



# Utilization of shrimp wastewater for poly- $\beta$ -hydroxybutyrate production by *Synechocystis* sp. PCC 6803 strain $\Delta$ SphU cultivated in photobioreactor

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## ABSTRACT

The wastewater discharge from the intensive shrimp aquaculture contains high concentration of nutrients, which can lead to eutrophication. This study aimed to reuse the shrimp wastewater for low cost cyanobacterial cultivation to produce biodegradable plastic poly- $\beta$ -hydroxybutyrate (PHB). The *Synechocystis* sp. PCC 6803 ( $\Delta$ SphU) lacking phosphate regulator (SphU) could utilize nutrients in shrimp wastewater for promoting biomass yield of 500 mg L<sup>-1</sup> after 14 days. The  $\Delta$ SphU showed the highest phosphate uptake rate of 20.16 mggDw<sup>-1</sup>d<sup>-1</sup> at the first day of photobioreactor running. In addition, the nutrient removal efficiencies were 96.99% for phosphate, 80.10% for nitrate, 67.90% for nitrite and 98.07% for ammonium. The reduction of nitrate in shrimp wastewater due to nitrogen assimilation could induce PHB accumulation in  $\Delta$ SphU. The highest PHB content was 32.48% (w/w) DW, with the maximum PHB productivity of 12.73 mg L<sup>-1</sup>d<sup>-1</sup>. The produced PHB of  $\Delta$ SphU had material properties similar to those of the commercial PHB.

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## 1. Introduction

Shrimp farming can be operated in either a marine or freshwater environment for human consumption. The world aquaculture production for crustaceans has increased over 60% from year 2006 to 2015 [1]. China, Norway, Vietnam, USA and Thailand are the top five countries for both aquaculture production and exportation of fishery commodities from 2013 to 2015 [1]. However, the continuous development of intensive shrimp aquaculture also brought some problems, especially the wastewater disposal. Every single tonne of typical shrimp aquaculture production can be translated into 5345–7157 m<sup>3</sup> of effluent discharge [2], which usually contains concentrated organic matter and nutrients (mostly phosphorus and nitrogen) as a result of metabolic waste from the food supplied to shrimp aquaculture system [3]. Therefore, wastewater generated in shrimp aquaculture needs treatment prior to its reuse or release in environment to avoid the eutrophication. To date several chemical and biological treatments have been successfully used to obtain a quality of shrimp aquaculture effluent such as the chemical precipitation

using ferrous chloride to remove phosphorus and the common biological nitrification/denitrification processes to remove nitrogen. Although these processes are effective, they are less environmentally friendly due to the generation of chemical waste or sludge as by product during the processes [4].

Cyanobacteria (blue green algae) are O<sub>2</sub>-evolving photosynthesizing prokaryotes that can be cultivated using aquaculture wastewater as sources of nutrient for biomass production [29]. The cultivation of cyanobacteria in aquaculture wastewater has double advantages, i.e. to reduce the cost of wastewater treatment as well as to produce valuable biomass which can be subsequently utilized for biofuels and feed application. However, there are scarce studies reporting the utilization of wastewater for biodegradable plastic production, most previous studies mainly focused on cyanobacterial cultivation for biomass and nutrient removal [29]. Poly- $\beta$ -hydroxybutyrate (PHB) is a biopolymer plastic consisting of  $\beta$ -hydroxybutyric acid monomers linked through ester bonds, which can be biosynthesized by several cyanobacterial strains [5]. PHB has the important properties of thermoplasticity, biodegradability, non-toxic and biocompatibility making it suitable for application in agricultural and biomedical fields [6]. In this present study, the cyanobacterium *Synechocystis* sp. PCC 6803 mutant strain  $\Delta$ SphU was used to investigate the efficiency of PHB production in a photobioreactor cultivation using shrimp wastewater as growth

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medium. The *Synechocystis* sp. PCC 6803 is a unicellular cyanobacterium, which is one of the most extensively studied species to be developed as a phototrophic cell factory for biofuels, biomaterials and commodity chemicals including PHB production (granules up to  $27 \text{ mg L}^{-1}$ ) [7,8]. The mutant strain  $\Delta\text{SphU}$  is *Synechocystis* sp. PCC 6803 with the deletion of its phosphate regulator gene (*SphU*). This strain efficiently removed phosphate in wastewater down to less than  $0.5 \text{ mg L}^{-1}$  [9]. Therefore, the purpose of this study was to reuse the discharge wastewater from shrimp pond for low cost cultivation of cyanobacteria and production of biodegradable PHB. The obtained PHB was also analyzed for its polymer structure and material properties.

## 2. Materials and methods

### 2.1. Wastewater collection and analysis

The shrimp wastewater was collected from discharge water of shrimp pond, Samut Songkhram, Thailand in 40 L containers and brought to laboratory immediately after collection. The collected discharge water was filtered through a membrane filter ( $0.45 \mu\text{m}$ ) to remove organic load and stored at  $4^\circ\text{C}$  for further utilization. The physiochemical water qualities; color, pH, conductivity, temperature, salinity, total suspended solid (TSS), total dissolved solid (TDS), nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ), ammonium ( $\text{NH}_4^+$ ), phosphate ( $\text{PO}_4^{3-}$ ) and sulfate ( $\text{SO}_4^{2-}$ ) were analyzed in triplicate according to standard method APHA [10]. The heavy metals detection was performed as described previously by Ansari et al. [11].

