Overexpression of *ACP* gene from cyanobacterium *Synechococcus elongatus* PCC 7942 modulates lipid profiles in expressing cells

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Abstract

Biofuel production directly from CO_2 is an attractive approach to solving the energy shortage and environmental problems. Accumulating evidence has been shown that photosynthetic microorganisms such as microalgae and cyanobacteria would serve as an attractive source because of several unique metabolisms for lipid biosynthesis. They synthesize and accumulate high lipids and triglycerides mostly in their cells, thus these lipids are further used as feedstocks for direct conversion to biodiesel. Here, we report the overexpression of Acyl Carrier Protein synthetase (*ACP*) into *Escherichia coli* as well as a fresh water cyanobacterium *Synechococcus elongatus* PCC 7942. ACP involves in the elongation of long chain free fatty acid, a precursor of lipid storage compound or triacylglycerol. The *ACP* from a freshwater cyanobacterium *Synechococcus elongatus* PCC 7942 driven under native promoter was cloned and transformed into the wild-type strain. Intracellular fatty acid profile in the heterologous expressor *Escherichia coli* strain DH5α was performed using Gas Chromatography. Our results showed that fatty acid profile in the expressing cells were changed, comparing with control ones. The results suggest that expression of ACP modulates lipid profile. Thus, metabolic engineering of *ACP* would be a potential way to enhance biofuel production in microalgae.

Keywords: biofuel, cyanobacteria, lipid biosynthesis, acyl carrier protein

Introduction

Nowadays, the demand of sustainable energy becomes one of the most concerns. There are many problems in both petroleum fuel production and consumption such as limitation sources and unstable cost. Moreover, petroleum fuel production can release high level of CO_2 that is not environmentally friendly (Radakovits et al. 2010). Therefore, sustainable energy study is highly challengeable. Biofuel is a well-known sustainable energy that is derived from a living or living organisms. Microalgae and cyanobacteria are oxygenic photosynthetic organisms that have several unique metabolisms. They have several advantages in biofuel production such as their high photosynthetic efficiency, their high growth rate and their ability to produce energy-rich compounds as potential biofuel precursors (Radakovits et al. 2010; Hu et al. 2008). Although they can be a resource in biofuel production, ability in lipid production of wild-type strains are limited. Lipid biosynthesis is attractive targets for strain improvement to enhance the efficiency in biofuel production by genetic engineering technique.
To date, many researches have been focused in expression of genes involved in lipid biosynthesis in cyanobacteria. For example, putative acyl-acyl carrier protein synthetase gene (*aas*) was isolated and identified from cyanobacteria *Synechocystis* sp. PCC 6803 and

Synechococcus elongatus PCC 7942 (Kaczmarzyk and Fulda 2010). AAS catalyzes fatty acid to thiol of acyl carrier protein. Loss-of-function of AAS reduced ability of exogenous fatty acids utilization. These results suggested that AAS enzyme is involved in the recycling of exogenous fatty acids (Kaczmarzyk and Fulda 2010). Ruffing et al. have been reported in 2012 that the engineered *S. elongatus* PCC 7942 using *ACP* and thioesterase (*tesA*) genes. The expressing cells could produce and secret free fatty acids in a small-scale production.
ACP encodes acyl carrier protein synthetase enzyme. This enzyme synthesizes acyl carrier protein that involves in free fatty acid (FFA) synthesis, thus it plays the important role in long chain free fatty acid elongation (Kaczmarzyk and Fulda 2010). Here, we examined the overexpression of ACP synthetase in *E. coli* as well as *S. elongatus* PCC 7942. The unicellular cyanobacterium is a genetic model of cyanobacteria because its genome database is available. Moreover, it can uptake exogenous entire genetic material directly by natural transformation (Nomura et al. 1995). FFA would be an important precursor of intracellular neutral lipid mainly triacylglycerol (TAG). In this study, *ACP* and its promoter were isolated from *S. elongatus* PCC 7942, and then expressed in *E. coli* as well as wild-type *S. elongatus* PCC 7942. Fatty acid profile was investigated in the overexpressing cells by gas chromatography. Our results revealed that lipid profiles were modulated in the overexpressing cells.

