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ORIGINAL PAPER

Increasing fatty acid production in *E. coli* by simulating the lipid accumulation of oleaginous microorganisms

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Abstract Unlike many oleaginous microorganisms, *E. coli* only maintains a small amount of natural lipids in cells, impeding its utility to overproduce fatty acids. In this study, acetyl-CoA carboxylase (ACC) from *Acinetobacter calcoaceticus* was expressed in *E. coli* to redirect the carbon flux to the generation of malonyl-CoA, which resulted in a threefold increase in intracellular lipids. Moreover, providing a high level of NADPH by overexpressing malic enzyme and adding malate to the culture medium resulted in a fourfold increase in intracellular lipids (about 197.74 mg/g). Co-expression of ACC and malic enzyme resulted in 284.56 mg/g intracellular lipids, a 5.6-fold increase compared to the wild-type strain. This study provides some attractive strategies for increasing lipid production in *E. coli* by simulating the lipid accumulation of oleaginous microorganisms, which could aid the development of a prokaryotic fatty acid producer.

Keywords Fatty acid · Lipids · Oleaginous microorganisms · ACC · NADP ME

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Introduction

Fatty acid is an important platform chemical that is widely used in different fields, especially in the production of biodiesel (fatty acid ester) [11]. There are two main processes that are utilized to produce fatty acids: chemical synthesis derived from fossil fuel and biosynthesis using organisms [5, 28]. Due to the gradual and inescapable exhaustion of the Earth's fossil energy resources, the use of biocatalysts to synthesize fatty acid in microbial cells as an alternative to traditional synthetic routes has become a research hotspot in recent years [6, 30].

Oleaginous microorganisms that are used for single-cell oil (SCO) production, including yeast and fungi, have been well studied because they can accumulate a large number of lipids [5, 17, 18]. However, fatty acid production from yeast and fungi in order to produce bulk chemicals such as biodiesel has been hampered by the slow growth rates and complicated regulation mechanisms of these organisms. *Escherichia coli* could be an alternative fatty acid producer, and it has been successfully used to generate many valuable platform chemicals and biofuels [1, 12, 13] because it is the most popular host cell for genetic manipulation and has one of the fastest growth rates among common microorganisms. However, *E. coli* is not an oleaginous microorganism, so its low yield of intracellular lipids restricts its utility for lipid production. The mechanism of lipid accumulation can be considered from at least two perspectives. One is the ability to produce a continuous supply of acetyl-CoA directly in the cytosol of the cell as a necessary starting material for malonyl-CoA synthesis; the other is the ability to produce enough NADPH to provide reducing force for fatty acid synthesis (Fig. 1). To convert *E. coli* into an effective producer of fatty acids, malonyl-CoA and NADPH levels need to be regulated based on the

