Effect of Increased Supply of Carbon Dioxide on Biomass Production, Bioplastic Accumulation, Carbon Content and Nitrogen Content in Cyanobacterium *Synechocystis* sp. PCC6803.

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ABSTRACT

Increasing level of carbon dioxide (CO_2) gas severely intensifies global climate change. One of biological methods to reduce CO_2 is using photosynthetic cyanobacteria to consume CO_2. This study determined effects of CO_2 supply on biomass and carbon content of cyanobacterium *Synechocystis* sp. PCC6803. The Na_2CO_3 was used as a source of CO_2 donor. The normal Na_2CO_3 supply (0.2 mM) yielded biomass level at 1.0 g/L. The increased Na_2CO_3 supplies at 9.5 or 38 mM, significantly enhanced biomass level to 1.3-1.4 g/L, and increased carbon: nitrogen ratio by 1.3-1.4 fold, however; these Na_2CO_3 supply did not enhanced bioplastic poly-3-hydroxybutyrate level. Further increased Na_2CO_3 to 95-190 mM reduced biomass and poly-3-hydroxybutyrate level. This optimal Na_2CO_3 supply that increased biomass and carbon: nitrogen content may be applicable in other cyanobacteria to reduce CO_2.

Keywords: Cyanobacteria, Carbon dioxide, Biomass

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Introduction

Increasing carbon dioxide (CO_2) concentration in the atmosphere has been creating several negative environmental impacts, such as the climate change and global warming. There are several ways to mitigate CO_2 in the atmosphere such as physical or chemical methods but the cost of these methodologies are expensive. One of effective methods to reduce CO_2 is biological approaches such as using photosynthetic cyanobacteria that consume CO_2 as a sole carbon substrate to produce biomass and valuable products (Singh et al., 2015).

Cyanobacterial photosynthesis pathway consists of two parts. The first part is light reaction that water is received the energy from light and is converted to ATP and NADPH. Then ATP and NADPH are transferred to the second part of the dark reaction of Calvin cycle that combines CO_2 in to organic molecules (Nicholas, Noel ,1992).

In green eukaryotic algae (*Chlorella* sp. and *Scenedesmus* sp.) and cyanobacteria (*Spirulina platensis*), increased CO_2 supply from 0.06% to 6% (v/v) enhanced biomass levels about 1.5-1.9 times and this also increased particular bioproduct levels. For significant increases of bioproduct levels, under 6% (v/ v) CO_2, *Chlorella* sp. increased carbohydrate content by 3 times, *Scenedesmus* sp. increased lipid content by 1.3 times, cyanobacterium *Spirulina platensis* increased protein content by 1.1 times (Singh et al., 2015) as show in Table 1. Carbohydrates and lipids are carbon-containing compounds, while proteins consist of both carbon and nitrogen. Nevertheless, effects of increased CO_2 supplies on carbon and nitrogen content of cyanobacteria have not been reported.

Table 1. Biomass and biochemical contents of *Chlorella* sp. (microalgae) , *Scenedesmus* sp. (microalgae) and *Spirulina platensis* (cyanobacteria) cultured under normal CO_2 and elevated CO_2 supply (Singh et al., 2015).

Strain	Biomass and biochemical content	Normal CO_2 supply (0.06% v/v)	High CO_2supply (6% v/v)
Chlorella sp. (Green algae)	Biomass (g $L^{-1}d^{-1}$)	0.60	0.94
	Protein (% dry weight)	53	35
	Carbohydrate (% dry weight)	17	50
	Lipid (% dry weight)	9	16
Scenedesmus sp. (Green algae)	Biomass (g $L^{-1}d^{-1}$)	0.45	0.84
	Protein (% dry weight)	16	24
	Carbohydrate (% dry weight)	41	46
	Lipid (% dry weight)	19	25
Spirulina platensis (Cyanobacteria)	Biomass (g $L^{-1}d^{-1}$)	0.70	1.03
	Protein (% dry weight)	57	66
	Carbohydrate (% dry weight)	22	31
	Lipid (% dry weight)	4	5

Poly(3-hydroxybutyrate) (PHB) is the biodegradable plastic. PHB has comparable material properties to those of chemical plastics. PHB was produced from renewable resource such as cyanobacteria. Cyanobacteria used CO_2 and light energy to produce PHB via photosynthesis. However, the effect of increased CO_2 supply to PHB production by cyanobacteria is still unknown.

