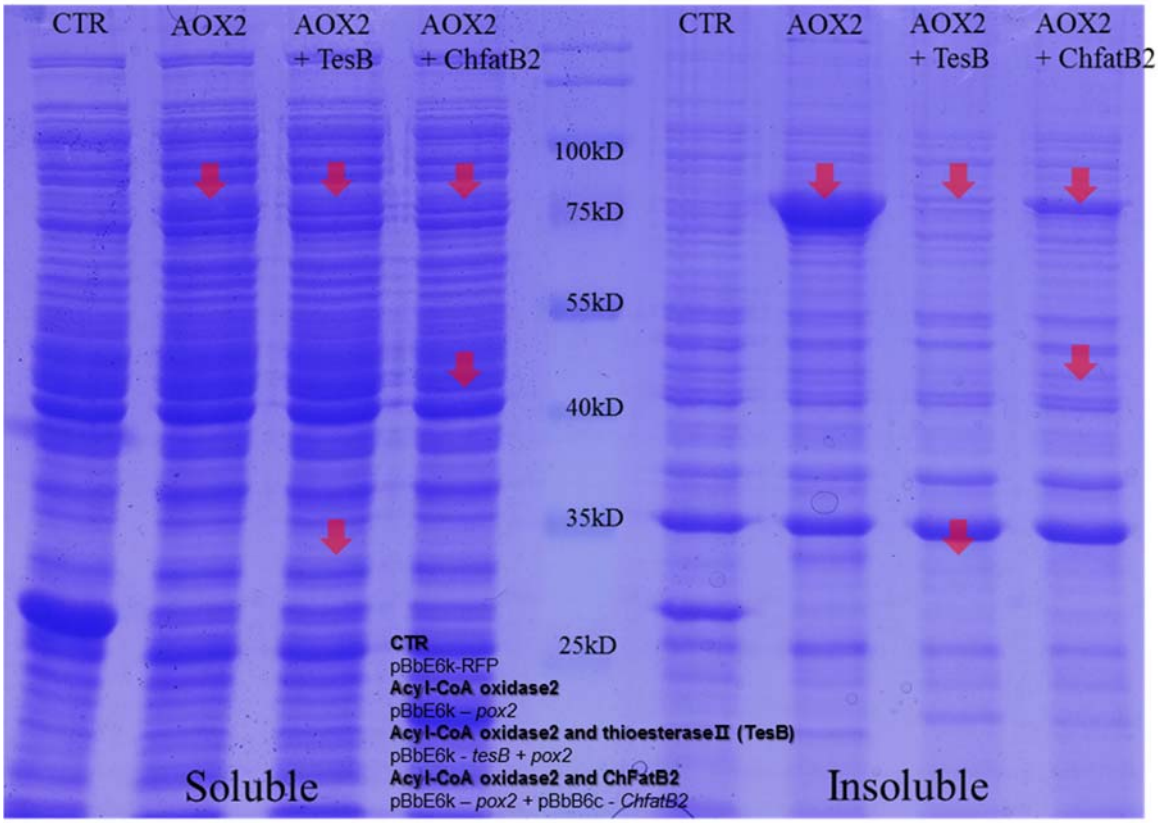

Figure 3. SDS-PAGE of total proteins of engineered *E. coli*

CTR indicates RFP expression as control, AOX2 indicates Aox2 expression, AOX2+TesB indicates Aox2 and TesB expression and AOX2+ChfatB2 indicates Aox2 and ChfatB2 expression. Red arrow indicates protein

3.2 Growth of the engineered strains

Growth of the engineered strain was to confirm growth effect by genetic modification. These tests was performed in M9 medium and LB broth medium respectively. The M9 medium contain 0.1 % oleic acid and 0.05 % glucose. The reason of glucose addition is that *fadE* knockout strain has defective growth on the condition of fatty acids, thus it difficult to induction of expression protein. Except for MGR strain as control, all strains showed growth deficiency in M9 medium with oleic acid (Fig.4A). In the LB broth condition, all strains showed fine growth. However, it was observed that the growth of MGRECA2 strain is slower than other strains. This strain has two kinds of plasmids. Therefore, two kinds of antibiotics (50 μg/mL of kanamycin and 30 μg/mL chloramphenicol) was injected in the medium. This result is decided from effect of antibiotics[30]. After 48 hours, all strain showed similar growth peaks.

