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Correlations Between FAS Elongation Cycle Genes Expression and Fatty Acid Production for Improvement of Long-Chain Fatty Acids in *Escherichia coli*

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Abstract Microorganisms have been used for biodiesel (fatty acid methyl ester) production due to their significant environmental and economic benefits. The aim of the present research was to develop new strains of *Escherichia coli* K-12 MG1655 and to increase the content of long-chain fatty acids by overexpressing essential enzymes that are involved in the fatty acid synthase elongation cycle. In addition, the relationship of β-ketoacyl-acyl carrier protein (ACP) synthase (*fabH*), β-ketoacyl-ACP reductase (*fabG*), β-hydroxyacyl-ACP dehydrase (*fabZ*), and β-enoyl-ACP reductase (*fabI*) with respect to fatty acid production was investigated. The four enzymes play a unique role in fatty acid biosynthesis and elongation processes. We report the generation of recombinant *E. coli* strains that produced long-chain fatty acids to amounts twofold over wild type. To verify the results, NAD^+/NADH ratios and glucose analyses were performed. We also confirmed that FabZ plays an important role in producing unsaturated fatty acids (UFAs) as *E. coli* SGJS25 (overexpressing the *fabZ* gene) produced the highest percentage of UFAs (35 % of total long-chain fatty acids), over wild type and other recombinants. Indeed, *cis*-9-hexadecenoic acid, a major UFA in *E. coli* SGJS25, was produced at levels 20-fold higher than in wild type after 20 h in culture. The biochemically engineered *E. coli* presented in this study is expected to be more economical for producing long-chain fatty acids in quality biodiesel production processes.

Keywords Long-chain fatty acids · FAS elongation cycle genes · FabZ · Fatty acid composition

Introduction

Recently, microbial long-chain fatty acids and their derivatives have received increased attention as an alternative source of fuel. Biodiesel is defined as containing fatty acid methyl esters (FAME) and fatty acid monoalkyl esters, and is a typical hydrocarbon fuel. It can be used instead of petroleum such as gasoline, FAME offer the advantage of being compatible

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with existing transport vehicles, and being used as jet fuel suitable for combustion systems [1–6]. Fatty acids are produced in biosynthetic reactions involving fatty acid synthase (FAS) until a molecule of 16 or 18 carbons is produced by the FAS elongation cycle [7–9].

The enzymatic reactions involved in the FAS elongation cycle are shown in Fig. 1 and are catalyzed by β-ketoacyl-acyl carrier protein (ACP) synthase (FabB, FabF, and FabH), β-ketoacyl-ACP reductase (FabG), β-hydroxyacyl-ACP dehydrase (FabA, FabZ), and β-enoyl-ACP reductase (FabI). The first phase in the elongation of fatty acid synthesis begins with the formation of acetyl-ACP and malonyl-ACP. Elongation is initiated through the condensation of these two molecules, which are catalyzed by FabB, FabF, and FabH [10–12]. The second elongation step is an NADPH-dependent reduction of β-ketoacyl-ACP into β-hydroxyacyl-ACP, which is catalyzed by FabG [13]. Next, the hydroxy group is removed by the action of FabZ, which converts the chain into a trans-2-enoyl group. FabA, an isomerase with similar properties to FabZ, is also responsible for converting the β-hydroxy intermediate into a trans-2-enoyl group [12]. The final step in the elongation cycle is performed by FabI (encoded by the *fabI* gene): a NADPH-dependent reduction of the double bond in the trans-2-enoyl intermediate to form acyl-ACP [7, 14].

Escherichia coli has an advantage over other industrial bacteria in that it has a more rapid growth rate and is relatively easy to genetically manipulate. The genes related to the FAS elongation cycle and related metabolic pathways have been studied in *E. coli*, and this bacterium has been shown to efficiently produce fatty acids from glucose [15]. In addition, the enzymes involved in the FAS pathway have been well characterized in *E. coli* and include the following observations: FabA and FabB can efficiently catalyze the dehydration of short-chain and long-chain saturated fatty acids (SFAs) and unsaturated fatty acids (UFAs) [3, 12]; FabH has specificity for carbon substrates that are two to four carbons in length, dictating the type of fatty acid made by the microorganism [16, 17]; overproduction of FabH

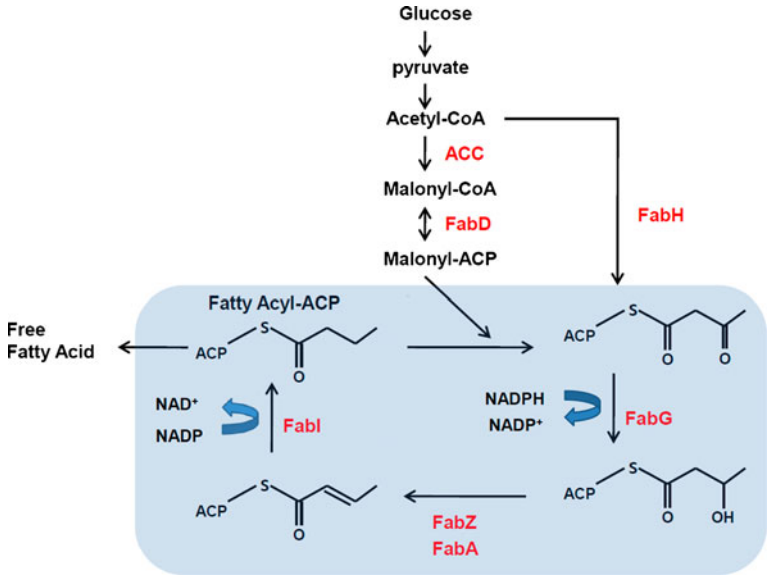


Fig. 1 Metabolic pathway of fatty acid synthesis (FAS) from glucose in *Pseudomonas aeruginosa* PA14. *ACC* acetyl-CoA carboxylase, *FabD* malonyl-CoA:ACP transacylase, *FabH* β-ketoacyl-ACP synthase, *FabG* β-ketoacyl-ACP reductase, *FabA FabZ* β-hydroxyacyl-ACP dehydrase, *FabI* β-enoyl-ACP reductase