### 2.2. Cyanobacterial stock and growth condition

The cyanobacterium *Synechocystis* sp. PCC 6803 strain  $\Delta\text{SphU}$  was used for the cultivation in shrimp wastewater. The cells were

grown in BG11 medium containing  $30 \mu\text{g mL}^{-1}$  chloramphenicol and incubated aerobically under continuous illumination of  $40 \mu\text{Em}^{-2}\text{s}^{-1}$  on a rotatory shaker at 160 rpm and  $30^\circ\text{C}$  until reaching log phase. The cells were harvested by centrifugation ( $2790 \times g$ , 10 min) and washed twice with BG11 without antibiotic before inoculation into a photobioreactor with initial cell concentration of  $250 \text{ mg L}^{-1}$ .

### 2.3. Photobioreactor system operation

The flat-plate photobioreactor made from acrylic plastic had internal dimensions of 30 cm (length) x 10 cm (width) x 50 cm (height) as shown in Fig. 1. The reactor was filled to a depth of 30 cm for effective volume. A steel plate was equipped with cool white fluorescent lamps placed at a distance of 2.5 cm from the 30 cm wall of the reactor. The light intensity was  $40 \mu\text{Em}^{-2}\text{s}^{-1}$  on the wall of photobioreactor. The 10 L volume of shrimp wastewater was supplied as a culture medium for bath photobioreactor cultivation. The reactor was continuously purged with atmospheric air (filtered through  $0.22 \mu\text{m}$  cellulose acetate membrane) supplied from the bottom of the reactor. The flow rate of supplied air was  $1.0 \text{ L min}^{-1}$ . Temperature was maintained under ambient condition in the range of  $27\text{--}30^\circ\text{C}$  and the pH was in the range of 7.0–9.0. The experiment was run for 14 days after cell inoculation.

### 2.4. Fluorescence microscopy

PHB granules in *Synechocystis* sp. PCC 6803 strain  $\Delta\text{SphU}$  were visualized by staining with the fluorescent dye Nile red. The  $20 \mu\text{L}$  of cell culture was mixed in  $100 \mu\text{L}$  of Nile red solution containing NaCl ( $0.9\% \text{ w/v}$ ) and Nile red dye ( $3 \text{ ng mL}^{-1}$ ) and incubated overnight under darkness before observation under fluorescence

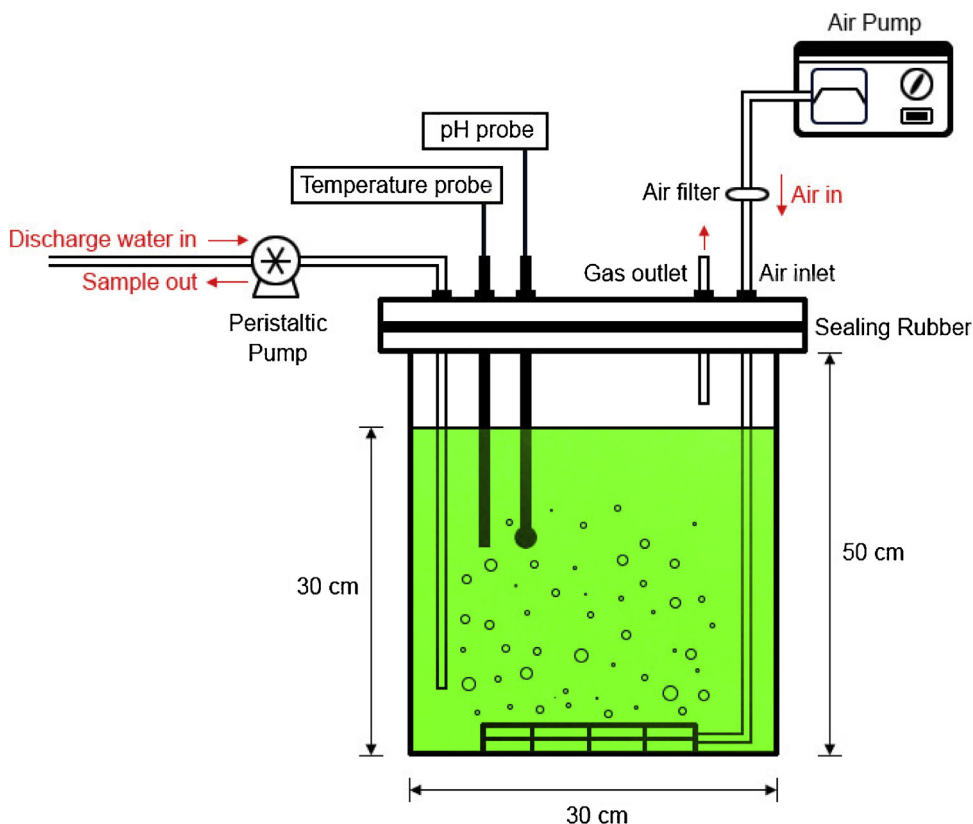


Fig. 1. Schematic diagram of the flat-plate photobioreactor for batch microalgal cultivation using discharge wastewater from shrimp pond.

microscope equipped with a digital camera (Olympus BX60, Japan), using a filter cube with 559 excitation wavelengths.

## 2.5. Analytical methods

### 2.5.1. Biomass and specific growth rate determination

Cyanobacterial growth was monitored at time intervals by measuring optical density at 730 nm ( $OD_{730}$ ) using a spectrophotometer. Total amount of chlorophyll *a* was measured spectrophotometrically at 665 nm in 90% (v/v) methanol extracts. Biomass dry weight (DW) was performed by separating cells from culture using 0.45  $\mu$ m glass microfiber filter and dried in a 60 °C oven until a constant weight was obtained. The specific growth rate ( $\mu$ ), biomass yield and biomass productivity were calculated as follows:

$$\text{Specific growth rate, } \mu \left( d^{-1} \right) = \frac{\ln N_2 - \ln N_1}{t_2 - t_1} \quad (1)$$

Where  $N_1$  and  $N_2$  are the  $OD_{730}$  at the beginning ( $t_1$ ) and at the time of  $t_2$ , respectively.

$$\text{Biomass yield (mg L}^{-1}\text{)} = X_2 - X_1 \quad (2)$$

$$\text{Biomass productivity (mg L}^{-1}\text{d}^{-1}\text{)} = \frac{X_2 - X_1}{t_2 - t_1} \quad (3)$$

Where  $X_1$  and  $X_2$  are the biomass dry weight (DW) at the beginning ( $t_1$ ) and at the time of  $t_2$ , respectively.