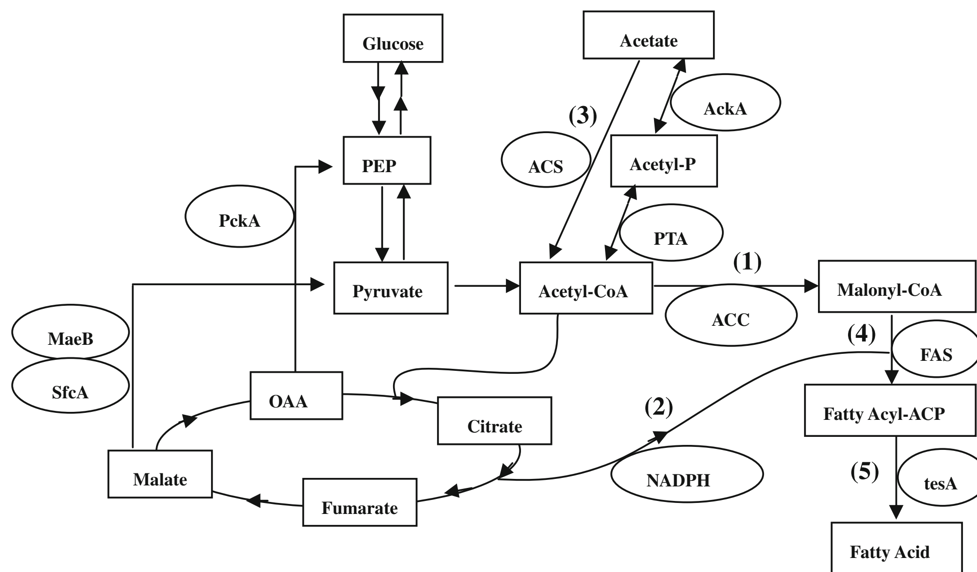

Fig. 1 Fatty acid biosynthetic pathway in *E. coli.* (*1*) ACC, which catalyzes the carboxylation of acetyl-CoA to produce malonyl-CoA; (*2*) NADPH, the reductant required for fatty acid synthesis, which can be supplied by the TCA and PPP pathways; (*3*) ACS, which catalyzes the formation of acetyl-CoA from acetate; (*4*) FAS, which plays a key role in converting malonyl-CoA to the final fatty acid

oleaginous microbial lipid accumulation mechanism. Acetyl-CoA carboxylase (ACC, EC 6.4.1.2) catalyzes the first step in the fatty acid biosynthetic pathway through the formation of malonyl-CoA from acetyl-CoA, which is thought to be the rate-limiting step in fatty acid biosynthesis [7]. Nevertheless, *E. coli* maintains only a small amount of cellular malonyl-CoA according to its natural metabolism [26]. However, it was recently shown that the overexpression of *E. coli* ACC resulted in an increase in the rate of fatty acid synthesis [8], although most of these results focused on the function of *E. coli* ACC, and fatty acid production was greatly limited by host feedback inhibition [4]. The function of the heterologous expression of ACC from other microorganisms in *E. coli* during flavonoid production has been reported [13]. On the other hand, fatty acid synthesis also needs a ready supply of NADPH as a reductant [18]. The major supplier of NADPH is currently considered to be malic enzyme (ME, $NADP^+$-dependent, EC 1.1.1.40; see Fig. 1) [27]. Malic enzyme catalyzes the irreversible decarboxylation of malate to pyruvate with the formation of NADPH from $NADP^+$, which was postulated to be the rate-limiting step for lipid accumulation in oleaginous fungi. It was supposed that malic enzyme activity is ubiquitous among most oleaginous microorganisms and has a positive effect on lipid accumulation. For example, overexpressing malic enzyme in *Mucor cicinelloides* resulted in a 2.5-fold increase in lipid accumulation [32]. In *E. coli*, there are two homologous genes for fungi malic enzyme [2], *maeA* (coding NAD ME) and *maeB* (coding NADP ME), which are part of the phosphoenolpyruvate (PEP)-pyruvate-OAA node that links glycolysis/gluconeogenesis to the tricarboxylic acid (TCA) cycle [21]. Both have been expressed in *E. coli*, but their effects on lipid synthesis in vivo have not been reported.

In this study, in order to test whether *E. coli* could be used as an alternative producer of fatty acids, we simulated the mechanism of oleaginous microorganisms and engineered *E. coli* from two points: (1) overexpressing *Acinetobacter calcoaceticus* ACC to improve malonyl-CoA synthesis; (2) improving the level of NADPH by overexpressing *E. coli* malic enzyme (NADP ME). Furthermore, ACC and NADP ME were co-expressed to improve lipid production in *E. coli*.

Materials and methods

Bacterial strains, plasmids and growth media

Acinetobacter calcoaceticus (ATCC 33530) and *E. coli* K-12 DH5α were used as donors for ACC and NADP ME cloning, respectively. *E. coli* BL21 (DE3) was used as a general expression host. The plasmids pACYCDuet-1 (Novagen) and pET-30a(+) (Novagen) were used for cloning in this study. Luria broth (LB) and M9 minimal media (1 L M9 salts, 20 g glucose, 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 0.24 g $MgSO_4$) were applied as culture media.

DNA manipulations

Recombinant DNA techniques were performed according to standard procedures [20]. ACC gene fragments, including *accA*, *accBC* and *accD*, were cloned from *A. calcoaceticus*, while the *maeB* gene was amplified from *E. coli* K-12.

