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RESEARCH PAPER

Production of *cis*-Vaccenic Acid-oriented Unsaturated Fatty Acid in *Escherichia coli*

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Abstract Biodiesel is produced worldwide as an alternative energy fuel and substitute for petroleum. Biodiesel is often obtained from vegetable oil, but production of biodiesel from plants requires additional land for growing crops and can affect the global food supply. Consequently, it is necessary to develop appropriate microorganisms for the development of an alternative biodiesel feedstock. *Escherichia coli* is suitable for the production of biodiesel feedstocks since it can synthesize fatty acids for lipid production, grows well, and is amenable to genetic engineering. Recombinant *E. coli* was designed and constructed for the production of biodiesel with improved unsaturated fatty acid contents *via* regulation of the FAS pathway consisting of initiation, elongation, and termination steps. Here, we investigated the effects of *fabA, fabB,* and *fabF* gene expression on the production of unsaturated fatty acids and observed that the concentration of *cis*-vaccenic acid, a major component of unsaturated fatty acids, increased 1.77-fold compared to that of the control strain. We also introduced the genes which synthesize malonyl-ACP used during initiation step of fatty acid synthesis and the genes which produce free fatty acids during termination step to study the effect of combination of genes in elongation step and other steps. The total fatty acid content of this strain increased by 35.7% compared to that of the control strain. The amounts of unsaturated fatty acids and *cis*-vaccenic acid increased by 3.27 and 3.37-fold, respectively.

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1. Introduction

Biodiesel is a mono-alkyl ester of long-chain fatty acids commonly derived by the transesterification of vegetable oils or animal fats. Feedstocks vary by country and region. Soybean oil (USA), rapeseed oil (Europe), and palm oil (equator region) have been used as vegetable oil feedstocks. Among these renewable resources, rapeseed oil consisting of 65% oleic acid accounts for 80% of biodiesel feedstocks worldwide [1]. However, there is currently a need to substitute vegetable oils with other raw materials due to problems such as additional farmland requirements and concerns over rising grain prices. Biodiesels must satisfy the standard requirements set forth by ASTM D6751 or EN 14214 before being used as a transportation fuel. Biodiesel properties are determined based on fatty acid profiles such as chain length and number of double bonds. By increasing saturated fatty acid content, biodiesels can have a shorter ignition time for combustion, higher oxidative stability, and higher viscosity. An increase in the proportion of unsaturated fatty acids content lowers the freezing point of biodiesel, whereas a higher number of double bonds reduces oxidative stability [2]. In terms of fatty acid composition, it has been proposed that oleic acid methyl ester is a suitable major component of biodiesel. However, palmitoleic acid methyl ester has the advantage of a lower freezing point compared to oleic acid methyl ester [3,4]. The properties of biodiesel depend on the fatty acid profiles of biomass feedstocks. Several researchers have investigated and attempted to overcome the limits of raw materials. Genetic or other methods were proposed to modify the

fatty acid profiles of biomass [5].

The lipid content of *E. coli* is approximately 10% [6]. Biosynthesis of fatty acids and regulation in *E. coli* has been investigated by many researchers [7,8]. Although the lipid content of *E. coli* is lower than that of algae, yeast, or fungi, it is suitable for genetic modification to achieve increased lipid content since information on its genome as well as host strains and cloning vectors are widely available. Further, *E. coli* grows rapidly on inexpensive medium. In contrast to plants or other bacteria, *E. coli* does not possess desaturase which is involved in the synthesis of fatty acids containing two or more double bonds [9,10]. The fatty acid composition of *E. coli* includes saturated fatty acids (miristic acid and palmitic acid), unsaturated fatty acids (palmitoleic acid and *cis*-vaccenic acid), and cyclopropane fatty acids (*cis*-9,10-methylene hexadecanoic acid and phytomonic acid) derived from unsaturated fatty acids. The total contents and compositions of fatty acids are influenced by the culture conditions. Fatty acid synthesis consists of three steps: initiation, elongation, and termination steps (Fig. 1). In the initiation step, acetyl-CoA is carboxylated by four subunits of AccABCD to form malonyl-CoA, which is then converted into malonyl-ACP. Fatty acid synthesis is initiated by β-ketoacyl-ACP synthase III (FabH), which condenses acetyl-CoA with malonyl-ACP to form β-ketoacyl-ACP. This product is reduced by β-ketoacyl-ACP reductase (FabG) into β-hydroxyacyl-ACP, which acts a substrate for β-hydroxyacyl-ACP dehydrases (FabA or FabZ). FabA is a bifunctional enzyme that catalyzes the removal of water to produce *trans*-2-decenoyl-ACP as well as the isomerization of intermediates to form *cis*-3-decenoyl-ACP [11]. The final reaction is carried out by enoyl-ACP reductase (FabI), and subsequent rounds of elongation cycles are initiated by β-ketoacyl-ACP synthase I (FabB) and β-ketoacyl-ACP synthase II (FabF). The elongation step terminates when the acyl-ACP chain reaches 14 ~ 18 carbons, and thioesterases (TesA and TesB) release fatty acid chains from acyl carrier protein to produce free fatty acids. Fatty acid biosynthesis occurs in the cytosol of *E. coli* while TesA is located in the periplasm. Consequently, free fatty acids are not overproduced even though TesA is overexpressed. To solve this problem, many researchers have assessed the efficacy of heterologous thioesterase genes as well as genetically mutated '*tesA* lacking the leader sequence of *tesA* of *E. coli.* 'TesA is maintained in the cytosol and is involved in the release of large amounts of free fatty acids [12]. Cyclopropane fatty acids, *cis*-9,10-methylene hexadecanoic acid and phytomonic acid, are the cyclopropanation products of structurally homologous unsaturated fatty acids such as palmitoleic acid and *cis*-vaccenic acid [8,13,14].