In this study, Na_2CO_3 was used as a source of CO_2. Upon dissolving in water, Na_2CO_3 generates bicarbonate ion (HCO^-_3). This bicarbonate ion (HCO^-_3) is the same ion form that was obtained from the dissolved CO_2 in the water. In this study, cyanobacterium *Synechocystis* sp. PCC6803, the laboratory model organism of cyanobacteria, was cultured under various Na_2CO_3 supplies up to 40 days and the biomass level, PHB content, carbon content and nitrogen content were determined.

Objectives of the study

1. To identify optimal CO_2 supply that yields maximum biomass of cyanobacterium *Synechocystis* sp PCC6803.
2. To determine effect of increased CO_2 supplies on PHB content, carbon content and nitrogen content of cyanobacterium *Synechocystis* sp PCC6803.

Methodology

Strain, growth conditions and biomass measurement

Cyanobacterium *Synechocystis* sp. PCC6803 was obtained from Pasture Institute, France. Cells were cultured using 500-ml erlenmeyer flasks containing 220 ml of BG11 medium (Rippka et al., 1979) under 25 μmol/m^2/s continuous white light upon 160 rpm shaking at 30 °C (hereafter, the standard growth condition) for 7 days. Next, the 7-day old cells were diluted to be approximate 0.15 of the optical density at 730 nm, in the BG11 medium with different concentrations of sodium carbonate (Na_2CO_3) as follows: the normal concentration at 0.2 mM as the control, and the increased concentrations at 9.5, 38, 95 and 190 mM. The cultures were then grown under the standard growth condition up to 40 days. Cells density was monitored by measuring the optical density at 730 nm. Biomass was harvested by centrifugation at 5,500 rpm for 10 minutes and immediately dried at 80°C. Biomass was determined gravitro metrically using the scale with 0.1 mg resolution.

PHB analysis

The quantity of PHB in the biomass was determined by High Performance liquid chromatography (HPLC) following the method of Karr et al. (1983). About 5 mg dry biomass was grinded and hydrolyzed in 1 ml of 98% (v/v) sulfuric acid for 1 hour at 100°C to hydrolyzed PHB to crotonic acid. The obtained hydrolyzates were 50-fold diluted in water, and adipic acid (3 mg/ml) was added to the samples as an HPLC marker. The samples were filtered by 0.45 μm membrane, and analyzed by HPLC. HPLC machine Shimadzu HPLC LGE (Japan), using carbon-18 column (Inert-Sustain 3-μm HPLC column, GL Sciences, USA). The phosphate buffer (10 mM KH_2PO_4, pH 2.3) containing 30% acetonitrile was used as a mobile phase at the flow rate of 1.0 ml/min and the 210 nm UV detection was used to detect crotonic acid. For quantification, the peak area of crotonic acid (PHB-hydrolyzed product) obtained from

cyanobacteria were compared to the peak area of the hydrolyzed product obtained from commercial PHB (Sigma, Chemical Company, USA) and crotonic acid standard (Sigma, Chemical Company, USA).

Analysis of carbon and nitrogen content in biomass

The analysis was performed by Scientific and Equipment Centre, Chulalongkorn University. The carbon and nitrogen content was analyzed using CHNS elemental analyzer (Thermo Fisher 2000, USA). Essentially, about 5 mg dry biomass was grinded and subjected for the combustion at 1800 °C in an oxygen-rich environment. Carbon was converted to carbon dioxide, and nitrogen was altered to nitrogen gas. The combustion gasses were then analyzed by gas chromatography. (Michael, 2008)

Results

Biomass growth of *Synechocystis* was enhanced under optimal Na_2CO_3 supply

Synechocystis sp. PCC 6803 was cultured in BG11 medium with various concentration of Na_2CO_3: 0.2 mM as normal level, and the increased levels at 9.5-190 mM.

Under normal Na_2CO_3 supply (0.2 mM), biomass level reached approximately the peak at 1.0 g/L (Figure 1). Increased Na_2CO_3 supply to 9.5 and 38 mM significantly enhanced biomass to the maximum levels of 1.3-1.4 g/L, the 30-40% increased from that obtained from normal Na_2CO_3 supply (Figure 1). Unexpectedly, further increased Na_2CO_3 supply to 95 and 190 mM sharply reduced the biomass level.

