Science and Technology

RMUTT Journal

Vol.6 No.2 (2016) : 29-36

www.sci.rmutt.ac.th/stj

Online ISSN 2229-1547

Suitable Cell Age for Enhanced Poly-ß-Hydroxybutyrate Accumulation under Photoautotrophic Nutrient Deprivation of *Synechocystis* sp. PCC 6803

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Abstract

Synechocystis sp. PCC 6803 can convert atmospheric CO_2 and water into carbohydrates by photosynthesis and store them as glycogen, lipid or poly-ß-hydroxybutyrate (PHB) when nutrient limitation. In this study, under normal photoautotrophic condition, *Synechocystis* cells could utilize nitrate and phosphate in medium for supporting growth, but cells did not show high PHB accumulation (0.1 – 6.5 %(w/w) DW) in all cultivated times although the concentration of nitrate and phosphate were low levels. The age of cell was found to have a major effect on PHB accumulation in *Synechocystis* cell. The maximum PHB content was 15.2 % (w/w) DW when cells at mid-stationary phase adapted in photoautotrophic nutrients (nitrogen and phosphorus) deprivation for 8 days. However, cells in all stationary phase were also found to accumulate of glycogen at higher levels than PHB. Therefore the main carbon storage component under photoautotrophic nutrient deprivation might be glycogen and PHB as a co-component, respectively.

Keywords: Cell age, Poly-ß-hydroxybutyrate, Nutrient deprivation, *Synechocystis* sp. PCC 6803

1. Introduction

Poly-ß-hydroxybutyrate (PHB) is the most common type of polyhydroxyalkanoic acid (PHA), which have attracted the interest of chemical industry because of their biodegradable property for applications in various technical,

Received: December 17, 2016

Revised: December 30, 2016

Accepted: December 30, 2016

medical and pharmaceutical sectors [1]. There are several microorganisms capable of PHB production under different modified nutrient conditions, such as fermentative or chemoautotrophic bacteria and microalgae including cyanobacteria [2-4].

Synechocystis sp. PCC 6803 is a unicellular non-nitrogen fixing cyanobacterium which its entire genome has been sequenced [5]. This strain can accumulate PHB up to 15% (w/w) DW under nitrogen deprivation in the presence of acetate [6]. Carbon dioxide (CO_2) is abundant carbon source and cheaper than acetate, accumulated PHB using CO_2 as only one of carbon source has also been reported in cyabobacteria, but it content is very low, i.e. maximum up to 4.5% (w/w) DW of early-stationary phase cells [7]. The PHB content was increased up to 13.5% (w/w) when transferred early-stationary phase cells into nitrogen deprivation [8]. Therefore, in this study the effect of cell age on PHB accumulation has been performed for enhanced PHB level using CO_2 as source of carbon under nutrient deprivation.

2. Materials and Experiment

2.1 Strain, culture condition and growth determination

The cyanobacterium *Synechocystis* sp. PCC 6803 (Pasteur Institute, France) was cultured photoautotrophically in 1000 ml of BG11 medium [9] buffered with 20 mM HEPES-NaOH (pH = 7.5). The cultures were bubbled continuously with air and incubated under continuous illumination of 40 $\mu E/m^2/s$ at 28^oC. The growth rate was determined spectrophotometrically by measuring the optical density of cells at 730 nm. Total amount of chlorophyll *a* determination was performed in 90% methanol according to MacKinney [10]. The cell dry weight (DW) was performed by drying wet cells in a 60^oC oven until a constant weight was obtained. Nitrogen deprivation was achieved by adaptation of cells in medium devoid of $NaNO_3$ and replacing ferric ammonium citrate and $Co(NO_3)_2 \cdot 6H_20$, with an equimolar equivalent of ferric citrate and $CoCl_2 \cdot 6H_20$, respectively. Phosphorus deprivation by replacing K_2HPO_4 with KCl in an equimolar substitution.

2.2 Analysis of nitrate (NO_3^--N) and phosphate (PO_4^{3}-P) concentrations

The 30 ml of cell suspensions were passed through a filter (Grade GF/C Glass Microfiber Filter; GE Healthcare) to remove cells and some other organic particles before analysis of nitrate (NO_3^--N) and phosphate (PO_4^{3}-P) remaining in medium in every 2 days according to standard methods [11].