and FabI leads to a decrease in the chain length of fatty acids [14, 18–20]. However, research to improve the production of long-chain fatty acids by overexpressing elongation enzymes, such as FabG and FabZ, in *E. coli* has been insufficient. Additionally, studies have focused on individual enzymes and their activities, but the simultaneous overexpression of these enzymes in the context of fatty acid elongation has not yet been attempted. It is unclear if overexpression of such enzymes will enhance long-chain fatty acid production in the FAS elongation pathway.

In this study, we endeavored to improve the synthesis of long-chain fatty acids in *E. coli* by overexpressing various combinations of essential genes that are involved in the FAS elongation phase of fatty acid biosynthesis. Four enzymes, targeted to overproduce fatty acids, were systematically introduced into the *E. coli* host. We further analyzed fatty acid production and composition of the long-chain fatty acids (SFAs and UFAs) and confirmed the results using a NAD^+/NADH ratio and glucose analysis.

Materials and Methods

Bacterial Strains

E. coli K-12 MG1655 [6, 21] was used as a host for the production of fatty acids and as the source for the *fabG*, *fabZ*, *fabI*, and *fabH* genes. Competent *E. coli* AG1 cells were used to construct and stabilize the recombinant plasmids. The strains that were used in this study are listed in Table 1.

Plasmid Construction and Transformation

The *fabG*, *fabZ*, *fabI*, and *fabH* genes were cloned from the genome of *E. coli* K-12 MG1655. A DNeasy Blood and Tissue Kit (Qiagen) was used to isolate total genomic DNA from the bacteria. The primers used for polymerase chain reaction (PCR) amplification of the genes (Takara) are shown in Table 1. The PCR products were purified by gel extraction and cloned into the pGEM-T vector (Promega). All cloning was performed in *E. coli* AG1 cells (Stratagene), and all enzymes for cloning were purchased from Takara. To screen for clones containing the *fabG*, *fabZ*, *fabI*, and *fabH* genes in the *E. coli* AG1 transformants, we used a plasmid miniprep (Dynebio) for isolation. Following digestion of the DNA with restriction enzymes, as detailed in Table 1, the genes were ligated into the pTrc99A expression vector [22]. The resulting plasmids were named pJS14, pJS15, pJS16, pJS17, pJS18, and pJS19, respectively (Table 1 and Fig. 2). Standard methods were used to manipulate the vectors and transform the bacteria [23]. Transformation of *E. coli* K-12 MG1655 cells with the plasmid constructs was performed using electroporation with a Gene Pulser Xcell (Bio-Red) electroporator.

Media and Culture Conditions

Luria-Bertani (LB) medium (containing 10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract) was used to culture *E. coli* AG1 and *E. coli* K-12 MG1655 cells. *E. coli* K-12 MG1655 cells were electroporated with recombinant plasmids and incubated on LB agar containing 50 μg/ml ampicillin for 18 h at 37 °C. For inoculum preparation, wild-type or engineered *E. coli* strains were cultivated overnight at 37 °C with shaking at 200 rpm in 3 ml LB or LB-ampicillin media,

Table 1 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer names	Relevant characteristic or sequence	Reference or enzyme site
E. coli		
AG1	F^- endA1 hsdR17 [Kn^-mk^+] supE44 thi-1 recA1gyrA96 relA1	Stratagene
K-12 MG1655	F^- lambda-ilvG- rfb-50 *rph-1*	ATCC
SGJS34	K-12 MG1655 containing pTrc99A	This study
SGJS24	K-12 MG1655 containing pJS14	This study
SGJS25	K-12 MG1655 containing pJS15	This study
SGJS26	K-12 MG1655 containing pJS16	This study
SGJS27	K-12 MG1655 containing pJS17	This study
SGJS28	K-12 MG1655 containing pJS18	This study
SGJS29	K-12 MG1655 containing pJS19	This study
		This study
Plasmids		
pGEM-T	T-easy vector	Promega
pTrc99A	Expression vector : $Ap^rP_{trc}rrBT_1T_2$ *ori* (pBR322) $lacI^q$	22
pJS14	pTrc99A containing *fabG* gene of *E. coli*	This study
pJS15	pTrc99A containing *fabZ* gene of *E. coli*	This study
pJS16	pTrc99A containing *fabI* gene of *E. coli*	This study
pJS17	pTrc99A containing *fabG* and *fabZ* genes of *E. coli*	This study
pJS18	pTrc99A containing *fabG, fabZ* and *fabI* genes of *E. coli*	This study
pJS19	pTrc99A containing *fabG, fabZ ,fabI* and *fabH* genes of *E. coli*	This study
Primers		
fabG-F	5′-GAATTCGGAAAATCATGAATTTTGAAGGAA-3′	EcoRI
fabG-R	5′-GAGCTCTTADGDGTAACCGTAGCTCATC-3′	SacI
fabZ-F	5′-GAGCTCGGAAGAGTATCTTGACTACTAAC-3′	SacI
fabZ-R	5′-GGATCCTCAGGCCTCCCGGCTACGAGC-3′	BamHI
fabH-F	5′-GGATCCCCGAAAAGTGACTGAGCGTACA-3′	BamHI
fabH-R	5′-TCTAGACTAGAAACGAACCAGCGCGGAG-3′	XbaI
fabI-F	5′-GGATCCAAGGATTAAAGCTATGGGTTTTCT-3′	XbI
fabI-R	5′-TCTAGATTATTTCAGTTCGAGTTCGTTCAT-3′	SalI

