

COMPARISON OF POLYHYDROXYALKANOATES BIOSYNTHESIS, MOBILIZATION AND THE EFFECTS ON CELLULAR MORPHOLOGY IN *SPIRULINA PLATENSIS* AND *SYNECHOCYSTIS* SP. UNIWG

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Abstrak: *Spirulina platensis* filamentus dan *Synechocystis* sp. UNIWG uniselular menunjukkan kebolehan mensintesis poli(3-hidroksibutirat) [P(3HB)] di bawah keadaan bebas nitrogen dengan akumulasi maksimum hingga 10% dan 14% P(3HB) masing-masing daripada berat kering sel (CDW) di bawah keadaan kultur mikсотrofik. Pendegradan intraselular (mobilisasi) granul P(3HB) oleh kedua-dua strain dimulakan dengan pembekalan semula sumber nitrogen. Mobilisasi P(3HB) dalam *S. platensis* adalah lebih cepat (80% telah didegradasikan dalam masa 8 hari) berbanding *Synechocystis* sp. (57% telah didegradasikan dalam masa 10 hari). Mikroskop elektron transmisi (TEM) menunjukkan mobilisasi P(3HB) melibatkan perubahan dalam kuantiti dan morfologi granul P(3HB). Bentuk granul P(3HB) berubah menjadi tidak sekata dan sempadannya tidak jelas kelihatan. Analisis ultrastruktur juga menunjukkan sel *Synechocystis* sp. UNIWG daripada fasa pertumbuhan diselaputi oleh struktur seperti pilus. Walau bagaimanapun, struktur seperti pilus ini tidak kelihatan pada sel semasa fasa akumulasi polihidroksialkanoat (PHA). Adalah dicadangkan bahawa struktur seperti pilus mungkin memainkan peranan yang penting dalam kemandirian sianobakteria ini di bawah keadaan stres.

Kata kunci: Polihidroksialkanoat (PHA), *Spirulina platensis*, *Synechocystis* sp. UNIWG

Abstract: Filamentous *Spirulina platensis* and unicellular *Synechocystis* sp. UNIWG showed capability of synthesizing poly(3-hydroxybutyrate) [P(3HB)] under nitrogen starvation conditions with a maximum accumulation of up to 10 and 14 wt% of the cell dry weight (CDW), respectively under mixotrophic culture conditions. Intracellular degradation (mobilization) of P(3HB) granules by both strains was initiated by the restoration of a nitrogen source. The mobilization of P(3HB) was faster in *S. platensis* (80% degradation in a period of 8 days) than in *Synechocystis* sp. (57% degradation in a period of 10 days). Transmission electron microscopy (TEM) revealed that the mobilization of P(3HB) involved changes in granule quantity and morphology. P(3HB) granules became irregular in shape and the boundary region was less defined. Ultrastructural analysis also revealed that *Synechocystis* sp. UNIWG cells from the growth phase was covered with pilus-like structures. However, these pilus-like structures were not observed in cells from the polyhydroxyalkanoate (PHA) accumulation phase. It is suggested that these pilus-like structures may play an important role for the survival of this cyanobacterium under stress conditions.

Keywords: Polyhydroxyalkanoate (PHA), *Spirulina platensis*, *Synechocystis* sp. UNIWG

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INTRODUCTION

It is known that some cyanobacteria can accumulate PHA, which is a storage material abundant in prokaryotes, under phototrophic or mixotrophic growth conditions with acetate. Cyanobacteria are of particular interest as PHA producers because of their minimal nutrient requirements for growth and capability of accumulating PHA by oxygenic photosynthesis. Cyanobacteria recover CO₂ from atmosphere by using natural biological processes and turn it into environmental-friendly P(3HB), which is the raw material for biodegradable plastics. However, not much effort is being directed to understand the PHA biosynthesis in cyanobacteria mainly because most of the known cyanobacteria that are capable of synthesizing PHA usually accumulate PHAs amounting to less than 6 wt% of their CDW (Carr 1996; Vincenzini *et al.* 1990; Stal 1992; Arino *et al.* 1995).

Since PHA plays a role as carbon and energy storage material in prokaryotic cells, synthesis is coupled with mobilization. Synthesis and mobilization of PHAs are part of a cycle in which the acyl coenzyme A (acyl-CoA) precursors are converted into PHAs by different metabolic pathways. The mobilization produces acyl-CoA and acetyl coenzyme A (acetyl-CoA), which are metabolized via the tricarboxylic acid cycle (TCA) as a source of carbon and energy (Kessler *et al.* 1998). The intracellular depolymerase is only active towards amorphous PHAs. However, intracellular degradation of PHA, i.e., the mobilization of accumulated amorphous PHA, is poorly understood. In fact, to our knowledge there have been no studies on the mobilization of PHA by cyanobacteria. But, it has been shown that PHA accumulation increases bacterial survival in nutrient-depleted conditions (Matin *et al.* 1979; López *et al.* 1995). Therefore, we have started a systematic study on the mobilization of PHA in cyanobacteria (Jau *et al.* 2005).