Prior studies on fatty acid biosynthesis metabolism have studied the introduction of various thioesterase genes for the modification of fatty acid profiles as well as the regulation of synthetic pathways for improvement of fatty acid production. Introduction of heterologous thioesterases from plants such as *Cinnamomum camphora* (14:0-ACP), *Umbellularia califonica* (12:0-ACP), and *Arabidopsis thaliana* (18:0-ACP) has been studied by several researchers [10]. Other bacterial thioesterase genes from *Streptococcus pyogenes* have been assessed to alter the amounts and compositions of fatty acids [15]. Codon-optimized *Streptococcus pyogenes* acyl-ACP thioesterase was previously studied to assess the utility of codon-optimized heterologous acyl-ACP thioesterase genes in *E. coli* [16]. Regarding the regulatory genes involved in fatty acid synthesis, overexpression of four genes encoding endogenous ACC (acetyl-CoA carboxylase encoded by *accA*, *accB*, *accC*, and *accD*) in the initiation step of the FAS pathway resulted

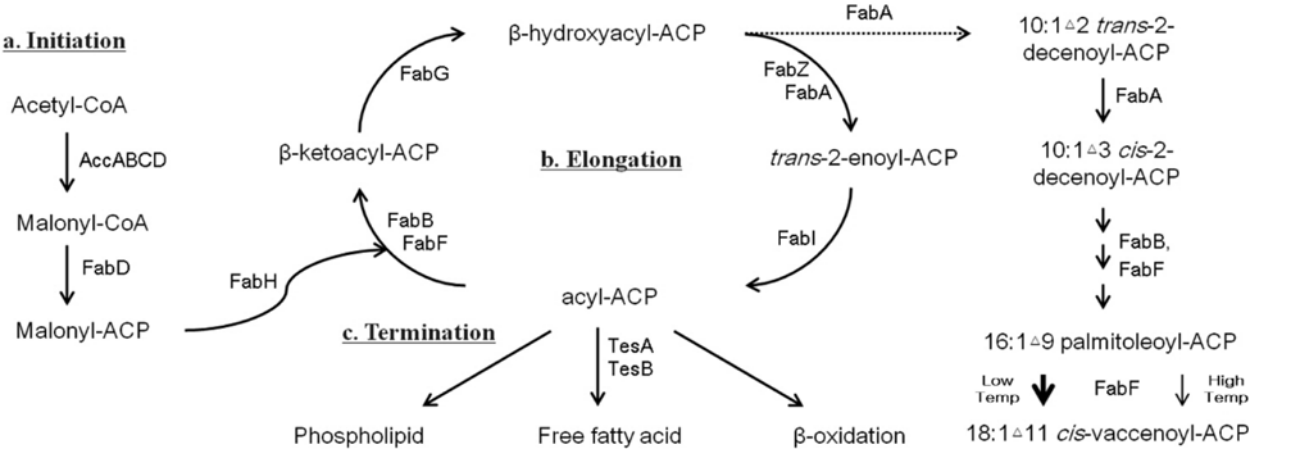

Fig. 1. General fatty acid biosynthesis in *E. coli.* This pathway is divided up into initiation, elongation, and termination steps. Unsaturated fatty acid pathway in the elongation step is indicated by using round dotted arrows, separately. (CoA: coenzyme A, ACP: acyl carrier protein).

in increased fatty acid productivity [17]. Introduction of *accA* and *fabD* from *Pseudomonas aeruginosa* PA14 for heterogeneous expression was investigated [15], and an increase in unsaturated fatty acid content was reported upon overexpression of *fabA* and *fabB*, which are involved in the elongation step [8]. However, the effects of combination of each step in the FAS pathway of *E. coli* on fatty acid profiles is not elucidated.

