


รายงานวิจัยฉบับสมบูรณ์

โครงการการคัดแยกไซยาโนแบคทีเรีย และสาหร่ายสีเขียว ที่ผลิตพลาสติกชีวภาพปริมาณสูง

Isolation of cyanobacteria and microalgae with high bioplastic content

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Isolation of cyanobacteria and green algae with high bioplastic content

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บทคัดย่อ

ไกลโคเจน (GI) ไขมัน (LP) และ polyhydroxybutyrate (PHB) เป็นสารที่สะสมคาร์บอนในโพรคาริโอต โดยสารสองชนิดแร ก เป็นสารตั้งต้นที่มีประสิธิภาพสูงในการผลิตพลังงานชีวภาพ และสารชนิดหลังเป็นพลาสติกที่ย่อยสลายได้ทางชีวภาพที่มีคุณสมบัติดี งานวิจัยนี้มีจุดประสงค์เพื่อเพิ่มการสะสมร่วมของสารทั้งสามชนิดดังกล่าวโดยการปรับสภาวะสรีรวิทยาในเซลล์ไซยาโนแบคทีเรีย *Synechocystis* sp. PCC 6803 ซึ่งเป็นสายพันธุ์ที่ใช้ในการศึกษากันอย่างกว้างขวางในห้องทดลอง ในภาวะ autophototrophic growth โดยใช้ CO_2 จากอากาศ *Synechocystis* มีผลผลิตร่วมของสารทั้งสามชนิดสูงสุดที่ระยะ mid-stationary growth phase ซึ่งมีค่าเท่ากับ 39.2% ของน้ำหนักเซลล์แห้ง (22.7% GI, 14.1% LP, 2.4% PHB) ในภาวะขาดไนโตรเจนพบว่าเซลล์สามารถเพิ่มผลผิตร่วมได้ถึง 61.5% (36.8% GI, 11.2% LP, 13.5% PHB) ซึ่งมากกว่าผลผลิตร่วมที่ได้จากภาวะขาดฟอสฟอรัส ซัลเฟอร์ เหล็ก หรือ แคลเซียม เมื่อควบรวมภาวะขาดไนโตรเจนเข้ากับการให้กลูโคส 0.4% (w/w) และการเพิ่มความเข้มแสง พบว่าสามารถเพิ่มผลผลิตร่วมได้ถึง 71.1 % ของชีวมวล (41.3% GI, 16.7% LP, 13.1% PHB) ซึ่งเป็นที่มีค่าสูงกว่าปริมาณผลผลิตร่วมที่เคยมีรายงานมาก่อนหน้านี้ในไซยาโนแบคทีเรีย อย่างไรก็ตามค่า productivity ของผลผลิตร่วมมีค่าสูงสุดในภาวะที่ให้กลูโคส 0.4% (w/w) โดยไม่มีการขาดธาตุอาหาร ซึ่งมีค่าเท่ากับ 0.72 $g.L^{-1}$ สำหรับการเลี้ยง 12 วัน เนื่องจากในสภาวะนี้ให้ชีวมวลในปริมาณที่สูงกว่าภาวะอื่น นอกจากนี้ยังสามารถคัดแยกไซยาโนแบคทีเรีย *Synechococcus* sp. ที่มีผลผลิตร่วมของสารทั้งสามชนิดเท่ากับ 34.2% และสาหร่ายยูคาริโอตสีเขียว *Chlorella* sp. ที่มีผลผลิตร่วมของ GI และ LP เท่ากับ 54.6% ของน้ำหนักเซลล์แห้งได้จากแหล่งน้ำจืดในประเทศไทย โครงการนี้ได้เสนอแนวคิดและวิธีการในการเพิ่มผลผลิตร่วมของสารชีวพลังงานและชีววัสดุกลุ่มหลักในเซลล์จุลชีพสังเคราะห์ด้วยแสง

คำสำคัญ: ไซยาโนแบคทีเรีย สาหร่ายสีเขียว ไกลโคเจน ไขมัน พอลิไฮดรอกซีบิวทิเรท พลาสติกชีวภา

Abstract

Glycogen (GI), lipids (LP) and polyhydroxybutyrate (PHB) are carbon storages in various prokaryotes which the first two storages are efficient substrates for biofuel production, while the later is a potent bio-degradable plastic. This study aims to increase cellular co-accumulations of such three storages by adjusting various physiological conditions in one of the best studied cyanobacteria, *Synechocystis* sp. PCC 6803. At the normal autophototrophic growth with atmospheric CO_2 concentration, the co-production of the three storages reached the maximum at 39.2% of dry weight (22.7% GI, 14.1% LP and 2.4% PHB) at the mid-stationary phase of growth. Nitrogen deprivation condition significantly increased the co-accumulation to 61.5% (36.8% GI, 11.2% LP and 13.5% PHB) higher than that caused by a nutrient deprivation of Phosphorus, Sulphur, Iron or Calcium. Combining the Nitrogen deprivation with the 0.4% (w/w) glucose supplementation altogether with optimizing light intensity enhanced the co-production to 71.1 % of biomass (41.3% GI, 16.7% LP and 13.1% PHB), the higher accumulation of the three storages than previous reports in cyanobacteria. However, the maximum productivity of the three storages at 0.72 $g.L^{-1}$ after 12-day culture was obtained at the condition supplemented with 0.4% (w/w) glucose and without a nutrient deprivation, due to its high biomass yield. In this, the cyanobacterium *Synechococcus* sp. with the three storage production at 34.2% and the eukaryotic green algae *Chlorella* sp. with the co-accumulation of GI and LP at 54.6% of dry weigh were isolated from Thailand natural resources. This study introduced conceptual and experimental tools to simultaneously enhance co-accumulations of the major bioenergy and biomaterial molecules in photosynthetic microorganisms.