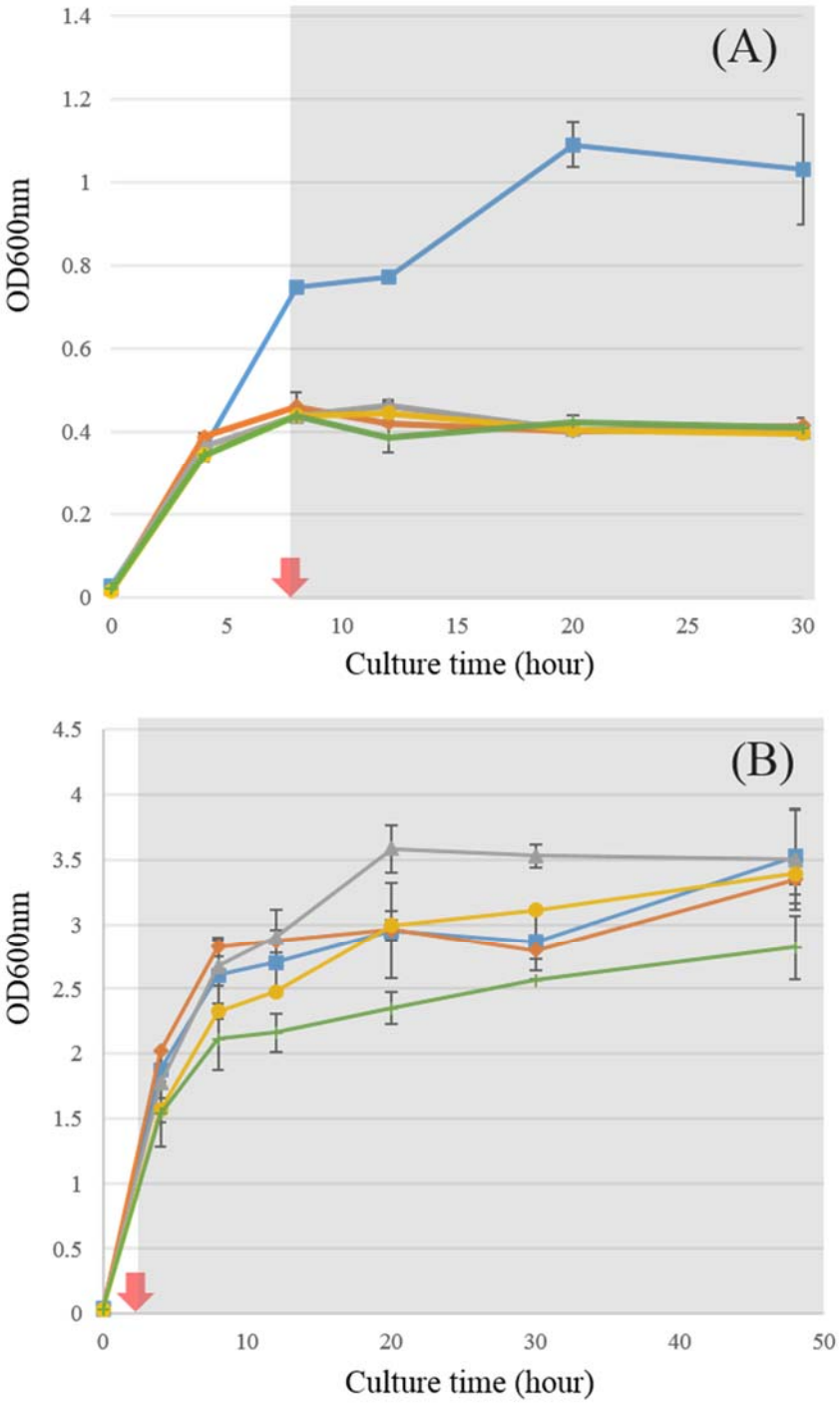

Figure 4. Cell growth in M9 medium with 0.1 % oleic acid and 0.05 % glucose (A) and in LB broth (B)

MGR (square, sky blue), MGRE (diamond, orange), MGREA2 (triangle, grey), MGREBA2 (circle, yellow), MGRECA2 (+, green) was measured. Red arrow indicates addition IPTG for expression induction. Grey box signify at 30 ℃ incubation. Error bars indicate the standard deviation of experiments performed in triplicate.