In this study, we investigated the feasibility of *E. coli* for the production of biodiesel by increasing production of total fatty acids, unsaturated fatty acids, and especially *cis*-vaccenic acid based on regulation of genes involved in the fatty acid biosynthesis pathway in *E. coli*. To enhance production of total fatty acids, targeted genes for the recombinant plasmid were *accA*, *accB*, *accC*, *accD*, and *fabD* in the initiation step as well as *'tesA* lacking the leading sequence of *tesA* in the termination step. Genes in the elongation step, *fabA* and *fabB*, were used for the synthesis of unsaturated fatty acids, and *fabF* was used to produce *cis*-vaccenic acid at low temperatures. We constructed three plasmids according to the three stages of the fatty acid synthesis pathway and introduced them into *E. coli* using various combinations.

2. Materials and Methods

2.1. Strains and culture condition

Target genes were amplified by polymerase chain reaction (PCR) using genomic DNA of *E. coli* K-12 MG1655 (ATCC 47076, USA). *E. coli* XL 1-Blue (Stratagene, USA) was used as a competent strain for plasmid construction and maintenance. *E. coli* BL21(DE3) was used as a host strain for the expression of target genes. Recombinant *E. coli* BL21(DE3) was grown in 200 mL of Luria-Burtani medium by using shake flasks. Kanamycin (30 μg/mL), ampicillin (100 μg/mL), and streptomycin (50 μg/mL) were used for the selection of recombinant plasmids and were purchased from Sigma-Aldrich, USA. One percent inoculum was used for bacterial culture, and cells were grown at 37°C and 200 rpm. When cells reached an optical density of about 0.6 ~ 0.8 at a wavelength of 600 nm, isopropyl *β*-D-1-thiogalactopryanoside (IPTG) (Acros Organics, USA) was added to the medium at a final concentration of 1 mM. After induction, culture temperature was changed to 17°C to increase the unsaturated fatty acid contents of *E. coli*.

2.2. Plasmid construction

Expression vectors used in this study were pCOLADuet™-1 vector (Merck, Germany), pEcoli-Nterm 6xHN vector (Clontech, USA), and pCDF-1b vector (Merck, Germany). Vectors were used for the expression of related genes involved in the steps of initiation, elongation, and