Underlined nucleotides represent the restriction enzyme sites

respectively. For the analysis of fatty acids, 500-ml Erlenmeyer flasks containing 200 ml of LB media were inoculated with 1 ml (0.5 %) of a seed culture and were incubated at 37 °C with agitation at 170 rpm (JeoTech). At an optical density (λ=600 nm) of 0.3–0.6, the cells were induced by the addition of 0.5 mM isopropyl-β-d-thiogalactopyranoside (IPTG). Glucose served as a carbon source at concentrations of 0.5, 1, and 2 % with a 0 % sample serving as control. Based on our previous studies, samples were taken at five time points (4, 8, 12, 16, and 20 h) after induction for fatty acid and extracellular metabolite analyses [21].

Fatty Acid Extraction and Analysis

Fatty acid extraction was based on protocols from the National Institute of Agricultural Science of Korea [6]. Fatty acids were analyzed by gas chromatography/mass spectrometry

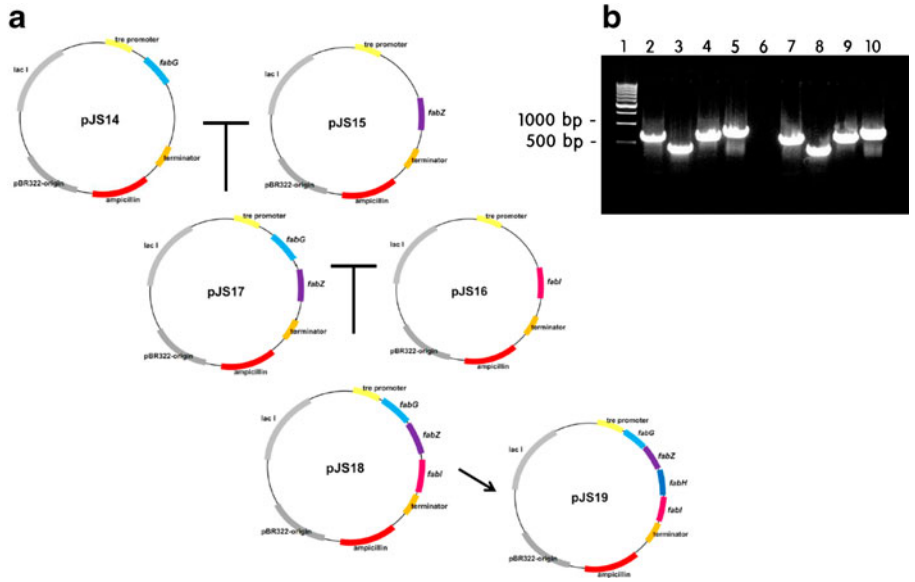


Fig. 2 **a** The sequential modifications in pJS vectors for long-chain fatty acid production. The recombinant plasmids pJS14, pJS15, and pJS16 were prepared by inserting the *fabG*, *fabZ*, and *fabI* genes, respectively, into the pTrc99A vector. pJS17 was constructed by inserting the *fabZ* gene from pJS15 into pJS14, and pJS18 was constructed by inserting the fabI gene from pJS16 into pJS17, and pJS19 was constructed by inserting the *fabI* gene into pJS18. **b** Identification of the inserted genes in the pTrc99A vector by PCR product gel electrophoresis of the *E. coli* SGJS29 strain. *Lane 1* 500-bp DNA marker (Takara), *lanes 2 and 7 fabG* gene, *lanes 3 and 8 fabZ* gene, *lanes 4 and 9 fabI* gene, *lanes 5 and 10 fabH* gene

using an Agilent model 5975 series MS and a 7890A GC (equipped with a HP-5 column at a film thickness of 30 m×0.32 m×0.25 μm; Agilent Technologies, Inc). Helium was used as the carrier gas. Peak identification was achieved by comparison with a mass spectral database. Quantification was accomplished by comparing integrated peaks with the calibration curve of a fatty acid methyl ester standard (F.A.M.E. Mix, C8-C24, Sigma). Samples were injected at a split ratio of 1:5. The following temperature program was applied: maintained at 100 °C for 5 min, increased 5 °C per min to 220 °C, and maintained at 220 °C for 5 min. The detector was maintained at a temperature of 280 °C.

Analytical Techniques

The glucose was measured using high-performance liquid chromatography with a LC-10AT unit (Younglin), RI 750F detector (Younglin), and HPX-87-H organic acids columns (Bio-Rad). Sulfuric acid (0.01 M) was used as the mobile phase (flow rate of 0.6 $ml min^{-1}$; column temperature of 60 °C). All solutions were filtered with a 0.2-μm membrane before use. To detect intracellular NADH and NAD^+ cycling enzyme, we was used NAD^+/NADH Assay Kit (Abcam). Briefly, this process involves removing cells from the culture media and extracting intracellular components by freezing and thawing. After extraction, to prevent unwanted degradation of NADH, enzymes were filtered with a 10-kDa molecular weight filter (Abcam) and removed. The NAD^+ and NADH were then measured by a LT-5000MS ELISA reader (Labtech).