2.3 Analysis of PHB levels

The quantification of PHB, as %(w/w) DW, was analyzed by high performance liquid chromatography (HPLC) (Shimadzu, Japan) with a 5 µm InsertSustain C18 reverse phase column (I.D. 4.6 x 150 nm) equipped with a SPD-20A UV/VIS detector at 210 nm as described [12]. Dry cells were boiled in H_2SO_4 to hydrolyze the PHB into crotonic acid and then analyzed the content of crotonic acid by HPLC using adipic acid as internal standard. The commercial PHB (Sigma-Aldrich, USA) was

analyzed in parallel, where an 84 % (w/w) conversion of PHB to crotonic acid was obtained.

2.4 Analysis of glycogen levels

The cellular glycogen content, as %(w/w) DW, was measured using a glucose hexokinase assay (Sigma, USA), using glycogen from bovine liver as standard as previously published by the method of Bandyopadhyay et al. [13]. Glycogen content is expressed as %(w/w) DW, which calculated using a standard calibration curve.

2.5 Fluorescence microscopy

PHB granules in cells were visualized by staining with the fluorescent dye Nile red. The 20 µl of cell culture was added with 100 µl of 0.9 % (w/v) NaCl and 0.3 µl of Nile red solution (1.0 µg/ml in ethanol), mixed and incubated overnight under darkness before observation under fluorescence microscope equipped with a digital camera (Olympus DP72, Japan), using a filter cube with 535 excitation wavelength.

2.6 Statistical analysis

Data were analyzed by using one-way analysis of variance (ANOVA). The differences in means values were identified by Duncan's multiple range tests to determine whether significant difference ($P < 0.05$) existed among different treatments. All the statistical analyses were carried out using SPSS software version 15.0.

3. Results and Discussion

The unicellular cyanobacterium *Synechocystis* sp. PCC 6803 were cultured in BG11 medium with continuous light and atmospheric CO_2 (the normal photoautotrophic condition). Cell sample was taken from a culture bottle every day of cultivation to determine growth rate by measuring the optical density of cell culture and chlorophyll *a* content as shown in Figure 1.

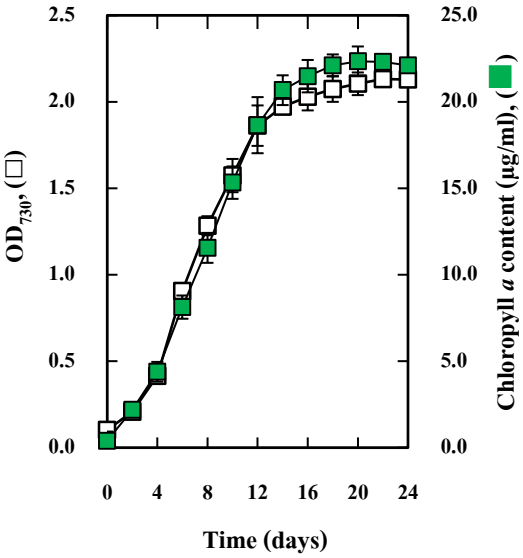

Figure 1 Growth curve of *Synechocystis* sp. PCC 6803 when cells were grown in BG11 medium (normal photoautotrophic condition). The error bars represent standard deviations of means (mean ± S.D., $n = 3$).

The growth rate pattern could be divided into different growth phases, log phase (4-10 days of cultivation), early-stationary phase (12-16 days of cultivation), mid-stationary phase (16-20 days of cultivation) and late-stationary phase (more than 20 days of cultivation), respectively. In BG11 medium containing 105.6 mg/l of nitrate and 33.8 mg/l of phosphate as the main nitrogen and phosphorus sources, *Synechocystis* cells could utilize nitrate and phosphate for supporting cell growth since the

optical cell density and chlorophyll *a* content were rapidly increased during 12 days of cultivation (Figure 1, 2), but thereafter the constant growth rate was found when the concentrations of nitrate and phosphate were dropped to 12.8 and 2.0 mg/l, respectively.