Table 1 Plasmids, strains and primers used in this study

	Characteristics	References
Plasmids		
pACYCDuet-1	P15A (pACYC184), Cm^r	Novagen
pET30a (+)	F1 (pBR322), Kan	Novagen
pMX1	pACYCDuet-1 carrying *accA* from *Acinetobacter calcoaceticus*	This study
pMX2	pACYCDuet-1 carrying *accBC* from *Acinetobacter calcoaceticus*	This study
pMX3	pACYCDuet-1 carrying *accD* from *Acinetobacter calcoaceticus*	This study
pMX4	pMX1 carrying T7*lac-accD* fusion	This study
pMX7	pACYCDuet-1 carrying *accABCD* from *Acinetobacter calcoaceticus*	This study
pMX20	pET30a (+) carrying *maeB* from *E. coli* K-12	This study
Strains		
E. coli BL21(DE3)	F^- *ompT hsd*S_B ($r_B^- m_B^-$) *gal dcm rne*131(DE3)	Invitrogen
A. calcoaceticus	Type strain	ATCC
E. coli K-12	Type strain	ATCC
MX7	*E.coli* BL21(DE3)/pMX7	This study
MX20	*E.coli* BL21(DE3)/pMX20	This study
MX32	*E.coli* BL21(DE3)/pMX7/pMX20	This study

Primers	Sequences (5′–3′)	Genes
accA-F	CGCGGATCCAAAAATAAAGCTGCTCAGTCTAAAG	*accA*
accA-R	AATCGCGAGCTCTTAAGAGGCGATGCCTAAAT	
accBC-F	GGAATTCCATATGGATATTCGCAAAATTAAGAAACTC	*accBC*
accBC-R	CCGCTCGAGTTAGGCTTTTTCTGTGGTTTCAG	
accD-F	CGCGGATCCAAAATCAAGAAGTGAAATCAGGTAAAATC	*accD*
accD-R	ATAAGAATGAGCTCTCAAGGTAAATTCATCAGTTTAG	
T7accD-F	ACGCGTCGACTAATACGACTCACTATAGGGGA	T7*accD*
T7accD-R	AATCCCCTTAAGTCAAGGTAAATTCATCAGTTTAG	
ME-F	CATGCCATGGCTGATGACCAGTTAAAACAAAGT	*maeB*
ME-R	ACGCGTCGACTTACAGCGGTTGGGTTTGC	

All PCR primers and plasmids used in this study are described in Table 1. The four ACC subunits were cloned into pACYCDuet-1. First, the amplified products of *accA*, *accBC* and *accD* were cloned into pACYCDuet-1, resulting in the plasmids pMX1, pMX2 and pMX3, respectively. Subsequently, a PCR reaction was performed using the plasmid pMX3 as template and a primer pair that allowed the amplification of the T7 lac sequence along with the *accD* structural gene. T7*accD* was then cloned into pMX1 between the restriction sites of SalI and AflII to create pMX4. Finally, pMX4 and pMX2 were digested with NdeI and XhoI to construct the plasmid pMX7. The NADP ME (*maeB*) gene was amplified from *E. coli* K-12 by PCR and cloned into the pET30a plasmid between the NcoI and SalI sites, resulting in plasmid pMX22.

Cultivation and expression

The strains used in this work are listed in Table 1. The plasmids pMX1, pMX2, pMX3, pMX7 and pMX22 were transformed into *E. coli* BL21 (DE3) for expression. Plasmids pMX7 and pMX22 were co-transformed into *E. coli* BL21 (DE3) to build the recombinant strain MX32 (with ACC and NADP ME) and cultivated in LB medium. M9 medium was used for gene expression and lipid accumulation. Strains were grown in rotary shaker flasks at 37°C with corresponding antibiotics. Biomass was determined by optical density (OD) measurements at 600 nm. An OD_{600} value of 1.0 corresponds to 0.43 g dry weight/l. When OD_{600} reached 0.6, the cultures were induced by 0.5 mM IPTG, and malate was added as mentioned. After being induced for 12 h, the cells were harvested by centrifugation and washed with distilled water for later analysis. The harvested cells were resuspended in phosphate buffer (pH 7.8) and broken by ultrasonic treatment. The broken mixture was centrifuged, and the supernatant was analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie brilliant blue staining.

