ผลของการเพิ่มการแสดงออกของยีน *sll1848* และ/หรือ *slr1510/sll1848* ต่อปริมาณลิพิด
ในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803

นางสาวอุมาพร โตวิจิตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECT OF OVEREXPRESSION OF *sll1848* AND/OR *slr1510*/*sll1848* GENES ON LIPID CONTENT IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803

Miss Umaporn Towijit

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	EFFECT OF OVEREXPRESSION OF *sll1848* AND/OR *slr1510*/*sll1848* GENES ON LIPID CONTENT IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803
By	Miss Umaporn Towijit
Field of Study	Biotechnology
Thesis Advisor	Assistant Professor Saowarath Jantaro, Ph.D.
Thesis Co-Advisor	Professor Aran Incharoensakdi, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Associate Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Kanoktip Packdibamrung, Ph.D.)
.....Thesis Advisor
(Assistant Professor Saowarath Jantaro, Ph.D.)
.....Thesis Co-Advisor
(Professor Aran Incharoensakdi, Ph.D.)
.....Examiner
(Assistant Professor Sanit Piyapattanakorn, Ph.D.)
.....External Examiner
(Assistant Professor Wuttinun Raksajit, Ph.D.)

อุมาพร โตวิจิตร : ผลของการเพิ่มการแสดงออกของยีน *sll1848* และ/หรือ *slr1510*/*sll1848* ต่อปริมาณลิพิดในไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 (EFFECT OF OVEREXPRESSION OF *sll1848* AND/OR *slr1510*/*sll1848* GENES ON LIPID CONTENT IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. เสาวรัตน์ จันทะโร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. อรัญ อินเจริญศักดิ์, 118 หน้า.

การศึกษานี้ได้สร้างสายพันธุ์ *Synechocystis* sp. PCC 6803 ที่เพิ่มการแสดงออกของยีน *sll1848* (OE+C) และ *slr1510*/*sll1848* (OE+XC) เป็นผลสำเร็จ และได้ศึกษาผลของสภาวะดัดแปรสารอาหารต่อการเจริญเติบโต ปริมาณคลอโรฟิลล์ เอ และคาโรทีนอยด์ ประสิทธิภาพการสังเคราะห์ด้วยแสง การแสดงออกของยีนที่เกี่ยวข้องกับการสังเคราะห์กรดไขมัน ปริมาณกรดไขมันและลิพิดในสายพันธุ์ปกติ (WT) สายพันธุ์ควบคุม (WT+pE) สายพันธุ์ OE+C และ OE+XC การเปรียบเทียบลำดับของกรดอะมิโนของ *sll1848* โปรตีน ของ *Synechosystis* เปรียบเทียบกับลำดับกรดอะมิโนของ *Synechosystis* sp. PCC 6714 และ *Synechococcus* sp. PCC 7002 โปรตีน แสดงเอกลักษณ์ประมาณ 89% และ 56.4% ตามลำดับ อย่างไรก็ตามเอกลักษณ์ที่ต่ำของมันกับลำดับ กรดอะมิโนของ 1-เอซิล-เอสเอ็น-กลีเซอรอล-3-ฟอสเฟต เอซิลทรานสเฟอเรสของพืช *Arabidopsis thaliana* มีประมาณ 18.7% การเพาะเลี้ยงเซลล์ทั้ง 4 สายพันธุ์ ในอาหารสูตร BG_{11} สามารถยืนยันได้ว่าสายพันธุ์ที่เพิ่มการแสดงออกของยีนทั้งสองสายพันธุ์มีการเจริญเติบโตและรงควัตถุภายในเซลล์ รวมถึงประสิทธิภาพการสังเคราะห์ด้วยแสง ที่ไม่แตกต่างจากสายพันธุ์ปกติและสายพันธุ์ควบคุมอย่างมีนัยสำคัญ ปริมาณลิพิดรวมของสายพันธุ์ OE+C และ OE+XC มีค่าสูงกว่า WT ประมาณ 1.24 เท่า และ 1.49 เท่า ตามลำดับ ระดับสูงสุดของปริมาณลิพิดภายใต้สภาวะปกติคือ 24.3% ต่อน้ำหนักเซลล์แห้งเมื่อเทียบกับสายพันธุ์ปกติ นอกจากนี้ในสายพันธุ์ OE+XC พบกรดไขมันไม่อิ่มตัว 1.3% ต่อน้ำหนักเซลล์ หลังจากนั้นสามารถทำให้ปริมาณลิพิดรวมเพิ่มขึ้นอีกได้โดยสภาวะดัดแปรสารอาหาร สภาวะการขาดไนโตรเจนสามารถทำให้ลิพิดเพิ่มสูงขึ้นเป็น 26.5 และ 32.7% ต่อน้ำหนักเซลล์แห้ง และมีกรดไขมันไม่อิ่มตัว 2.0 และ 2.4% ต่อน้ำหนักเซลล์แห้งในสายพันธุ์ OE+C และ OE+XC ตามลำดับ เป็นที่น่าสนใจว่าสภาวะเสริมอะซีเทตเหนี่ยวนำปริมาณลิพิดได้สูงถึง 39% ต่อน้ำหนักเซลล์แห้งอย่างมีนัยสำคัญ ขณะที่กรดไขมันไม่อิ่มตัวเพิ่มสูงถึง 1.6% ต่อน้ำหนักเซลล์แห้ง การแสดงออกเกินปกติแบบคู่ของยีน *slr1510*/*sll1848* ของ OE+XC แสดงการควบคุมที่เพิ่มขึ้นในระดับ mRNA ของ *sll1848* และ *slr1510* นอกจากนี้ระดับ mRNA ของยีน *AccA* และ *lipA* เพิ่มขึ้นเล็กน้อย ผลการทดลองจึงบ่งชี้ว่าระดับการทรานสคริปต์ของยีน *sll1848* และ *slr1510* สัมพันธ์กับปริมาณลิพิดในสายพันธุ์ OE+XC โดยเฉพาะการเสริมอะซีเทต 0.4% จากผลการทดลองทั้งหมดนี้สรุปได้ว่าการเสริมการผลิตลิพิดใน *Synechocystis* sp. PCC 6803 สามารถทำสำเร็จได้โดยการเพิ่มการแสดงออกของยีน *sll1848* และ *slr1510*/*sll1848* การผลิตที่สูงกว่าของปริมาณลิพิดสูงขึ้นได้เช่นเดียวกันโดยสภาวะขาดไนโตรเจนและการเสริมอาหารด้วยอะซีเทต

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS:

UMAPORN TOWIJIT: EFFECT OF OVEREXPRESSION OF *sll1848* AND/OR *slr1510*/*sll1848* GENES ON LIPID CONTENT IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803. ADVISOR: ASST. PROF. SAOWARATH JANTARO, Ph.D., CO-ADVISOR: PROF. ARAN INCHAROENSAKDI, Ph.D., 118 pp.

In this work, *sll1848*-overexpressing (OE+C) and *slr1510/sll1848* overexpressing (OE+XC) strains of *Synechocystis* sp. PCC 6803 were successfully constructed. The effects of nutrient-modified conditions on growth rate, chlorophyll *a* and carotenoid contents, oxygen evolution rate, transcript level of genes related to fatty acid biosynthetic pathway, total lipid and unsaturated lipid contents of cells WT, control WT+pE, OE+C and OE+XC strains were studied. The alignment of the amino acid sequences of the putative Sll1848 protein of *Synechosystis* sp. PCC 6714 and of *Synechococcus* sp. PCC 7002 showed the identities of 89% and 56.4%, respectively to that of *Synechocystis* sp. PCC 6803. However, it showed low identity with 1-acyl-sn-glycerol-3-phosphate acyltransferase of *Arabidopsis thaliana* plant with about 18.7%. The cultivated cells of four strains in BG_{11} medium could confirm that two overexpressing strains had insignificant changes on their growth, pigment contents and oxygen evolution rate when compared to WT and control WT+pE. Importantly, the total lipid contents of those OE+C and OE+XC strains were significantly higher than WT of about 1.24 fold and 1.49 fold, respectively when compared to WT. The maximum level of total lipid content under normal condition was 24.3% w/dcw observed in OE+XC strain whereas its maximum unsaturated lipid content was 1.3% w/dcw. Additionally, the total lipid content could be further enhanced by nutrient-modified condition. The nitrogen deficiency condition could increase total lipid contents of OE+C and OE+XC strains to 26.5 and 32.7% w/dcw, respectively, whereas unsaturated lipid contents of both strains were increased to 2.0 and 2.4% w/dcw, respectively. Interestingly, 0.4% acetate supplementation significantly induced the total lipid content of OE+XC up to 39% w/dcw whereas its unsaturated lipid content was increased to 1.6% w/dcw. Double overexpression of *slr1510*/*sll1848* genes of OE+XC strain showed up-regulation on both *sll1848* and *slr1510* mRNA levels. In addition, *AccA* and *lipA* mRNA levels were slightly induced in OE+XC strain. The results suggest that transcript levels of *sll1848* and *slr1510* mRNA were related to the total lipid content in OE+XC strain, especially under 0.4% acetate supplementation. Altogether, the enhancement of lipid production in *Synechocystis* sp. PCC 6803 was achieved by overexpressing *sll1848* and *slr1510/sll1848* genes. The higher production of lipid content also occurred by nitrogen deficiency and acetate supplementation.

Field of Study: Biotechnology
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

bp	Base pair
°C	Degree Celsius
DNA	Deoxyribonucleic acid
DMF	*N, N*-dimethylformamide
EDTA	Ethylenediamine tetraacetic acid
dNTP	Deoxynucleotides triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
g	Gram
HEPES	Hydroxyethyl piperazineethanesulfonic acid
kb	Kilobase
L	Liter
mM	Millimolar
mRNA	messenger ribonucleic acid
min	Minute
µg	Microgram
µl	Microliter
µmol	Micromole
ml	Milliliter
M	Molar
nm	Nanometer
OD	Optical density

PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolution per minute
RT-PCR	Reverse transcription- polymerase chain reaction
sec	Second
w/v	Weight by volume
w/w	Weight by weight

CHAPTER I

INTRODUCTION

1.1 Lipid and fatty acid

1.1.1 Structure and functions

Lipids are generally hydrophobic in nature and soluble in organic solvents which may fully generate or in part by carbanion based condensations of thioesters (such as fatty acids and polyketides) and/or by carbocation based condensations of isoprene units (such as prenols and sterols) (Subramaniam *et al.*, 2011). Based on this classification system, lipids have been divided into eight categories: fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), saccharolipids (SL), and polyketides (PK) as shown in Figure 1.1. These lipids are found in both eukaryotic and prokaryotic sources (Fahy *et al.*, 2009). The main biological functions of lipids include energy storage, structural components of cell membranes, and important signalling molecules.

Triacylglycerols (TAG), triesters of glycerol with fatty acids, are major forms of energy storage compounds in most eukaryotic organisms. In the case of plants, TAG are commonly stored in the seeds (Murphy, 1993) while adipocytes (fat cells) in animals are used for biosynthesis and storage of TAG (Voet and Voet, 1990). In contrast, most bacteria, which are able to accumulate storage lipids, produce specialized lipids such as poly (3-hydroxybutyric acid), or other polyhydroxyalkanoic acids (PHA) (Steinbüchel, 1991) PHA comprise a complex class of storage polyesters occurring as inclusions within cells (Steinbüchel and Valentin, 1995)

The biological membranes are consisted of glycerophospholipids as main structure component. Moreover, other non-glyceride lipid components such as sphingomyelin and sterols are also found in membranes. In plants and algae, the galactosyldiacylglycerols, and sulfoquinovosyldiacylglycerol, which lack a phosphate group, are important components of chloroplast membranes and are the most abundant lipids in photosynthetic tissues (Ohlrogge and Browse, 1995; Harwood, 1998).

Signalling lipids control important cellular processes, including cell proliferation, apoptosis, metabolism and migration (Wymann and Schneiter, 2008) These lipids may occur via activation of a variety of receptors, including G protein-coupled and nuclear receptors, and members of several different lipid categories have been identified as signalling molecules and cellular messengers. For example, a sphingolipid is a potent messenger molecule involved in regulating calcium mobilization (Yasui and Palade, 1996), cell growth, and apoptosis (Spiegel and Merrill, 1996; Spiegel *et al.*, 1998) and a diacylglycerol (DAG) involved in calcium-mediated activation of protein kinase C (Nishizuka, 1992).

1.1.2 Fatty acids

Fatty acids are parts of a lipid which synthesized by chain-elongation of an acetyl-Co A primer with malonyl-Co A or methylmalonyl-Co A groups in a process called fatty acid synthesis. They are made of a hydrocarbon chain that terminates with a carboxylic acid group. This arrangement confers the molecule with a polar, hydrophilic end, and a nonpolar, hydrophobic end that is insoluble in water. The carbon chain, typically between 4 and 24 carbons long may be saturated or unsaturated, and may be attached to functional groups containing oxygen, halogens, nitrogen, and sulphur.

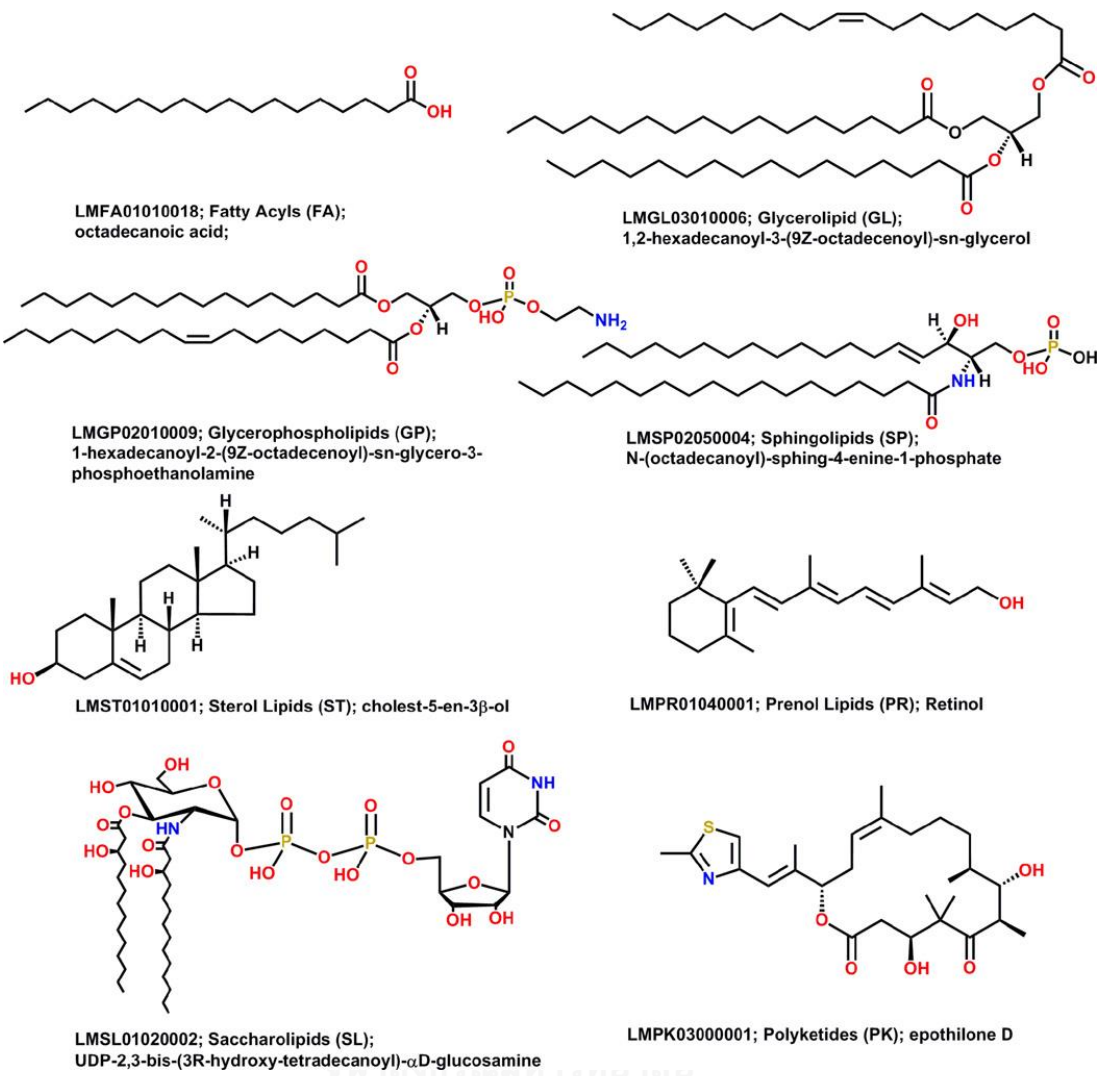


Figure 1. 1 Representative structures from each lipid category shown with LM ID, category name, category abbreviation, and systematic name (Subramaniam *et al.*, 2011).

1.1.3 Fatty acid biosynthesis

The de novo biosynthesis of fatty acids in plant, shown in Figure 1.2, from acetyl-CoA involves two enzyme systems: (i) acetyl-CoA carboxylase (ACCase) which is catalyzes the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, an essential substrate for FAS and (ii) fatty acid synthase (FAS). In higher plants both of these systems are located in the chloroplast. In seeds de novo fatty acid biosynthesis occurs in proplastids, which might have fundamentally different properties from chloroplasts.

In plants, fatty acid synthase (FAS) is similar to that present in most bacterial species and synthesizes saturated fatty acids of generally 16 or 18 carbons. Malonyl-CoA: ACP transacylase transfers the 3-carbon malonyl-group from malonyl-CoA onto the essential thiol of ACP as the source of carbon atoms for fatty acid elongation. The initial condensation reaction is catalyzed by 3-ketoacyl synthase III (KASIII) which uses malonyl-ACP to form a four-carbon 3-ketoacyl-ACP. The ketoacyl-ACP is subsequently reduced by NADPH in a reaction catalyzed by 3-ketoacyl-ACP reductase (KR) to generate a 3-hydroxyacyl-ACP. 3-Hydroxyacyl-ACP dehydratase (DH) hydrolyzed this to generate an enoyl-ACP, which is finally reduced by NADH to butyryl-ACP by enoyl-ACP reductase (ENR). The product of this first synthetic cycle, butyryl-ACP, is the substrate for further elongation rounds, each of which uses one molecule of malonyl-ACP and releases carbon dioxide. Elongation is catalyzed by the same enzymes used for generation of butyryl-ACP from acetyl-CoA and malonyl-ACP with the exception of subsequent condensation reactions. These occur in a similar way to the initial 3-ketoacyl-ACP synthase III reaction, with transfer of the acyl-primer onto cysteine and reaction with malonyl-ACP, but condensations from C4 to C16 are carried

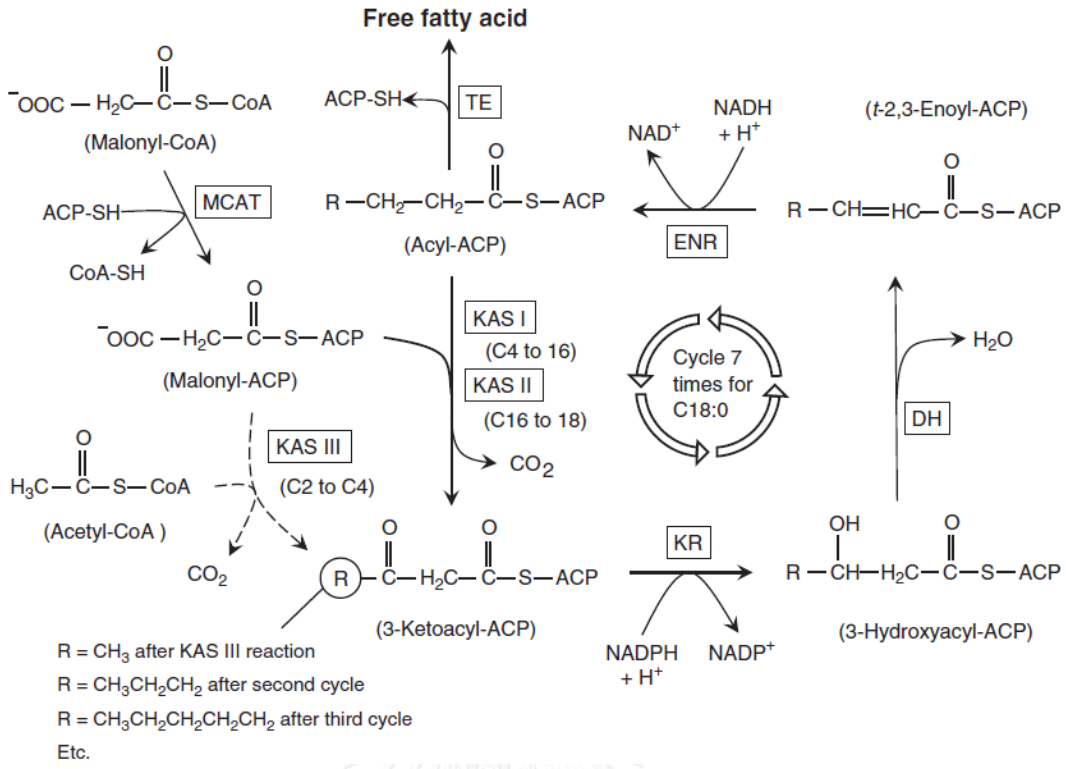


Figure 1. 2 Core reactions of fatty acid synthase. The cyclical reactions adding two-carbon units during fatty acid synthesis in plant plastids are shown. The dashed arrows indicate the reaction catalyzed by 3-ketoacyl-ACP synthase III, which is specific for the synthesis of butyryl-ACP. Thereafter the four-step cycle is repeated until the appropriate chain-length is reached and a thioesterase releases the fatty acid from ACP. Enzyme abbreviations: MCAT, malonyl-CoA: ACP transacylase; KAS, 3-ketoacyl-ACP synthase; KR, 3-ketoacyl-ACP reductase; DH, 3-hydroxyacyl-ACP dehydratase; ENR, enoyl-ACP reductase and TE, acyl-ACP thioesterase (Brown *et al*., 2009).

out by 3-ketoactyl-ACP synthase I (KASI) and from C16 to C18 by 3-ketoactyl-ACP synthase II (KASII).

Once a saturated acyl-ACP has been through an appropriate number of FAS, three alternative reactions are possible (Joyard *et al.*, 1998) a double bond can be introduced, commonly at the Δ9 position, to give mono-unsaturated 18:1-ACP; the acyl group can be incorporated into plastid glycerolipids by sequential transfer to the sn-1 and sn-2 positions of glycerol-3-phosphate–catalyzed by a soluble glycerol-3-phosphate-1-acyltransferase (GPAT) and membrane bound 1-acyl-sn-glycerol-3-phosphate acyltransferase (LPAT) respectively, the acyl group can be cleaved to form a free fatty acid and ACP which catalyzed by acyl-ACP thioesterase (TE).

1.3 Phospholipid Biosynthesis

In cyanobacterium *Synechocystis* sp. PCC 6803, the main intermediate for phospholipid is acetyl Co-A from glycolysis pathway and acetyl Co-A flux is direct to many pathways such as TCA cycle, PHB biosynthesis, glycogen biosynthesis and fatty acid biosynthesis. Acetyl Co-A which is converted to malonyl-CoA in a rate-limiting reaction catalysed by a multi-subunit acetyl-CoA carboxylase consisting of *AccA* (encoded by *slr0728* gene). First, the malonyl subunit from malonyl-CoA is transferred to ACP by the malonyl-CoA:ACP transacylase (*fabD*, EC 2.3.1.39). The resulting malonyl-ACP is then condensed to acetyl CoA with the activity of 3-ketoacyl-ACP synthase (*fabH*, EC 2.3.1.41). The formed fatty acyl ACPs are later directed to the synthesis of membrane glycerolipids (Figure 1.3). The enzyme lysophosphatidic acid acyltransferase or 1-sn-glycerol-3-phosphate acyltransferase (LPAAT; *pls*C; EC 2.3.2.51) catalyzes the second step in phospholipid biosynthesis, and its function might close proximity to the first step catalyzed by glycerol-3-phosphate acyltransferase

(GPAT) (Kessels, 1983).This enzyme can be utilize either acyl-Coenzyme A or acyl-acyl carrier protein as the fatty acyl donor at *sn*-2 position (Rock and Gutman, 1981). Nowadays, the LPAAT of *Synechocystis sp*. PCC 6803 has already identified as *sll1752* and *sll1848* genes (Weier *et al.*, 2005; Okazaki *et al.*, 2006) while the gene encoded GPAT has yet been identified. Okazaki and co-worker (2006) reported that disruption of *sll1848* (*Δsll1848*) dramatically decreased the relative levels of palmitate (16:0) acids at the *sn*-2 position which is replaced by C18 acids. Moreover, the product of *sll1848* that was overexpressed in *E. coli* had 130-fold higher specific activity, as LPAAT, for 16:0-CoA than for 18:0-CoA when examined with acyl-CoAs as substrates instead of acyl-ACPs. These results indicated that *sll1848* encodes the major LPAAT, which has strong specificity for 16:0-ACP. Another LPAAT, *sll1752* in *Synechocystis*, is a minor LPAAT that its activity prefer 18:0-CoA rather than 16:0-CoA (Okazaki *et al.*, 2006). The chloroplast LPAAT that is encoded by the *ATS2* gene of a higher plant *Arabidopsis thaliana* (Yu *et al.*, 2004) is structurally similar to *sll1848* gene.

In microorganisms, the fatty acyl-ACP is directly added into a PG molecule (backbone for the glycerolipid synthesis) by a sn-glycerol-3-phosphate acyl- transferase (EC 2.3.1.15 encoded by *GPAT* or *PlsB*) or by a newly discovered two-reaction system catalyzed by the enzymes, PlsX and PlsY (Zhang and Rock, 2008). Phosphate is added into the fatty acyl group derived from a fatty acyl-ACP chain by PlsX catalyzing and then transferred into G-3-P molecule catalyzed by PlsY (Lu *et al.*, 2006).

In *Bacillus subtilis*, the roles of three genes consist of *plsX, plsY* and *plsC* were investigated. Long-chain acyl-ACPs are the end products of the bacterial dissociated type II fatty acid synthase system (FAS II). *B. subtilis* uses PlsX to convert these acyl-ACPs to acyl-PO_4 via a phosphotransacylase-type reaction. The PlsX step is reversible.

The next step is catalyzed by the membrane-associated PlsY (acylglycerol-P acyltransferase) encoded by the *yneS* gene that transfers the acyl moiety to the 1 position of glycerol-P to form acyl-G3P. Acylation of the 2 position is catalyzed by PlsC (YhdO), a membrane-bound 1-acyl-glycerol-P acyltransferase that specifically uses acyl-ACP as the acyl donor to form phospholipid (Figure 1.4). The functions of those three genes were studied by the constructions of knockout strains of three genes in *B.subtilis*. The *plsX*-depleted cells were inactivated on fatty acid and phospholipid systems. Thus *B. subtilis* mutant could not produce long chain acyl-ACP end product of fatty acid synthesis. On the other hand, *plsY*-depleted cells also blocked phospholipid synthesis whereas *plsC*-depleted cells accumulated monoacylglycerol content and high amount of fatty acid (Paoletti *et al.*, 2007). The plsC enzyme catalyzed the transfer of fatty acid to the 2-position of acyl-G3P via acyl-ACP into phosphatidic acid (PtdOH), the key intermediate of phospholipid synthesis.

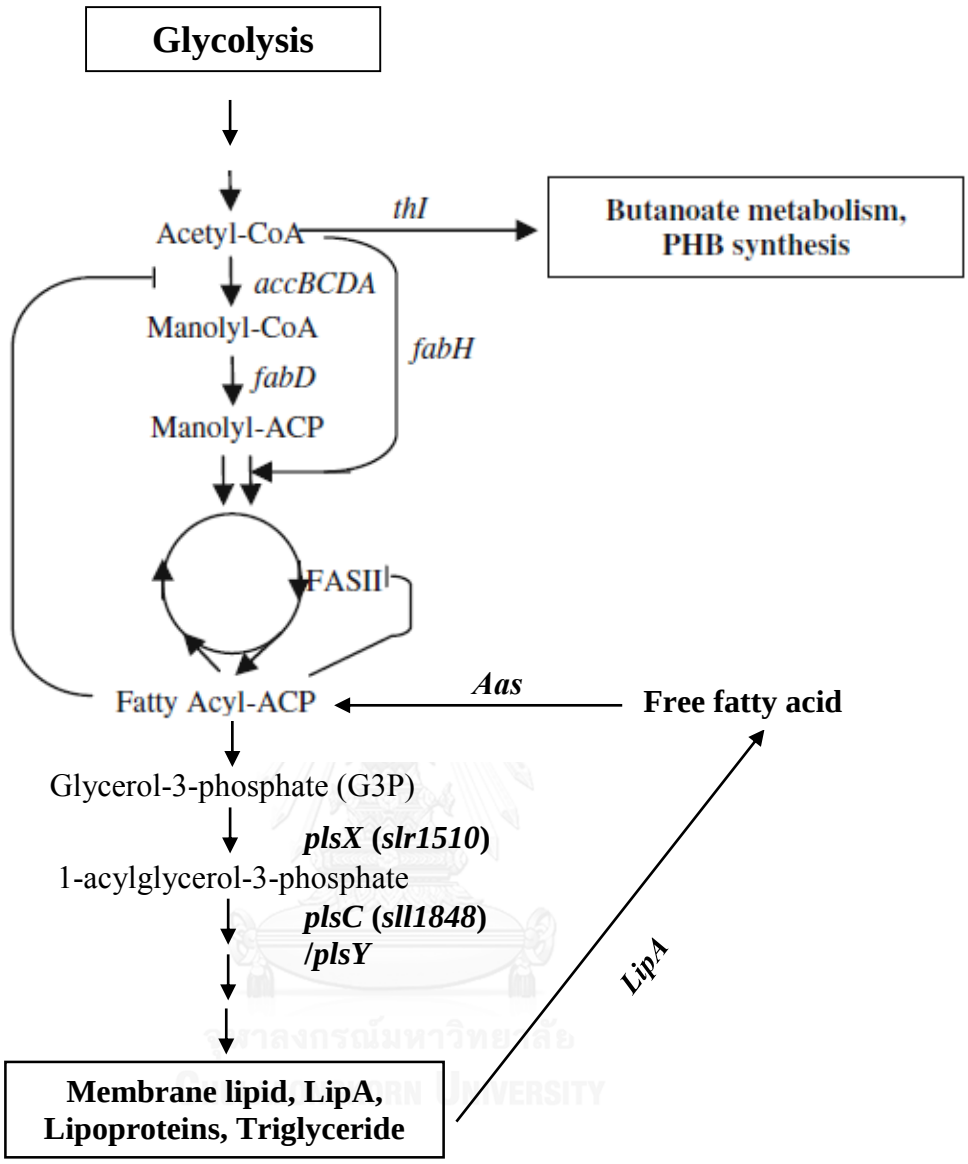


Figure 1. 3 Simplified overview of the lipid biosynthesis and some of the competing pathways in cyanobacteria. Genes encoding metabolic enzymes: *thI* (PHA-specific beta-ketothiolase), *accBCDA* (acetyl-CoA carboxylase subunit BCDA), *fabD* (ACP transacylase), *fabH* (3-ketoacyl-ACP synthase), FASII (fatty acid synthase II), *plsX* (or *slr1510*) (putative glycerol-3-phosphate acyltransferase PlsX), *plsC* (or *sll1848*) (putative 1-acyl-sn-glycerol-3-phosphate acyltransferase), *LipA* (putative lipase) and *aas* (acyl-acyl carrier protein synthetase) (modified from Quintana *et al*., 2011).