The photoautotrophic cyanobacteria can convert CO_2 and water into carbohydrates by photosynthesis using sunlight as energy source and store them as glycogen, fatty acid or poly-ß-hydroxybuterate [14].

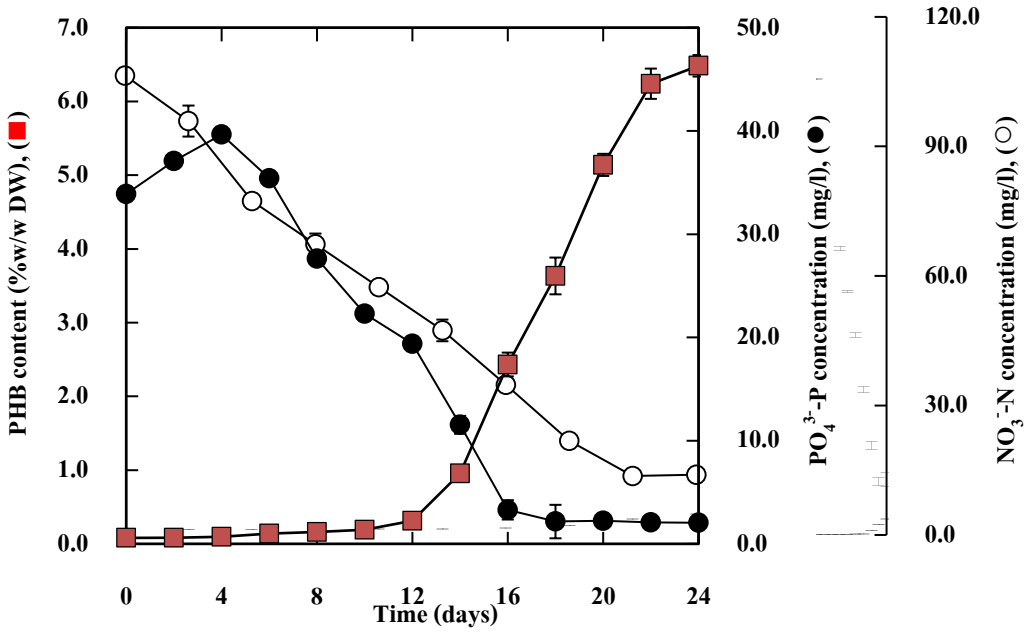

Figure 2 Phosphate and nitrate remaining in BG11 medium and PHB accumulation of *Synechocystis* sp. PCC 6803 when cells were grown under photoautotrophic growth condition. The error bars represent standard deviations of means (mean ± S.D., $n = 3$).

Under photoautotropic growth, PHB accumulation of *Synechocystis* cell was low (0.1 - 0.3 %(w/w) DW) during the log to early-stationary phases (4 - 12 days of cultivation) and then continually increased up to 6.5 %(w/w) DW during mid to late-stationary phases (18 - 24 days of cultivation), respectively. The nitrate and phosphate remaining in the medium was also found in cells during mid to late-stationary period (Figure 2). Nutrient-limited growth has previously reported to increase the accumulation of PHB and glycogen in many cyanobacteria [4, 15, 16]. However, nutrient-deprived growth has known that able to induce more PHB accumulation than nutrient-limited growth [7, 8]. Therefore, the different cell ages adapted under nitrogen and phosphorus deprivations to enhance PHB accumulation under photoautotrophic condition has been investigated in this study. The

result as shown in Figure 3 was revealed that cell at mid-stationary phase (18 days of cultivation) showed highest PHB accumulation under nitrogen and phosphorus deprivations which the maximum PHB content was 15.2 %(w/w) DW. Whereas, log phase cells did not show significantly different contents of PHB when compared with the control BG11 (Figure 3). The accumulated PHB granules (bright yellow particles as shown in Figure 4) observed under fluorescence microscope were evidently appeared in cells at mid log phase adapted under nitrogen and phosphorus deprivations when compared with cells in other phases. These results suggested that the age of cell has a major effect on PHB accumulation under photoautotrophic nutrient deprived condition. In addition, cells in all stationary phase could accumulated more glycogen than PHB, Glycogen content was increased significantly up to 33.12 %(w/w) when adapting early-stationary phase cells in nitrogen and phosphorus