Fatty acid analysis

The lipid was extracted from the harvested wet biomass with chloroform/methanol (2:1, v/v) and evaporated to dryness under N_2 protection [10]. It was then measured by a colorimetric procedure based on the formation of cupric soaps [14], which can be described briefly as follows. Total lipids of the whole cell were saponified at 60°C for 1 h [19]. Then a portion of the reaction mixture (at least 0.1 mmol of FFA) was extracted with chloroform/*n*-heptane/methanol (56:42:2, v/v) and colorimetric measurements were performed on the upper layer at 550 nm to determine fatty acid quantitatively. The fatty acid molar yield was given as the molar ratio of fatty acids to glucose consumed (based on the molecular weight of palmitic acid). Different forms of fatty acids were separated by thin-layer chromatography (TLC). Chromatographic separation was developed on silica-coated glass plates.

For fatty acid composition analysis, the total lipids extracted from the biomass were saponified using the procedure described above and converted to methyl esters by methanolysis with BF_3/CH_3OH (1:4, v/v) for 30 min at 60°C [16]. The fatty acid methyl esters (FAMEs) were then analyzed in an Agilent 6897 GC-MS equipped with a TR-Wax MS GC column (30 m, 0.25 mm, 0.25 μm); the elution conditions were as follows: the starting temperature was 100°C (hold for 2 min), and then 10°C/min to 250°C (hold for 5 min). Eicosanoic acid (20:0) was spiked in as an internal standard in all samples.

Determination of NADP ME activity

The wet cells were broken apart in a sonicator, and then the resultant mixture was centrifuged at 4°C and 13,000×*g* for 20 min. The supernatant was used immediately to determine the ME activity at 340 nm at room temperature with a UV-visible spectrophotometer [29]. A standard reaction mixture containing 50 mM pH 7.5 Tris-HCl, 1 mM $MgCl_2$, 0.5 mM $NADP^+$, and 10 mM L-malate, to a final volume of 0.5 ml, was used. The supernatant containing NADP ME was added to the mixture to start the reaction. One unit of the enzyme was defined as the amount of enzyme that catalyzed the production of 1 μmol of NAD(P)H/min. Protein was determined using the Bradford method, with BSA used as a standard [3].

Results

Improving fatty acid production by overexpressing ACC

A. calcoaceticus is a Gram-negative oleaginous microorganism (TAG accounts for nearly 70% of its dry cell weight). Its ACC has four subunits. The four subunits expressed in *E. coli* were checked by SDS-PAGE, and ACC activity was detected via phosphate measurements using colorimetric method [23] (data not shown). The in vivo activity of ACC and its effect on fatty acid production in *E. coli* were examined at three different ACC expression levels (Fig. 2). The results showed that the lipid accumulation of the recombinant strain was greatest when it was induced by 0.5 mM IPTG. After being induced for 24 h, the intracellular fatty acid content reached 177.66 mg/g and a molar yield of 1.82%, corresponding to a threefold enhancement in lipids compared to the control strain, and providing evidence to back up our assumption.

Lipid accumulation by overexpressing malic enzyme

In order to achieve a further increase in fatty acid production, native *E. coli* NADP ME was expressed to