Keywords: cyanobacteria; green algae, glycogen; lipid; polyhydroxybutyrate; bioplastic

ไซยาโนแบคทีเรีย สาหร่ายสีเขียว ไกลโคเจน ไขมัน พอลิไฮดรอกซีบิวทิเรท พลาสติกชีวภาพ

OBJECTIVES

1. To enhance the co-accumulation of glycogen, lipids and bioplastic polyhydroxybutyrate in the model strain of cyanobacteria, *Synechocystis* sp. PCC 6803
2. To isolate cyanobacteria with high cellular contents of glycogen, lipids and bioplastic from Thailand natural resources

MATERIALS AND METHODS

Organism, culture conditions and growth determination

Wild type *Synechocystis* sp. PCC 6803 (Pasteur Institute, France) and fresh water cyanobacteria and microalgae strains isolated from Thailand were cultured *autophototrophically* in 250 ml flasks containing 150 ml BG-11 media [21] supplement with 20 mM HEPES-NaOH (pH 7.5). The cultures were shaken at 150 rpm at a continuous illumination of 50 µmol photon.m^{-2}.s^{-1} at 28 ^{o}C with atmospheric CO_2 concentration (hereafter: the normal photoautotrophic growth condition). Nitrogen depletion was obtained by culturing the cells in the media devoid of $NaNO_3$ and replacing ferric ammonium citrate and $Co(NO_3)_2.6H_2O$ with equimolar of ferric citrate and $CoCl_2.6H_2O$. The following nutrient deprivations in the BG11 medium were prepared using an equimolar substitution basis: Phosphorus depletion, replacing K_2HPO_4 by KCl; Sulphur depletion, replacing $MgSO_4$ and $CuSO_4$ by $MgCl_2$ and $CuCl_2$, respectively; Iron depletion, substituting ferric ammonium citrate by ammonium citrate; Calcium depletion, replacing $CaCl_2$ by KCl. For heterophototrophic growth, 0.4 % glucose (w/w) was added into the media. Cell growth was monitored by determining dry weight gravitrometrically (0.1 mg resolution). Wet cells were dried at 60 ^{o}C until constant weight to prevent lipid loss.

Glycogen analysis

The extraction was carried out by a method of Ernst [11]. Grinded dry cells were resuspended in 300 µl of 30% (w/v) KOH and were heated at 100 °C for 90 min. The supernatant was collected and ice-cold 900 µl ethanol were added and placed on

ice for 2 h. Cyanobacterial glycogen was harvested by centrifugation and the pellet was washed twice by 70% and 90% ethanol, respectively and dried at 60 °C. Glycogen was resuspended in 300 µl of 100 mM sodium acetate pH 4.75 and enzymatically hydrolyzed to glucose by the treatment with 0.2 mg amyloglucosidase ml^{-1} (Sigma) and 0.2 mg amylase ml^{-1} (Sigma) at 50 ^{o}C for 14 h. The reaction was terminated by heating at 100 °C for 10 min and the obtained glucose solution in the supernatant was quantified using glucose oxidase assay (GLUCOSE Liquicolor Kit, Human Gesellschaft fur Biochemica und Diagnostica mbH, Wiesbaden, Germany). A glycogen standard from oyster (Sigma) was also analyzed to correlate glucose concentrations to the initial glycogen concentrations.

Lipid analysis

Lipids were extracted by two-step method. The first extraction was done as described [25]. Grinded dry cells were resuspended in 5 ml methanol which has been shown to efficiently dissolve *Synechocystis* total lipids [25] but not PHB polymer [27]. Then, the mixtures were vortexed for 24 h at room temperature, filtrated and dried at 50 ^{o}C. Next, the second extraction were conducted according to [8]. The extracts were resuspended in 3 ml chloroform: methanol (2:1 v/v), vortexed for 3 h at room temperature, and then 3 ml of 0.88% KCl was mixed to the combined chloroform–methanol solution and centrifuged. An 0.3 ml aliquot of the bottom phase containing lipids was dried at 50 ^{o}C and the remaining crude lipid extracts were subjected for total lipid quantification by as described [3]. Lipids were quantitatively oxidized by 2 ml acidic dichromate reagent (*2.5 g*.l^{-1} $K_2Cr_2O_7$ in 36 N H_2SO_4) at 100 ^{o}C for 45 min. The oxidized lipids were quantified from the amount of reduced dichromates as determined spectrophotometrically at 350 nm. Palmitic acid (Sigma) was used as a standard for the lipid quantification.