### 2.5.2. Nutrient removal efficiency analysis

Nutrient uptake rate which refers to the nutrient taken up by unit mass of cells and nutrient removal efficiency were calculated using the following equations:

$$\text{Nutrient uptake rate (mg gDW}^{-1}\text{ d}^{-1}\text{)} = \frac{(C_0 - C_t)V}{DW(t_2 - t_1)} \quad (4)$$

$$\text{Nutrient removal efficiency (\%)} = 100 - \frac{(100 \times C_t)}{C_0} \quad (5)$$

Where  $C_0$  and  $C_t$  are the initial and final concentrations ( $\text{mg L}^{-1}$ ) of nutrient and  $V$  is the volume of culture (L).

### 2.5.3. Quantitative analysis of PHB production by HPLC

The quantitative PHB was analyzed by high performance liquid chromatography (HPLC) essentially as described by Khetkorn et al. [12]. Briefly, biomass dry weight was boiled in concentrated  $\text{H}_2\text{SO}_4$  for 1 h to hydrolyze PHB polymer into crotonic acid, and then 25-fold diluted with water and filtered through a 0.45  $\mu$ m polypropylene membrane filter to remove cell debris. The crotonic acid content was determined by HPLC using adipic acid as an internal HPLC standard. Commercial PHB (Sigma-Aldrich, USA) was analyzed in parallel, where  $84.6 \pm 4.0\%$  (w/w) conversion of PHB to crotonic acid was obtained. The PHB productivity was calculated according to Eq. (6):

$$\text{PHB productivity (mg L}^{-1}\text{d}^{-1}\text{)} = \frac{(\text{PHB content}) \times (\text{biomass productivity})}{100} \quad (6)$$

Where PHB content is in percentage per biomass dry weight (% w/w DW)

### 2.5.4. Photosynthesis efficiency and PHB synthase activity analysis

The photosynthesis efficiency was analyzed in terms of  $\text{O}_2$ -evolving ability of cells ( $10 \mu\text{g chl } a \text{ mL}^{-1}$ ) under saturating

white light ( $500 \mu\text{E m}^{-2} \text{ s}^{-1}$ ) with a Clark-type  $\text{O}_2$ -electrode (Hansatech instruments, UK) at 25 °C as described by [12]. The PHB synthase activity was determined as previously described by [13]. The reaction (1 mL) contained 0.2 mg of crude protein, 1.5 mM of  $\beta$ -hydroxybutyryl-CoA substrate and 0.5 mM of 2-nitrobenzoic acid in 25 mM Tris-HCl buffer pH 7.5 containing 5% (v/v) glycerol. The reaction was initiated by adding  $\beta$ -hydroxybutyryl-CoA and incubated at 30 °C for 10 min. The thiobenzoate anion resulted from the reaction was measured spectrophotometrically at 412 nm. Protein content was estimated using the Lowry method.

### 2.5.5. PHB extraction, polymer structure and material properties analysis

Total biomass after 14 days of cultivation was harvested and centrifuged ( $2790 \times g$ , 10 min) before freeze dried using lyophilizer (OPERON, Korea). The dried cells were soaked in methanol to remove pigments before extracting PHB using hot chloroform and precipitated with diethyl ether according to Yellore and Desai [14]. The chemical structure of the polymer was analyzed using  $^1\text{H}$  and  $^{13}\text{C}$  nuclear magnetic resonance (NMR). The PHB  $2 \text{ mg mL}^{-1}$  in deuteriochloroform ( $\text{CDCl}_3$ ) was analyzed at 25 °C using a FT-NMR spectrometer 500 MHz (Bruker Avance III HD, Germany). For thermal properties, 10 mg of PHB was analyzed using a differential scanning calorimetry (Netzsch DSC-204 F1 Phoenix, Germany) and thermogravimetric analyzer (Netzsch 209 F3 Tarsus, Germany). For mechanical analysis, the polymer film was cut into rectangles (according to the standard ASTM) and analyzed at 25 °C using a material testing machine (Hounsfield H10KM, UK). The commercial PHB (Sigma-Aldrich, USA) was used to compare their properties with extracted polymer.

## 2.6. Statistical analysis

All experiments were performed with three biological replicates. 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

### 3.1. Shrimp wastewater characterization

Shrimp wastewater collected from discharge water of shrimp pond was characterized for the presence of nutrients required for cyanobacterial cultivation. The physiological water qualities are shown in Table 1. The shrimp wastewater initially contained  $35\text{--}60 \text{ mg L}^{-1}$  of suspended solids; however, after filtration through a membrane filter it contained colored organic compounds lower than  $30 \text{ mg L}^{-1}$  with no negative effect on the growth of cyanobacteria due to the decrease of light penetration reaching the cells inside the culture. The nitrogen and phosphorus compounds are the most important nutrients for cyanobacterial growth and their metabolism [15]. This shrimp wastewater has high content of nitrogen and phosphorus compounds present in various inorganic forms which can be taken up by cells such as nitrate ( $55.28 \text{ mg L}^{-1}$ ), nitrite ( $3.71 \text{ mg L}^{-1}$ ), ammonium ( $2.77 \text{ mg L}^{-1}$ ) and phosphate ( $8.22 \text{ mg L}^{-1}$ ). The pH of the collected wastewater was 8.51. Moreover, the essential micronutrients such as calcium, magnesium, iron, sulfur, zinc, copper required for various physiological activities of cyanobacteria were available in this wastewater (Table 1).

