ปริมาณพอลิไฮดรอกซีบิวทิเรตในสายพันธุ์ปกติและสายพันธุ์กลายยีน *adc1*
ของไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803

นางสาวสุฐิรา อุทาน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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POLYHYDROXYBUTYRATE CONTENTS IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803 WILD TYPE AND *adc1* MUTANT

Miss Suthira Utharn

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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Thesis Title	POLYHYDROXYBUTYRATE CONTENTS IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803 WILD TYPE AND *adc1* MUTANT
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สุฐิรา อุทาน : ปริมาณพอลิไฮดรอกซีบิวทิเรตในสายพันธุ์ปกติและสายพันธุ์กลายยีน *adc1* ของไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 (POLYHYDROXYBUTYRATE CONTENTS IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803 WILD TYPE AND *adc1* MUTANT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. เสาวรัตน์ จันทะโร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. อรัญ อินเจริญศักดิ์, 97 หน้า.

ในการศึกษานี้ ได้ใช้เซลล์ไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 สายพันธุ์ปกติ (WT) และสายพันธุ์กลายยีน *adc1* (*Δadc1*) สำหรับการผลิต PHB ภายใต้สภาวะที่หลากหลายของการขาดสารอาหารและการเสริมแหล่งคาร์บอน นอกจากนี้ ยังศึกษาผลของภาวะเครียดจากรังสีอัลตราไวโอเลต (UV) ต่อการผลิต PHB ของเซลล์ WT การเพาะเลี้ยงเซลล์ของทั้งสองสายพันธุ์ที่การเพาะเลี้ยง 15 วัน ซึ่งระบุเป็นระยะปลายของช่วงล็อก ผ่านการเก็บเกี่ยวและถ่ายโอนไปยังอาหารสูตร BG_{11} ที่ขาดไนโตรเจนและฟอสฟอรัส (BG_{11}-N-P) นอกจากนี้ ยังมีการเสริมของแหล่งคาร์บอน รวมถึง อะซีเทต (A) และ กลูโคส (G) เติมใน BG_{11}-N-P สำหรับเซลล์ซึ่งถูกทดสอบ กล่าวคือ BG_{11}-N-P+A และ BG_{11}-N-P+G ตามลำดับ การเจริญของ WT และสายพันธุ์กลายภายใต้สภาวะซึ่งดัดแปลงสารอาหารทั้งหมดลดลงอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับเซลล์ที่เจริญในสภาวะ BG_{11} ปกติ สำหรับรงควัตถุภายในเซลล์ของทั้งสองสายพันธุ์ลดลงอย่างมีนัยสำคัญเช่นกัน อย่างไรก็ตาม การเติมของอะซีเทตใน BG_{11}-N-P +A เพิ่มรงควัตถุภายในเซลล์อย่างมีนัยสำคัญเปรียบเทียบกับภายใต้สภาวะ BG_{11}-N-P ขณะที่การเติมกลูโคสไม่พบการเพิ่มของรงควัตถุ นอกจากนี้ การเจริญของเซลล์และรงควัตถุภายในเซลล์ลดลงอย่างเห็นได้ชัดโดยการได้รับรังสียูวี โดยเฉพาะอย่างยิ่ง การฉายรังสียูวี-ซี ในทางกลับกัน เซลล์ที่ย้อม Nile-red แสดงการสะสมที่กระจายทั่วของแกรนูล PHB ภายใต้สูตรอาหาร BG_{11}-N-P+A ทั้งใน WT และสายพันธุ์กลาย *Δadc1* ปริมาณ PHB ที่สูงสุดของ WT ได้มาในเชิงปริมาณภายใต้สูตรอาหาร BG_{11}-N+A ณ วันที่ 9 ของการทดลอง ด้วยค่าเฉลี่ย 24.95 ± 18.31% PHB/DCW ขณะที่สายพันธุ์กลาย *Δadc1* ให้ปริมาณที่สูงสุดประมาณ 36.07 ± 9.24% PHB/DCW ณ วันที่ 7 ของการทดลอง นอกจากนั้น เซลล์ WT ที่ทดสอบโดยการฉายรังสียูวี-ซี เพิ่มปริมาณ PHB อย่างมีนัยสำคัญที่การทดสอบ 6 ชั่วโมง ประมาณ 13.69 ± 3.09 % PHB/DCW เปรียบเทียบกับภายใต้แสงปกติ ในทางกลับกัน ยีนที่เกี่ยวข้องกับวิถีชีวสังเคราะห์ PHB รวมถึง *phaA, phaB, phaE* และ *phaC* ตรวจสอบโดย RT-PCR พบว่าระดับทรานสคริปต์ของ *phaC* เพิ่มขึ้นในทุกสภาวะที่ดัดแปลงสารอาหารของทั้งสองสายพันธุ์ เปรียบเทียบกับระดับเหล่านั้นของยีน *pha* ตัวอื่น จากภาพรวมทั้งหมด ผลบ่งชี้ว่าปริมาณ PHB ที่เพิ่มขึ้นได้รับการเหนี่ยวนำเป็นหลักจากอาหารที่ขาด-N และ -P ที่ประกอบด้วย อะซีเทต 0.4% (w/v) ใน WT และสายพันธุ์กลายยีน *adc1* ภาวะเครียดจากรังสียูวี โดยเฉพาะ รังสียูวี-ซี เพิ่มปริมาณ PHB ด้วยเช่นกัน ในเซลล์ WT

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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SUTHIRA UTHARN: POLYHYDROXYBUTYRATE CONTENTS IN CYANOBACTERIUM *Synechocystis* sp. PCC 6803 WILD TYPE AND *adc1* MUTANT. ADVISOR: ASST. PROF. SAOWARATH JANTARO, Ph.D., CO-ADVISOR: PROF. ARAN INCHAROENSAKDI, Ph.D., 97 pp.

In this study, cyanobacterium *Synechocystis* sp. PCC 6803 wild type (WT) and *adc1* gene mutant cells (*Δadc1*) were used for PHB production under nutrient deficiencies and carbon source supplementation. Moreover, the effect of ultraviolet (UV) stress on PHB production of WT cells were also investigated. Cell cultures of both strains at late-log phase were harvested and transferred to nitrogen and phosphorus deficient BG_{11} medium (BG_{11}-N-P). Carbon sources including acetate (A) and glucose (G), was also added in BG_{11}-N-P for treated cells, namely BG_{11}-N-P+A and BG_{11}-N-P+G, respectively. Cell growths of WT and *Δadc1* under all nutrient modified conditions were significantly decreased when compared with cells grown under normal BG_{11} condition. For intracellular pigments of both strains were significantly decreased. However, the addition of acetate in BG_{11}-N-P+A significantly enhanced the intracellular pigments compared with those under BG_{11}-N-P condition, whereas glucose addition did not. Furthermore, cell growth and intracellular pigments were obviously decreased by UV exposure, especially UV-C radiation. Additionally, Nile-red stained cells showed widespread accumulation of PHB granules under BG_{11}-N-P+A in both WT and *Δadc1*. The highest PHB content of WT was quantitatively obtained under BG_{11}-N-P+A condition at 9 day-treatment with the average value of 24.95 ± 18.3 %PHB/DCW whereas *Δadc1* gave the highest amount of about 36.07 ± 9.24 %PHB/DCW at 7 day-treatment. Furthermore, WT cells treated by UV-C radiation significantly increased PHB content at 6 hour-treatment of about 13.69 ± 3.09 %PHB/DCW compared with cells treated by normal light. Moreover, genes related to PHB biosynthetic pathway including *phaA*, *phaB*, *phaE* and *phaC* were determined by RT-PCR. The *phaC* transcript level was increased by all nutrient modified conditions of both strains compared to those levels of other *pha* genes. Altogether, the results indicate that the increased PHB content was mainly induced by N- and P-deficient medium containing 0.4% (w/v) acetate condition in WT and *adc1* mutant. The UV stress, in particular, UV-C, also enhanced PHB content in WT cells.

Field of Study: Biotechnology
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

ADC	Arginine decarboxylase
bp	Base pair
^{o}C	Degree celsius
EDTA	Ethylene diamine tetraacetic acid
g	Gram
hrs	Hour
KCL	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
L	Liter
min	Minute
mL	Milliliter
mg	Milligram
mM	Millimolar
µg	Microgram
µL	Microliter
nm	Nanometer

$NaNO_3$	Sodium nitrate
OD	Optical density
ODC	Ornithine decarboxylase
PCR	Polymerase chain reaction
PHB	Polyhydroxybutyrate
RT-PCR	Reverse transcription polymerase chain reaction
TAE	Tris-acetate-EDTA
T_g	Transition temperature
T_m	Melting temperature

CHAPTER I

Introduction

On the 20th year, plastics gain more roles of function than the previous applications (Panda et al., 2006). Their common materials are useful in daily life, such as beverage containers, toys, furniture, soft drink bottles, lids, bottles, appliances, bags, cups and utensils and medical devices. Recently, environmental problems of petrochemical derived plastic materials were concerned mainly from harmful waste pollution (Reddy et al., 2003). In order to find replaced materials, many researchers were interested and developed on biodegradable plastics, such as polyhydroxyalkanoates (PHAs) (Verlinden et al., 2007). It is environmentally compatible and able to be decomposed by nature microorganisms (Liebergesell et al., 1994, Peña et al., 2011). However, the wide use of the biological production of PHB in bacteria are limited by high costs compared to widely employed petroleum derived plastics (Balaji et al., 2013). Cyanobacteria can be considered as sustainable and alternative sources of PHB production due to their photoautotrophic nature, capacity to fix CO_2 and minimal nutrient consumption.

1.1 Poly β-hydroxybutyrate (PHB)

Polyhydroxyalkanoates (PHAs) (Figure 1) are polyesters that accumulate as inclusions in an extensive array of bacteria (Poirier et al., 1995). They are able to made into plastic materials which are similar to petrochemical derived plastics and can replace these materials in wider applications (Nishioka et al., 2001).

Poly β-hydroxybutyrate or P(3HB) or PHB (Figure 2) is the most common type of PHAs (Madison and Huisman 1999, Wu et al., 2002). PHB is widely spread stored in granule typically found in prokaryotic organisms (Liebergesell et al., 1994), such as *Alcaligenes eutrophus* (Anderson and Dawes, 1990), *Ralstonia eutropha* (Kichise et al., 1999). The important properties of pure PHB are resistance to water, thermoplastic ability and complete biodegradability (Hrabak 1992, Lee 1996, Panda et al., 2006). Actually, it is efficiently produced not only from bacteria and heterotrophic bacteria, but also from many organisms, such as algae and cyanobacteria (Hein et al., 1998, Tokiwa et al., 2009). The properties of PHB compared with polypropylene (PP) are shown in Table 1. The PHB structure is mainly as similar as polypropylene structure (Marchessault and Yu, 2005). From Table 1, properties were compared between PHB and PP including melting temperature of both type were similar. The glass transition temperature of PHB plastics was higher than that of PP which indicated that PHB can easily change some features, such as density and young's modulus. PHB had higher crystalline form than PP but it was broken easier than PP. Moreover, the co-polymers of PHB, such as poly(3-hydroxyhexanoate (PHHx) as shown in Table 2, had better properties in terms of thermo-durable and strength.

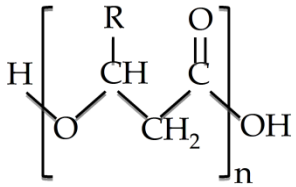
1.2 PHB biosynthetic pathway

PHB biosynthetic pathway is shown in Figure 4. The PHB biosynthetic pathway is mainly started from acetyl-CoA which is catalyzed by several enzymes including beta-ketothiolase (encoded by *phaA*), acetoacetyl-CoA reductase (encoded by *phaB*) and PHB synthase (encoded by *phaC* and *phaE*). The β-ketothiolase (*phaA*; EC2.3.1.9), catalyzes two acetyl-CoA converted to acetoacetyl-CoA, and further acetoacetyl-CoA reductase (*phaB*; EC 1.1.1.36) which is responsible for reducing acetoacetyl-CoA with NADPH to hydroxybutyryl-CoA. PHB synthase (EC 2.3.1.B4) is the enzyme catalyzing the polymerization reaction to polyhydroxybutyrate (PHB). *Synechocystis* PCC 6803 contains two subunits of PHA synthase, *phaC* and *phaE* that are located in the same operon. After two years, another two genes in PHB biosynthesis pathway, *phaA* and *phaB* were identified and the sequence analysis showed that both genes are also located neighboring in the same orientation of chromosome (Steinbuchel et al., 1992, Hauf et al., 2013).

1.3 PHB degradation

Inside the cell, the degradation is occurred in the reverse reaction of the synthesis. PHB are stored in subcellular complex, which contain amorphous PHB coated with a phospholipidic monolayer and various proteins involved in PHB production. These PHB carbonosomes are also known as native PHB in granules. When these granules are released into the extracellular medium as a consequence of cell lysis, PHB become denatured, acquiring a semi-crystalline structure, known as denatured PHB (García-Hidalgo et al., 2013).

The properties of PHB mostly possess its biodegradability and hydrophobicity in nature. PHB are degraded which found on exposure to soil, or marine sediment (Reddy et al., 2003). Microorganisms in nature are able to degrade PHAs by PHB hydrolases and PHB depolymerases like inside the cells in Figure 5 (Jendrossek and Handrick 2002, Choi et al., 2004). The activities of these enzymes may vary and depend on the type of the polymer and the environmental circumstance (Verlinden et al., 2007). Biodegradation is mainly dependent on a number of factors, such as microbial activity of the environment the exposed surface area, moisture, temperature, pH and molecular weight (Boopathy 2000). PHB have been reported previously that being degraded in aquatic environments (Lake Lugano, Switzerland) within 254 days even at temperatures under 6 ^{o}C (Johnstone 1990).



PHAs

Figure 1 Chemical structure of PHAs (Madison and Huisman, 1999). Where R = CH_3 in PHB. The foremost other polyesters presently identified have R = CH_3CH_2 (polyhydroxyvalerate, PHV) and R = $CH_3(CH_2)_4$ (polyhydroxyoctanoate, PHO) (Dawes, 1988).

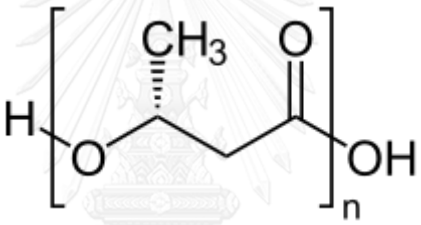


Figure 2 Structure of poly-(R)-3-hydroxybutyrate (PHB) monomer
(Source: [cited 2015 June]. https://en.wikipedia.org/wiki/Polyhydroxybutyrate.2015)

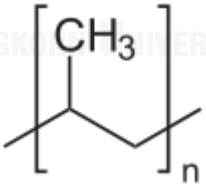


Figure 3 Structure of polypropylene (PP)
(Source: [cited 2015 June].https://en.wikipedia.org/wiki/Polypropylene)

Table 1 Properties of PHAs and polypropylene (Modified from Doi et al., 1990).

Parameter	Value for	
	PHB	PP
T_m (°C)[c]	177	176
T_g (°C)[d]	2	-10
Crystallinity (%	70	60
Extension to break (%)	5	400

[c]T_m is melting temperature.

[d]T_g is glass transition temperature.

Table 2 Chemical structures of PHB and their copolymer (Verlinden et al., 2007).

R-Group	Full name	Short
CH_3	Poly(3-hydroxybutyrate)	PHB
CH_2CH_3	Poly(3-hydroxyvalerate)	PHV
$CH_2CH_2CH_3$	Poly(3-hydroxyhexanoate)	PHHx

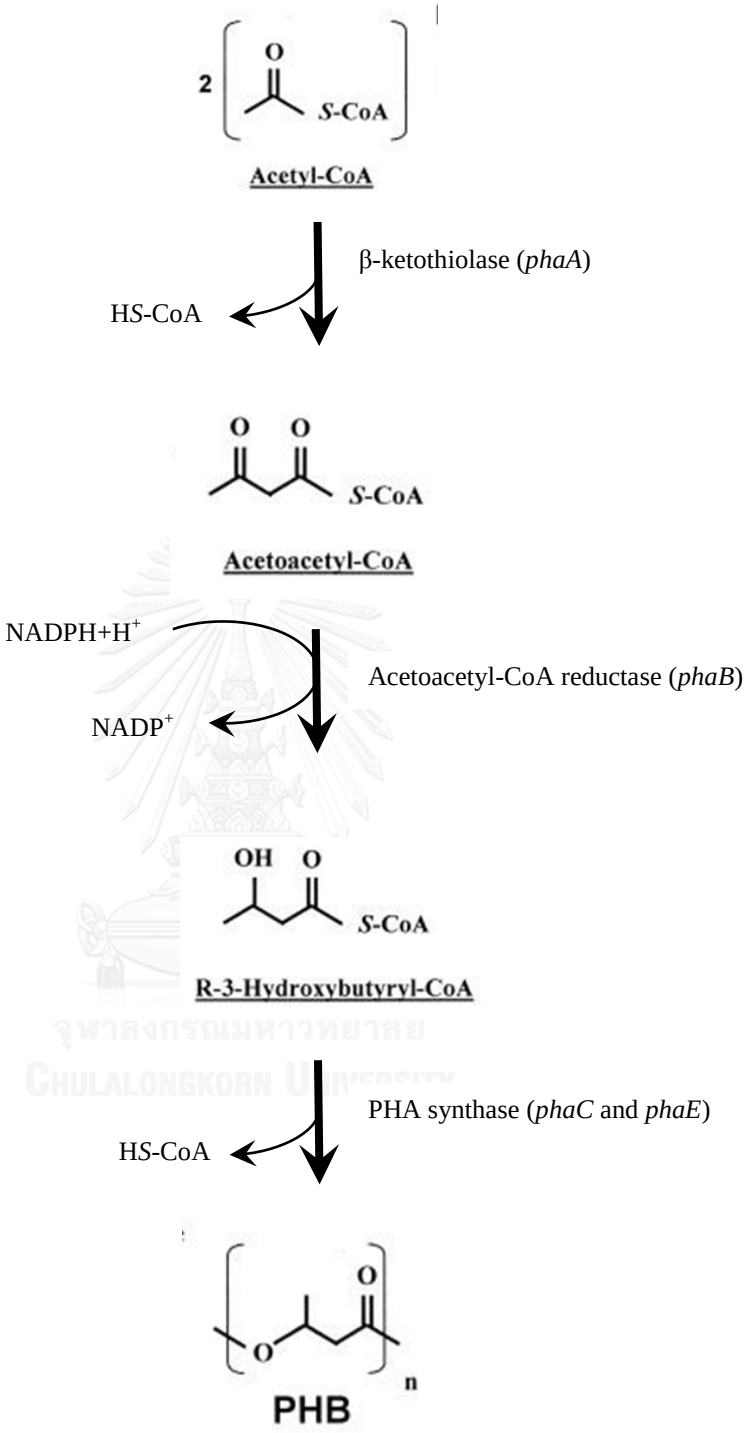


Figure 4 PHB biosynthetic pathway (Taroncher-Oldenburg et al., 2000).

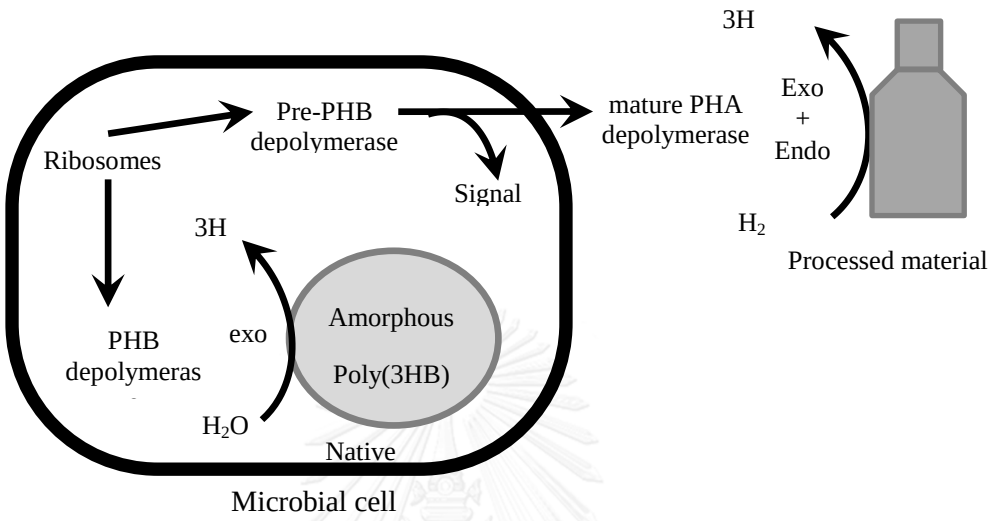


Figure 5 Biodegradation of PHB. In microbial cells, PHB was broken down to release the monomers of 3-hydroxybutyrate (3-HB) by PHB depolymerase enzyme (encoded by *phaZ*).
(Source:[cited 2015 June] http://mibi1.unimuenster.de/Biologie.IMMB/Biologie. IMMB. Steinbuechel/ en/Forschung/Syntpoly.html)

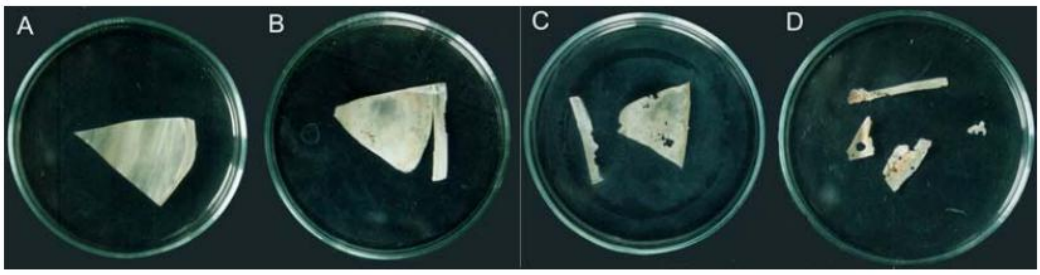


Figure 6 Undegraded PHB film (A) and PHB films with different degrees of degradation after 2 months incubation in soil suspension: anaerobic conditions without nitrate (B), microaerobic conditions without nitrate (C), and microaerobic conditions with nitrate (D)(Bonartsev et al., 2007).

It was shown the relation between the degree of PHB degradation and the molecular weight of degraded PHB (Figure 6). The levels of aeration were similar in each flask, the presence of nitrate boosted the degradation of PHB films and increased the decline in the molecular weight of PHB (Bonartsev et al., 2007).

1.4 PHB storage

PHB are insoluble in water, the polymers are mainly accumulated in intracellular granules inside the cells reserved as energy and carbon source (Taguchi et al., 2003, Verlinden et al., 2007). It is profitable for bacteria to store excess nutrients inside their cells, exceptionally as their general physiological fitness is not influenced. By polymerizing soluble intermediates into insoluble molecules, the cell does not endure alterations of its osmotic state. Thus, leakage of these valuable compounds out of the cell is prevented and the nutrient stores will remain safely available at a low maintenance cost (Peters and Rehm 2005). The three strains including *Spirulina* sp., *Synechocystis* sp. and *Ralstonia eutropha* gave the positive results for PHB accumulation visualized through Nile red staining method. The PHB inclusions were seen as light intracellular granules (shown in the Figures 7, 8 and 9) (Balaji et al., 2013).

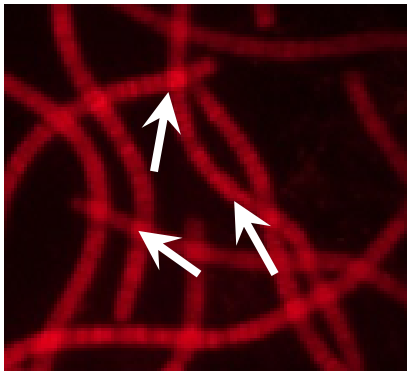


Figure 7 Nile red stained *Spirulina* sp. containing PHB inclusions (Balaji et al., 2012).

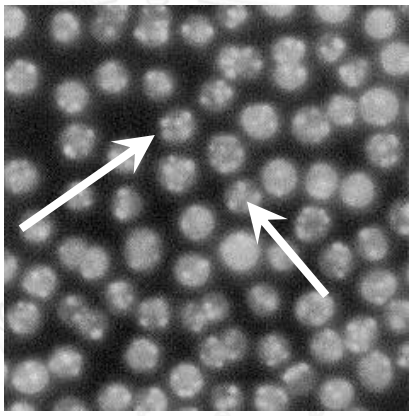


Figure 8 Nile red stained *Synechocystis* sp. containing PHB inclusions (Sudesh et al., 2002).

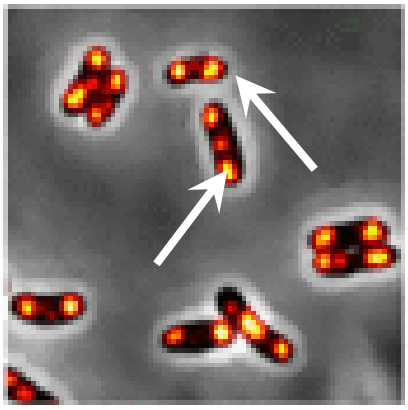


Figure 9 Nile red stained *Ralstonia eutropha* containing PHB inclusions (Wahl et al., 2012).

1.5 The effect of nutrient modification on PHB accumulation

PHB accumulation in most microorganisms was mainly induced by several factors including environmental stresses, sources of carbon, nutrient deficiencies and recently genetic engineering and gene manipulation technologies. In case of nutrient deficiencies, it had a significant impact to PHB accumulation in cells. Previous study in *Synechocystis* sp. PCC 6803, culture media were modified by depleting some nutrients which efficiently boosted up PHB accumulation and also designed the combined effects of P-deficiency and gas-exchange limitation (GEL). The supplement of carbon source into culture medium was also performed in order to direct carbon structure to PHB, such as acetate which lead to increase the flux of an key intermediate acetyl-CoA catalyzed by acetyl-CoA synthetase towards PHB synthesis. PHB accumulation was increased up to 38% (w/w) of dry cell weight (dcw) in *Synechocystis* sp. PCC 6803 under the presence of both fructose and acetate (Panda and Mallick, 2007) which was about eight-fold higher as compared with the accumulation under photoautotrophic growth condition. On the other hand, *Nostoc muscorum*, grown under a mixotrophic condition with 0.4% (w/v) glucose and acetate, was increased PHB content up to 35% whereas gas-exchange limitations under mixotrophy and chemoheterotrophy with 0.4% (w/v) acetate enhanced the accumulation up to 40-43% (w/w) (Sharma and Mallick, 2005). Moreover, *Nostoc muscorum* was boosted up PHB content to 35% (w/w) of dry cells when cells supplemented with 0.2% acetate and subjected to dark incubation for 7 days (Sharma and Mallick, 2005). In another bacteria, *Bacillus thuringiensis* IAM 12077 strain

grown under nitrogen-deficient medium condition gave high PHB accumulation from 24% to 43.4% (Pal et al., 2009).