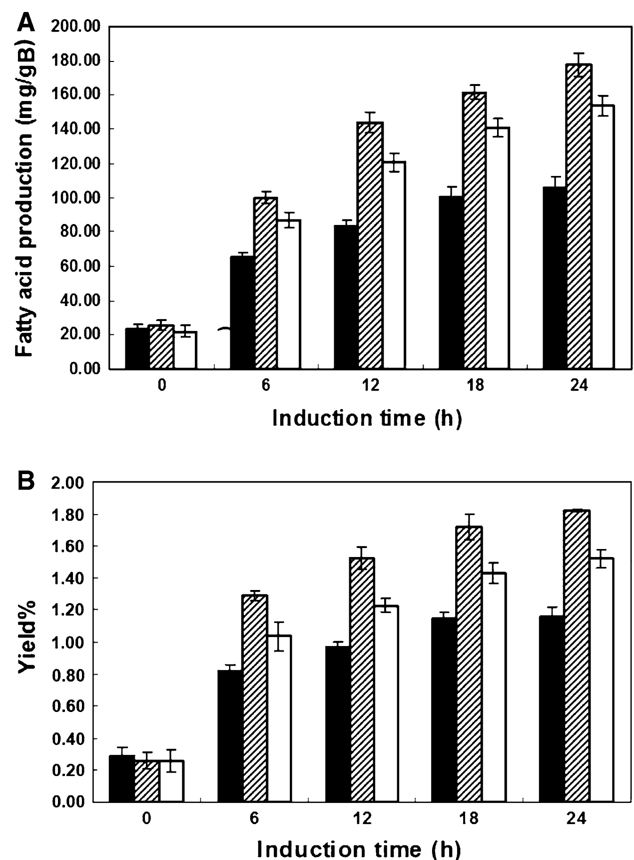

Fig. 2 Fatty acid accumulation at different expression levels of ACC. IPTG levels: 0.1 mM (*black bars*); 0.5 mM (*hatched bars*); 1 mM IPTG (*open bars*). Fatty acid production (**a**) and fatty acid yield on glucose (**b**) are shown. MX7 was cultured in M9 at 37°C, as mentioned in the "Methods" section. When OD_{600} reached 0.6, IPTG was added at various concentrations to the culture medium, and the expression time of ACC was as shown. All experiments were done in triplicate, and the standard deviations were less 10% of the values of the points

improve NADPH levels for fatty acid production. The expressed ME was first checked by SDS-PAGE, and in vitro activity was confirmed (the enzyme activity was 40.79 U/mg). However, fatty acid production did not change significantly with in vivo expressed malic enzyme (data was not shown), probably because there was not enough malate serving as a substrate for NADPH synthesis [21]. Different concentrations of malate were then added to the culture to test substrate availability. All of the engineered strains accumulated more lipids than the control in the presence of malate at levels of up to 40 mM. Total fatty acid in the recombinant strain MX20 was greatest (169.67 mg/g and 14.14 mg/g/h), with a molar yield of 1.69%, for 15 mM malate (Fig. 3). However, lipid production did not increase with greater malate concentrations. Furthermore, we found that the amount of fatty acid in the recombinant strain MX20 increased with cultivation time for 15 mM malate. After 30 h of cultivation, the total amount of cellular fatty acid reached its maximum. The fatty acid production of this strain was 197.74 mg/g, which is fourfold greater than that of the wild-type strain, and the molar yield of fatty acid (moles of fatty acid per mole of glucose) was 1.89%.

Increasing fatty acid production by co-expression of ACC and NADP ME

With the aim to accumulate more lipids, ACC and NADP ME were co-expressed in *E. coli* BL21 (DE3). As shown in Fig. 4, recombinant strain MX32 (with the ACC gene and the NADP ME gene) gave the highest fatty acid concentration among the four strains. This strain accumulated 284.56 mg/g fatty acids after induction for 30 h under shake-flask conditions, which was much greater than strains MX7 and MX20. The molar yield of fatty acid of strain MX32 reached 2.7%.

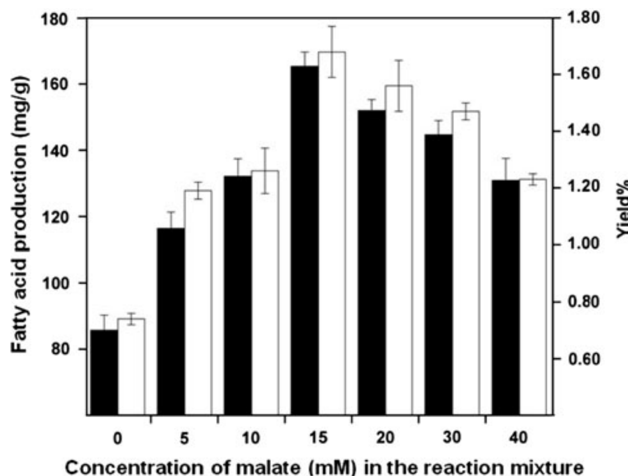

Fig. 3 Fatty acid production at various concentrations of malate. *Black bars* show fatty acid production (mg/g); *open bars* show fatty acid yield on glucose (mol/mol %). MX20, which affords ME, was cultured in M9 at 37°C, as mentioned in the "Methods" section. When OD_{600} reached 0.6, IPTG and different concentrations of malate were added to the culture media, and the resulting cultures were run at 37°C for a further 16 h. All experiments were done in triplicate, and the standard deviations were less 10% of the values of the points