Results

Generating Recombinant *E. coli*

The metabolic pathway for the FAS elongation cycle is shown in Fig. 1. The genes (*fabG*, *fabZ*, *fabI*, and *fabH*) encoding the essential enzymes involved in the elongation cycle were cloned. The *fabG* gene (encoding FabG) has a 930-bp open reading frame (ORF), and the *fabZ* and *fabI* genes (encoding FabZ and FabI) have a 456- and a 789-bp ORF, respectively. Acetyl-CoA reacts under catalyzed conditions with FabH, encoded by the *fabH* gene with a 954-bp ORF.

In this study, pJS14, pJS15, and pJS16 were prepared by inserting the *fabG*, *fabZ*, and *fabI* genes, respectively, into the pTrc99A vector. The pTrc99A vector is stable and controllable with a strong *trc* promoter [6, 22]. The pJS17 plasmid (containing *fabG* and *fabZ* genes) was constructed by inserting the *fabZ* gene from pJS15 into pJS14. The pJS18 plasmid (containing *fabG*, *fabZ*, and *fabI* genes) was constructed by inserting the *fabI* gene from pJS16 into pJS17. Finally, the pJS19 plasmid (containing *fabG*, *fabZ*, *fabI*, and *fabH* genes) was constructed by inserting the *fabH* gene into pJS18. Because multiple genes were inserted into the vector, the genes were constructed sequentially to increase stability. The recombinant plasmids produced in this study are summarized in Table 1. Maps of the constructs and all genes contained in the plasmids were confirmed by electrophoresis as shown in Fig. 2.

Analysis of Long-Chain Fatty Acids in Recombinants

The recombinant *E. coli* strains were cultivated in LB media and induced by the addition of IPTG (0.5 mM/L). Samples were then collected for analysis at 4 and 20 h to examine the influence of IPTG and to determine the fatty acid composition and content. We compared the cell biomass production from the newly developed strains, measured as dry cell weight (DCW), to that of wild-type *E. coli* (Table 2). The data indicate that the growth of the

Table 2 The long-chain fatty acid content of recombinant *E. coli* strains and wild-type *E. coli*

Strains	Fatty acid content (mg/L)								DCW (g/L)[a]	DCW (g/L)[b]
	Hexadecanoic acid (C16)				Octadecanoic acid (C18)					
	4 h	20 h	mg/g DCW[a]	mg/g DCW[b]	4 h	20 h	mg/g DCW[a]	mg/g DCW[b]		
Wild-type *E. coli* K-12 MG1655	104.0±1.2	122.3±1.3	130	74	42.8±0.1	36.6±0.2	54	22	0.80	1.63
E. coli SGJS24	123.3±1.5	101.2±1.2	152	57	54.5±0.5	36.2±0.3	67	20	0.81	1.77
E. coli SGJS25	119.3±1.7	133.4±1.9	140	83	63.9±0.7	47.2±0.3	75	30	0.85	1.60
E. coli SGJS26	127.7±0.5	107.3±0.8	150	77	65.3±0.5	46.9±0.5	77	34	0.85	1.39
E. coli SGJS27	106.9±0.7	121.0±0.9	134	92	58.8±0.8	62.0±0.7	74	47	0.80	1.32
E. coli SGJS28	154.4±3.1	110.9±2.5	182	69	74.4±1.2	31.0±0.2	88	19	0.85	1.60
E. coli SGJS29	111.7±2.0	154.4±2.7	131	101	119.0±1.5	60.4±0.9	140	39	0.85	1.53

[a] DCW (dry cell weight) was measured 4 h after plasmid induction by IPTG

[b] DCW (dry cell weight) was measured 20 h after plasmid induction by IPTG

recombinant strains was not inhibited by the overexpression of the various gene combinations or the insertion of the recombinant plasmids.

Hexadecanoic acid (C16) and octadecanoic acid (C18) had previously been determined to be the major fatty acid products in the newly developed strains [6, 21]; as such, C16 and C18 were measured preferentially in this study. As shown in Table 2, all recombinant strains had long-chain fatty acid (C16 and C18) content enhanced by up to 1.6-fold at 4 h and 2-fold at 20 h versus wild-type *E. coli.* C16 content was increased at 20 h compared with at 4 h in *E. coli* SGJS25, SGJS27, and SGJS29 (in which the *fabZ* gene was overexpressed); however, in *E. coli* SGJS24, SGJS26, and SGJS28, the C16 content was reduced. The highest level of C16 content was observed in *E. coli* SGJS28 (182 mg/g DCW) at 4 h and in *E. coli* SGJS29 (101 mg/g DCW) at 20 h. With the exception of *E. coli* SGJS27, which had overexpressed the *fabG* and *fabZ* genes, the C18 content decreased over the cultivation period. The C16 and C18 products were more abundant in *E. coli* SGJS27, SGJS28, and SGJS29 (which had two or more overexpressed genes) versus *E. coli* strains SGJS24, SGJS25, and SGJS26 (which had only one overexpressed gene).