1.6 Genetic manipulation of PHB production

At present, enhancement strategy of PHB production in biosources includes not only nutrient modification but also genetically engineering manipulation that enables cells to produce PHB higher than its nature ability. Previous study of PHB production in term of increased products by molecular genetics, Tyo and coworker (2009) employed the inverse metabolic engineering (ME) to produce higher PHB by constructing transposon library of *Synechocystis* sp. PCC 6803. An inverse ME was employed once as a combinatorial approach of transposon-based gene disruption, nutrient modification screening and genotype characterization on *Synechocystis* sp. PCC 6803 clones with increased PHB production. They found that the high PHB-mutant lacking *sll0461* gene could produce higher PHB content. This *sll0461* gene encodes gamma-glutamyl phosphate reductase enzyme (*proA*) which related to glutamate biosynthesis. Moreover, the genetic engineering in other bacteria was performed in a recombinant *Escherichia coli* using glycerol as the main carbon source. It was found that PHB content was increased up to 51% in fed-batch microaerobic cultures (Nikel et al., 2008). Moreover, by overexpressing transforming gene from *Ralstonia eutropha* into *Synechocystis* sp. PCC 6803, the 2-fold increased PHA synthase activity in the presence of acetate whereas a 3% increase of PHB accumulation was reported (Sudesh et al., 2002). Other main neighboring routes for PHB biosynthesis, that utilized the same acetyl-CoA intermediate, are TCA cycle, fatty acid biosynthetic pathway and glycogen pathway. The *rre37* overexpression

functioned as a pathway-level regulator that stimulates the metabolic flow from glycogen to PHB and the hybrid TCA and ornithine cycle (Osanai et al., 2014), as well as its regulation on accumulation of TCA cycle metabolites (Joseph et al.,2013).

On the other hand, we have proposed new hypothesis for PHB enhancement in this study via the blockade of polyamine pathway (Figure 10). Polyamines are polycationic molecules found in all organisms which exists in two biosynthetic pathways. Direct pathway is started with ornithine to give putrescine via ornithine decarboxylase (ODC) (Davis et al., 1992). The other indirect pathway converts arginine to agmatine by arginine decarboxylase (ADC) (EC 4.1.1.19) by additional steps to produce putrescine (Watson et al., 1998). The roles of polyamines are not only in the regulation of cell division and morphogenesis in plants, but they are also known to affect the folds of DNA by their binding (Flink and Pettijohn 1975), protein synthesis, membrane stability, and stress responses of plants and cyanobacteria (Raksajit et al., 2006). In Figure 11, the hypothesis of the study has been designed when disrupted arginine decarboxylase gene (*adc1*), arginine potentially redirect to other pathway, herein urea cycle to ornithine via arginase enzyme. Additionally, ornithine is converted to many intermediates, such as glutamate semialdehyde or pyrroline-5-carboxylate, proline and glutamate. Since we have known that glutamate is the main amino acid that enables to flow into TCA cycle via gamma-aminobutyrate shunt and converted to succinate (Xiong et al., 2015). When glutamate is pooled from polyamine synthesis blockade, its excess amount will be sufficient to feed TCA cycle and produce energy. Consequently, acetyl-CoA potentially redirect proceeding to PHB production instead of TCA cycle.

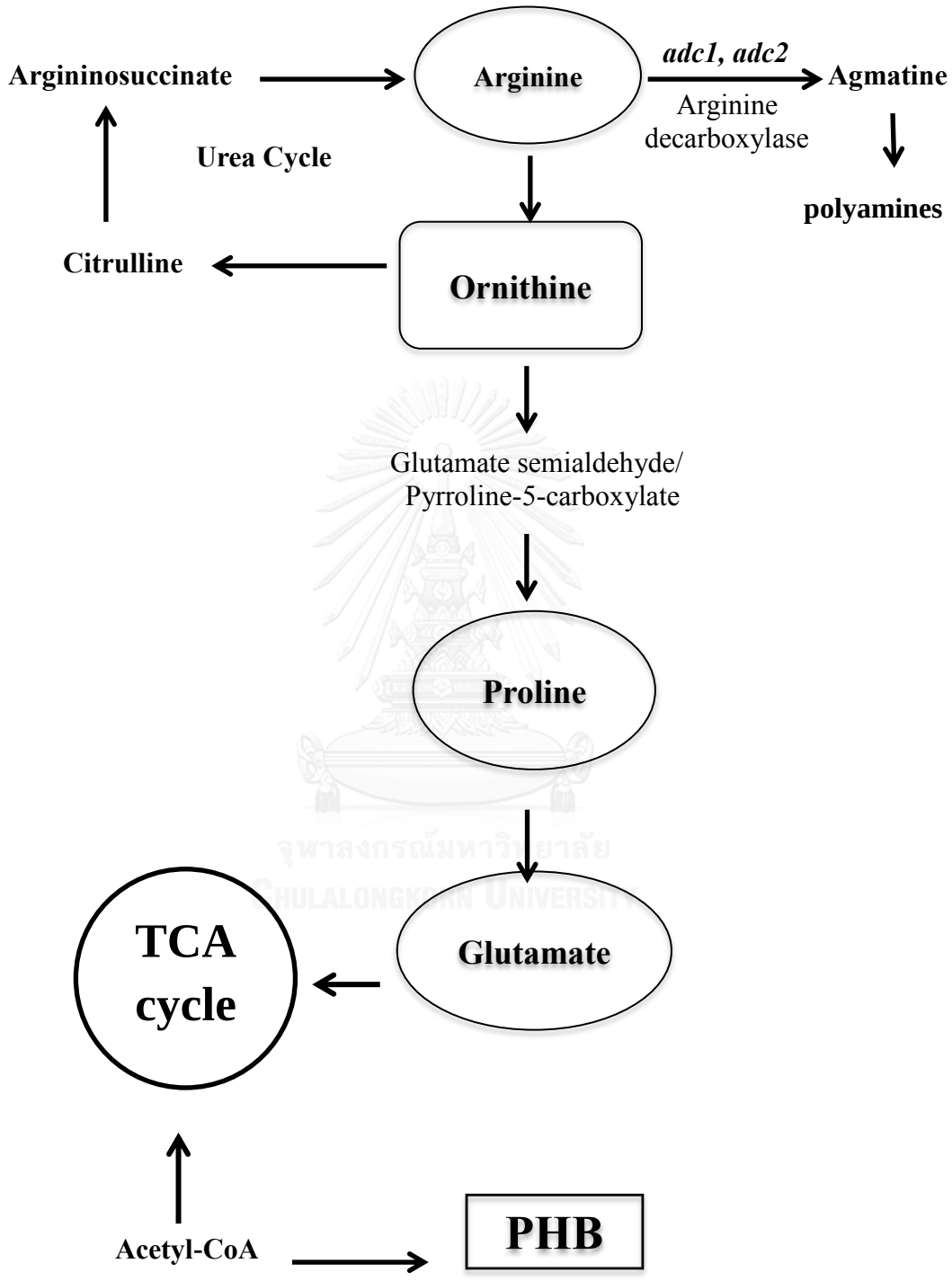


Figure 10 The proposed relation between PHB biosynthesis and polyamine pathway via proline-glutamate production (modified from (Quintero et al., 2000, Tyo et al., 2009)).

1.7 *Synechocystis* sp. PCC 6803

Cyanobacteria (blue-green-algae) are probably the largest, most divers and most widely distributed group of photosynthetic prokaryotes (Allen 1984). Cyanobacteria are clearly separated from other photosynthetic bacteria, such as purple and green bacteria, because they utilize H_2O as an electron donor; others do not. It has generally been accepted that the ancestors of cyanobacteria which acquired the ability to conduct oxygen-producing photosynthesis in the early stage of evolution gave rise to plant plastids by endosymbiotic events, thereby conferring the ability for photosynthesis to the extant algae and plants. Cyanobacteria have long been used as model organisms for studying photosynthesis in higher plants, where a more complex genetic system regulates the whole photosynthetic process. (Kaneko and Tabata 1997).

Synechocystis sp. PCC 6803 is a unicellular non-nitrogen (N_2)-fixing cyanobacterium, as well as a ubiquitous inhabitant of fresh water. It has been one of the most popular organisms for genetic and physiological studies of photosynthesis for two major reasons; it is naturally transformable by exogenous DNA (Grigorieva and Shestakov 1982) and the complete nucleotide sequence of *Synechocystis* sp. PCC 6803 was carried out in 1996. This was the first photoautotrophic organism to be fully sequenced. Sequencing was carried out using a clone-by-clone strategy based on the physical map of the genome, resulting in a highly accurate sequence. The circular genome was originally deduced to be 3,573,470 bp long (Kaneko et al., 1996).

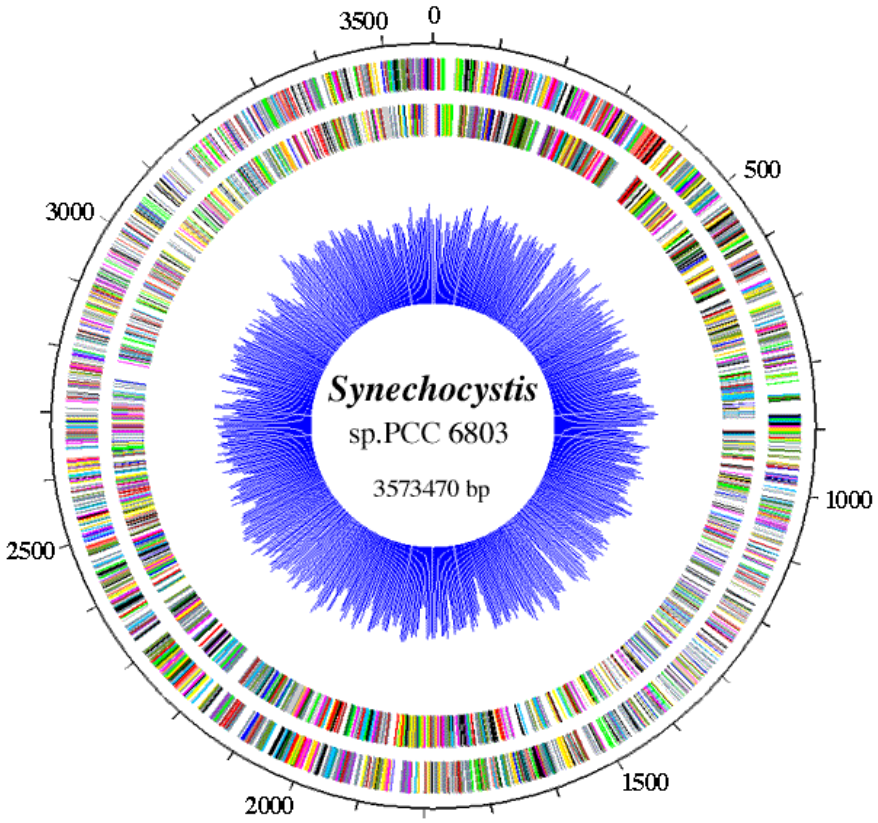


Figure 11 The circular genome of *Synechocystis* sp. PCC6803 from Cyanobase. (Source:[cited 2015 June] www.

http://genome.microbedb.jp/cyanobase/Synechocystis /map/Chr)

1.8 Objectives

1.8.1 To investigate the nutrient deficiency effect on growth, pigment contents, oxygen evolution, Nile-red stained visualization and PHB production in cyanobacterium *Synechocystis* sp. PCC 6803 wild type and mutant lacking arginine decarboxylase gene.

1.8.2 To investigate transcript levels of genes (*phaA*, *phaB*, *phaC* and *phaE*) related to PHB-biosynthetic pathway in cyanobacterium *Synechocystis* sp. PCC 6803 wild type and mutant lacking *adc1* gene under various nutrient modified treatments.

1.8.3 To investigate the UV radiation effect on growth, pigment contents, Nile-red stained visualization and PHB production in cyanobacterium *Synechocystis* sp. PCC 6803 wild type.

CHAPTER II

Materials and Methods

2.1 Materials

2.1.1 Equipments

Autoclave HV-100	:	Ta Chang Medical instrument, Taiwan
Autopipette	:	Pipetman, Gilson, France
Digital balance	:	Mettler Toledo, USA
Filtration equipment	:	Pyrex, USA
Fluorescent microscope	:	Olympus DP72
Laminar flow cabinet	:	Boss tech, HBV120
Microcentrifuge	:	MIKRO120, Germany
pH meter	:	Mettler Toledo, USA
Thermal cycler	:	Eppendorf Mastercycler gradient, USA
High performance liquid chromatography	:	Shimadzu HPLC LGE System, Japan
Hood	:	Science Technology
Hot plate	:	Cole-Parmer, Malaysia
Vortex	:	Scientific Industries, USA

2.1.2 Chemicals and reagents

Acetonitrile (HPLC grade)	:	RCI Labscan, Thailand
Adipic acid	:	Sigma, USA
Ammonium chloride	:	Katayama Chem, Japan
Crotonic acid	:	Sigma, USA
Ethylenediaminetetraacetic acid (EDTA)	:	Sigma, USA
Ethanol	:	Katayama Chem, Japan
Ferric sulfate	:	Mallinckrodt Chemical, USA
Glucose	:	Sigma, USA
HEPES	:	Sigma, USA
Hexane	:	Fisher Scientific, UK
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	:	Sigma, USA
Manganese chloride ($MnCl_2 \cdot 4H_2O$)	:	Univar, Australia
Methyl alcohol (CH_3OH)	:	J.T. Baker, Australia
Methyl alcohol (CH_3OH) (HPLC grade)	:	RCI Labscan
Polyhydroxybutyrate	:	Sigma, USA
Potassium chloride (KCl)	:	Fisher Scientific, India
Potassiumdihydrogenphosphate (KH_2PO_4)	:	RANKEM, India
Potassiumhydrogenphosphate (K_2HPO_4)	:	Univar, Australia
Sodium acetate	:	BHD, England
Sodium carbonate (Na_2CO_3)	:	Sigma, USA
Sodium hydroxide (NaOH)	:	Merck, Germany
Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	:	BDH, England
Sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$)	:	Sigma, USA

Sodium nitrate	:	Ajax Finechem, Australia
Ortho-phosphoric acid (85%)	:	BHD, New Zealand
Sulphuric acid 98%	:	BHD, New Zealand
Zinc sulphate ($ZnSO_4 \cdot 7H_2O$)	:	Univar, Australia

2.1.3 Kit & supplies

SuperScript® III Reverse Transcriptase	:	Invitrogen, USA

2.1.4 Antibiotics

Ampicillin	:	Sigma, USA
Kanamycin	:	Sigma, USA

2.1.5 Oligonucleotides

Table 3 PCR primers for RT-PCR

Target gene	Name	Oligo sequences	Amplified fragment length (bp)
phaA	*phaAL*	5'-CATGATGGTTTGACGGACAG- 3'	310
	phaAR	5'-GACTACAGTTGCCCGCTGTT- 3'	
phaB	*phaBL*	5'-ATGCCGGTATCACCAAAGAC- 3'	390
	phaBR	5'-CAATTTCCTCCGGTTTACCA- 3'	
phaC	*phaCL*	5'-GGGCACATTTAGCCTGTGTT- 3'	346
	phaCR	5'-GTAAGTTTCCCCCGCTTGAT- 3'	
phaE	*phaEL*	5'-GAGCAATATACCGCCACCAC- 3'	371
	phaER	5'-TCTTCCATCAAAGCAGCAAA- 3'	
16S rRNA	*16L*	5'-AGTTCTGACGGTACCTGATGA- 3'	521
	16R	5'-GTCAAGCCTTGGTAAGGTTCT- 3'	

2.1.6 Organisms

Cyanobacterium *Synechocystis* sp. PCC 6803 wild type cells were provided from Ångström Laboratory, Molecular Biomimetics; Microbial Chemistry, Department of Chemistry, Uppsala University, Sweden.

Cyanobacterium *Synechocystis* sp. PCC 6803 mutant cells lacking *slr1312* (*adc1*) gene which encodes arginine decarboxylase enzyme (*adc*). This strain was constructed previously by Dr. Panatda Yodsang (unpublished).

2.2 Methods

2.2.1. Cell culture and treatments of various nutrient conditions

Cyanobacterium *Synechocystis* sp. PCC 6803 wild type cells were grown in BG_{11} medium (Rippka et al., 1979) for 7 days as a stock culture, then transferred into new BG_{11} medium, and further cultured until late-log phase of growth for 15 days. Growth condition was performed on the rotary shaker under a continuous light (40-50 μE) at 28-32 ºC. In order to measure cell growth, the cell culture was determined for the optical density at 730 nm with spectrometer. After that, the treatments with various nutrient modified conditions were carried out with those late-log cells under the same growth condition. For nutrient modified treatments, we used normal BG_{11} medium as a control whereas the deficiencies of nitrogen (N, without $NaNO_3$ and ferrous ammonium citrate) and phosphorus (P, KCl was used instead of KH_2PO_4) from medium were done, namely BG_{11}-N-P. In addition, the supplements of two carbon sources including 0.4% acetate (A) and 0.4% glucose (G) the optimum concentration reported by (Panda and Mallick 2007) were also carried out; nitrogen

and phosphorus deficient BG_{11} plus acetate (represented as BG_{11}-N-P+A), and nitrogen and phosphorus deficient BG_{11} plus glucose (represented as BG_{11}-N-P+G) respectively. These modified nutrient treatments were performed and cells were subsequently collected at interval of 0, 1, 3, 5, 7, 9 and 11 days, respectively.

Cyanobacterium *Synechocystis* sp. PCC 6803 mutant cells lacking *adc* gene were grown and treated with various nutrient modifications as same procedures as *Synechocystis* sp. PCC 6803 wild type cells.

2.2.2. Determination of intracellular pigments

Cultured cells were harvested and further determined for intracellular pigments. One mL of cell culture in an eppendoff tube was centrifuged for 10 min at 12,000 *xg*. Then, liqiud phase was discarded and added 1 mL of DMF (dimethyl formamide) to dissolve cell pellets. The sample tube was vortexed and centrifuged for 10 min, at 12,000 *xg*. Finally, the extracted sample was determined the optical density at 461, 625, 664 nm with spectrometer and further a calculated by following equations;

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

(Moran 1982)

Carotenoid content (μg/cells) = $[(OD_{461}-(0.046x\ OD_{664}))x4]$/total cells

(Chamovitz et al., 1993)

Total cells (cells/ml) = $(OD_{730}/0.25)x10^8$ (Jantaro et al., 2003)

2.2.3. Ultraviolet (UV) stressed treatment

Cyanobacterium *Synechocystis* sp. PCC 6803 wild type cells were grown on BG_{11} agar plates (Rippka et al., 1979) for 7 days as a stock culture, then re-streaked onto new BG_{11} agar plates, and further cultured for 7 days under a continuous light (40-50 μE) at 28-32 ºC. Then, cells were exposed under three types of UV radiation including UVA, UVB and UVC respectively. The intensities of each UV were 11.0 - 13.00 watts per square meter for UV-A (365 nm), 1.2 - 1.3 watts per square meter for UV-B (302 nm), and 1.0 - 1.3 watts per square meter for UV-C (254 nm) (Jantaro et al., 2011). The time course of UV treatment was done at 0, 3, 6, and 12 hours, respectively. Next, the UV stressed cells were collected for further experiment.

2.2.4. Nile red staining method

One ml of *Synechocystis* cell culture was harvested from control and treatments. Then, a small loop of cell pellets was taken and resuspended in 3 μL of Nile red staining solution (Sigma, USA). The 0.9% normal saline (100 μL) was added, mixed sample tube and further incubated overnight in the dark (Tyo et al, 2006). To monitor the stained cells, the fluorescent microscope (Olympus, DP72) was used with the magnification of 100X.

2.2.5. Polyhydroxybutyrate (PHB) extraction and analysis

Cultured cells (50 mL) were harvested and cell pellets were obtained after centrifuging for 10 min, at 6000*xg* speed. The pellets were hydrolyzed by boiling for 60 min with 98% (v/v) sulfuric acid (800 μL) and 20 mg/mL of adipic acid (100 μL) (Sigma, USA), an internal standard. Boiled samples were filtered by 0.45 μm polypropylene membrane filter. The PHB content was detected by high-pressure liquid chromatography instrument (Shimadzu HPLC LGE System, Japan) using carbon-18 column, Inert Sustain 3-μm (GL Sciences, Japan), and the flow rate was 1.0 ml per minute. The running buffer was 30% (v/v) acetonitrile in 10 mM KH_2PO_4 (pH 2.3). The UV detector was set at 210 nm in order to detect the PHB-hydrolyzed product (crotonic acid). The injection volume was 10 μL (Tyo et al., 2006)

The unit of PHB content was %PHB per dry cell weight (DCW). The dry cell weight (DCW) was performed by drying harvested cell pellets (from 50 mL cells culture) in 80 ^{o}C oven overnight.

2.2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

2.2.6.1 Total RNA extraction

Synechocystis cells were extracted for total RNA by TRIzol method. Cells were resuspended in 500 μl of TRIzol reagent and added 100 mg glass beads and incubated at 70ºC for 5 min. Then, the reaction mixture was immediately vortexed at max speed for 30 sec. One hundred μl of chloroform was then added and shortly vortexed. After that, centrifugation for 5 min at 12,000 *xg* at room temperature was performed. Then, transferred supernatant to new tube and added an equal volume of

isopropanol, mixed and stored at room temperature for 1 min. Next, centrifuged for 5 min at 12,000 *xg* at 4ºC, discarded supernatant and washed pellet once with 1 mL of 75% ice-cold ethanol. Then, centrifuged for 3 min at 12,000 *xg* and discarded supernatant, finally air dried the pellet. The dissolved RNA pellets was done with DEPC-treated water. The cDNA was synthesized later using SuperScript® III First Strand Synthesis Kit (Invitrogen) in next step.

2.2.6.2 RT-PCR

RNA from 6.1 was calculated for RNA purity ((A_{260}/A_{280}) = >1.8). And, cDNA was synthesized after calculating RNA dilution for preparing equal concentration (1µg RNA = cDNA) by [RNA] = A_{260} x 40 x dilution factor. Then, combined the following in a 0.2 mL tube. Added 8 µl of RNA, 1 µl of random hexamers primer and 1 µl of 10 mM dNTP mix, respectively. Then, incubated at 65^{o}C for 5 min, and placed on ice for 1 min. After that, added 10 µL of cDNA synthesis mix including 2µl of 10x RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µL of 0.1 M DTT, 1 µL of RNase and 1 µL of Super Script III RT, respectively. After that, incubated the reaction mixture at 25^{o}C for 10 min, 50^{o}C for 50 min and 85^{o}C for 5 min, respectively. Finally, added 1 µL of RNaseII and incubated at 37^{o}C for 20 min.

The cDNA was used as template for PCR as following; denature initialization 95 ^{o}C, 5 min, and further run for 19 cycles of denaturation at 95 ^{o}C, 30 sec, DNA annealing at 55 ^{o}C, 30 sec, extension at 72 ^{o}C, 35 sec, and then hold at 72 ^{o}C, 5 min. For *phaA, phaB, phaC, phaE* primers, the PCR condition was initialization at 95 ^{o}C, 5 min, and further run for 31 cycles of denaturation at 95 ^{o}C, 30 sec, DNA annealing at

50 ^{o}C, 30 sec, extension at 72 ^{o}C , 35 sec, and then hold 72 ^{o}C, 5min. PCR product was checked by agarose gel electrophoresis using 1.5% agarose gel in 0.5 TAE buffer.

2.2.7. Oxygen evolution measurement

Synechocystis cells were grown in BG_{11} medium for 15 days and transferred cells to new media including BG_{11}, BG_{11}-N-P, BG_{11}-N-P+A and BG_{11}-N-P+G, respectively. Cells culture (10 mL) of each medium was harvested at day 0, 1, 3, 5, 7, 9 and 11, respectively, and measured for chlorophyll *a* content. Then, harvested cells were concentrated to 2 ml by centrifuging for 10 min, at 6000*xg*. Then, the supernatant was discarded, cell pellets were collected and added 2 mL of 1x TE buffer. The sample was mixed and incubated under darkness for 30 min adaptation. Then, applied sample into the chamber of oxygen electrode - Clark type, closed chamber and incubated under the dark for 1 min. After that, saturated light was illuminated to the cell chamber. The oxygen evolution was performed at 25 ^{o}C. The unit of oxygen evolution was μmole O_2 per chlorophyll *a* content per hour.

CHAPTER III

Results

3.1 Growth of *Synechocystis* cells under normal condition of growth stage

Synechocystis sp. PCC 6803 wild type (WT) and *Δadc1* mutant cells were cultured in BG_{11} medium under normal growth condition. In Figure 12, growth of WT cells grown to mid-log phase at day 10 of cultivation and towards to late-log phase at day 15 of cultivation. On the other hand, *Δadc1* cells seemed to grow better than WT after day 18 of cultivation. From results, we chose WT and *Δadc1* cells grown at 15 day-culture for next phase of PHB production under adaptation in nutrient modification conditions.

3.2 Growth of *Synechocystis* sp. PCC 6803 under nutrient adaptation

After WT and *Δadc1* cells were grown to late-log phase. Cells were harvested and transferred into new media including normal BG_{11} medium as a control, BG_{11}-N-P, BG_{11}-N-P+A and BG_{11}-N-P+G, respectively. Growth curves of WT and *Δadc1* cells were shown in Figure 12. In adaptation stage under control BG_{11} medium, WT and *Δadc1* normally increased their growth (Figure 13A and 13B). Interestingly, cell growths of both WT and *Δadc1* were decreased significantly under all BG_{11}-N-P, BG_{11}-N-P+A and BG_{11}-N-P+G compared to normal BG_{11} control.

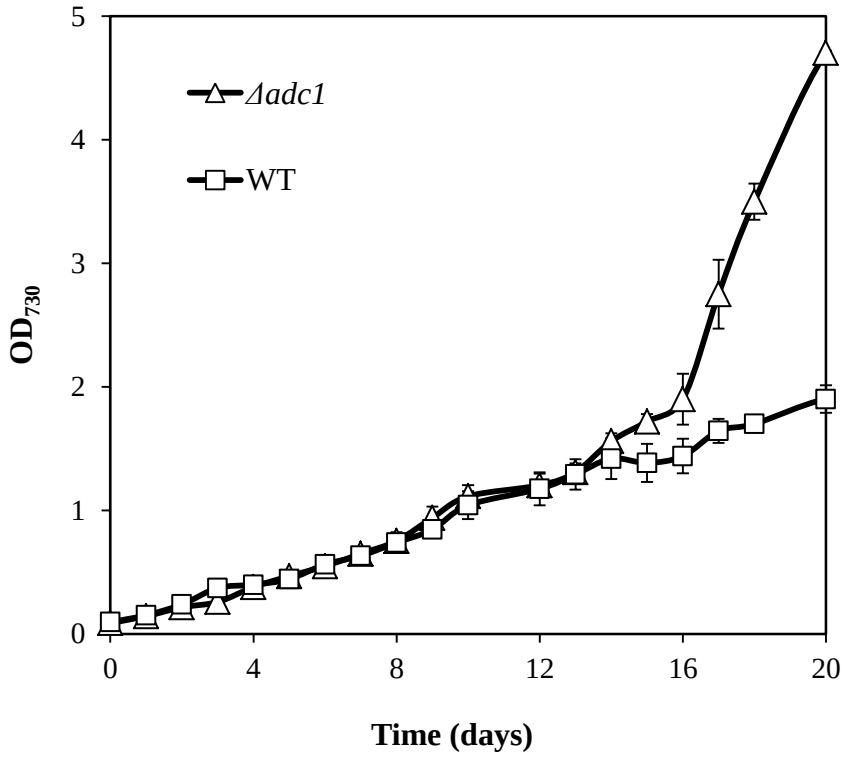


Figure 12 Growth curves of *Synechocystis* sp. PCC 6803 wild-type (WT) and *Δadc1* mutant cells were grown in BG_{11} medium under normal condition. Data represent Mean ± S.D. (n=6).