Fatty acid analysis

Glycerophospholipids and lipid A comprised the major lipids in *E. coli*. The fatty acid compositions of the recombinant strains are shown in Table 2. The wild-type *E. coli* strain synthesized four major fatty acids [25], including myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1) and oleic acid (18:1). After being induced for 12 h, the recombinant strains MX7 (ACC) and MX20 (NADP ME) produced 16:0 as their major fatty acid

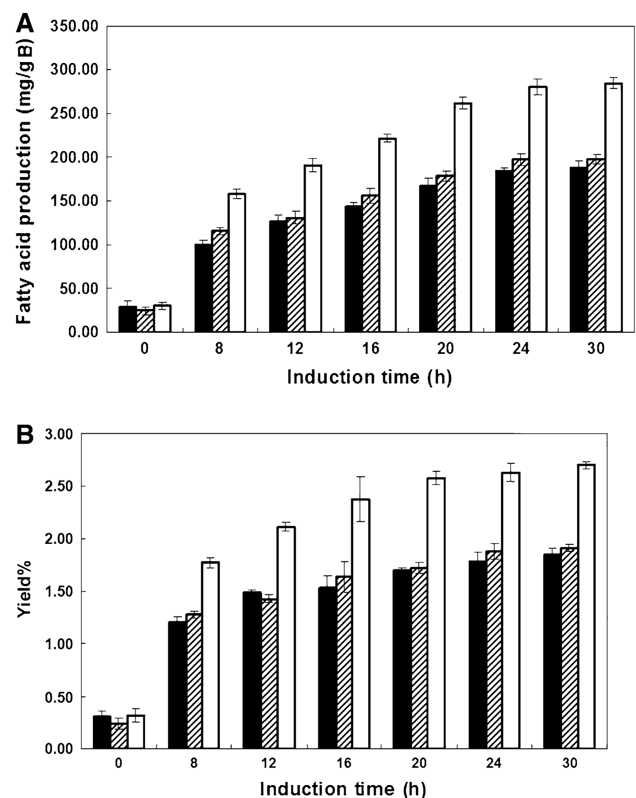

Fig. 4 Fatty acid production of *E. coli* which affords ME and ACC. *Black bars* MX7 (ACC); *hatched bars* MX20 (NADP ME); *open bars* MX32 (ACC and NADP ME). Fatty acid production (**a**) and fatty acid yield on glucose (**b**) are shown. The growth of each recombinant strain (MX7, MX20 and MX32) was monitored over time. All strains were cultured in M9 at 37°C, as mentioned in the "Methods" section. When OD_{600} reached 0.6, IPTG was added to the media and the cultures were run at 37°C for various durations. Malate was added to the media for MX20 and MX32 individually when IPTG was added. All experiments were done in triplicate, and the standard deviations were less 10% of the values of the points

Table 2 Fatty acid compositions of wild-type and engineered strains

Strain	C14:0	C16:0	C16:1	C18:1	Total (mg/g)[a]
BL21(DE3)/pET30a	6.5 ± 0.8	64.58 ± 4.6	10.65 ± 1.1	18.17 ± 2.5	50.17 ± 6.8
MX7 (ACC)	10.68 ± 1.4	52.91 ± 8.4	19.11 ± 2.4	17.29 ± 1.8	119.45 ± 8.7
MX20 (ME)	8.47 ± 1.6	57.73 ± 6.6	17.96 ± 2.8	15.83 ± 0.9	128.04 ± 7.3
MX32 (ACC and ME)	10.72 ± 0.7	57.21 ± 7.1	19.24 ± 1.7	12.83 ± 1.4	213.67 ± 9.2

Data show fatty acid production as a percentage of total fatty acid. Cells were grown in M9 at 37°C. When OD_{600} was about 0.6, the cells were induced with 0.5 mM IPTG at 37°C for 16 h

[a] The last column represents the total fatty acid in the cells. All experiments were done in triplicate, and the standard deviations were less 10% of the values of the points

constituent, and both of them were observed to produce more 16:1 and 18:1 than the control strain BL21 (DE3)/pET30a. When ACC and ME were co-expressed in MX32, fatty acid productivity was further increased to fourfold that of the wild-type strain (from 50.17 to 213.67 mg/g; the yield increased from 0.45 to 1.53%). High levels of 16:0, 16:1 and 18:1 were obtained.