Methodology

Strain and Growth Conditions
Fresh water cyanobacterium, *S. elongatus* PCC 7942 cells were grown photoautotrophically (70 µE m^{-2} s^{-1}) in BG11 liquid medium with shaking 150 rpm (Rippka et al. 1979) at 30°C. *S. elongatus* PCC 7942 cells overexpressing putative *ACP* were grown in solid medium under the same conditions as the wild-type cells but were supplemented with 50 µg/ml of streptomycin. *Escherichia coli* strain DH5α cells were grown in Luria-Bertani (LB) medium at 37°C and use as general purpose on cloning. *E. coli* transformed cells were grown under the same conditions as the wild-type cells but were supplemented with 50 µg/ml of ampicillin and streptomycin when they were transformed with pGEM-T Easy vector (Promega, Wisconsin, USA) and shuttle vector pUC303 (Nomura et al. 1995), respectively. The growth of cyanobacterial and bacterial cells were monitored by measuring the absorbance at 730 and 600 nm respectively with a Shimadzu UV-160A spectrophotometer.

DNA isolation
Genomic DNA was extracted from 100 mg fresh weight of *S. elongatus* PCC 7942 cells using the DNeasy Plant mini kit (Qiagen, Hilden, Germany) as per the manufacturer's instruction. The genomic DNA quantity was electrophoresed on 1.0 % (w/v) agarose gels with 1: 10,000 dilution of SYBR safe DNA gel stain (Life technologies, CA, USA). Extracted DNA was monitored by measuring the absorbance at 260/280 nm with NanoDrop 2000 UV-Vis Spectrophotometer (Thermo scientific, MA, USA).

Cloning and expression of *S. elongatus* PCC 7942 *ACP*
Putative *ACP* together with its native promoter was amplified by PCR from genomic DNA of *S. elongatus* PCC 7942 using specific primer pair: GTGACTGGAACCGCCCTCGCG, ACP_7942_F, and TTAACTCGCCGATTCAAACAT, ACP_7942_R. PCR products were clone into pGEM-T Easy vector as per the manufacturer's instruction and sequenced to exclude PCR errors. DNA fragments of *ACP* with its native promoter were prepared by digestion with *Bam*HI and *Xho*I and ligated into the corresponding sites of pUC303. The recombinant plasmid, pUC_ACP7942_303 was used to transform into *E. coli* DH5α cells as

standard protocol (Sambrook et al. 1989) for propagation. The pUC_ACP7942_303 was extracted from *E. coli* cells using PureLink Quick Plasmid Miniprep Kit (Invitrogen, CA, USA). Restriction enzyme analysis was performed to confirm the insertion of correct plasmid (approximately 2.3 kb).

Transformation into *S. elongatus* PCC 7942

The recombinant plasmid, pUC_ACP7942_303 was transformed into wild-type strain *S. elongatus* PCC 7942 as previously describe (Nomura et al. 1995; Waditee et al. 2005). Five milliliters of *S. elongatus* PCC 7942 (Optical density at 730 nm approx. 0.5) cells were harvested by centrifugation at 4,500 rpm for 10 minutes. Cells pellet then were washed by BG11 medium for at least three times. The washed cells were re-suspended in one milliliter of BG11 medium. One hundred microliters of cells suspension were mixed with 300 ng DNA plasmids, pUC_ACP7942_303 as overexpressing cells and pUC303 as control cells. Then, they were incubated at 30°C for overnight in dark. BG11 medium was added into the mixture, the volume up to 300 µl. Then, the cell mixtures were laid onto BG11 plates. Transformed cells were grown under the same conditions as the wild-type cells for 5 days. Then, the transformant colonies were selected by addition of 50 µg/ml streptomycin. After 21 days, several single colonies were re-streaked on BG11 supplemented with streptomycin. To check candidate transformants, cells of candidate clones were used as a template DNA in colony PCR analysis by using specific primers for *ACP* amplification.