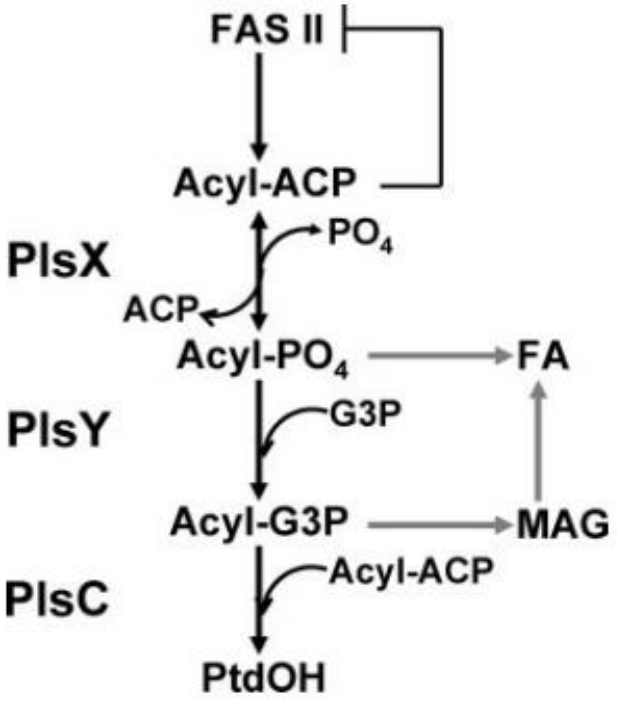


Figure 1. 4 Coupling of fatty acid and phospholipid syntheses in *B. subtilis*. Genes encoding metabolic enzymes: *PlsX* (phosphotransferase), *PlsY* (acylglycerol-phosphate-acyltransferase) encoded by *yneS* gene and *PlsC* (1-acylglycerol-phosphate-acyltransferase) or encoded by *yhdO* gene. The intermediates of phospholipid synthesis: Acyl-PO_4 (acyl phosphate), Acyl-G3P (acyl glycerol-3-phosphate), PtdOH (phosphatidic acid), FA (fatty acid), Acyl-ACP (acyl-acyl carrier proteins) and MAG (monoacylglycerol) (Paoletti *et al.*, 2007).

1.4 Membrane lipid component in cyanobacteria

Membrane lipids consisted of glycerolipid as major component (Wada and Murata, 1998). The glycerolipid structure which characterizes each individual class of lipid have two acyl groups esterified at the *sn*-1 and *sn*-2 positions of the glycerol moiety and a polar head group at the *sn*-3 position (Figure 1.5). In membrane lipid of cyanobacteria have three classes of glycolipids, namely, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG) and the other class of phospholipid, namely, phosphatidylglycerol (PG). The most abundant lipid in cyanobacteria is MGDG (40–60% of total lipids), followed by DGDG, SQDG, and PG (each of which represents 10–20% of total lipids) (Murata *et al.*, 1992; Wada and Murata, 1998). In addition, cyanobacteria also contain monoglucosyldiacylglycerol (MGlcDG) as minor glycolipid which relatively low as compare to the levels of other glycolipids (less than 1%) (Sato and Murata, 1982; Wada and Murata, 1998). *Synechocystis* sp. PCC 6803 has glycerolipids in the thylakoid membrane, cytoplasmic membranes and intact cells which mainly consist of MGDG more than 50% and the relative levels of DGDG, SQDG and PG about 5-25% (Wada and Murata, 1989; Gombos *et al.*, 1996).

Thylakoid membranes are the predominant membrane system in cyanobacteria and, thus, their lipid composition is similar to that of the total cellular membranes (Sakurai *et al.*, 2006) The thylakoid membranes of chloroplasts contain the same lipid classes as those of cyanobacterial cells. This similarity provides evidence that suggests that cyanobacteria might be the evolutionary ancestors of chloroplasts (Dorne *et al.*, 1990).

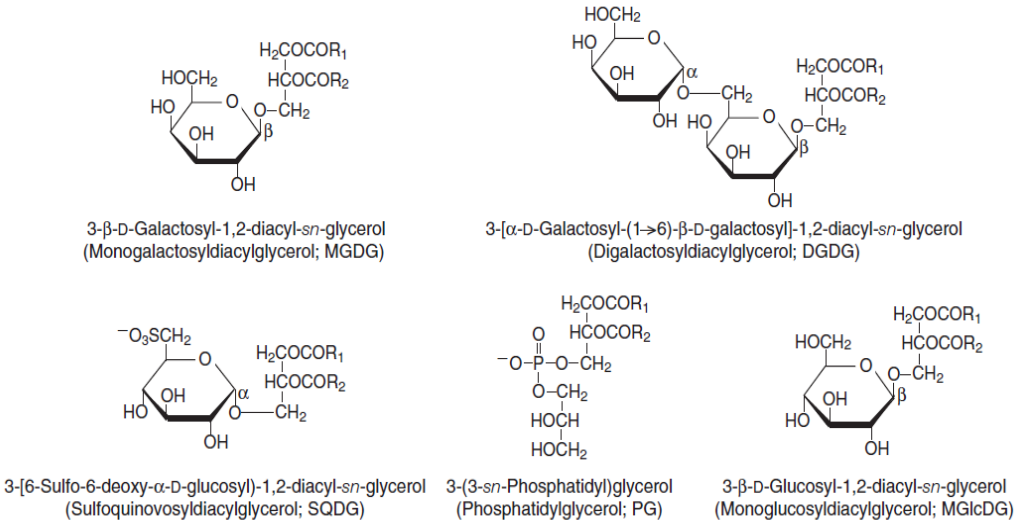


Figure 1. 5 Structures of membrane lipids in cyanobacteria. R1 and R2 denote hydrocarbon chains of fatty acids (Sato and Wada, 2009).

1.5 Environmental stress and lipid production

1.5.1 Abiotic stress

Abiotic stress conditions, such as drought, salinity, heat, nutrient cause extensive losses to agricultural production worldwide. One negative result from abiotic stress shows changes in the fatty acid composition of plant membrane lipids which mainly occurred through the metabolic regulation of fatty acid desaturase activity (Testerink and Munnik, 2005). The major membrane lipids of organisms were found their significantly responsive to environmental stresses (Olie and Potts, 1986).

1.5.2 Changes in membrane lipids in response to environmental factors

There are many environmental stresses that limit growth and productivity of microorganisms. The hyperosmotic stress and salt stress induced the inactivation of photosynthetic machinery, particularly the oxygen evolving machinery of the photosystem II complex, in *Synechococcus* sp. PCC 7942 (Allakhverdiev *et al.*, 2001). There are many reports suggested that lipids might be involved in the protection against salt stress (Huflejt *et al.*, 1990; Khomutov *et al.*, 1990; Ritter and Yopp, 1993). When photosynthetic organisms were exposed to salt stress, the fatty acids of membrane lipids are desaturated (Allakhverdiev *et al.*, 2001). The targeted mutagenesis altered genes for fatty acid desaturases in *Synechocystis* sp. PCC 6803 gave the decreased levels of unsaturated fatty acids on their membrane lipids as well as decreased their salt tolerance (Allakhverdiev *et al.*, 1999).

At low temperatures, plant membranes undergo transition from a liquid crystalline to a gel-like phase with reduced fluidity and ion leakage and deactivation of membrane proteins occurs. Observations suggest that saturated phosphatidylglycerol (PG) content in chloroplast membranes may be related to the phase transition

temperature and thus related to the low-temperature adaptability of plants (Upchurch, 2008). In cyanobacteria, they respond to a decrease in ambient growth temperature by desaturating the fatty acids of membrane lipids to compensate for the decrease in membrane fluidity at low temperatures (Murata and Nishida, 1987). Fatty acid desaturases are the enzymes that introduce the double bonds into the hydrocarbon chains of fatty acids, and thus these enzymes play an important role during the process of cold acclimation of cyanobacteria (Sato and Murata, 1980; Wada and Murata, 1990). The unsaturation of fatty acids occurs without de novo synthesis of fatty acids during low temperature acclimation of cyanobacterial cells (Sato and Murata, 1981; Wada and Murata, 1990). Most of the cyanobacterial desaturases are intrinsic membrane proteins that act on acyl-lipid substrates. Many reports demonstrated that the unsaturation of membrane lipids was essential for low temperature tolerance of cyanobacteria by genetic manipulation of the *desA* gene, which was isolated from the transformable cyanobacterium *Synechocystis* sp. PCC 6803. The mRNA level for the *desA* gene was apparently regulated in response to temperature (Vigh *et al.*, 1993; Murata and Los, 1997), and *desB* transcripts accumulated in cells grown below 26°C (Sakamoto *et al.*, 1994). It should be addressed that cold-sensitive *Synechocystis* with monounsaturated fatty acids was only become cold-tolerant by introduction of the gene for Δ12 desaturase that allowed cells to synthesize di-unsaturated fatty acids (Wada *et al.*, 1990). On the other hand, directed mutation of desaturases in cold-tolerant *Synechocystis* led to production of monounsaturated fatty acids which made this strain to cold-sensitive (Tasaka *et al.*, 1996).

Al-Hasan *et al.* (1989) studied the light effect on the fatty acid composition of five species of marine cyanobacteria. In *Phormidium jenkelianum* and *Synechocystis*

sp., the fatty acid compositions of cells from light- and dark-incubated cultures were similar whereas in the dark incubation of *Anabaena constricta*, *Phormidium corium* and *Spirulina subsalsa* cells caused an increase in the level of 18:1 in all lipid classes. Transformation of the cyanobacterium *Synechococcus* sp. PCC 7942 with the *desA* gene for a Δ12 desaturase apparently increased the unsaturation of membrane lipids and thereby enhancing the tolerance of cyanobacterium to high light (Gombos *et al.*, 1997). These findings demonstrate that the ability of membrane lipids to desaturate fatty acids is important for enhanced tolerance of photosynthetic organism to high light stress by accelerating the synthesis of the D1 protein de novo.

In plants, deficiencies of nitrogen and some minerals led to a decline in nutritional status, metabolism, and lipid synthesis. In general, the negative effects are mainly influenced on fatty acid desaturation and the level of polyunsaturated fatty acids. In bacteria, the ionic composition of the growth medium generally had little effect on the lipid composition unless there was a very marked deficiency. Still, in those bacteria that facultatively fixing nitrogen, there were large changes in lipid metabolism depending upon the nitrogen source. Two cyanobacteria studied in strain M2 of *Pseudanabaena* sp. and strain L3 of *O. splendid* were treated under nitrogen deficiency during growth. These two cyanobacteria strains do not seem to be particularly affected by nitrogen deficiency. The major acyl lipids found were MGDG, DGDG, SQDG, and PG. For chloroplasts, the galactolipids are the main lipid components. Nitrogen deficiency did not increase the neutral lipid content of the cells. In the case of two strains studied, the results here obtained would indicate that the third step of desaturation performed by different enzymes which recognized the polar head of lipid (galactose or phosphoglycerol and sulfoquinovose) and introduced the double bond in

a different position. Marine cyanobacterium *Oscillatoria willei* BDU 130511 cells grown under nitrogen deprivation stress could decrease total lipid content (26.08%g/DW) (Saha *et al.*, 2003).

1.5.3 Effect of organic carbon source on lipid content

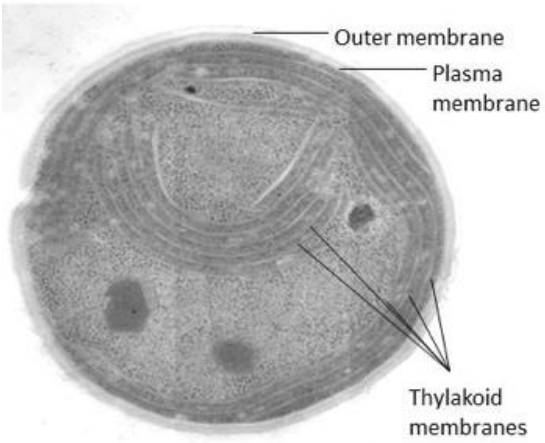
Under autotrophic growth conditions, both biomass and lipid productivities were low compared with those from heterotrophic growth *C. vulgaris* can grow on acetate in the dark and in the light with acetate being directly converted to fatty acids. (Nichols *et al.*, 1967; Harris and James, 1969). Similar acetate uptake was also reported for *Nannochloropsis sp.* (Hu and Gao, 2003) and *C. protothecoides* (Xu *et al.*, 2006). Several studies have recently been conducted to increase the growth rate of *H.pluvialis*, and have report that this alga can grow mixotrophically as well as photoautotrophically and that for the mixotrophic culture, acetate has been shown as a good organic carbon source (Jeon *et al.*, 2006). In previous studies in *Chlorella pyrenoidosa* was grown in mixotrophic condition in presence of sodium acetate showed that six fold enhancement in biomass productivity and a remarkable 32 fold increment in lipid productivity were recorded in cultures grown with sodium acetate in comparison to autotrophic culture (Rai *et al.*, 2013).

1.6 *Synechocystis* sp. PCC 6803

Cyanobacteria being photosynthetic organism, use the sun energy, H_2O and CO_2 to synthesize the energy storage component, i.e. carbohydrates, lipids and proteins. These energy storage components from a potential feed – stock can be converted to bioenergy. Of these three biochemical fractions, lipids have the highest energy content (Quintana *et al.*, 2011). Among prokaryotes, cyanobacteria are the only organisms to engage in oxygenic photosynthesis, and there is an evidence to suggest that they are the progenitor (s) of plant plastids. Their phylogenetic position in the bacterial kingdom is still obscure, although recent analysis of ancient genes has indicated a genetic relationship with Gram-positive bacteria (Hansmann and Martin, 2000). One model of cyanobacterial species is the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 which is unicellular non-nitrogen (N_2) - fixing cyanobacterium and gas vesicles (Figure 1.6). It was ubiquitous inhabitant of fresh water using binary fission for reproduction. Importantly, it is the first cyanobacterium whose genome was sequenced since 1996. This was the first photoautotrophic organism to be fully sequenced. The circular genome was originally deduced to be 3,573,470 bp long (Figure 1.7) whereas the average GC content is 47.7 % (Kaneko *et al.*, 1996). *Synechocystis* will have four culture sub strains of PCC, ATCC, GT and Kazusa, which all derived from the Berkeley strain 6803 and was isolated from freshwater in California by R. Kunisawa (Stanier *et al.*, 1971). It was originally believed that these subcultures were the same. For this reason, they were grouped together under the name of *Synechocystis* sp. PCC strain number 6803 (Rippka and Herdman, 1992). However, the four substrains show certain differences in phenotype (Figure 1.8). Consequently, *Synechocystis* has been one of the most popular organisms for genetic and physiological studies of photosynthesis for

reasons. This strain is naturally transformable by exogenous DNA (Grigorieva and Shestakov, 1982), grow easily with basic nutritional requirement. There are able to survive if supplied with air, water and mineral salts with light as the only energy source, and its cultivation is therefore relatively simple and inexpensive. *Synechocystis* sp. PCC 6803 is popularly in many topic such as high- throughput system biology for genome wild analysis, metabolic modeling for physiological prediction and rational metabolic engineering and applications in producing diverse chemical (Yu *et al.*, 2013). The generally membranes in cyanobacteria is similar to that of higher plant. Therefore, *Synechocystis* sp. PCC 6803 might serve as a powerful model for studying the molecular mechanisms of stress response and long-term adaptation (Lehel *et al.*, 1993; Jantaro *et al.*, 2003).

(A)



(B)

Figure 1. 6 Ultrastructure of a cyanobacterial cell under electron micrograph (A) and *Synechocystis* cells culture both in liquid media and solid media (B) (Source:https://newunderthesunblog.wordpress.com/the-basics/cyanobacteris/)

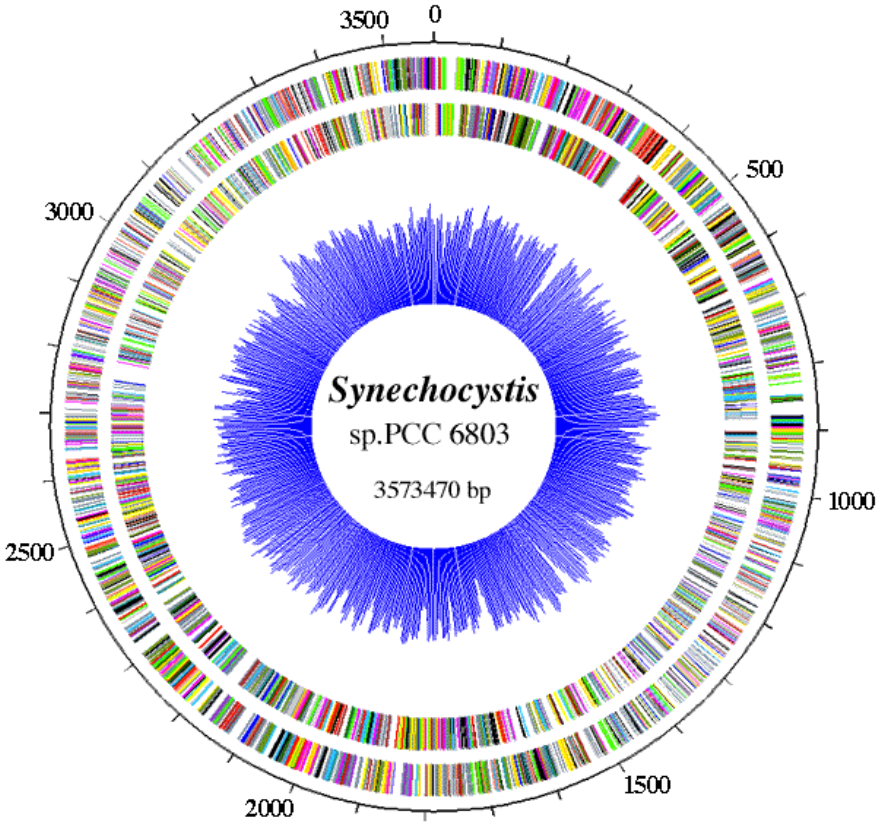


Figure 1. 7 The cellular genome of the *Synechocystis* sp. PCC 6803 according to Cyanobase. (Source:http://genome.microbedb.jp/cyanobase/Synechocystis/map/Chr)

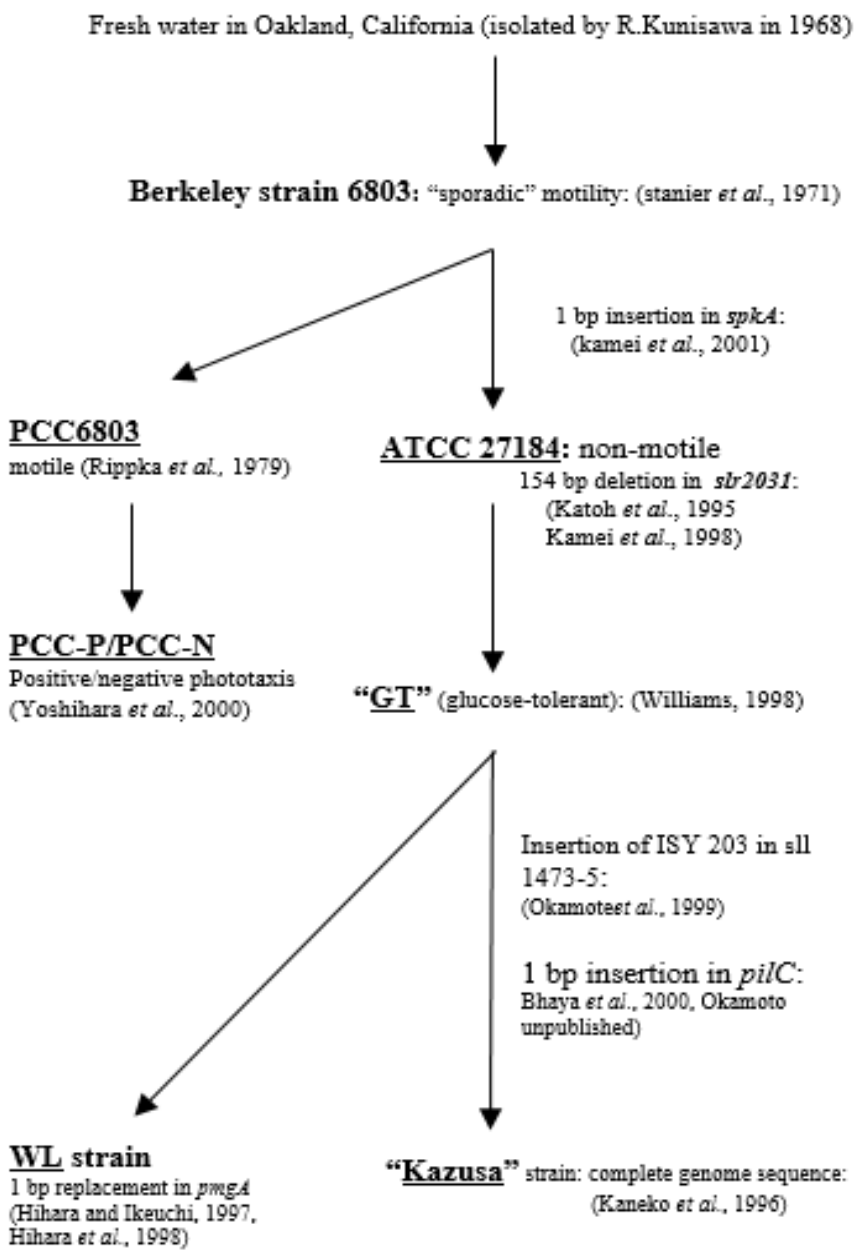


Figure 1. 8 Strain history of *Synechocystis* sp. PCC6803 (Ikeuchi and Tabata, 2001).

1.7 Gene overexpression in *Synechocystis* cells

Genetic recombination in *Synechocystis* sp. PCC 6803 is almost exclusively carried out by double crossover, while the integrative single crossover of circular DNA molecules is very rare (Golden, 1987; Williams, 1988). The short homologous regions are insufficient for double crossover recombination. Both upstream and downstream regions of more than 400 bp are required for the double crossingover event involved in the introduction of an antibiotic resistance cassette into a target site. This makes gene disruption laborious because of the time required to prepare each ORF construct (Stanier *et al.*, 1971).

To simplify and standardize the genetic enginerring required for biosynthesis of 13R-MO in *Synechoystis*, a series of integrative vector, named pEERM were contracted. The base pEERM vector are ready made for transgenic overexpression in the genome, and contain all genetic parts needed for integration and expression shown in Figure 1.9 (Englund *et al.*, 2015).

1.8 Objectives of this research

1. To overexpress s*ll1848* (*plsC*) or *slr1510/sll1848* (*plsX/plsC*) genes in cyanobacterium *Synechocystis* sp. PCC 6803.

2. To investigate fatty acid and lipid contents in overexpressing strain compare with wild type under normal and modified nutrient conditions.

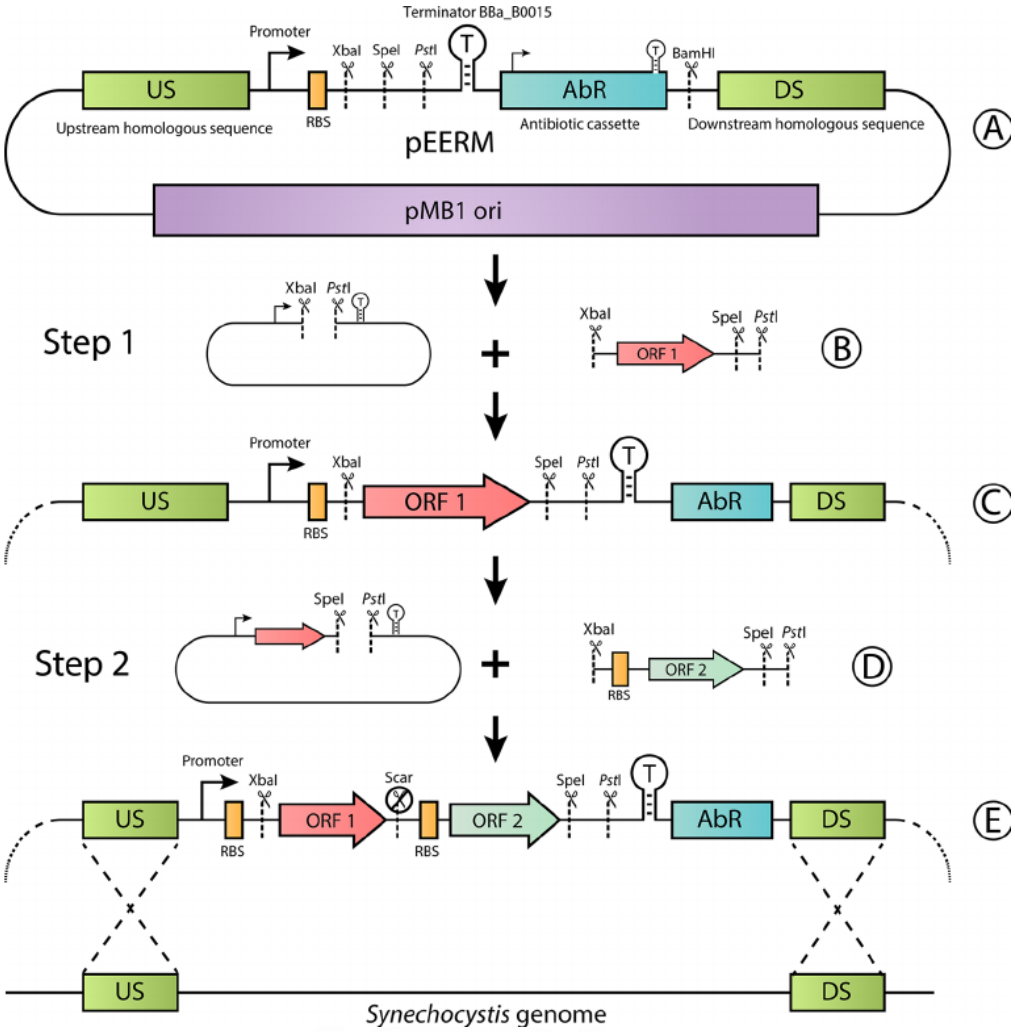


Figure 1. 9 Schematic overview and utilization of the pEERM vectors. The base pEERM vector (A) and the open-reading frame (ORF) to be inserted (B) are cut with *Xba*I and *Pst*I and ligated together (step 1). Additional genes can be cloned downstream of first gene by cutting the new vector (C) with *Spe*I and *Pst*I and the next insert (D) with *Xba*I and *Pst*I, and ligating them (step 2). A *SpeI*/*Xba*I scar will form between the two inserts in the resulting plasmid. When all genes have been inserted, the final construct (E) can be directly transformed into the *Synechocystis* genome through homologous recombination (Englund *et al.*, 2015)

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Autoclave	Hirayama Manufacturing Cooperation, Japan
Balances	METTER TOLEDO, USA
Centrifuge	HETTICH zentrifugen, UK5417C
	Eppendorf, Germany
Fume Hood	Science technology protection laboratory
	hood, USA
Gel Document	DNA visualisation UV light, USA
Gel Electrophoresis System	Gibthai, Thailand
Incubator shaker	Innova 4000 PLATFORM SHAKER, USA
Laminar flow	BVT-124 international Scientific Supply,
	Thailand
PCR apparatus	Thermo Cycler, Japan
pH meter	Metter Toledo, USA
Power supply	BIO-RAD POWER PAC 1000, USA
Spectrophotometer	Jenway UV/Vis 6400, USA
Vortex mixer	Scientific Industries, USA
Water bath	THERMOMIX® B B.BRAUN, USA

2.1.2 Chemicals

Acetic acid	Lab Scan, Poland
Agarose	Invitrogen, Spain
Ammonium persulfate (APS)	Merck, Germany
Bacto agar	Scharlau, Spain
Boric acid	Scharlau Chemie S.A., Spain
Bromophenol blue	Sigma, USA
Calcium chloride	Sigma, USA
Citric acid	Ajax Finechem, Australia
Chloramphenicol	Sigma, USA
Chloroform	Merck, Germany
Cobalt nitrate	UNIVAR, Australia
Copper (II) sulfate	Carlo Erba Reagents, France
Dimethlyformamide	Lab Scan, Ireland
EDTA	Ajax Finechem, Australia
Ethanol	Katayama Chem, Japan
Ethidium bromide	Sigma, USA
Ferric ammonium citrate	Sigma, USA
Glycerol	Ajax Finechem, Australia
HEPES	USB Corporation, USA
Isoamylalcohol	Sigma, USA
Isopropanol	Sigma, USA
Magnesium chloride	Ajax Finechem, Australia
Magnesium sulfate heptahydrate	Ajax Finechem, Australia

Methanol	Scharlau, Spain
Ortho-phosphoric acid (85%)	BHD, New Zealand
Phenol	Merck, Germany
Potassium acetate	Ajax Finechem, Australia
Potassium sodium tartrate	Merck, Germany
Sodium chloride	Carlo Erba reagent, China
SDS, Sodium dodecyl sulfate	Ajax Finechem, Australia
Sodium hydroxide	Merck, Germany
Sodium nitrate	Ajax Finechem, Australia
Sodium thiosulphate	Sigma, USA
Sulphuric acid 98%	BHD, New Zealand
Tris(hydroxymethyl)-aminomethane	Barcelona, Spain
Tryptone	HiMedia, India
Triol®reagent	Invitrogen, USA
Yeast Extract	Barcelona, Spain

2.1.3 Kits and supplies

GeneRuler™ 1 kb DNA Ladder	Fermentas, Canada
PCR purification kit	Machery-Nagel, USA
Plasmid extraction kit	Invitrogen, USA
SuperScrip™ III	Invitrogen, USA
DNA polymerase	Fermemtas, Canada
T4 DNA ligase	Fermemtas, Canada
Taq DNA polymerase	Invitrogen, USA

RNase A	Fermemtas, Canada
RNase-Free DNase	Fermentas, Canada
Restriction enzyme *Pst*I	Fermemtas, Canada
Restriction enzyme *Spe*I	Fermemtas, Canada
Restriction enzyme *Xba*I	Fermemtas, Canada

2.1.4 Expression vectors

2.1.4.1 The pEERM vector

The pEERM vector was used for gene overexpression in this study, which provided by Professor Peter Li nblad from the Photochemistry and Molecular Science, Department of Chemistry – ÅngstrÖm Laboratory, Uppsala University, Sweden. This vector contains a strong promoter (P_{psbA}). It was created to consist of multiple cloning sites *Xba*I, *Spe*I and *Pst*I and a selective chloramphenicol antibiotic cassette gene. The size of pEERM vector is 3,595 bp (Appendix A).