3.3 Comparison between *fadE* knockout and *fadR*/*fadE* double knockout strain

In order to form hexanoic acid from long chain fatty acids, biotransformation on LB broth with 0.1% oleic acid was performed, because all strain of *fadE* knockout has growth deficiency on sole carbon source of fatty acids. Before the biotransformation, because the composition of product as hexanoic acid could be affected by the growth on LB medium, pure LB as not adding any carbon source was used for culture during 48 hour about all strain in this study. This results is no detection of product as hexanoic acid. In the LB medium condition, whole strain was grown well, 0.1 % oleic acid was injected shortly before stationary phase. Zero time point for measurement of titer hexanoic acid is injection time. In order to confirm the influence from *fadR* deletion, MGE and MGREBA2 strain was selected with control strains. Generally, FadR work on repressor *fad* genes as related β-oxidation[25].

MGR strain, which is *fadR* gene deleted, showed fine growth on oleic acid as sole carbon source. In comparison with MG1655 strain as wild type, a considerable difference was observed (Fig. 5A). Wild type strain showed beginning growth after 48 hours, because it does take time to adaptation for regulation of β-oxidation while inactivated FadR is not.

In order to confirm the effect of *fadR* deletion for Aox2 activity, MGEA2, which is acyl-CoA dehydrogenase removed, and MGREA2, which is also FadR removed from MGEA2, strains was used for biotransformation in LB broth with 0.1 % oleic acid. The results showed that MGREA2 was produced more hexanoic acid than MGEA2 after 48 hours reaction (Fig. 5B). MGREA2 was converted to approximately 110 mg/L of hexanoic acid from 1 g/L of oleic acid, whereas MGEBA2 showed approximately 80 mg/L of hexanoic acid converted during 72 hours. Moreover, MGREA2 strain formed hexanoic acid faster than MGEA2. This results supported that hexanoic acid production strain based on *fadR* deletion.