Long-Chain Fatty Acid Accumulation as a Function of Glucose Concentration and Determination of Total Fatty Acid Content

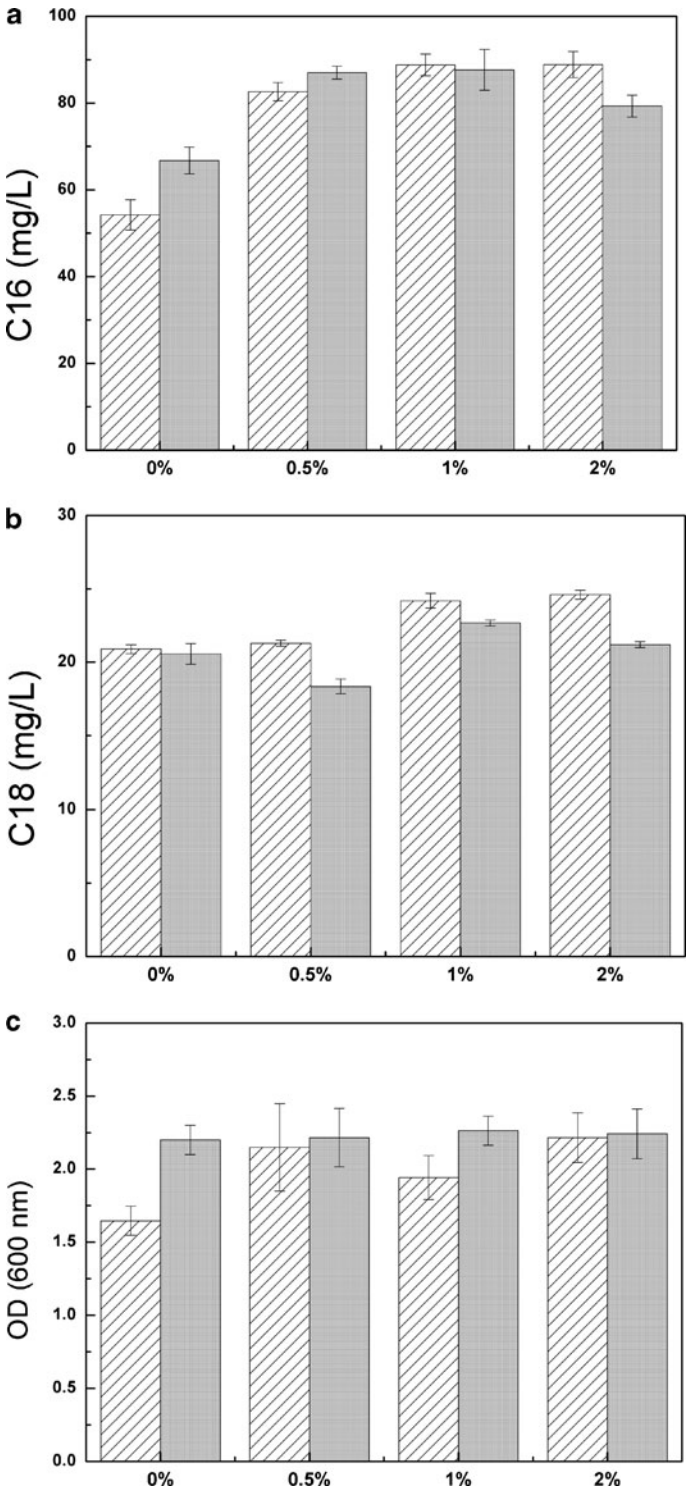
The fatty acid biosynthesis pathway of the recombinant *E. coli* strains produces fatty acids from glucose, and the degree of fatty acid production depends on the glucose content of the culture medium (Fig. 1). *E. coli* SGJS34 (pTrc99A) had minor differences in growth rates in LB media containing 0, 0.5, 1, or 2 % glucose. Following the addition of glucose, the initial growth rate rapidly increased, but after 20 h, the final optical density was similar at all glucose concentrations. It appears that *E. coli* SGJS34 used glucose in growth simultaneously with using it for fatty acid production in the early cultivation stages. Increasing the glucose content resulted in further increases in the production of C16 and C18 compounds until a steady state was reached at glucose concentrations above 1 %. However, in 2 % glucose LB media, C16 and C18 compounds declined at 20 h (Fig. 3).

The growth of recombinants and the intracellular total fatty acid (TFA) content were measured in cells grown in 1 % glucose LB media. Under these conditions, it was observed that *E. coli* SGJS34 increased fatty acid content most efficiently (Fig. 3). Wild-type *E. coli* and *E. coli* SGJS25, SGJS28, and SGJS29 appeared to behave similarly after IPTG induction, as TFAs were increased (about 1.2-fold) and maintained after 4 h, but levels decreased after 16 h. In contrast, *E. coli* SGJS27 had the highest level of TFA content at all sampling time points, and such levels increased twofold after 12 h (Fig. 4).

Glucose Utilization and Intracellular NAD^+/NADH Ratios

Glucose consumption confirmed that all of the strains grew with increasing incubation time (Fig. 5a). There were not many differences with respect to the pattern of glucose consumption between wild-type and recombinant *E. coli* strains; however, *E. coli* SGJS27 consumed the most glucose at 12 and 20 h, totaling 33 and 38 mM, respectively, also corresponding to the highest level of fatty acid production.

Because several genes were expressed simultaneously in the plasmid constructs, it was necessary to confirm that the function of these enzymes was maintained. This was determined by assessing the intracellular NAD^+/NADH ratios (Fig. 5b). This ratio varied widely depending on strain. In general, recombinant strains produced a lower NAD^+/NADH ratio than that of wild-type strains. This result demonstrated that recombinant strains had


a
C16 (mg/L)
100
80
60
40
20
0
0%
0.5%
1%
2%
b
C18 (mg/L)
30
20
10
0
0%
0.5%
1%
2%
c
OD (600 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0%
0.5%
1%
2%

◀ **Fig. 3** Effect of glucose concentration (0, 0.5, 1, and 2 %) on long-chain fatty acid [C16 (**a**) and C18 (**b**)] production and cell growth (**c**) in *E. coli* SGJS34 (pTrc99A). *Striped bar* and *gray bar* represent the results obtained at 4 and 20 h, respectively

increased enzymatic activity (in particular FabI) in order to produce fatty acids. The NAD^+/NADH ratios in *E. coli* SGJS27 and SGJS29 decreased with time, while the ratio increased in *E. coli* SGJS28 with time.