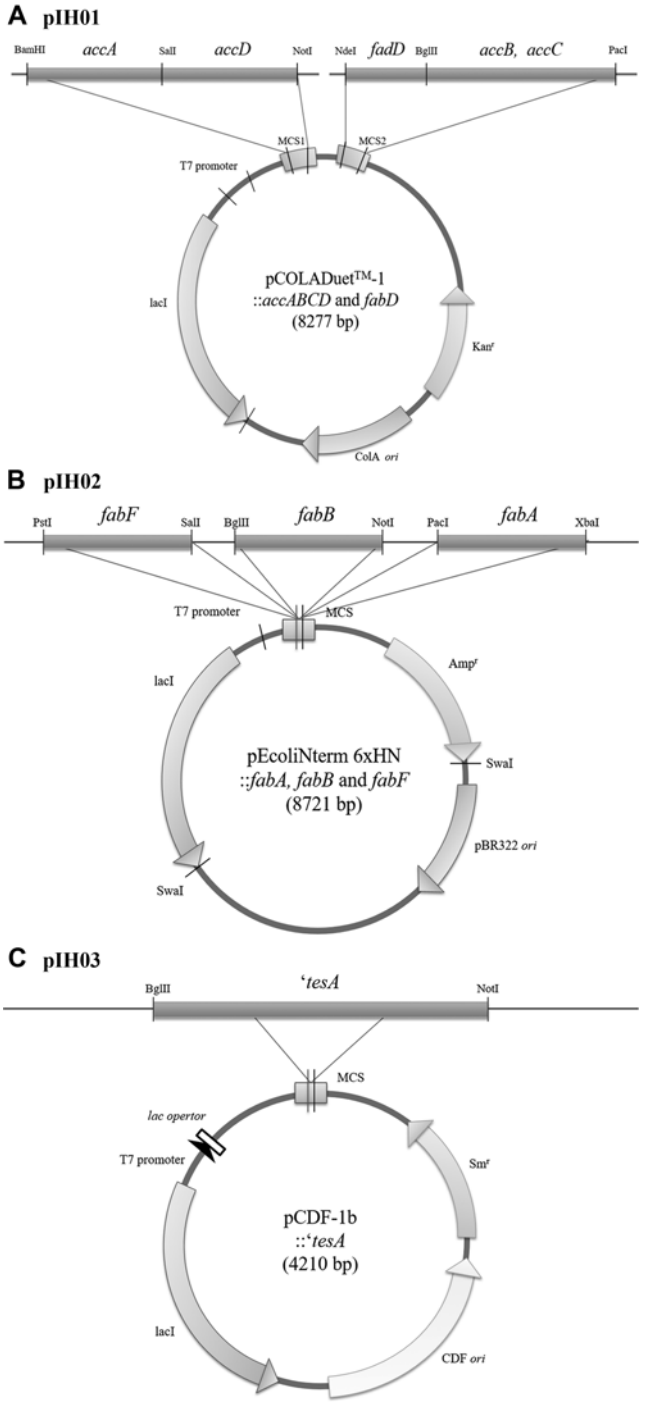

Fig. 2. Plasmid construction in this study. (A) Plasmid pIH01 contains kanamycin resistance genes as well as *accA*, *accB*, *accC*, *accD*, and *fabD*, which perform the initiation step. *accB* and *accC* were obtained by PCR simultaneously since the two regions are contiguous to one another. (B) Plasmid pIH02 possesses ampicillin resistance genes as well as *fabA*, *fabB*, and *fabF*. (C) pIH03 harbors streptomycin resistance genes and mutated *tesA* gene (named *'tesA*), which conducts the termination step.

termination. Genomic DNA was isolated from *E. coli* K-12 MG1655 using a Core-One™ Bacterial Genomic DNA Isolation Kit (CoreBio Co., Ltd., Korea). Information on target genes (*accA*, *accB*, *accC*, *accD*, *fabD*, *fabA*, *fabB*, *fabF*, and *tesA*) was obtained by KEGG [18], and primers were prepared by Cosmo Genetech (Korea). DNA polymerase used was TaKaRa Ex Taq™ (TaKaRa Biotechnology Co., Ltd., Japan), and nine genes were amplified by PCR. QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, USA) was used to delete the leader sequence from *tesA*, and used oligonucleotides were F-5' GTTAAC CTTCCGTGCCGCTGCAGCGGACACGTTATTG 3' and R-5' CAATAACGTGTCCGCTGCAGCGGCACGGAA GGTTAAC 3' (mutation underlined) [12].

All enzymes for cloning were purchased from NEB (New England Biolabs Inc., USA), and plasmid DNA was isolated by using a LaboPass Plasmid Mini Purification Kit (Cosmo Genetech, Korea). Detailed molecular cloning techniques were performed as described by Sambrook and David [19], and recombinant plasmids used in this study are shown in Fig. 2.

2.3. Fatty acids analysis

Analysis of prepared fatty acids was performed using the method described by Lepage *et al.* [20]. Cells were harvested by centrifugation and dried in a freeze-dryer (Ilshin Lab. Co., Korea). Dried cells were resuspended with 3 mL of sulfuric acid-methanol (5:100) solution. Nitrogen gas was added for methylation at 90°C, and the reaction was performed for 30 min. The sample was mixed with 800 μL of hexane after methylation. After centrifugation at 3,000 rpm for 5 min, the reagent mixture was separated into two layers. The upper phase was used for analysis of fatty acid methyl esters (FAMEs) with the GS Agilent 6890 N (Agilent Technologies, USA) System. Column used for the analysis of fatty acids was an HP-INNOWax capillary column (30 m × 0.25 mm × 0.25 μm) (Agilent Technologies, USA), and the carrier gas was helium. The injection port temperature was 250°C, and the temperature of the FID detector was 280°C. The oven temperature program was as follows: held initially at 120°C for 1 min after injection, increased to 250°C at a rate of 10°C/min for 13 min, and held at 250°C for 1 min. GC standards were purchased from SIGMA (Sigma-Aldrich Co., USA). Most FAMEs were identified by comparing the retention times of samples to those of standards. The FAME standards were lauric acid methyl ester (C12:0), myristic acid methyl ester (C14:0), myristoleic acid methyl ester (C14:1), palmitic acid methyl ester (C16:0), palmitoleic acid methyl ester (C16:1Δ9), stearic acid methyl ester (C18:0), *cis*-vaccenic acid methyl ester (C18:1Δ11), and phytomonic acid methyl ester (C19:0 cyclo). *cis*-9,10-methylene hexadecanoic acid methyl ester (C17:0 cyclo) was obtained from bacterial acid methyl esters mix (BAMEs).