Lipid profile analysis

For esterification of extracted fatty acid, two milliliters of methanol-hydrochloric 95:5 (v/v) was added to approximately 100 mg harvested cells then mixed and incubated at 80 - 85 °C for 90 min. One milliliter of distilled water was added to the mixture. For fatty acid methyl esters (FAME) extraction, FAMEs were extracted by hexane containing 0.01 % butylated hydroxytoluene (BHT) at room temperature. Reaction products were centrifuged at 4,000 rpm for 5 min to separate the organic and aqueous phases. Then, aqueous phase was selected and mixed with Na_2SO_4 for remaining water absorption. FAMEs were evaporated to dryness at room temperature under a gentle stream of nitrogen. The residue was solubilized again in 1 ml of hexane and analyzed it by gas chromatography.

FA were chromatographed as methyl esters on a 3 meter of HP-INNOWax column with an internal diameter of 0.25 µm. Agilent 6890N gas chromatograph equipped with a flame ionization detector (FID). Analysis was performed as following condition, helium carrier gas was performed as a mobile phase. The injector and detector temperature were maintained at 250 °C. The oven temperature was started at 150 °C and then increased to 250 °C with increasing rate 10 °C per min. For analysis, fatty acids were compared with fatty acids standard (sigma).

Results

Cloning and expression of *ACP* from *S. elongatus* PCC 7942

To generate expressing plasmid, *ACP* together with its native promoter was amplified from genomic DNA of *S. elongatus* PCC 7942 by using specific primers as described in methodology. PCR products approximately 2.3 kb were subcloned into pGEM-T Easy vector. A recombinant plasmid harbouring *ACP* gene was confirmed by restriction analysis.

To construct *ACP* with its native promoter in shuttle vector. The pUC303 was use as expression vector, DNA fragments covering *ACP* and its native promoter from cloning vector were prepared by digestion with *Bam*HI and *Xho*I from pGEM-T Easy vector. Then, these

fragments were ligated into corresponding sites of pUC303. To confirm the insertion, restriction enzyme analysis was performed by *Bam*HI and *Xho*I digestion. Electrophoresis result shows the inserted DNA fragment of *ACP* and its native promoter size approx. 2.3 kb after double digestion (Fig. 1). The correct generated plasmid was transferred to *S. elongatus* PCC 7942 cells.

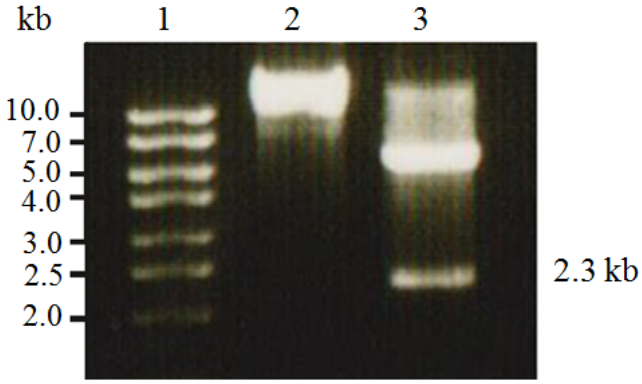

Figure 1: Restriction enzyme analysis of *ACP* with its native promoter in pUC303. Lane 1: DNA ladder, lane 2: non-digestion of pUC_ACP7942_303, lane 3: double digestion of pUC_ACP7942_303 with *Bam*HI and *Xho*I.

Transformation of *ACP* into *S. elongatus* PCC 7942

To overexpress *ACP* in a wild-type strain *S. elongatus* PCC 7942, natural transformation was performed as described in methodology. After streptomycin addition, only candidate transformants could grow on selective BG11 plates. The single colony that obtained empty vector pUC303 or generated plasmid would appear on selective BG11 plates after growing for 3 weeks (Fig. 2). For checking generated plasmid transformants, candidate clones were re-streaked on selective plates. Then, colony PCR analysis was performed by using specific primers for *ACP* amplification. The electrophoresis result shows in Fig. 3. The overexpressing cells which obtained *ACP* generated plasmid (pUC_ACP7942_303), had stronger PCR products signal when compared with wild-type strain or control cells which *ACP* had only native expression.