Long-Chain Fatty Acid Compositions

The long-chain fatty acid compositions of the five strains are shown in Fig. 6. The C16 and C18 SFA compounds were produced mostly during the incubation period for wild-type and recombinant strains. Furthermore, the ratio of SFAs to UFAs varied widely between strains. The wild-type *E. coli* produced UFAs (23 % of TFAs) until 4 h of cultivation. The main product was the C16:1 molecule (*cis*-9-hexadecenoic acid). However, after 4 h, C16:1 was rarely produced and the percentage of UFAs (4 % of TFAs) decreased. *E. coli* SGJS25 had the highest percentage of UFAs (35 % of TFAs) at 12 and 20 h, higher than wild-type *E. coli* and other recombinant strains. For this strain, the percentage of UFAs (C16:1) increased with cultivation time while the percentage of SFAs (C16 and C18) decreased. The UFA, C16:1 content in *E. coli* SGJS25 was 20-fold higher than that in wild type at 20 h.

The fatty acid compositions of *E. coli* strains SGJS27 and SGJS28 demonstrated a similar pattern over time. The two strains had the highest productivity of fatty acids (Fig. 4). The percentage of UFAs (C16:1 and C18:1) and SFAs (C12, C14, C16, and C18) increased with cultivation time. At 20 h, C16 and C18 compounds from *E. coli* strains SGJS27 and SGJS28 were about twofold higher than wild-type and other engineered strains.

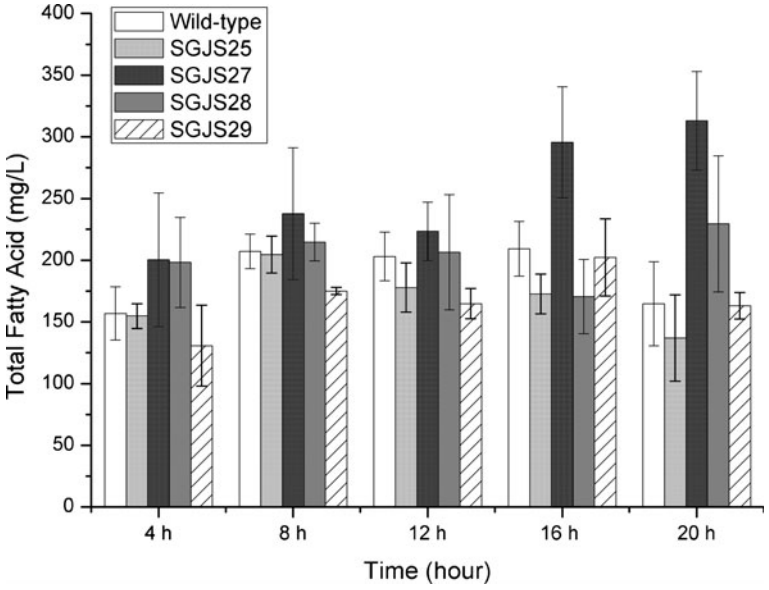


Fig. 4 The total long-chain fatty acid production of wild-type *E. coli* and the recombinant strains (*E. coli* SGJS25, SGJS27, SGJS28, and SGJS29). Strains incubated for 20 h after IPTG induction at 37 °C in LB media which contains 1 % glucose. Samples were then collected for analysis at 4, 8, 12, 16, and 20 h to determine total long-chain fatty acid content