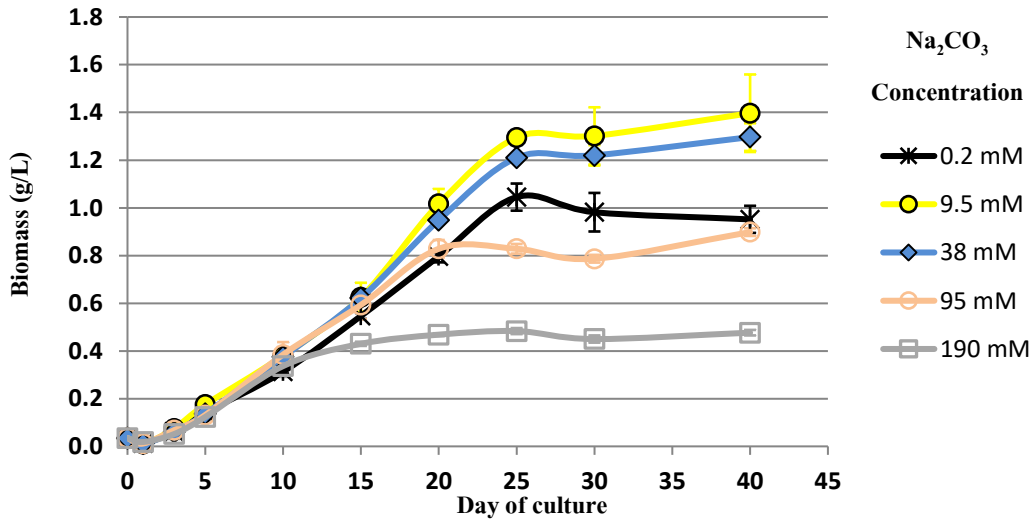

Figure 1. Biomass levels of *Synechocystis* sp. PCC 6803 under increased concentration of Na_2CO_3. The 0.2 mM is the normal Na_2CO_3supply. The 9.5-190 mM are increased Na_2CO_3 supply. Data are averages of three independent cultures.

PHB content of *Synechocystis* was not enhanced under increased Na_2CO_3 supply

Biomass of *Synechocystis* sp. PCC 6803 was determined for PHB contents. Biomass was hydrolyzed in sulfuric acid to convert PHB to crotonic acid (PHB-hydrolyzed product) and the obtaining crotonic acid was subsequently quantified using high performance liquid chromatography (HPLC). Commercial PHB (positive control), sulfuric acid (negative control) and adipic acid (HPLC marker) were included in HPLC. Figure 2 showed that HPLC fractionated these above mentioned acids at the specific retention times as follows: sulfuric acid at 1.4 min, adipic acid at 2.1 min and crotonic acid (PHB-hydrolyzed product) at 2.9 min.

PHB content was determined in the cells cultured under normal Na_2CO_3 supply and under the elevated Na_2CO_3 supplies at day 0 (initial culture time), day 10 (early exponential growth phase), day 20 (mid exponential growth phase), day 30 and day 40 (stationary growth phase). Overall resulted in Figure 3 showed that increased Na_2CO_3 supplies (9.5-190 mM) did not enhanced PHB contents of the cells when compared to that obtained under the normal Na_2CO_3 supply (0.2 mM). In particular significance, the maximal PHB content (3.2% w/w dry weight) was found under the normal Na_2CO_3 supply which this value is approximate 2-12 times higher than those obtained under the increased Na_2CO_3supplies.

Elevated Na_2CO_3 supply increased carbon: nitrogen ratio of *Synechocystis*

Synechocystis sp. PCC 6803 was analyzed for carbon and nitrogen content (% w/w dry weight). The cells were cultured under normal Na_2CO_3 supply (0.2 mM). Results showed that increased Na_2CO_3 supply at 9.5 or 38 mM yielded the maximal biomass levels of 1.3-1.4 g/L (Figure 1). The cells were analyzed at day 0 (initial culture time), day 20 (mid exponential growth phase) and day 40 (stationary growth phase, Figure 1).