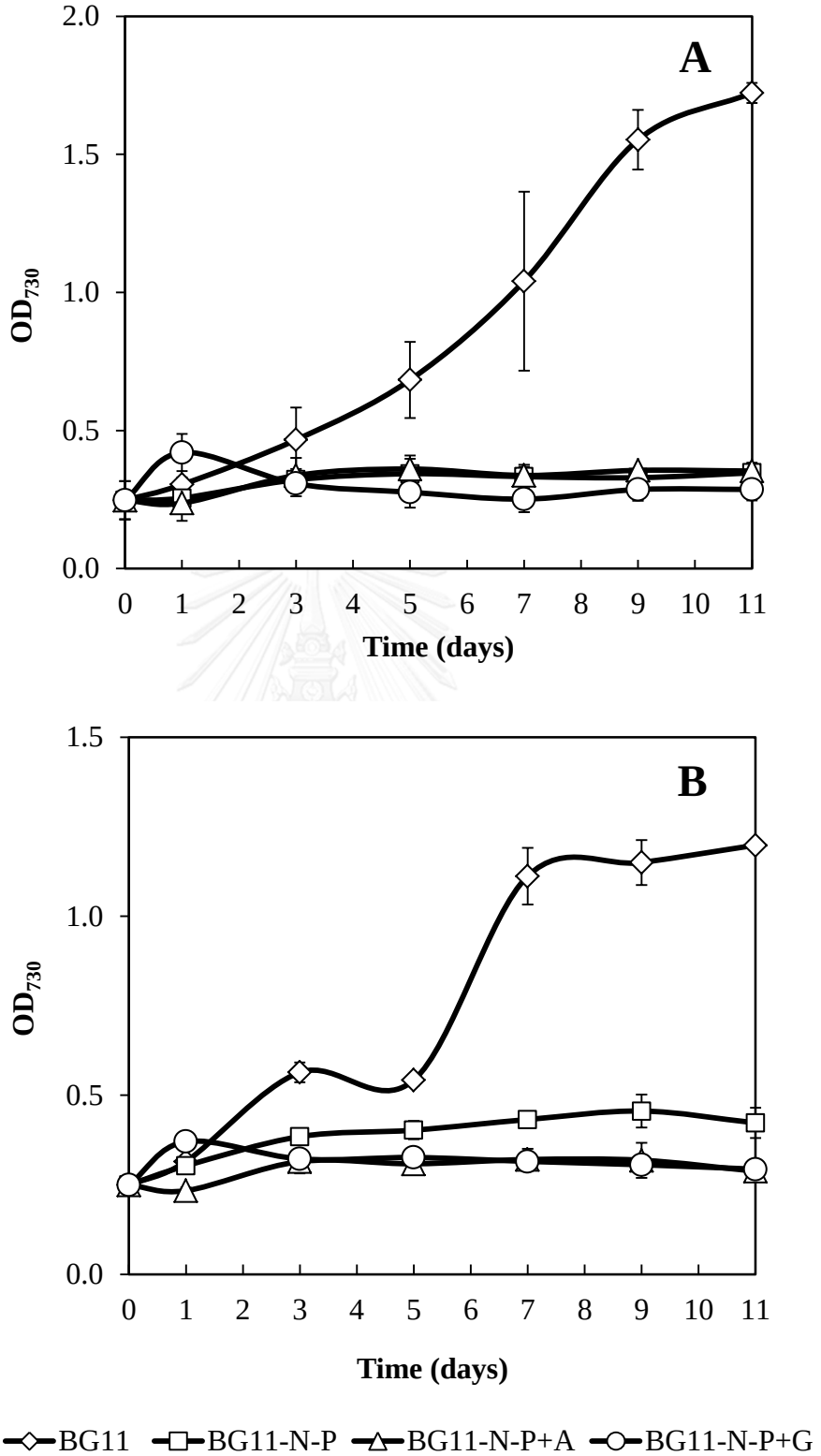


Figure 13 Growth curves of *Synechocystis* PCC6803 wild type (A) and *Δadc1* mutant (B) cells after adaptation under various nutrition modifications. Data represent Mean ± S.D. (n=5).

3.3 Growth of cells under UV radiation

After exposure to UV radiation, WT cell growth was determined as shown in Figure 13. UV-B and UV-C radiations obviously inhibited growth of WT whereas UV-A slightly increased growth of cells after exposure for 6 to 12 hours compared to cells exposed under normal light condition.

3.4 Intracellular pigments under normal condition of growth stage

Chlorophyll *a* and carotenoid contents of WT and *Δadc1* were monitored and shown in Figure 14. The chlorophyll *a* contents of both WT and *Δadc1* showed insignificant differences (Figure 14A). However, *Δadc1* cells had a reduction of chlorophyll *a* amount after 16 day-culture. For carotenoid content in WT and *Δadc1* (Figure 14B), WT cells accumulated higher content than that of *Δadc1* cells.

3.5 Intracellular pigments under UV radiation

In Figure 16A, UV-B and UV-C radiations significantly inhibited the accumulation of chlorophyll *a* amount along 12 hours compared to that of control cells. UV-A radiation did not affect the chlorophyll *a* amount. In Figure 16B, UV-B and UV-C obviously inhibited carotenoid accumulation when compared to cells grown under normal light. UV-A radiation did not harm to carotenoid contents along 12 hours. Then, UV-B and UV-C seemed to generate severe effect to intracellular pigments rather than that UV-A.

3.6 Chlorophyll *a* content under adaptation in various nutrient modifications

In Figure 17A, WT cells normally maintained their chlorophyll *a* contents under normal BG_{11} along 11 days-adaptation. Under BG_{11}-N-P condition, there was a reduction of chlorophyll *a* accumulation, as well as under BG_{11}-N-P+A, compared to that under control. The acetate supplementation in BG_{11}-N-P+A condition did not enhance the chlorophyll *a* content. On the other hand, BG_{11}-N-P+G condition significantly decreased the accumulation of chlorophyll *a*. For chlorophyll *a* content in *Δadc1* cells as shown in Figure 17B, the acetate supplementation in BG_{11}-N-P+A condition could enhanced the chlorophyll *a* contents compared to those under BG_{11}-N-P condition. The apparent decrease of chlorophyll *a* content of *Δadc1* cells under BG_{11}-N-P+G condition was observed.

3.7 Carotenoid content under adaptation in various nutrient modifications

From WT and *Δadc1* cells were adapted in various nutrient modifications and further measured their carotenoid contents (Figure 18). Under BG_{11}-N-P treatment, the carotenoid contents were decreased in both WT and *Δadc1* cells compared to those under normal BG_{11} control. The acetate supplementation in BG_{11}-N-P+A treatment slightly increased carotenoid content compared with that under BG_{11}-N-P in both WT and *Δadc1* strains. In contrast, carotenoid contents in both WT and *Δadc1* strains were decreased significantly under BG_{11}-N-P+G treatment. Glucose supplementation in BG_{11}-N-P treatment seemed to have severe effect on chlorophyll *a* accumulation.

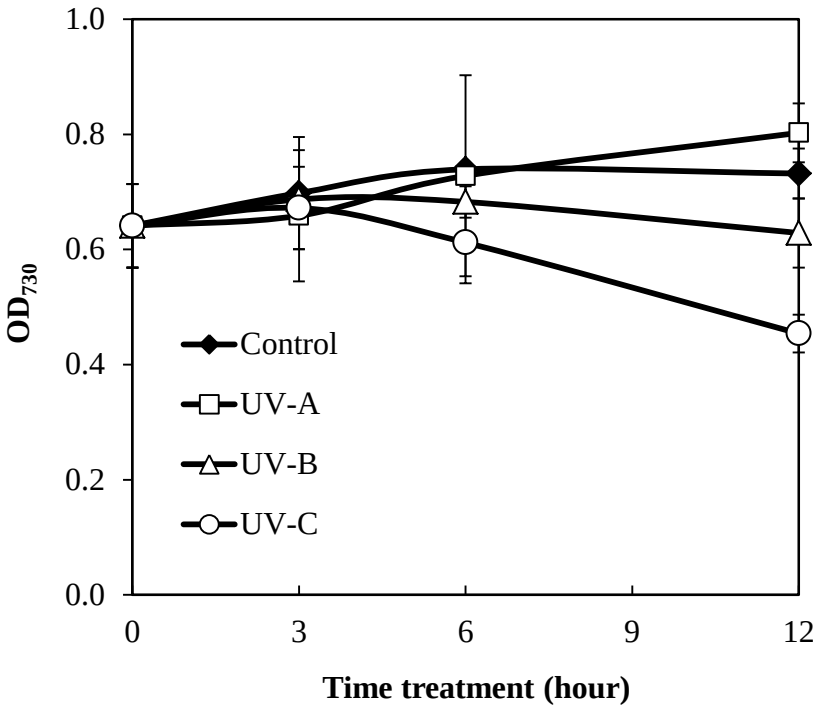


Figure 14 Growth curve of cyanobacterium *Synechocystis* sp. PCC 6803 wild type cells under UV radiations. Data represent Mean ± S.D. (n=3).

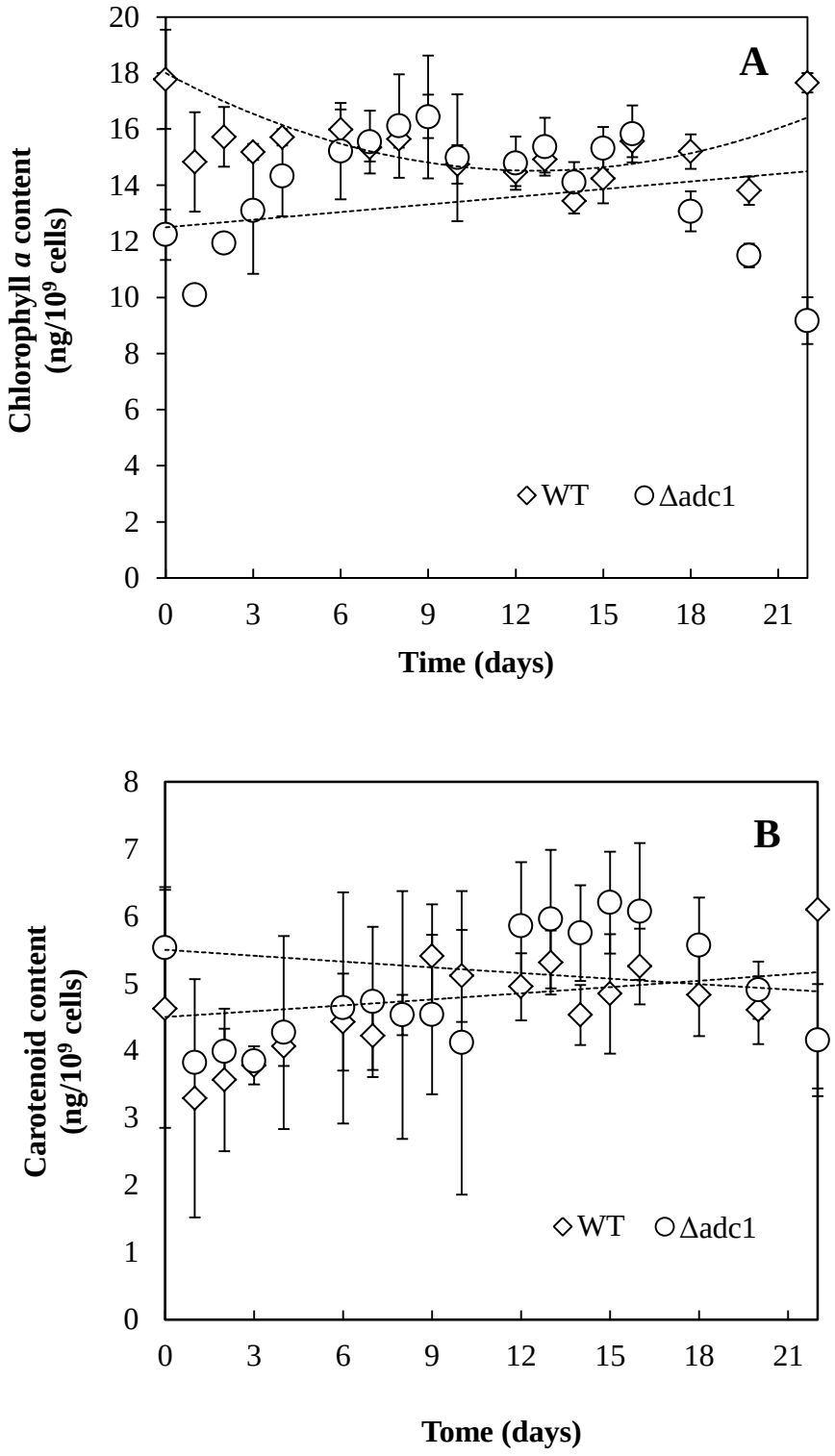


Figure 15 Intracellular pigments including chlorophyll *a* content (A) and carotenoid content (B) from *Synechocystis* PCC6803 wild-type and mutant. Data represent Mean ± S.D. (n=3)

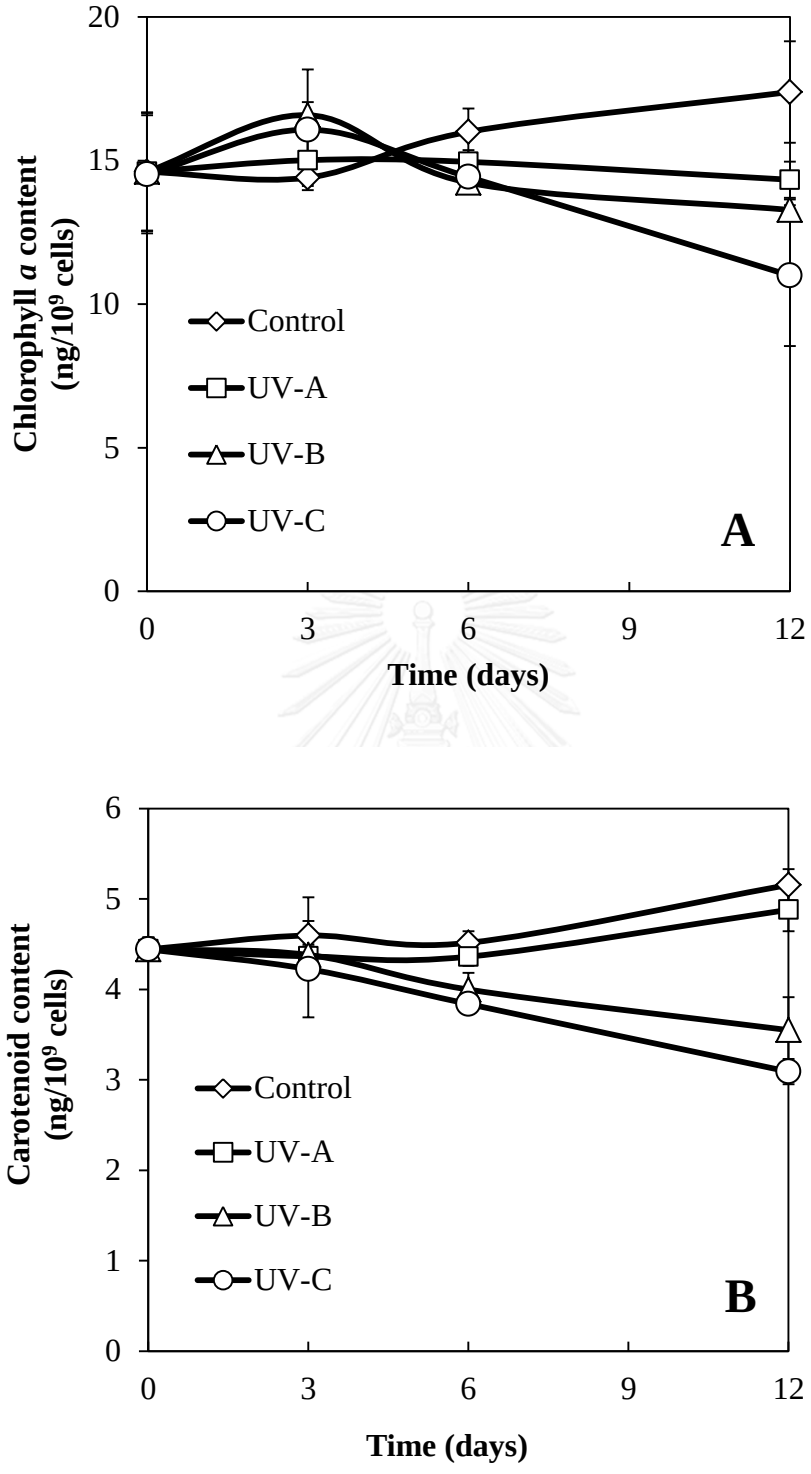


Figure 16 Intracellular pigments from *Synechocystis* PCC6803 wild type under UV radiation including chlorophyll *a* content (A) and carotenoid content (B). Data represent Mean ± S.D. (n=3)

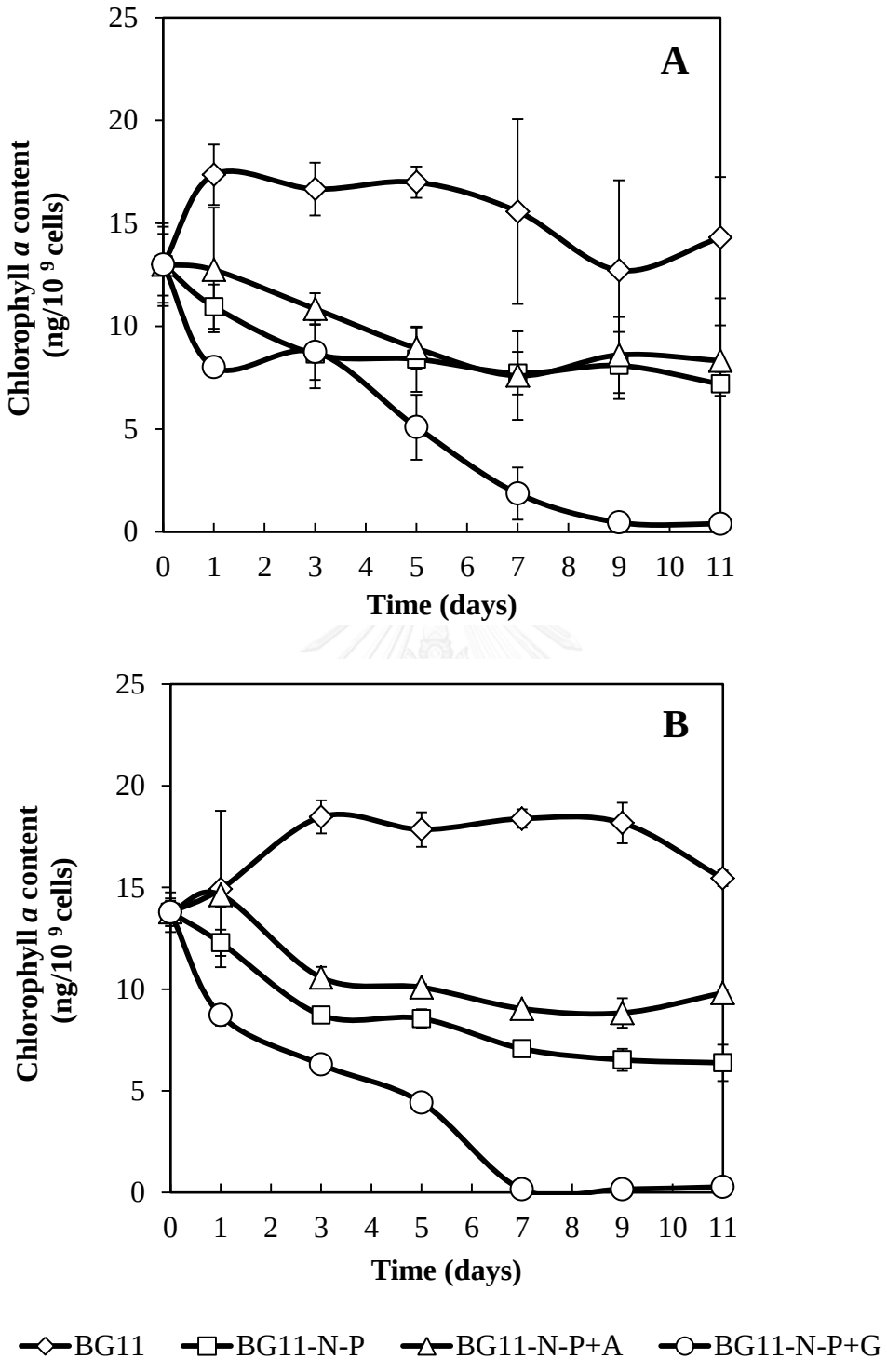


Figure 17 Chlorophyll *a* contents from *Synechocystis* PCC 6803 wild type (A) and *Δadc1* mutant (B) were measured under various nutrient modifications. Data represent Mean ± S.D. (n=3)

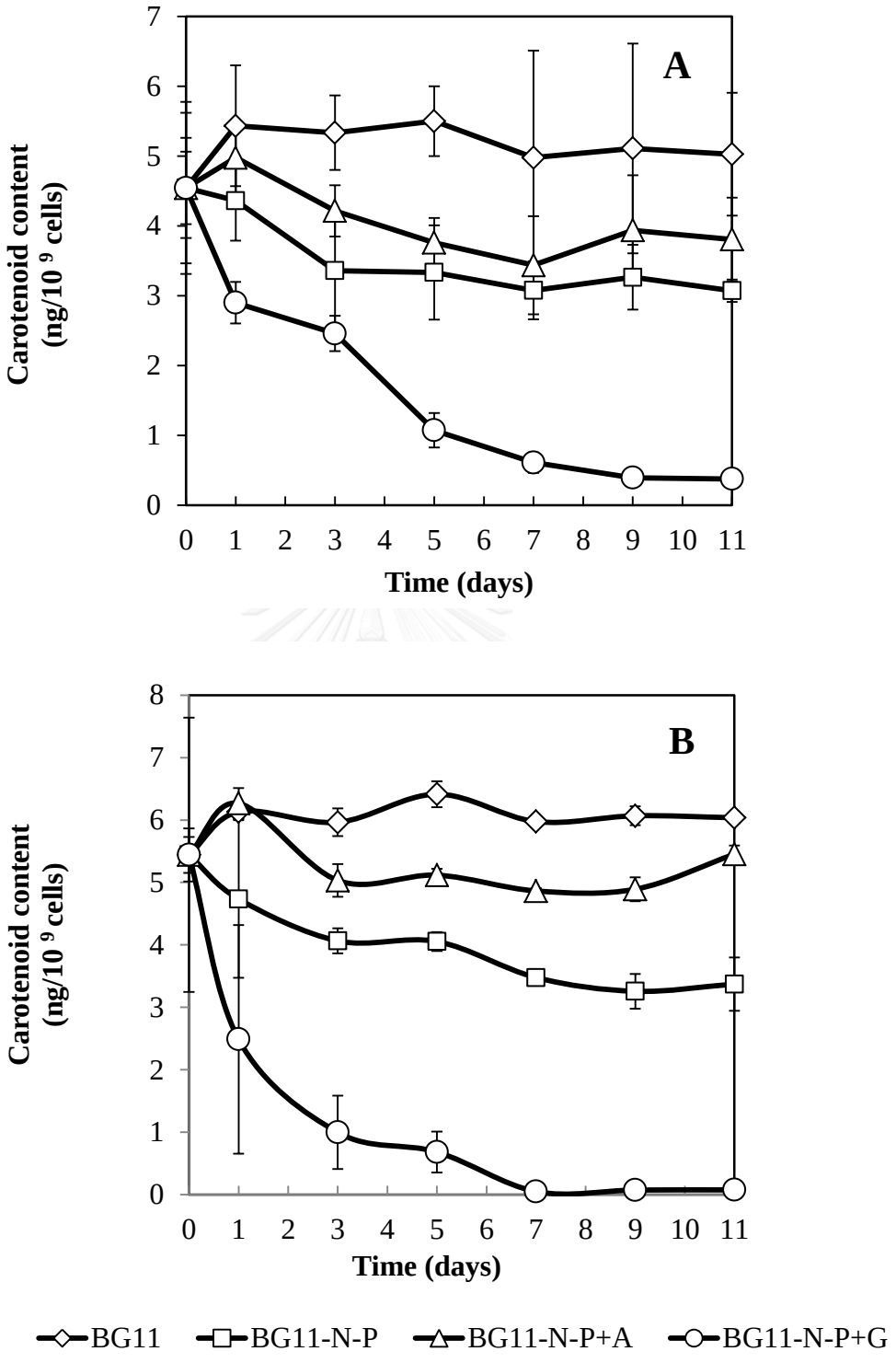


Figure 18. Carotenoid contents of *Synechocystis* PCC 6803 wild type (A) and *Δadc1* mutant (B) were measured under adaptation in various nutrient modifications. Data represent Mean ± S.D. (n=3)

3.8 Oxygen evolution under nutrient modified conditions

In Figure 19A, oxygen evolution rate in WT cells under BG_{11} control were significantly increased whereas cells adapted under all nutrient modified treatments were gradually declined. In Figure 19B, oxygen evolution rate in *Δadc1* cells under normal BG_{11} sharply increased whereas photosynthetic efficiencies of cells under BG11-N-P showed an increase at day 1 of treatment and later gradually decreased. Moreover, the oxygen evolution rates of *Δadc1* cells were decreased significantly under BG11-N-P+A and BG11-N-P+G conditions.