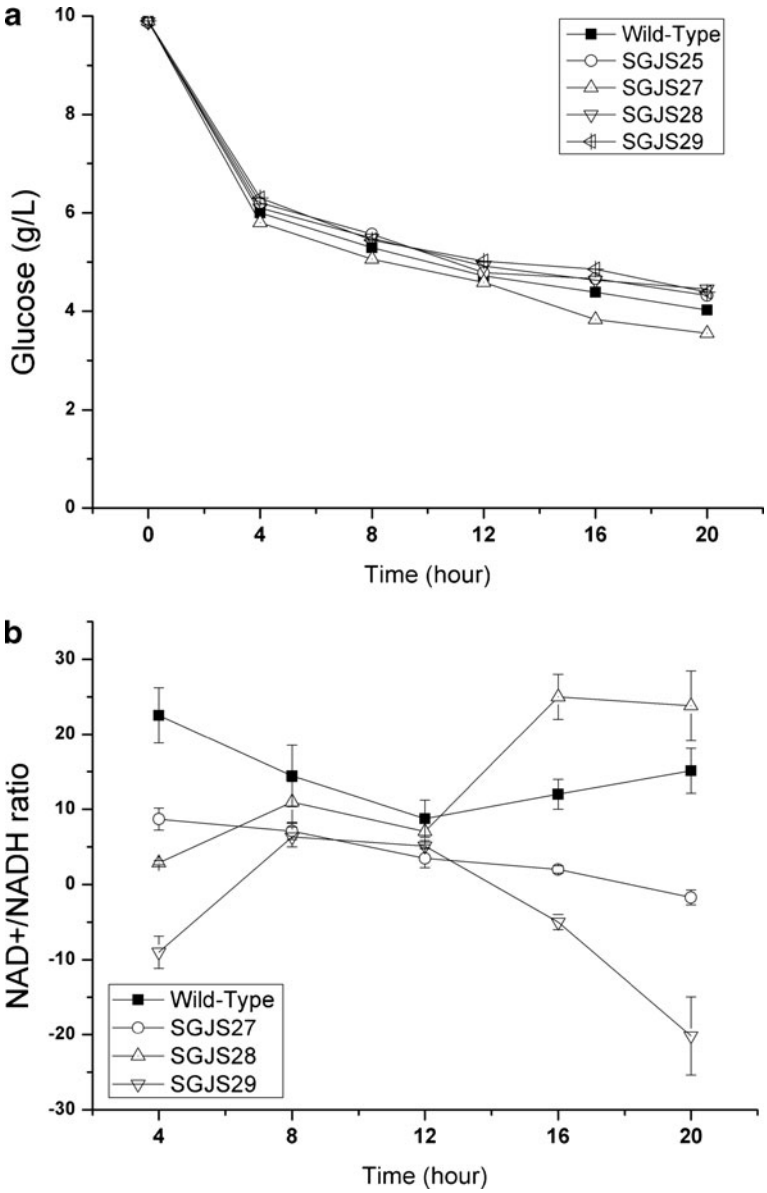


Fig. 5 Glucose utilization (**a**) and intracellular NAD^+/NADH ratios (**b**). Wild-type and recombinant strains containing the *fabI* gene (*E. coli* SGJS27, SGJS28, and SGJS29) incubated for 20 h after IPTG induction at 37 °C in LB media which contains 1 % glucose

Discussion

Many enzymes are needed to produce fatty acids in *E. coli*. In fact, *E. coli* expresses many genes simultaneously and repetitively during fatty acid biosynthesis (Fig. 1). Previous studies have focused on increasing fatty acid production in bacteria by individually changing the expression of each relevant gene [12, 13, 24, 25]; however, little to no research has

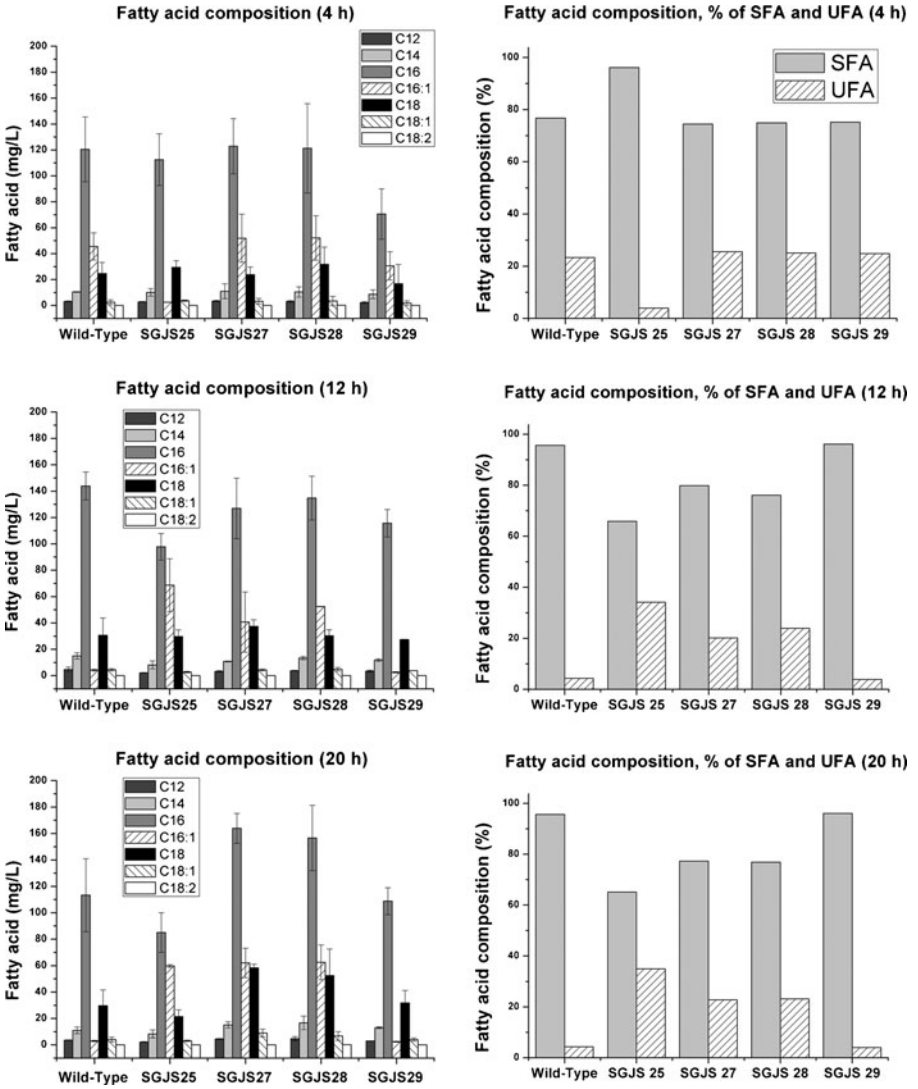


Fig. 6 Effect and correlation of FAS elongation genes on long-chain fatty acid compositions, and the percentage of saturated fatty acid (SFA) and unsaturated fatty acid (UFA), in total fatty acid (TFA) at 4, 12, and 20 h. Wild-type *E. coli* and recombinant strains (*E. coli* SGJS25, SGJS27, SGJS28, and SGJS29) incubated for 20 h after IPTG induction at 37 °C in LB media which contains 1 % glucose

focused on expressing patterns of multiple genes in order to enhance production of long-chain fatty acids. In this study, we manipulated genes related to fatty acid biosynthesis, and we observed a correlation between genes expressed and increased production of fatty acids.