**Table 1**  
Physiological water qualities of shrimp wastewater used for  $\Delta$ SphU cultivation in a photobioreactor.

Parameters	Initial cultivation	Unit
Conductivity	12.34 ± 0.32	mScm <sup>-1</sup>
Salinity	6.09 ± 0.09	ppt
pH	8.51 ± 0.01	-
Temperature	28.0 ± 0.05	°C
Total suspended solids; TSS	19.37 ± 0.07	mgL <sup>-1</sup>
Total dissolved solids; TDS	8.13 ± 0.42	ppt
Calcium	144 ± 0.45	mgL <sup>-1</sup>
Magnesium	259 ± 0.33	mgL <sup>-1</sup>
Iron	2.06 ± 0.22	mgL <sup>-1</sup>
Manganese	0.15 ± 0.02	mgL <sup>-1</sup>
Copper	0.10 ± 0.01	mgL <sup>-1</sup>
Zinc	0.03 ± 0.05	mgL <sup>-1</sup>
Sulfate; SO <sub>4</sub> <sup>2-</sup>	318 ± 5.29	mgL <sup>-1</sup>
phosphate; PO <sub>4</sub> <sup>3-</sup>	8.22 ± 0.13	mgL <sup>-1</sup>
Nitrate; NO <sub>3</sub> <sup>-</sup>	55.28 ± 0.49	mgL <sup>-1</sup>
Nitrite; NO <sub>2</sub> <sup>-</sup>	3.71 ± 0.08	mgL <sup>-1</sup>
Ammonium; NH <sub>4</sub> <sup>+</sup>	2.77 ± 0.06	mgL <sup>-1</sup>
Total phosphorus	11.46 ± 0.12	mgL <sup>-1</sup>

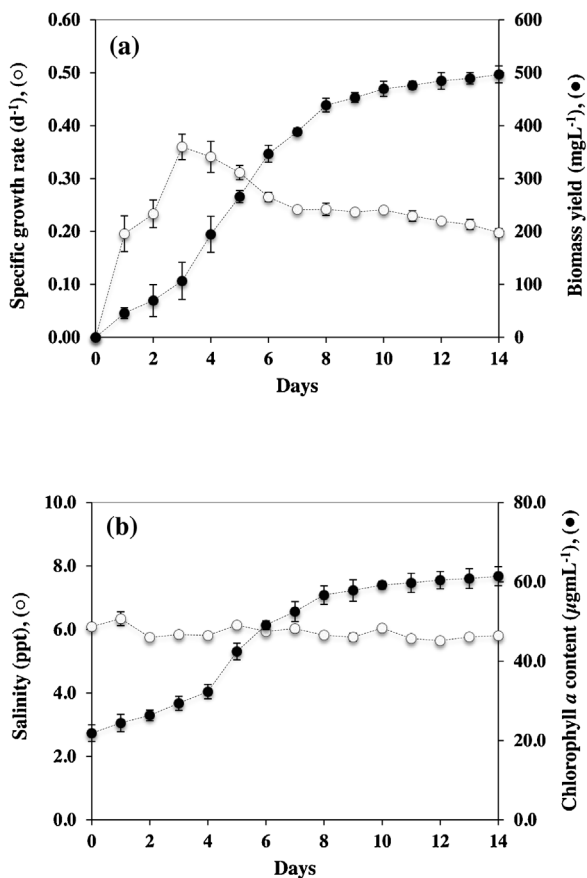
3.2. *Synechocystis* strain  $\Delta$ SphU cultivated in shrimp wastewater

To utilize shrimp wastewater for cyanobacterial growth, *Synechocystis* sp. PCC 6803 strain  $\Delta$ SphU was cultured with shrimp wastewater as growth medium in a batch photobioreactor (Fig. 1). The changes in physiological shrimp wastewater

characteristics such as pH, temperature and salinity were determined every day during cultivation as shown in Fig. 2. The biomass yield of  $\Delta$ SphU strain was continuously increased during the first week of cultivation in shrimp wastewater and then the culture entered the stationary phase. After 14 days of cultivation,  $\Delta$ SphU strain showed the maximum biomass yield of approximately 500 mgL<sup>-1</sup> (Fig. 2a), and the highest biomass productivity was 57.80 mgL<sup>-1</sup>d<sup>-1</sup> after 7 days of cultivation (Table 2). Previously, the cyanobacterium *Spirulina platensis* was shown to grow well in aquaculture wastewater with a specific growth rate of 0.623 day<sup>-1</sup> at day 3 of cultivation [16], indicating that shrimp wastewater can be reused for cyanobacterial cultivation. Moreover, the changes of pH in culture was an important effector of the cyanobacterial CO<sub>2</sub> concentrating mechanism since the amount of dissolved inorganic carbon depends on pH, temperature and salinity [17]. The results of this study showed that temperature and salinity were not significantly changed irrespective of cultivation times, but pH of the culture was apparently increased from 8.5 to 9.9 within 3 days of cultivation (data not shown). The pH is a main factor influencing the abundance of inorganic carbon. Under alkaline condition, the HCO<sub>3</sub><sup>-</sup> is a major inorganic carbon leading to the reduced level of CO<sub>2</sub> and causing the growth inhibition of cyanobacteria [15,17]. Interestingly, the  $\Delta$ SphU cells showed no negative effect of high pH on their growth, the highest specific growth rate was 0.36 day<sup>-1</sup> at day 3 (Fig. 2a). Low inorganic carbonate concentration has been reported to activate CO<sub>2</sub> concentrating mechanism of *Synechocystis* sp. PCC 6803 by induction of a highly efficient bicarbonate transporter for taking up HCO<sub>3</sub><sup>-</sup> into *Synechocystis* cell [8,18]. This might explain why *Synechocystis* sp. PCC 6803 strain  $\Delta$ SphU has the capability to grow under low CO<sub>2</sub> concentrations. In addition, the increase of chlorophyll a content in Fig. 2b revealed that a high salinity (5.75–6.34 ppt) in shrimp wastewater had no influence on the photosynthesis of  $\Delta$ SphU strain.