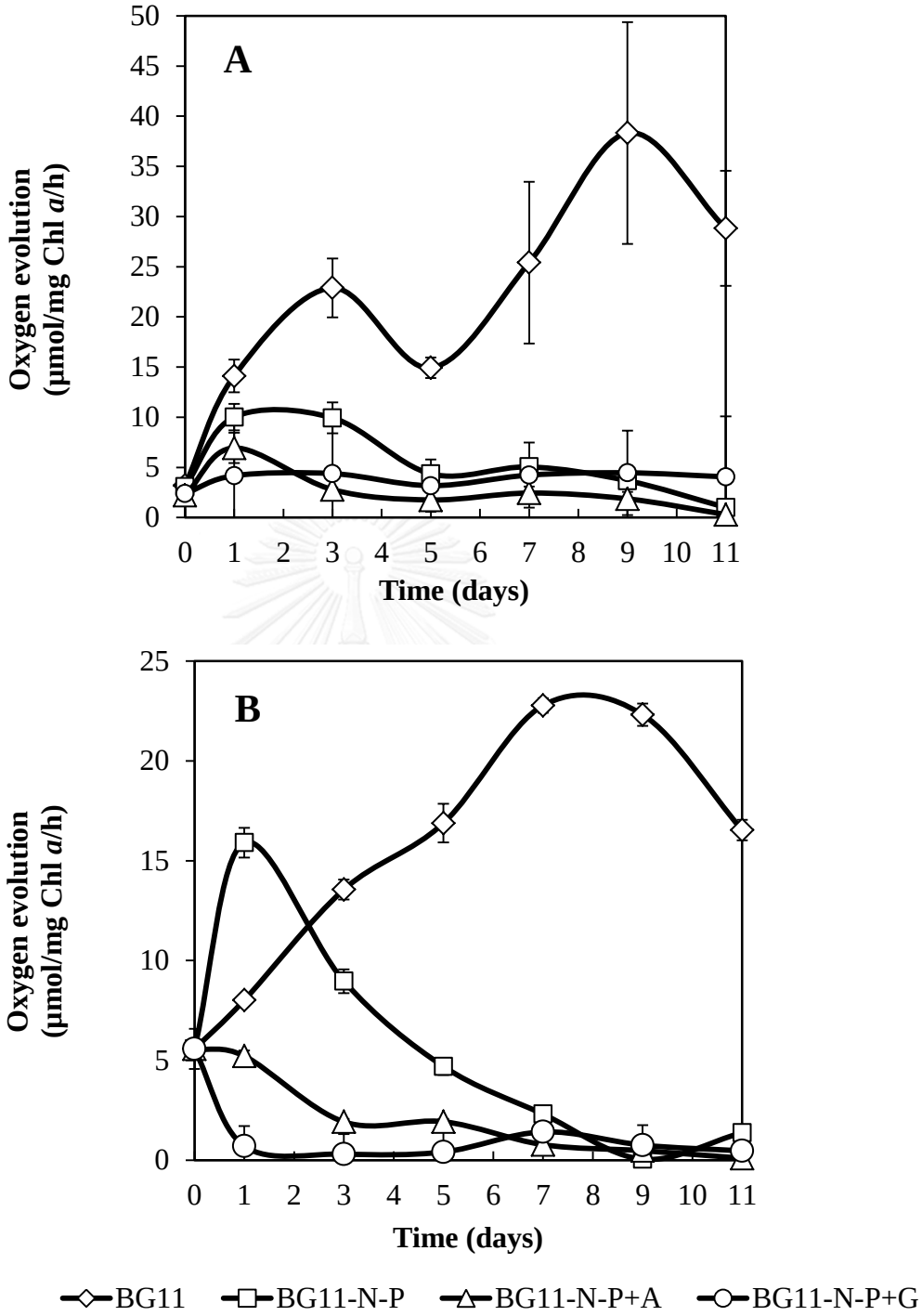


Figure 19 Oxygen evolution rates of cyanobacterium *Synechocystis* PCC 6803 wild type cells (A) and *Δadc1* cells (B). Data represent Mean ± S.D. (n=3)

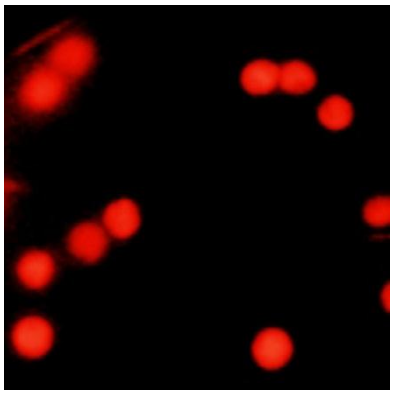
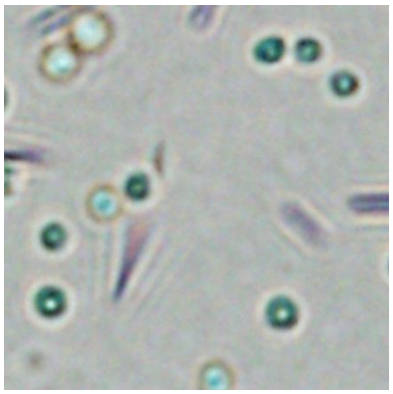
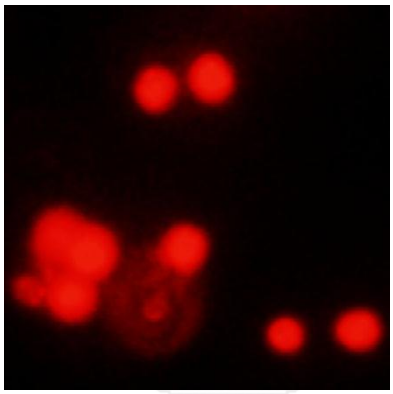
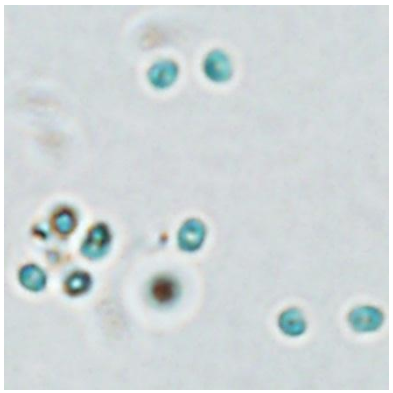
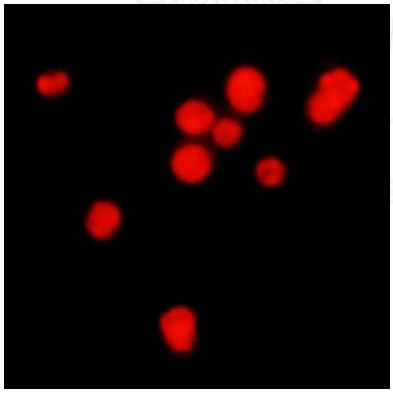
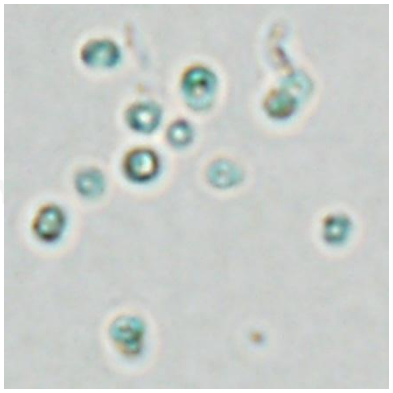
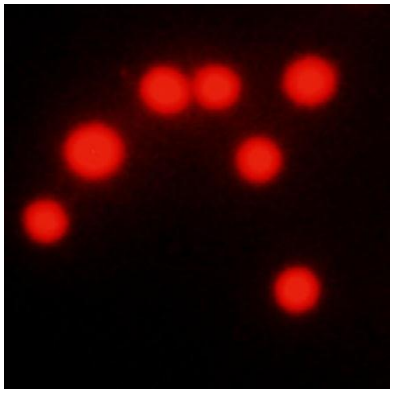
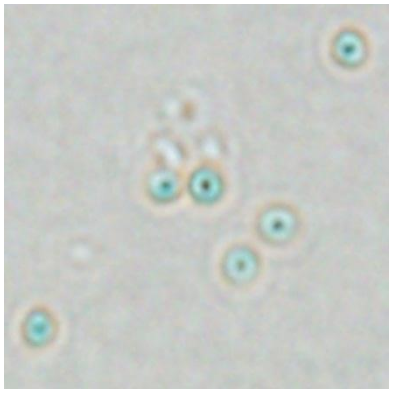
3.9 Nile red staining of *Synechocystis* PCC 6803 wild type cells

Synechocystis sp. PCC 6803 (WT) cells adapted in various nutrient modified conditions were stained for neutral lipid granule, herein namely PHB in golden-yellow spot, by Nile red solution and monitored by the fluorescent microscope as shown in Figures 20-23 WT cells accumulated some golden-yellow spots at day 9 under normal BG_{11} condition. Normally, WT cells rarely accumulated neutral lipids (or PHB) under normal condition.

In Figure 21, WT cells adapted under BG_{11}-N-P condition accumulated high amounts of golden-yellow spots in cells at day 7, 9 and 11. The highest amount of golden-yellow spots was seen apparently at day 9 when compared to those of WT under normal BG_{11} in Figure 20

In Figure 22, WT cells adapted under BG_{11}-N-P+A condition also accumulated highly PHB granules within cells under microscope monitoring. There were clearly seen those granules at day 5, 7 and 9 of treatment compared to those of WT cells under BG_{11} condition.

In Figure 23, WT cells adapted in BG_{11}-N-P+G condition also some showed golden-yellow spots in cells clearly at day 7, 9 and 11 of treatments. This condition enhanced the accumulation of PHB granules less than those under BG_{11}-N-P and BG_{11}-N-P+A conditions.

BG_{11}	Nile red staining	Light microscope
day 0		
day 1		
day 3		
day 5		

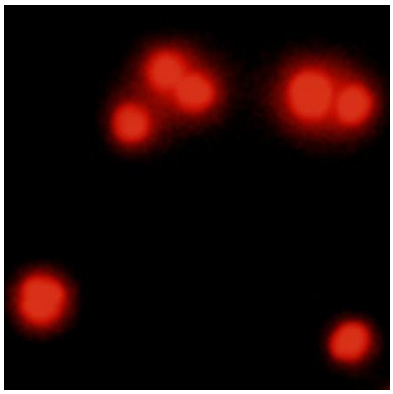
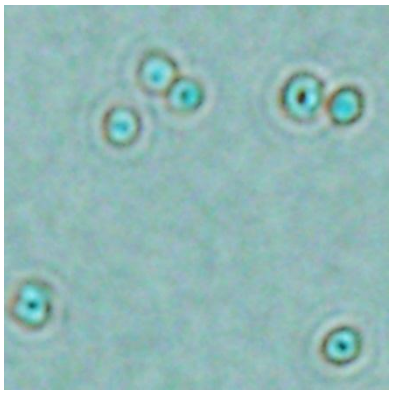
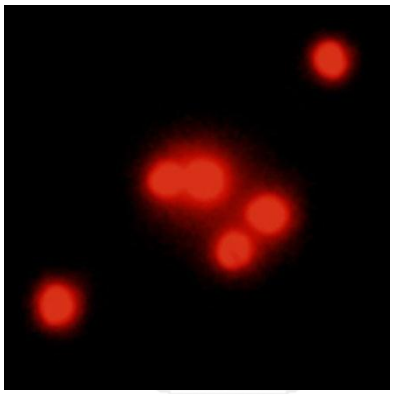
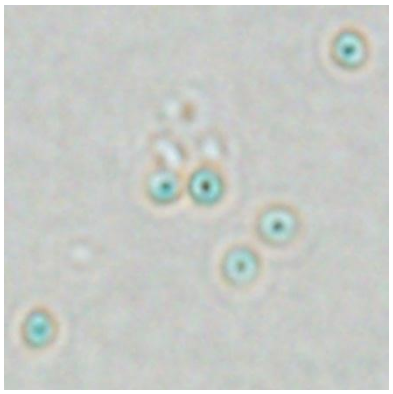
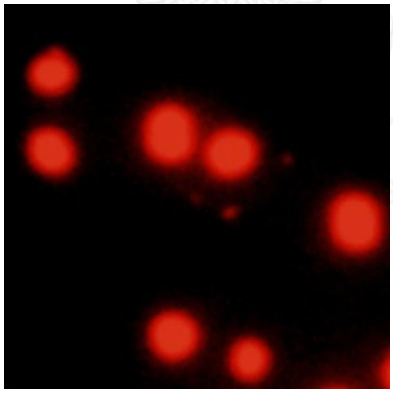
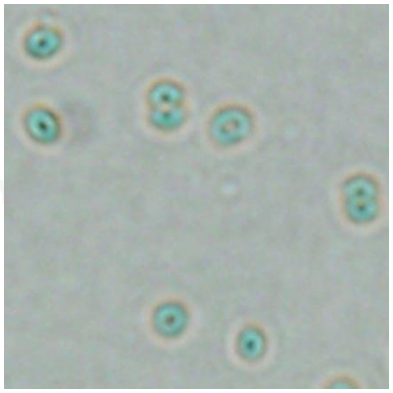
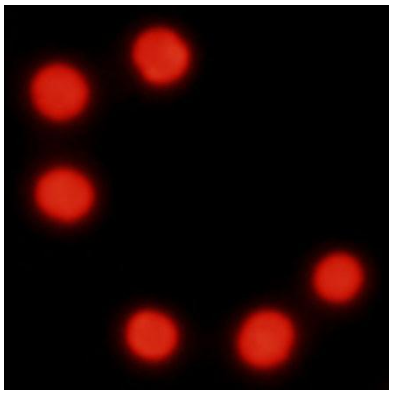
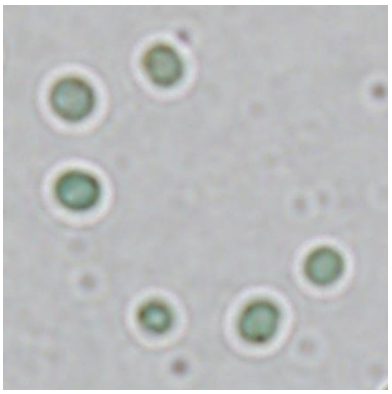
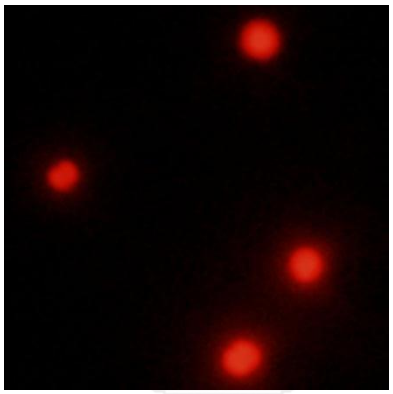
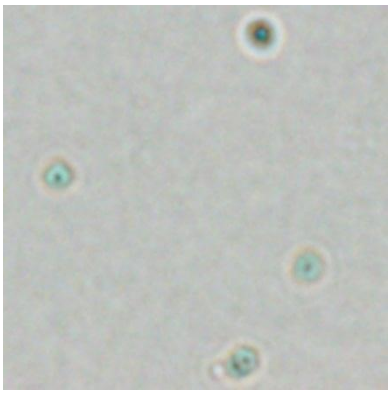
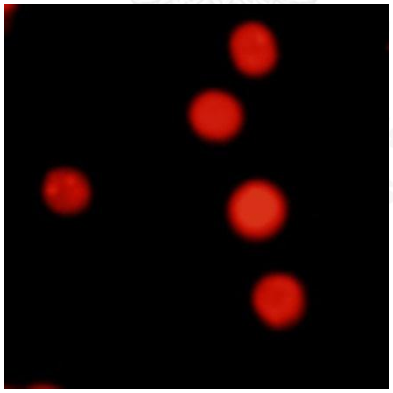
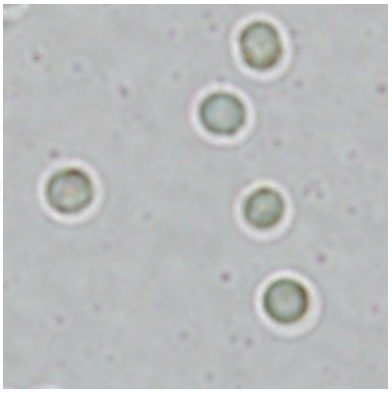
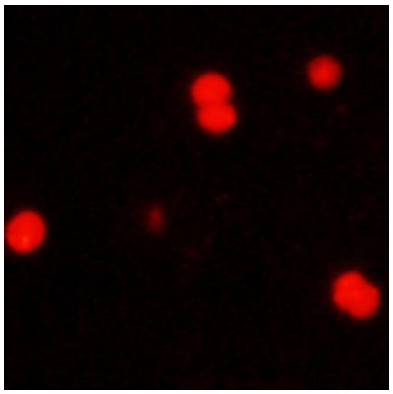
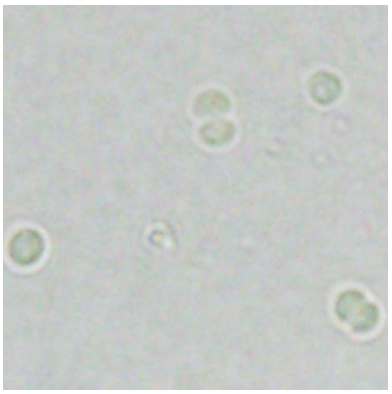
BG_{11}	**Nile red staining**	**Light microscope**
day 7		
day 9		
day 11		

Figure 20 Images of Nile-red stained cells of *Synechocystis* sp. PCC 6803 wild type under normal BG_{11} medium. Cells were stained by Nile red solution and visualized under fluorescent microscope with a magnification of 100x

BG_{11}-N-P	**Nile red staining**	**Light microscope**
day 0		
day 1		
day 3		
day 5		

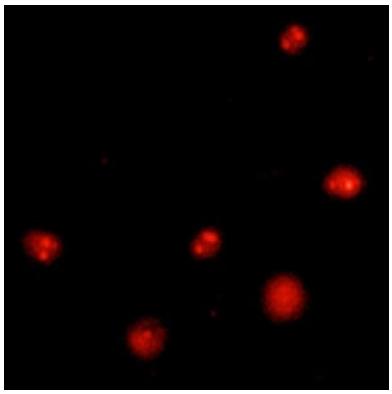
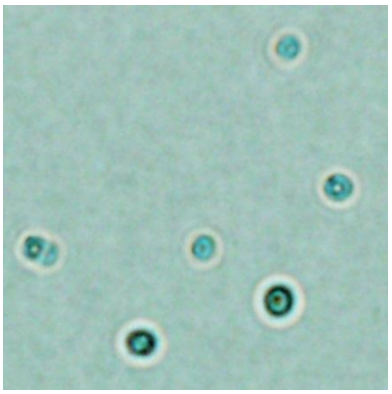
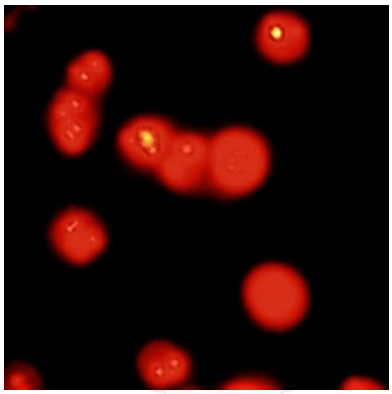
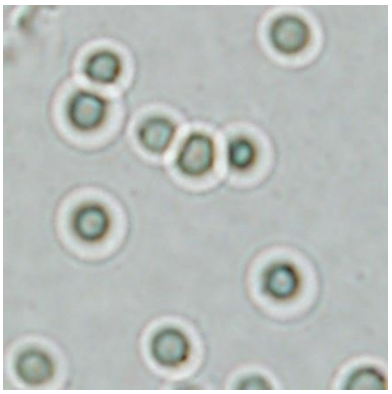
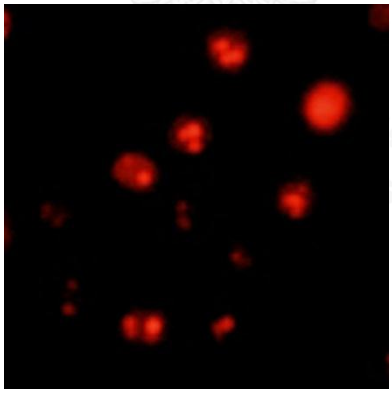
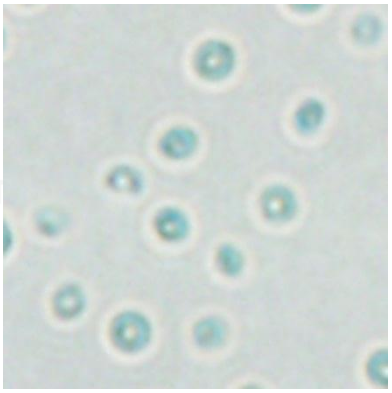
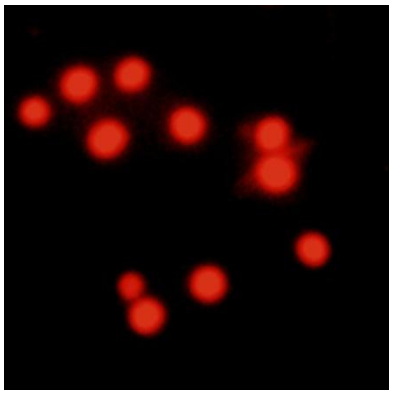
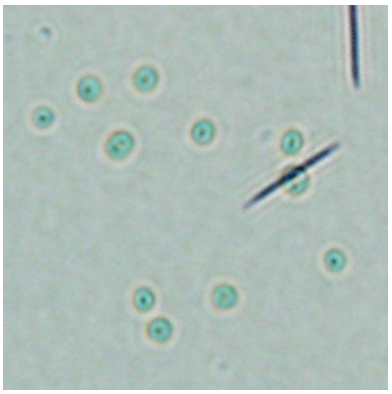
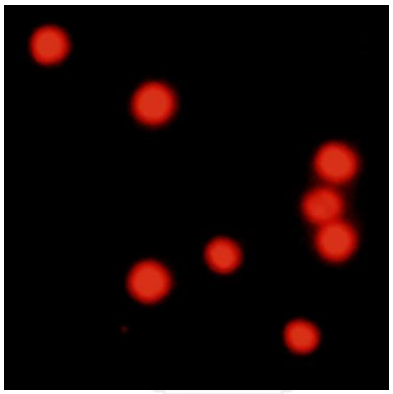
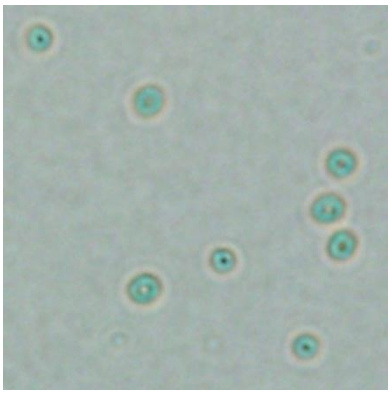
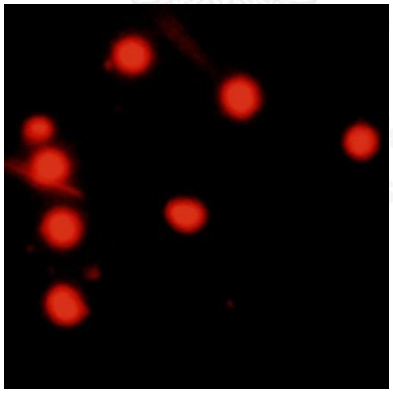
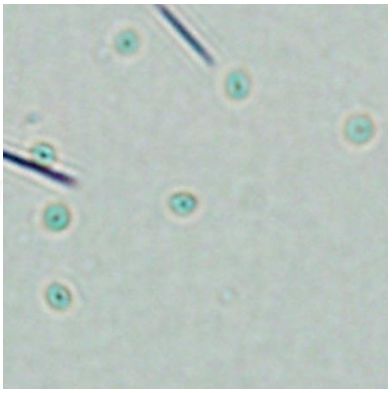
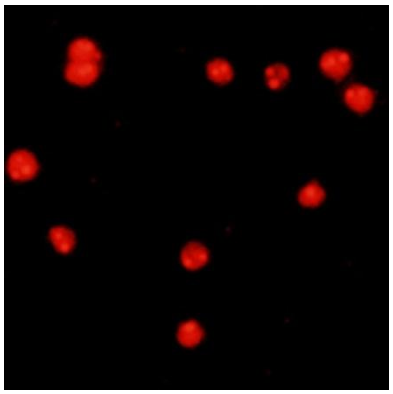
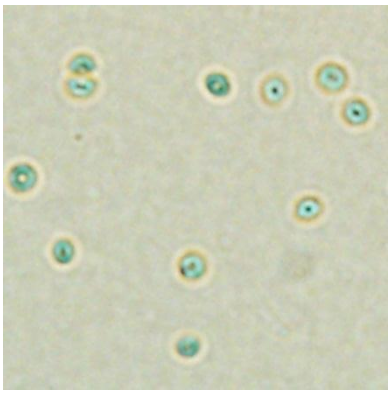
BG_{11}-N-P	**Nile red staining**	**Light microscope**
day 7		
day 9		
day 11		

Figure 21 Images of Nile-red stained cells of *Synechocystis* sp. PCC 6803 wild type under BG_{11}-N-P medium. Cells were stained by Nile red solution and visualized under fluorescent microscope with a magnification of 100x

BG_{11}-N-P+A	**Nile red staining**	**Light microscope**
day 0		
day 1		
day 3		
day 5		