For carbon content, the normal Na_2CO_3 supply yielded carbon content at 46-47% w/w dry weight during day 20-40. The increased Na_2CO_3 supplies subtly reduced (but not significantly reduced) carbon content to 44-45% w/w dry weight during day 20-40. (Figure 4A).

For nitrogen content, the normal Na_2CO_3 supply showed nitrogen content at 10-11% w/w dry weight during day 20-40. Interestingly, the increased Na_2CO_3 supplies moderately reduced (significantly reduced) nitrogen content to 7-8% w/w dry weight during day 20-40. (Figure 4B).

For carbon: nitrogen ratio (C:N ratio hereafter), the normal Na_2CO_3 supply exhibited the C:N ratio at 4.3-4.6 while the increased Na_2CO_3 supplies at 9.5 and 38 mM significantly raised the C:N ratio to 5.4-5.8 and 6.0-6.3 %, respectively (Figure 4C).

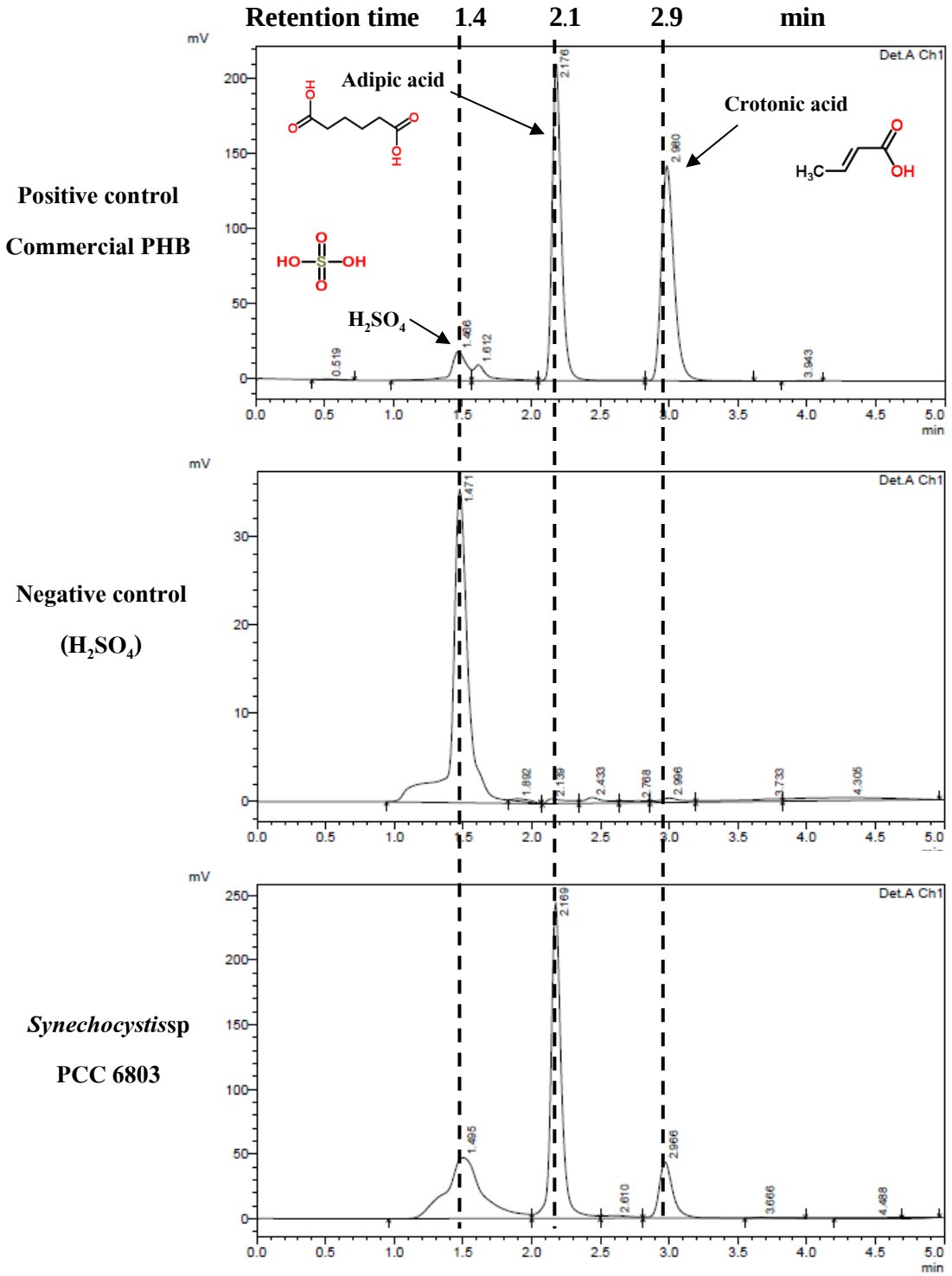

Figure 2. HPLC chromatogram for PHB analysis. Sample (commercial PHB or *Synechocystis* biomass) were hydrolyzed in sulfuric acid to convert PHB to crotonic acid (PHB-hydrolyzed product) and analyzed using high performance liquid chromatography (HPLC). Commercial PHB (positive control), sulfuric acid (negative control) were included in the HPLC. Adipic acid was added in every sample as HPLC marker.

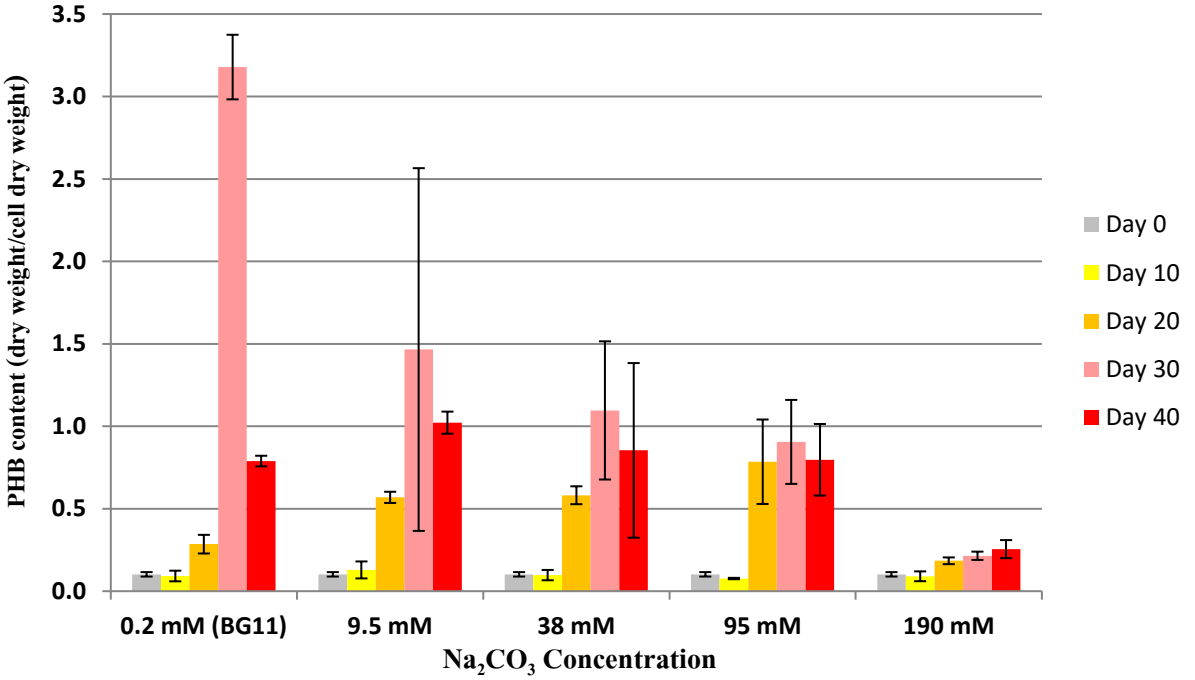

Figure 3. Cellular PHB contents of *Synechocystis* sp. PCC 6803 cultured under different Na_2CO_3 supply. The 0.2 mM is the normal Na_2CO_3 supply and 9.5-190 mM is increased Na_2CO_3 supply. Cells were cultured up to 40 days. Data are mean of three independent experiments.