3.3. The efficiency of nutrient removal by  $\Delta$ SphU strain under batch photobioreactor

With regard to water qualities, the discharge of shrimp pond wastewater containing high level of nutrients such as nitrogen and phosphorus is one of the main causes of eutrophication. Fig. 2a shows that *Synechocystis* strain  $\Delta$ SphU utilized the nutrients present in the shrimp wastewater for promoting its growth which has an advantage in reducing the cost of wastewater treatment. However, the nutrient removal efficiencies depend on the cyanobacterial strain applied and the initial concentration of nutrients in the wastewater [19]. The nutrient removal efficiency of  $\Delta$ SphU was determined as shown in Table 2. After 14 days cultivation, the removal efficiencies were found to be 96.99% for phosphate, 80.10% for nitrate, 67.90% for nitrite and 98.07% for ammonium. It should be noted that  $\Delta$ SphU has a high efficiency to remove phosphate and ammonium from shrimp wastewater. Similarly, *Chlorella sorokiniana* and *Scenedesmus obliquus* also had high removal efficiency (over 95%) of both phosphate and ammonium present in aquaculture wastewater [11]. Surprisingly,  $\Delta$ SphU strain effectively decreased phosphate concentration down to 2.37 mgL<sup>-1</sup> with as high as 71.16% of removal efficiency occurring within the first day of batch photobioreactor running where the maximum phosphate uptake rate was 20.16 mg g<sup>-1</sup>d<sup>-1</sup> (Table 2). Shrimp wastewater is plentiful with various forms of inorganic phosphorus arising from shrimp feed medium. Total phosphorus concentration in shrimp effluent was initially analyzed as 11.46 mgL<sup>-1</sup> (Table 1). After cultivation, the total phosphorus in the photobioreactor was reduced to 0.27 mgL<sup>-1</sup> (data not shown). According to the regulations for pollution control in many countries including Thailand, the total phosphorus



**Fig. 2.** Specific growth rate and biomass yield (a) and effect of salinity variation on chlorophyll a content (b) of *Synechocystis* sp. PCC 6803 strain  $\Delta$ SphU in batch photobioreactor using shrimp wastewater as growth medium for 14 days. The error bars represent standard deviations of means (means ± S.D., n = 3).



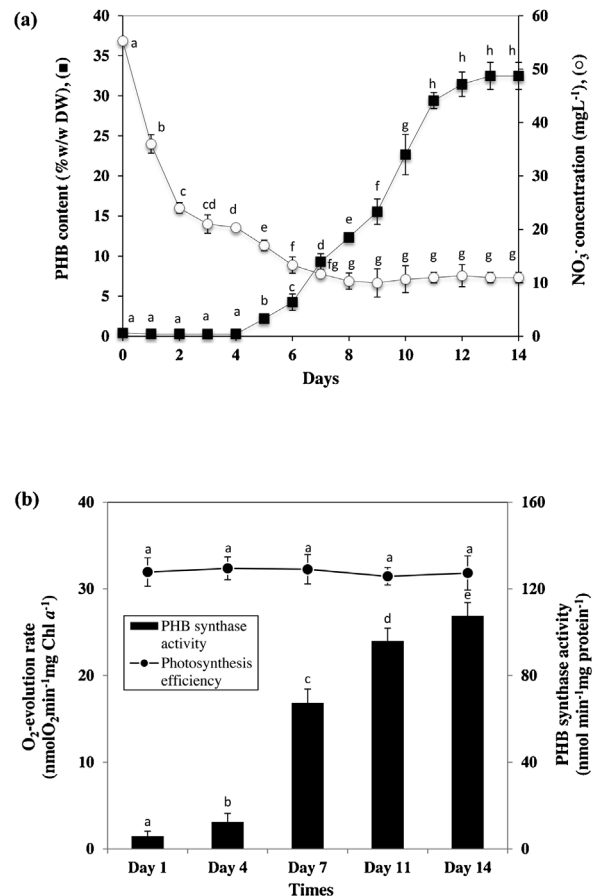
**Table 2**The efficiency of nutrient uptake, nutrient removal, biomass and PHB productivities of  $\Delta$ SphU grown in shrimp wastewater at various times. Means  $\pm$  S.D. ( $n=3$ ).