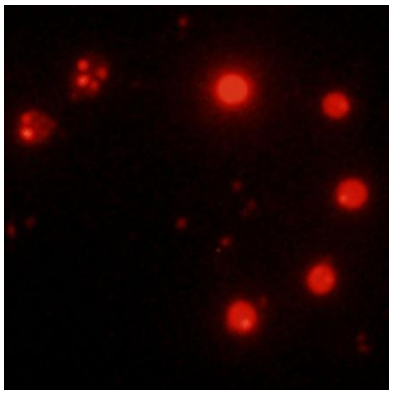
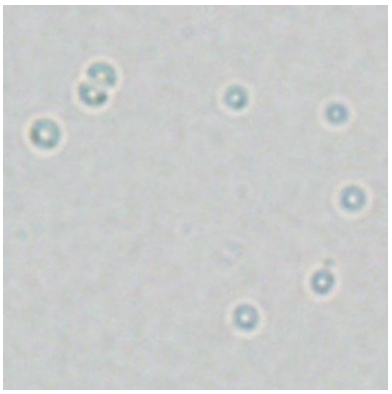
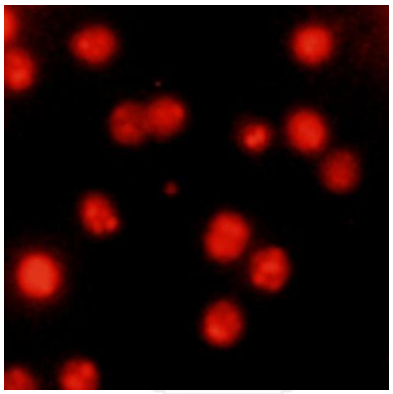
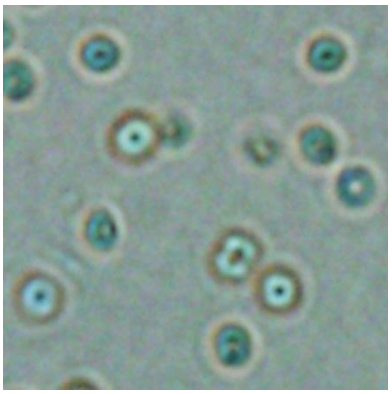
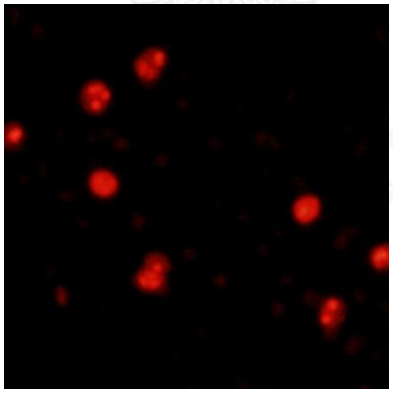
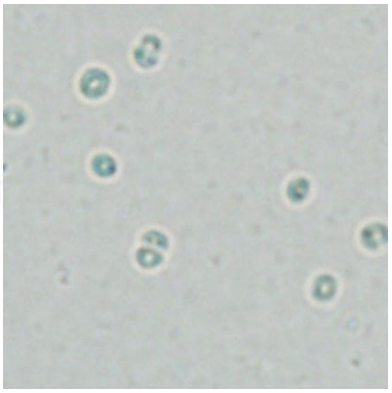
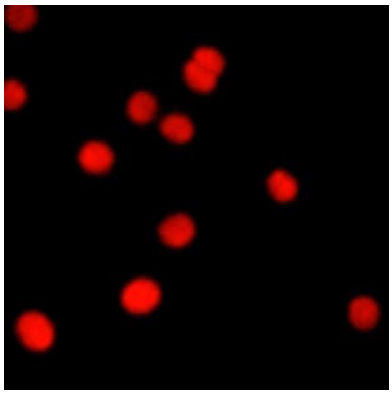
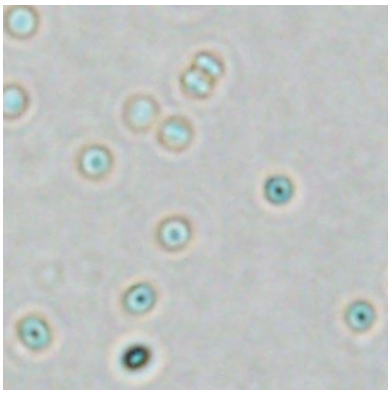
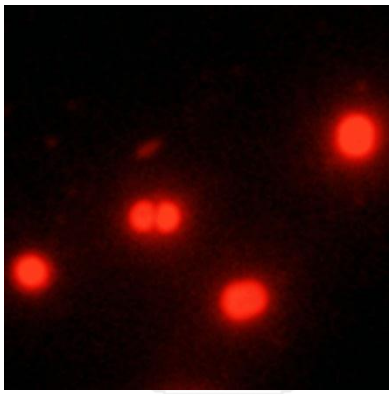
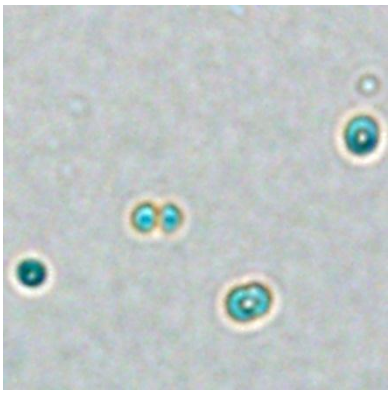
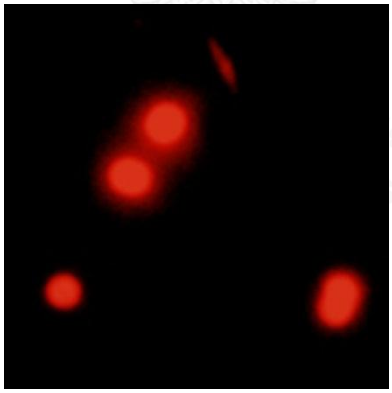
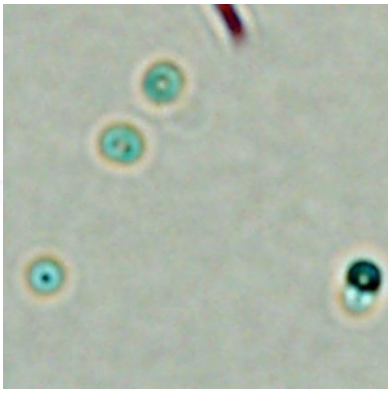
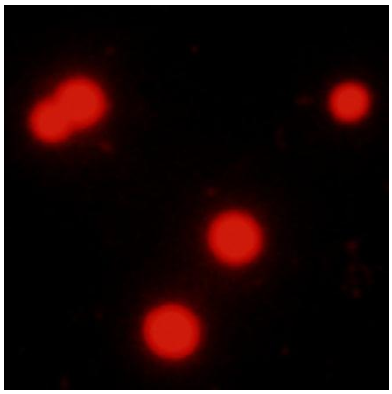
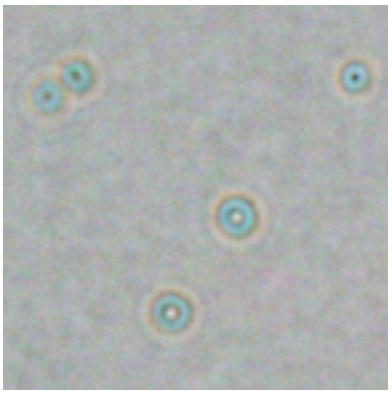
BG_{11}-N-P+A	**Nile red staining**	**Light microscope**
day 7		
day 9		
day 11		

Figure 22 Images of Nile-red stained cells of *Synechocystis* sp. PCC 6803 wild type under BG_{11}-N-P+A medium. Cells were stained by Nile red solution and visualized under fluorescent microscope with a magnification of 100x

BG_{11}-N-P+G	**Nile red staining**	**Light microscope**
day 0		
day 1		
day 3		
day 5		

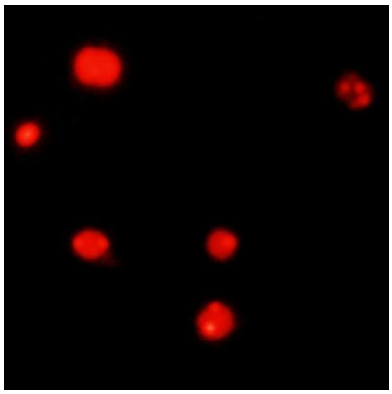
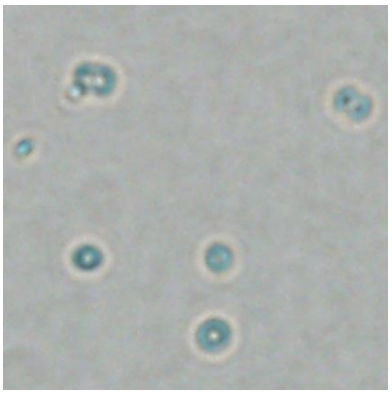
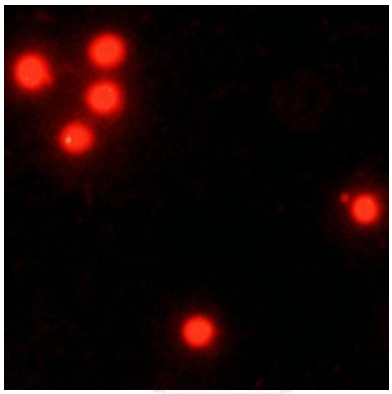
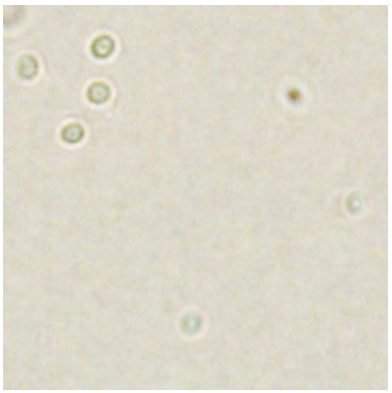
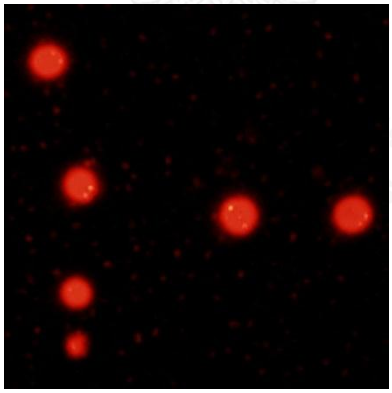
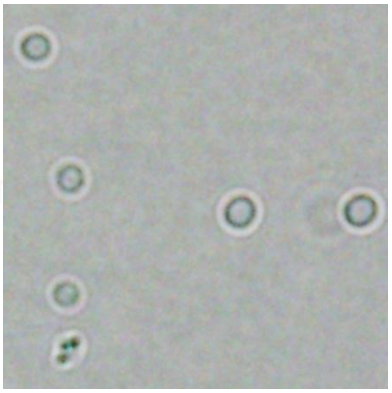
BG_{11}-N-P+G	Nile red staining	Light microscope
day 7		
day 9		
day 11		

Figure23 Images of Nile-red stained cells of Synechocystis sp. PCC 6803 wild type under BG11-N-P+G medium. Cells were stained by Nile red solution and visualized under fluorescent microscope with a magnification of 100x

3.10 Nile red staining of *Synechocystis* PCC6803 mutant *(Δadc1)* cells

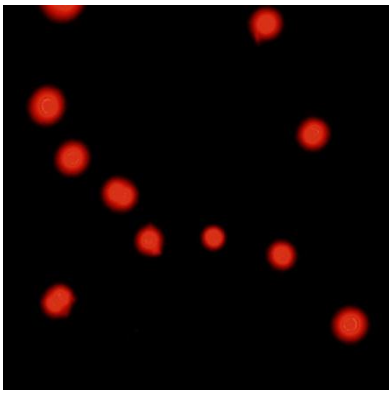
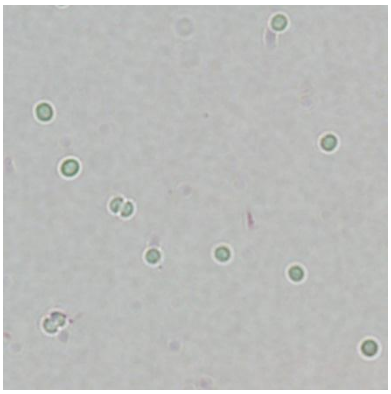
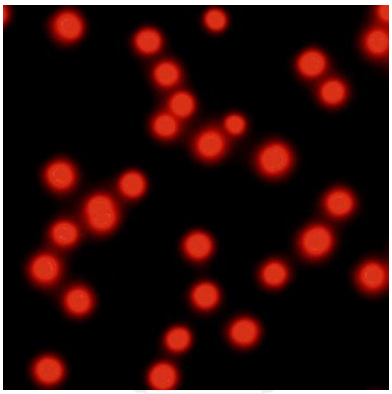
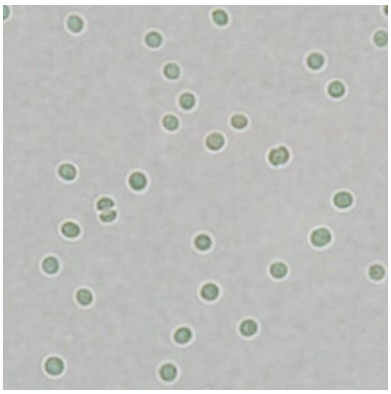
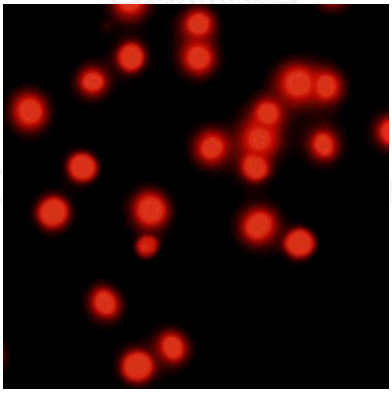
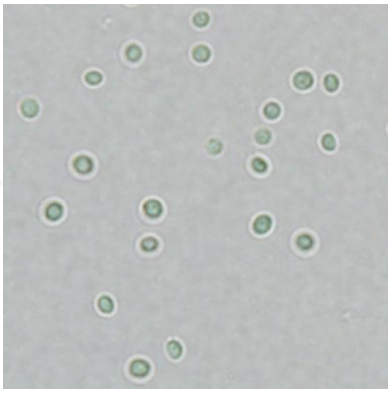
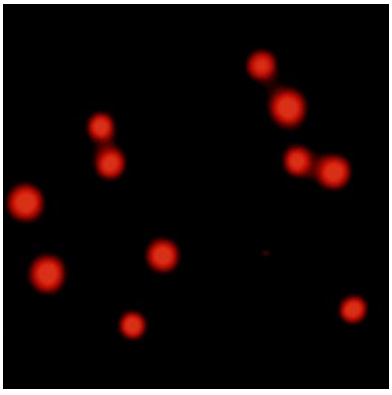
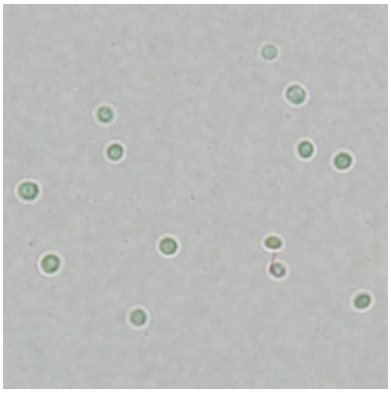
From Figure 24-27, *Synechocystis* sp. PCC 6803 mutant *(Δadc1)* were grown under BG_{11} medium and later stained with Nile red solution and monitored by fluorescent microscope. There was no any golden-yellow spots founded under normal condition in *Δadc1* mutant cells.

From Figure 25, *Δadc1* cells adapted under BG_{11}-N-P condition accumulated widespread of PHB granules dispersed nearly whole cells. Starting from day 7 of treatment, the PHB granules were clearly observed when compared with those under normal condition in Figure 24.

From Figure 26, BG_{11}-N-P+A condition obviously induced the accumulation of PHB granules in whole cells. At day 7 of treatment, the most neutral lipid granules were closely existed inside granules when compared with another days in the same condition. The higher amount of golden-yellow granules was shown apparently than WT cells under BG_{11}-N-P+A condition (Figure 22).

From Figure 27, *Δadc1*cells adapted under BG_{11}-N-P+G condition formed unclear golden-yellow spots at day 11 of treatment. This condition did not induce the accumulation of PHB granules.

From Figures 28, the highest amounts of PHB granules were observed at day 7 under BG_{11}-N-P+A treatment, followed by BG_{11}-N-P, BG_{11} control and BG_{11}-N-P+G, respectively. When comparing WT and mutant strains of *Synechocystis* sp. PCC 6803, Nile red stained cells under BG_{11}-N-P+A at day 7 of treatment of *Δadc1* dramatically accumulated neutral lipids or PHB granules higher than WT under the same condition.

BG_{11}	Nile red staining	Light microscope
day 0		
day 1		
day 3		
day 5		

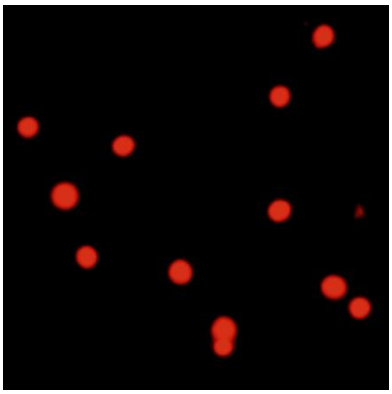
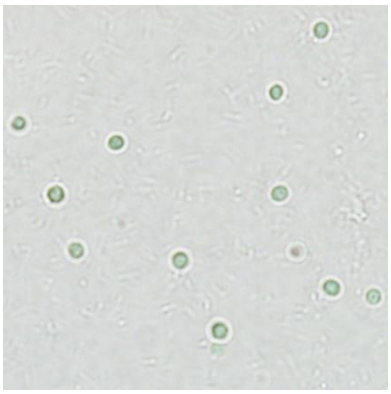
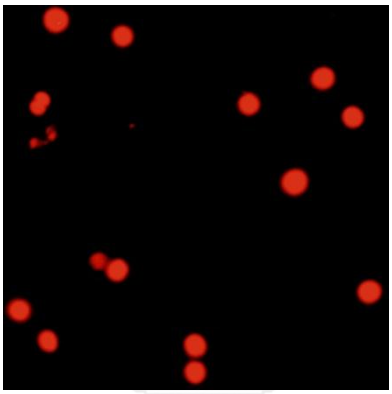
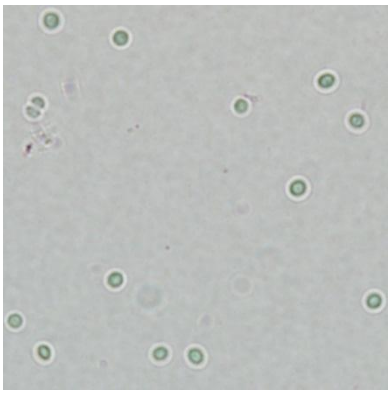
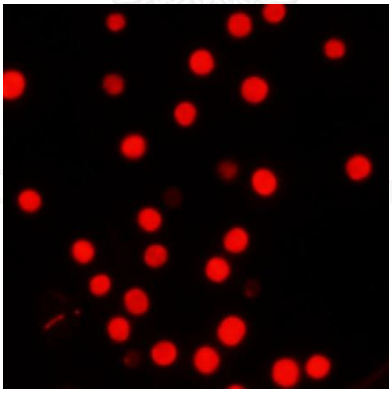
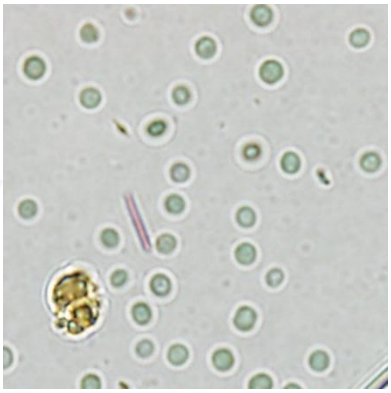
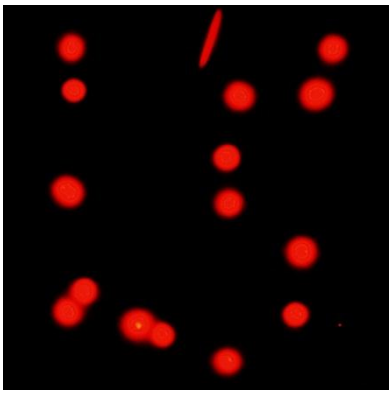
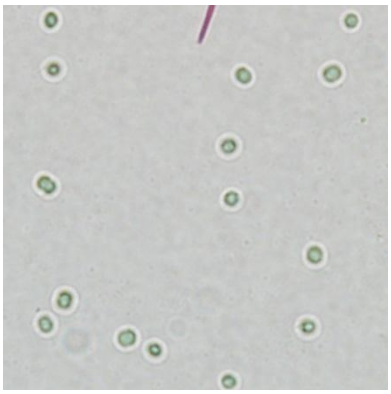
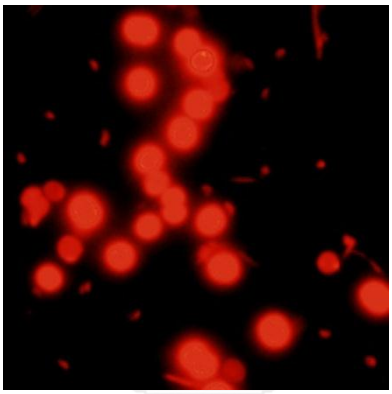
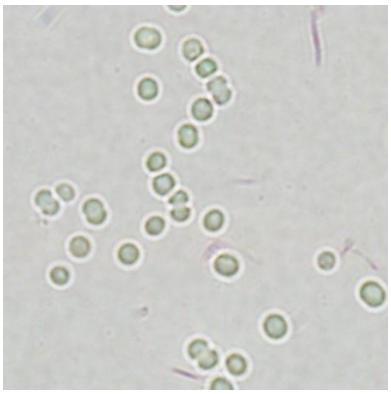
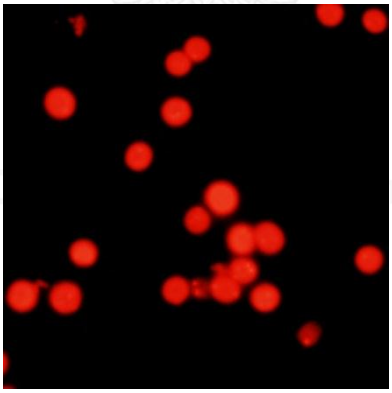
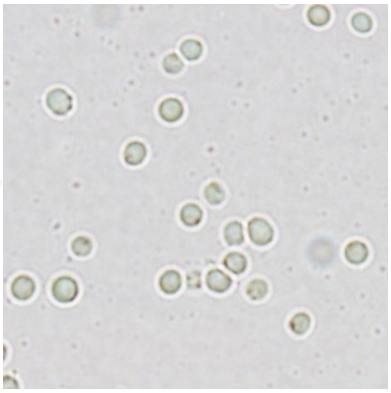
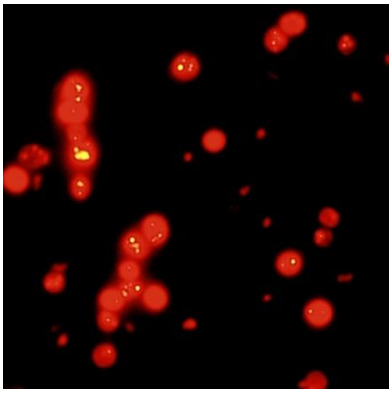
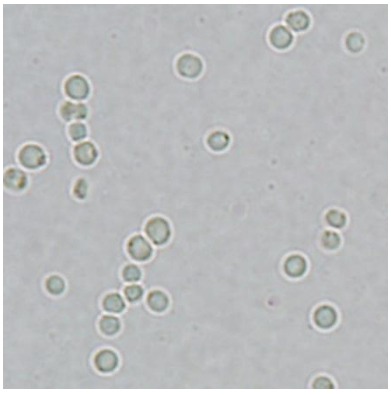
BG_{11}	Nile red staining	Light microscope
day 7		
day 9		
day 11		

Figure 24 Images of Nile-red stained cells of *Synechocystis* sp. PCC 6803 mutant *Δadc1* under BG_{11} medium as control. Cells were stained by Nile red solution and visualized under fluorescent microscope with a magnification of 100x

BG_{11}-N-P	**Nile red staining**	**Light microscope**
day 0		
day 1		
day 3		
day 5		

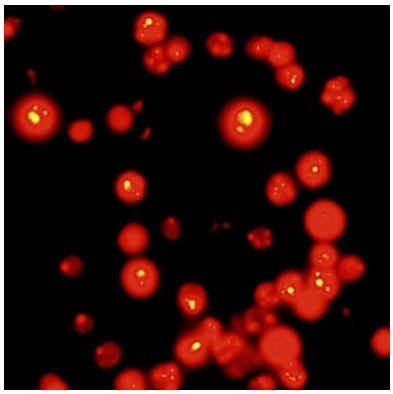
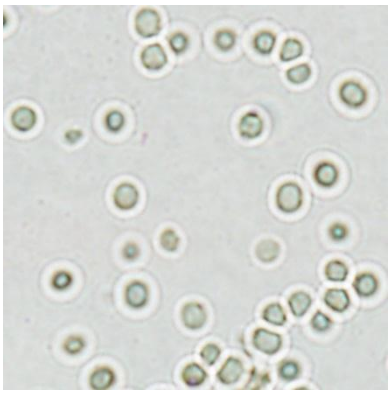
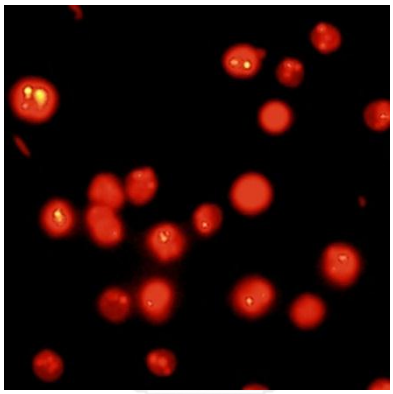
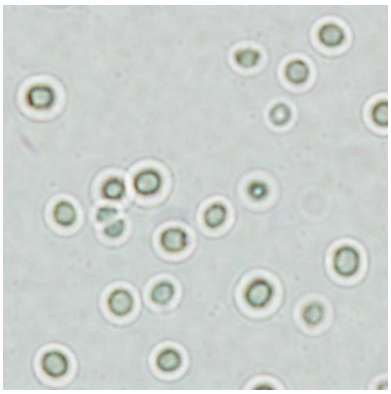
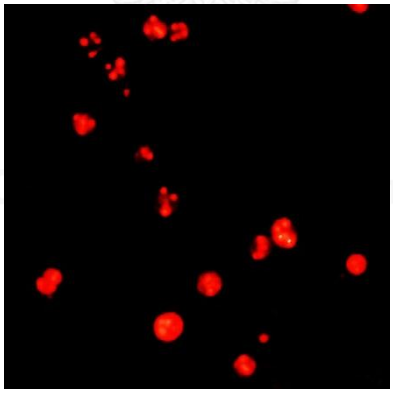
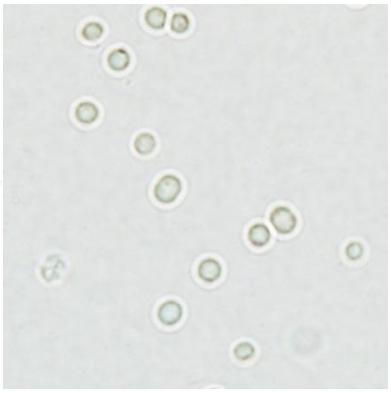
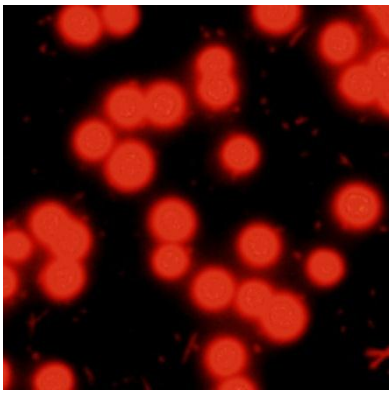
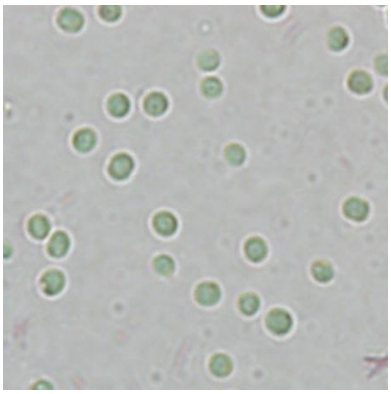
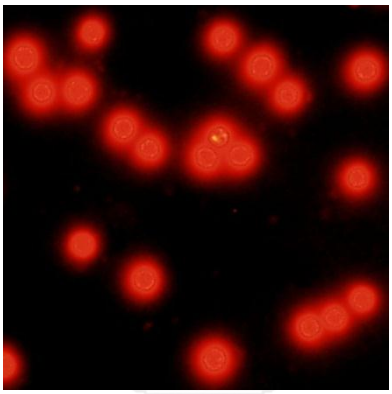
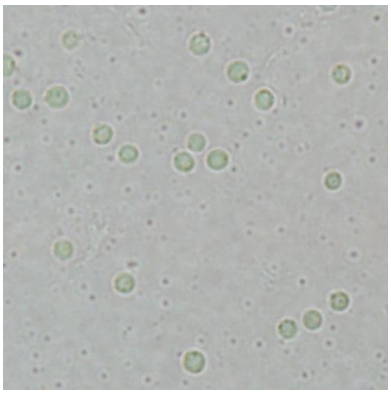
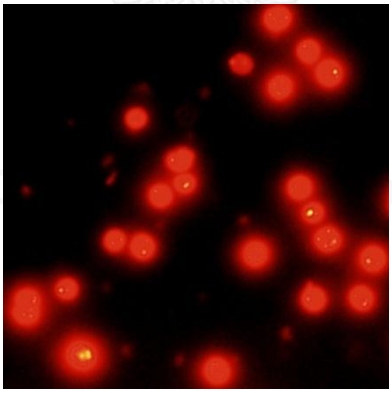
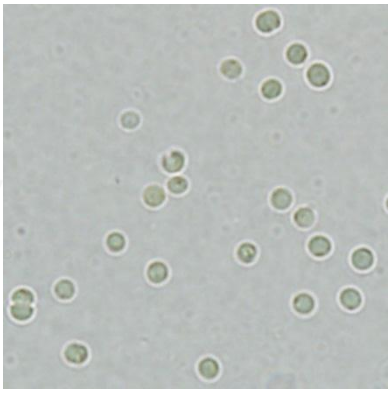
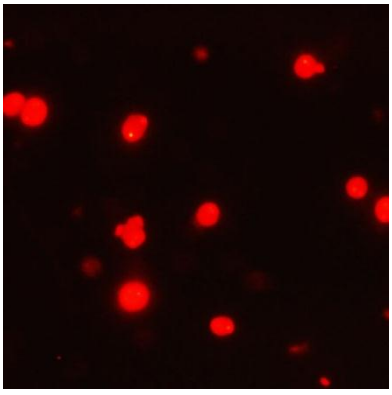
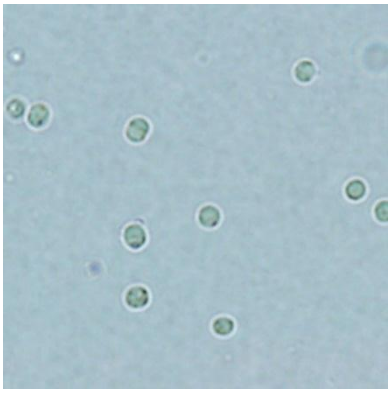
BG_{11}-N-P	**Nile red staining**	**Light microscope**
day 7		
day 9		
day 11		