PHB analysis

PHB quantification was done by high-performance liquid chromatography essentially as described [23]. Grinded dry cells were boiled in 1 ml of 95% H_2SO_4 for 1 h to hydrolyze PHB polymer, then 25-fold diluted with 0.014 M H_2SO_4 and filtered. Samples containing the PHB-hydrolyzed product, crotonic acid, were analyzed by HPLC using InertSustain 3-µm *C18* column (GL Sciences, USA). Commercial PHB (Sigma) was analyzed in parallel with the samples as quantification standards. Adipic

acid and crotonic acid (Sigma) were also used as an internal standard and crotonic standard, respectively for the HPLC analysis.

INTRODUCTION

Sustainable production of energy and materials are required to cope with reducing global petroleum reserves. Cyanobacteria are one of potential microorganisms capable of producing renewable bioenergy and environmental-friendly materials. These oxygenic photosynthetic prokaryotes are able to assimilate carbon dioxide green house gas to their metabolisms and synthesize glycogen (GI), a major cellular carbohydrate which is a polymer of glucoses linked through α-1,4- and α-1,6 glycosidic bonds [4,5,9], an efficiently substrate for bioethanol production [14,28]. Cyanobacteria also synthesize lipids (LP), mainly composed of acylglycerols [25,22] which can be readily transformed to biodiesel [15,25]. In addition, some cyabobacteria are able to accumulate polyhydroxybutyrate (PHB), the bio-degradable plastic comprised of 3-hydroxybutyric acid monomers linked through ester bonds [6,17,19,24]. Thus, enhancing the co-accumulation of such carbon storages in cyanobacteria is desirable for biofuel and biopolymer productions.

In the well-studies cyanobacterial model *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), cellular GI content under a normal autophototrophic growth was reported in various quantification units ranging from 1 µg.mg chlorophyll [26], 1 mg.g wet cell^{-1} [30] to 18.5% of dry weight [16]. One study significantly enhanced *Synechocystis* GI accumulation from 1 to 19 mg.g wet cell^{-1} after adjusting nutrient supplies [30]. Increased *Synechocystis* PHB production from a few percent to 38% of dry weight by adjusting various external factors was also described previously [18,19]. Attempts to increase *Synechocystis* LP is still limited; the content was

reported to ranges from 10% to 15% of dry weight which diacylglycerols are major constituents [25].

Form above information, the studies in determining the GI, LP and PHB contents in cyanobacterial cells grown under distinct physiological conditions are still limited. We asked questions: what are relative amounts of the three carbon storages accumulated in cyanobacteria upon adapting to different nutrients and light intensities and whether optimizing the nutrients and light intensities could simultaneously enhance the co-production of such three carbon storages in cyanobacteria. To address those questions, here we quantified the cellular amount of GI, LP and PHB as in the same quantification unit (% dry weight) in *Synechocystis*. We optimized various nutrient supplies and light intensities with the aim to enhance the co-production of the three carbon storages of *Synechocystis* cells. In addition, we also isolated natural strains from Thailand natural resources and determined for their PHB and lipids accumulation. This study has a goal to maximally utilize cyanobacterial biomass for the productions of renewable energy and biomaterials.

RESULTS

Synechocystis carbon storages under the normal autophototrophic growth

Cells grown in the standard BG11 media with continuous light and atmospheric CO2 concentration (the normal autophototrophic condition, hereafter) were quantified for their glycogen (GI), lipids (LP) and polyhydroxybutyrate (PHB) as percent to cell dry weight, used throughout this study. During the lag to the log phase of growth (day 0-20), *Synechocystis* accumulated both GI and LP as major carbon storages at the comparable amounts in the range of 9.8% to 13.3% (Fig. 1). Then, at the early to the mid stationary phases (day 24-28), GI increased to 22.7%,

while LP remained at about 14 %, indicating that GI is the major carbon storage at this growth period. However, at the late stationary phase (day 32-40), GI reduced to about 17%, comparable to the level of LP accumulation.

For PHB, the content ranges from only 0.4% to 5.6% which the accumulation increased during the log phase and reduced at the stationary phase (Fig. 1). Thus, PHB is a minor carbon storage of *Synechocystis* in all autophototrophic growth phases. When combine the total three storage content, [GI + LP + PHB], it continually increased from the lag to the mid stationary phase (25.8% to 39.2%), and slightly reduced and maintained at ~35% when the cells reached late stationary period.