3. Results and Discussion

Many reports have shown that the properties of biodiesel are influenced by the fatty acid profiles of feedstock [21-23]. Biodiesel derived from saturated fatty acids has a high cetane number and higher oxidative stability [22,24]. On the other hand, unsaturated fatty acids have a lower cold filter plugging point. The features of ideal biodiesel are a high content of monounsaturated fatty acids, a low content of polyunsaturated fatty acids, and an appropriate level of saturated fatty acids [25]. As a result, we attempted to increase the amount of total fatty acids and content of monounsaturated fatty acids, mainly *cis*-vaccenic acid, in *E. coli*. AccABCD (encoded by *accA, accB, accC*, and *accD*), FabD (encoded by *fabD*), and `TesA (encoded by '*tesA*) were used to increase the amount of total fatty acids. FabA (encoded by *fabA*), KAS I (encoded by *fabB*), and KAS II (encoded by *fabF*) were selected since they are involved in the synthesis of unsaturated fatty acids. Strains and plasmids used in this study are described in Table 1.

Table 1. Strains and plasmids used in this study

Strains	Relevant characteristics		Reference
Control	*E. coli* BL21(DE3)		Stratagene
IH101	*E.coli* BL21(DE3) harboring pIH02		This study
IH102	*E.coli* BL21(DE3) harboring pIH01 and pIH02		This study
IH103	*E.coli* BL21(DE3) harboring pIH02 and pIH03		This study
IH104	*E.coli* BL21(DE3) harboring pIH01, pIH02, and pIH03		This study
Plasmids	Desription	Replication origin	Antibiotics
pIH01	pCOLADuet™-1 harboring *E. coli accA*, *accB*, *accC*, *accD*, and *fabD*	pColA	Kanamycin
pIH02	pEcoli-Nterm 6xHN harboring *E. coli fabA, fabB, and fabF*	pBR322	Ampicillin
pIH03	pCDF-1b harboring *E. coli `tesA*	pCDF	Streptomycin

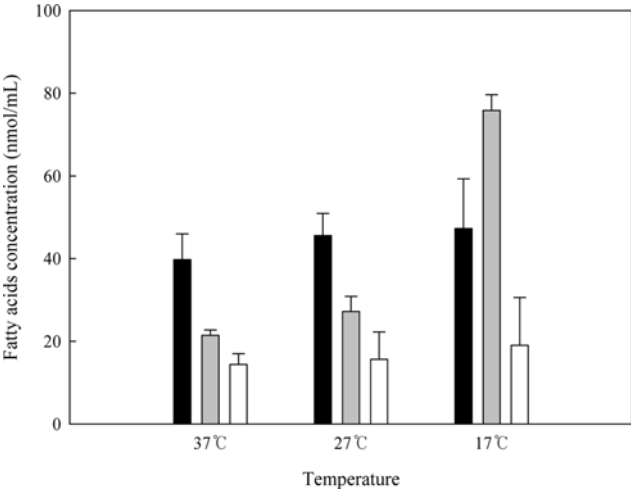

Fig. 3. Concentrations of fatty acids in IH101 strain. The black bar is the amount of palmitic acid, the gray bar is the amount of *cis*-vaccenic acid, and the white bar is the amount of phytomonic acid.

IH101 strain was used to investigate the effect of the elongation step in the FAS pathway on production of unsaturated fatty acids. Thus, we designed IH101 strain containing the three genes involved in the elongation step: *fabA* and *fabB*, which are essential for unsaturated fatty acid synthesis, and *fabF*, which regulates the fatty acid composition of the cell membrane according to temperature. IH101 strain was cultured at 37, 27, and 17°C for selecting appropriate culture temperature conditions.