2.1.4.2 The pEERM vector containing *slr1510* gene

The pEERM vector containing *slr1510* gene fragment was constructed and obtained from Miss Nutchaya Songruk, a master student in 2014, Laboratory of Cyanobacterial Biotechnology, Department of Biochemistry, Faculty of Science, Chulalongkorn University.

2.1.5 Primers

Table 2. 1 The information of primers used in this work

Target gene	Primer	Sequence (5´→3´)	Product size(bp)
sll1848	Forward	CTAGTCTAGAGTGGATTCCGAGATTAAT	678
sll1848	Reverse	CTAGACTAGTCTAATCCCTGCCTAAATCCAGCAT	
Slr1510	Forward	TAGAGAACTAGTATGGCTGTAACGCGG	1,047
Slr1510	Reverse	TAGAGACTGCAGCTAGATATTCTGTAATTCCTC	
CmR	Forward	GAGTTGATCGGGCACGTAAG	899
CmR	Reverse	CTCGAGGCTTGGATTCTCAC	
UpsbA2	Forward	TGCCTGTCAGCAAAACAACTT	2,841
DpsbA2	Reverse	CGAGGGCAATCATCAATTCCG	

Table 2. 2 Sequences of the primers for RT-PCR

Target gene	Primer	Sequence (5´→3´)	Product size(bp)
16s	Forward	AGTTCTGACGGTACCTGATGA	521
16s	Reverse	GTCAAGCCTTGGTAAGGTTAT	
sll1848	Forward	TCTCTACCGGGGCTTGAAATG	508
sll1848	Reverse	CGCCTTACCAATGCGAATAGT	
Slr1510	Forward	AAGGGGTGGTGGAAATGGAA	488
Slr1510	Reverse	AAGTAGGTCCCTTCCTTCGG	
AccA	Forward	ATGCACGGCGATCGAGGAGGT	428
AccA	Reverse	TGGAGTAGCCACGGTGTACAC	
Aas	Forward	CCCATTGAAGATGCCTGTTT	304
Aas	Reverse	GTGCTGGGATAAAACGGAAA	
phaA	Forward	CATGATGGTTTGACGGACAG	310
phaA	Reverse	GACTACAGTTGCCCGCTGTT	
lipA	Forward	TTGGCGGAGCAAGTGAAGCAAT	379
lipA	Reverse	ATTTTGCCTGTGCTGGTCCATG	

2.1.6 Organisms

Escherichia coli strain DH5-α was used as a host for plasmid propagation.

Synechocystis sp. PCC 6803 wild type strain was kindly obtained from Microbial Chemistry, Department of Chemistry, Ångström Laboratory, Uppsala University, Sweden**.**

2.2 Methods

2.2.1 The construction and transformation of overexpressing strains of *Synechocystis* sp. PCC 6803

2.2.1.1 The genomic DNA extraction

Synechocystis sp. PCC 6803 cells were grown in 100 ml of normal BG_{11} medium (Appendix B). The growth condition was performed at 30 °C, under continuous white light intensity of 50 μmole photon $m^{-2}s^{-1}$. Cultivated cells with optical density at 730 nm about 0.5, cells were harvested by centrifugation at 6,000 rpm (4,025 × *g*), 4°C for 10 min. The obtained pellet was resuspended in 400 μl of 1X TE pH 7.5 buffer. After that, glass beads (0.2 g) were added. Cell disruption was performed by vortexing for 2-3 times until cells were completed broken and transferred the supernatant fraction into a new tube. Then, the extracted chromosomal DNA was obtained by phenol-chloroform method (Sambrook *et al.*, 1989). Other tube was added 1 volume of the phenol:chloroform:isoamyl alcohol mixture of sample and mixed solution by shaking for 2-3 times. The sample tube was centrifuged at 12,000 rpm (21,009× *g*), 25°C for 10 min and transferred the upper aqueous phase containing DNA into a new tube. One volume of chloroform:isoamyl alcohol mixture was add into the tube and centrifuged again at 12,000 rpm (21,009 × *g*), 25°C for 10 min. Later, 0.1 volume of 3M NaOAC and 2.5 volumes of absolute ethanol were added and mixed that solution by shaking for 2-3 times. After that, the reaction tube was incubated at -20°C for 2 hours and centrifuged at 12,000 rpm (21,009 × *g*), 4°C for 10 min. In order to get DNA pellet, the supernatant was discarded and washed pellets by 70% ice-cold ethanol. Next step, the DNA sample tube was centrifuged at 12,000 rpm (21,009 × *g*), 4°C for 10 min. The genomic DNA pellet was dried at room temperature and dissolved in TE buffer (20 μl).

The content of genomic DNA was checked by 0.8% agarose gel electrophoresis in 0.5X TAE buffer (Appendix C).

2.2.1.2 The construction of recombinant plasmid

2.2.1.2.1 *sll1848* gene information and primer design

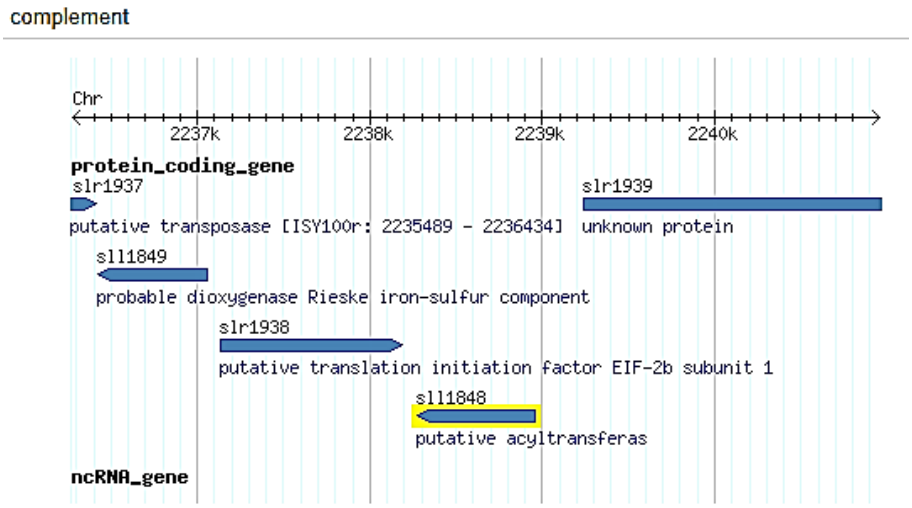
The information of *sll1848* gene was searched from the genome database of cyanobacteria (Cyanobase, http://kazuza.or.jp/cyanobase/). The *sll1848* gene from cyanobase was found that gene encodes a putative acyltransferase which composed of 678 nucleotides with deduced 225 amino acids (Figure 2.1A).

In order to design primers, the nucleotide sequence of additional restriction enzyme site was added as flanking regions to primers. The sequence for restriction enzyme site of *XbaI* was connected to forward primer whereas that of *SpeI* was connected to reverse primer (Figure 2.1B). After that, both primers were checked for their self-complementarity using program OligoCalc.

2.2.1.2.2 Polymerase chain reaction (PCR)

Synechocystis sp. PCC 6803 genomic DNA prepared form 2.2.1.1 was used as the DNA template for amplifying *sll1848* gene fragment. The *sll1848 gene* fragment was amplified by PCR method using a primer pair of F_*sll1848* and R_*sll1848* containing *Xba*I and *Spe*I digestion site sequences. The PCR mixture and its reaction procedure were shown in following Tables 2.3 and 2.4, respectively. The PCR products was checked by 0.8% gel electrophoresis (Appendix D).

(A)



(B)

GTGGATTCCGAGATTAATCATCGTGGTGGTTTGAGTGCTCCCCGCCCAAGGGAAACG
TCACTTAATTTAGCTCTCTACCGGGGCTTGAAATGGGGGGTGGTGCGGCCACTGCTC
CATGGATTGTTCCAGGCCCAGGTATATGGTCAGGAATTGGTGCCAACCCGGGGGCCG
GCCTTGGTGGTGAGCAACCATGCCAGTTATTTTGACCCCCCATTTTTGTCCTGTGCC
ATGGCCCGGCCGGTGGCCTTTATGGCCAAGGAAGAGTTATTTAATGTGCCCCTGCTG
GGTCCAGCCATTCGCCTCTATGGGGCCTATCCAGTCAAACGGGGCAGTGGCGATCGG
GGAGCATTGCGGGCCGCCTTGACGGCGCTGGGGGATGGTTGGTTAGTGGGGGTCTTT
CTGGAGGGAACCAGAACAAAGGATGGCCGCATTCACCAGCCAAAATTGGGGGCTGCC
ATGATTGCAGCTAAAGCCCAAGTGCCCATTATTCCCGTCAGCCTAGGGGGAGTAGAG
CAAATTTTTCAGCCCGGTTCCCCCTGGCCCCATCCTGTGCCTTTAACTATTCGCATT
GGTAAGGCGATCGCCCCTCCAGTAAAGAATAGGAAACCCGAATTGGAAGCGGTTACT
AAAGCTTGCCAAGCCCAAATTCACGAGATGCTGGATTTAGGCAGGGATTAG

name	Primer sequence	Restriction enzyme	GC content
*F*_sll1848	CTAGTCTAGAGTGGATTCCGAGATTAAT	*Xba*I	39%
*R*_sll1848	CTAGACTAGTCTAATCCCTGCCTAAATCCAGCAT	*Spe*I	44%

Figure 2. 1 The outlined map (A) and nucleotide sequences of *sll1848* gene retrieved from Cyanobase, as well as its designed pair of primers (B).

Table 2. 3 The components of PCR mixture

PCR mixture (total volume of 50 µl)	Volume (µl)
5X Phusion HF buffer	10
Template DNA (50-500ng)	1
10 mM dNTP mixed	1
10 mM Forward primer	1
10 mM Reverse primer	1
Phusion DNA Polymerase	0.5
Autoclaved DI water	36.5

Table 2. 4 PCR procedure for amplification

Step	Temperature (°C)	Time
1. Initial denaturation	98	30 sec
2. Denaturation	94	10 sec
3. Primer annealing	55	30 sec
4. DNA Extension	72	25 sec
5. Repeated steps 2-4	for 29 cycles	
6. Final Extension	72	5 min
7. Hold	16	over

2.2.1.3 Preparation of competent cells for calcium chloride method

E.coli strain DH5-*α* was used as the completent cell. Cells were grown in 3 ml of liquid LB medium and then incubated at 37 ˚C by shaking at 250 rpm for 16 hour. The inoculum was thansferred into 100 ml of fresh liquid LB medium and cultivated until OD at 600 nm reaching about 0.3-0.4. The cell culture was harvested by centrifugation at 4,000×g for 10 min at 4 ˚C. The cell pellets were resuspended in 15 ml of TFBI solution (Appendix E). After that, centrifuged again at 4,000×g for 10 min at 4 ˚C. The completent cells were gently mixed with 2 ml of TFBII solution (Appendix E) and then made an aliquot each 100 ml into the microcentifuge tube for further use in transformation step and storage at -80˚C.

2.2.1.4 Construction of *sll1848*-overexpressing strain

After PCR amplification, the *sll1848* gene fragment for PCR product was digested with the *Xba*I and *Spe*I enzymes (Appendix F), and then the digested fragment was ligated into the multiple cloning sites of double digest pEERM vector using T4 DNA ligase. The sample tube was incubated at 22 ˚C for 2 hours and inactivated the enzyme reaction at 65˚C for 10 min. This obtained recombinant plasmid was then transformed into *Escherichia coli* DH5-*α* strain using calcium chloride method. Completent cells were mixed with the ligation mixture and placed for 30 min on ice. After that, the sample mixture was incubated at 42 ˚C for 2 min, then hold on ice for 3 min. After that, added 900µl of LB medium into the test tube and incubated at 37˚C on the shaker for 1 hour. Then, cells were spread on LB agar containing 30 µg/ml chloramphenicol. Next, incubated at 37 ˚C for 16 hours and extracted the recombinant plasmid. The sample was checked by restriction enzyme digestion and agarose gel

electrophoresis. Finally, the recombinant plasmid was also confirmed by DNA sequencing.

2.2.1.5 Construction of *slr1510/sll1848*-overexpressing strain

After PCR amplification, the *sll1848* gene fragment was digested with the *Xba*I and *Spe*I enzymes (Appendix G) and further cloned into the recombinant plasmid containing *slr1510* gene insertion using T4 DNA ligase. The sample tube was incubated at 22 ˚C for 2 hours and inactivated the enzyme reaction at 65˚C for 10 min. This obtained double recombinant was then transformed into *Escherichia coli* DH5-*α* strain using calcium chloride method. Cells were spread on LB agar containing 30 µg/ml chloramphenicol and checked by restriction enzyme digestion and agarose gel electrophoresis. Lasty, the recombinant plasmid was also confirmed by DNA sequencing.

2.2.1.6 Transformation of recombinant plasmid into *Synechocystis* cells

Synechocystis cell culture (100 ml) was carried out in normal BG_{11} medium until OD at 730 nm reaching about 0.5. The growth condition was set at 30 ˚C, under continuous white light intensity of 50 µmole photon $m^{-2}s^{-1}$. After that, cells were harvested by centrifugation at 6,000 rpm (4,025 × *g*), 4˚C for 10 min. The cell pellets were resuspended in fresh BG_{11} medium and transferred those cells into a new tube with 500 µl of cell suspension. The recombinant plasmid and empty vector were independently transformed into *Synechocystis* sp. PCC 6803 cells by natural transformation method. Then, 10 µl of the recombinant plasmid solution was added into the tube and incubated under normal growth light condition at 30 ˚C for 6 hours. Next, a reaction mixture was spread on BG_{11} agar containing 10 µg/ml chloramphenicol. Incubation at 30 ˚C for 2-3 weeks was performed until single green colony was

appeared. Next, the cell tranformants were selected on BG_{11} agar containing higher concentration of chloramphenicol up to 30 μg/ml. After that, the obtained transformant was used as a template for checking both size and gene location by PCR method with different pairs of primers.

2.2.2 *Synechocystis* cell culture

Those *Synechocystis* cells wild type, control WT strain (wild type cell containing empty pEERM vector) and both overexpressing strains of *sll1848* and *slr1510/sll1848* were cultivated in liquid BG_{11} medium at 30 ºC under continuous light intensity of 50 μmol photons m^{-2} s^{-1} for 20 days. Cell growth was monitored by a measurement of optical density (OD) at 730 nm using a spectrophotometer. For the overexpressing strains, they were grown in BG_{11} medium with the presence of antibiotic chloramphenicol (30 $\mu g.mL^{-1}$).

For nutrient modified treatments, the cell culture with mid-logarithmic phase of growth was harvested by centrifuging at 6,000 rpm (4,025 × *g*), 25°C for 10 min and transferred cell pellets to modified BG_{11} media using normal BG_{11} medium as a control. The modified BG_{11} medium was carried out by two factors of acetate supplementation 0.4% (w/v) acetate or 6.7 mM concentration) and the deficiency of nitrogen (BG_{11}-N, without $NaNO_3$**)**. Cells were grown in indicated media and subsequently collected at interval times of 0, 2, 4, 6 and 8 days, respectively. The experiments were repeated independently for at least three times.

2.2.3 Determination of pigment contents

The contents of chlorophyll *a* and carotenoids of all *Synechocystis* strains were extracted by N, N-dimethylformamide (DMF) method. One ml of cell culture was harvested by centrifuging at 10,000 rpm (17,507 *x g*) at 25 ˚C for 10 min and discarded supernatant. The obtained pellet was further carried out by extracting with *N*, *N*-dimethylformamide (DMF) and incubated under darkness for 10 min. Then, centrifugation at 10,000 rpm (17,507 *x g*) 25 ˚C for 10 min was performed. Later, the supernatant was measured its absorbances at 461, 625, and 664 nm, respectively. The pigment contents were calculated according to (Moran, 1982) and (Chamovitz *et al.*, 1993) equations.

Chlorophyll *a* content (μg/cells) = $[(12.1 \times OD_{664}) - (0.17 \times OD_{625})]$ / total cells

Carotenoid content (μg/cells) = $[(OD_{461} - (0.046 \times OD_{664})) \times 4]$ / total cells

Total cells (cells/ml) = $(OD_{730} / 0.25) \times 10^8$

2.2.4 Measurement of oxygen evolution

The cell culture (5 ml) harvested by centrifuging at 8000xg for 10 min and cell pellets were resuspended in 2 ml of fresh BG_{11} medium. The incubation under darkness about 30 min was performed before measuring oxygen evolution by Oxygraph plus oxygen electrode (Hansatech Instruments, U.K.). The oxygen evolution measurement was done at 25 ºC using fluorescent light as a saturated light source. The unit of oxygen evolution rate represented as μmol O_2 mg Chl$a^{-1}h^{-1}$.

2.2.5 Total lipid screening

2.2.5.1 Sudan Black B staining (modified from (Wei *et al*., 2011))

Firstly, 0.1 ml of cell culture was spread and smeared on the slide. Later, the slide was air-dried and flood the entire slide with Sudan black B solution for 10 min, then washed slide with distilled water. After that, drained off excess staining and counterstained with safranin for 1 min and washed slide again by distilled water, followed by air-drying. Then, stained cells were visualized under the light microscope.

2.2.6 Determination of total lipid content

The whole cell was measured for total lipid content by dichromate oxidation method (Fales, 1971). Standard lipid stock was prepared using commercial canola oil. *Synechocystis* cells were cultivated at OD_{730} nm and harvested (5 ml) by centrifuging at 8000×*g* for 10 min. The cell pellet were collected and added 2 ml of concentrated sulfuric acid (98%) and 2 ml of potassium dichromate solution (Appendix H) and boiled that mixture for 30 min at 100 ºC, followed by cooling for 10 min on ice bath. After the incubation at room temperature for 10 min, two ml of distilled water was added and mixed. The sample was measured its absorbance at 600 nm by spectrophotometer. Unit of total lipid content was %w/w of dry cell weight (dcw). The dry cell weight was performed by incubating at 80 ºC for 48 hours until reaching the constant dry cell weight.

2.2.7 Determination of unsaturated lipid content

The unsaturated lipid content was measured by colorimetric sulfo-phospho-vanillin (SPV) method (Cheng *et al.*, 2011). The standard lipid stock was prepared using commercial gamma-linolenic acid. Cell culture (5 ml) for lipid quantification was harvested by centrifugation at 8000×*g* for 10 min. The 2 ml of concentrated sulfuric acid (98%) was added into the sample before boiling for 30 min at 100 ˚C and cooling later for 10 min on ice bath. Two ml of freshly prepared phospho-vanillin reagent (Appendix H) was then added. The sample mixure was then incubated for 10 min at room temperature. After that, an absorbance reading at 540 nm was performed in order to measure the unsaturated lipid content. Unit of total unsaturated lipid content was % w/w of dry cell weight (dcw).

2.2.8 Reverse transcription-polymerase chain reaction (RT-PCR)

2.2.8.1 Extraction of total RNA

The cell culture (15 ml) was harvested by centrifuging at 5,500 rpm (17,507 *x g*), 4˚C for 10 min. The cell pellets were resuspended in 1 ml of Trizol® reagent mixing with 0.2 g of acid washed-glass beads. After that, incubated at 70˚C for 5 min and vortexed cells, then incubated 2 min on ice. After the homogenization centrifuging at 12,000 *x* g, 10 min at 4 ˚C, the aqueous phase was transferred into a new tube. Then, the addition of 0.2 ml of chloroform was done and mixed that solution by shaking for 15 second. Later, that reaction tube was incubated at room temperature for 5 min and centrifuged the sample tube at 12,000×*g* for 15 min at 4 ˚C. The upper phase about 400 µl was transferred into new tube, and added 400 µl of isopropanol, followed by mixing it before incubating at room temperature for 10 min. Centrifuging at 12,000×*g* for 15

min, at 4 ˚C, the obtain RNA was precipitated as gel-like pellets. The supernatant was discarded and washed pellets by 1 ml of 75% ethanol and centrifuged again at 12,000×g for 15 min. The RNA pellet was dried for 10 min and DepC water 20 µl was added and incubated at 55˚C for 5 min. The total RNA solution was treated with DNaseI and incubated at 37 ˚C for 20 min. Then, stopping the reaction by adding 2.5 µl of EDTA and incubated at 75 ˚C for 10 min. The RNA concentration was measured on the absorbance at 260 nm by spectrophotometer and calculated as following formula:

RNA concentration (µg/µl) = OD_{260}×0.04 µg/µl of RNA × dilution factor

2.2.8.2 Reverse transcription-polymerase chain reaction (RT-PCR)

The first stand cDNA synthesis was used in one microgram of total extracted RNA. The reaction was performed by SuperScript™ III Frist-Strand Synthesis System kit. Firstly, one µl of 50 ng/µl random hexamers and 1 µl of 10 mM dNTP were mixed and adjusted the final volume by DepC-treated water into 10 µl. After that, the mixture was incubated at 65˚C for 5 min and placed on ice for at least 1 min. Secondly, the reaction mixture was added into cDNA synthesis prepared as shown in Table 2.5.

Table 2. 5 The cDNA synthesis mixture

Ingredients	Volume (µl)
10x RT buffer	2
25 mM $MgCl_2$	4
0.1 M DTT	2
RNaseOUT™III RT (40 U/µl)	1
SuperScript™ III RT (200 U/µl)	1

The reaction was incubated at 25°C for 10 min, followed by 50°C for 50 min and terminated the reaction at 85°C for 5 min before placing on ice. Later, added 1 µl of RNase H and incubated at 37 °C for 20 min in order to remove RNA. Lastly, the reaction was kept in - 20°C for future experiment. The cDNA was amplified by PCR method. The PCR product was analyzed using 1.5 % agarose gel electrophoresis in 1X TAE buffer (Appendix I)

2.2.9 Phylogenetic profiling of *sll1848* gene

The amino acid sequences of *sll1848* gene in various cyanobacteria strain were obtained from the cyanobacteria database, namely Cyanobase (http://genome .kazusa.or.jp/ caynobase). The other amino acid sequences of plant, bacteria and animal were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). All full-length amino acid sequence were aligned using multiple alignment and phylogenetic profiling of *sll1848* gene with ClustalW2 version 1.2.1 program (http://www.ebi.ac.uk/services) by Clustal Omega - Multiple Sequence Alignment. Phylogenetic tree was built using TreeView software and dendroscope program for demonstrating the tree picture.

CHAPTER III

RESULTS

3.1 PCR optimization of *sll1848* gene fragment

The *sll1848* gene fragment was amplified by PCR method using F_*sll1848* and R_*sll1848* primers. The annealing temperature was optimized varying between 50-65 ˚C shown in Figure 3.1A. The PCR product of *sll1848* gene fragment was separated by 0.8% agarose gel electrophoresis and used the optimum annealing temperature at 55˚C. The expected size of *sll1848* gene fragment was 678 bp (Figure 3.1B).

3.2 Recombinant plasmid and overexpressing strain constructions

3.2.1 Recombinant plasmid containing *sll1848* gene

The PCR product of *sll1848* gene fragment was ligated into pEERM vector system between *Xba*I and *Spe*I restriction sites. The recombinant plasmid containing *sll1848* gene (rpOE+C) was transformed into E.*coli* DH5-α strain. The colony containing recombinant plasmid could grow on LB plate containing chloramphenicol. The colony PCR method was used to verify the *sll1848* gene fragment in recombinant plasmid (Figure 3.2A). From result, four colonies (numbers 2, 5, 8 and 11) contained *sll1848* gene fragment in the construct recombinant plasmid. After that, each colony was grown in LB medium and extracted from E.*coli* DH5-α strain followed by confirming on double digestion with *XbaI* and *SpeI*. The result showed the correct size of *sll1848* gene located in pEERM vector. The two digested fragments were demonstrated at about 3,595 bp of pEERM vector and

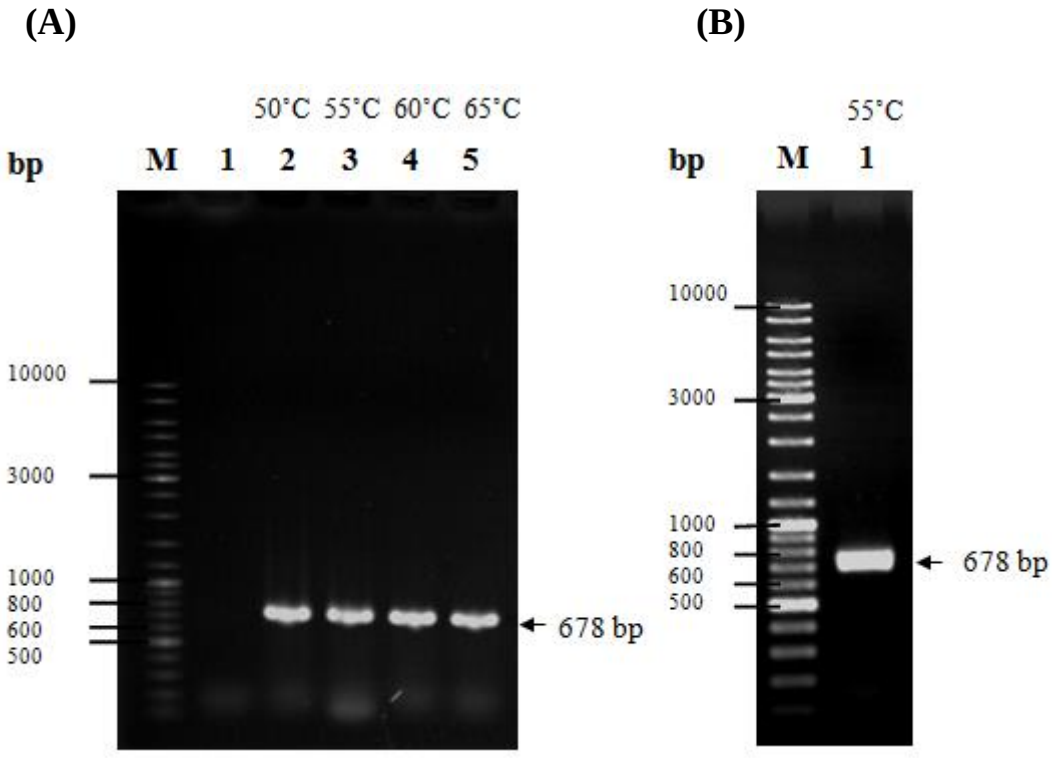


Figure 3. 1 Agarose gel electrophoresis of the amplified DNA fragment. The PCR product was run on 0.8% agarose gel electrophoresis.

A: The amplification of *sll1848* gene fragment of gradient annealing temperatures.

Lane M	DNA Marker Ladder 1 kb
Lane 1	Negative control
Lane 2-5	PCR products of *sll1848* gene fragment at 50, 55, 60 and 65°C, respectively

B: The PCR product of *sll1848* gene fragment at optimum annealing temperature.

Lane M	DNA Marker Ladder 1 kb
Lane 1	PCR product of *sll1848* gene fragment at 55°C

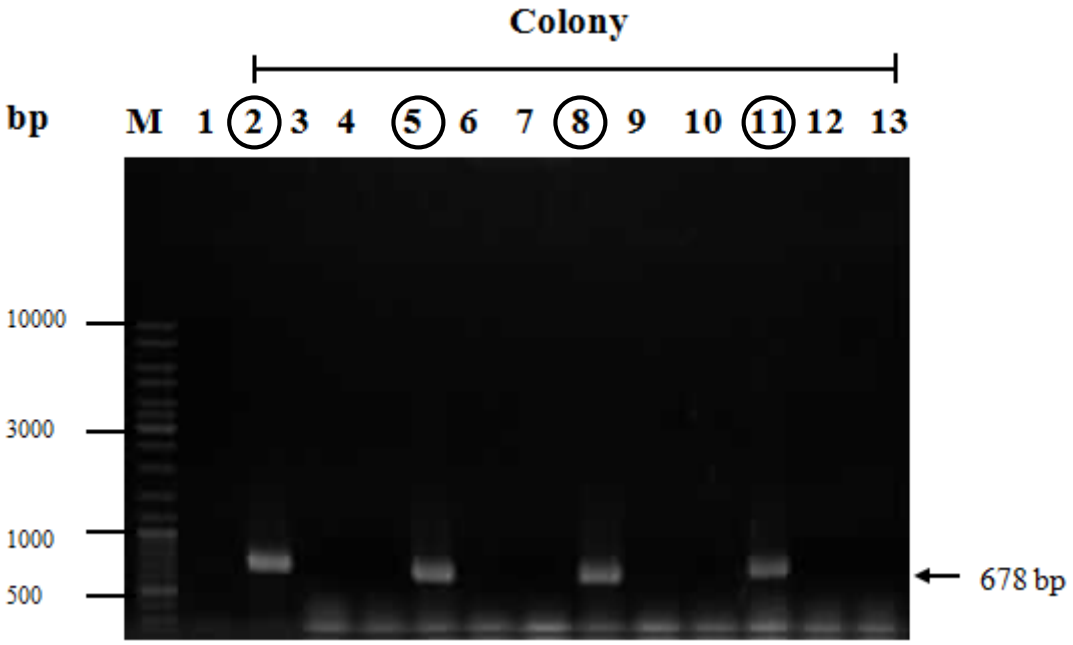


Figure 3. 2 Agarose gel electrophoresis of the amplified DNA fragment. The PCR product was run on 0.8% agarose gel of the recombinant plasmid containing *sll1848* gene (rpOE+C).

Lane M	DNA Marker Ladder 1 kb
Lane 1	Negative control
Lane 2-13	Colony PCR of *sll1848* gene fragment in different clones

678 bp of *sll1848* gene fragment, respectively (Figure 3.3), here in after the recombinant plasmid was called as rpOE+C.

3.2.2 Recombinant plasmid containing *slr1510/sll1848* genes

The PCR product of *sll1848* gene fragment was ligated into pEERM vector containing *slr1510* gene fragment (rpOE+XC) between *Xba*I and *Spe*I restriction sites. The recombinant plasmid was then transformed into *E.coli* DH5-α strain. The colony PCR method was used for verifying the *sll1848* gene fragment whether it was located on that recombinant plasmid on the new recombinant plasmid (Figure 3.4). Colonies in number 2, 4, 5 and 11 showed the amplified *sll1848* gene fragment, at a size product 678 bp. Then that recombinant plasmid (rpOE+XC) was extracted from those competent cells. After that, the confirmation of obtain recombinant plasmids was carried out by different enzyme digestions with *Xba*I and *Spe*I, *Spe*I and *Pst*I, and *Xba*I and *Pst*I, respectively. The result showed the correct sizes of various gene fragments. The digested fragments of about 3,595 bp of pEERM vector, 678 bp of *sll1848* gene, 1,047 bp of *slr1510* gene, and 1,725 bp of a fragment from *sll1848* to *slr1510* gene location, respectively (Figure 3.5) here in after the recombinant plasmid was named as rpOE+XC.