Fig. 1

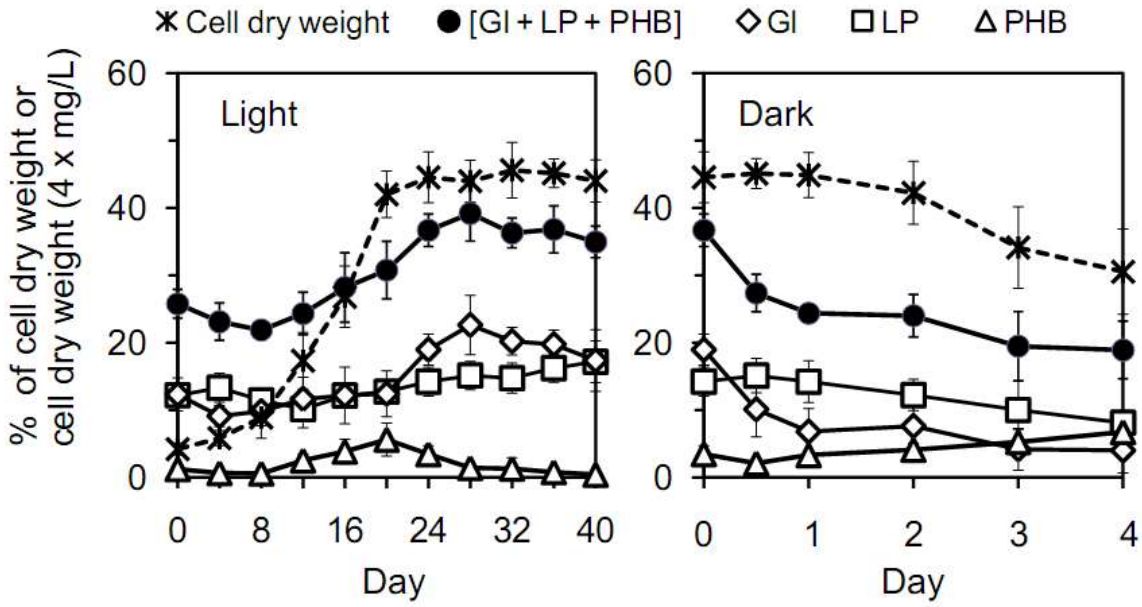


Fig. 1. Accumulations of *Synechocystis* carbon storages in the light and in the dark. Cells were cultured in the normal autotrophic condition using the standard BG11 media and continuous 50 µmol photon.m^{-2}.s^{-1} (Light). In another experiments, the stationary 24-day old cultures from (Light) were transferred to the darkness (Dark). GI, glycogen; LP, total lipids; PHB, polyhydroxybutyrate. Data are means ± 1SD of at least three independent experiments.

Effects of the darkness

Synechocystis that had been grown in the normal autophototrophic condition were placed into the darkness. Cellular GI rapidly decreased from 19.0% to 10.1% after 12 h, then dropped to 6.8% and 4.0 % after one day and four day in the dark, respectively (Fig. 1). LP also reduced from 14.2 % to 8.1% within four day in the dark. In contrast with GI and LP, PHB was found to increase from 3.5% to 6.8% after four days in the dark. The overall [GI + LP + PHB] content decreased from 36.7% to 24.4% after one day, and slightly fell to 18.9% after four days in the dark.

Effects of light intensities

Synechocystis were grown under different light intensities as follows (the numbers are in µmol photon.m^{-2}.s^{-1}): 10, low light; 50, moderate-low light; 200, moderate-high light; 500, high light. *Synechocystis* accumulated GI only 8.7% at the low light, but GI significantly increased to 22.6% at the moderate-low light and peaked at 29.6% at the moderate-high light, but reduced to 9.4% at the high light (Fig. 2). In the similar fashion, LP was only 10.6% at the low light and raised to14.3% - 15.1% at the moderate lights, but reduced to 9.3 % at the high light.

In contrast with GI and LP, PHB levels were found to be consistent at the range of 1.1% - 1.8% at all light intensities. We also found that the total [GI + LP + PHB] content of *Synechocystis* grown at the moderate lights were approximate two-fold higher than those grown at the low light or at the high light (Fig. 2).