Changes in the major fatty acid compositions of IH101 strain according to incubation temperature are shown in Fig. 3. Major fatty acids, including palmitic acid, *cis*-vaccenic acid, and phytomonic acid, in the fatty acid compositions of IH101 strain were more elevated at 17°C than at other temperatures. Fatty acids in IH101 strain at 17°C were composed mainly of 10.12% palmitic acid, 43.99% *cis*-vaccenic acid, and 26.56% phytomonic acid. The other fatty acids were 3.46% lauric acid, 4.21% myristic acid, 0.29% myristoleic acid, 4.09% palmitoleic acid, 5.39% cis-9, 10-methylene hexadecanoic acid, and 1.88% stearic acid. Based on a maximum 3.51-fold increase in unsaturated fatty acid content at 17°C, cultivation of cell was performed at 17°C in this study. Table 2 shows the unsaturated fatty acid contents of the control and IH101 strains during cultivation at 17°C. Changes in fatty acid profiles were analyzed by sampling at 5, 10, 15, and 20 h after induction. At 20 h after induction, the amount of total fatty acids gradually increased during the late stage of culture. The amount of unsaturated fatty acids in IH101 strain was 1.77-fold higher than that of the control strain, and the UFA:SFA ratio of IH101 strain was 2.32-fold higher than that of the control strain. At 20 h compared with 5 h after induction, saturated fatty acid content of IH101 strain had increased 1.80-fold, which was 12% less than that of the control strain (1.92-fold increase), whereas the unsaturated fatty acid content was 41% higher than that of the control strain. The amount of *cis*-vaccenic acid, which is the most abundant unsaturated fatty acid, increased from 62.52 nmol/mL to 97.35 nmol/mL (1.56-fold) from 5 to 20 h after induction. The ratio of UFA:SFA in IH101 strain at 10 h after induction was 27.2% lower than that at 12 h after induction in a study by Cao *et al*. [8], which attempted to increase the amount of unsaturated fatty acids using *fabA* and *fabB* at 37°C. However, in that study, the content of *cis*-vaccenic acid increased 1.27-fold compared to the control strain (from 30.3 to 38.5%), whereas the content of *cis*-vaccenic acid of IH101 strain in this study increased 1.61-fold compared to the control strain (from 30.98 to 49.92%). This result can be attributed to production of *cis*-vaccenic acid due to increased FabF activity at low temperature [7,26,27]. Despite low temperature, IH101 strain also produced 168.52 nmol/mL of total fatty acids at 10 h after induction compared with about 120 nmol/mL of total fatty acids produced at 12 h after induction in the previous study [8]. This result suggests that there was no restriction on production of lipids required for growth of *E. coli* since *cis*-vaccenoyl-ACP produced by FabF was synthesized as a lipid component. This can be inferred from the description of Davis and Cronan [28].

After confirming the increase in the amount of unsaturated fatty acids by genes involved in the elongation step of the FAS pathway, we investigated changes in the amount of unsaturated fatty acids during the initiation, elongation, and

Table 2. Contents of unsaturated fatty acids in the control and IH101 strain

	Control			IH101		
	UFA%	*cis*-vaccenic acid (18:1Δ11) %	UFA/SFA	UFA%	*cis*-vaccenic acid (18:1Δ11) %	UFA/SFA
5 h	40.51	36.55	1.02	60.24	50.28	1.82
10 h	34.19	30.98	0.86	55.76	49.92	1.73
15 h	26.33	24.19	0.66	51.63	47.01	1.60
20 h	23.15	21.12	0.54	48.76	44.53	1.44

UFA%; sum of unsaturated fatty acids (nmol/mL) / total fatty acids (nmol/mL) × 100 (%), *cis*-vaccenic acid (%); *cis*-vaccenic acid (nmol/mL) / total fatty acids (nmol/mL) × 100, UFA/SFA; sum of unsaturated fatty acids (nmol/mL) / sum of saturated fatty acids (nmol/mL).

termination steps. For this, we designed IH102 strain containing *accA*, *accB*, *accC*, *accD*, *fabD*, *fabA*, *fabB,* and *fabF* involved in the initiation and elongation steps of the FAS pathway, IH103 strain containing *fabA*, *fabB*, *fabF,* and '*tesA* involved in the elongation and termination steps of the FAS pathway, and IH104 strain containing *accA*, *accB*, *accC*, *accD*, *fabD*, *fabA*, *fabB*, *fabF,* and '*tesA* involved in the initiation, elongation, and termination steps of the FAS pathway. The UFA:SFA ratios of four strains (IH101, IH102, IH103, and IH104) overexpressing *fabA*, *fabB,* and *fabF* were all higher than that of the control strain (data not shown). Based on this result, it can be assumed that *fabA*, *fabB,* and *fabF* play important roles in the production of unsaturated fatty acids. Regarding each strain, IH102 strain produced less total fatty acids than other strains even though ACC, which increases the rate of fatty acid synthesis, was overexpressed (Fig. 4). This result seems to be related to the fact that accumulated acyl-ACP inhibits the activity of ACC [29]. In the case of IH103 strain, the proportion of unsaturated fatty acids was 48.85%, which was 2.11 times higher than that of the control strain. The amount of *cis*-vaccenic acid reached a stationary state at 10 h after induction, after which the production of phytonomic acid increased (Fig. 5). Based on this result, *cis*-vaccenic acid content reached a saturation point due to the genes involved in the elongation and termination steps in IH103 strain at 10 h after induction, and the amount was sufficient to react with CFA synthase as a substrate. IH104 strain, the target strain designed to express enzymes in the three stages of the FAS pathway, showed a 36% increase in the amount of total fatty acids compared with the control strain until 20 h after induction. This strain produced the highest amount of total fatty acids at 20 h after induction compared to other strains (Fig. 4). According to previous studies, overexpression of ACC and 'TesA resulted in a 100-fold increase in malonyl-CoA as well as 6-fold increase in the amount of free fatty acids [30]. Since previous studies specifically measured free fatty acids, they cannot be compared with our results. Nonetheless, the amount of total fatty acids increased by only 36% compared to the control strain in our study despite co-expression of ACC, 'TesA, and FabD, which synthesizes malonyl-ACP using malonyl-CoA as a substrate. Thus, it can be assumed there was a limitation in the FAS pathway. The amounts of total