Figure 25 Images of Nile-red stained cells of *Synechocystis* sp. PCC 6803 mutant *Δadc1* under BG_{11}-N-P medium, Cells were stained by Nile red solution and visualized under fluorescent microscope with a magnification of 100x

BG_{11}-N-P+A	**Nile red staining**	**Light microscope**
day 0		
day 1		
day 3		
day 5		

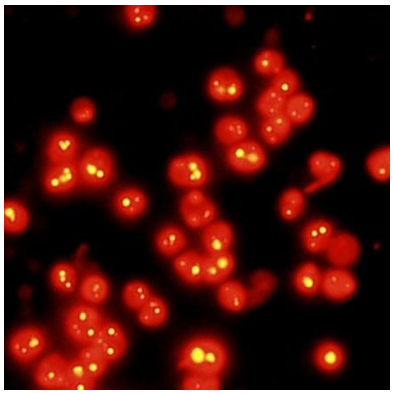
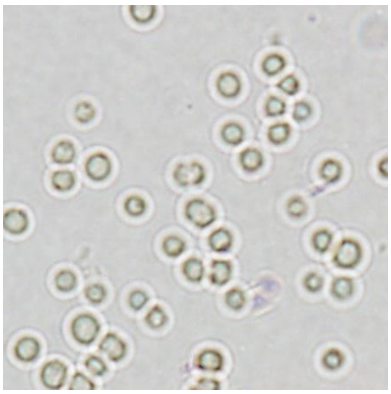
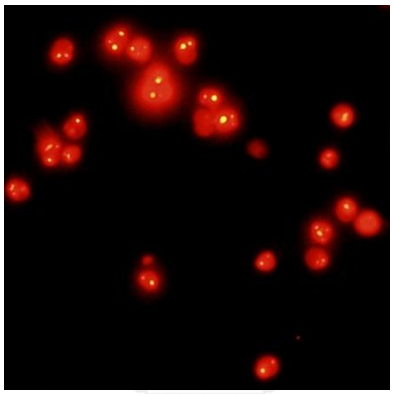
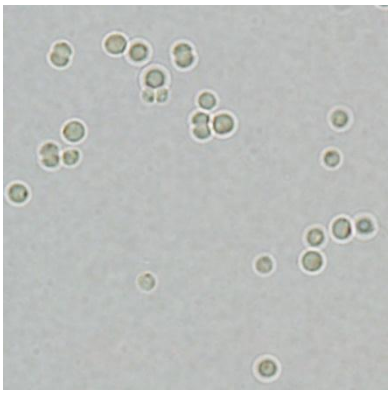
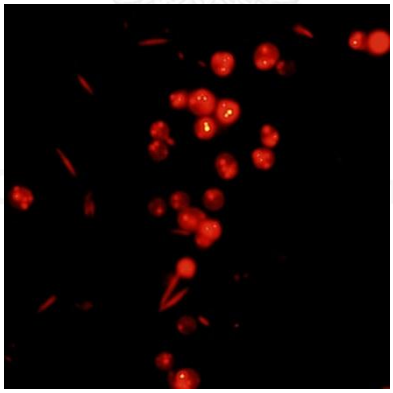
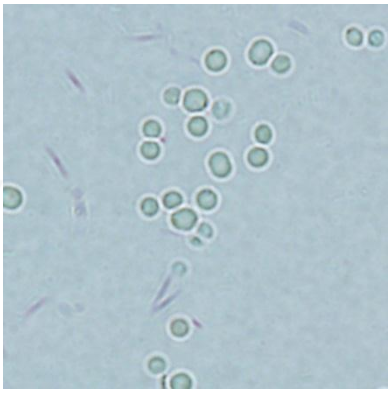
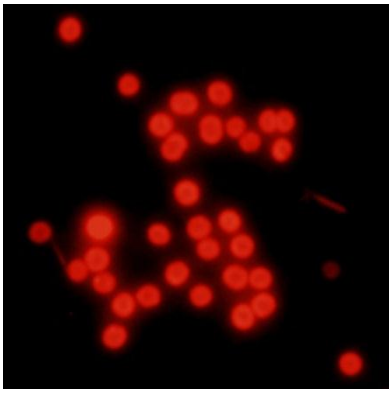
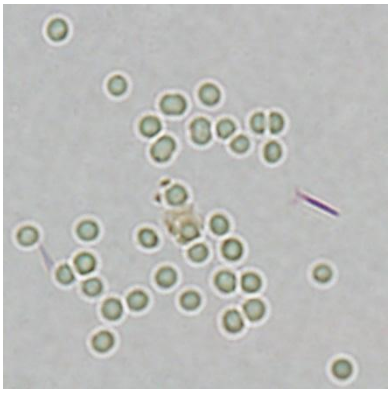
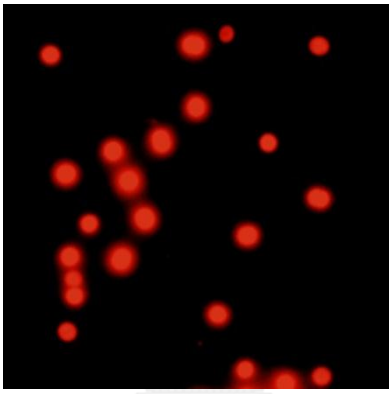
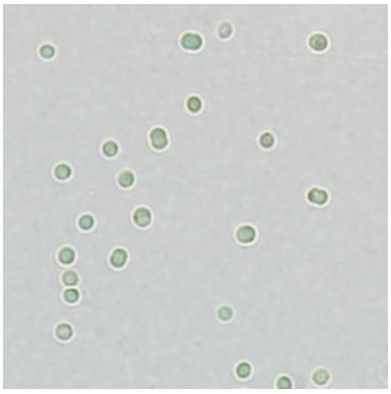
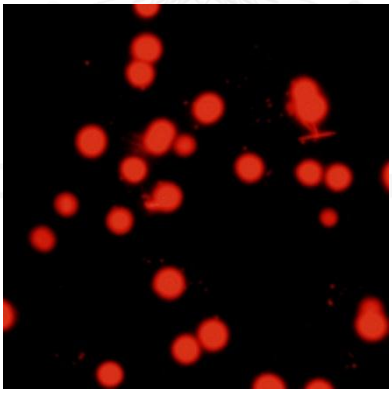
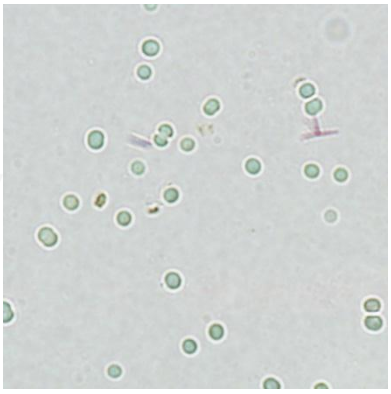
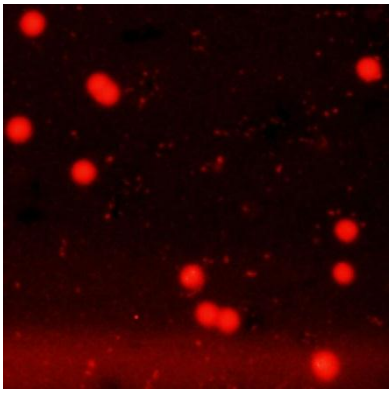
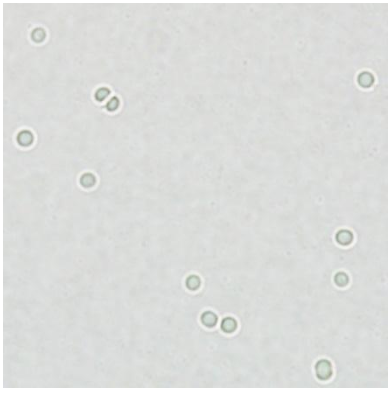
BG_{11}-N-P+A	Nile red staining	Light microscope
day 7		
day 9		
day 11		

Figure26 Images of Nile-red stained cells of *Synechocystis* sp. PCC 6803 mutant *Δadc1* under BG_{11}-N-P+A medium. Cells were stained by Nile red solution and visualized under fluorescent microscope with a magnification of 100x

BG_{11}-N-P+G	**Nile red staining**	**Light microscope**
day 0		
day 1		
day 3		
day 5		

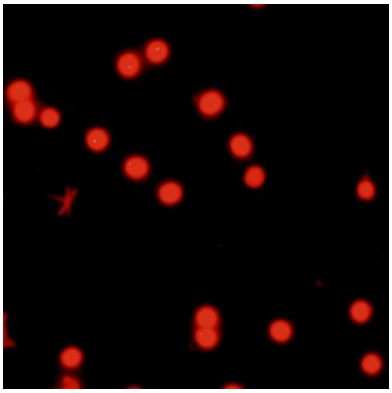
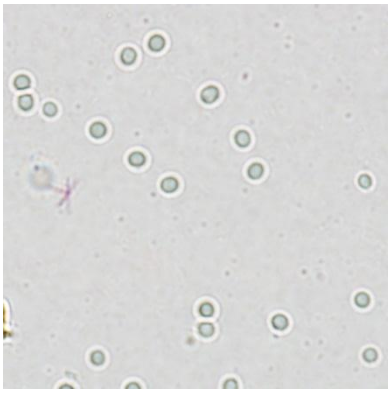
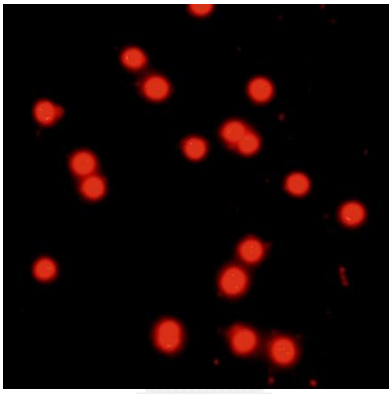
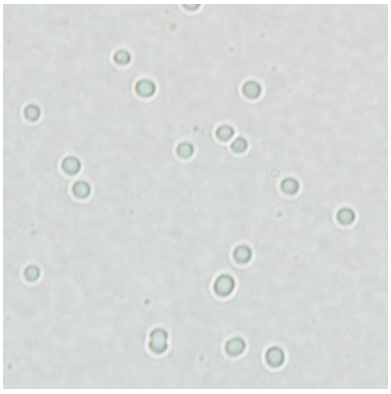
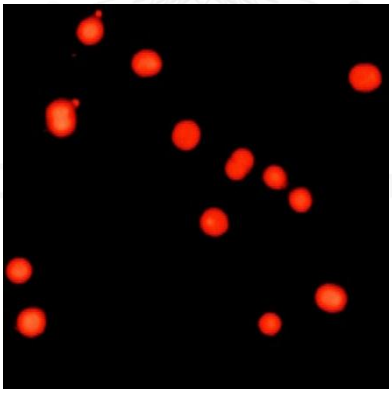
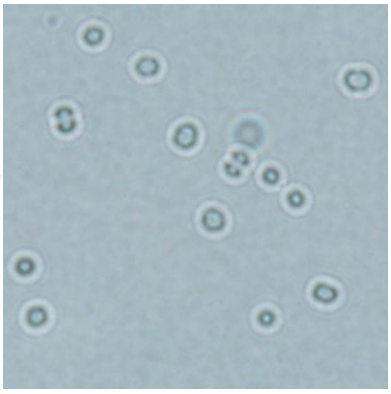
BG_{11}-N-P+G	Nile red staining	Light microscope
day 7		
day 9		
day 11		

Figure 27 Images of Nile-red stained cells of Synechocystis sp. PCC 6803 mutant *Δadc1* under BG11-N-P+G medium. Cells were stained by Nile red solution and visualized under fluorescent microscope with a magnification of 100x

3.11 Nile red staining of Synechocystis sp. PCC 6803 wild-type cells under UV radiation

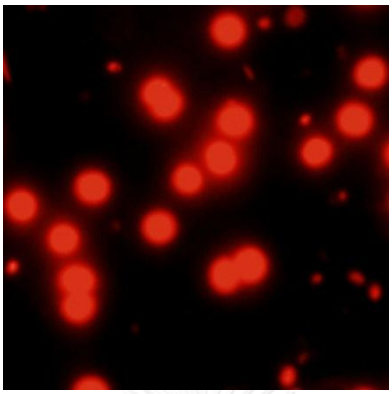
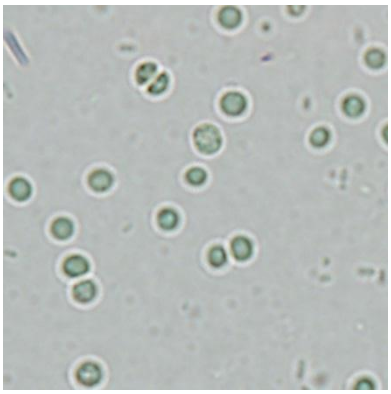
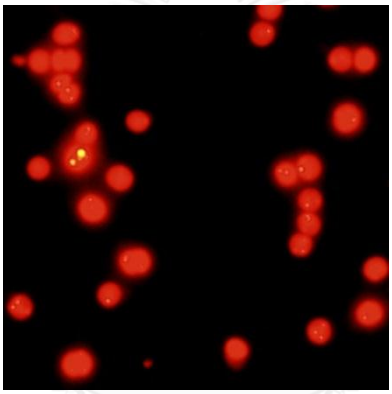
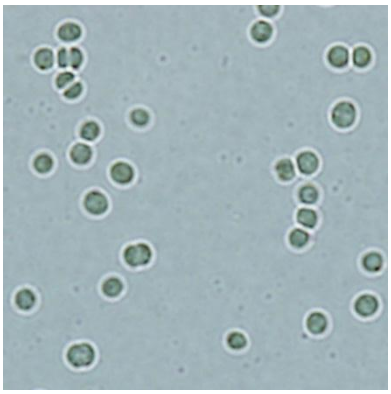
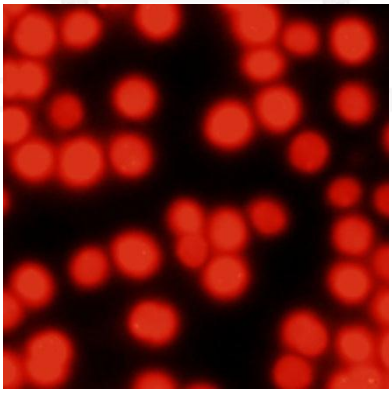
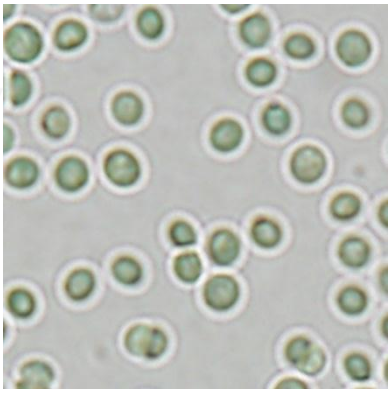
According to Figures 28-31, *Synechocystis* sp. PCC 6803 wild type (WT) cells were treated by various UV radiation including UV-A, UV-B and UV-C, respectively, compared with normal growth light as control and further stained with Nile red solution. The highest amounts of golden-yellow spots in granules were observed under UV-C treatment at 12 hours, followed by UV-A treatment at 3 hours and UV-B treatment at 6 hours, respectively.

From Figure 28, UV-A treated cells slightly induced PHB granules which occurred at 3 and 6 hours of treatments.

Cells treated with UV-B radiation (Figure 29) accumulated apparently PHB granules at 6 and 12 hours of treatments.

In Figure 30, UV-C radiation obviously induced the amounts of PHB granules at 12 hours of treatment. However, there also showed few of them at 3 and 6 hours of treatments.

WT cells adapted in BG_{11} medium under normal growth light (Figure 31) rarely accumulated PHB granules although few of them could be seen at 3, 6 and 12 hours.

UV-A	Nile red staining	Light microscope
0 hr		
3hrs		
6hrs		

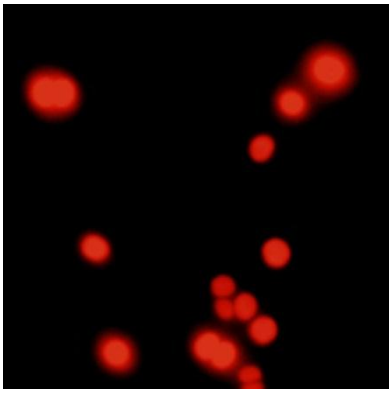
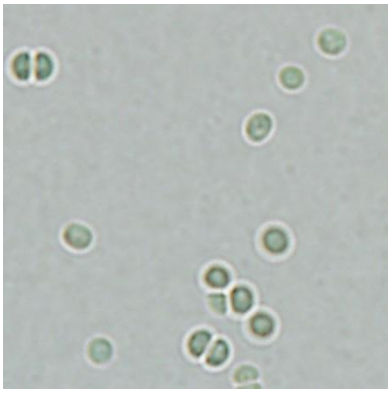
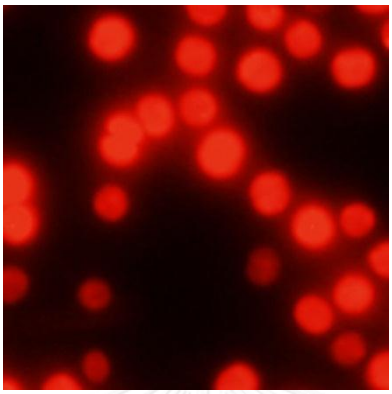
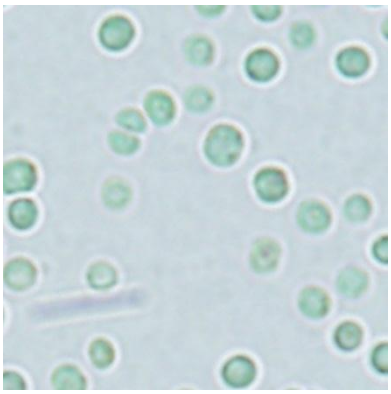
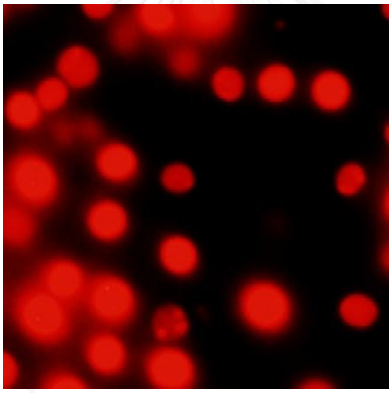
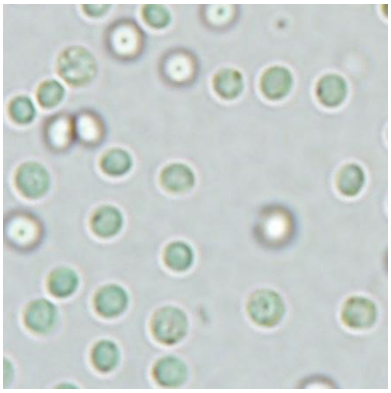
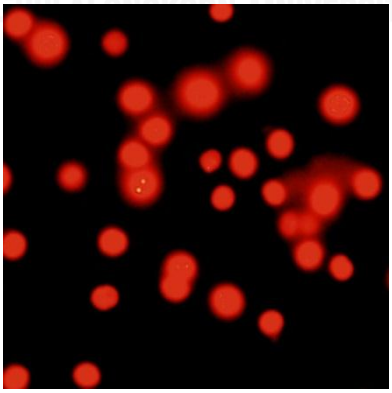
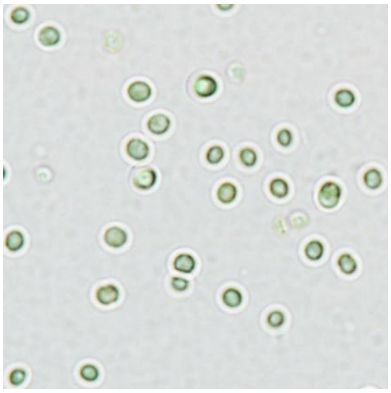
UV-A	Nile red staining	Light microscope
12hrs		

Figure 28 Images of Nile-red stained cells of *Synechocystis* sp. PCC 6803 wild type under UV-A radiation. Cells were stained by Nile red solution. The fluorescent microscope with a magnification of 100x was used for visualization.

UV-B	Nile red staining	Light microscope
0 hr		
3hrs		
6hrs		

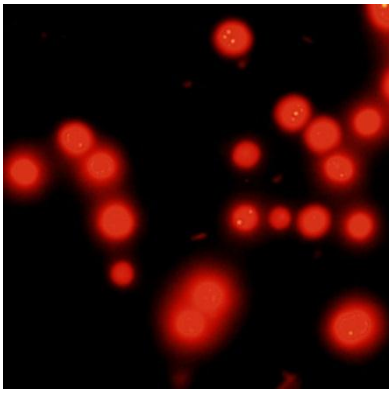
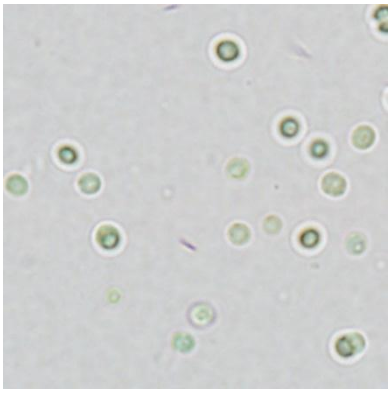
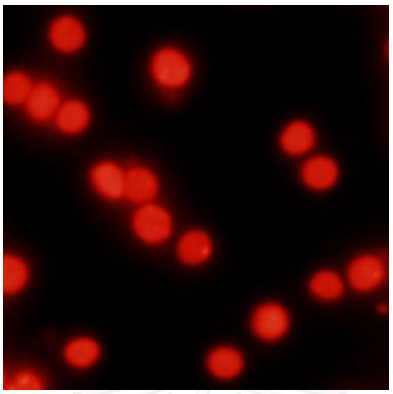
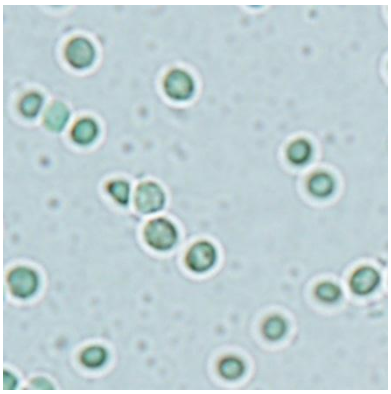
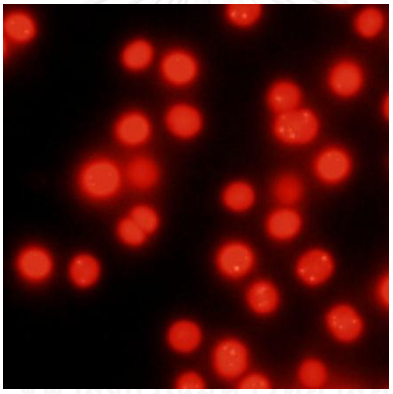
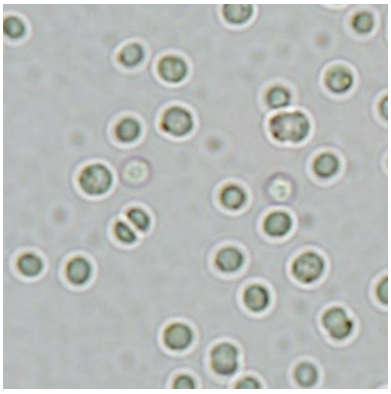
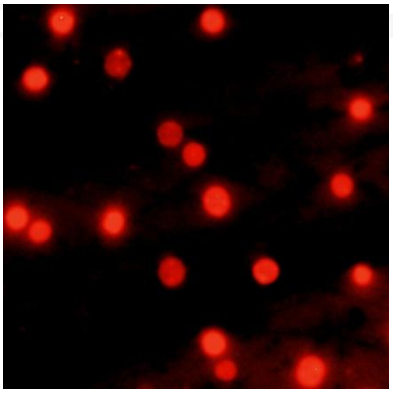
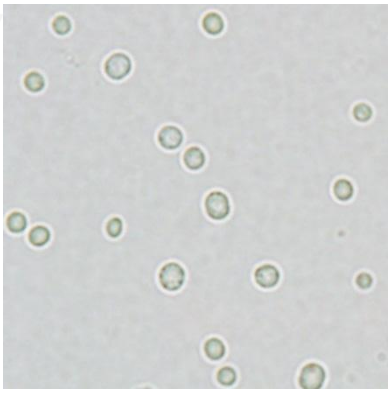
UV-B	Nile red staining	Light microscope
12hrs		

Figure 29 Images of Nile-red stained cells of *Synechocystis* sp. PCC 6803 wild type under UV-B radiation. Cells were stained by Nile red solution. The fluorescent microscope with a magnification of 100x was used for visualization.