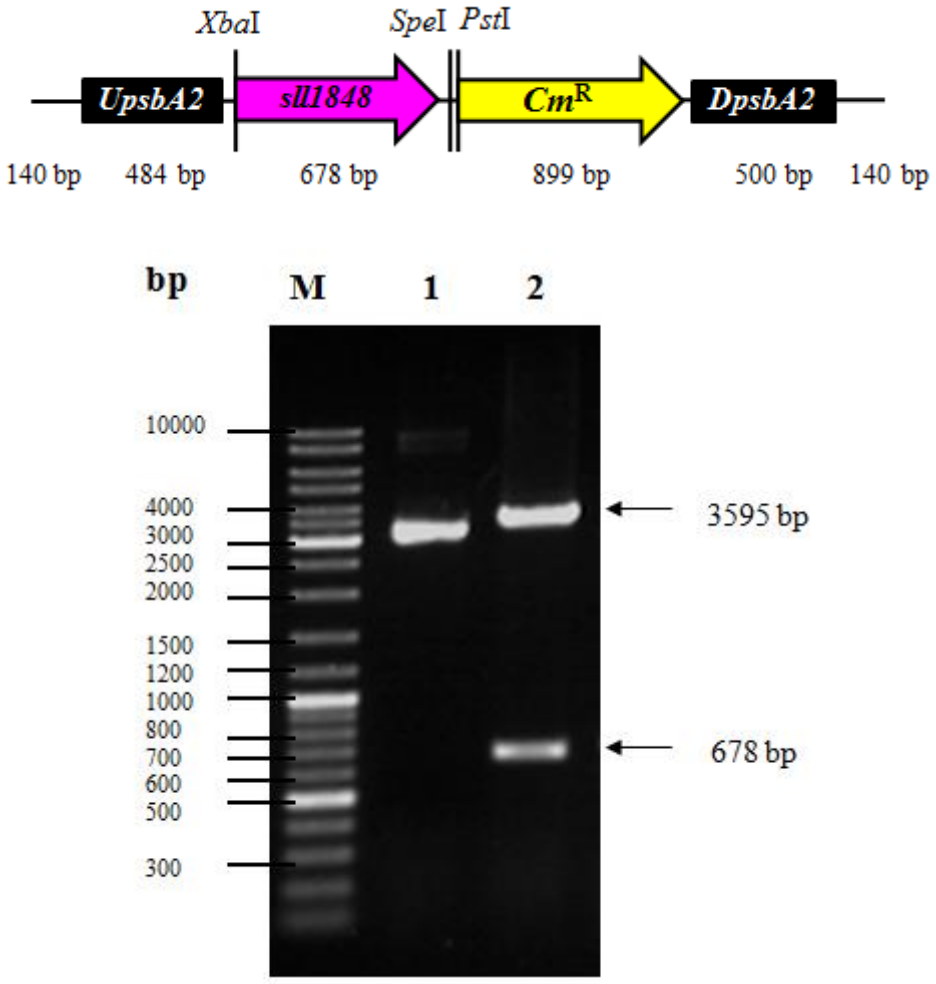


Figure 3. 3 Agarose gel electrophoresis of the the digested fragment from *Xba*I and *Spe*I enzymes of rpOE+C.

Lane M	DNA Marker Ladder 1 kb
Land 1	Recombinant plasmid extraction without enzyme digestion
Lane 2	Gene fragments digested with *Xba*I and *Spe*I enzymes

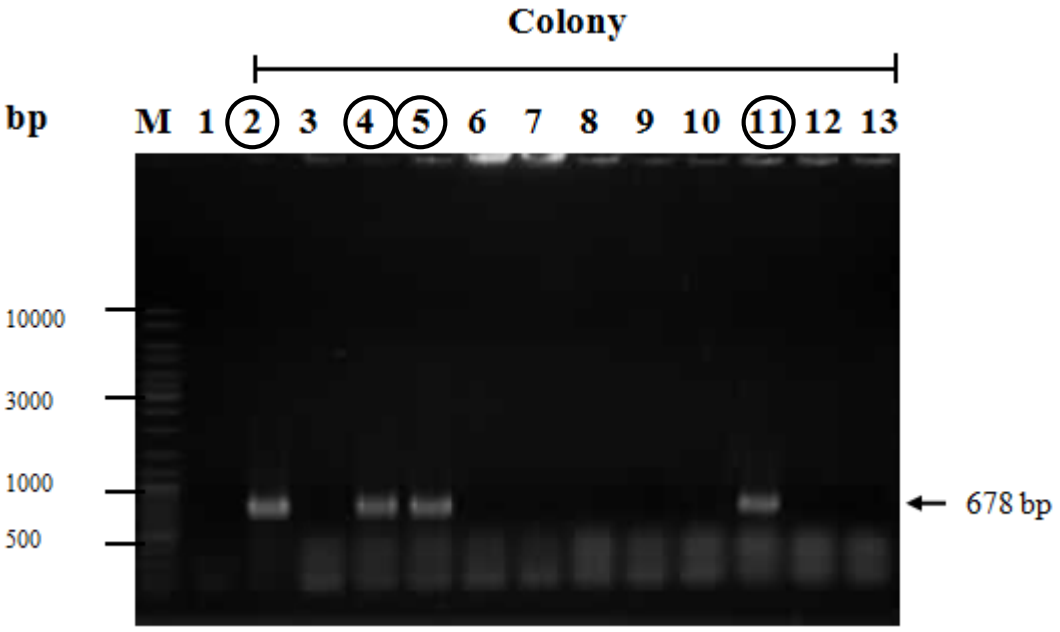


Figure 3. 4 Agarose gel electrophoresis of the amplified DNA fragment. The PCR product was run on 0.8% agarose gel of the recombinant plasmid containing *sll1848* gene (rpOE+XC).

Lane M	DNA Marker Ladder 1 kb
Lane 1	Negative control
Lane 2-13	Colony PCR of *sll1848* gene fragment in different clones

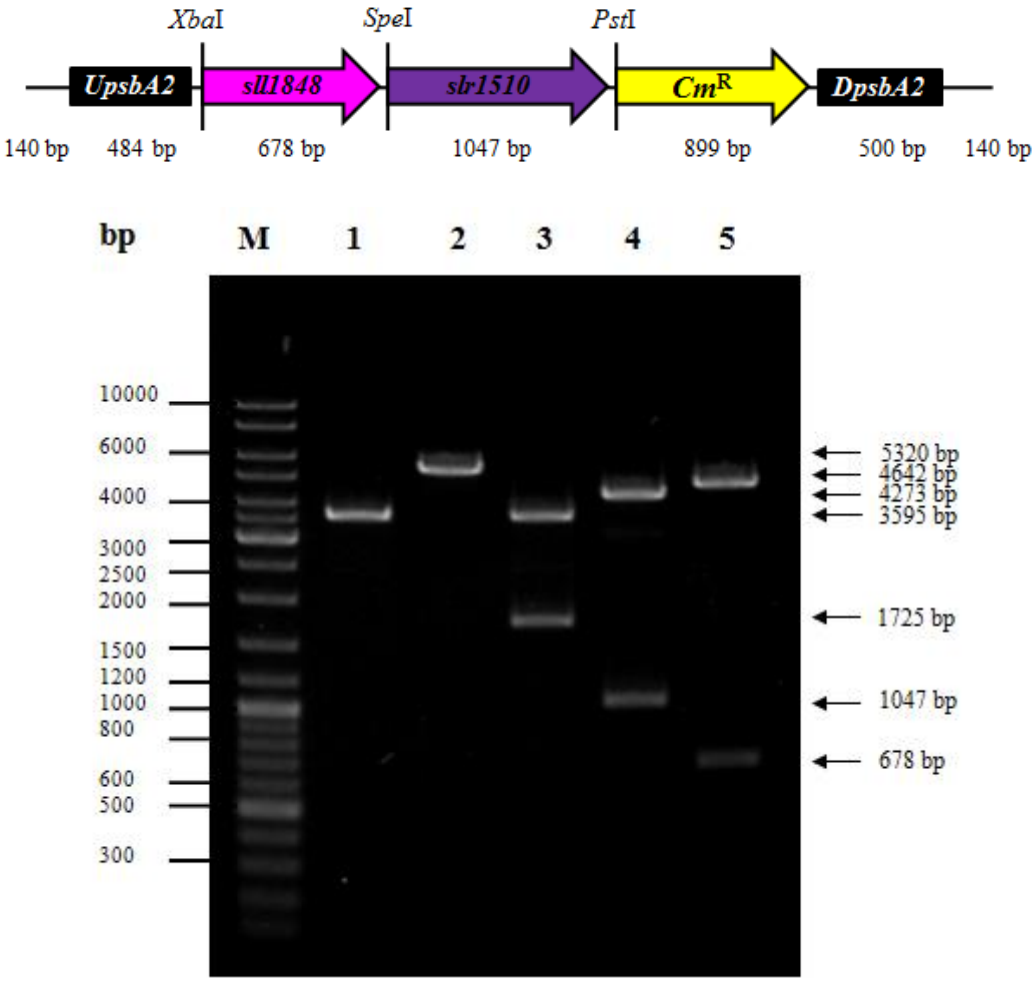


Figure 3. 5 Agarose gel electrophoresis of the gene fragment from restriction enzyme digestion.

Lane M	DNA Marker Ladder 1 kb
Lane 1	pEERM vector digested with *Xba*I enzyme
Land 2	Recombinant plasmid rpOE+XC digested with *Xba*I enzyme
Lane 3	Gene fragments digested with *Xba*I and *Pst*I enzymes
Lane 4	Gene fragments digested with *Spe*I and *Pst*I enzymes
Lane 5	Gene fragments digested with *Xba*I and *Spe*I enzymes

3.3 Single and double overexpressing strains

The empty pEERM vector and two recombinant plasmids rpOE+C and rpOE+XC were separately transformed into *Sysnechocystis* cells by the natural transformation method. The *Sysnechocystis* transformants were selected on BG_{11} medium containing antibiotic chloramphenicol. After that, the *sll1848*-overexpressing strain or OE+C strain was confirmed by PCR method with different primer pairs including chloramphenicol –resistance gene fragment amplified by F_*Cm*/R_*Cm* of about 899 bp, *sll1848* gene fragment amplified by F_*sll1848*/R_*sll1848* of about 678 bp, *sll1848* to chloramphenicol-resistance gene fragment amplified by F_*sll1848*/R_*Cm* of about 1,577 bp (Figure 3.6). The complete segregation integrated into *Synechocystis* genome by double homologous recombination of OE+C strain were checked by PCR method with a gene fragment of *psbA2* and amplified by F_*UpsbA2* /R_D*psbA2* of about 2,841 bp, a gene fragment from *sll1848* to *DpsbA2* gene amplified by F_*sll1848* /R_*DpsbA2* of about 2,217 bp, a gene fragment from *UpsbA2* gene to *sll1848* gene amplified by F_*UpabA2* /R_*sll1848* of about 1,302 bp (Figure 3.7). For wild type , the *psbA2* gene fragment studied had a size of about 2,217 bp whereas the control of wild type contains the empty vector (only chloramphenicol-resistance gene) had a size of about 2,163 bp (Figure 3.7, lane 1 and lane 2). The single band in lane 3 of Figure 3.7 represented the complete segregation of OE+C strain.

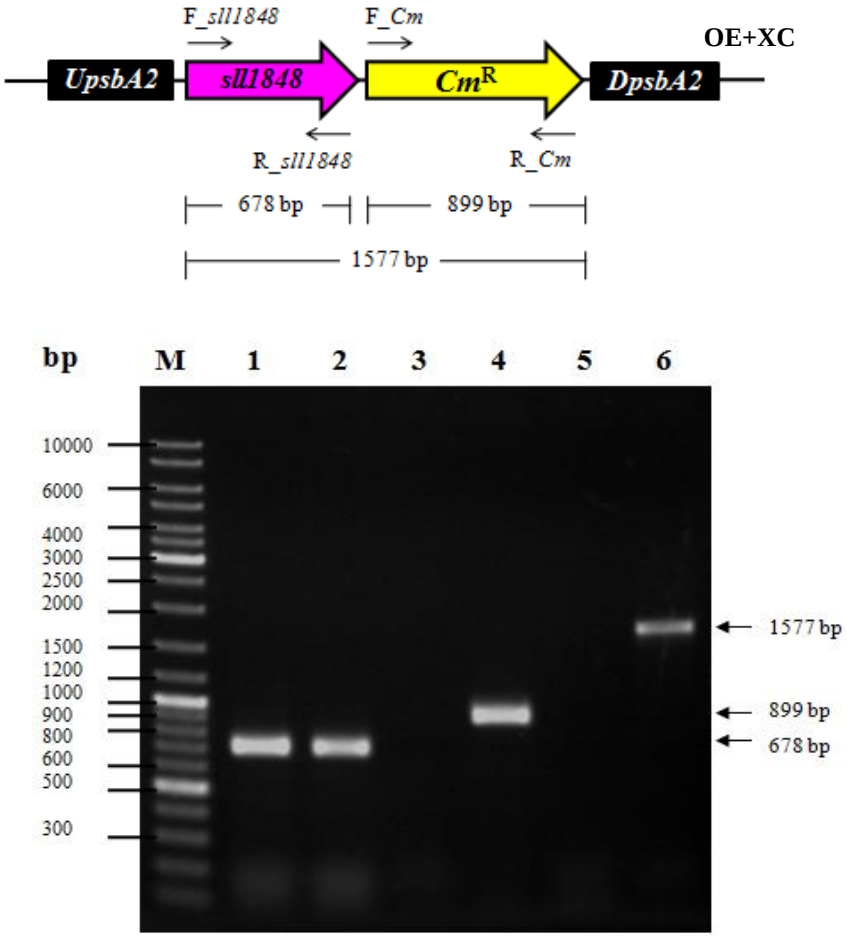


Figure 3. 6 Confirmation of the constructed OE+C and WT strains by Colony PCR method and 0.8% agarose gel electrophoresis.

Lane M DNA Marker Ladder 1 kb

Lane1 and 2 *sll1848* gene fragment of WT and OE+C amplified by F_*sll1848*/ R_*sll1848*

Lane3 and 4 Cm^R gene fragment of WT and OE+C amplified by F_*Cm* /R_*Cm*

Lane5 and 6 *sll1848* gene to *Cm* gene fragment of WT and OE+C amplified by F_*sll1848* /R_*Cm*

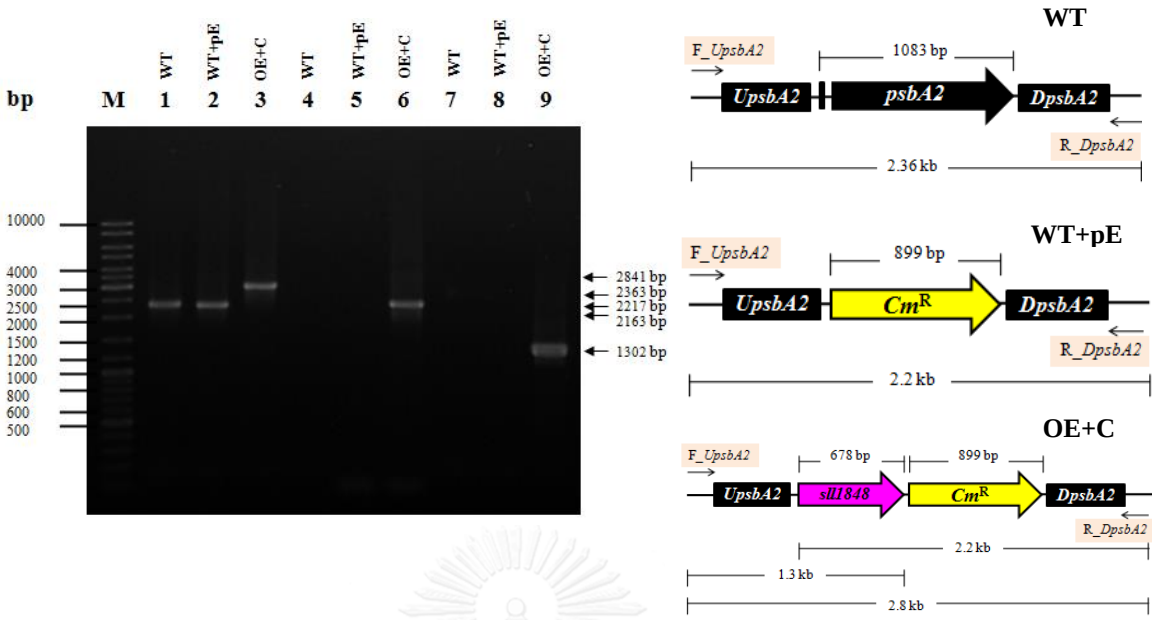


Figure 3. 7 Confirmation of complete segregation of *Synechocystis* cells WT and OE+C strains by Colony PCR method and 0.8 % agarose gel electrophoresis.

Lane M DNA Marker Ladder 1 kb

Lane1,2,3 PCR product of Up-*PsbA2* to Down-*PsbA2* gene fragment in 3 strains of WT, WT+pE and OE+C strains, respectively

Lane4,5,6 PCR product of *sll1848* gene to Down-*PsbA2* gene fragment in 3 strains of WT, WT+pE and OE+C strains, respectively

Lane7,8,9 PCR product of *Up-PsbA2* gene to *sll1848* gene fragment in 3 strains of WT, WT+pE and OE+C strains respectively

On the other hand, *slr1510/sll1848*-overexpressing strain or OE+XC strain was confirmed by PCR method with different primer pairs including *sll1848* gene fragment amplified by F_*sll1848*/R_*sll1848* of about 678 bp, *slr1510* gene fragment amplified by F_*slr1510*/R_*slr1510* of about 1,047 bp, chloramphenicol-resistance gene fragment amplified by F_*Cm*/R_*Cm* of about 899 bp, a fragment from *slr1510* gene to chloramphenicol-resistance gene amplified by F_*slr1510*/R_*Cm* of about 1,946 bp, a fragment from *sll1848* to *slr1510* gene amplified by F_*sll1848*/ R_*slr1510* of about 1,725 bp and *sll1848* to chloramphenicol-resistance gene fragment amplified by F_*sll1848*/R_*Cm* of about 2,624 bp (Figure 3.8). The gene location of OE+XC strain was corrected as expected design. On the other hand, the complete segregation into *Synechocystis* genome by double homologous recombination were checked by PCR method with a gene fragment of *psbA2* and amplified by F_*UpsbA2*/R_D*psbA2* of about 3,888 bp, a gene fragment from *psbA2* to *slr1510* amplified by F_*UpsbA2* /R_*slr1510* of about 2,389 bp. (Figure 3.9). The gene fragment amplified by PCR using *psbA2* primer had confirmed the complete segregation of OE+XC strain (Lanes 3-5, Figure 3.9).

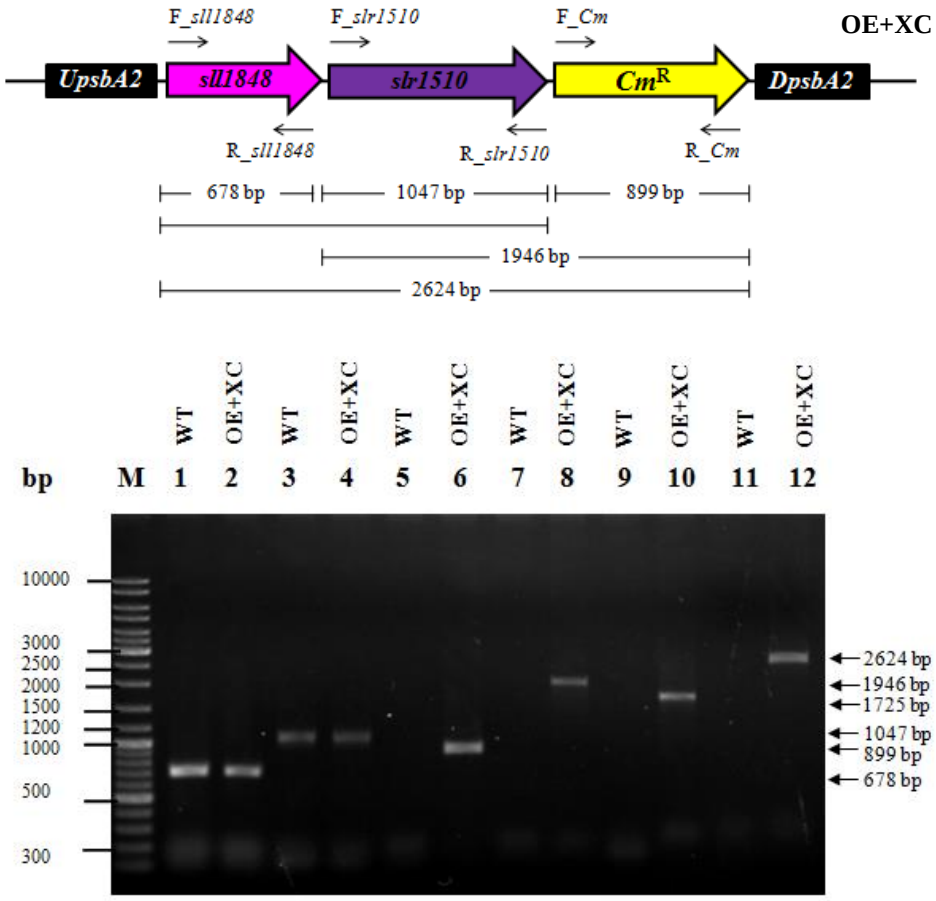


Figure 3. 8 Confirmation of gene location of WT and OE+XC strains by Colony PCR method and 0.8% agarose gel electrophoresis.

Lane M	DNA Marker Ladder 1 Kb
Lane1,2	*sll1848* gene fragment of WT and OE+XC strains
Lane3,4	*slr1510* gene fragment of WT and OE+XC strains
Lane5,6	*Cm*^{R}gene fragment of WT and OE+XC strains
Lane7,8	*slr1510* gene to *Cm*^{R} gene fragment of WT and OE+XC strains
Lane9,10	*sll1848* gene to *slr1510* gene fragment of WT and OE+XC strains
Lane11,12	*sll1848* gene to *Cm*^{R} gene fragment of WT and OE+XC strains

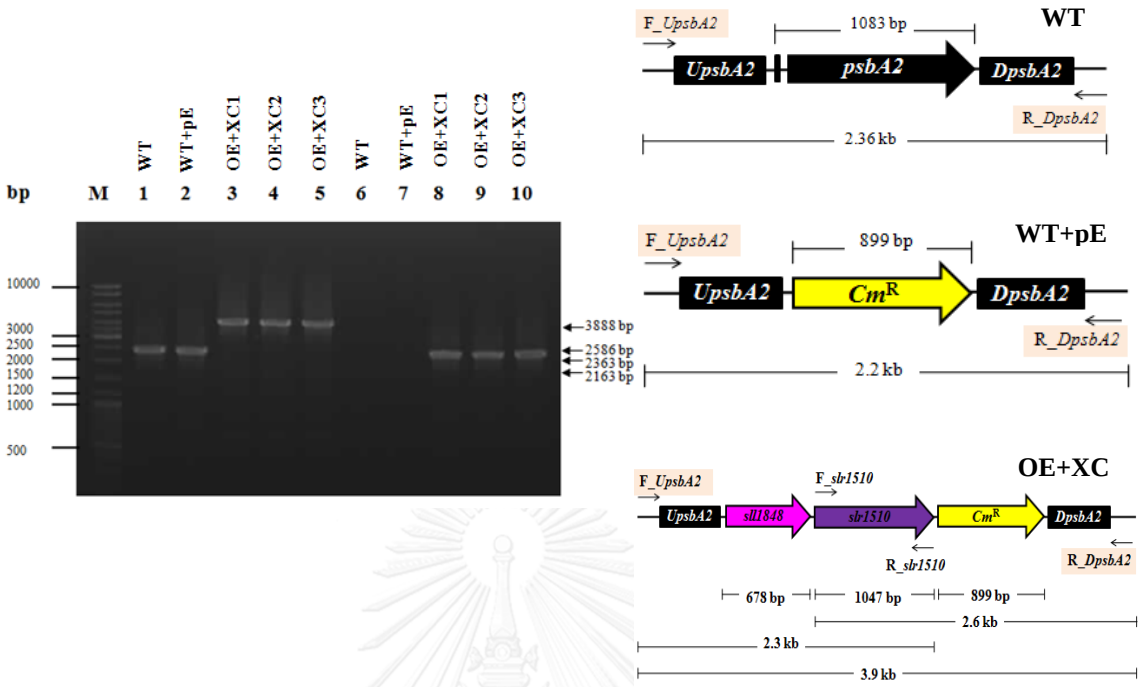


Figure 3. 9 Confirmation of the complete segregation of *Synechocystis* OE+XC strains compared with WT by Colony PCR method and 0.8% agarose gel electrophoresis.

Lane M DNA Marker Ladder 1 kb

Lane1-5 PCR products of Up-*PsbA2* to Down-*PsbA2* gene fragment in 3 strains of lane 1: WT, lane 2: WT+pE, lane 3: OE+XC1, lane 4: OE+XC2 and lane 5: OE+XC3 strains, respectively

Lane6-10 PCR products of Up-*PsbA2* to *slr1510* gene fragment in 3 strains of lane 1: WT, lane 2: WT+pE, lane 3: OE+XC1, lane 4: OE+XC2 and lane 5: OE+XC3 strains, respectively

3.4 Cell growth of wild type and overexpressing strains

3.4.1. Cell growth under normal condition BG_{11} medium

Synechocystis sp. PCC 6803 wild type (WT) and two overexpressing strains, OE+C and OE+XC, respectively, were grown in BG_{11} medium and their cell growth was determined for 20 days (Figure3.10). The growth of WT was higher than those of OE+C and OE+XC strains. Interestingly, the control WT+pE strain was slightly lower than WT. Although the growth of two overexpressing cells was slightly less than WT, their cell color was still in green culture (Figure 3.11A). The stage of cell growth was classified into 4 phases including lag phase (0-4 days), log phase (4-12 days), late-log phase (12-16 days) and stationary phase (16-20 days).

3.4.2. Cell growth under nutrient modification

Synechocystis wild type and two overexpressing strains were grown in BG_{11} medium until reaching mid-log phase and transferred into new media including BG_{11} medium (control condition), 0.4 % acetate supplementation into BG_{11} medium and nitrogen deficiency of BG_{11} medium (Figure 3.11B). Cell growth after each modified-nutrient treatment was determined for 8 days. Results are shown in Figures 3.12 and 3.13. For WT, control WT+pE and two overexpressing strains cells, their growth were increased after treating with 0.4% acetate supplementation whereas the decrease of cell growth was observed under nitrogen-deprived condition (-N) (Figure 3.12A). It was coincident with all strains that after 4 day-treatment the changes of increased growth was clearly observed whereas –N condition significantly reduced cell growth (Figures 3.12B, C, D). On the other hand, results of cell growth in different media are present in Figure 3.13.

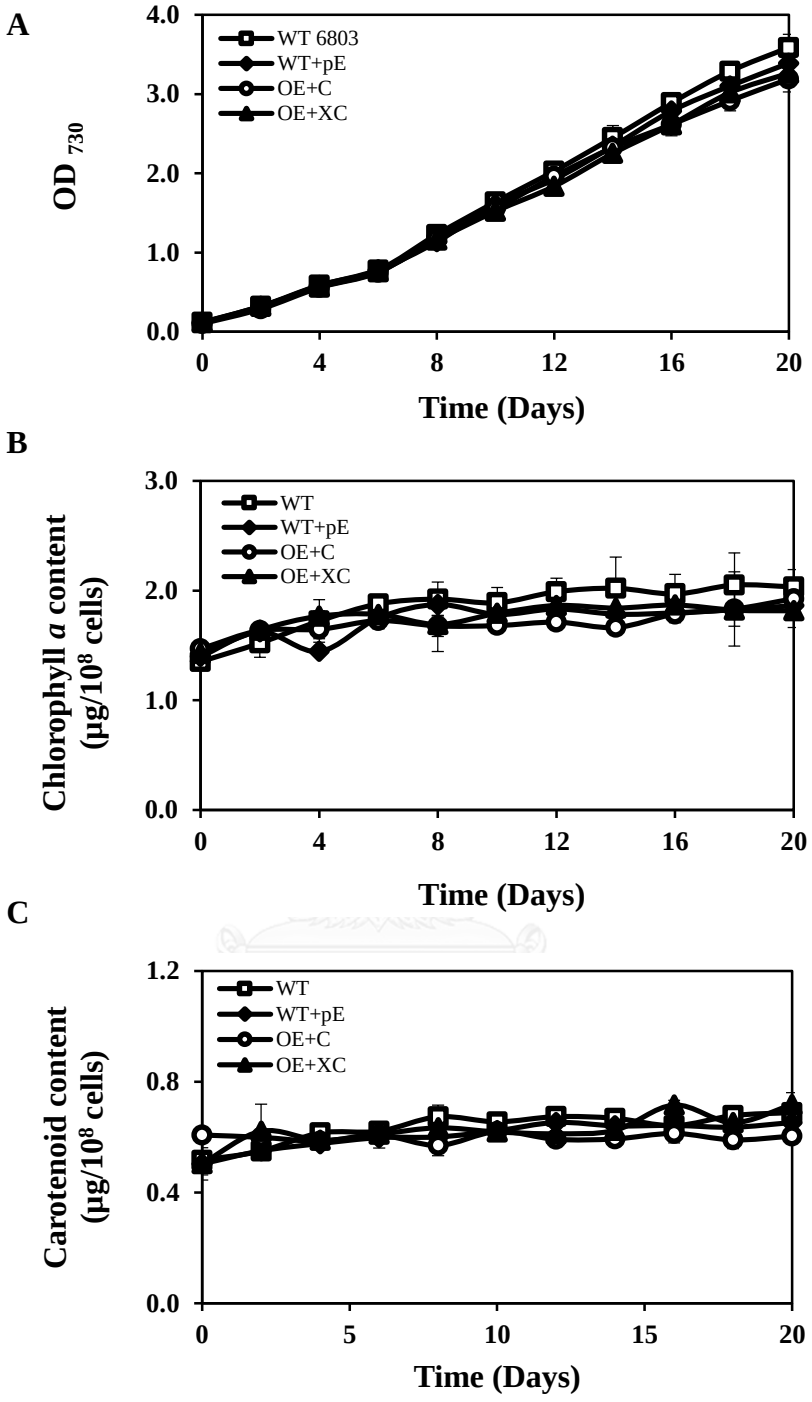


Figure 3. 10 The growth curve (A), chlorophyll *a* content (B) and carotenoid content (C) of *Synechocystis* wild type (□), WT+pE (♦), OE+C (○) and OE+XC (▲) overexpressing strains grown in BG_{11} medium. Data represent mean±S.D., n=3.