Time	Nutrient uptake rate (mg gDW <sup>-1</sup> d <sup>-1</sup> )		Nutrient removal (%)		Biomass productivity (mgL <sup>-1</sup> d <sup>-1</sup> )	PHB productivity (mgL <sup>-1</sup> d <sup>-1</sup> )
	PO <sub>4</sub> <sup>3-</sup>	NO <sub>3</sub> <sup>-</sup>	PO <sub>4</sub> <sup>3-</sup>	NO <sub>3</sub> <sup>-</sup>		
Day 1	20.16 $\pm$ 0.6	64.92 $\pm$ 5.8	71.17 $\pm$ 3.5	35.55 $\pm$ 3.1	45.65 $\pm$ 9.9	0.13 $\pm$ 0.03
Day 7	1.79 $\pm$ 0.01	9.74 $\pm$ 0.4	95.91 $\pm$ 0.5	79.11 $\pm$ 1.0	57.80 $\pm$ 1.0	5.16 $\pm$ 0.6
Day 11	1.01 $\pm$ 0.02	5.53 $\pm$ 0.2	96.76 $\pm$ 0.3	79.71 $\pm$ 4.5	43.23 $\pm$ 0.7	12.73 $\pm$ 1.2
Day 14	0.77 $\pm$ 0.03	4.23 $\pm$ 0.09	96.99 $\pm$ 0.5	80.10 $\pm$ 1.8	35.49 $\pm$ 1.1	11.49 $\pm$ 0.7

in wastewater from aquaculture ponds before its release into the environment must not exceed 0.4 mg L<sup>-1</sup>. The  $\Delta$ SphU is therefore a promising cyanobacterium for use as a phosphorus remover. Interestingly, the  $\Delta$ SphU could take up phosphate with the rate higher than those from a cyanobacterium *Anabaena* sp. PCC 7120 and a green microalga *Chlorella* sp. GD under aquaculture wastewater utilization [20,21]. The  $\Delta$ SphU is a *Synechocystis* mutant strain lacking phosphate regulator gene (*sphU*). This strain was capable of accumulating phosphate inside the cell at higher concentration than that of the wild type [9], indicating that the deletion of phosphate regulator gene enabled the  $\Delta$ SphU strain to remove phosphate from shrimp wastewater at high efficiency.

### 3.4. PHB accumulation under low concentration of nitrate in shrimp wastewater

The PHB production has been widely investigated in *Synechocystis* strain, but the previous studies focused on optimum conditions for enhanced PHB accumulation in cells grown in algal medium [22,23]. Therefore, this study firstly reported the utilization of shrimp wastewater for inducing PHB production in *Synechocystis* cells. In this batch photobioreactor, *Synechocystis* strain  $\Delta$ SphU was harvested for analysis of PHB accumulation as shown in Fig. 3a. Low levels of PHB were detected in cells during the growth promoting phase (0–4 days of cultivation), after which the levels of PHB rapidly increased from 0.29 to 29.40% (w/w) before reaching a constant level. The highest PHB content was 32.48% (w/w), with the maximum PHB productivity of 12.73 mg L<sup>-1</sup>d<sup>-1</sup> after 11 days cultivation (Table 2). It should be noted that *Synechocystis* strain  $\Delta$ SphU had lower PHB content when cultivated in BG11 medium as compared to that in shrimp wastewater (Table 4). The limitation of nitrogen was reported to have high impact on PHB induction in several cyanobacteria when compared to other nutrient factors [5]. In addition, the low level of nitrate in BG11 medium under photoautotrophic condition could induce the accumulation of PHB in *Synechocystis* sp. PCC 6803 wild type [6,24]. The Fig. 3a shows that PHB content was significantly increased when the concentration of nitrate in shrimp wastewater was lower than 20 mg L<sup>-1</sup> caused nitrate assimilation of cyanobacteria. However, when cells were cultured longer than 7 days there were no significant changes in nitrate levels with the average level of 10.76 mg L<sup>-1</sup>. The cell biomass was also constant during the second week of cultivation (Fig. 2a), suggesting that the limited availability of nitrate in shrimp wastewater is one of important factors affecting the growth of cyanobacterial cells. In contrast, the O<sub>2</sub>-evolution of cells under saturated light exposure was not significantly different under nitrogen limited condition (Fig. 3b), indicating that the photosynthesis was still active and cells continued to fix CO<sub>2</sub>. Therefore, the PHB with high content in  $\Delta$ SphU cells might serve as a storage compound under stress condition which correlated well with higher PHB synthase activity (Fig. 3b) and increased PHB granules inside the cells (Supplementary Fig. S1).



**Fig. 3.** The effect of nitrate limitation on PHB content (a) and the PHB synthase activity relative with photosynthesis efficiency (b) of  $\Delta$ SphU in a photobioreactor (a) when cells were cultured in shrimp wastewater for various times (means  $\pm$  S.D.,  $n=3$ ). The different letters within the same parameter represent significant difference according to Duncan's test ( $p < 0.05$ ).

### 3.5. The material properties of PHB extracted from $\Delta$ SphU grown in shrimp wastewater

Dried cells as shown in Supplementary Fig. S2 were collected to extract intracellular polymer before analyzing chemical structure. The <sup>1</sup>H and <sup>13</sup>C NMR spectra of polymer extracted from  $\Delta$ SphU cells matched the respective spectra of the commercial PHB (Supplementary Fig. S3, S4). The thermal and mechanical properties of extracted PHB were comparable to those of the commercial PHB (Table 3). Unfortunately, the extracted PHB from  $\Delta$ SphU grown in shrimp wastewater had a weaker tensile strength than the commercial PHB. In contrast, the PHB from other cyanobacteria such as *Nostoc muscorum* and *Calothrix scytonemica* had a greater tensile strength than the commercial PHB [28].