Discussion and Conclusions

Biomass levels of *Synechocystis* under the optimal Na_2CO_3 supplies

Increased Na_2CO_3 supplies to 9.5 mM and 38 mM enhanced biomass levels by 30-40% compared to that acquired by the normal Na_2CO_3 supply (0.2 mM). However, further increased Na_2CO_3 supply to 95 mM and 190 mM reduced the biomass level. The results were corresponding to the previous study that cyanobacterium *Spirulina platensis* cultured under the increased CO_2 concentration (from 0.06% to 6% v/v) increased biomass level by 47%, but further increased CO_2, reduced the biomass level (Singh et al., 2015).

In other study, bicarbonate was used as carbon source for *Spirulina platensis* and the result show that increased bicarbonate supply from 0.038% to 0.5% (w/v) increased biomass level about 100%, but under increased bicarbonate more than 0.5% (w/v), the biomass level was slightly decreased (Zhang et al., 2015).

In this study, we used Na_2CO_3 as a source of CO_2 to enhance the biomass because cells can uptake bicarbonate ion (HCO^-_3) that is the disassociation product from Na_2CO_3 in the water at pH 7.5-10. The reducing of biomass when further increased CO_2 concentration may be the effects of high pH in the medium which is corresponding to the previous study that found elevated pH under high Na_2CO_3 supply (Touloupakis et al., 2016). The optimal pH for *Synechocystis*is growth is between 7.5-9, and in this study, the Na_2CO_3 supplies was found to increase pH of the media to pH11(data not shown) which might be toxic to the cells. Another negative drawback of high levels of Na_2CO_3 supplies is the presence of sodium ion (Na^+) that may be toxic to the cells.