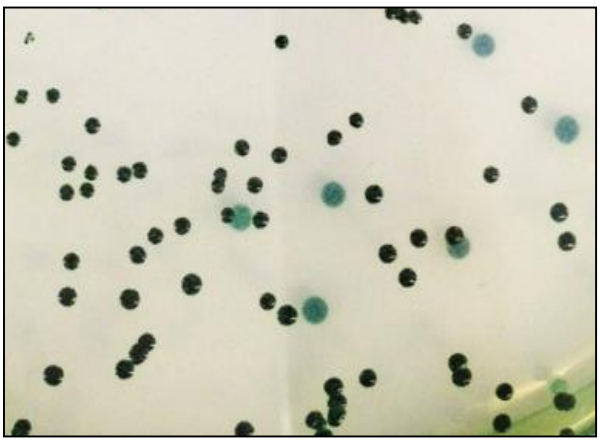

Figure 2: Single colony of candicate transformants on BG11plate supplemented with streptomycin (50µg/mL)

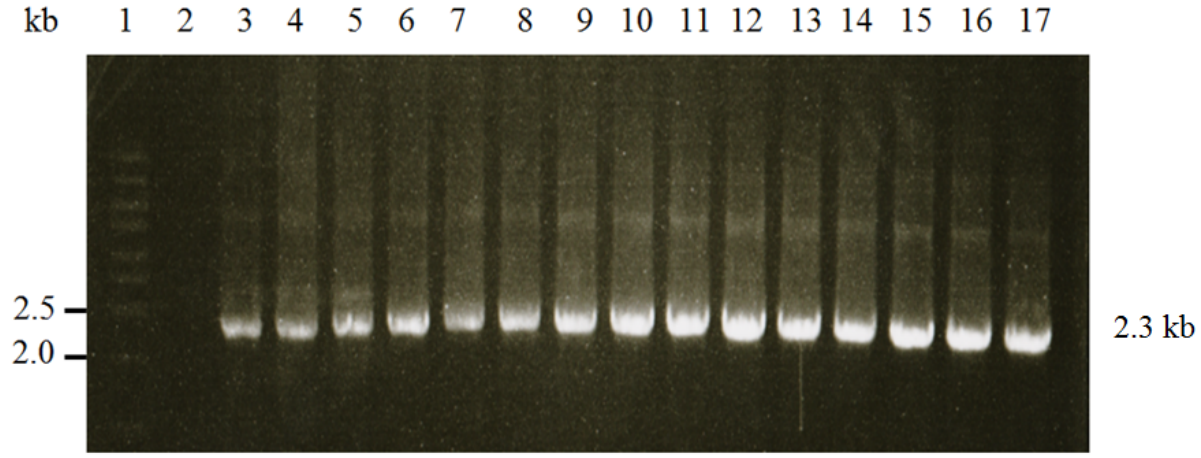

Figure 3: Colony PCR analysis of *ACP* of *S. elongatus* PCC 7942 overexpressing cell. Lane 1: DNA ladder, lane 2: negative control (no template DNA), lane 3: empty vector pUC303 (control cells), lane 4: wild-type strain cells, lane 5-17: candidate transformants of pUC_ACP7942_303.

Lipid profile of the *E. coli* DH5α expressing cells

Lipid profile analysis was firstly conducted in *E. coli* expressing cells. For this, *E. coli* control cells (empty vector) and expressing cells (harbouring *ACP* gene) were grown in LB at 37 ^{o}C for overnight. Cells were harvested by centrifugation. Then, intracellular fatty acids were extracted and esterificated as described in methodology. FAMEs were extracted by hexane containing 0.01 % BHT. After processing, extracts were analyzed by gas chromatography with a flame ionization detector and using helium as carrier gas.
As shown in Fig. 4, the profile of esterified fatty acids showed some differences between control and heterologous expressing cells. In both control and expressing cells found several kinds of fatty acids between C12 to C18 and 16:0 (palmitic acid) was a majority of these fatty acids. As the results, both unsaturated and saturated fatty acids were found. There were slightly increasing of 12:0, 17:0 and 18:2 in the expressor when compared with control ones. Significantly, amount of 16:0, 16:1 (palmitoleic acid) and 18:1 (oleic acid) in the expressing cells were clearly higher than control cells. On the other hand, only 18:3 (linolenic acid) slightly decreased in the expressing cells (Fig. 4). These data indicated that the expression of *ACP* from *S. elongatus* PCC 7942 could modulate fatty acid compositions in *E. coli* DH5α cells.