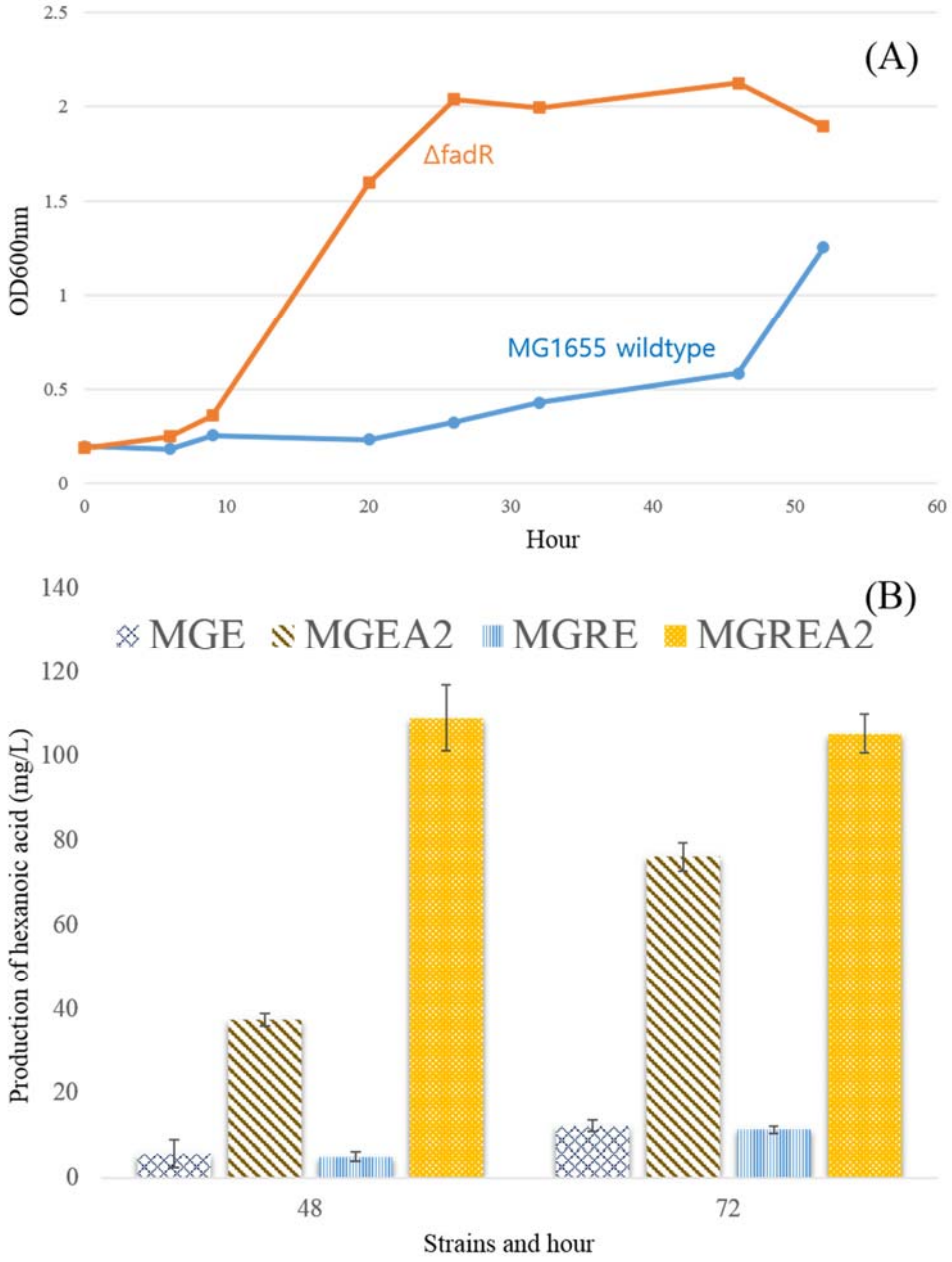

Figure 5. Growth curve between wild type strain and *fadR* knockout strain (A), biotransformation to hexanoic acid from oleic acid in LB broth (B)

Growth curve was measured in OD600 nm. MG1655 strain (circle, sky blue) and MGR strain (square, orange) was cultured on M9 medium with fatty acid with 0.5 % oleic acid (A). Biotransformation was performed in LB medium with 0.1 % oleic acid. Error bars indicate the standard deviation of experiments performed in triplicate. X-axis indicates used strains and reaction time of each strain. Y-axis indicates titer (mg/L) of hexanoic acid (B).

3.4 Biotransformation to hexanoic acid

Before biotransformation, the hexanoic acid titer between supernatant and whole medium was checked for measurement of product secretion out of cell. This experiment was conducted through whole cell biotransformation using MGREA2 strain on M9 medium with 0.1 % oleic acid. In order to measure the secretion of hexanoic acid to medium from cell, identical sample, which is reacted during 48 hours by biotransformation, was prepared, and then it was divide into supernatant and whole culture medium. Both of each sample were analyzed through GC/FID. The GC/FID results showed similar titer of hexanoic acid (Fig. 6A). This result supposed that hexanoic acid is diffused out cell through cell wall.

In order to compare effect of hexanoic acid production by thioesterase, MGREBA2 was used. Biotransformation in LB broth with 0.1 % oleic acid was performed for formation of hexanoic acid. MGE and MGRE as control strain hardly formed hexanoic acid (Fig. 6B). After 48 hours, MGREA2 and MGREBA2 strains showed the highest production of hexanoic acid. However, MGREBA2 strain produced hexanoic acid earlier than MGREA2 strain. Non-*fadR* knockout strain (MGEA2 and MGEBA2) also showed similar tendency (Fig. 6B).

Based on these results, whole cell biotransformation in M9 medium with 0.1% oleic acid as feedstock was conducted using MGREA2, MGREBA2 and MGRECA2 containing ChfatB2 derived *Cuphea hookeriana* with control strain of MGRE and MGR. ChfatB2 as plant thioesterase has substrate specificity for C8 to C10 acyl-CoA[31]. After 48 hours reaction, the control cells as MGR and MGRE were observed to no-production of hexanoic acid. MGREA2 containing sole of Aox2 showed approximately 75mg/L hexanoic acid production (Fig. 7A), and it also approximately 45 mg/L titer of octanoic acid production (Fig. 7B). However, this strain showed decrease titer of hexanoic acid after 24hours (Fig 7A). MGRECA2 produced approximately 140mg/L of hexanoic acid and it also formed approximately 30 mg/L of octanoic acid. In the case of MGREBA2, the hexanoic acid was produced to the highest titer (Fig. 7A). From this results, suitable strain for production of hexanoic acid was selected.