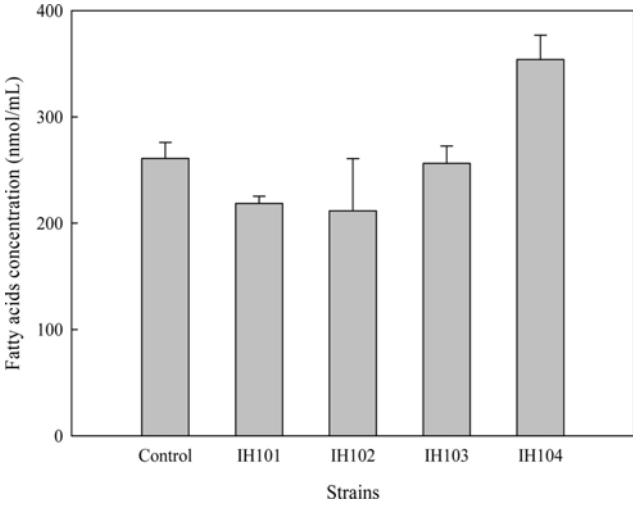

Fig. 4. Concentrations of total fatty acids in the control, IH101, IH102, IH103, and IH104 strains.

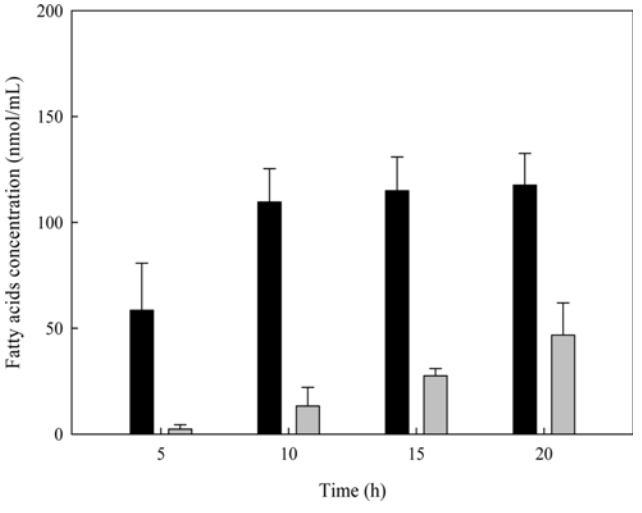

Fig. 5. Concentrations of *cis*-vaccenic acid and phytomonic acid in IH103 strain. The black bar is the amount of *cis*-vaccenic acid, and the gray bar is the amount of phytomonic acid.

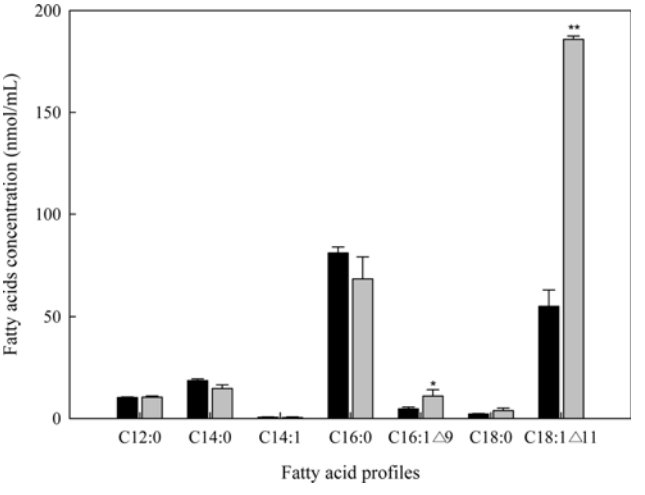

Fig. 6. Concentrations of fatty acid profiles in the control and IH104 strains. The black bar is control strain, and the gray bar is IH104 strain. * indicates $p < 0.05$ and ** indicates $p < 0.01$ compared with each fatty acid components of the control strain (C12:0: lauric acid, C14:0: myristic acid, C14:1: myristoleic acid, C16:0: palmitic acid, C16:1Δ9: palmitoleic acid, C18:0: stearic acid and C18:1Δ11: *cis*-vaccenic acid).