Fig. 2

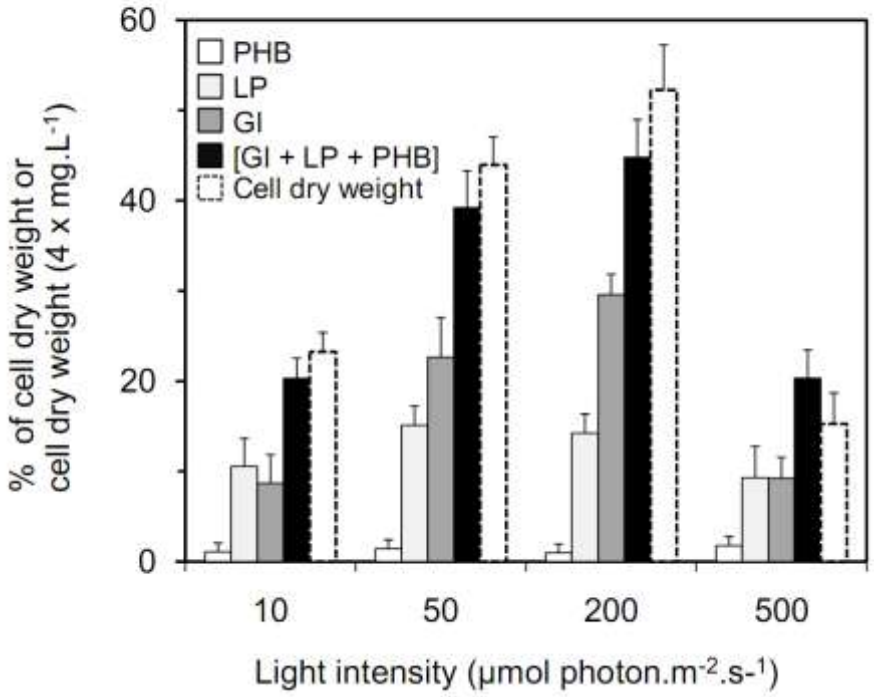


Fig. 2. Effects of light intensities to the carbon storages of *Synechocystis*. Cells were cultured in the standard BG11 media for 28 days under different light intensities: 10, 50, 200 and 500 µmol photon.m^{-2}.s^{-1}, defined as low, moderate-low, moderate-high and high light intensity, respectively. Data are averages ± 1SD derived from three independent analyses.

Accumulation of carbon storages upon nutrient depletions

Synechocystis that had been grown at the normal autophototrophic condition (complete nutrients) were harvested and further cultured in the fresh media without one of the following nutrients: Nitrogen (-N); Phosphorus (-P); Iron (-Fe); Sulphur (-S); Calcium (-Ca). The cellular carbon storages under such a deprivation were determined (Fig. 3). When compared with the cells grown under the normal condition, we found that – N drastically enhanced the accumulations of GI (15.1% to 36.8%) and

PHB (2.4% to 13.5%), but did not significantly altered LP content. In addition,–N increased the total [GI+LP+PHB] content up to 61.5 % of biomass, 1.7-fold higher than that content of the normal condition.

– P moderately increased GI (15.1% to 28.9%) and PHB (2.4% to 14.6%). We noted that these increased GI and PHB content caused by –P occurred slower than those caused by – N (Fig. 3). However, –P was found to slightly reduced LP. The overall [GI+LP+PHB] content at –P peaked at 49.7 %, 1.4 fold of that accumulated in the normal condition.

–Fe moderately increased GI (15.1% to 29.7%), but not LP nor PHB. However, the maximum [GI+LP+PHB] content under –Fe was 40.6 %, not significantly different from that found in the normal condition. For – S and –Ca, each depletion did not significantly alter the levels of GI and LP, but slightly reduced PHB in *Synechocystis* (Fig. 3).

Carbon storages under heterophototrophic growth combined with nutrient depletion(s) and the elevated light intensity

Synechocystis cells were adapted from the normal autophototrophic to heterophototrophic conditions by transferring the cells to the fresh medium with 0.4% (w/w) glucose addition (+Glu) and the cultures were placed under the moderate-high light intensity at 200 µmol photon.m^{-2}.s^{-1} for 12 days. When compared with the normal autophototrophic condition, +Glu treatment slightly raised GI (21.8% to 28.3%), LP (10.2% to 14.2%), but significantly increase PHB content (3.3% to 9.2%) which altogether made up total [GI+LP+PHB] increased from 35.4% to 51.7% (Table 1). +Glu also enhanced the biomass production up to 6.1 folds (228 to 1394 mg.L^{-1}, highest in this work) as well as the [GI+LP+PHB] productivity to 8.9 folds (81 to 722 mg.L^{-1}, highest in this study).