UV-C	Nile red staining	Light microscope
0 hr		
3hrs		
6hrs		

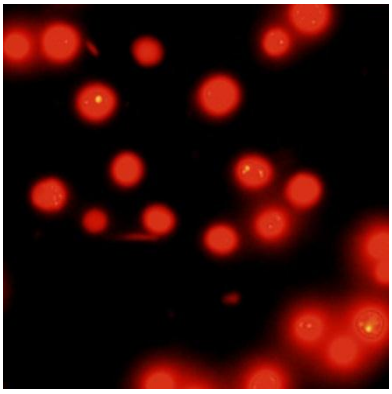
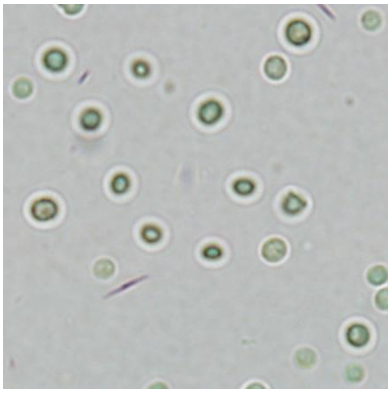
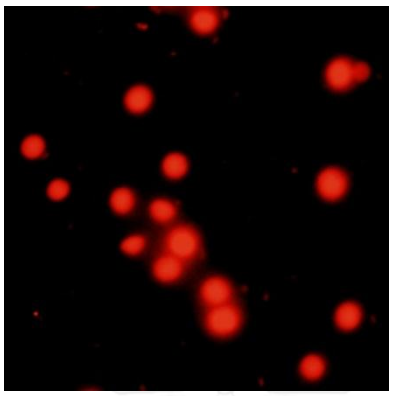
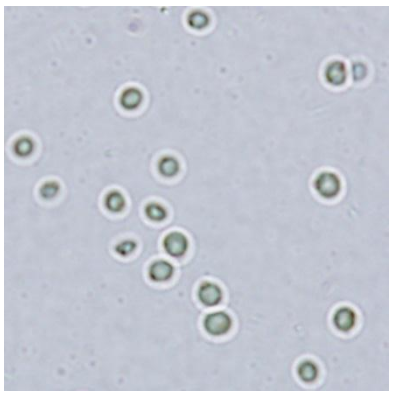
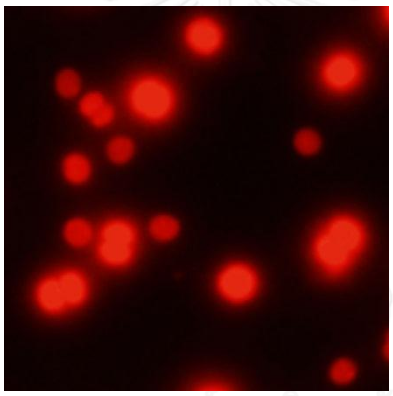
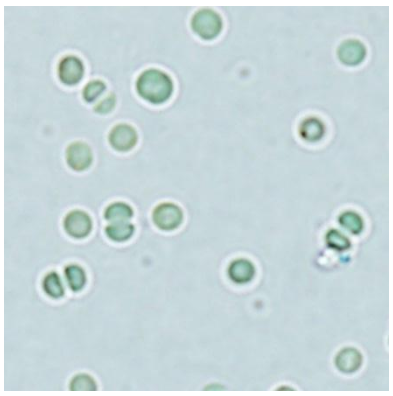
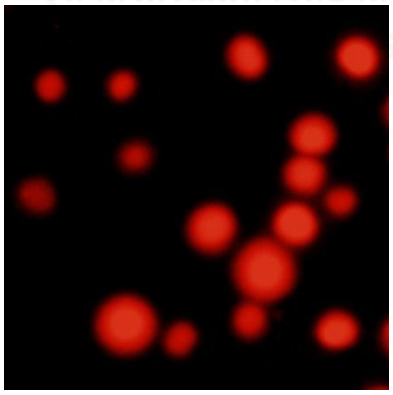
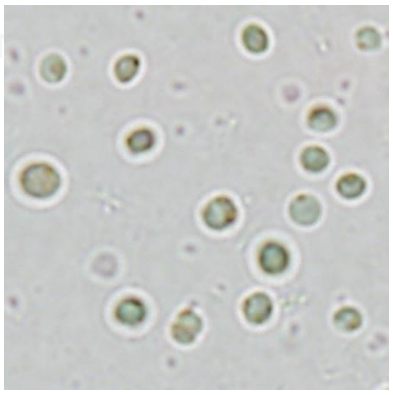
UV-C	Nile red staining	Light microscope
12hrs		

Figure 30 Images of Nile-red stained cells of *Synechocystis* sp. PCC 6803 wild type under UV-C radiation. Cells were stained by Nile red solution. The fluorescent microscope with a magnification of 100x was used for visualization.

Normal light	Nile red staining	Light microscope
0 hr		
3hrs		
6hrs		

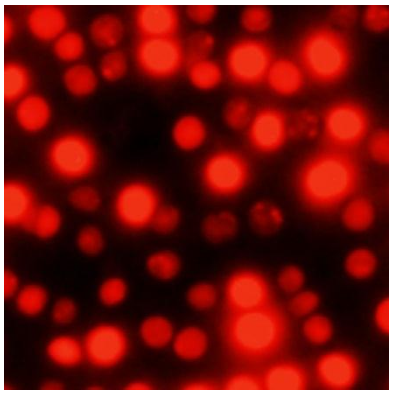
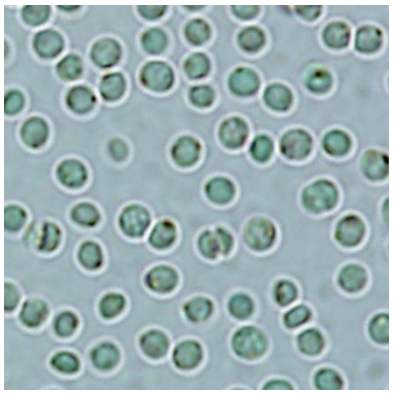
Normal light	Nile red staining	Light microscope
12hrs		

Figure 31 Images of Nile-red stained cells of *Synechocystis* sp. PCC 6803 wild type under normal growth light as control. Cells were stained by Nile red solution and visualized under fluorescent microscope with a magnification of 100X.

3.12 PHB contents in *Synechocystis* sp. PCC 6803 wild type cells

Synechocystis sp. PCC6803 (WT) cells with late-log phase of growth were adapted with various nutrient modified treatments, and further harvested for monitoring PHB accumulation and detection. The PHB contents under various nutrient treatments were shown in Figure 32. Under normal BG_{11} medium condition, the PHB content was constant along 11 day-treatment. For BG_{11}-N-P treatment, the higher PHB content was observed from day 5 to day 11 of treatment whereas BG_{11}-N-P+G increased PHB contents from day 7 to 11 of treatment. The highest content of PHB was observed in WT cells under BG_{11}-N-P+A condition with an average of 24.95%PHB/DCW at day 9 of treatment.

3.13 PHB contents in *Synechocystis* sp. PCC 6803 mutant cells lacking *adc1* gene

The highest PHB content of *Δadc1* cells obtained at day 7 of treatment under BG_{11}-N-P+A with an average of 36.07 % PHB/DCW (Figure33). The *Δadc1* cells significantly increased PHB content higher than WT cells of about 1.62 time increase in a shorter time. On the other hand, PHB contents under BG_{11}-N-P condition were increased significantly at day 7 and kept constantly up to day 11 of treatment. It was also found that glucose supplementation in BG_{11}-N-P+G condition could not enhance PHB content along 11 days of treatment.

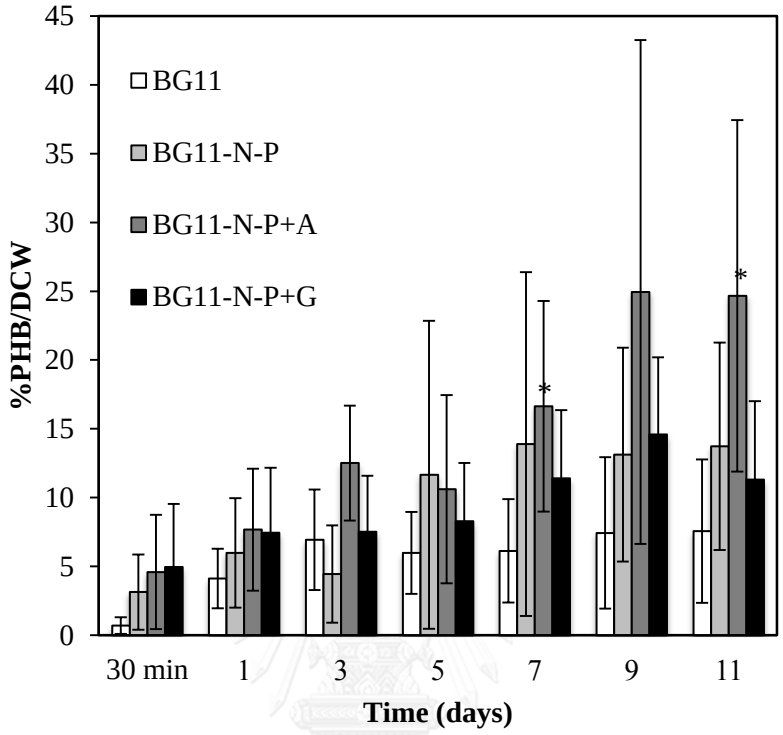


Figure 32. PHB contents in cyanobacterium *Synechocystis* sp. PCC 6803 wild type. Cells were adapted in various nutrients modified media and further extracted for PHB content and detected by HPLC instrument. Data represent Mean ± S.D. (n=5)**.** (**P* < 0.05 compared with BG_{11} medium).

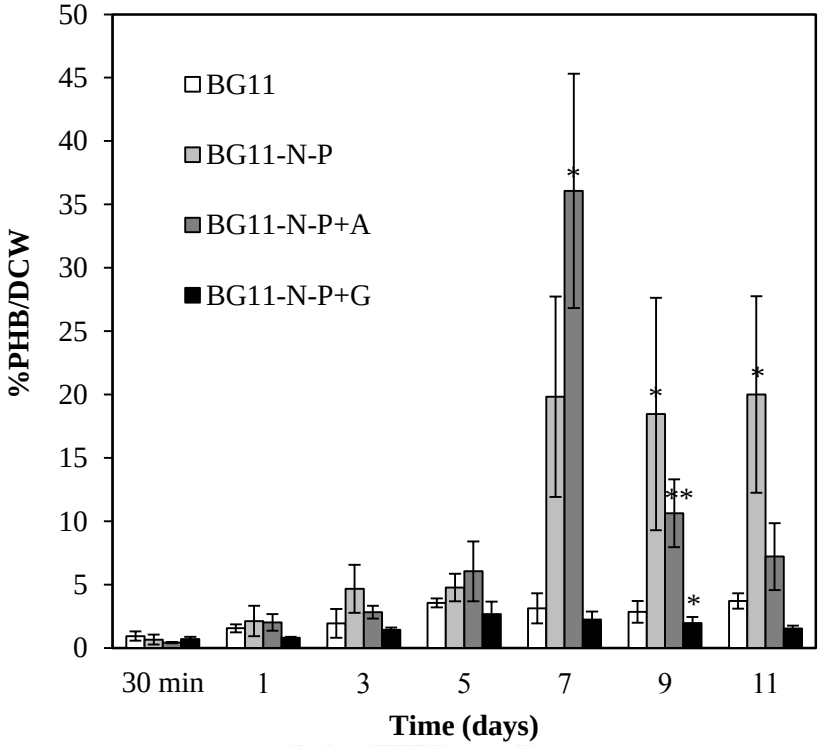


Figure 33 PHB contents in cyanobacterium *Synechocystis* sp. PCC 6803 *Δadc1* cells. Cells were adapted in various nutrients modified media and further extracted for PHB content and detected by HPLC instrument. Data represent Mean ± S.D. (n=4). (* $P < 0.05$, ** $P < 0.01$ compared with BG_{11} medium).

3.14 PHB contents of *Synechocystis* sp. PCC 6803 wild type cells under UV treatment

Synechocystis sp. PCC 6803 WT cells were also treated with three types of UV radiations and further harvested for PHB extraction and detection. The results of PHB contents in WT cells treated by UVs were shown in Figure 34. UV-A and UV-B radiations induced the PHB contents at 3 and 6 hours of treatment whereas UV-C radiation significantly increased the PHB content at 6 hours treatment of about 13.69 %PHB/DCW.

3.15 Expression of genes related to PHB biosynthetic pathways from *Synechocystis* sp. PCC 6803 wild type

The PHB biosynthetic gene expressions of *phaA, phaB, phaC* and *phaE* in *Synechocystis sp.* PCC 6803 WT cells are shown in Figure 35. It was interesting that *phaC* transcript level was highest among those *pha* genes under all conditions studied. *phaA* transcript levels were not changed under BG_{11} control, BG_{11}-N-P and BG_{11}-N-P+A whereas the decrease was observed under BG_{11}-N-P+G. For *phaB* transcript, its level was slightly increased by BG_{11}-N-P condition. The *phaE* transcript amounts showed no differences under all conditions studied.

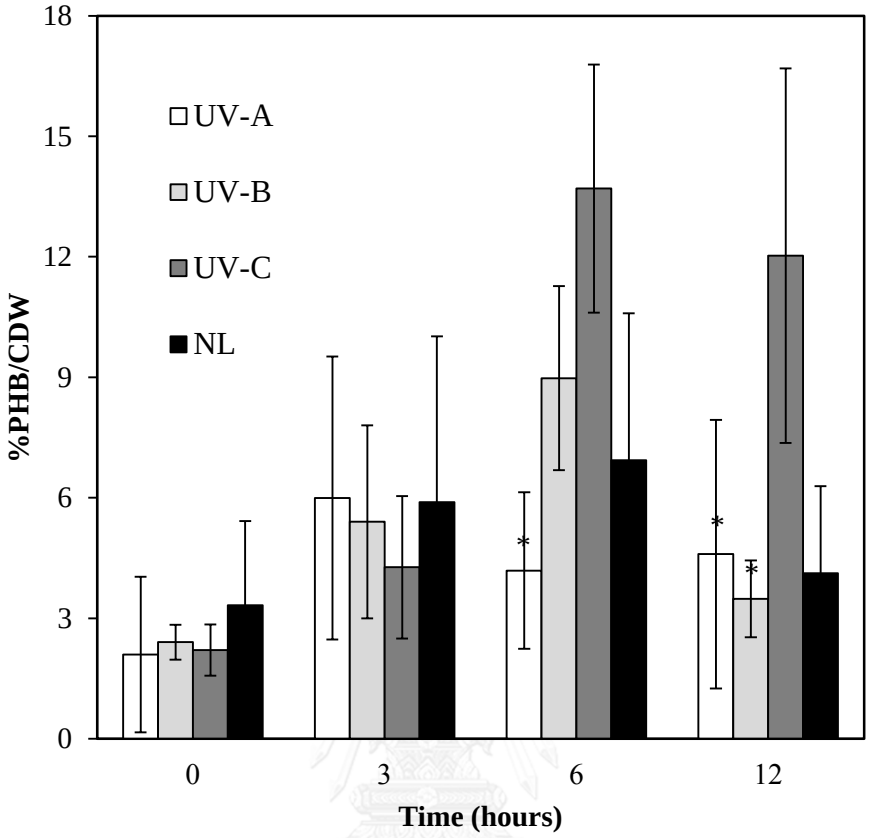


Figure 34 PHB contents in cyanobacteria *Synechocystis* sp. PCC 6803 WT cells after treating with UV-A, UV-B, UV-C radiations and normal growth light (NL). Treated cells were harvested for further PHB extraction and detection by HPLC. Data represent Mean ± S.D. (n=5)**.** (* $P < 0.05$ compared with BG_{11} medium).

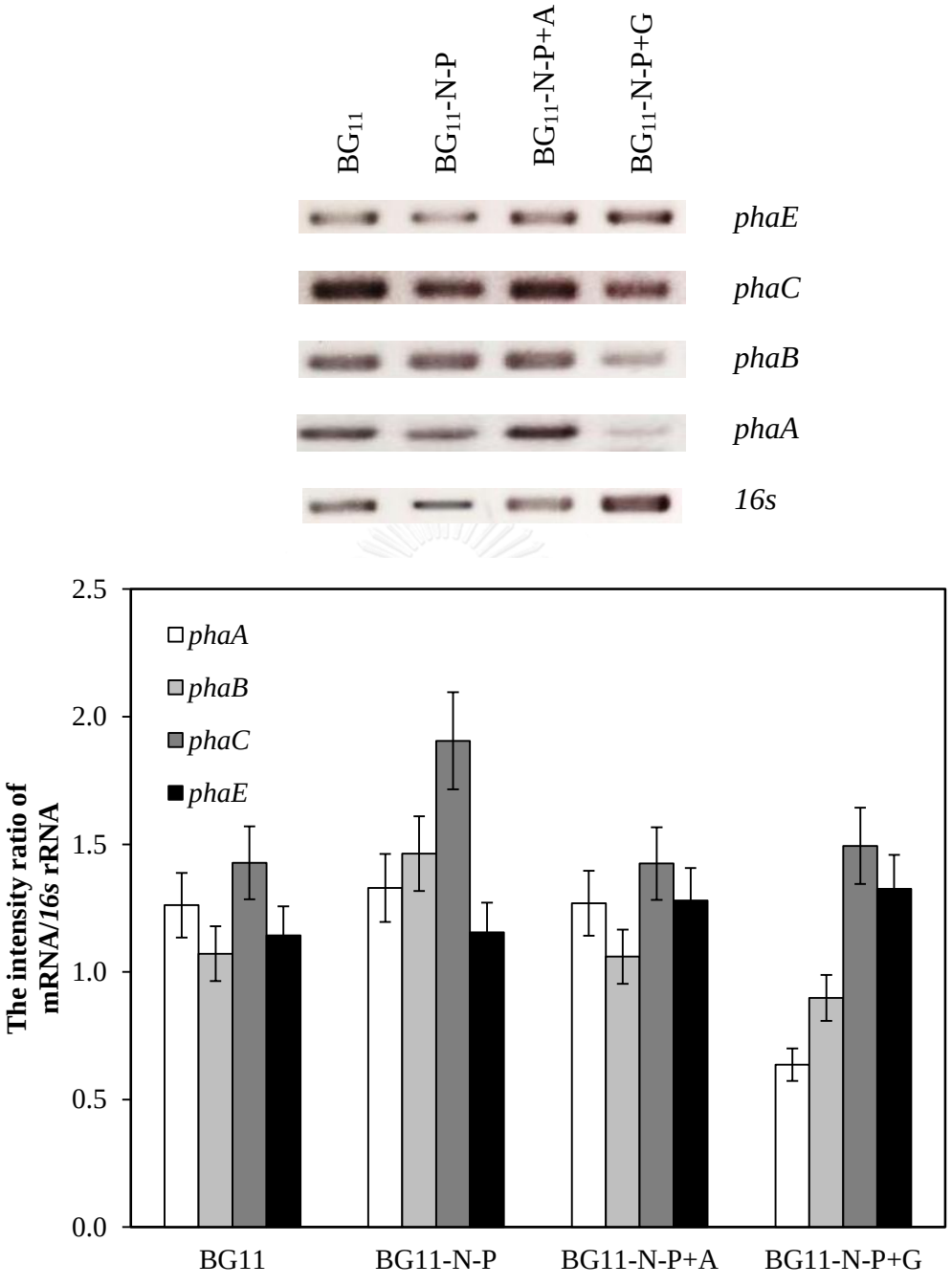


Figure 35 Expressions of *phaA, phaB, phaC* and *phaE* transcripts in *Synechocystis* sp. PCC 6803 WT cells under BG_{11}, BG_{11}-N-P, BG_{11}-N-P+A, and BG_{11}-N-P+G, respectively, at 9-day treatment. The *16s* transcript was used as the reference. Data represent Mean ± S.D. (n=3)

3.16 Expression of genes related to PHB biosynthetic pathways from *Synechocystis* sp. PCC 6803 mutant

Gene expressions of PHB-biosynthetic pathways in *Δadc1* mutant were shown in Figure 36. The *phaC* mRNA showed in highest levels under all conditions. *PhaA* transcripts were not different under all conditions whereas *phaB* transcripts was decreased under BG_{11}-N-P+G. On the other hand, the constant levels of *phaE* transcripts were also observed when compared with all conditions studied.

When we compared the intensity ratio between WT and mutant (as shown in Figure 37). Under normal BG_{11} condition (Figure 37A), all *pha* transcripts were not different between WT and mutant. On the other hand, *phaC* and *phaE* transcripts levels of mutant were increased under BG_{11}-N-P condition when compared to those of WT cells (Figure 37B). The *phaC* transcript level of mutant was insignificantly increased when compared to that of WT whereas there were no significant changes for *phaA* and *phaE* transcript levels (Figure 37C). In contrast, the levels of *phaA*, *phaC* and *phaE* transcripts of mutant strain showed increases under BG_{11}-N-P+G condition compared to those of WT (Figure 37D).

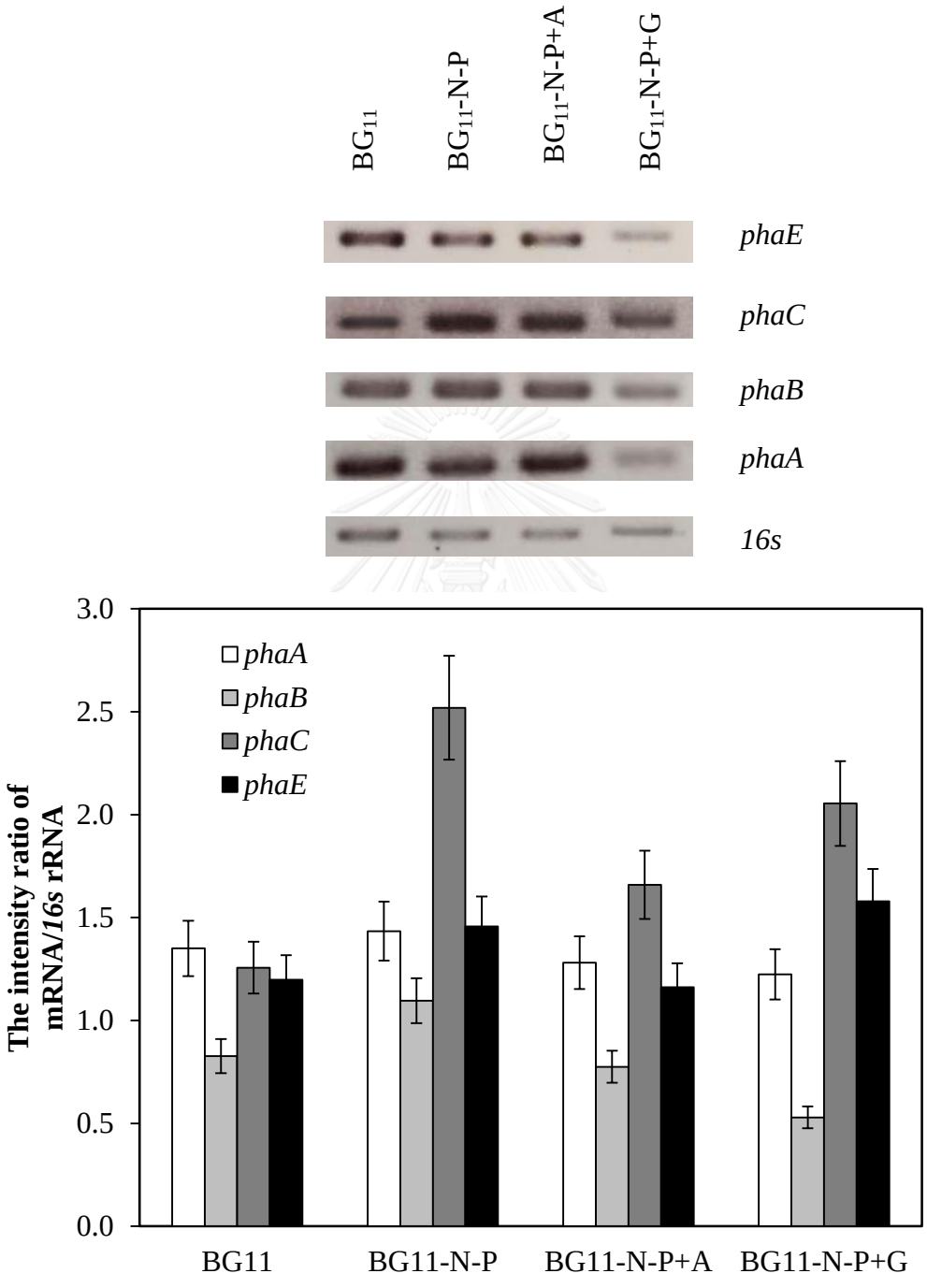


Figure 36 Expressions of *phaA,phaB, phaC* and *phaE* transcripts in *Synechocystis* sp. PCC 6803 mutant *(⊿adc1)* under BG_{11}, BG_{11}-N-P, BG_{11}-N-P+A, and BG_{11}-N-P+G, respectively, at 7-day treatment. The *16s* transcript was used as the reference. Data represent Mean ± S.D. (n=3)

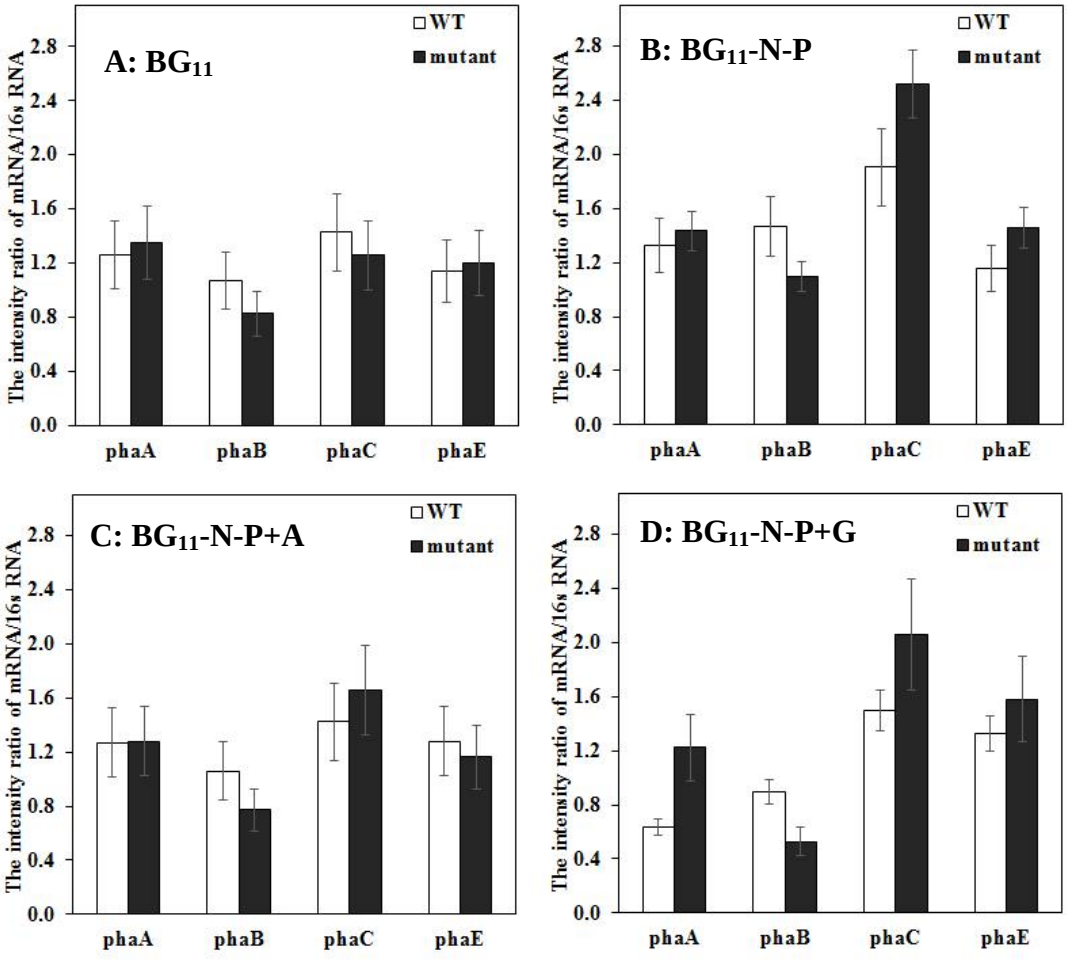


Figure 37 Comparison of intensity ratio of gene transcripts of *phaA, phaB, phaC* and *phaE* between *Synechocystis* sp. PCC 6803 wild type (data from Figure 36) and mutant (data from Figure 36) at the treatment day obtained highest PHB amount. The error bar was set at 20%.