fatty acids and unsaturated fatty acids in IH104 strain were 353.99 and 197.21 nmol/mL, respectively, which were the highest among the five strains used in this study. The ratio of UFA:SFA in IH104 strain was 2.02, which was 3.76 times that of the control strain. The detailed fatty acid compositions compared to the control strain are shown in Fig. 6. The amount of *cis*-vaccenic acid in IH104 strain was 185.84 nmol/mL, which was 3.37-fold higher than that of the control strain, and the composition of unsaturated fatty acids significantly increased. On the other hand, the amount of *cis*-9, 10-methylene hexadecenoic acid and phytonomic acid, which are cyclopropane fatty acids in IH104 strain, decreased 0.7 times and 0.66 times, respectively, compared with the control strain. The amount of palmitic acid, the representative saturated fatty acid, in IH104 strain also decreased 0.84 times compared with the control strain. These results demonstrate an increase in the ratio of UFA:SFA. Regarding the fatty acid compositions of IH104 strain from 15 to 20 h, the amount of *cis*-vaccenic acid increased 1.28 times while the amount of phytonomic acid increased 3.25 times. This result suggests that CFA synthase activity increased since it reached a mid-stationary state in IH104 strain at 20 h after induction. This result is consistent with the reference of Chang *et al*. [31].

There have been many attempts to increase the amount of fatty acids using *E. coli* until now [32-36], but there have been few efforts to increase the amount of unsaturated fatty acids. Nevertheless, there was an attempt to increase the amount of unsaturated fatty acids by using three genes (*fabA*, *fabB*, and *AtFatA* (*Arabidopsis thaliana*)) by Cao *et al*. [8]. In that study, the content of *cis*-vaccenic acid and the ratio of UFA:SFA at 12 h after induction in a strain co-expressing three genes were 35.2% and 2.2, respectively, at 37°C. Compared with the above results, the content of *cis*-vaccenic acid and the ratio of UFA:SFA at 10 h after induction in IH103 strain overexpressing four genes (*fabA*, *fabB*, *fabF*, and '*tesA*) were 53.50% and 1.82, respectively. These differences may suggest that *fabF*, which is regulated by low temperatures, and temperature play large roles in increasing the content of unsaturated fatty acids. Moreover, despite low temperature, the strains overexpressing *fabA*, *fabB*, and *fabF* showed similar growth rates compared to the control strain. Based on this result, it can be assumed that the growth rates were not reduced since *fabB* and *fabF* were largely involved in the production of palmitic acid, palmitoleic acid, and *cis*-vaccenic acid, which are major fatty acids constituting the cell membrane. However, the contents of *cis*-vaccenic acid as well as unsaturated fatty acids did not continuously increase. Specifically, the contents of unsaturated fatty acids and *cis*-vaccenic continued to decrease from 5 to 20 h after induction in the control and IH101 strains and decreased after 20 h after induction in the IH102, IH103, and IH104 strains (data not shown). These results imply limitations to increasing the amount of unsaturated fatty acids based on temperature and the activity of FabF. We expect that the above issues can be solved through further research on FabR, a transcription factor regulating unsaturated fatty acid content [37].

The objective of this study was to improve the amount of unsaturated fatty acids and contents of *cis*-vaccenic acid by using recombinant *E. coli*, which produces fatty acids in accordance with quality standards as a raw material of biodiesel. The quality of *cis*-vaccenic acid in *E. coli* needed to replace oleic acid is not significantly different from that of oleic acid [23,38,39]. We confirmed the production and up-regulation of unsaturated fatty acids by controlling the initiation, elongation, and termination steps of the fatty acid synthesis pathway in *E. coli*.

4. Conclusion

This research investigated the effect of the elongation step in the *E. coli* FAS pathway on enhancement of unsaturated fatty acid production and *cis*-vaccenic acid content through various combinations with initiation and termination steps. The three target genes in the elongation step were *fabA* and *fabB*, which are involved in increasing the amount of unsaturated fatty acids, and *fabF*, which shows increased activity at lower temperature leading to improvement of *cis*-vaccenic acid content. Recombinant strains were cultivated at 17°C, and analysis of fatty acid profiles was performed. The content of *cis*-vaccenic acid in IH101 strain containing the *fabA*, *fabB*, and *fabF* was 50.28% (*cis*-vaccenic acid/ total fatty acids %). For improvement of fatty acid amount, recombinant strains were constructed using combinations of various recombinant plasmids such as pIH01 containing *accA*, *accB*, *accC*, *accD*, and *fabD* involved in the initiation step of the FAS pathway as well as pIH03 containing '*tesA* involved in the termination step. IH104 strain containing all plasmids showed the highest *cis*-vaccenic acid production (185.84 nmol/mL), which was 3.37 times higher than that of the control strain and approximately 94% of the total content of unsaturated fatty acids. The ratio of UFA:SFA was 2.02, which was 3.76 times higher than that of the control strain.

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