**Table 3**  
Material properties of PHB produced by ΔSphU compared to commercial PHB.

Sources of PHB	Thermal properties			Mechanical properties		
	T <sub>m</sub> (°C)	T <sub>g</sub> (°C)	T <sub>d</sub> (°C)	Elongation at break (%)	Tensile strength (MPa)	Young's modulus (MPa)
Commercial PHB	172.5	3.5	276.1	5.8	26.9	820
ΔSphU <sup>a</sup>	179.4	3.2	279.8	5.9	15.4	679

<sup>a</sup> ΔSphU cells were cultured in shrimp wastewater for 14 days before cells were collected to extract PHB. T<sub>m</sub>, melting temperature; T<sub>g</sub>, glass transition temperature; T<sub>d</sub>, decomposition temperature.

**Table 4**  
Comparison of PHB content of cyanobacterial strains grown on synthetic medium and wastewaters under photobioreactor.

Cyanobacterial strains	Growth media	Conditions of PHB production	PHB contents (% w/w DW)	References
<i>Synechocystis</i> sp. PCC 6714	BG11	Jacketed glass photobioreactor (1 L), under nitrogen and phosphorus limitation for 15 days	16.4 ± 2.0	[25]
<i>Synechocystis</i> sp. CCALA192	BG11	Tubular photobioreactor (200 L), under complete BG11 medium cultivation for 20 days	12.7	[26]
<i>Synechocystis</i> sp. PCC 6803 strain ΔSphU	BG11	Flat-plate photobioreactor (10 L), under complete BG11 medium cultivation for 13 days (control condition)	6.42 ± 1.8	This study
<i>Synechocystis</i> sp. PCC 6803 strain ΔSphU	Shrimp wastewater	Flat-plate photobioreactor (10 L), under shrimp wastewater cultivation for 14 days	32.48 ± 1.7	This study
<i>Synechocystis salina</i>	Low-solid digestate from urban wastewater	Tubular photobioreactor (200 L), under digestate supernatant (1/3) cultivation for 40 days	5.5 ± 0.3	[27]

#### 4. Conclusion

In conclusion, the cells of *Synechocystis* strain ΔSphU could utilize the nutrients present in the discharge of shrimp pond for supporting their growth, which has double advantages in reducing the cost of wastewater treatment and obtaining algal feedstock. The maximum biomass yield was approximately 500 mg L<sup>-1</sup>. The lack of phosphate regulator (SphU) in *Synechocystis* sp. PCC 6803 enabled the cells to efficiently remove phosphate present in the shrimp wastewater with the rate of 20.16 mg g<sup>-1</sup> d<sup>-1</sup> at the first cultivating day. In addition, the limitation of nitrate in shrimp wastewater due to nitrogen assimilation could induce PHB accumulation 32.48% (w/w) DW in ΔSphU cells. The obtained PHB polymer showed material properties comparable to those of the commercial PHB. Until now, a few studies focusing on bath photoautotrophic cultivation of PHB in large-scale, optimizing the nutrients mainly has been done in flask scale. Table 4 represents the comparison of PHB content of cyanobacteria grown under bath photobioreactor from previous study. Batch synthetic medium cultivation of *Synechocystis* sp. PCC 6714 under nitrogen and phosphorus limitation, showed 16.4% (w/w) DW [25]. While, the maximum PHB content of 5.5% (w/w) DW was obtained for low-solid digestate from urban wastewater cultivation of *Synechocystis* sp. CCALA192 in a 200 L tubular photobioreactor [27]. Therefore, photobioreactor systems are more flexible depending on the cultivation process and the desired species. However, our study has firstly revealed that shrimp wastewater can be used for biodegradable plastic production by *Synechocystis* strain ΔSphU under large scale photobioreactor.

#### Declaration of interest

The authors declare that there are no conflicts of interest.

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#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2019.e00345>.

#### References

- [1] FAO, Fisheries and Aquaculture Statics. FAO Fishery and Aquaculture Statistics Yearbook, Food and Agriculture Organization of the United Nations, Room. Italy, 2017.
- [2] P.T. Anh, C. Kroeze, S.R. Bush, A.P.J. Mol, Water pollution by intensive brackish shrimp farming in south-east Vietnam: causes and options for control, *Agric. Water Manag.* 97 (6) (2010) 872–882.
- [3] R. Crab, Y. Avnimelech, T. Defoirdt, P. Bossier, W. Verstraete, Nitrogen removal techniques in aquaculture for a sustainable production, *Aquaculture* 270 (2007) 1–14.
- [4] W.T. Mook, M.H. Chakrabarti, M.K. Aroua, G.M.A. Khan, B.S. Ali, M.S. Islam, M.A. Abu Hassan, Removal of total ammonia nitrogen (TAN), nitrate and total organic carbon (TOC) from aquaculture wastewater using electrochemical technology: a review, *Desalination* 285 (2012) 1–13.
- [5] S. Balaji, K. Gopi, B. Muthuvelan, A review on production of poly β hydroxybutyrate from cyanobacteria for the production of bioplastics, *Algal Res.* 2 (2013) 278–285.
- [6] R. Carpine, F. Raganati, G. Olivieri, K.J. Hellingwerf, A. Pollio, P. Salatino, A. Marzocchella, Poly-β-hydroxybutyrate (PHB) production by *Synechocystis* PCC 6803 from CO<sub>2</sub>: model development, *Algal Res.* 29 (2018) 49–60.
- [7] G.F. Wu, Q.Y. Wu, Z.Y. Shen, Accumulation of poly-β-hydroxybutyrate in cyanobacterium *Synechocystis* sp. PCC6803, *Bioresour. Technol.* 76 (2) (2001) 85–90.
- [8] Y. Yu, L. You, D. Liu, W. Hollinshead, Y.J. Tang, F. Zhang, Development of *Synechocystis* sp. PCC 6803 as a phototrophic cell factory, *Mar. Drugs* 11 (2013) 2894–2916.
- [9] S. Burut-Archanai, J.J. Eaton-Rye, A. Incharoensakdi, S. Powtongsook, Phosphorus removal in a closed recirculating aquaculture system using the cyanobacterium *Synechocystis* sp. PCC 6803 strain lacking the SphU regulator of the Pho regulon, *Biochem. Eng. J.* 74 (2013) 69–75.
- [10] APHA, Standard Methods for the Examination of Water and Wastewater, 22 nd., American Public Health Association, Washington DC, 2012.
- [11] F.A. Ansari, P. Singh, A. Gulde, F. Bux, Microalgal cultivation using aquaculture wastewater: integrated biomass generation and nutrient remediation, *Algal Res.* 21 (2017) 169–177.
- [12] W. Khetkorn, A. Incharoensakdi, P. Lindblad, S. Jantaro, Enhancement of poly-3-hydroxybutyrate production in *Synechocystis* sp. PCC 6803 by overexpression of its native biosynthetic genes, *Bioresour. Technol.* 214 (2016) 761–768.
- [13] T. Monshupanee, P. Nimdach, A. Incharoensakdi, Two-stage (photoautotrophy and heterotrophy) cultivation enables efficient production of bioplastic poly-3-hydroxybutyrate in auto-sedimenting cyanobacterium, *Sci. Rep.* 6 (2016) 37121.
- [14] V. Yellore, A. Desai, Production of poly-3-hydroxybutyrate from lactose and whey by *Methylobacterium* sp. ZP24, *Lett. Appl. Microbiol.* 26 (1998) 391–394.