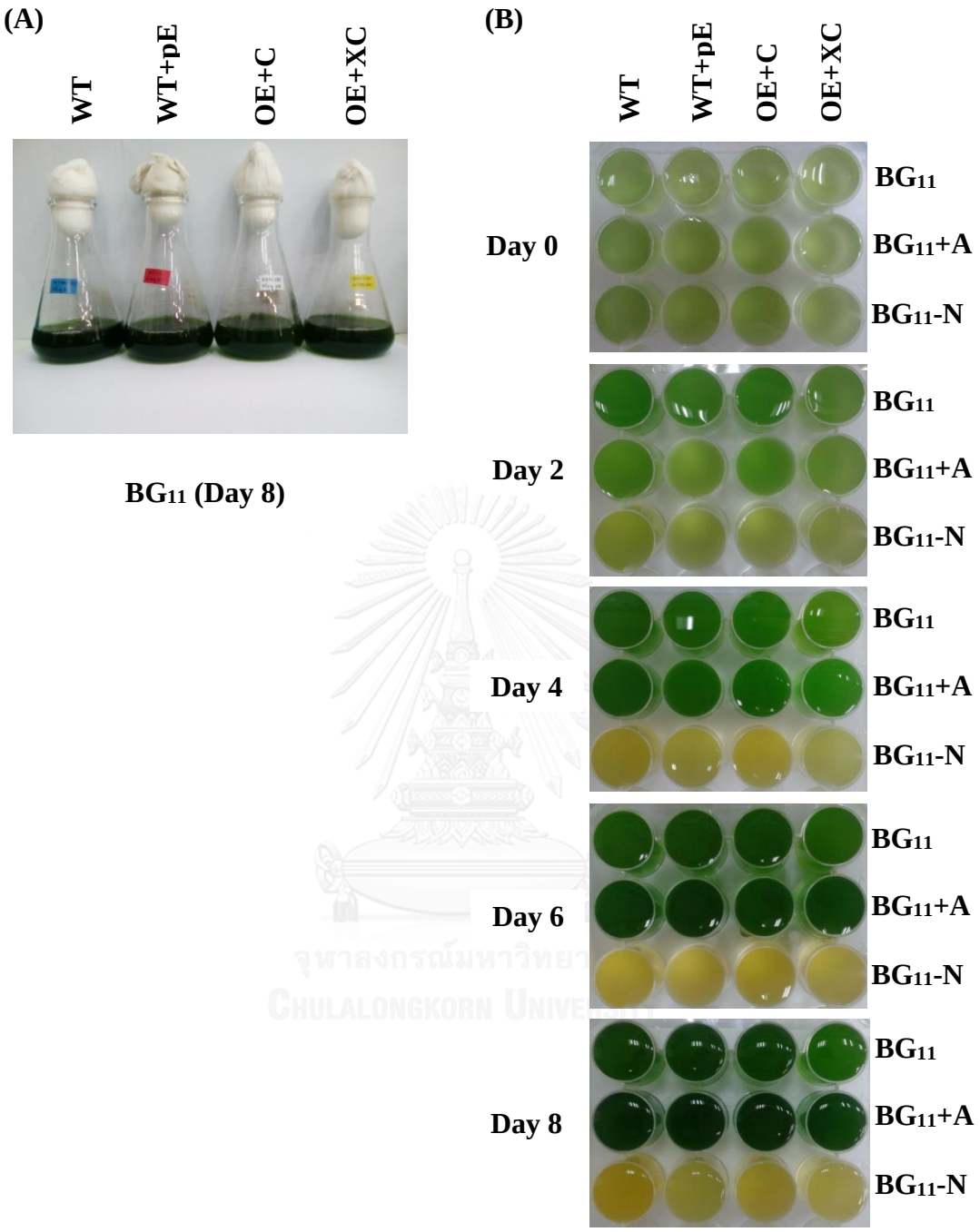


Figure 3. 11 Color of cell culture. Cells were grown in BG_{11} medium for 8 days before adapting in new media (A) of various nutrient modifications including BG_{11}, 0.4% acetate supplementation (BG_{11}+A) and nitrogen deficiency (BG_{11}-N) (B).

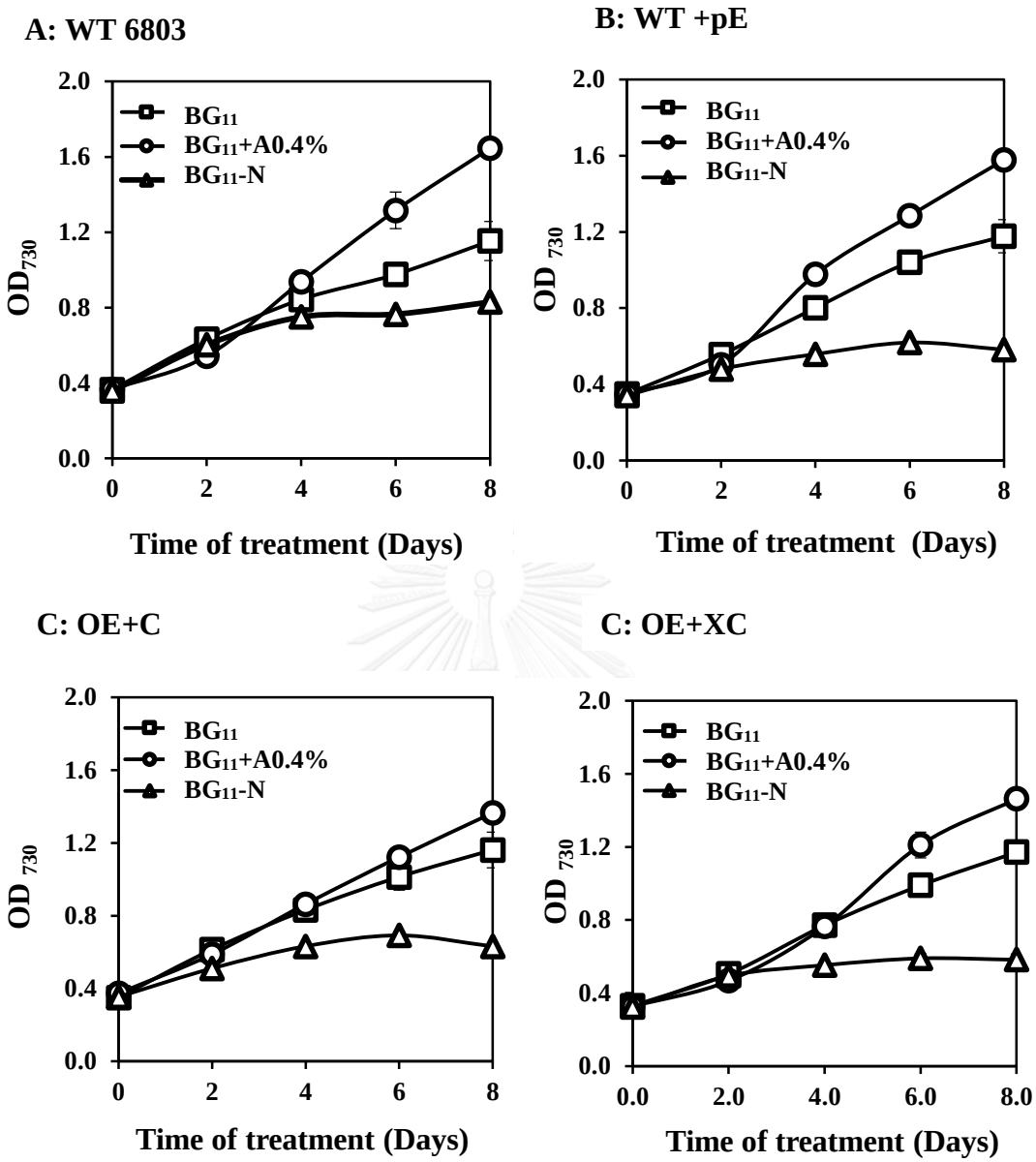


Figure 3. 12 The growth curves of WT 6803 (A), WT+pE (B), OE+C (C) and OE+XC (D) strains. Mid-log phase cells were treated in various BG_{11} media including normal BG_{11} medium (BG_{11}), containing 0.4% acetate (BG_{11}+A 0.4%) and nitrogen deprived BG_{11} medium (BG_{11}-N). Data represent mean ± S.D., n=3.

Under BG_{11} treatment, there was no differences on cell growth in all strains studied (Figure 3.13A). The slight decreases of cell growth were observed after treating overexpressing cells with BG_{11}+0.4% acetate condition along 8 day-treatment (Figure 3.13B). For nitrogen deficiency condition, control WT+pE and two overexpressing strains showed lower growth when compare to WT, starting at 2 day-treatment (Figure 3.13C).

3.5 The contents of intracellular pigments

3.5.1 Intracellular pigment content under normal condition of BG_{11} medium

The chlorophyll *a* content (Figure 3.10B) under normal condition of control WT+pE and two overexpressing strains showed slight decreases when compared to WT. Likewise, there were no differences on carotenoid content in each strain under normal BG_{11} condition (Figure 3.10C). Only OE+C strain showed insignificant reduction of chlorophyll *a* and carotenoid contents (Figures 3.10B and C).

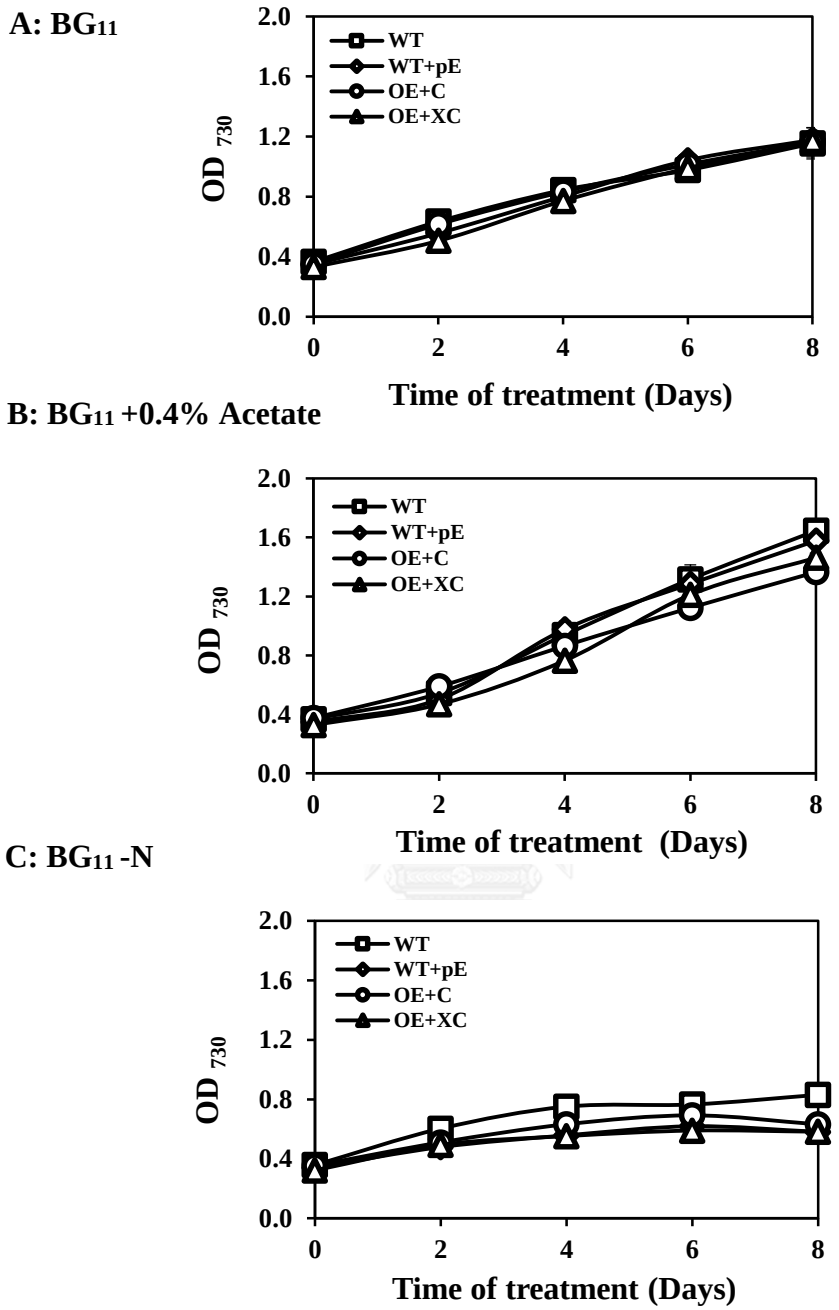


Figure 3. 13 The growth curve of *Synechocystis* wild type (□), WT+pE (◊), OE+C (○) and OE+XC (Δ) strains. Mid-log phase cells were treated in various BG_{11} media including normal BG_{11} (A), BG_{11}+0.4% acetate (B) and BG_{11}-N (C) conditions. Data represent mean ± S.D., n=3.

3.5.2. Intracellular pigment content under nutrient modification

The determination of chlorophyll *a* and carotenoid contents were carried out after nutrient modified–treatment (Figures 3.14 - 3.17). The 0.4% acetate supplementation significantly increased the accumulation of chlorophyll *a* content in all strains (Figure 3.14A-D). In contrast, all four strains showed significant decreases on chlorophyll *a* contents under nitrogen deficiency starting after 2 day-treatment compared to normal condition. When the comparison on each treatment was carried out (Figure 3.15), the significant decreases of chlorophyll *a* were observed in WT and WT+pE strains under BG_{11}-N condition.

The carotenoid contents of wild type and overexpressing strains is shown in Figure 3.16. The highest amount of carotenoid content was observed under 0.4% acetate–supplemented condition starting at day 4 treatment when compared to normal condition. The significant decreases of carotenoid contents in all strains were demonstrated clearly under BG_{11}-N condition, starting at 2 day treatment. In Figure 3.17, the carotenoid content in each strain showed no differences under all conditions studied. However, the higher accumulation of carotenoid level was observed in OE+C and OE+XC strains under BG_{11}-N condition when compared to WT.

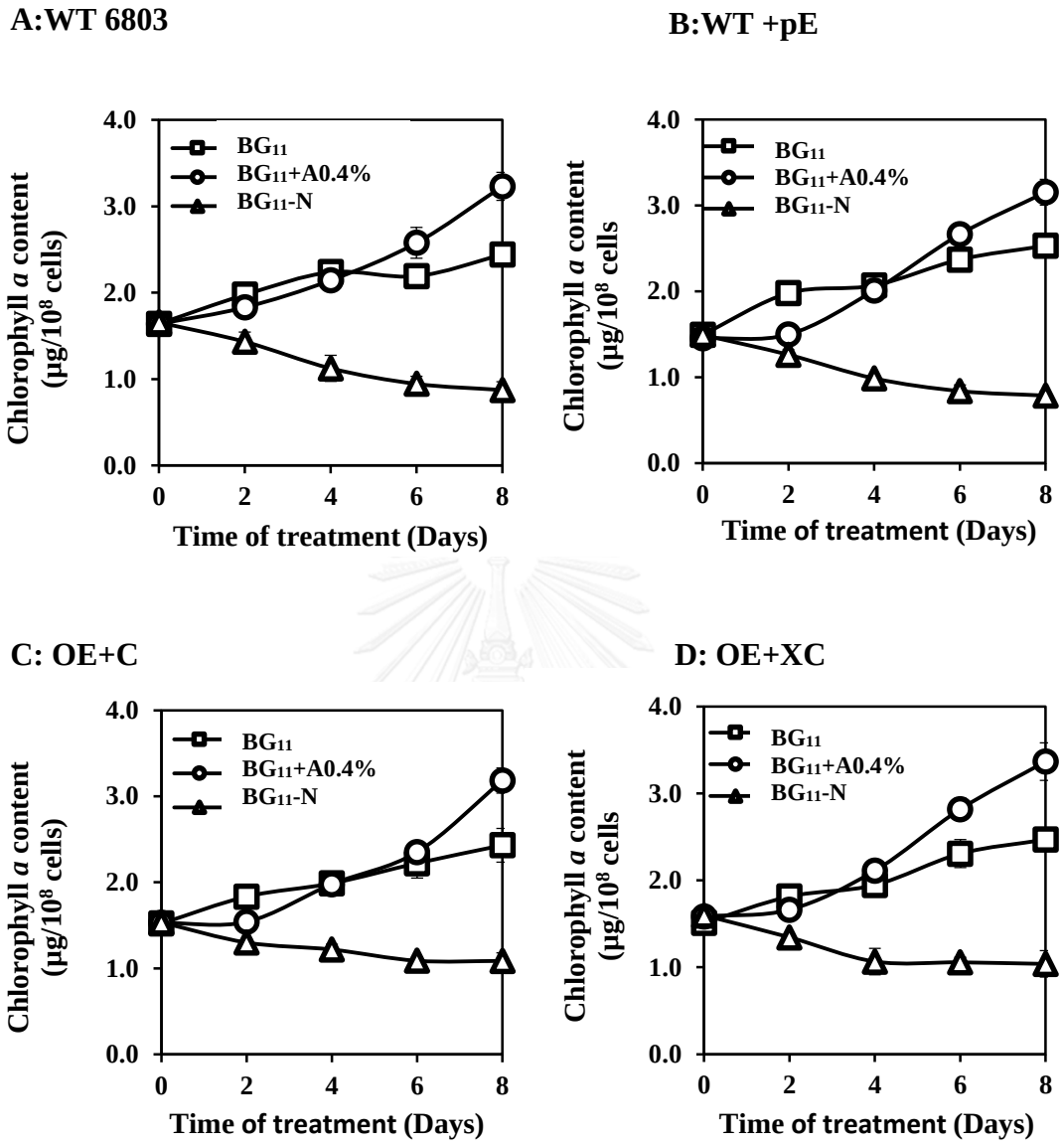


Figure 3. 14 The chlorophyll *a* contents of WT 6803 (A), WT+pE (B), OE+C (C) and OE+XC (D). Mid-log phase cells were grown in BG_{11} media under nutrient modification including BG_{11}, 0.4% acetate supplementation (BG_{11}+A 0.4%) and nitrogen deficiency (BG_{11}-N). Data represents mean ± S.D., n=3.

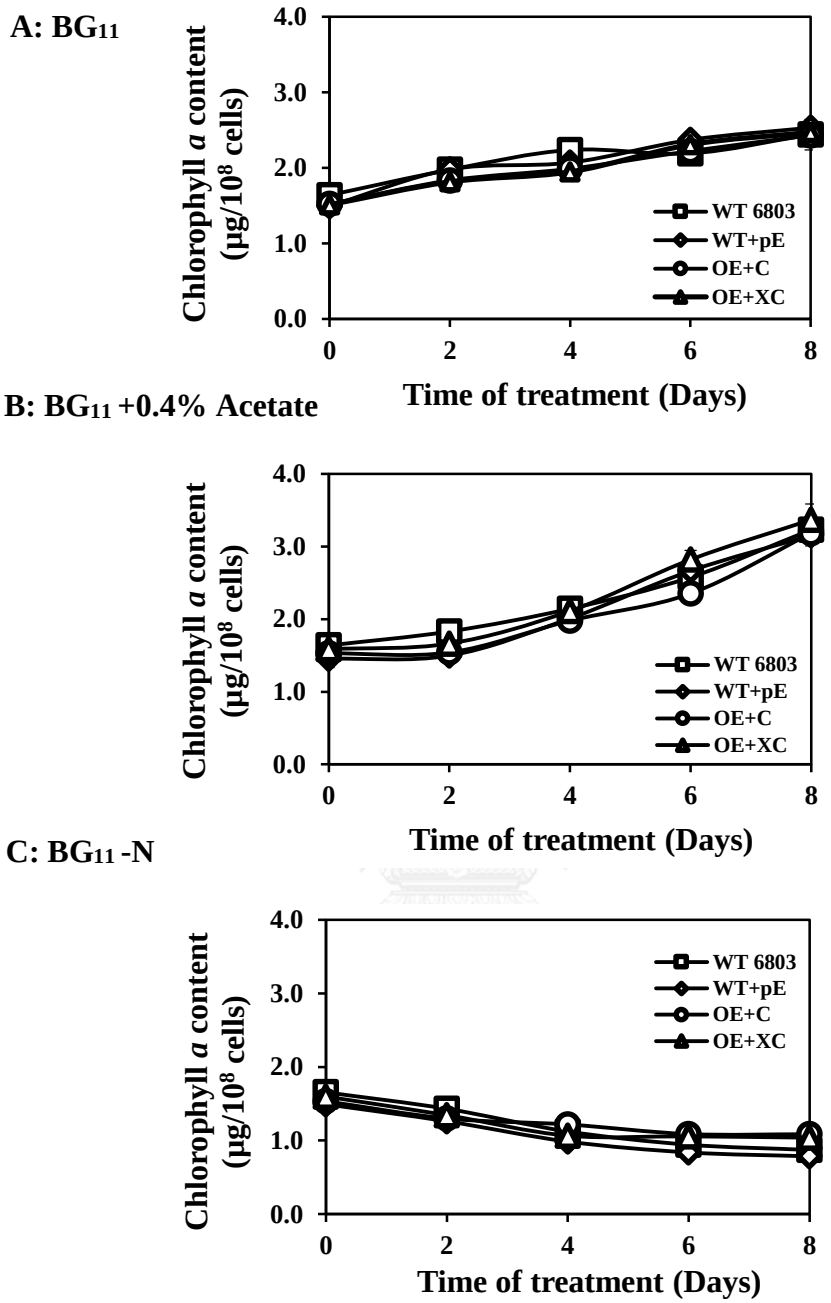


Figure 3. 15 The chlorophyll *a* contents of *Synechocystis* wild type (□), WT+pE (◊), OE+C (○) and OE+XC (Δ) strains. Mid-log phase cells were treated in various BG_{11} media including BG_{11} (A), BG_{11}+0.4% acetate (B) and BG_{11}-N (C) conditions. Data represent mean ± S.D., n=3.

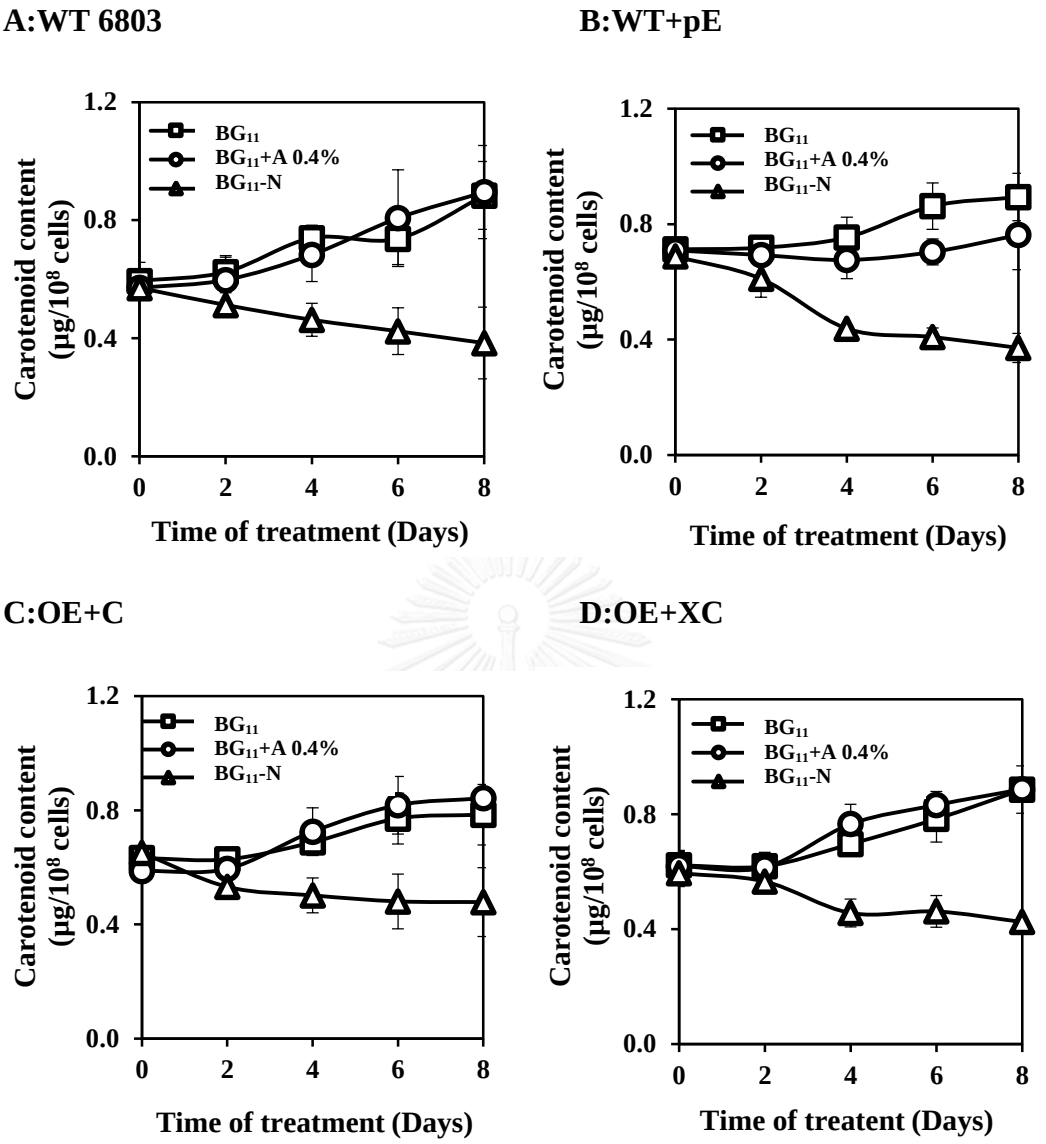


Figure 3. 16 The carotenoid contents of WT 6803 (A), WT+pE (B), OE+C (C) and OE+XC (D) strains. Mid-log phase cells were treated in various BG_{11} media including BG_{11}, BG_{11}+0.4% acetate (BG_{11}+A 0.4%) and BG_{11}-N conditions (BG_{11}-N). Data represent mean ± S.D., n=3.

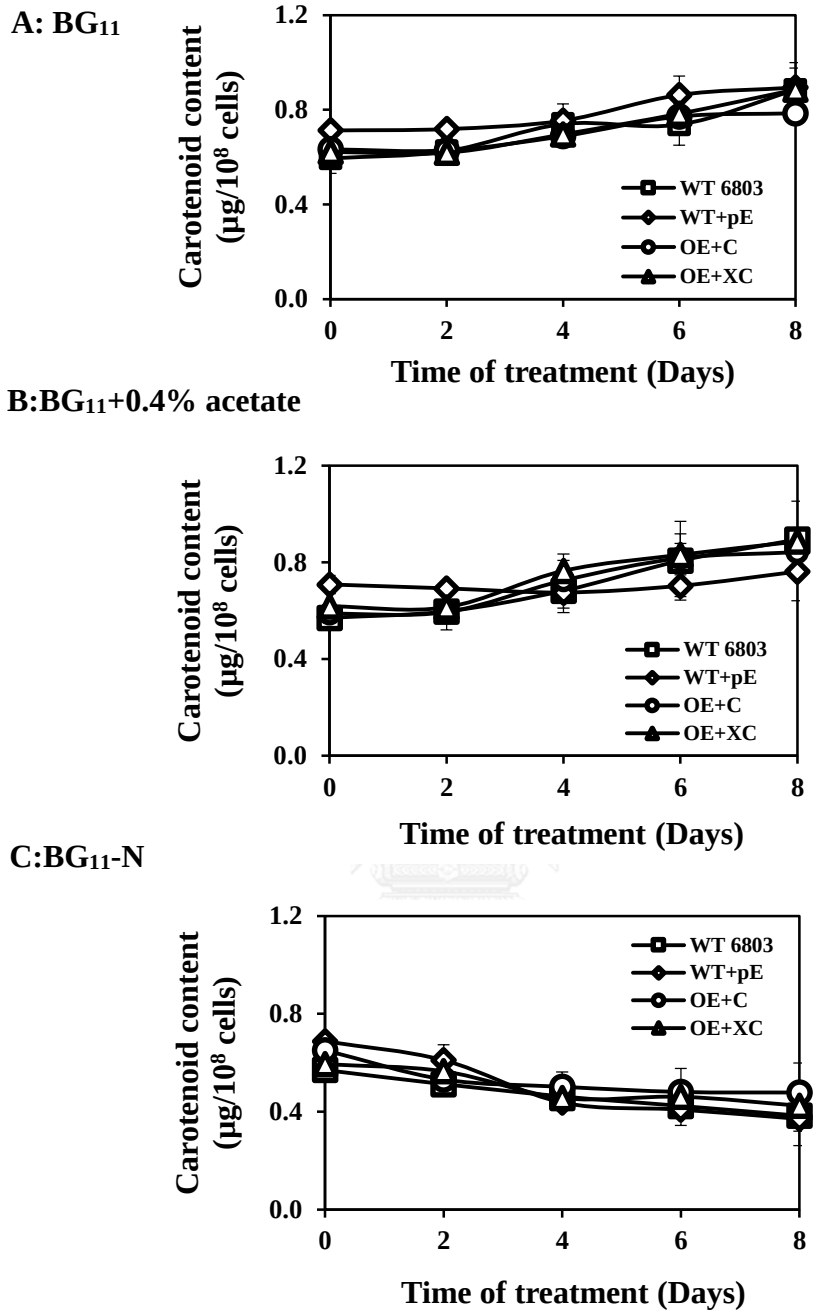


Figure 3. 17 The carotenoid contents of *Synechocystis* wild type (□), WT+pE (◊), OE+C (○) and OE+XC (Δ) strains. Mid-log phase cells were treated in various BG_{11} media including BG_{11} (A), BG_{11}+0.4% acetate (B) and BG_{11}-N (C) conditions. Data represent mean ± S.D., n=3.

3.6 Oxygen evolution rate of *Synechocystis* sp. PCC 6803 wild type and overexpressing strains

3.6.1 Oxygen evolution rate of cells under normal growth condition

The oxygen evolution rates in each growth phase of WT and overexpressing strains are shown in Figure 3.18. This oxygen evolution rate normally represented the photosynthetic efficiency of photosynthetic organisms, especially in cyanobacteria. Interestingly, the oxygen evolution rates of OE+C and OE+XC overexpressing strains showed no significant changes when compared to WT. Interestingly, overexpressing cells grown under late-lag phase gave high oxygen evolution rates than those other growth phase.

3.6.2 Oxygen evolution rate of cells under nutrient modification

The oxygen evolution rates of *Synechocystis* WT and overexpressing strains were determined under nutrient-modified conditions (Figure 3.19 and 3.20). In Figure 3.19A, WT cells were significantly decreased on their photosynthetic efficiency under BG_{11}-N condition whereas a slight increase was observed under BG_{11}+acetate (0.4%). For control WT containing empty vector (WT+pE), it showed the same tendency as that in BG_{11}-N condition which had lower oxygen evolution rate when compared to normal BG_{11} medium (Figure 3.19B). Other two overexpressing strains (Figure 3.19C and D) also possessed the lower photosynthetic efficiency under BG_{11}-N condition as also shown in Figure 3.20. Interestingly, underBG_{11}+acetate (0.4%) after day 4-treatment of all strains found that the photosynthetic efficiency was slightly decreased.