The MGREBA2 sample as the highest titer was measured for production of hexanoic acid for time unit. In this result, hexanoic acid production had been on the increase gradually for 48hours (Fig 7A). Finally, over the 200 mg/L of hexanoic acid with some of the octanoic acid was produced after 48hours reaction in *E. coli* (Fig 7B).

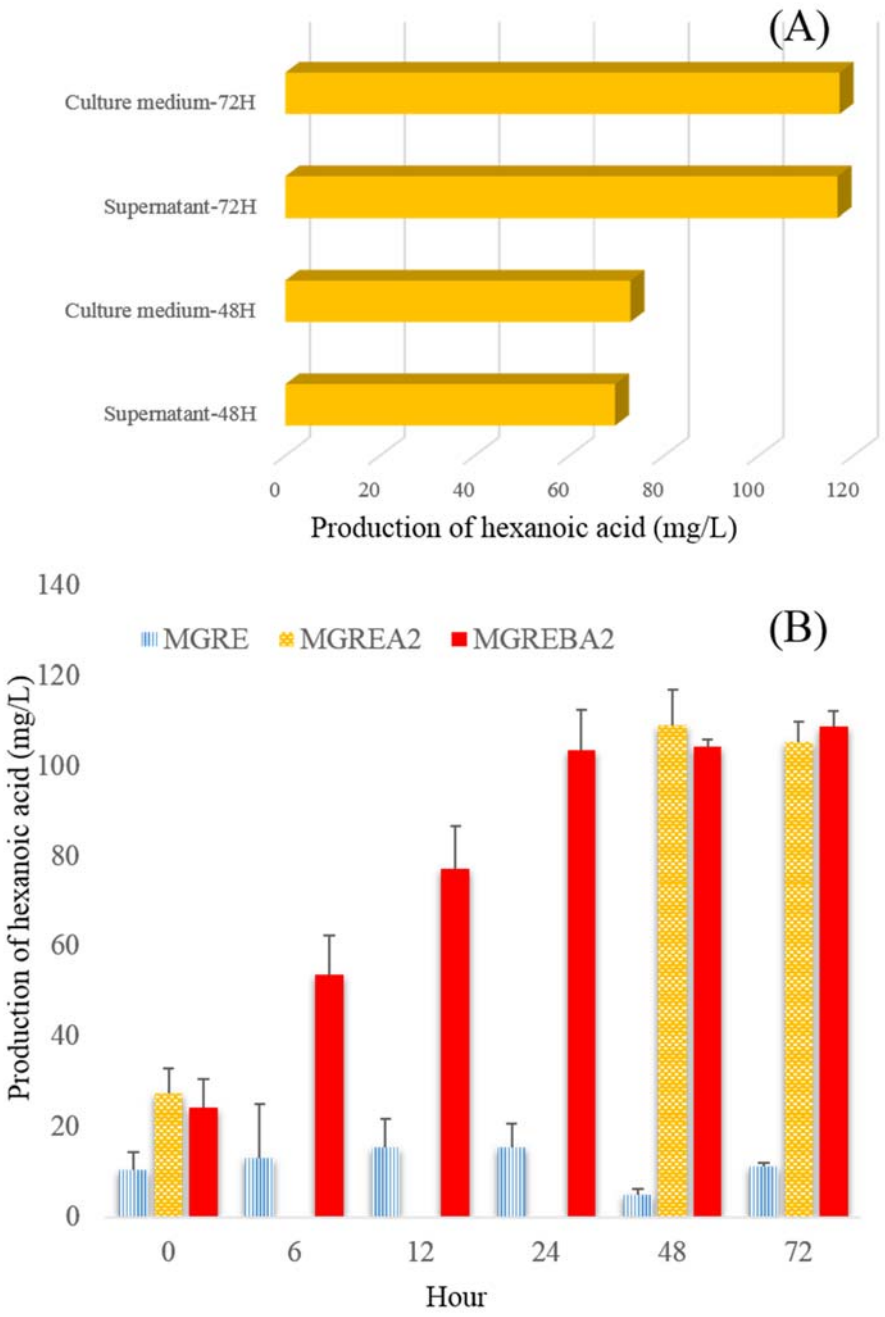

Figure 6. Hexanoic acid titer of supernatant and culture medium (A), production of hexanoic acid by biotransformation in LB broth (B)

On the condition of biotransformation in LB, supernatant and whole culture medium was measured to titer of hexanoic acid using MGEA2 strain (A). The titer of hexanoic acid from biotransformation in LB with 0.1% oleic acid was measured for time unit. Error bars indicate the standard deviation of experiments performed in triplicate. X-axis indicates reaction time of each strain. Y-axis indicates titer (mg/L) of hexanoic acid (B).