- [15] J. Zhu, J. Rong, B. Zong, Factors in mass cultivation of microalgae for biodiesel, *Chinese J. Catal.* 34 (2013) 80–100.
- [16] S.C. Wuang, M.C. Khin, P.Q.D. Chua, Y.D. Luo, Use of *Spirulina* biomass produced from treatment of aquaculture wastewater as agricultural fertilizers, *Algal Res.* 15 (2016) 59–64.
- [17] G. Markou, D. Vandamme, K. Muylaert, Microalgal and cyanobacterial cultivation: the supply of nutrients, *Water Res.* 65 (2014) 186–202.
- [18] T. Hasunuma, F. Kikuyama, M. Matsuda, S. Aikawa, Y. Izumi, A. Kondo, Dynamic metabolic profiling of cyanobacterial glycogen biosynthesis under conditions of nitrate depletion, *J. Exp. Bot.* 64 (2013) 2943–2954.
- [19] A. Guldhe, F.A. Ansari, P. Singh, F. Bux, Heterotrophic cultivation of microalgae using aquaculture wastewater: a biorefinery concept for biomass production and nutrient remediation, *Ecol. Eng.* 99 (2017) 47–53.
- [20] S. Burut-Archanai, S. Powtongsook, Identification of negative regulator for phosphate-sensing system in *Anabaena* sp. PCC 7120: a target gene for developing phosphorus removal, *Biochem. Eng. J.* 125 (2017) 129–134.
- [21] C.M. Kuo, J.F. Jian, T.H. Lin, Y.B. Chang, X.H. Wan, J.T. Lai, J.S. Chang, C.S. Lin, Simultaneous microalgal biomass production and CO<sub>2</sub> fixation by cultivating *Chlorella* sp. GD with aquaculture wastewater and boiler flue gas, *Bioresour. Technol.* 221 (2016) 241–250.
- [22] T. Monshupanee, A. Incharoensakdi, Enhanced accumulation of glycogen, lipids and polyhydroxybutyrate under optimal nutrients and light intensities in the cyanobacterium *Synechocystis* sp. PCC 6803, *J. Appl. Microbiol.* 116 (4) (2014) 830–838.
- [23] B. Panda, N. Mallick, Enhanced poly-beta-hydroxybutyrate accumulation in a unicellular cyanobacterium, *Synechocystis* sp. PCC 6803, *Letts. Appl. Microbiol.* 44 (2) (2007) 194–198.
- [24] W. Khetkorn, S. Suphan, S. Pongswat, Suitable cell age for enhanced poly-β-hydroxybutyrate accumulation under photoautotrophic nutrient deprivation of *Synechocystis* sp. PCC 6803, *Sci&Tech RMUTT J.* 6 (2016) 29–36.
- [25] D. Kamravamesh, S. Pflugl, W. Nischkauer, A. Limbeck, M. Lackner, C. Herwig, Photosynthetic poly-beta-hydroxybutyrate accumulation in unicellular cyano bacterium *Synechocystis* sp. PCC 6714, *AMB Express* 7 (2017) 143.
- [26] C. Troschl, K. Meixner, I. Fritz, K. Leitner, A.P. Romero, A. Kovalcik, P. Sedlacek, B. Drosig, Pilot-scale production of poly-β-hydroxybutyrate with the cyanobacterium *Synechocystis* sp. CCALA192 in a non-sterile tubular photobioreactor, *Algal Res.* 34 (2018) 116–125.
- [27] K. Meixner, I. Fritz, C. Daffert, K. Markl, W. Fuchs, B. Drosig, Processing recommendations for using low-solids digestate as nutrient solution for poly-β-hydroxybutyrate production with *Synechocystis salina*, *J. Biotechnol.* 240 (2016) 61–67.
- [28] R. Bhati, S. Samantaray, L. Sharma, N. Mallick, Poly-beta-hydroxybutyrate accumulation in cyanobacteria under photoautotrophy, *Biotechnol. J.* 5 (2010) 1181–1185.
- [29] R. Wang, B. Peng, K. Huang, The research progress of CO<sub>2</sub> sequestration by algal bio-fertilizer in China, *J. CO<sub>2</sub> Utilization* (11 2015) 67–70.