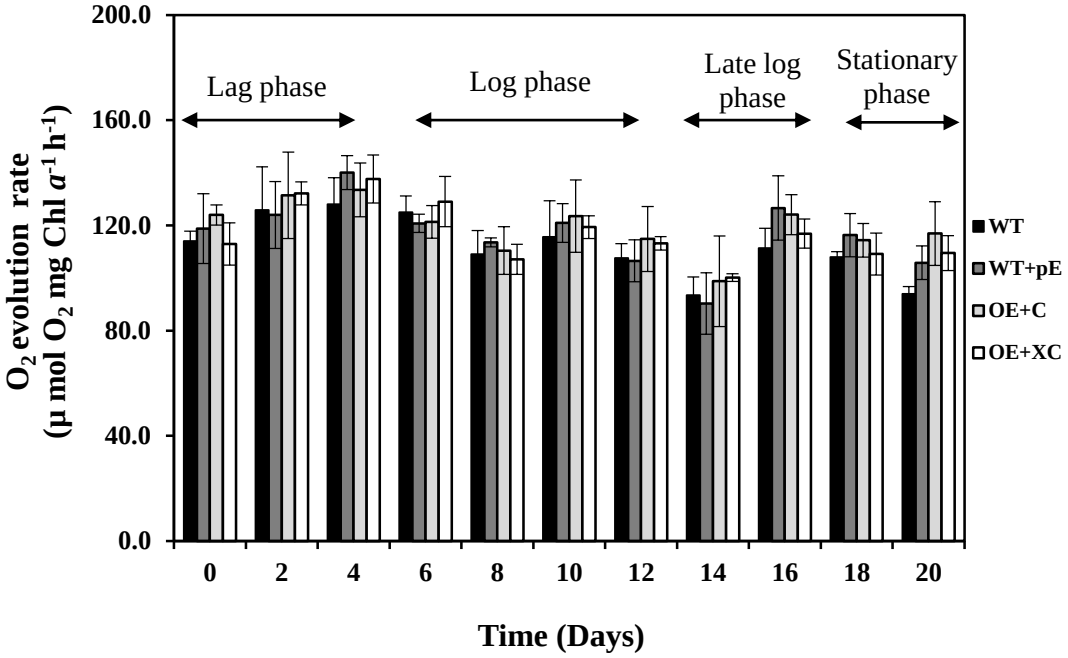


Figure 3. 18 The oxygen evolution rates of wild type, control WT+pE and two overexpressing strains under BG_{11} medium. Cells grown under each growth stage were harvested and measured for their oxygen evolution rate. Data represent mean ± S.D., n=3.

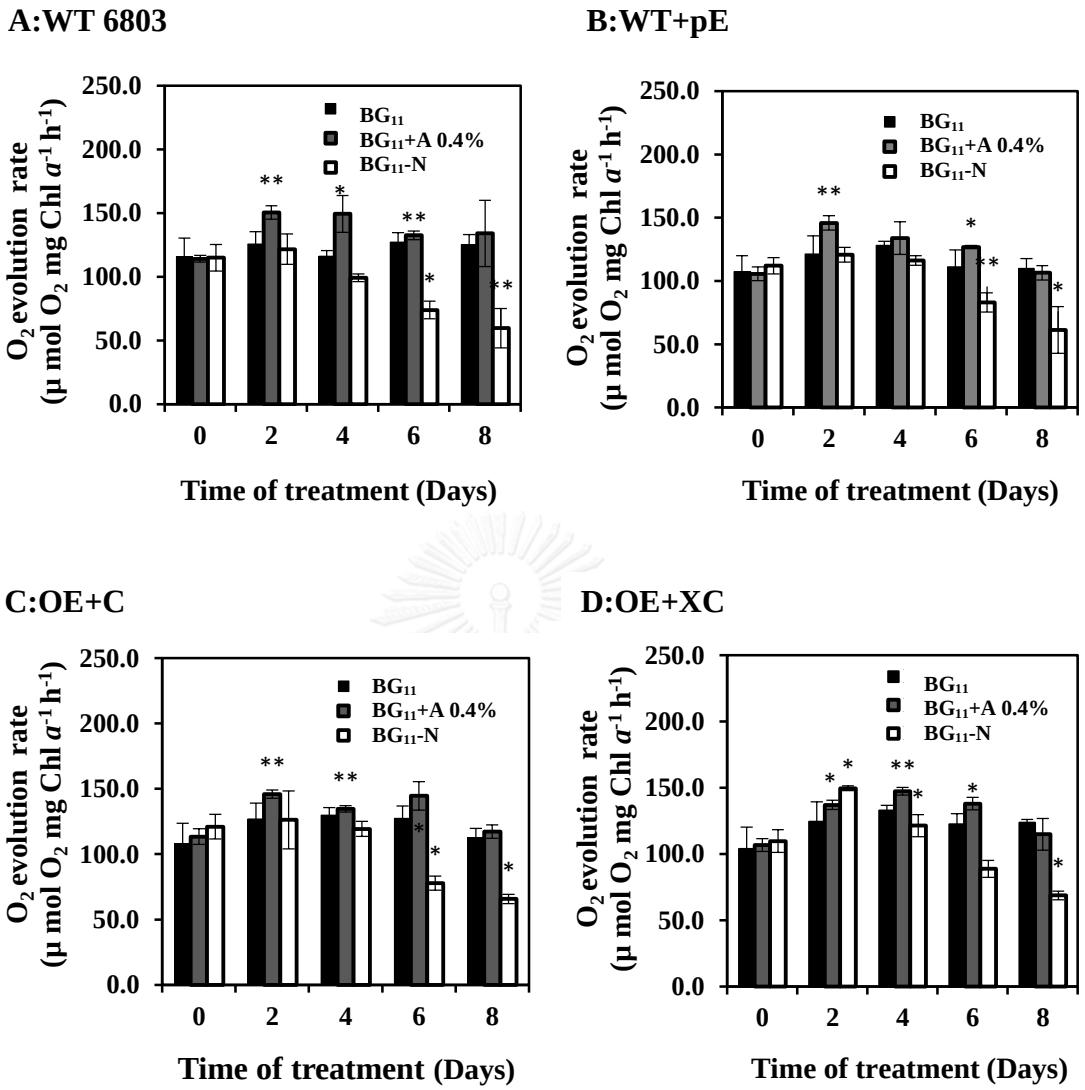


Figure 3. 19 The oxygen evolution rates of wild type, control WT+pE and two overexpressing strains under BG_{11} medium, BG_{11}+0.4% acetate and BG_{11}-N conditons. Data represent mean ± S.D., n=3.

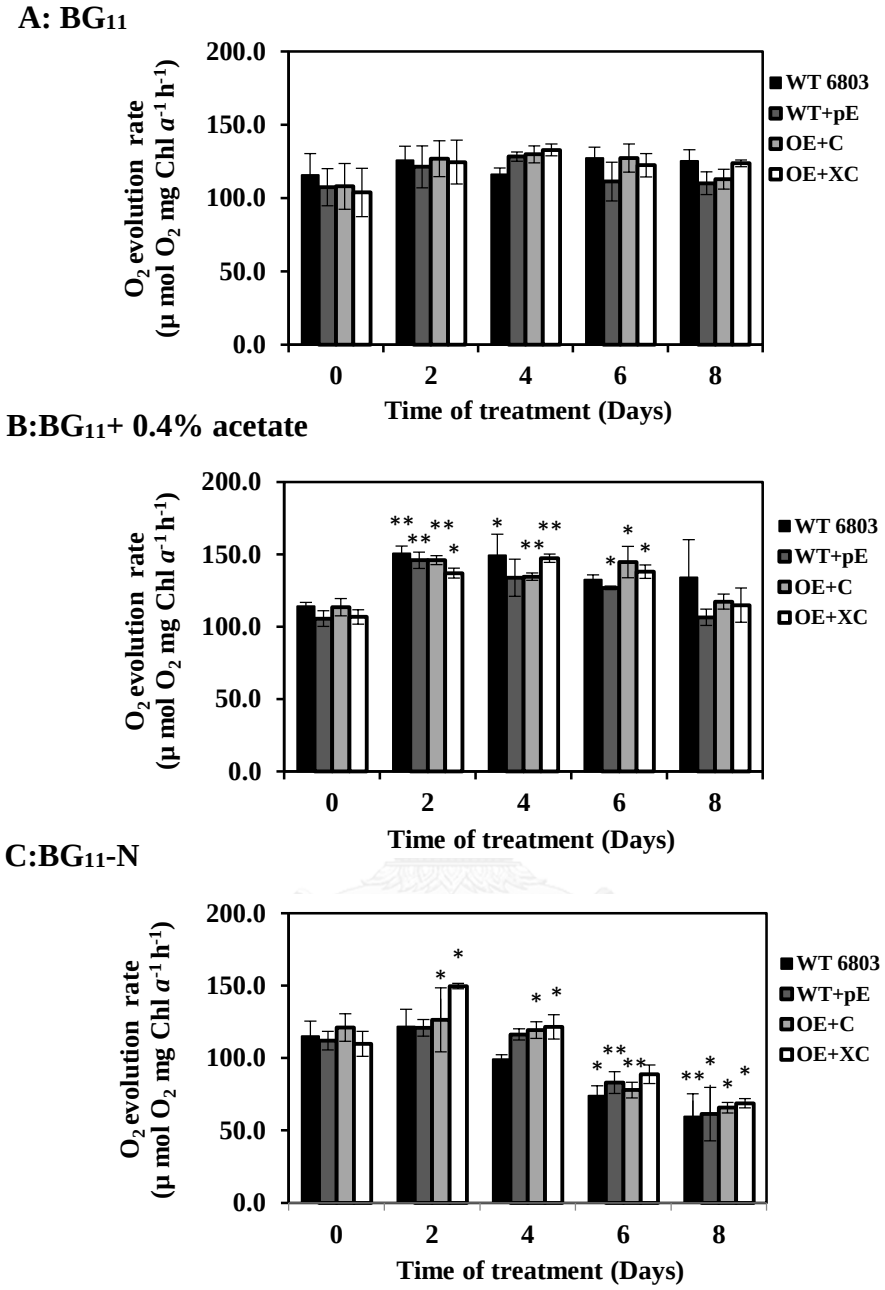


Figure 3. 20 The oxygen evolution rates of *Synechocystis* wild type (■), WT+pE (■), OE+C (■) and OE+XC (□). Cells were grown in BG_{11} media under various nutrient modifications including BG_{11} (A), 0.4% acetate supplementation (B) and nitrogen deficiency (C). Data represent mean ± S.D., n=3.

3.7 Effect of nutrient modification on mRNA levels in *Synechocystis* sp. PCC 6803 wild type and overexpressing strains

The gene expression levels and the ratio of band intensity (gene/*16s*RNA) are shown in Figure 3.21. The mRNA levels of *sll1848* or *plsC* gene (encoding putative 1-acyl-sn-glycerol-3-phosphate acyltransferase) of both OE+C and OE+XC overexpressing cells under normal BG_{11} medium showed higher transcript amount than WT. The *sll1848* transcripts of WT cells and WT+pE (control cell) were slightly decreased under BG_{11}+A0.4% and BG_{11}-N conditions. Moreover the BG_{11}+A0.4% and BG_{11}-N did not induce the *sll848* transcripts increase of overexpressing strains when compare to these at start day. However, the *sll1848* transcript showed the highest levels in OE+C strains in all conditions.

The transcript level of *slr1510* or *plsX* encoding fatty acid/phospholipid synthesis protein PlsX (Figure 3.21) was increased in OE+XC overexpressing cells compare to those of WT and WT+pE cells. Interestingly, the *slr1510* transcripts in WT were induced by BG_{11}+A0.4% and BG_{11}-N conditions. The significant increase on *slr1510* transcripts of OE+XC strain were observed under BG_{11}+A 0.4% and BG_{11}-N conditions.

The transcript levels of *AccA* gene encoding acetyl-CoA carboxylase alpha subunit, a key enzyme catalyzing the conversion of acetyl-CoA to malonyl-CoA are shown in Figure 3.21. The increase of *AccA* transcript of overexpressing strains inducing OE+C and OE+XC were existed comparing to WT and WT+pE strains. The BG_{11}+A 0.4% and BG_{11}-N did not affect to *AccA* transcript levels in all strains.

The transcript levels of *Aas* encoding acyl-ACP synthetase converted free fatty acids to fatty acyl-ACP were not different in all WT, WT+pE, and two overexpressing strains.

The *lipA* gene transcript encoding putative lipase was depicted in Figure 3.21. Interestingly, overexpressing strains OE+C showed an increase of *lipA* transcript when compared to other strains studied. The decrease of *lipA* transcript level of WT was observed under BG_{11}+A0.4% whereas its increase was shown under BG_{11}-N. Also, WT+pE strains showed up-regulation on *lipA* transcript level under BG_{11}-N condition. The OE+XC strain had high level of *lipA* transcript induce by BG_{11}-N condition.

The transcript levels of *phaA* encoding beta-ketothiolase which catalyzes two acetyl-CoA converted to acetoacetyl-CoA, and further acetoacetyl-CoA reductase (*phaB*) which is responsible for reducing acetoacetyl-CoA with NADPH to hydroxybutyryl-CoA to the PHB were also shown in Figure 3.21. Interestingly, the *phaA* transcript amounts in WT and WT+pE strains were up-regulated by BG_{11}-N condition whereas those transcript levels of OE+C and OE+XC were decreased when compared to WT and WT+pE control strains. Moreover, the acetate supplementation had a decreased effect on *phaA* transcript in both OE+C and OE+XC strains.

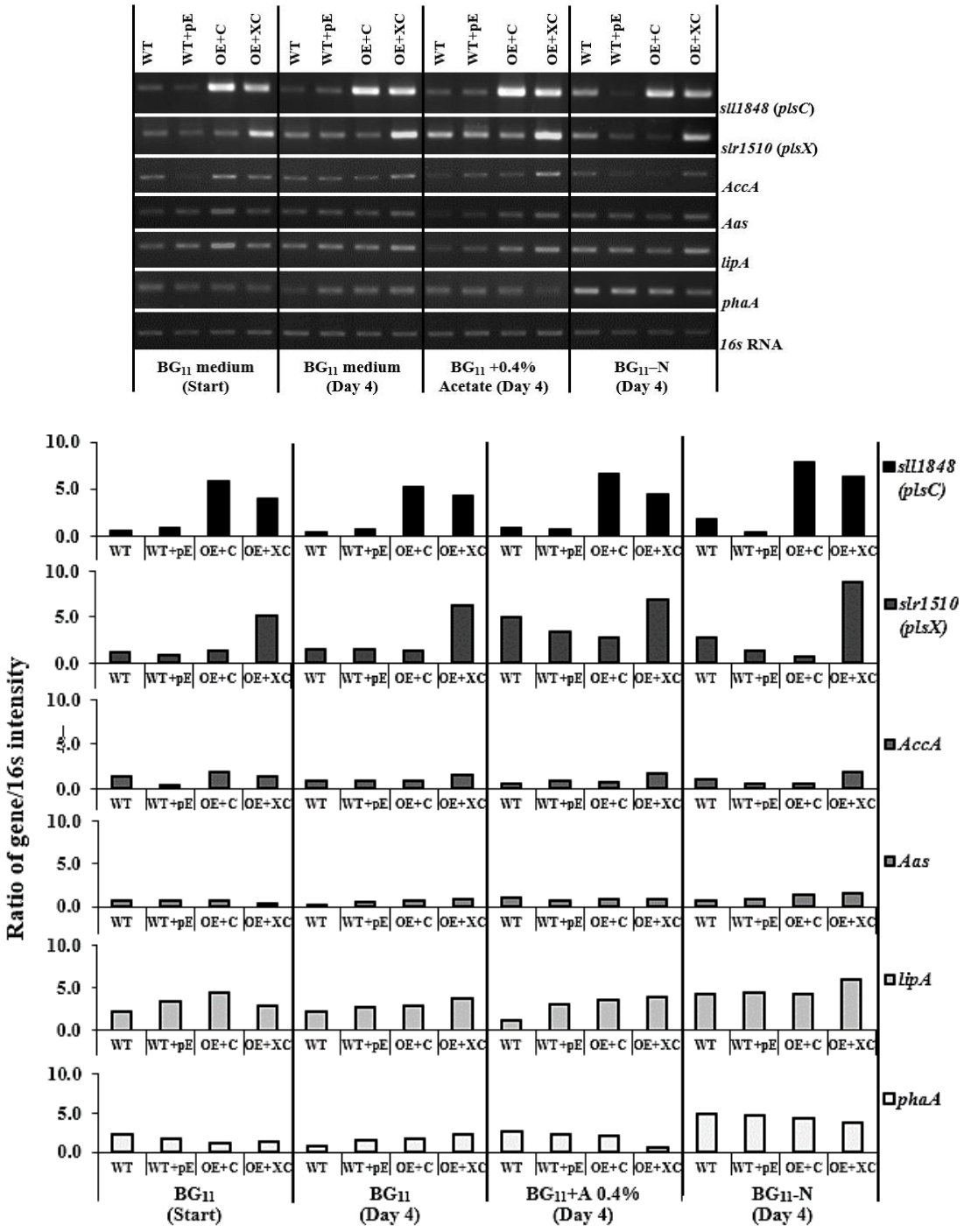


Figure 3. 21 The gene expressions of wild type, control WT+pE and two overexpressing strains. Cells treated in different nutrient conditions were harvested and extracted their RNA for transcript determination using RT-PCR.

3.8 Total lipid screening by Sudan black B staining

The total lipid staining was determined by Sudan black B solution and showed in Figure 3.22. The stained membrane lipid was appeared in dark brown color. It was found that after adaptation in nutrient-modified treatments, the stained membrane showed higher dark brown color in all strains. Although the total lipid screening by Sudan black B staining was obviously stained on cell polar lipids, the visual observation under light microscope could not differentiate between WT and overexpressing cells.

3.9 Total lipid and unsaturated lipid contents in *Synechocystis* sp. PCC 6803 wild type and overexpressing strains

The total lipid and unsaturated lipid contents under normal condition for 20 days of cultivation shown in Figure 3.23. The OE+C and OE+XC overexpressing strains significantly accumulated higher content of total lipid than wild type with the highest level at day 8 (Figure 3.23). The total amount of intracellular lipid content in log-phase growing cell (at day 8) of overexpressing strains under normal condition was in a range of about 20.3% w/dcw in OE+C strain and 24.3% w/dcw in OE+XC strain whereas their unsaturated lipid content was in a range of about 1.1% w/dcw in OE+C strain and 1.3 % w/dcw in OE+XC strain (Figure 3.23 A and B). However, those unsaturated lipid contents of two overexpressing strains were slightly higher than those of WT and control WT+pE in all days of cultivation (Figure 3.23B). The total lipid content was then 20 fold-higher than unsaturated lipid content.

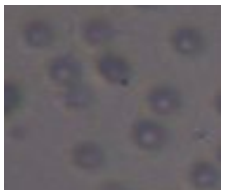
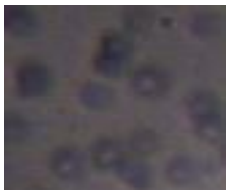
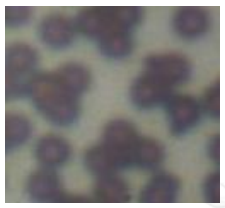
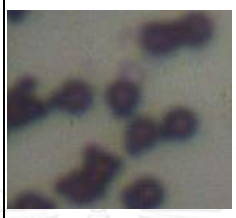
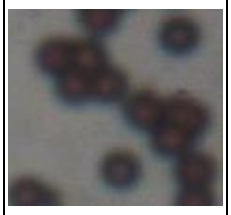
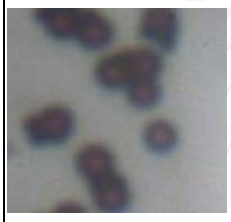
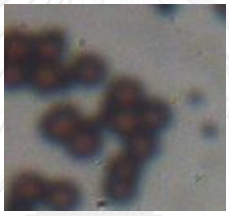
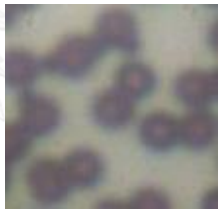
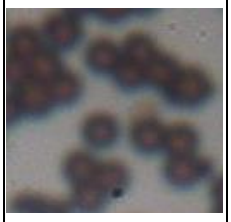
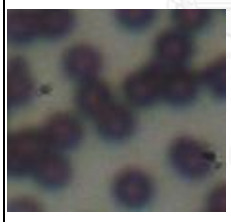
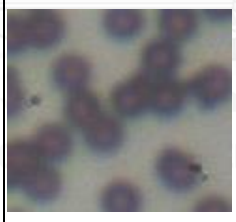
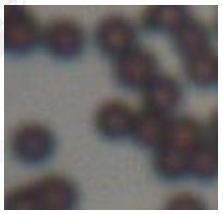
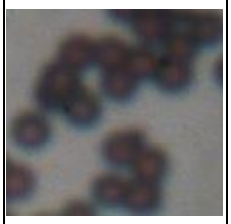
Conditions	WT	WT+pE	OE+C	OE+XC
BG_{11} medium (Start)				
BG_{11} medium (Day4)				
BG_{11}+0.4 % acetate (Day4)				
BG_{11}–N (Day4)				

Figure 3. 22 The Sudan black B staining of membrane lipids in *Synechocystis* sp. PCC 6803 wild type (WT), WT+pE, OE+C and OE+XC overexpressing strains. Cells grown in BG_{11} medium at start day as the control, BG_{11} medium, BG_{11} medium adding 0.4% acetate and BG_{11} medium with nitrogen deficiency, at day 4-treatment, respectively. The stained cells were visualized under light microscope with a magnification of 100X.

3.9.1 Total lipid contents under nutrient modified conditions

Log-phase cells were used to further treatments with nutrient-modified conditions consisting of BG_{11} medium as the control, 0.4% acetate supplementation and nitrogen deficiency. The total lipid contents under nutrient-modified conditions are shown in Figures 3.24-3.26. It was found that total lipid contents in all strains treated under BG_{11} medium were occurred at day 4-treatment in a range of about 24.3% w/dcw in OE+C strain and 27.9% w/dcw in OE+XC strain. Both OE+C and OE+XC overexpressing strains had a slight increase of total lipids at day 6 and day 8 of treatments. It was similarly in unsaturated lipid pattern with the highest levels found in day 4-treatment (Figure 3.24).

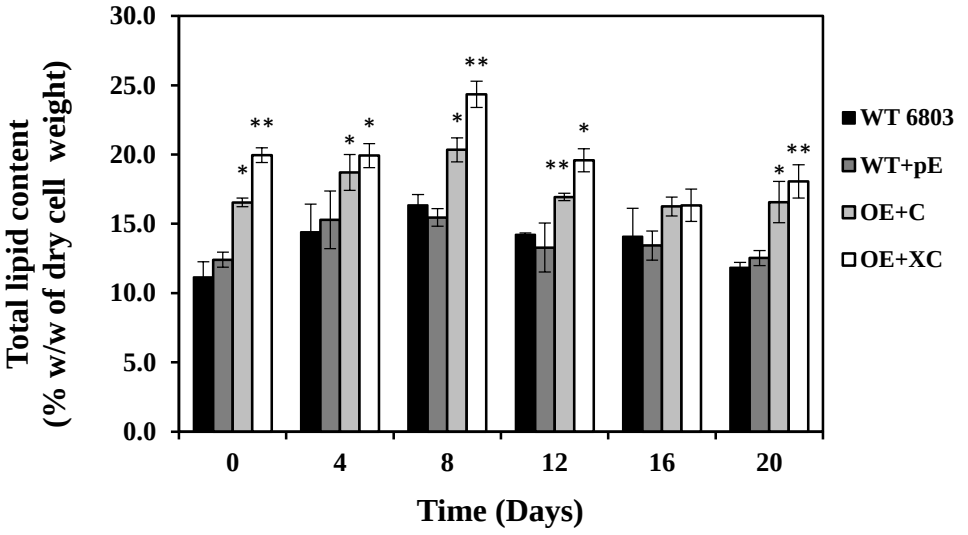
For 0.4% acetate supplementation, the OE+XC strain had the highest content of total lipids at day 4 of treatment of about 39% w/dcw (Figure 3.25A) whereas OE+C strain showed the highest content of total lipids of about 29.4% w/dcw. On the other hand, the high level of total lipid content was also observed in nitrogen deficiency condition at day 4 and day 6 of treatments when compared with WT. The OE+XC and OE+C strains gave the highest levels of about 32.6% w/dcw and 26.5% w/dcw at day 4 treatment, respectively (Figure 3.26A).

3.9.2 Unsaturated lipid content under nutrient modified conditions

Under control condition (Figure 3.24B), the total unsaturated lipid contents of OE+C and OE+XC strains were accumulated higher than WT and WT+pE strains. The unsaturated lipid content with the highest levels found in day 4-treatment in a range of about 1.2% w/dcw in OE+C strain and 1.5 %w/dcw in OE+XC strain. On the other hand, WT and control WT+pE showed gradual decrease of unsaturated lipid content after day 4-treatment (Figure 3.24 B).

Results of total unsaturated lipid contents under 0.4% acetate supplementation are shown in Figure 3.25B. The treatment with 0.4% acetate supplementation highly induced the accumulation of the unsaturated lipid contents in overexpressing strains. The OE+C had about 1.2% w/dcw at day 4-treatment whereas OE+XC had about 1.6% w/dcw at the same day of treatment. On the other hand, for nitrogen deprived condition increased the unsaturated lipid contents along treatment of all four strains (Figure 3.26B). The highest level of unsaturated lipid content was observed in day 8-treatment in overexpressing strains. Both OE+C and OE+XC gave the unsaturated lipid content higher than WT about 2.0% w/dcw and 2.4% w/dcw, respectively. In addition, results are indicated that nitrogen deficient significantly induced the highest accumulation of unsaturated lipid content among those treatments.

A



B

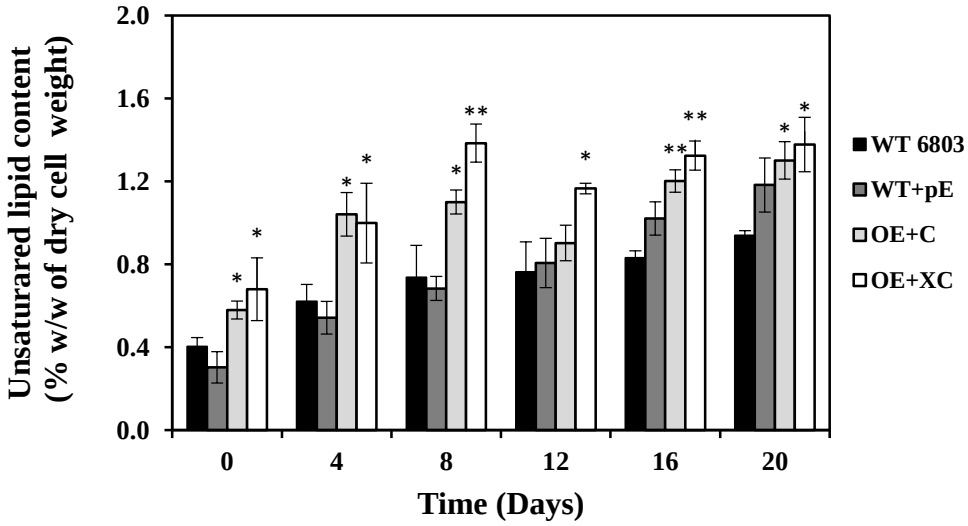
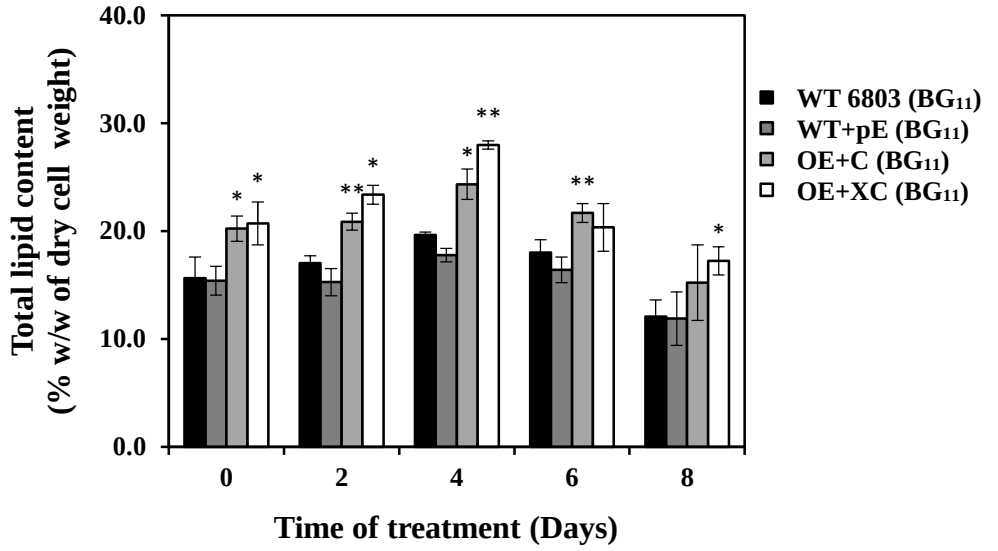


Figure 3. 23 Total lipid (A) and unsaturated lipid (B) contents in *Synechocystis* sp. PCC 6803 wild type (WT), control WT+pE and two overexpressed strains of OE+C and OE+XC. Cells grown in BG_{11} medium for 20 day. Data represent mean ± S.D., n=3 and significant levels of *P≤0.05 and **P≤ 0.01 compared with WT 6803 in each day.