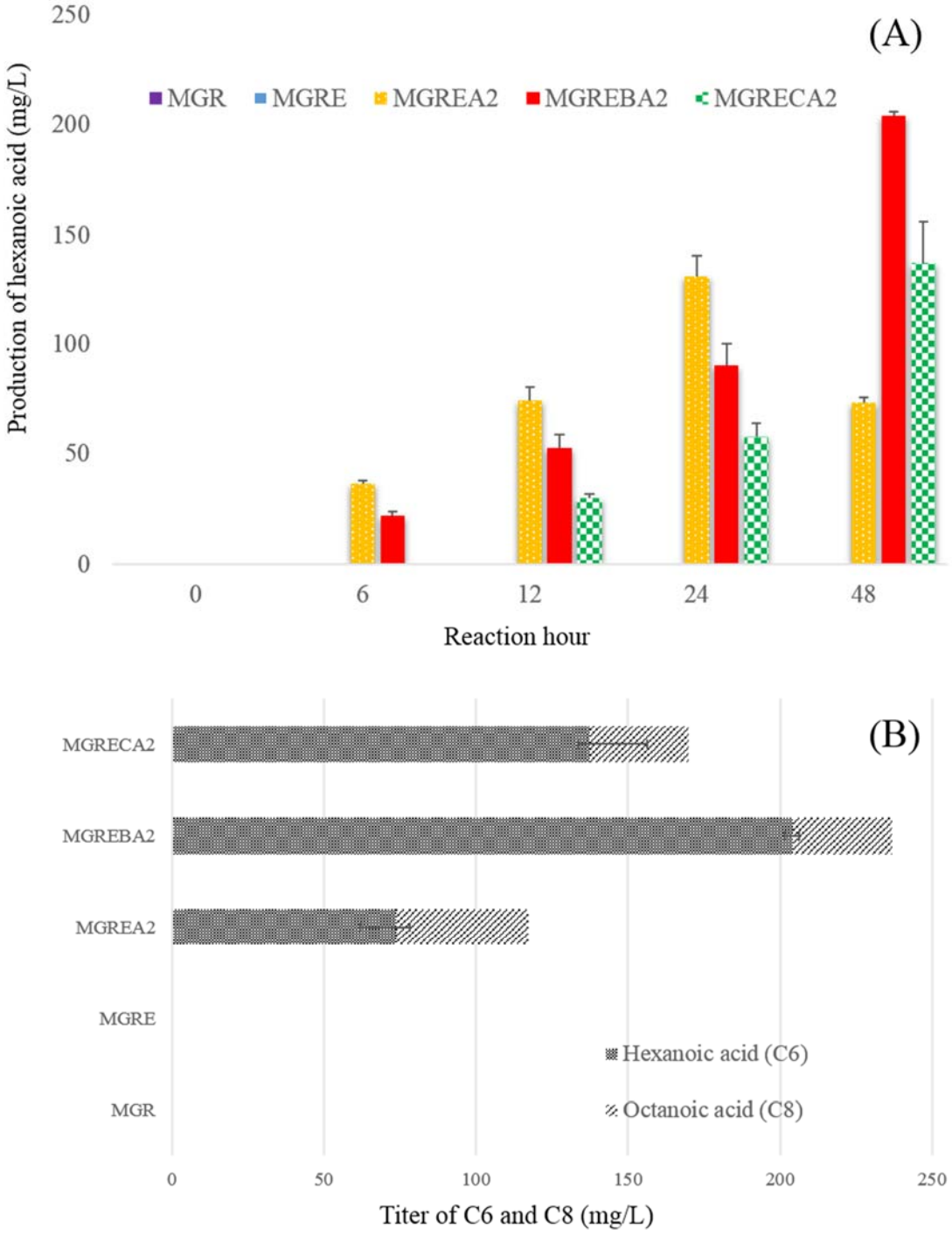

Figure 7. Production of hexanoic acid by biotransformation in M9 medium

The titer of hexanoic acid from biotransformation in M9 with sole carbon source of 0.1 % oleic acid was measured for time unit. Error bars indicate the standard deviation of experiments performed in triplicate. X-axis indicates reaction time of each strain. Y-axis indicates titer (mg/L) of hexanoic acid (A). Total production of hexanoic acid and octanoic acid was measured at 48 hour. Error bars indicate the standard deviation of experiments performed in triplicate. X-axis indicates titer (mg/L) of hexanoic acid and octanoic acid. Y-axis indicates strain name (B).

4. Discussion

In this study, we engineered β-oxidation pathway in order to specifically produce hexanoic acid. Many of existing studies about fatty acids production have been focused on engineering of the fatty acid synthetic pathway using feedstock derived carbohydrate [6c, 6d, 11]. However, SCFAs production through this pathway engineering has limitation as control of fatty acid chain length under 8. Because it is regulated depending on acyl-ACP thioesterase specificity[6c]. Therefore, we targeted to the production of hexanoic acid from long chain fatty acid. In order to fortify the foundation that various chemical derived fatty acid will be produced using waste oil in the future study, this study was began.

In order to produce hexnoic acid, heterologous expression of Aox2 from *Y.Lipolytica* in *E. coli* firstly. Then thioesterase Ⅱ was overexpressed in engineered *E. coli*, that hexanoyl-CoA from shortening limit product of Aox2 convert to hexanoic acid[23]. Unfortunately, whole *fadE* knockout strains showed growth deficiency on the sole carbon source of fatty acid (Fig. 4A). Therefore, biotransformation was performed in order to confirm the enzyme activity. Biotransformation was progressed on LB broth condition and M9 medium condition separately with 0.1 % oleic acid. In case of biotransformation in LB broth, the reaction was started shortly before stationary phase after expression induction. Because sufficient amount of the cell is required