Fig. 3

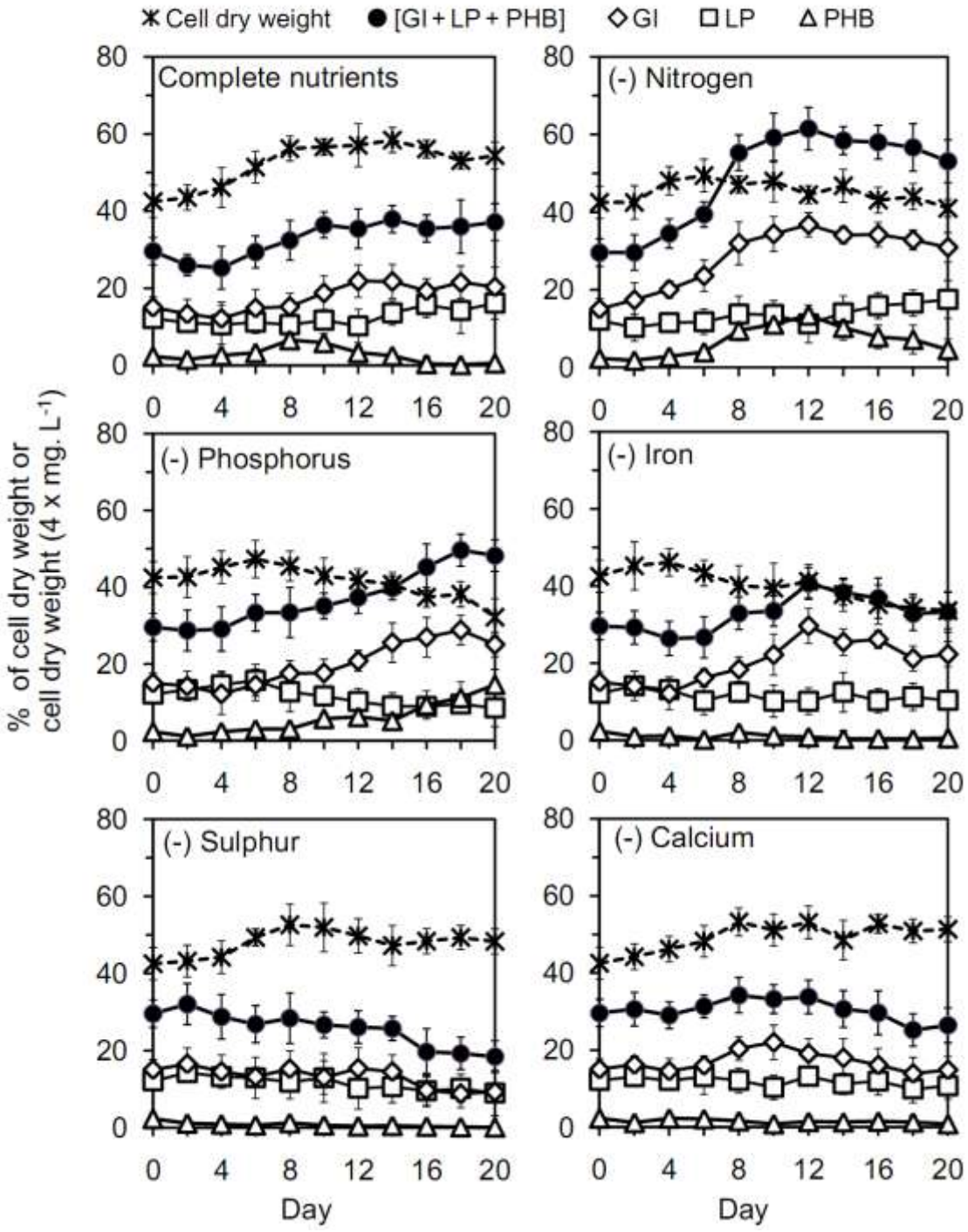


Fig. 3. Accumulation of *Synechocystis* carbon storages upon nutrient depletions. Stationary-growth cells that had been grown in the standard BG11 media with complete nutrient were transferred to the fresh BG11 or the BG11 without a specific

nutrient (-) as indicated and then grown at 50 µmol photon.m^{-2}.s^{-1}. Data are means ± 1SD of at least three independent experiments.

+Glu in the combination with –N (+Glu –N) sharply increased the levels of GI (21.8% to 41.3%, highest in this work), LP (10.2% to16.7%) and PHB (3.3% to 13.1%) which made up [GI+LP+PHB] co-accumulation raised from 35.4% to 71.1%, the highest in this study (Table 1). We noted that this (+Glu –N) condition yield more cellular [GI+LP+PHB] co-accumulation than that of +Glu condition by 1.4 fold; however, (+Glu –N) condition yielded only 0.32-fold biomass and 0.46-fold [GI+LP+PHB] productivity when compare to those of +Glu condition.

(+Glu –P) condition sharply increased PHB accumulation (3.3% to 15.1%), but did not considerably altered GI and LP contents. For (+Glu –N –P) condition, it significantly increased PHB accumulation up to 21.8% of dry weight, the highest in this study; however this condition yielded lower levels of GI and LP than those found in (+Glu –N) condition. We also noted that extending the culturing time from 12 to 18 days reduced all storages content in heterophototrophically-grown *Synechocystis* (Table 1), and that glucose additions above or below 0.4 % (w/w) did not yield a higher [GI+LP+PHB] accumulation and productivity than those found in the 0.4% glucose conditions (data not shown).

Table 1.

Co-production of the carbon storages in *Synechocystis* under heterophototrophic condition combined with nutrient depletion and elevated light intensity. Exponential-phase cells under the normal growth condition were collected and further cultured in the indicated condition using the light intensity 200 µmol photon.m^{-2}.s^{-1}. Data are averages from at least three independent analyses.