Spirulina platensis and a newly isolated unicellular *Synechocystis* sp. UNIWG (Abed *et al.* 2002) are the subject of interest in this study due to their natural ability to accumulate PHA as a storage product of CO₂ fixation (Vincenzini *et al.* 1990). *S. platensis* and *Synechocystis* sp. PCC 6803 have been reported to accumulate a maximum of 6 wt% (Campbell *et al.* 1982) and 7 wt% (Sudesh *et al.* 2001) of CDW of P(3HB), respectively under mixotrophic conditions. Early studies done in our laboratory showed that these two strains are capable of accumulating much higher PHA content, which has then become the topic of interest and is discussed here. In this study, we also report the mobilization of accumulated P(3HB) by *S. platensis* and *Synechocystis* sp. The mobilization of accumulated P(3HB) by *S. platensis* was faster than its synthesis process. Therefore, we were keen on comparing the P(3HB) mobilization process in other cyanobacterial cells.

The ultrastructural or morphological effects of nitrogen starvation on cyanobacteria have been studied in unicellular (Stevens *et al.* 1981; Yew *et al.* 2005), filamentous (de Vasconcelos & Fay 1974; Giesy 1964; Kulasooriya *et al.* 1972; Neilson *et al.* 1971) and aggregate forming genera (Peat & Whitton 1967). Nitrogen starvation is known to stimulate certain cellular responds including heterocyst differentiation in filamentous cyanobacteria (Kulasooriya *et al.* 1972;

Neilson *et al.* 1971) like *Anabaena* sp. or chlorosis in *Synechococcus* sp. PCC 7002 (Sakamoto & Bryant 1998). In this paper, we also discuss the changes in cellular morphology along with the accumulation and mobilization of P(3HB) granules in the two cyanobacteria from different genera, i.e., *S. platensis* and *Synechocystis* sp.

MATERIALS AND METHODS

Cultures and Growth

Synechocystis sp. UNIWG (Abed *et al.* 2002) isolated from Palestine was cultured in BG-11 medium (pH 7.1) (Stanier *et al.* 1971) meanwhile *S. platensis* UMACC 161 obtained from University Malaya Algae Culture Collection, Malaysia was cultured in Kosaric medium (pH 9). Compared to the commonly used Zarrouk's medium, Kosaric medium used in this study contains 9 g l⁻¹ of NaHCO₃ instead of 16 g l⁻¹. Both strains were cultured at 25°C under continuous illumination with cool white fluorescent light (1020 lux). The cultures were continuously bubbled with filter-sterilized atmospheric air. pH of the medium was adjusted with 1 M HCl or NaOH. For nitrogen free conditions, both BG-11 and Kosaric media without sodium nitrate were prepared. Cell growth was monitored by measuring the optical density of the culture at 730 nm.

Biosynthesis and Mobilization of the Accumulated PHA

In order to promote the biosynthesis of P(3HB) in both strains, cells grown in complete medium for 10 days were transferred to nitrogen-free medium. In addition to CO₂ from atmospheric air, reduced carbon source (sodium acetate) was added at a concentration of 0.5% (w/v). The cells were then incubated for 15 days in the same conditions as mentioned above. For mobilization studies, the cells containing P(3HB) granules were recovered by centrifugation and transferred to complete medium. Both strains were incubated at 25°C and bubbled with sterile atmospheric air under continuous illumination. The cells were sampled every two days for qualitative and quantitative analysis of P(3HB).

Qualitative Analysis of PHA

Both *S. platensis* and *Synechocystis* sp. cells were heat-fixed onto glass slides and stained with 1% (w/v) aqueous Nile blue A (Ostle & Holt 1982) solution at 55°C. Excessive stain was washed with water and 8% (v/v) acetic acid. The stained samples were observed by fluorescent microscope at an excitation wavelength of 450–490 nm. For TEM, cells were first fixed in McDowell-Trump fixative (McDowell & Trump 1976) prepared in 0.1 M sodium phosphate buffer (pH 7.2) and then fixed by 1% osmium tetroxide. After dehydration using a graded ethanol series, the cells were embedded in Spurr's low viscosity resin (Spurr 1969). Ultrathin sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and then observed using Philip CM-12 TEM.

Quantitative Analysis of PHA

Cells of both strains were harvested by centrifugation, washed at least twice with sterile distilled water and freeze-dried before subjecting to standard methanolysis reaction. 30 mg of freeze-dried cells were methanolized in 10 ml screw-cap test tube for 140 min at 100°C (Braunegg *et al.* 1978) in a thermostat-equipped heating block. The methanolysis solution consisted of 15% (v/v) sulfuric acid and 85% (v/v) methanol. Upon completion of the methanolysis reaction, 1 ml of distilled water was added to the cooled mixture and the test tube was shaken vigorously for 1 min. The organic phase (bottom layer), consisting of the hydroxyacyl methyl esters was then subjected to gas chromatography (GC) analysis (Brandl *et al.* 1988) employing a Shimadzu GC-14B instrument equipped with flame-ionization detector and SPBTM-1 (Supelco) fused silica capillary column (30 m x 0.25 mm x 0.25 µm).