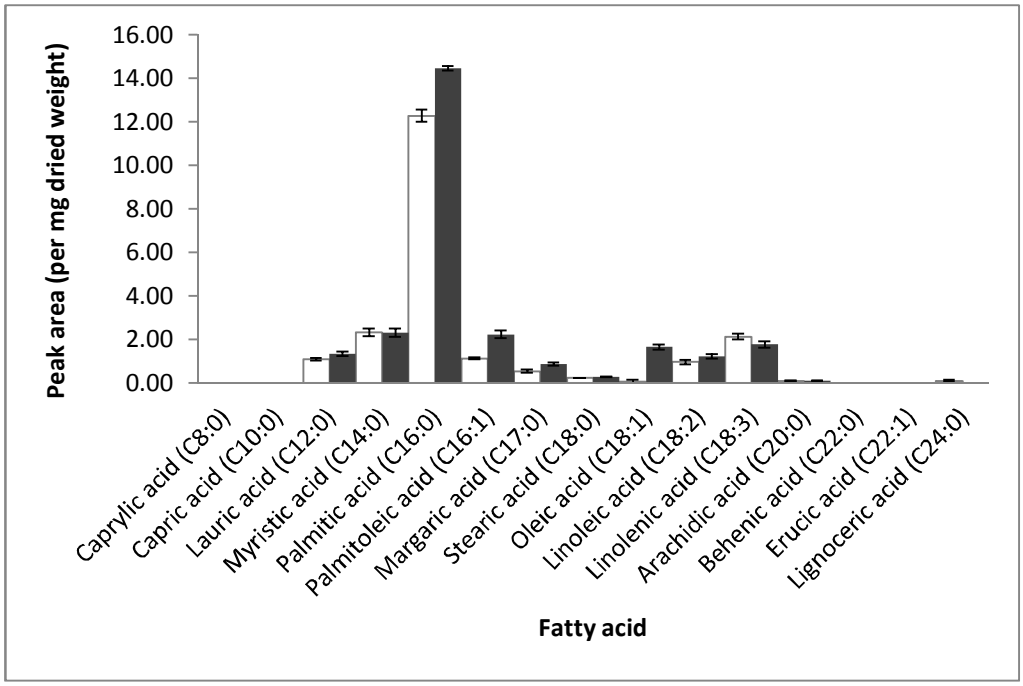

Figure 4: Intracellular fatty acid profiles of control and expressing cells from *E. coli* DH5α. White bars: fatty acids from control cells, black bars: fatty acids from expressing cells.

Discussion

In microalgae, lipid metabolism has been extensively studied for biofuel production. Particularly, fatty acid synthesis is attractive approach. In this study, we aim to overexpress *ACP* which is important for free fatty acid elongation in cyanobacteria. Our results showed that *ACP* with its native promoter was successfully overexpressed in *S. elongatus* PCC 7942. We confirmed the overexpressors by (1) Ability to grow on BG11 supplemeted with streptomycin plates and (2) Colony PCR analysis with specific primers of ACP and streptomycin genes. For lipid profile analysis, we firstly evaluated in *E. coli* DH5α expressing cells. As shown here, fatty acid compositions were changed when compared with control ones. The results indicate that palmitic acid (16:0), palmitoleic acid (16:1) and oleic acid (18:1) were clearly increased in the expressing cells (Fig.4). In expressing cells, palmitic acid increased approximately 1.2 fold compared with control cells. Palmitic acid is a dominant fatty acid in *E. coli* DH5α cells. As mentioned above, it corresponded to Kaczmarzyk and Fulda (2010) that C16 fatty acid was specific substrate for acyltransferases in prokaryotic organisms. Lipid profile of overexpressors *S. elongatus* PCC 7942 will be further analyzed.

Conclusion

Here, *ACP* from *S. elongatus* PCC 7942 could be successfully cloned and expressed in *E. coli* DH5α or overexpressed in wild-type strain. The fatty acid analysis demonstrated that ACP could modulate lipid profile in heterologous expressing cells *E. coli* DH5α. Therefore, *ACP* would be a potential way to develop biofuel production in microalgae by metabolic engineering. Further, lipid profile will be analyzed in the overexpressor *S. elongatus* PCC 7942.

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