for *in vivo* reaction using exogenous fatty acid. Through biotransformation in LB broth, the formation of hexanoic acid was confirmed from oleic acid (Fig. 5B). In addition, the result in pure LB cultivation showed no-hexanoic acid production, it could support hexanoic acid formation from oleic acid. From biotransformation LB, it was confirmed also that Aox2 has limitated substrate specificity for hexanoyl-CoA. Finally, we produced over the 200 mg/L of hexanoic acid from oleic acid by biotransformation method in M9 medium (Fig 7A, B).

4.1 Effect of hexanoic acid production for growth

SCFAs such as hexanoic acid have been reported that it diffused out of the cell not accumulated on the inside of the cell[4]. In the result of comparison between supernatant and whole culture medium, mostly hexanoic acid was detected on the supernatant part (Fig. 6A). This result could support to reported theory, which short. In contrast with yeast species, SCFAs have higher toxicity because of its membrane leakage. Therefore, *E. coli* is negatively affected on SCFA condition, it is caused to cell death consequently. This problem is critical for produce hexanoic acid. It is considered that growth deficiency on sole carbon source of fatty acids is caused by SCFA toxicity (Fig 4A). Therefore, adaptation will be performed on SCFA condition in further study, thereby this problem will overcome by modification of the lipid composition on the cell membrane[4].

4.2 Effect of *fadR* knockout on hexanoic acid production

Through biotransformation through LB broth, the titer of hexanoic acid was measured between MGREA2 and MGEA2 strain. Resultingly, MGREA2 strain, which is *fadR* deleted, showed higher titer of hexanoic acid (Fig. 5B), and it is considered to cause by major repressor removal of β-oxidation. Due to *fadR* deletion, fatty acid transporter (FadL) has no effect by FadR protein. Therefore, higher amount hexanoic acid was produced through enzyme reaction within cell because *fadR* knockout strain could take more oleic acid than non-*fadR* deleted strain[32]. However, final titer was limitation because cell death was caused (Fig. 6B).