CHAPTER IV

Discussion

4.1 Growth, pigment contents and oxygen evolution of wild type and mutant cells

Based on our study, we divided stages of cell culture for PHB production into two stages. First stage was defined as "growth cultivation" which needed to obtain cell biomass sufficient for PHB production. The second stage was then defined as "PHB induction". We demonstrated that growth cultivation chosen for further PHB induction stage was 15 day-culture which cell growth was reached to late-log phase of growth. Although the growth phase of *⊿adc1* mutant cells at 15 day-culture was not their late-log phase, we decided to harvest samples in the same period of wild type. In *Nostoc muscorum*, it was found that the highest PHB production obtained from cells grown in stationary phase (Sharma and Mallick, 2005). Panda et al. (2006) used cells grown about stationary phase-21 day-culture in normal medium for further PHB induction under nutrient deficient conditions in order to create starvation condition which exerted cells to accumulate energy reserves, herein PHB granules. In second stage of PHB induction, late-log phase cells were transferred into BG_{11} medium lacking some nutrients including nitrogen and phosphorus. Nitrogen and phosphorus are important minerals for most microorganisms, even in cyanobacteria (Rippka et al., 1979). Miyake and co-workers (1997) found that when the cells were transferred into BG_{11}-N (nitrogen deficiency), cell growth was ceased. In our study, although there was glucose in BG_{11}-N-P+G condition, cell growths of both WT and mutant were decreased. It was contrast with a report from Wu et al. (2002) that the addition of

glucose in BG_{11} significantly stimulated the cell growth. On the other hand, the supplementation of acetate in BG_{11}-N-P+A condition also showed decreases in both WT and mutant cells which was agreeable with a report of Wu et al. (2002) that the addition of acetate did not show the stimulation to cell growth.

On the other hand, intracellular pigment contents including chlorophyll *a* and carotenoids (in Figures 15 and 16) showed the significant decreases under all nutrient conditions. The amounts of intracellular pigments were mainly corresponded to growth tendency. However, the addition of acetate into BG_{11}-N-P medium seemed to enhance the chlorophyll *a* and carotenoid accumulations in both WT and mutant cells whereas the addition of glucose into BG_{11}-N-P medium even decreased their intracellular pigments. Although we could observe the increases of intracellular pigments under BG_{11}-N-P+A condition compared to those of BG_{11}-N-P, the photosynthetic efficiency represented by oxygen evolution rate was decreased. This result was consistent with Wu et al. (2002) which revealed that the lower oxygen evolution was in reply of the addition of exogenous carbon sources.

4.2 Effect of nutrient modification on PHB content and expressions of gene related to PHB-biosynthetic pathway

PHB is the most widespread and thoroughly found in bacteria. It is accumulated as an energy storage material in various microorganisms usually under limiting nutritional conditions (Steinbuchel et al., 1992, Liebergesell et al., 1994, Yu 2001). Under nitrogen and phosphorus deficiency (BG_{11}–N-P condition), PHB contents in WT and *Δadc1* were increased up to 12.08 and 20.01 %PHB/DCW,

respectively. It was discussed previously that NADPH uptake under nitrogen deficiency was decreased due to limitation of nitrogen sources, which blocked the amino acid synthesis pathways, particularly the reaction from α-ketoglutarate to glutamate that could lead to a decrease on cell growth. This residual NADPH might be responsible then for the enhanced PHB accumulation in nitrogen-deficient cells (Panda et al., 2006). Under BG_{11}–N-P+A condition (nitrogen and phosphorus deficiency and addition of 0.4% acetate), it was found that induced PHB accumulations were up to 24.95 %PHB/DCW in wild type cells at 9 day-treatment whereas mutant *Δadc1* mutant gave up to 36.07 %PHB/DCW at 7 day-treatment. PHB accumulations showed the highest levels under BG_{11}–N-P+A condition of both strains. It was clearly observed that acetate supplementation efficiently boosted up PHB accumulation from induced PHB contents by BG11-N-P medium. This result was consistent with result in *Nostoc muscorum* which significantly raised PHB pool under acetate addition in light-dark cycle (Sharma and Mallick, 2005). The previous works in *Synechocystis* cells demonstrated that the acetate addition increased PHB content up to 19%/DCW in a *Synechocystis* mutant lacking ADP-glucose pyrophosphorylase, in glycogen biosynthesis, when compared to wild type cells with 10% per dry cell weight (Wu et al., 2002). A possible explanation for acetate advantage related to formed acetyl-CoA, the key substrate for PHB biosynthetic pathway (Wu et al., 2002). Under BG_{11}–N-P+G condition (nitrogen and phosphorus deficiency and addition of 0.4% glucose) in this study, PHB contents in WT and mutant were increased up to 6.36 and 2.24 %PHB/DCW, respectively. The addition of glucose did not enhance PHB accumulation due to *Synechocystis* cells could utilize

glucose as a carbon source for cell growth do not need to reserve energy source or PHB.

Synechocystis sp. PCC 6803 mutant disrupting *adc*1 gene that involved in polyamine pathway. This *Synechocystis* mutant (*⊿adc*1) rapidly accumulated higher PHB amount than WT after inducing by BG_{11}–N-P+A. There were no any previous reports before that studied the relation between polyamine and PHB. Our proposed hypothesis has been created based on the correlation of polyamine biosynthetic pathway and TCA cycle via proline-glutamate intermediates. The *adc1* gene encodes arginine decarboxylase which catalyzes a conversion of arginine to agmatine (see in Figure 10). Arginine is utilized also to urea cycle and proline-glutamate production. This glutamate product is one of major amino acids directly entered to proceed TCA cycle. After the disruption of *adc1* gene, the polyamine pathway was blocked which may lead arginine directed to another pathway to produce higher glutamate amounts that sufficient to proceed TCA cycle. In the consequence of this situation, the precursor acetyl-CoA might be needed in less amount towards to TCA cycle, then it could be re-directed to PHB biosynthetic pathway. Previously, Tyo and co-workers (2009) employed the inverse metabolic engineering for selecting cells containing higher PHB amounts in *Synechocystis* sp. PCC 6803 by transposon insertion technique. They reported that increased PHB cells were obtained from some gene disruptions including gamma-glutamyl phosphate reductase (*proA*; *sll0461*), an enzyme catalyzed the conversion of glutamate to proline, and *sll0565*, a hypothetical protein.

Transcript abundance analysis of genes involved in PHB biosynthesis in the WT and *⊿adc1* strain demonstrated that the *phaC* transcript level was dominantly induced by all conditions (Figures 35-37). It was interestingly that the pattern of gene expression did not correspond with PHB contents and visualization of Nile-red stained cells. However, we found the up-accumulation of *phaC* transcript, encoding PHA synthetase, in mutant when compared to that of WT. Previously, the heterologous expression of PHA-synthesizing gene operon from the bacterium *Ralstonia eutropha* in *Synechocystis* PCC 6803 could increase PHB content from 7 to 11 % of DCW when grown in N-deprived medium with 10 mM acetate for 5days (Sudesh et al., 2002). Moreover, when *phaA* and *phaB* genes were disrupted and grown in BG_{11} media which acetate supplement, PHB content were absent in *Synechoctstis* sp. PCC6803 *⊿phaA-B* strains (Taroncher-Oldenburg et al., 2000).

CHAPTER V

Conclusion

Cell growths and intracellular pigments under all nutrient modifications including BG_{11}-N-P, BG_{11}-N-P+A and BG_{11}-N-P+G, respectively, were significantly decreased compared to that under normal BG_{11} condition in both WT and *Δadc1* mutant strains. Acetate supplementation in BG_{11}-N-P+A condition could enhance the accumulation of intracellular pigments when compared to those under BG_{11}-N-P condition. Visualization on Nile red stained cells of wild type strain showed highest PHB granules inside the cells under BG_{11}-N-P+A condition at day 5, 7 and 9-treatments whereas *Δadc1* mutant accumulated widespread PHB granules at 7 day-treatment under the same condition. The highest content of PHB was obtained in wild type cells under BG11-N-P+A condition with an average of 24.95%PHB/DCW at day 9 of treatment whereas the highest PHB content of *Δadc1* mutant obtained at day 7 of treatment was an average of 36.07 % PHB/DCW under the same condition. The abiotic stress of UV-C treatment apparently induced the highest PHB content of about 13.69 %PHB/DCW at 6 hour-exposure when compared to other UV exposure and normal growth light. Moreover, expression of *phaC* mRNA level was highest when compared to other *pha* transcript levels under all conditions in both wild type and mutant cells.

REFERENCES

Allen, M. M. (1984). "Cyanobacterial cell inclusions." *Annual Review of Microbiology* 38: 1-25.

Anderson, A. J. and Dawes, E. A. (1990). "Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates." *Microbiological Reviews* 54(4): 450-472.

Balaji, S., Gopi, K. and Muthuvelan, B. (2013). "A review on production of poly β hydroxybutyrates from cyanobacteria for the production of bio plastics." *Algal Research* 2(3): 278-285.

Bonartsev, A., Myshkina, V., Nikolaeva, D., Furina, E., Makhina, T., Livshits, V., Boskhomdzhiev, A., Ivanov, E., Iordanskii, A. and Bonartseva, G. (2007). "Biosynthesis, biodegradation, and application of poly (3-hydroxybutyrate) and its copolymers-natural polyesters produced by diazotrophic bacteria." *Communicating Current Research and Educational Topics and Trends in Applied Microbiology* 1: 295-307.

Boopathy, R. (2000). "Factors limiting bioremediation technologies." *Bioresource Technology* 74(1): 63-67.

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.

Choi, G. G., Kim, H. W. and Rhee, Y. H. (2004). "Enzymatic and non-enzymatic degradation of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) copolyesters

produced by *Alcaligenes* sp. MT-16." *Journal of Microbiology* 42(4): 346-352.

Davis, R. H., Morris, D. R. and Coffino, P. (1992). "Sequestered end products and enzyme regulation: the case of ornithine decarboxylase." *Microbiological Reviews* 56(2): 280-290.

Dawes, E. A. (1988). "Polyhydroxybutyrate: an intriguing biopolymer." *Bioscience Reports* 8(6): 537-547.

Doi, Y., Segawa, A. and Kunioka, M. (1990). "Biosynthesis and characterization of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in *Alcaligenes eutrophus*." *International Journal of Biological Macromolecules* 12(2): 106-111.

Flink, I. and Pettijohn, D. E. (1975). "Polyamines stabilise DNA folds." *Nature* 253(5486): 62-63.

García-Hidalgo, J., Hormigo, D., Arroyo, M. and de la Mata, I. (2013). "Novel extracellular PHB depolymerase from *Streptomyces ascomycinicus* PHB copolymers degradation in acidic conditions." *PLoS ONE* 8(8): e71699.

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

Hauf, W., Schlebusch, M., Hüge, J., Kopka, J., Hagemann, M. and Forchhammer, K. (2013). "Metabolic changes in *Synechocystis* PCC6803 upon nitrogen-starvation: excess NADPH sustains polyhydroxybutyrate accumulation." *Metabolites* 3(1): 101.

Hein, S., Tran, H. and Steinbuchel, A. (1998). "*Synechocystis* sp. PCC6803 possesses a two-component polyhydroxyalkanoic acid synthase similar to that of anoxygenic purple sulfur bacteria." *Archives of Microbiology* 170(3): 162-170.

Hrabak, O. (1992). "Industrial production of poly-β-hydroxybutyrate." *FEMS Microbiology Letters* 103(2-4): 251-255.

Jantaro, S., Maenpaa, 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.

Jantaro, S., Pothipongsa, A., Khanthasuwan, S. and Incharoensakdi, A. (2011). "Short-term UV-B and UV-C radiations preferentially decrease spermidine contents and arginine decarboxylase transcript levels of *Synechocystis* sp. PCC 6803." *Current Microbiology* 62(2): 420-426.

Jendrossek, D. and Handrick, R. (2002). "Microbial degradation of polyhydroxyalkanoates." *Annual Review of Microbiology* 56: 403-432.

Johnstone, B. (1990). "A throw away answer." *Far Eastern Economic Review* 147(6): 62-63.

Joseph, A., Aikawa, S., Sasaki, K., Teramura, H., Hasunuma, T., Matsuda, F., Osanai, T., Hirai, M. Y. and Kondo, A. "Rre37 stimulates accumulation of 2-oxoglutarate and glycogen under nitrogen starvation in *Synechocystis* sp. PCC 6803." *FEBS Letters* 588(3): 466-471.

Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M. and Tabata, 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 (supplement)." *DNA Research* 3(3): 185-209.

Kaneko, T. and Tabata, S. (1997). "Complete genome structure of the unicellular cyanobacterium *Synechocystis* sp. PCC6803." *Plant and Cell Physiology* 38(11): 1171-1176.

Kichise, T., Fukui, T., Yoshida, Y. and Doi, Y. (1999). "Biosynthesis of polyhydroxyalkanoates (PHA) by recombinant Ralstonia eutropha and effects of PHA synthase activity on in vivo PHA biosynthesis." *International Journal of Biological Macromolecules* 25(1-3): 69-77.

Lee, S. Y. (1996). "Bacterial polyhydroxyalkanoates." *Biotechnology and Bioengineering* 49(1): 1-14.

Liebergesell, M., Sonomoto, K., Madkour, M., Mayer, F. and Steinbuchel, A. (1994). "Purification and characterization of the poly(hydroxyalkanoic acid) synthase from chromatium vinosum and localization of the enzyme at the surface of poly(hydroxyalkanoic acid) granules." *European Journal of Biochemistry* 226(1): 71-80.

Madison, L. L. and Huisman, G. W. (1999). "Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic." *Microbiology and Molecular Biology Reviews* 63(1): 21-53.

Marchessault, R. H. and Yu, G.-e. (2005). Crystallization and material properties of polyhydroxyalkanoates PHAs). *Biopolymers Online*, Wiley-VCH Verlag GmbH & Co. KGaA.

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

Nikel, P. I., Pettinari, M. J., Galvagno, M. A. and Mendez, B. S. (2008). "Poly(3-hydroxybutyrate) synthesis from glycerol by a recombinant *Escherichia coli* arcA mutant in fed-batch microaerobic cultures." *Applied Microbiology and Biotechnology* 77(6): 1337-1343.

Nishioka, M., Nakai, K., Miyake, M., Asada, Y. and Taya, M. (2001). "Production of poly-β-hydroxybutyrate by thermophilic cyanobacterium, *Synechococcus* sp. MA19, under phosphate-limited conditions." *Biotechnology Letters* 23(14): 1095-1099.

Osanai, T., Oikawa, A., Numata, K., Kuwahara, A., Iijima, H., Doi, Y., Saito, K. and Hirai, M. Y. (2014). "Pathway-level acceleration of glycogen catabolism by a response regulator in the cyanobacterium *Synechocystis* species PCC 6803." *Plant Physiology* 164(4): 1831-1841.

Pal, A., Prabhu, A., Kumar, A. A., Rajagopal, B., Dadhe, K., Ponnamma, V. and Shivakumar, S. (2009). "Optimization of process parameters for maximum poly(-beta-)hydroxybutyrate (PHB) production by *Bacillus thuringiensis* IAM 12077." *Polish Journal of Microbiology* 58(2): 149-154.

Panda, B., Jain, P., Sharma, L. and Mallick, N. (2006). "Optimization of cultural and nutritional conditions for accumulation of poly-beta-hydroxybutyrate in *Synechocystis* sp. PCC 6803." *Bioresource Technology* 97(11): 1296-1301.

Panda, B. and Mallick, N. (2007). "Enhanced poly-β-hydroxybutyrate accumulation in a unicellular cyanobacterium, *Synechocystis* sp. PCC 6803." *Letters in Applied Microbiology* 44(2): 194-198.

Peña, C., Castillo, T., Núñez, C. and Segura, D. (2011). "Bioprocess design: fermentation strategies for improving the production of alginate and poly-β-

hydroxyalkanoates (PHAs) by *Azotobacter vinelandii*." *Rijeka, Croatia: INTECH-Open Access Publisher*: 217-242.

Peters, V. and Rehm, B. H. (2005). "In vivo monitoring of PHA granule formation using GFP-labeled PHA synthases." *FEMS Microbiology Letters* 248(1): 93-100.

Poirier, Y., Nawrath, C. and Somerville, C. (1995). "Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants." *Biotechnology (N Y)* 13(2): 142-150.

Quintero, M. J., Muro-Pastor, A. M., Herrero, A. and Flores, E. (2000). "Arginine catabolism in the cyanobacterium *Synechocystis* sp. strain PCC 6803 involves the urea cycle and arginase pathway." *Journal of Bacteriology* 182(4): 1008-1015.

Raksajit, W., Maenpaa, P. and Incharoensakdi, A. (2006). "Putrescine transport in a cyanobacterium *Synechocystis* sp. PCC 6803." *Journal of biochemistry and molecular biology* 39(4): 394-399.

Reddy, C. S., Ghai, R., Rashmi and Kalia, V. C. (2003). "Polyhydroxyalkanoates: an overview." *Bioresource Technology* 87(2): 137-146.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. and Stanier, R. Y. (1979). "Generic assignments, strain histories and properties of pure cultures of cyanobacteria." *Journal of General Microbiology* 111(1): 1-61.

Sharma, L. and Mallick, N. (2005). "Accumulation of poly-beta-hydroxybutyrate in *Nostoc muscorum*: regulation by pH, light-dark cycles, N and P status and carbon sources." *Bioresource Technology* 96(11): 1304-1310.

Sharma, L. and Mallick, N. (2005). "Enhancement of poly-beta-hydroxybutyrate accumulation in *Nostoc muscorum* under mixotrophy, chemoheterotrophy and limitations of gas-exchange." *Biotechnology Letters* 27(1): 59-62.

Steinbuchel, A., Hustede, E., Liebergesell, M., Pieper, U., Timm, A. and Valentin, H. (1992). "Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria." *FEMS Microbiological Reviews* 9(2-4): 217-230.

Sudesh, K., Taguchi, K. and Doi, Y. (2002). "Effect of increased PHA synthase activity on polyhydroxyalkanoates biosynthesis in *Synechocystis* sp. PCC6803." *International Journal of Biological Macromolecules* 30(2): 97-104.

Taguchi, S., Nakamura, H., Kichise, T., Tsuge, T., Yamato, I. and Doi, Y. (2003). "Production of polyhydroxyalkanoate (PHA) from renewable carbon sources in recombinant *Ralstonia eutropha* using mutants of original PHA synthase." *Biochemical Engineering Journal* 16(2): 107-113.

Taroncher-Oldenburg, G., Nishina, K. and Stephanopoulos, G. (2000). "Identification and analysis of the polyhydroxyalkanoate-specific beta-ketothiolase and acetoacetyl coenzyme A reductase genes in the cyanobacterium *Synechocystis* sp. strain PCC6803." *Applied and Environmental Microbiology* 66(10): 4440-4448.

Tokiwa, Y., Calabia, B. P., Ugwu, C. U. and Aiba, S. (2009). "Biodegradability of Plastics." *International Journal of Molecular Sciences* 10(9): 3722-3742.

Tyo, K. E., Jin, Y. S., Espinoza, F. A. and Stephanopoulos, G. (2009). "Identification of gene disruptions for increased poly-3-hydroxybutyrate accumulation in *Synechocystis* PCC 6803." *Biotechnology Progress* 25(5): 1236-1243.

Tyo, K. E., Zhou, H. and Stephanopoulos, G. N. (2006). "High-throughput screen for poly-3-hydroxybutyrate in *Escherichia* coli and *Synechocystis* sp. strain PCC6803." *Applied and Environmental Microbiology* 72(5): 3412-3417.

Verlinden, R. A., Hill, D. J., Kenward, M. A., Williams, C. D. and Radecka, I. (2007). "Bacterial synthesis of biodegradable polyhydroxyalkanoates." *Journal of Applied Microbiology* 102(6): 1437-1449.

Wahl, A., Schuth, N., Pfeiffer, D., Nussberger, S. and Jendrossek, D. (2012). "PHB granules are attached to the nucleoid via PhaM in *Ralstonia eutroph*a." *BMC Microbiology* 12(1): 1-11.

Watson, M. B., Emory, K. K., Piatak, R. M. and Malmberg, R. L. (1998). "Arginine decarboxylase (polyamine synthesis) mutants of *Arabidopsis thaliana* exhibit altered root growth." *The Plant Journal* 13(2): 231-239.

Wu, G., Bao, T., Shen, Z. and Wu, Q. (2002). "Sodium acetate stimulates PHB biosynthesis in *Synechocystis* sp. PCC 6803." *Tsinghua Science and Technology* 7(4): 435-438.

Xiong, W., Morgan, J. A., Ungerer, J., Wang, B., Maness, P.-C. and Yu, J. (2015). "The plasticity of cyanobacterial metabolism supports direct CO_2 conversion to ethylene." *Nature Plants* 1.

Yu, J. (2001). "Production of PHA from starchy wastewater via organic acids." *Journal of Biotechnology* 86(2): 105-112.

APPENDICE

APPENDIX A

BG_{11} medium

Normal medium (BG_{11}) volume 1 liter

Standard BG-11		Broth	Agar
$MgSO_4 \cdot 7H_2O$	(15g / 500ml)	1 ml	1 ml
$CaCl_2 \cdot 2H_2O$	(7.2g /200 ml)	1 ml	1 ml
Citric acid	(1.2g / 200 ml)	1 ml	1 ml
EDTA	(0.2g / 200 ml)	1 ml	1 ml
1000x trace element*		1 ml	1 ml
Na_2CO_3	(4g / 200ml)	1 ml	1 ml
Ferric ammonium citrate	(6mg / ml)	1 ml	1 ml
KH_2PO_4	(8g / 200ml)	1 ml	1 ml
$NaNO_3$	(75g / 500ml)	10 ml	10 ml
1M Hepes-NaOH		10 ml	10 ml
30% $Na_2S_2O_3 \cdot 5H_2O$	(240g / 800 ml)	-	10 ml
Distilled H_2O		To 1000 ml	-

*1000x trace element (1000 ml)

H_3BO_3	2.86 g
$MnCl_2 \cdot 4H_2O$ $NaNO_3$	1.81 g
$ZnSO_4 \cdot 7H_2O$	0.221 g
$Na_2MoO_4 \cdot 2H_2O$	0.390 g
$CuSO_4 \cdot 5H_2O$	0.080 g
$Co(NO_3)_2 \cdot 6H_2O$	0.049 g

Nitrogen and phosphorus limited medium (-N-P) modified from BG_{11} medium

- $NaNo_3$ and KH_2PO_4 were removed.
- Ferric ammonium citrate was replaced by $FeSO_4$ (1.2 g/200 ml)
- KH_2PO_4 was replaced by KCl (4.47 g/200 ml)

Acetate supplementation from BG_{11}-N-P medium

- Added Na-acetate 0.4% (5.5 g/1000 ml)

Glucose supplementation from BG_{11}-N-P medium

- Added glucose 0.4%

APPENDIX B

PHB content standard graph

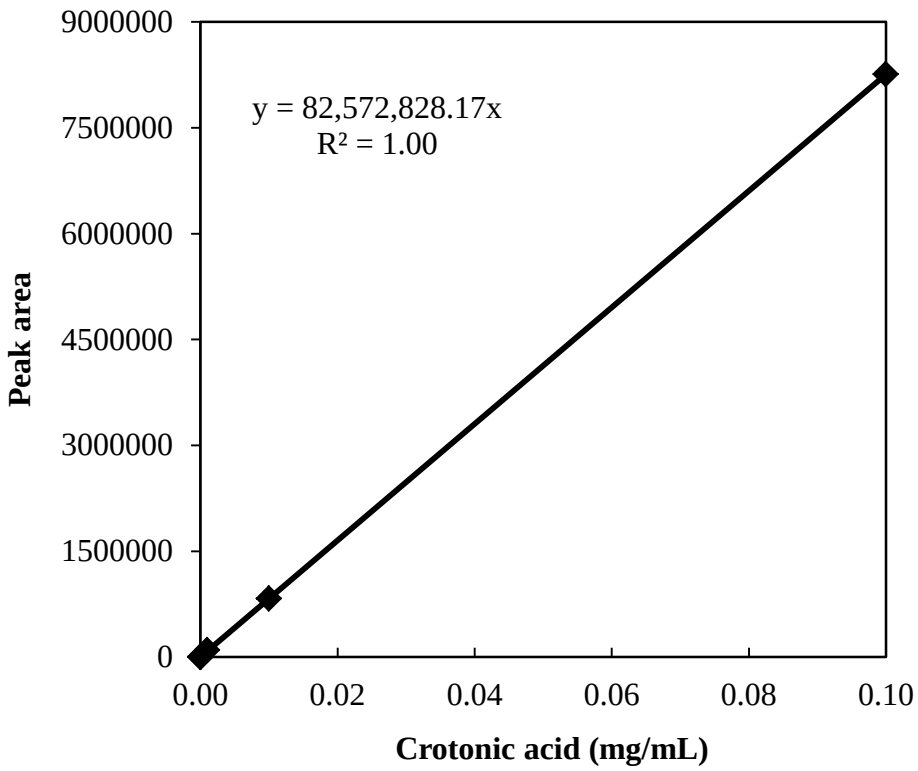


Figure A.1 Standard curve of PHB

APPENDIX C

PHB content calculation

$$\%\ \text{PHB content} = \left(\frac{\text{amount of crotonic}}{\text{Total of dry cell weight}} \times 250\ \text{dilution factor}\right) \times \%\text{recovery}$$

$$\text{amount of crotonic} = \frac{\text{peak area of crotonic}}{\text{slope from standard graph above}}$$

$$\%\ \text{recovery} = \frac{\text{peak area of adipic}}{\text{Choose peak area of adipic one value for stable calculation}} \times 100$$

APPENDIX D

TAE buffer

Working solution

1 X 0.04 M Tris-acetate

0.01 M EDTA

Concentrated stock solution (per liter)

50: 242 g Tris base

57.1 mL glacial acetic acid

100 mL 0.5 M EDTA (pH8.0)

APPENDIX E

Preparation of agarose gel electrophoresis and quantification

1. The edges of clean, dry, glass were sealed and then molded on a horizontal section of the bench.
2. The 0.5 TAE electrophoresis buffer (100 mL) was prepared.
3. The 1.5 g of agarose powder was weighed and transferred into the TAE buffer.
4. The mixture was boiled for 2-3 minutes with microwave.
5. The comb was placed in a suitable position.
6. Agarose gel solution was poured onto tray.
7. After gel was completely set (30-45 minutes at room temperature), the comb was removed carefully.
8. The gel was placed into electrophoresis tank.
9. The 6x loading dye was added into each sample and the sample were loaded into the slot well.
10. The lid of gel tank was closed and then the electrical status was adjusted to 80 volt.
11. The electric current was turned off and the gel was removed into bowl for stained with ethidium bromide (a stock solution of 1mg/mL adjusting final concentration to 0.5 µg/mL) and later destained with water about 10 minutes.
12. The gel was placed into UV-light machine to quantify their bands.

VITA

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