A



B

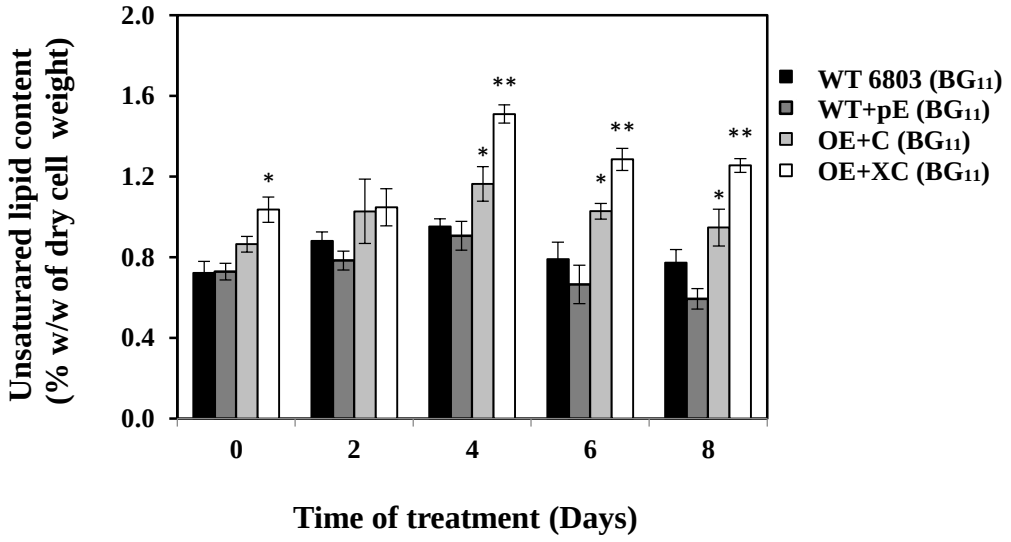
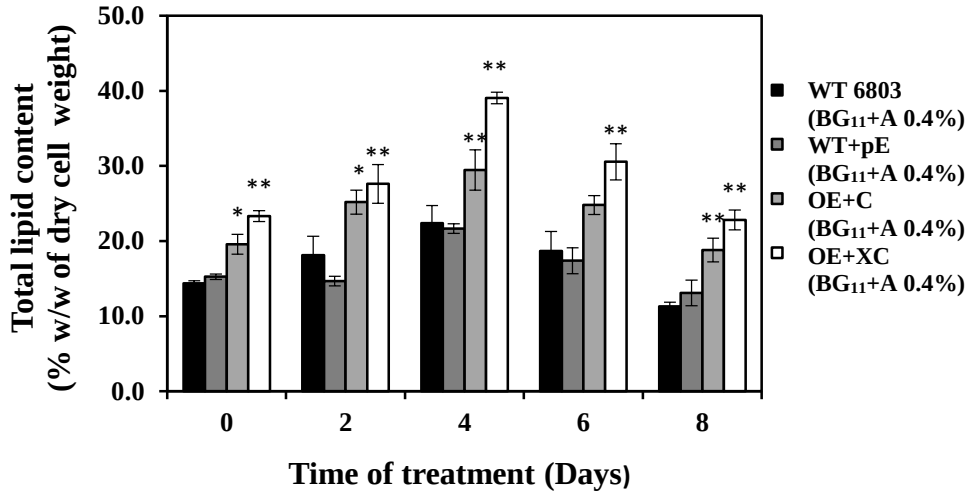


Figure 3. 24 Total lipid contents (A) and unsaturated lipid content (B) in *Synechocystis* sp. PCC 6803 wild type (WT), control WT+pE and two overexpressing strains. Cells grown in BG_{11} medium for 8 day. Data represent mean ± S.D., n=3 and significant levels of *P≤0.05 and **P≤ 0.01 when compared it with that of WT in the same condition.

A



B

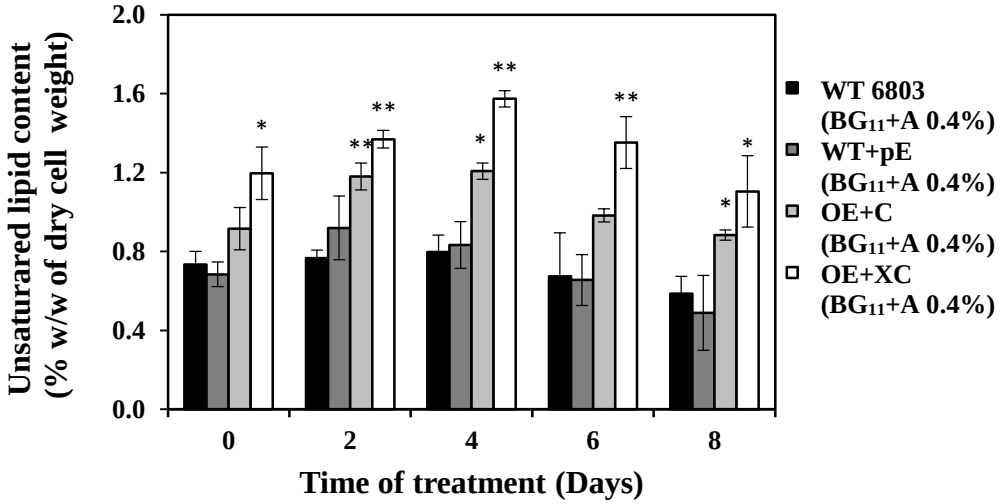
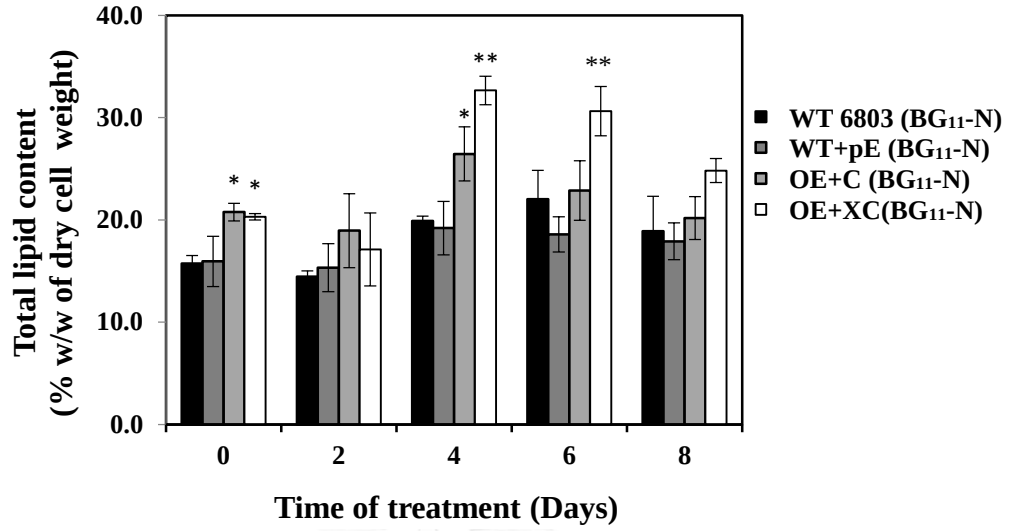


Figure 3. 25 Total lipid contents (A) and unsaturated lipid contents (B) in *Synechocystis* sp. PCC 6803 wild type (WT), control WT+pE and two overexpressing strains. Cells grown in BG_{11} medium adding 0.4% acetate for 8 day. Data represent mean ± S.D., n=3 and significant levels of *P≤0.05 and **P≤ 0.01 when compared it with that of WT in the same day of treatment.

A



B

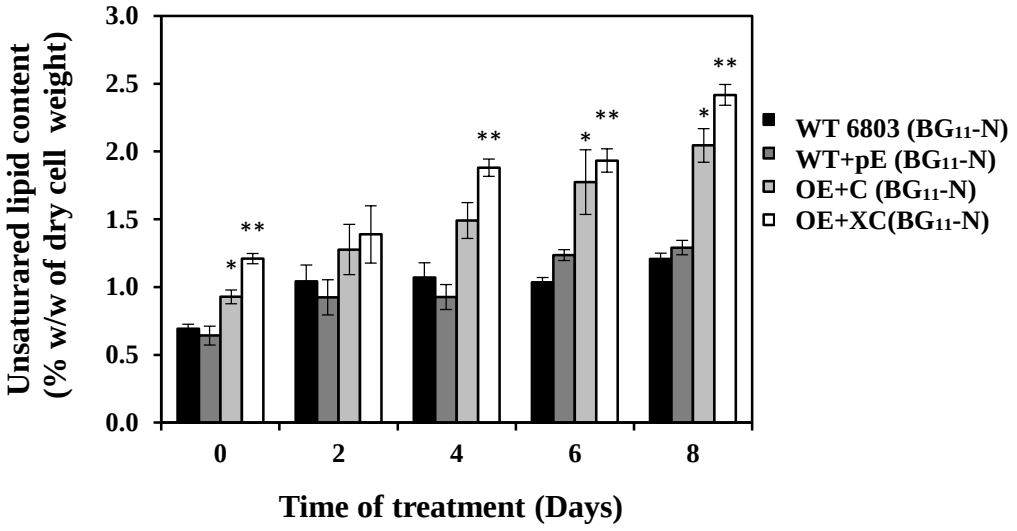


Figure 3. 26 Total lipid (A) and unsaturated lipid (B) contents in *Synechocystis* sp. PCC 6803 wild type (WT), control and two overexpressing strains of OE+C and OE+XC. Mid log phase cells were grown in BG_{11} medium with nitrogen deficiency for 8 day of treatment. Data represent mean ± S.D., n=3 and significant levels of *P≤0.05 and **P≤ 0.01 when compared it with that of WT in the same day of treatment.

3.10 Phylogenetic tree and sequence alignment of *sll1848* gene

The *sll1848* gene with consisted of 225 amino acid when submitted in Interproscan protein sequence analysis (Figure 3.27) showed that residue 42-170 similar to domain of phospholipid/glycerol acyltransferase and this family contains acyltransferases involved in phospholipid biosynthesis and domain 1-acyl-sn-glycerol-3-phosphate acyltransferase is also called 1-AGP acyltransferase, lysophosphatidic acid acyltransferase, and LPA acyltransferase which is to convert acyl-CoA and 1-acyl-sn-glycerol -3-phosphate to CoA and 1,2-diacyl-sn-glycerol 3-phosphate.

The multiple alignment and phylogenetic tree (Figure 3.28 and 3.29) from *Synechocystis* sp. PCC 6803 and other species consisted of cyanobacteria, algae, bacteria, fungus, plant and animal were performed using ClustalW2. The database for cyanobacteria from cyanobase and other species from NCBI with encode 1-acyl-sn-glycerol 3-phosphate acyltransferase (LPAAT or plsC) were analyzed in Table 3.1. The amino acid sequence encoded by *sll1848* gene were predicted in the same group of putative acyltransferase (Figure 3.28). The result showed that *sll1848* gene of *Synechosystis* sp. PCC 6803 was similar with unicellular cyanobacteria, such as *Synechosystis* sp. strain PCC 6714 with identity of 89%, *Synechococcus* sp. PCC 7002 with identity of 56.4%, *Synechococcus sp*. WH 8102 with identity of 42.4% (Figure 3.28). Moreover, the amino acid sequence of *sll1848* from *Synechosystis* sp. PCC 6803 has the identity percentages when aligned with algae in *Auxenochlorella protothecoides* with identity 25.0% and plant *Arabidopsis thaliana* (18.7%). Interestingly, the amino acid sequence of *sll1848* gene when aligned with bacteria such as *Bacillus subtilis* had identity about 23.6%.

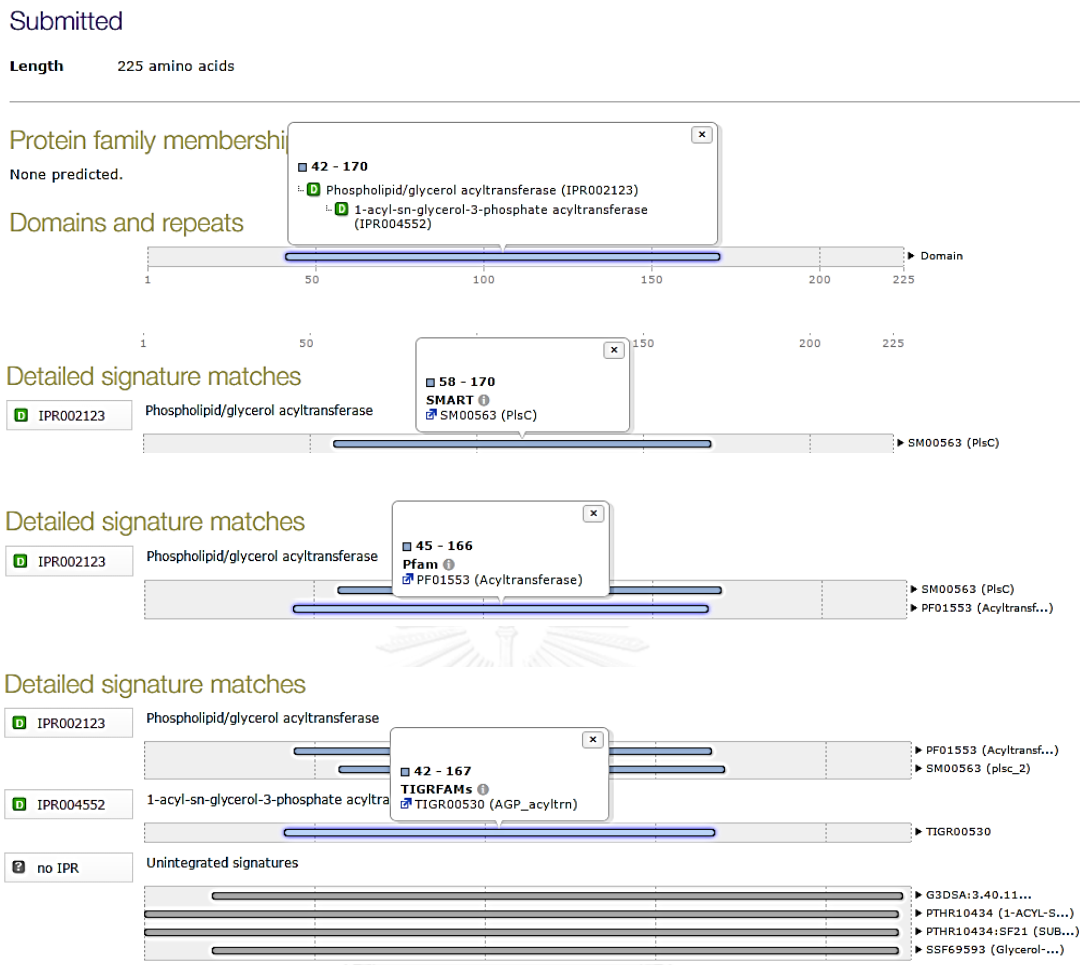


Figure 3. 27 Interproscan of amino acid sequence analysis of *sll1848* gene.

(http://www.ebi.ac.uk/interpro/search/sequence-search)

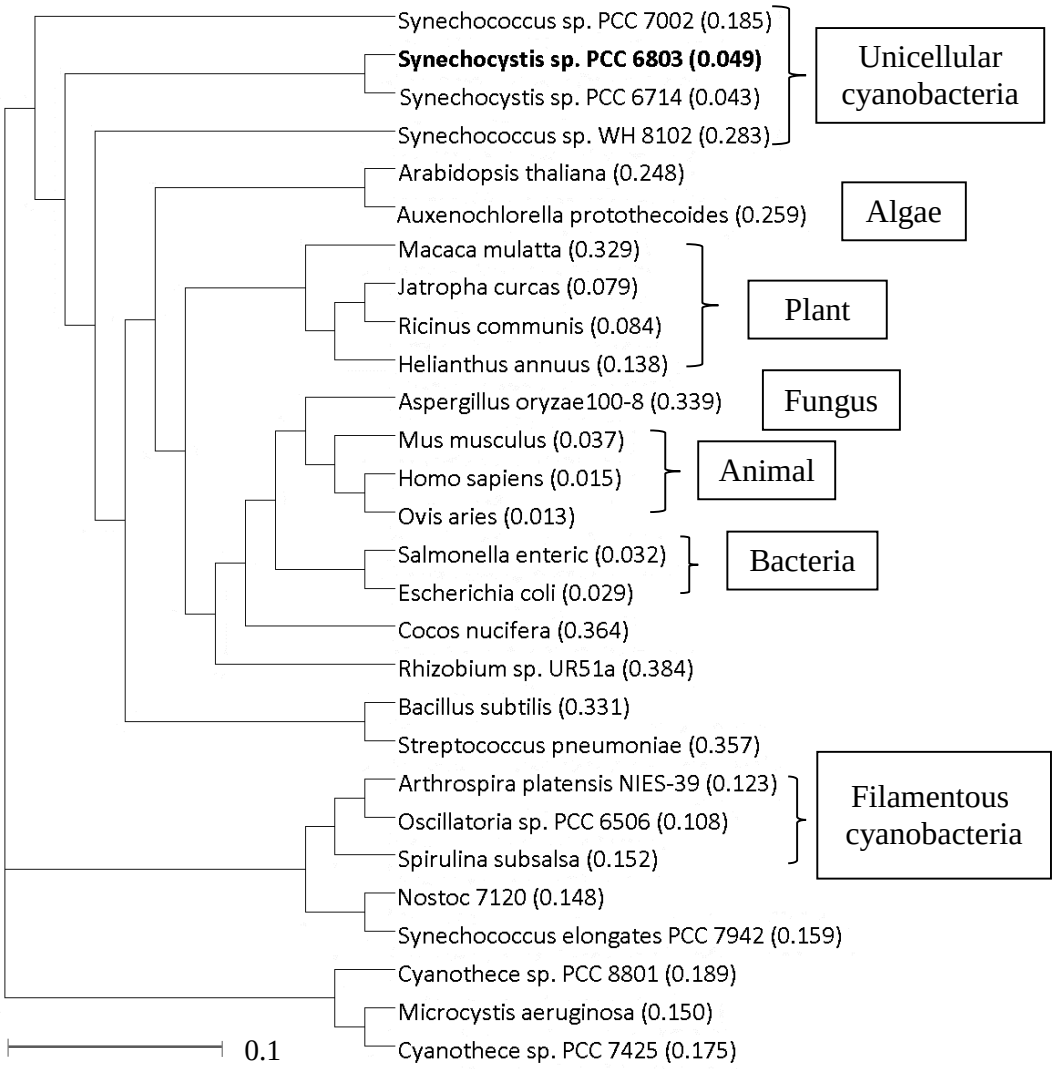


Figure 3. 28 Unrooted phylogenetic tree analysis of the amino acid sequences of 1-acyl-sn-glycerol-3-phosphate acyltransferase from 28 strains of cyanobacteria, and other known species (shown in Table 3.1).

Figure 3. 29 ClustalW2 amino acid alignment of *sll1848* (plsC) of *Synechocystis* sp. PCC 6803 and 1-acyl-sn-glycerol-3-phosphate acyltransferase in *Synechocystis* sp. PCC 6803 and other species.

Table 3. 1 Gene information of cyanobacteria and other species

No.	Accession	Organisms	Predicted as	Sources
		Cyanobacteria		
1	WP_010873224.1	*Synechocystis* sp. PCC 6803	1-acyl-sn-glycerol-3-phosphate acyltransferase	Cyanobase
2	WP_051738848.1	*Synechocystis* sp. PCC 6714	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
3	ACL46701.1	*Cyanothece* sp. PCC 7425	1-acyl-sn-glycerol-3-phosphate acyltransferase	Cyanobase
4	ACK65339.1	*Cyanothece* sp. PCC 8801	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
5	WP_002792013.1	*Microcystis aeruginosa*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
6	ABB57487.1	*Synechococcus elongates* PCC 7942	1-acyl-sn-glycerol-3-phosphate acyltransferase	Cyanobase
7	ACB00374.1	*Synechococcus sp.* PCC 7002	1-acyl-sn-glycerol-3-phosphate acyltransferase	Cyanobase
8	NP_898339	Synechococcus sp. WH 8102	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
9	WP_026080032.1	*Spirulina subsalsa*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
10	CBN57772.1	*Oscillatoria* sp. PCC 6506	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
11	BAI94386.1	*Arthrospira platensis* NIES-39	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
12	NP_484285	*Nostoc* sp. PCC 7120	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
		Algae		
13	KFM23593.1	*Auxenochlorella protothecoides*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
		Bacteria		
14	Q8DNY1.1	*Streptococcus pneumoniae*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
15	BAM49875.1	*Bacillus subtilis*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI

No.	Accession	Organisms	Predicted as	Sources
16	P0A257.1	*Salmonella enteric*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
17	P26647.1	*Escherichia coli*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
		Fungus		
18	KDE82468.1	*Aspergillus oryzae*100-8	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
19	WP_004439605.1	*Rhizobium* sp. UR51a	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
		Plants		
20	Q8GXU8.1	*Arabidopsis thaliana*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
21	ABU50327.1	*Helianthus annuus*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
22	ACC59198.1	*Ricinus communis*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
23	NP_001295696.1	*Jatropha curcas*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
24	Q42670.1	*Cocos nucifera*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
		Animals		
25	O35083.1	*Mus musculus*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
26	Q99943.2	*Homo sapiens*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
27	Q95JH0.1	*Ovis aries*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI
28	AFE79273.1	*Macaca mulatta*	1-acyl-sn-glycerol-3-phosphate acyltransferase	NCBI

CHAPTER IV

DISCUSSION

We identified *Synechocystis* sp. PCC 6803 *sll1848* gene by Interproscan program, multiple alignment and phylogenetic tree compared the annotated gene encoding putative 1-acyl-sn-glycerol-3-phosphate acyltransferase of other species including cyanobacteria, green algae, bacteria, fungi, plants and animals. The multiple alignment demonstrated that *sll1848* gene of *Synechocystis* was similar to predicted *PlsC* gene of *Synechosystis* sp. strain PCC 6714 with an identity of 89% and that of *Synechococcus* sp. PCC 7002 with an identity of 56.4%. On the other hand, it showed low identity with 1-acyl-sn-glycerol-3-phosphate acyltransferase or lysophosphatidic acid acyltransferase (LPAAT) in *Arabidopsis thaliana* plant of about 18.7 %. Recently, (Yu *et al.*, 2004) studies on phosphatidic acid biosynthesis in *Arabidopsis* plant with the multiple alignment of the ATS2 gene of *Arabidopsis* which encoded putative lysophosphatidic acid acyltransferase (LPAAT). They found that the ATS2 gene clustered with two plastidic LPAATs on a branch alongside the cyanobacterial cluster. ATS2 appears to present a highly conserved plastidic LPAAT. It shared 68% and 91% identities, respectively, with *Oryza sativa* and *Brassica napus* over 207 amino acids representing the domain aligning with the bacterial sequence encoded by *plsC.* In the course of de novo glycerol biosynthesis, this prokaryotic pattern is established by the sequential acylation of glycerol-3-phosphate. Previous research in 2005, identified the putative *Synechocystis* acyltransferases involved in glycerolipid metabolism. Their result annotated the functional expression of *sll1848* gene encoding lysophosphatidic acid acyltransferase (LPAAT) with a high specificity with fatty acid 16:0 ACP (Weier

et al., 2005). The *sll1848* gene was also reported to encode an indispensable palmitoyl –specific LPAAT (Okazaki *et al.*, 2006).

In this study, we successfully constructed two overexpressing strains of *Synechocystis* sp. PCC 6803, namely OX+C and OE+XC strains. Also, we demonstrated that two overexpressing strains were slightly decreased of growth when compared with WT. However, the acetate supplementation effectively enhanced growth of all strains. Moreover, the decrease of cell growth was observed under nitrogen-deprived condition (-N) of all strains. Similar results were shown in *Phaeodactylum tricornutum* cells that organic carbon sources of acetate addition significantly increased their specific growth rate. The addition of acetate significantly enhanced the maximal biomass concentrations of about 1.28 times in *P. tricornutum* (Liu *et al.*, 2009). Moreover, with the presence of acetate (1%, w/v) in dark, cell growth rates of *Chlorella vulgaris* were increased up to 7 times in 12 days of culture. On the other hand, the condition of nitrate deprivation could decrease the growth of *Chlorella vulgaris* but not to a significant extent when compared with that under nitrate presence (Liang *et al.*, 2009).

In this study, the chlorophyll *a* contents (Figure 3.10B) of control WT+pE and two overexpressing cells under normal BG_{11} medium condition were slightly decreased when compared to WT. On the other hand, there was no difference on carotenoid content in each strain under normal BG_{11} condition (Figure 3.10C). After treatment of 0.4% acetate supplementation, the significant increase of chlorophyll *a* content in all strains was observed (Figure 3.14 A-D). Under nitrogen deficiency, all four strains showed significant decreases on chlorophyll *a* contents. This result was coincident with previous report that addition of organic carbon sources resulted in significant decreases

of chlorophyll *a* and carotenoid contents in *Phaeodactylum tricornutum* (Liu *et al*., 2009).

Moreover, we also demonstrated the oxygen evolution rates of OE+C and OE+XC overexpressing strains. They did not significantly change when compared to WT. Our result confirms that the overexpressions of both *sll1848* and *slr1510* gene did not affect the efficiency of photosynthesis of *Synechocystis* cells. The BG_{11}-N condition significantly decreased their photosynthetic efficiency whereas a slight increase was occurred by BG_{11}+acetate (0.4%) condition. Previously, the study of *Phaeodactylum tricornutum* photosynthetic O_2 evolution rate under photoautotrophic and mixotrophic conditions was reported. The results showed that organic carbon sources (namely, acetate) decreased the net photosynthetic O_2 evolution of *P. tricornutum* but significantly increased their respiration rate (Liu *et al.*, 2009). In mixotrophic culture, cells could assimilate organic carbon sources to produce energy leading to a lesser dependence on light. Therefore, the saturation irradiance (Ik) was decreased in a mixotrophic culture. Likewise in *C. reinhardtii* cells, growth under the increased acetate concentrations reduced the photosynthetic CO_2 fixation and net O_2 evolution, without effects on respiration and PSII efficiency (Heifetz *et al.*, 2000). Moreover, acetate also repressed the activities of *rbcL* and *rbcScah-1* encoding Rubisco, and psbA encoding protein D1 (Kroymann *et al.*, 1995).

We demonstrated that the OE+C and OE+XC overexpressing strains accumulated higher contents of total lipid than wild type along normal culture under BG_{11} medium. After treatments with various nutrient-modified conditions, the 0.4% acetate condition significantly induced the total lipid content. In this study, it was found that the OE+XC strain had the highest accumulation of total lipid content under 0.4%

acetate supplementation of about 39 %W/DCW whereas OE+C strain had the total lipid content about 29.4% W/DCW when compared with WT. Coincidently, *Chlorella vulgaris* which grown on acetate both under darkness and light conditions gave higher levels of fatty acids. The lipids with rapid fatty acid turnover may be involved in the sequences of saturated and unsaturated fatty acid synthesis (Nichols *et al.*, 1967). Morever, *Chlorella pyrenoidosa* grown under mixotrophic condition in presence of sodium acetate showed almost six fold enhancement in biomass productivity and a remarkable 32 fold increment in lipid productivity when compared with those productions under autotrophic culture (Rai *et al.*, 2013). Similarly, studies on mixotrophy effect of *P. tricornutum* reported the maximum lipid productivity of 0.02 g m^{-3} d^{-1} on NaAc which was 1.7 fold of photoautotrophic control culture (Wang *et al.*, 2012). In this study, the acetate supplementation significantly increased higher lipid content in overexpressing strain compared with wild type cells

Moreover, the BG_{11}-N condition could also exert the OE+C and OE+XC overexpressing strains to accumulate higher lipid content which increased from 24 % to 26.5% W/DCW and 28 % to 32.6 %W/DCW at day 4 treatment, respectively, when compared with those under BG_{11} medium. The previous report of *Chlorella vulgaris* demonstrated the increased cellular lipid content from 33% to 38% due to nitrogen limitation (Liang *et al.*, 2009). In *Pseudochlorococcum* sp. grown under high light and nitrogen-limited conditions, starch synthesis was transiently up-regulated (Li *et al.*, 2011). After nitrogen depletion, starch content was decreased while neutral lipids were rapidly increased up to 52.1% of cell dry weight. Additionally, the nitrogen deficiency obviously affected on microalga lipid contents ranging from 20–30% to 60–70%

whereas their growth rate was greatly reduced resulting in the overall lipid productivity being unchanged (Benemann and Oswald, 1996).

In this study, we also showed that two overexpressing strains accumulated unsaturated lipid contents higher than WT and control WT+pE under BG_{11} medium. The unsaturated lipid content gave the highest accumulation at day 4-treatment with 0.4% acetate supplementation, as well as with nitrogen deficiency. Previously, the bacterium *Nitratireductor* sp. OM-1, which could produce lipids from lower organic acids in treated wastewater. That strain was proliferated in propionic, acetic, butyric or valeric acid-containing media, and primarily produced higher TAG content in media containing acetic and propionic acids or glycerol (Okamura *et al.*, 2016). Furthermore, the nutrient limitation caused the increased amounts of saturated fatty acids, monounsaturated, and diunsaturated fatty acids in *Chlamydomonas reinhardtii* (Weers and Gulati, 1997).

Moreover, in this study also showed the transcript level of genes involved in fatty acid biosynthetic pathway compared with both WT and control WT+pE strains. The result indicated that these WT and control WT+pE strains had no significant differences on transcription levels of *sll1848* (*plsC), slr1510 (plsX), AccA, Aas* and *lipA* mRNAs, except an up-regulated transcript level of *phaA* mRNA found in control WT+pE when compared to that of WT (pathway shown in Figure 1.3). On the other hand, the OE+C strain showed up-regulated level of *sll1848* mRNA of about 7 fold when compared to control WT+pE. Interestingly, the OE+XC strain showed the higher transcript levels of *sll1848* and *slr1510* mRNA of about 6 fold and 4 fold-increase, respectively. Therefore, double overexpression of *sll1848* and *slr1510* had an impact on gene expression in lipid biosynthetic pathway. Previously, the depletion strain of

PlsX (namely, Δ*plsX* mutant) was engineered and led to the cessation of both fatty acid synthesis and phospholipid synthesis in *Bacillus subtilis*. Moreover, the phospholipid synthesis was also ceased following PlsC depletion whereas fatty acid synthesis was continuously performed at a high rate, leading to the accumulation of fatty acids arising from the dephosphorylation of 1-acylglycerol-3-P followed by the deacylation of monoacylglycerol (Paoletti *et al.*, 2007).

Moreover, the expression level of *phaA* mRNA in control WT+pE in this study was enhanced about 1.2 fold under 0.4 % acetate supplementation when compared with control BG_{11} medium at day 4 treatment. Previously, *Synechocystis* cells pre-grown in glucose (0.1%)-supplemented BG_{11} medium when subjected to P-deficiency in presence of acetate (0.4%), PHB accumulation was boosted up to 29% (w/w of dry cells). Then, the nutrient-modified conditions, especially acetate supplementation and nitrogen deficiency, seemed to enhance PHB production rather than fatty acid production since they both used acetyl Co-A as a main substrate. However in OE+C strain of this study, the 0.4% acetate supplementation significantly induced the level of *sll1848* mRNA which boosted up to 10 fold when compared with that of control WT+pE. Additionally, this OE+C strain accumulated higher *sll1848* mRNA level of 1.27 fold, higher *lipA* mRNA level of 1.25 fold, and higher *slr1510* mRNA level of 2.2 fold when compared with that under BG_{11} medium (Figure 3.21). For OE+XC strain, both *sll1848* and *slr1510* mRNA levels were up-regulated about 7 fold and 3 fold, respectively, when compared with those of control WT+pE strain. For *AccA* and *LipA* mRNA levels, their expressions were slightly up-regulated except only *phaA* mRNA level with showing down-regulation. Interestingly, our results of the transcript levels of *sll1848* and *slr1510* genes clearly corresponded to the total lipid content in OE+XC

overexpressing strain, especially under 0.4% acetate addition (Figure 3.25). On the other hand, the BG_{11}-N condition obviously increased *phaA* mRNA levels of both WT and control WT+pE of about 5.8 fold and 3.2 fold increase, respectively, when compared with that at day 4 treatment of BG_{11} medium. The OE+C strain showed up-regulated level of *sll1848* mRNA whereas the OE+XC strain showed up-regulated levels of both *sll1848* and *slr1510* mRNAs. Previously, three *Chlamydomonas* genes encoding acyltransferases, *DGAT1*, *DGTT1*, and *PDAT1*, were induced by nitrogen starvation (Boyle *et al.*, 2012). In oleaginous green microalga *Pseudochlococcum* sp., it utilized starch as a primary carbon and energy storage under N-repleted conditions whereas the organism shifted the fixed carbon into neutral lipid as a secondary storage product under N-depleted conditions (Li *et al.*, 2011).