	12-day cultured cells						18-day cultured cells					
	% of dry weight				Cell dry weight ($mg.L^{-1}$)	[GI+LP+PHB] productivity ($mg.L^{-1}$)	% of dry weight				Cell dry weight ($mg.L^{-1}$)	[GI+LP+PHB] productivity ($mg.L^{-1}$)
Conditions[a]	PHB	LP	GI	[GI+LP+PHB]			PHB	LP	GI	[GI+LP+PHB]		
Control	3.3 ± 1.4	10.2 ± 3.4	21.8 ± 4.2	35.4 ± 5.1	228.5 ± 30.5	81.0 ± 0.03	0.2 ± 0.1	14.2 ± 3.9	21.5 ± 4.2	36.0 ± 4.9	212.4 ± 24.2	76.5 ± 8.2
+Glu	9.2 ± 2.6	14.2 ± 3.4	28.3 ± 3.1	51.7 ± 6.7	1394.2 ± 115.7	722.1 ± 0.07	5.3 ± 1.5	12.1 ± 2.1	30.2 ± 3.7	47.6 ± 6.2	1492.5 ± 98.7	711.6 ± 65.4
+Glu -N	13.1 ± 2.2	16.7 ± 3.1	41.3 ± 4.2	71.1 ± 5.4	448.5 ± 44.2	332.4 ± 0.08	8.6 ± 1.7	10.8 ± 2.6	34.1 ± 4.2	53.6 ± 6.5	420.9 ± 25.2	226.0 ± 23.7
+Glu -P	15.1 ± 2.5	10.2 ± 1.7	25.1 ± 3.5	50.4 ± 4.3	367.3 ± 36.3	185.4 ± 0.04	9.1 ± 2.3	8.9 ± 2.2	27.1 ± 3.6	45.2 ± 3.3	305.4 ± 21.8	138.1 ± 14.2
+Glu -N -P	21.8 ± 3.0	10.8 ± 2.0	30.7 ± 3.1	63.4 ± 6.2	333.4 ± 45.6	211.5 ± 0.05	10.2 ± 2.1	9.8 ± 1.3	27.8 ± 2.1	47.9 ± 5.4	278.5 ± 19.5	133.4 ± 12.6

[a] Control, the standard BG11 media; +Glu, the BG11 with 0.4% (w/v) glucose supplementation; -N, Nitrogen depletion; -P, Phosphorus depletion.

Isolation of cyanobacteria with high GI and LP content from Thailand natural resources

In this work,we isolated cyanobacteria and eukaryotic green algae from fresh water resources in Thailand. From 122 isolated we screened for their LP and PHB contents using Nile Red fluorescence staining method. The potential strains were analyzed for their GI, LP and PHB accumulation. We obtained two cyanobactrial strains that can produce such three carbon storages and they were morphologically identified as *Synechococcus sp. and Nostoc sp.* which the later species yielded the co-accumulation at 34.3% (15.2 GI, 9.6 LP and 9.4% PHB) (Table 2 and 4).

We also obtained three eukaryotic green algae for high LP accumulation as observed by Nile Red fluorescence microscopy (Table 3). However such strains did not produce PHB. We analyzed one isolated green algae, *Chlorella sp.* and found the co-accumulation at 54.6% (12.1% GI, 42.5% LP, 0.0% PHB) (Table 4).

Table 2. Thai-isolate cyanobacteria capable of accumulating PHB and LP.
Nile Red fluorescence dye binds both LP and PHB and illuminated golden color while cellular chlorophyll eluminated red color under fluorescence microscope.

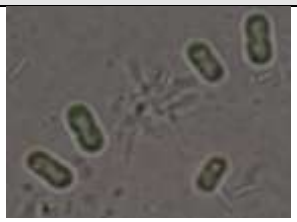
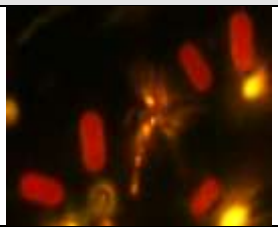
Isolate no.	Cyanobacteria	Non staining	Nile red staining
TM1 TM2 TM3	*Synechococcus sp.* (< 3 µm)		
TM9	*Nostoc sp.* (> 20 µm)		

Table 3. Thai-isolate eukaryotic green algae with high LP content but not producing PHB. Nile Red dye illuminated as golden while chlorophyll appears as red under fluorescence microscope.

Isolate no.	Eukaryotic green algae	Non - staining	Nile red staining
TM4, TM5, TM6	*Chlorella sp.* (3-5 µm)		
TM7	*Ankistrodesmus sp*. (5-10 µm)		
TM8	*Nitzschia sp.* (10-20 µm)		

Table 4. Carbon storages in Thai-isolated cyanobacteria and green algae.

Cells were grown under the autotrophic growth with nitrogen deprivation under the light intensity 50 µmol photon.m^{-2}.s^{-1}. Data are averages from three independent analyses.

Strains	Characteristics	Content (% dry weight)			
		GI	LP	PHB	GI+LP+PHB
Synechococcus sp.	Unicellular Cyanobacteria	2.1 ± 0.9	8.7 ± 2.4	3.2 ± 1.1	14.0 ± 4.1
Nostoc sp.	Filamentous Cyanobacteria	15.2 ± 3.5	9.6 ± 3.3	9.4 ± 3.0	34.2 ±10.2
Chlorella sp.,	Unicellular Green Algae	12.1 ± 4.6	42.5 ± 10.1	None detectable	54.6 ± 12.3

DISCUSSIONS

We found that both GI and LP are the major carbon storages of *Synechocystis* in all autophototrophic growth phases with the only exception that during the early to the mid stationary phases, GI accumulation increased and became the main cellular carbon storage (Fig. 1). *Synechocystis* PHB content were found to be subtle in all autophototrophic growth. In the autophototrophic condition with –N, -P or -Fe, as well as at in the all heterophototrophic growths determined in this study, GI was the major carbon storage, while LP and PHB were the minors (Fig. 3 and Table 1). These results implied the role of GI as the major carbon storage of *Synechocystis* in adapting to the stationary phase of autophototrophic growth, heterophototrophic growth and the –N, -P or -Fe conditions.