Indeed, the recombinants which two or more genes were overexpressed simultaneously were higher fatty acids content than the recombinants which only one gene was overexpressed (Table 2 and Fig. 4). It is considered a synergy effect at the fatty acid production by influencing each other genes. *E. coli* SGJS27 (contain *fabG* and *fabZ* genes) produced the highest fatty acid level, and overexpression of these two genes was shown to increase fatty acid production,

especially the production of long-chain fatty acids, when cultured in media supplemented with glucose (Figs. 4 and 5a). However, such synergistic effects were not observed with all gene combinations. Previous reports have demonstrated that overexpression of the *fabI* and *fab*H genes inhibit the production of fatty acids [16, 17, 26]. This finding is consistent with the results in Fig. 4, whereby overexpression of *fabI* in addition to *fabG* and *fabZ* in *E. coli* SGJS28 produced the same amount of fatty acids as *E. coli* SGJS27 (which only contained *fabG* and *fabZ* genes) despite having more expressed genes. This result demonstrates that overexpression of the *fabI* gene helps to retain fatty acid production and completes a FAS cycle although it is an inhibitory factor [7, 12]. FabI is $NADP^+$ dependent and converts NADH into NAD^+ [26], and its role in fatty acid biosynthesis was confirmed here by observing an increased ratio of NAD^+/NADH (Fig. 5b).

Fatty acid production in *E. coli* SGJS29 (containing the *fabG, fabZ, fabI*, and *fabH* genes) declined more significantly than in *E. coli* SGJS27 and SGJS28, demonstrating that overexpression of the *fabH* gene exerts a greater inhibitory effect on fatty acid biosynthesis than overexpression of the *fabI* gene. However, FabH may be hindered when fatty acid elongation begins, thereby accelerating fatty acid production in *E. coli* SGJS29 during early stages of biosynthesis. In our previous research, overexpression of the *fabH* gene increased fatty acids when it revealed with the genes of FAS initial stage [21]; however, when this gene was overexpressed simultaneously with elongation cycle genes, fatty acid content decreased. We observed that each gene associated with fatty acid synthesis affected the production of fatty acids variably, depending on the overall gene expression pattern: in particular, the co-expression of genes involved in distinct stages of fatty acid synthesis.

In order to stabilize biodiesel as a fuel and use it as a premium fuel, improvement on quality is as important as production. SFAs solidify at a low temperature, even in a strong lipid as single bond. High-quality biofuel can be produced by maintaining a favorable UFA content in the lipid [3, 27]. We were most focused on determining how the ratio of UFAs to SFAs changed due to overexpression of other enzymes involved in fatty acid biosynthesis. As mentioned, *E. coli* SGJS27 produced the largest amount of fatty acids among the different strains, with the production of SFAs (C12, C14, C16, and C18) being most represented. In this strain, the content of UFAs was similar to that of *E. coli* SGJS25, and, despite a paucity of studies on FabG [12, 13]. It would be interesting to speculate a potential mechanism by which FabG may act to increase SFAs.

FabZ, an isomerase with similar properties to FabA, is responsible for converting a β-hydroxy intermediate into *trans*-2-enoyl and *cis*-3-enoyl molecule. These two enzymes affect SFA and UFA production. However, we elected to focus on the role of FabZ overexpression in fatty acid biosynthesis as overproduction of FabA also leads to a decrease in UFA content [12]. In *E. coli* SGJS25, SGJS27, and SGJS28, which contained the *fabZ* gene, UFAs were significantly increased versus in wild type (Fig. 6). In a previous paper, FabZ is known to produce most of the *trans*-2-enoyl [7], but measured UFAs were the *cis*-9-hexadecenoic acid (C16:1) and *cis*-9-octadecenoic acid (C18:1) in this study. Overproduction of fabZ affects the production of *cis*-3-enoyl, and may be increased with the reaction of FabA. This result demonstrated that FabZ is a very important enzyme in production and composition of the UFAs.

E. coli SGJS29, which, in addition to containing the *fabZ* gene, also contained the *fabH* gene. In this strain, we noted that the percentage of UFAs increased until 4 h, but the production of UFAs as well as SFAs significantly decreased by 20 h. This result showed that overexpression of FabH in particular led to a decrease in UFAs as well as overall fatty acid content compared with other strains. In addition, the composition of fatty acids changed depending on the time of incubation and growth. This result

could be due to differing activities of each enzyme during repetition of fatty acid biosynthesis elongation. It suggests that information of these composition changes and growth is needed in future fuel research.

Conclusion

In previous studies, when genes associated with fatty acid biosynthesis were expressed individually, a correlation between individual enzymes and fatty acid production proved difficult. The novelty of this present study lies in the fact that multiple biosynthetic genes were simultaneously overexpressed, allowing us to compare and analyze the production and composition of long-chain fatty acids in recombinant *E. coli* strains. In order to confirm successful fatty acid synthesis, NAD^+/NADH ratios and glucose utilization were investigated. The recombinant strains exhibited up to twofold increase in the production of long-chain fatty acids over wild type. In particular, we successfully developed strains to improve UFA content (about 20-fold). These results are significant because biochemically engineered bacterial strains are expected to be more feasible for the mass production of long-chain fatty acids in commercialized biodiesel production processes.

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