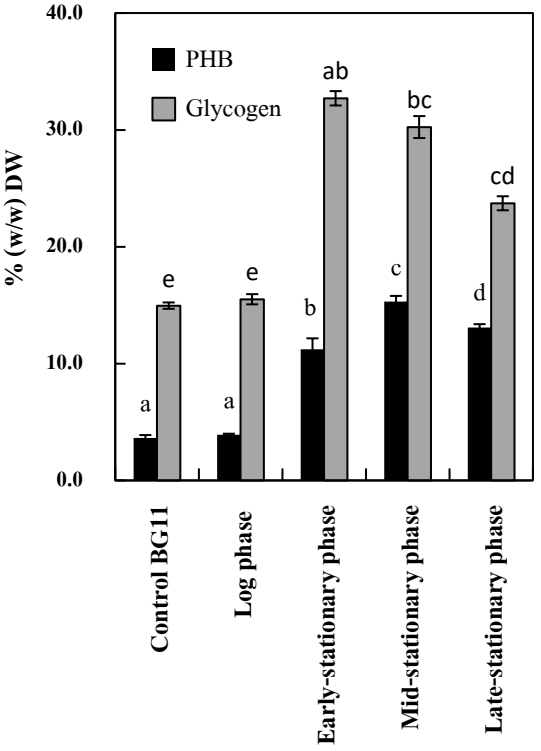

Figure 3 Effect of cell age on PHB and glycogen accumulations when cell in different phases were adapted under photoautotrophic nutrient (nitrogen and phosphorus) deprived condition for 8 days. Control BG11 was analyzed by using cells grown for 18 days in BG11 medium. The error bars represent standard deviations of means (mean ± S.D., $n = 3$). The different letter on the same value are significant different according to Duncan's test ($p < 0.05$).

deprivations for 8 days (Figure 4). It was indicated that glycogen is a major carbon storage component of *Synechocystis* cell under photoautotrophic nutrient deprived condition, which correlates with the results of Monshupanee and Incharoensakdi that nitrogen deficient under atmospheric CO_2 increased the glycogen level in all stationary phase cells [8].

However, the metabolic network of the central carbon metabolism together with the biosynthesis of PHB and glycogen would further help to gain more understanding in the role of cell age on accumulation of these carbon storage compounds.

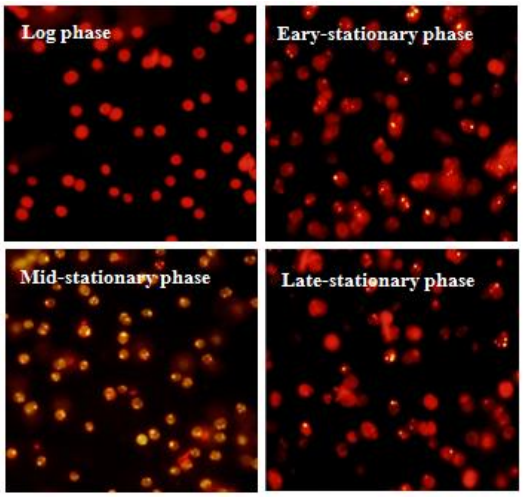

Figure 4 Fluorescence microscopy of *Synechocystis* sp. PCC 6803 when cells of different ages were adapted under photoautotrophic nutrient (nitrogen and phosphorus) deprived condition for 8 days and then stained with Nile red. The bright yellow particles represent the position of PHB granules accumulated in cell.

4. Conclusions

In conclusion, this study demonstrated the enhanced PHB accumulation in *Synechocystis* sp. PCC 6803 under photoautotrophic nutrient deprived condition. The age of cell has a major effect on PHB accumulation and the highest PHB level was 15.2 %(w/w) DW when cells in mid-stationary phase were adapted in nitrogen and phosphorus deprivations for 8 days. However, glycogen was also found as a major carbon storage than PHB under photoautotrophic nutrient deprivation in all stationary phase cell of this strain. Further studies to enhance the productivity, some selected environmental parameters playing a significant role will be examine.

5. Acknowledgements

The authors would like to thank the Rajamangala University of Technology Thanyaburi Research Foundation Scholarships Grant for New Researcher 2015 and Research Innovation and Invention 2016 for financial support.

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