It has been known that Nitrogen- or Phosphorus-limiting conditions increase accumulations of GI [1,12,29] or PHB [17-19,24] in various cyanobacterial species. Enhanced both GI and PHB contents by limited nitrogen supply was previously reported in cyanobacterium *Asthrospira maxima* which the GI content was estimated by phenol/sulfuric acid method to ranges from 60% to 70 %, and PHB was found to not exceed 0.7% of dry weight [20]. Another attempt to optimize co-production of the three carbon storages by increased CO_2 concentration was recently reported in the thermophilic cyanobacterium *Thermosynechococcus elongates* which the total accumulation was 50% of dry weight (22% GI, 15% PHB and 3 % LP) [10]. In this present study, we found that the autophototrophic –N condition with atmospheric CO_2 concentration, can increased the co-accumulation to 61.5 % (36.8% GI, 13.5% PHB and 17.5% LP, Fig 3) in *Synechocystis*. Further combined –N with +Glu and the elevated-light intensity synergistically enhanced the *Synechocystis* co-productions to 71% of biomass (41.3% GI, 13.1% PHB and 16.7% LP), the highest in this study in

term of % cell dry weight (Table 1). However, when consider the productivity of [GI+LP+PHB], +Glu condition yielded the maximum productivity at 722 $mg.L^{-1}$, about 2.2 and 6.6 folds higher than those found in (+Glu –N) and -N conditions, respectively, due to the superior biomass yield obtained under +Glu condition (Table 1 and Fig. 3). We also found that–S, -Fe or -Ca did not significantly increase the co-production of such three carbon storages.

In this study, we obtained the maximum PHB accumulations in *Synechocystis* at 21.8% under (+Glu –N –P) condition (Table 1). We noted that, significant cyanobacterial PHB content at 45% of dry weight was demonstrated previously in *Nostoc muscorum* grown under glucose and acetate addition, combined with elevated CO_2 concentration and limited K_2HPO_4 supply [24]. For LP, no significantly enhanced production was described in cyanobacteria; the LP content determined in various strains was found to ranges from 1% to the maximum at 20% of dry weight [2,7,13]. In this work, increased *Synechocystis* LP from ~10% to 16.7%-17.5% were obtained in both autophototrophic and heterophototrophic growths (Fig. 1, Fig. 3 and Table 1). Although the majority of *Synechocystis* LP is diacylglycerols as characterized previously [25], we noted that the LP contents reported in this study must be carefully interpreted as total lipids since we quantified LP by the dichromate method that reacts to various types of lipid molecules [3].

We also found that the optimized light intensities for the co-production of the three storages were at the moderate levels which yielded the co-accumulation bout two-fold higher than those found at the low and the high light level (Fig 2). This results is correlated to the previous study that 2.5-fold increased GI content in *A. platensis* was achieved at elevated light intensities [1].

In this study we also obtained two Thai-isolated species of cyanobacteria: *Synechococcus sp*. and *Nostoc sp*. capable of co-producing GI, LP and PHB; however the co-production was reached to only 34.2% under –N condition (Table 4). Three lipid-accumulating species of eukaryotic green algae from Thailand were obtained. One of them, *Chlorella sp*. accumulating up to 42.5% of cellular LP and 12.1 % GI, but did not produce PHB at –N condition (Table 4).

CONCLUSION

This work demonstrated enhanced co-accumulations of the two potent biofuel substrates: glycogen and lipid, as well as the biodegradable-plastic PHB in cyanobacteria which the total cellular co-accumulation reached 71% of biomass and the productivity was up to 722 $mg.L^{-1}$ at the 12-day culture period. Further enhancing the product productivity as well as utilizating the left over proteins, pigments and biomass which the later could be used in gasification/pyrolysis for fuel production, would create a mean for maximal utilization of cyanobacterial and microalgal bioresources.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

   ยังไม่ได้ตีพิมพ์ ผู้วิจัยได้เขียน Manuscript เสร็จแล้วและกำลังอยู่ระหว่างการ revise กับ mentor เพื่อจะ submit เพื่อตีพิมพ์ในวารสารนานาชาติ ISI กลุ่ม Biotechnology (IF. ประมาณ 1-2) ภายในหนึ่งเดือนจากนี้

2. การนำผลงานวิจัยไปใช้ประโยชน์

   ยังไม่มี

3. การเสนอผลงานในที่ประชุมวิชาการ

   3.1 การเสนอผลงานแบบ poster ในหัวข้อ Biohydrogen and Bioplastic Production from Cyanobacteria in Thailand ในการประชุมวิชาการระดับนานาชาติ EBEC: European Biofuels Exhibition & Conference, 5-8 November, 2011, LONDON, UK

   3.2 การเสนอผลงานแบบบรรยายในหัวข้อ Microalgae as Cell Factories to Produce Oil & Bioplastic ในการประชุมวิชาการประชุมวิชาการสาหร่ายและแพลงก์ตอนแห่งชาติครั้งที่ 6 วันที่ 28-30 มีนาคม 2556 จังหวัดเชียงใหม่

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