CHAPTER V

CONCLUSION

In this study, the single *sll1848*-overexpressing (OE+C) and double *slr1510/sll1848* overexpressing (OE+XC) strains of *Synechocystis* sp. PCC 6803 were successfully constructed. These two overexpressing strains showed insignificant changes on their growth, pigment contents and oxygen evolution rate. The total lipid contents of those engineering strains were significantly increased, in particular the maximum level of total lipid content in OE+XC strain under normal condition was 24.3% w/dcw whereas the maximum unsaturated lipid content was 1.3% w/dcw. Morever, the total lipid content could be enhanced by nutrient-modified condition. The nitrogen deficiency condition obviously increased total lipid contents of both OE+XC and OE+C strains to 32.7 and 26.5 % w/dcw, respectively, whereas unsaturated lipid contents of both strains were increased to 2.4 and 2.0 % w/dcw, respectively. Nevertheless, 0.4% acetate supplementation increased the total lipid content of OE+XC up to 39% w/dcw whereas its unsaturated lipid content was increased 1.6% w/dcw. On the other hand, OE+XC strain showed up-regulation of both *sll1848* and *slr1510* mRNA levels. In addition, *AccA* and *lipA* mRNA levels were slightly induced in OE+XC strain. Altogether, the enhancement of lipid production in *Synechocystis* sp. PCC 6803 was achieved by overexpressing both single *sll1848* and double *slr1510/sll1848* genes. The higher production of lipid content was also achieved by the growth-modified conditions on nitrogen deficiency and acetate supplementation. The phylogenetic profiling also confirmed that amino acid sequences of *plsC* or *sll1848* gene had genetic correlation with acyltransferase enzyme.

REFERENCES

Al-Hasan, R., Ali, A. and Radwan, S. (1989). "Effects of light and dark incubation on the lipid and fatty acid composition of marine cyanobacteria." Microbiology **135**(4): 865-872.

Allakhverdiev, S. I., Kinoshita, M., Inaba, M., Suzuki, I. and Murata, N. (2001). "Unsaturated fatty acids in membrane lipids protect the photosynthetic machinery against salt-induced damage in *Synechococcus*." Plant Physiology **125**(4): 1842-1853.

Allakhverdiev, S. I., Nishiyama, Y., Suzuki, I., Tasaka, Y. and Murata, N. (1999). "Genetic engineering of the unsaturation of fatty acids in membrane lipids alters the tolerance of *Synechocystis* to salt stress." Proceedings of the National Academy of Sciences **96**(10): 5862-5867.

Benemann, J. R. and Oswald, W. J. (1996). Systems and economic analysis of microalgae ponds for conversion of CO_2 to biomass. Final report, California University., Berkeley, CA (United States). Department of Civil and Environmental Engineering.

Boyle, N. R., Page, M. D., Liu, B., Blaby, I. K., Casero, D., Kropat, J., Cokus, S. J., Hong-Hermesdorf, A., Shaw, J. and Karpowicz, S. J. (2012). "Three acyltransferases and nitrogen-responsive regulator are implicated in nitrogen starvation-induced triacylglycerol accumulation in *Chlamydomonas*." Journal of Biological Chemistry **287**(19): 15811-15825.

Brown, A. P., Slabas, A. R. and Rafferty, J. B. (2009) Fatty acid biosynthesis in plants-metabolic pathways, structure and organization. Lipids in Photosynthesis 11-34

Chamovitz, D., Sandmann, G. and Hirschberg, J. (1993). "Molecular and biochemical characterization of herbicide-resistant mutants of cyanobacteria reveals that phytoene desaturation is a rate-limiting step in carotenoid biosynthesis." Journal of Biological Chemistry **268**(23): 17348-17353.

Cheng, Y.-S., Zheng, Y. and VanderGheynst, J. S. (2011). "Rapid quantitative analysis of lipids using a colorimetric method in a microplate format." Lipids **46**(1): 95-103.

Dorne, A.-J., Joyard, J. and Douce, R. (1990). "Do thylakoids really contain phosphatidylcholine?" Proceedings of the National Academy of Sciences **87**(1): 71-74.

Englund, E., Andersen-Ranberg, J., Miao, R., Hamberger, B. r. and Lindberg, P. (2015). "Metabolic engineering of *Synechocystis* sp. PCC 6803 for production of the plant diterpenoid manoyl oxide." ACS Synthetic Biology **4**(12): 1270-1278.

Fahy, E., Subramaniam, S., Murphy, R. C., Nishijima, M., Raetz, C. R., Shimizu, T., Spener, F., van Meer, G., Wakelam, M. J. and Dennis, E. A. (2009). "Update of the lipid maps comprehensive classification system for lipids." Journal of Lipid Research **50**: S9-S14.

Fales, F. W. (1971). "Evaluation of a spectrophotometric method for determination of total fecal lipid." Clinical Chemistry **17**(11): 1103-1108.

Golden, S. S. (1987). "Mutagenesis of cyanobacteria by classical and gene-transfer-based methods." Methods in Enzymology **167**: 714-727.

Gombos, Z., Kanervo, E., Tsvetkova, N., Sakamoto, T., Aro, E.-M. and Murata, N. (1997). "Genetic enhancement of the ability to tolerate photoinhibition by

introduction of unsaturated bonds into membrane glycerolipids." Plant Physiology **115**(2): 551-559.

Gombos, Z., Wada, H., Varkonyi, Z., Los, D. A. and Murata, N. (1996). "Characterization of the *Fad*12 mutant of *Synechocystis* that is defective in Δ12 acyl-lipid desaturase activity." Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism **1299**(1): 117-123.

Grigorieva, G. and Shestakov, S. (1982). "Transformation in the cyanobacterium *Synechocystis* sp. 6803." FEMS Microbiol Letters **13**(4): 367-370.

Hansmann, S. and Martin, W. (2000). "Phylogeny of 33 ribosomal and six other proteins encoded in an ancient gene cluster that is conserved across prokaryotic genomes: influence of excluding poorly alignable sites from analysis." International Journal of Systematic and Evolutionary Microbiology **50**(4): 1655-1663.

Harris, P. and James, A. (1969). "The effect of low temperatures on fatty acid biosynthesis in plants." Biochemical Journal **112**(3): 325-330.

Harwood, J. L. (1998). Membrane lipids in algae. Lipids in photosynthesis: structure, function and genetics, Springer**:** 53-64.

Heifetz, P. B., Förster, B., Osmond, C. B., Giles, L. J. and Boynton, J. E. (2000). "Effects of acetate on facultative autotrophy in *Chlamydomonas reinhardtii* assessed by photosynthetic measurements and stable isotope analyses." Plant Physiology **122**(4): 1439-1446.

Hu, H. and Gao, K. (2003). "Optimization of growth and fatty acid composition of a unicellular marine picoplankton *Nannochloropsis* sp. with enriched carbon sources." Biotechnology Letters **25**(5): 421-425.

Huflejt, M. E., Tremolieres, A., Pineau, B., Lang, J. K., Hatheway, J. and Packer, L. (1990). "Changes in membrane lipid composition during saline growth of the fresh water cyanobacterium *Synechococcus* 6311." Plant Physiology **94**(4): 1512-1521.

Ikeuchi, M. and Tabata, S. (2001). "*Synechocystis* sp. PCC 6803 a useful tool in the study of the genetics of cyanobacteria." Photosynthesis Research **70**(1): 73-83.

Jantaro, S., Mäenpää, P., Mulo, P. and Incharoensakdi, A. (2003). "Content and biosynthesis of polyamines in salt and osmotically stressed cells of *Synechocystis* sp. PCC 6803." FEMS Microbiology Letters **228**(1): 129-135.

Jeon, Y.-C., Cho, C.-W. and Yun, Y.-S. (2006). "Combined effects of light intensity and acetate concentration on the growth of unicellular microalga *Haematococcus pluvialis*." Enzyme and Microbial Technology **39**(3): 490-495.

Joyard, J., Teyssier, E., Miège, C., Berny-Seigneurin, D., Maréchal, E., Block, M. A., Dorne, A.-J., Rolland, N., Ajlani, G. and Douce, R. (1998). "The biochemical machinery of plastid envelope membranes." Plant Physiology **118**(3): 715-723.

Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M. and Sasamoto, S. (1996). "Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions." DNA Research **3**(3): 109-136.

Kessels, J. L. W. (1983). "On-the-fly optimization of data structures." Communications of the ACM **26**(11): 895-901.

Khomutov, G., Fry, I. V., Huflejt, M. E. and Packer, L. (1990). "Membrane lipid composition, fluidity, and surface charge changes in response to growth of the fresh water cyanobacterium *Synechococcus* 6311 under high salinity." Archives of Biochemistry and Biophysics **277**(2): 263-267.

Kroymann, J., Schneider, W. and Zetsche, K. (1995). "Opposite regulation of the copy number and the expression of plastid and mitochondrial genes by light and acetate in the green flagellate *Chlorogonium*." Plant Physiology **108**(4): 1641-1646.

Lehel, C., Los, D., Wada, H., Györgyei, J., Horvath, I., Kovacs, E., Murata, N. and Vigh, L. (1993). "A second groEL-like gene, organized in a groESL operon is present in the genome of *Synechocystis* sp. PCC 6803." Journal of Biological Chemistry **268**(3): 1799-1804.

Li, Y., Han, D., Sommerfeld, M. and Hu, Q. (2011). "Photosynthetic carbon partitioning and lipid production in the oleaginous microalga *Pseudochlorococcum* sp. (Chlorophyceae) under nitrogen-limited conditions." Bioresource Technology **102**(1): 123-129.

Liang, Y., Sarkany, N. and Cui, Y. (2009). "Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions." Biotechnology Letters **31**(7): 1043-1049.

Liu, X., Duan, S., Li, A., Xu, N., Cai, Z. and Hu, Z. (2009). "Effects of organic carbon sources on growth, photosynthesis, and respiration of *Phaeodactylum*

tricornutum." Journal of Applied Phycology **21**(2): 239-246.

Lu, Y.-J., Zhang, Y.-M., Grimes, K. D., Qi, J., Lee, R. E. and Rock, C. O. (2006). "Acyl-phosphates initiate membrane phospholipid synthesis in gram-positive pathogens." Molecular Cell **23**(5): 765-772.

Moran, R. (1982). "Formulae for determination of chlorophyllous pigments extracted with N, N-dimethylformamide." Plant Physiology **69**(6): 1376-1381.

Murata, N., Ishizaki-Nishizawa, O., Higashi, S., Hayashi, H., Tasaka, Y. and Nishida, I. (1992). "Genetically engineered alteration in the chilling sensitivity of plants." Nature **356**(6371): 710-713.

Murata, N. and Los, D. A. (1997). "Membrane fluidity and temperature perception." Plant Physiology **115**(3): 875.

Murata, N. and Nishida, I. (1987). "Lipids of blue-green algae (cyanobacteria)." The Biochemistry of Plants **9**: 315-347.

Murphy, D. J. (1993). "Structure, function and biogenesis of storage lipid bodies and oleosins in plants." Progress in Lipid Research **32**(3): 247-280.

Nichols, B., James, A. and Breuer, J. (1967). "Interrelationships between fatty acid biosynthesis and acyl-lipid synthesis in *Chlorella vulgaris*." Biochemical Journal **104**(2): 486.

Nishizuka, Y. (1992). "Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C." Science **258**(5082): 607-614.

Ohlrogge, J. and Browse, J. (1995). "Lipid biosynthesis." The Plant Cell **7**(7): 957.

Okamura, Y., Nakai, S., Ohkawachi, M., Suemitsu, M., Takahashi, H., Aki, T., Matsumura, Y., Tajima, T., Nakashimada, Y. and Matsumoto, M. (2016). "Isolation and characterization of bacterium producing lipid from short-chain

fatty acids." Bioresource Technology **201**: 215-221.

Okazaki, K., Sato, N., Tsuji, N., Tsuzuki, M. and Nishida, I. (2006). "The significance of C16 fatty acids in the sn-2 positions of glycerolipids in the photosynthetic growth of *Synechocystis* sp. PCC 6803." Plant Physiology **141**(2): 546-556.

Olie, J. J. and Potts, M. (1986). "Purification and biochemical analysis of the cytoplasmic membrane from the desiccation-tolerant cyanobacterium *Nostoc commune* UTEX 584." Applied and Environmental Microbiology **52**(4): 706-710.

Paoletti, L., Lu, Y.-J., Schujman, G. E., de Mendoza, D. and Rock, C. O. (2007). "Coupling of fatty acid and phospholipid synthesis in *Bacillus subtilis*." Journal of Bacteriology **189**(16): 5816-5824.

Quintana, N., Van der Kooy, F., Van de Rhee, M. D., Voshol, G. P. and Verpoorte, R. (2011). "Renewable energy from cyanobacteria: energy production optimization by metabolic pathway engineering." Applied Microbiology and Biotechnology **91**(3): 471-490.

Rai, M. P., Nigam, S. and Sharma, R. (2013). "Response of growth and fatty acid compositions of *Chlorella pyrenoidosa* under mixotrophic cultivation with acetate and glycerol for bioenergy application." Biomass and Bioenergy **58**: 251-257.

Rippka, R. and Herdman, H. (1992). "Pasteur culture collection of cyanobacteria: catalogue and taxonomic handbook. I." Catalogue of Strains **1**.

Ritter, D. and Yopp, J. H. (1993). "Plasma membrane lipid composition of the halophilic cyanobacterium *Aphanothece halophytica*." Archives of Microbiology **159**(5): 435-439.

Rock, I. and Gutman, D. (1981). "The effect of inattention on form perception." Journal of Experimental Psychology: Human Perception and Performance **7**(2): 275.

Saha, S. K., Uma, L. and Subramanian, G. (2003). "Nitrogen stress induced changes in the marine cyanobacterium *Oscillatoria willei* BDU 130511." FEMS Microbiology Ecology **45**(3): 263-272.

Sakamoto, T., Wada, H., Nishida, I., Ohmori, M. and Murata, N. (1994). "delta 9 Acyl-lipid desaturases of cyanobacteria. Molecular cloning and substrate specificities in terms of fatty acids, sn-positions, and polar head groups." Journal of Biological Chemistry **269**(41): 25576-25580.

Sakurai, I., Shen, J.-R., Leng, J., Ohashi, S., Kobayashi, M. and Wada, H. (2006). "Lipids in oxygen-evolving photosystem II complexes of cyanobacteria and higher plants." Journal of Biochemistry **140**(2): 201-209.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning, Cold spring harbor laboratory press New York.

Sato, N. and Murata, N. (1980). "Temperature shift-induced responses in lipids in the blue-green alga, *Anabaena variabilis*: the central role of diacylmonogalactosylglycerol in thermo-adaptation." Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism **619**(2): 353-366.

Sato, N. and Murata, N. (1981). "Studies on the temperature shift-induced desaturation of fatty acids in monogalactosyl diacylglycerol in the blue-green alga (cyanobacterium), *Anabaena variabilis*." Plant and Cell Physiology **22**(6): 1043-1050.

Sato, N. and Murata, N. (1982). "Lipid biosynthesis in the blue-green alga (cyanobacterium), *Anabaena variabilis* III. UDPglucose: diacylglycerol glucosyltransferase activity in vitro." Plant and Cell Physiology **23**(6): 1115-1120.

Sato, N. and Wada, H. (2009). Lipid biosynthesis and its regulation in cyanobacteria. Lipids in Photosynthesis, Springer**:** 157-177.

Spiegel, S., Cuvillier, O., Edsall, L. C., Kohama, T., Menzeleev, R., Olah, Z., Olivera, A., Pirianov, G., Thomas, D. M. and Tu, Z. (1998). "Sphingosine-1-phosphate in cell growth and cell deatha." Annals of the New York Academy of Sciences **845**(1): 11-18.

Spiegel, S. and Merrill, A. (1996). "Sphingolipid metabolism and cell growth regulation." The FASEB Journal **10**(12): 1388-1397.

Stanier, R., Kunisawa, R., Mandel, M. and Cohen-Bazire, G. (1971). "Purification and properties of unicellular blue-green algae (order *Chroococcales*)." Bacteriological Reviews **35**(2): 171.

Steinbüchel, A. (1991). Polyhydroxyalkanoic acids. Biomaterials, Springer**:** 123-213.

Steinbüchel, A. and Valentin, H. E. (1995). "Diversity of bacterial polyhydroxyalkanoic acids." FEMS Microbiology Letters **128**(3): 219-228.

Subramaniam, S., Fahy, E., Gupta, S., Sud, M., Byrnes, R. W., Cotter, D., Dinasarapu, A. R. and Maurya, M. R. (2011). "Bioinformatics and systems biology of the lipidome." Chemical Reviews **111**(10): 6452-6490.

Tasaka, Y., Gombos, Z., Nishiyama, Y., Mohanty, P., Ohba, T., Ohki, K. and Murata, N. (1996). "Targeted mutagenesis of acyl-lipid desaturases in *Synechocystis*:

evidence for the important roles of polyunsaturated membrane lipids in growth, respiration and photosynthesis." The EMBO Journal **15**(23): 6416.

Testerink, C. and Munnik, T. (2005). "Phosphatidic acid: a multifunctional stress signaling lipid in plants." Trends in Plant Science **10**(8): 368-375.

Upchurch, R. G. (2008). "Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress." Biotechnology Letters **30**(6): 967-977.

Vigh, L., Los, D. A., Horvath, I. and Murata, N. (1993). "The primary signal in the biological perception of temperature: Pd-catalyzed hydrogenation of membrane lipids stimulated the expression of the *desA* gene in *Synechocystis* PCC 6803." Proceedings of the National Academy of Sciences **90**(19): 9090-9094.

Voet, D. and Voet, J. (1990). "Lipid metabolism." Biochemistry: 618-677.

Wada, H., Gombos, Z. and Murata, N. (1990). "Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation." Nature **347**(6289): 200-203.

Wada, H. and Murata, N. (1989). "*Synechocystis* PCC 6803 mutants defective in desaturation of fatty acids." Plant and Cell Physiology **30**(7): 971-978.

Wada, H. and Murata, N. (1990). "Temperature-induced changes in the fatty acid composition of the cyanobacterium *Synechocystis* PCC 6803." Plant Physiology **92**(4): 1062-1069.

Wada, H. and Murata, N. (1998). Membrane lipids in cyanobacteria. Lipids in Photosynthesis: Structure, function and genetics, Springer**:** 65-81.

Wang, H., Fu, R. and Pei, G. (2012). "A study on lipid production of the mixotrophic microalgae *Phaeodactylum tricornutum* on various carbon sources." African

Journal of Microbiology Research **6**(5): 1041-1047.

Weers, P. M. and Gulati, R. D. (1997). "Growth and reproduction of *Daphnia galeata* in response to changes in fatty acids, phosphorus, and nitrogen in *Chlamydomonas reinhardtii*." Limnology and Oceanography **42**(7): 1584-1589.

Wei, Y.-H., Chen, W.-C., Wu, H.-S. and Janarthanan, O.-M. (2011). "Biodegradable and biocompatible biomaterial, polyhydroxybutyrate, produced by an indigenous *Vibrio* sp. BM-1 isolated from marine environment." Marine Drugs **9**(4): 615-624.

Weier, D., Müller, C., Gaspers, C. and Frentzen, M. (2005). "Characterisation of acyltransferases from *Synechocystis* sp. PCC 6803." Biochemical and Biophysical Research Communications **334**(4): 1127-1134.

Williams, J. G. (1988). "Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in *Synechocystis* 6803." Methods in Enzymology **167**: 766-778.

Wymann, M. P. and Schneiter, R. (2008). "Lipid signalling in disease." Nature Reviews Molecular Cell Biology **9**(2): 162-176.

Xu, H., Miao, X. and Wu, Q. (2006). "High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters." Journal of Biotechnology **126**(4): 499-507.

Yasui, K. and Palade, P. (1996). "Sphingolipid actions on sodium and calcium currents of rat ventricular myocytes." American Journal of Physiology-Cell Physiology **270**(2): 645-649.

Yu, B., Wakao, S., Fan, J. and Benning, C. (2004). "Loss of plastidic lysophosphatidic acid acyltransferase causes embryo-lethality in *Arabidopsis*." Plant and Cell Physiology **45**(5): 503-510.

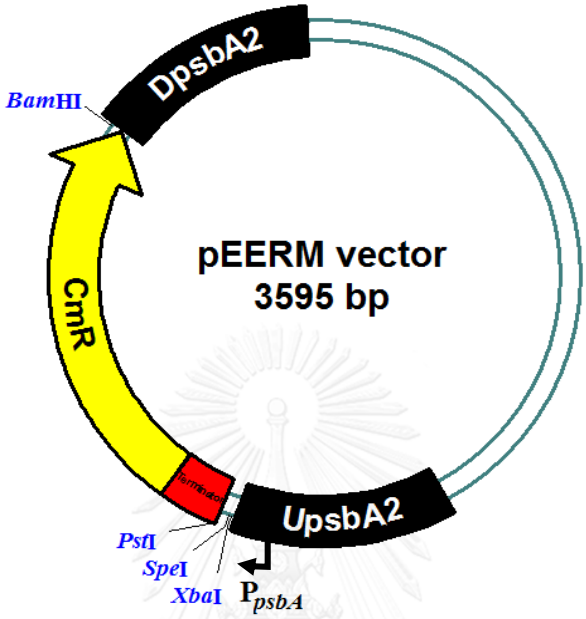
Yu, Y., You, L., Liu, D., Hollinshead, W., Tang, Y. J. and Zhang, F. (2013). "Development of *Synechocystis* sp. PCC 6803 as a phototrophic cell factory." Marine Drugs **11**(8): 2894-2916.

Zhang, Y.-M. and Rock, C. O. (2008). "Membrane lipid homeostasis in bacteria." Nature Reviews Microbiology **6**(3): 222-233.

APPENDICES

APPENDIX A

- **pEERM vector**



pEERM vector was used for overexpression gene in *Synechocystis* sp. PCC 6803 because it was created for useful integration into *Synechocystis* genome at photosyntem II, D1 protein region (*psbA2*). The physical map of pEERM vector containing strong promoter (P_{psbA}), selective chloramphenicol antibiotic resistance cassette gene and multiple cloning site; *Xba*I, *Spe*I and *Pst*I. The size of pEERM vector is 3,595 bp.

APPENDIX B

BG_{11} medium

Nutrients	Stock solution (1 L)	BG_{11} medium (1 L)	
		Liquid medium	Solid medium
KH_2PO_4	30 g	1 ml	1 ml
$MgSO_4.7H_2O$	75 g	1 ml	1 ml
$CaCl_2.2H_2O$	36g	1 ml	1 ml
$NaCO_3$	20 g	1 ml	1 ml
Citric acid	6 g	1 ml	1 ml
Ferric ammonium citrate	6 g	1 ml	1 ml
EDTA+Na_2O_3	1 g	1 ml	1 ml
Trace element A5	See below	1 ml	1 ml
$NaNO_3$	150 g	10 ml	10 ml
HEPES	238.3 g	10 ml	10 ml
30%$Na_2S_2O_3$+5·H_2O	30 g	-	10 ml
Bacto-agar	-	-	15 g
H_2O added up to	-	1000 ml	1000 ml

*** Trace element solution (1 L)

H_3BO_3	2.86 g	$CuSO_4.5H_2O$	0.079 g
$ZnSO_4.7H_2O$	0.22 g	$Co(NO_3)_2.6H_2O$	0.049 mg
$Na_2MoO_4.2H_2O$	0.39 g		
$MnCl_2.4H_2O$	1.81 g		

-Nitrogen modification from normal medium (BG_{11})

- Without $NaNO_3$ from BG_{11} medium
- Ferric ammonium citrate was replaced by $FeSO_4$ 6 g/L

-0.4% acetate supplementation

- Added Na-acetate 5.5 g/L

APPENDIX C

TAE buffer

1. Concentrate stock solution (1L)

50X :	Tris- base	240.0 g
	Glacial acetic acid	51.1 ml
	0.5 M EDTA, pH 8.0	100 ml

Added distilled water to make 1 liter.

2. Working solution

1X : 0.04 M Tris-acetate

0.01 M EDTA

APPENDIX D

Agarose gel electrophoresis for DNA

Preparation of agarose gel:

1. The edges of a clean, dry. Glass was sealed plate and then molded on a horizontal section of the bench
2. TAE electrophoresis buffer (100 ml) was prepared.
3. 1 g of agarose was weighed and put into the TAE buffer.
4. The mixture was boiled for 2-3 minute with microwave.
5. Ethidium bromide (a stock solution of 1 mg/ml) was added into gel solution by adjusting final concentration to 0.5 µg/ml and then mixed.
6. The comb was placed in suitable position.
7. Agarose solution was poured onto the tray.
8. After gel was completely set (30-45 min at room temperature), the comb was removed carefully.
9. The gel was placed into the electrophoresis tank.

APPENDIX E

LB medium

LB medium, composition per 1 L

Nutrients composition	Liquid medium	Solid medium
Bacto tryptone	10 g	10 g
NaCl	10 g	10 g
Yeast extract	5 g	5 g
Agar	-	15 g

Added distilled water to a total volume of 1 L. The medium was sterilized by autoclaving at 120 °C for 15 minute.

APPENDIX F

1. TfBI solution (500 ml)

Potassium acetate	1.47 g
$MnCl_2$	4.95 g
$CaCl_2$	0.74 g
Glycerol	75 ml

Added distilled water to make 500 ml, pH 5.8 store at 4℃.

2. TfBII solution (100 ml)

100 mM MOPS, pH 7.0	10 ml
$CaCl_2$	1.10 g
RbCl	0.12 g
Glycerol	15 ml

Added distilled water to make 100 ml, store at 4℃.

APPENDIX G

DNA digestion

1. Combine the reaction components by following below

Reaction components	Plasmid DNA	PCR product
Water, nuclease-free	15µL	17µL
10X restriction enzyme buffer	2µL	2µL
DNA	2µL (up to 1µg)	10µL (~0.2 µg)
Restriction enzyme	1µL(1U)	1µL(1U)
Total volume	20µL	30µL

2. Mix gently and spin down
3. Incubate at appropriate temperature of each restriction enzyme for optimum time
4. Inactivate the enzyme by heating of 10 min at 80 ℃ or depends on the enzyme recommendation
5. Double and multiple digestion of DNA are performed by using 1 µl of each enzyme and scale up the reaction condition appropriately. Using the proper buffer. If the enzyme require different reaction temperature, start with the enyme that required a lower temperature. Then add the second enzyme and incubate at the higher temperature.

APPENDIX H

- **Sudan black B solution**

  Sudan black B 3 g

  Added 70 % ethanol to a total volume of 100 ml.

- **Safranin O solution**

  Safranin O 5 g

  Added distilled water to a total volume of 100 ml.

- **Vanillin –phosphoric acid reagent**

  -Vanillin 100 ml

  Vanillin 20 g

  Added distilled water to a total volume of 100 ml.

  -17% phosphoric acid 100 ml

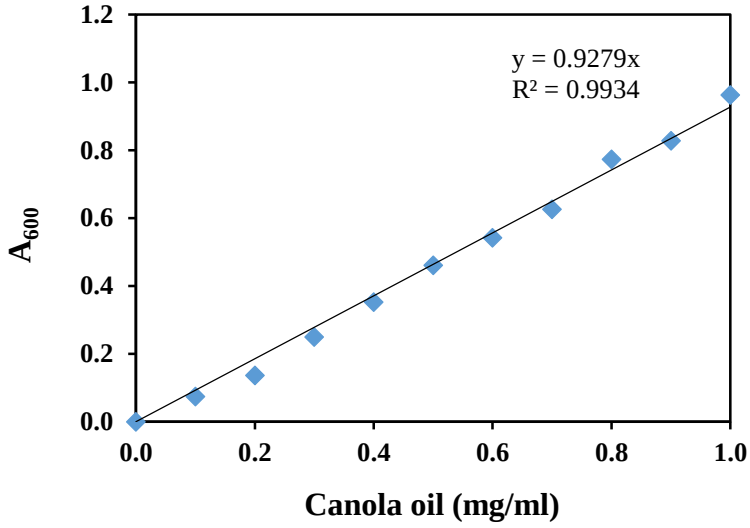
  Phosphoric acid 20 ml

  Added distilled water to a total volume of 100 ml.

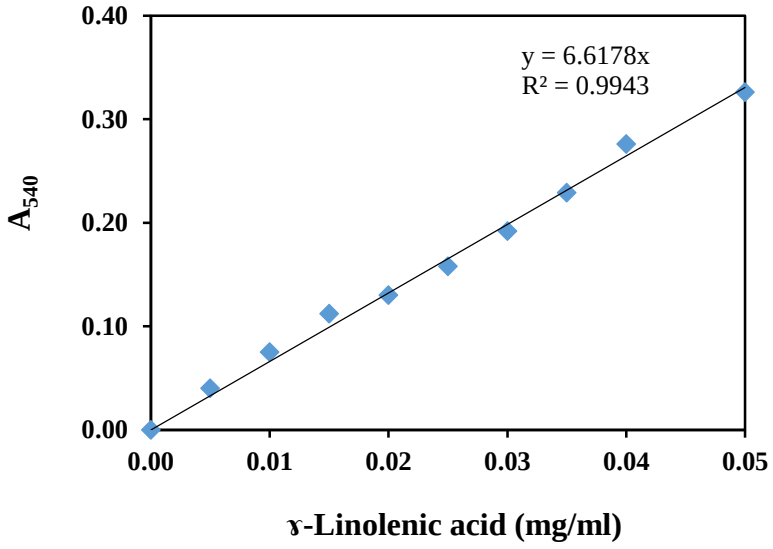
  The mixture volume contained 0.2 mg vanillin per ml 17 % phosphoric acid.

APPENDIX I

1. Standard curve for total lipid content using canola oil as standard.



2. Standard curve for total unsaturated lipid content using γ -Linolinic acid (18:3) as a standard.



VITA

Miss Umaporn Towijit was born on August 17, 1990 in Angthong, Thailand. She was graduated with a Bachelor of Science degree in Biotechnology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok in 2013. She has studied for M.Sc. (Program in Biotechnology) at Department of Biochemistry, Faculty of Science, Chulalongkorn University since 2013.

She was Teaching Assistant (TA) for General Biochemistry Laboratory in Biochemistry, Faculty of Science, Chulalongkorn University (2014-2015).

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