4.3 Effect of thioesterase Ⅱ (TesB) on hexanoic acid production

In order to find suitable thioesterase for producing hexanoic acid, TesB and ChfatB2 was separately expressed with Aox2, then hexanoic acid production titer was measured by GC/FID. As a result, the best strain of hexanoic acid was MGREBA2 containing Aox2 with TesB (Fig. 6, 7), it is considered that hexanoic acid production relate to substrate specificity of these enzymes. In case of TesB, it has been reported that the only thioesterase have broad chain length specificity including C6-CoA to C18-CoA in *E. coli* [22-23]. Moreover, Aox2 derived *Y.Lipolytica* has substrate specificity, which drop down remarkably on hexanoyl-CoA[33]. Because of this, it is assumed that entered oleic acid to β-oxidation cycle shorten to hexanoyl-CoA after 6cycles, then this limited production by Aox2 convert to hexanoic acid by overexpressed TesB inside of cell. This hexanoic acid diffuse out of the cell through membrane.

MGREBA2 strain showed the increase titer of hexanoic acid production in process of time (Fig. 6B). On the other hand, MGREA2 strain showed a sudden increase after 24 hours reaction (Fig. 6B). It is assumed that acyl-CoA accumulation is caused by Aox2. MGREBA2 strain could faster conversion to fatty acid from acyl-CoA, because it has overexpressed TesB. Moreover, MGREA2 strain showed decrease titer of hexanoic acid after 48 hours in LB result (Fig 6B), M9 result was also observed to decrease titer of hexanoic acid after 24 hours (Fig 7A). In this case, it is assumed that hexanoic acid shorten to butanoic acid and acetyl-CoA in β-oxidation without overexpressed TesB, because Aox2 has very little substrate specificity for C6. In the presence of overexpressed TesB, hexanoyl-CoA could convert to hexanoic acid better. Therefore, in the condition of C6- acyl-CoA presence inside the cell, this hexanoyl-CoA could react by TesB than Aox2, this supposition is supported by the results of MGRECA2 strain (Fig. 7A). MGRECA2 containing ChfatB2, which has substrate specificity for C8 to C10, slowly produced to hexanoic acid in result on M9. It is considered that octanoyl-CoA shortened

oleic acid in β-oxidation could catalyze by ChfatB2 to octanoic acid, it also react by Aox2 inside of the cell. It is assumed that the cause of slow production in MGRECA2 strain occurred at conversion from C8 to C6 in β-oxidation because ChfatB2 compete with Aox2 at that moment.

4.4 Future research plan

In order to apply for strain of industrial use, growing cell is necessary. In this study, all *fadE* knockout strain showed growth deficiency on sole carbon source of oleic acid. Therefore, future work will be focused on construction of growing cell. The growth deficiency is considered to two causes. 1) SCFAs has toxicity to cell, because it can membrane leakage by diffusion. Therefore, these SCFAs could negative effect for cell growth by damage of membrane integrity[4]. 2) Acyl-CoA oxidase form hydrogen peroxide from its output of oxidation catalysis. This has also toxicity to cell and negative effect for cell growth[34]. Therefore, future research work will be proceeded to overcome this problem. The strategy of overcoming SCFAs toxicity problem is that adaptation will be performed on the condition of SCFAs. Because adapted *E. coli* strain was reported that the negative effect of cell growth is decreased by their membrane lipid composition change. Thereby, the composition of unsaturated lipid increase and the average lipid length increase[4]. Furthermore, we will conduct adding thiourea in the culture medium for overcoming hydrogen peroxide toxicity. It was reported that *E. coli* is partially protected against the lethal effects of hydrogen peroxide by pretreatment with thiourea[35].

5. Conclusions

In conclusion, although wild type *E. coli* could not form hexanoic acid by β-oxidation, we produced hexanoic acid in E. coli through β-oxidation engineering. Aox2 is key enzyme for produce hexanoic acid, because it has substrate specificity for long chain fatty acyl-CoA as very low activity on C6. Using the strain containing *fadR* knockout with Aox2 produced approximately 75 mg/L of hexanoic acid, and then thioesterase Ⅱ overexpression strain as *fadR* knockout and Aox2 contained produced increase the 2.5 fold as over the 200 mg/L of hexanoic acid. In this study, we constructed basis strain related for production of chemicals derived fatty acid. However, this strain showed growth deficiency on sole carbon source of fatty acids. Therefore, further study focused on overcoming this growth problem. In order to overcome, 1) SCFAs adaptation will be performed for overcoming SCFA toxicity and 2) thiourea will be added to culture medium for elimination of hydrogen peroxide in the cell.

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