Discussion

Many "traditional" lipid-accumulating microorganisms, such as fungi and yeast, have already been well studied. However, given that fatty acids are increasingly being used in biofuels, none of these are ideal for producing the fatty acids intended for use in biofuels if we consider their oil contents, growth rates and fatty acid compositions [24]. The advantages of using *E. coli* as an alternative producer of fatty acids rather than oleaginous microorganisms include the well-known genetics of this bacterium and its popularity as a host cell for genetic manipulation. *E. coli* also rapidly grows to reach a high density in inexpensive substrates. It accumulates maximal biomass within 36 h under fed-batch conditions, while the cultivation of many other eukaryotes is often a time-consuming task. Engineered *E. coli* has been used for fatty acid production related to biodiesel [15]. However, increasing the fatty acid production of *E. coli* is still a challenge. The lipid accumulation mechanism employed by oleaginous microorganisms provides valuable information for constructing an effective *E. coli* fatty acid producer. In this work, we tried to reconstruct *E. coli* in order to enhance fatty acid production through metabolic engineering strategies.

A previous study showed that co-expression of *E. coli* ACC and thioesterase I (encoded by the *tesA* gene) resulted in a sixfold increase in the rate of fatty acid synthesis with 6.6 nmol of free fatty acid [8]. However, expressing the *E. coli* ACC alone did not cause an obvious increase in fatty acid production (0.08 nmol of free fatty acid), which was similar to our results (the intracellular lipid was not improved at all; it was only 57 mg/g). This could be because the overexpression of native *E. coli* ACC suffered from feedback inhibition by acyl-acyl carrier proteins [4, 9]. *tesA* may reduce this inhibition by forming free fatty acid, resulting in the increased production of fatty acid. Another way to reduce this inhibition could be to express a non-native ACC gene that is not recognized by the acyl-acyl carrier proteins of *E. coli*. In our work, the heterologous expression of ACC from *A. calcoaceticus* in *E. coli* caused a threefold enhancement in lipid levels and thus provided support for our assumption. A similar strategy was used in flavonoid production; the highest productivity was achieved when *Photorhabdus luminescens* ACC was expressed in *E. coli* [31].

NADPH plays an important role in fatty acid accumulation in oleaginous microorganisms, and these microbes have a unique mechanism to supply NADPH. Based on its performance in the lipid accumulation of oleaginous fungi, malic enzyme (ME, $NADP^+$-dependent) was considered as a way to establish a pathway for the supply of NADPH in *E. coli*. Initially, we overexpressed native *E. coli* NADP ME in order to increase the supply of NADPH. However, the fatty acid production did not change significantly. We then supposed that malate could be a limiting factor for NADPH synthesis. Based on this hypothesis, malate at different concentrations was added to the culture to improve the availability of substrate for malic enzyme. The results showed that all of the engineered strains accumulated more lipids than the control. This phenomenon may be caused by the two potentially linked consequences of overexpressing malic enzyme: increased NADPH levels and increased pyruvate levels from malate [22]. In addition, we found that increasing the malate concentration above 15 mM did not cause any further increase in lipid accumulation. One reason for this could be toxicity of malate to or its inhibition of malic enzyme or other related enzymes, because we did not observe cell growth inhibition at malate concentrations higher than that value. This phenomenon will be further studied in the future.

Co-expression of ACC and NADP ME resulted in a 5.6-fold increase in total fatty acid productivity compared to the control. However, the fatty acid yield obtained in this

initial study performed in shake-flask batch mode was still lower than that obtained with oleaginous microorganisms like microalgae and yeast. Therefore, further studies will focus on several points: (1) resolving the feedback inhibition caused by acyl-ACPs, by expressing *tesA* gene for example; (2) knocking out the *poxB* gene in order to reduce the main by-product of acetate and thus stop the carbon flux from branching in the metabolic pathway; (3) the overexpression of acetyl-CoA synthase (ACS) in order to improve acetyl-CoA levels while simultaneously reducing the toxic effects of acetate on cells, as used in the strategy employed for high-value plant flavonoid biosynthesis [13]. To sum up, this study provides some attractive strategies for increasing fatty acid production in *E. coli* by simulating the lipid accumulation of oleaginous microorganisms, which could aid the development of a prokaryotic fatty acid producer.

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