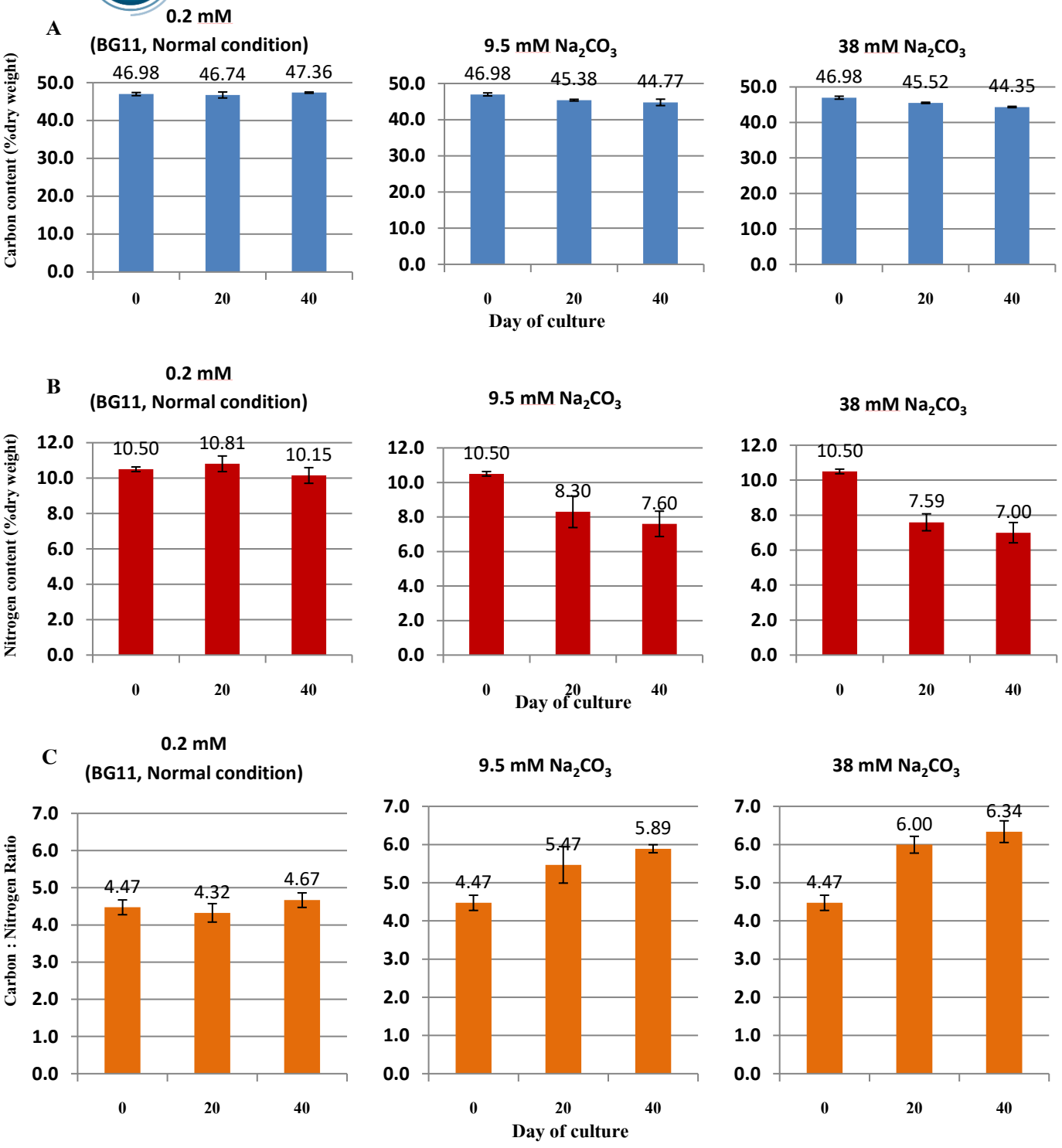

Figure 4. Carbon and nitrogen content of *Synechocystis* sp. PCC 6803 cultured under different Na_2CO_3 supply. (A) Carbon content. (B) Nitrogen content. (C) content: nitrogen ratio. Cells were harvested for the analysis at day 0 (starting period), day 20 (exponential growth phase) and day 40 (stationary phase). The increased Na_2CO_3 supply at 9.5 and 38 mM that yielded the maximal biomass levels were used in this section. The normal Na_2CO_3 supply at 0.2 mM was included as the control experiment. Data are average of three to four independent experiments.

PHB content of *Synechocystis* under increased Na_2CO_3 supply

In other study, increased CO_2 supply in cyanobacterium *Thermosynechococcus elongates* from the atmospheric 0.04 to 20%v/v, decreased PHB content from 14 to 2% (w/w DW) (Eberly, Ely, 2012). This result was similarly to this study that the increased Na_2CO_3 supplies (9.5-190 mM) decreased PHB contents of *Synechocystis* by 2-12 times.

The increased PHB accumulation in cyanobacterium *Synechocystis* was reported under the limitation of nutrients such as nitrogen or phosphorus that increased PHB content up to 4.8-5.5 times. This increased PHB levels may be the effect of the nutrients limitation that induced PHB accumulation as the cellular energy storage (Panda et al., 2006). In this study the cells were not cultured in the deprivation of such nutrient conditions. So, further experiments that cultured the cells in such nutrient deprivations may be able to induce PHB accumulation in *Synechocystis.*

Carbon: nitrogen ratio of *Synechocystis* under increased Na_2CO_3 supply

In this study, all increased Na_2CO_3 supplies did not significantly altered carbon content (C) but significantly decreased nitrogen content (N). As a result, C:N ratio was increased upon elevated Na_2CO_3 supplies. The changing of C: N ratio may be related to the accumulation of bioenergy molecules in the cell, particularly increasing carbon-containing biomolecules such as carbohydrates and lipids, and reducing nitrogen-containing molecules such as proteins. In the previous study of *Spirulina platensis*, increased CO_2 supply enhanced lipid and carbohydrate content by 1.2 and 1.4 times, respectively (Singh et al., 2015, Table 1.). This result implied that the increased lipid and carbohydrate content that is carbon-containing molecule may increase C:N ratio. Results in this study implied that the cells under increased CO_2 supply prefer to accumulate carbon-containing compounds rather than nitrogen-containing compounds. *Synechocystis* should be further determined for protein, carbohydrate and lipid content because different CO_2 concentrations may affect biomolecule compositions of cyanobacteria as suggested previously (Gonçalves et al., 2016).

In conclusion, the optimal Na_2CO_3 supply is 95 mM. This optimal Na_2CO_3 supply can enhance the biomass production of *Synechocystis* sp. PCC 6803 to the maximum level of 1.40 g/L and also significantly increase C:N ratio to 5.89. However, this optimal Na_2CO_3 supply significantly reduced PHB content. This increased Na_2CO_3 supply may be applicable to enhance biomass in other cyanobacteria.

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