RESULTS

Morphological Characteristics

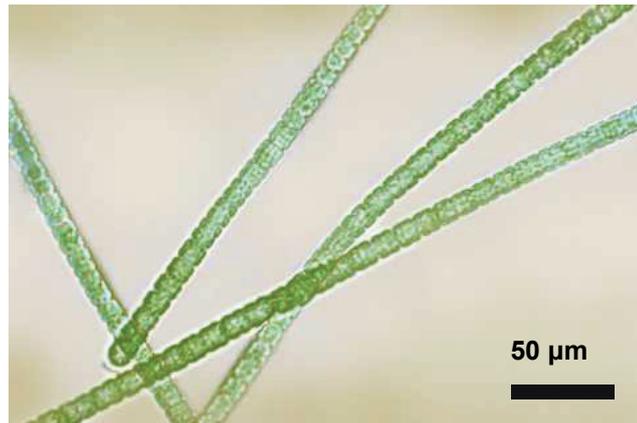
S. platensis UMACC 161 appeared under light microscope as multicellular open helics with long filaments. The size of a single cell (trichome) is about 1–2 µm (Fig. 1A). The cells reproduced by binary fission in single plane and not by producing akinetes or heterocysts. In comparison, *Synechocystis* sp. UNIWG was coccoid in shape but reproduced by binary fission just like *S. platensis*. The sizes of cells were about 2–3 µm in diameter (Fig. 1B). The growth profiles of *S. platensis* UMACC 161 and *Synechocystis* sp. UNIWG are shown in Figure 2. The cells grew photoautotrophically with a doubling time of approximately 17 h for *Synechocystis* sp. and 24 h for *S. platensis*.

Biosynthesis and Mobilization of PHA

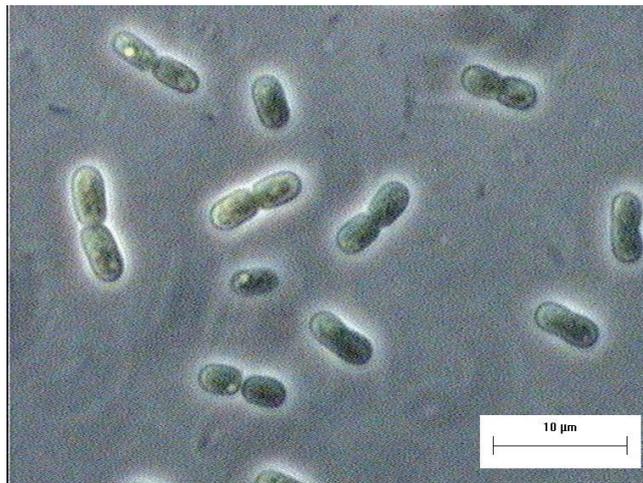
Table 1 shows the content and composition of PHAs accumulated by both strains of *S. platensis* and *Synechocystis* sp. when incubated first in complete and then followed by nitrogen free media for 15 days. P(3HB) accumulation was detected in both *S. platensis* and *Synechocystis* sp. UNIWG in the absence of nitrogen sources. Only trace amounts of P(3HB) (1 wt% of CDW) was accumulated in cells of both strains when cultivated under nitrogen free conditions using CO₂ from atmospheric air as the sole carbon source. The P(3HB) content can be significantly increased by the addition of 0.5% (w/v) sodium acetate to the nitrogen free medium. This resulted in the accumulation of 14 wt% P(3HB) of the CDW in *Synechocystis* sp. while *S. platensis* produced about 10 wt% P(3HB) of the CDW.

After 15 days of cultivation in mixotrophic conditions, the P(3HB) containing cells were harvested by centrifugation and transferred into complete medium to induce P(3HB) mobilization. The incubation conditions were maintained exactly the same as the P(3HB) synthesis process. It was noted that the mobilization process was initiated with the restoration of nitrogen source. As shown in Table 2, the P(3HB) content in cells decreased when nitrogen source was provided in the culture medium. The mobilization of P(3HB) in *S. platensis*

appeared to be relatively faster compared to its biosynthesis process. Results showed that the P(3HB) content decreased from 10 wt% to 2 wt% of CDW within 8 days of incubation in the complete Kosaric medium. On the other hand, the mobilization of P(3HB) in *Synechocystis* sp. was a slower process compared to *S. platensis* as only 4 wt% of P(3HB) was degraded within 10 days of incubation in the complete BG-11 medium.



(a)



(b)

Figure 1: (a) Cell morphology of filamentous *S. platensis* UMACC 161 and (b) unicellular *Synechocystis* sp. UNIWG observed under light microscopy.

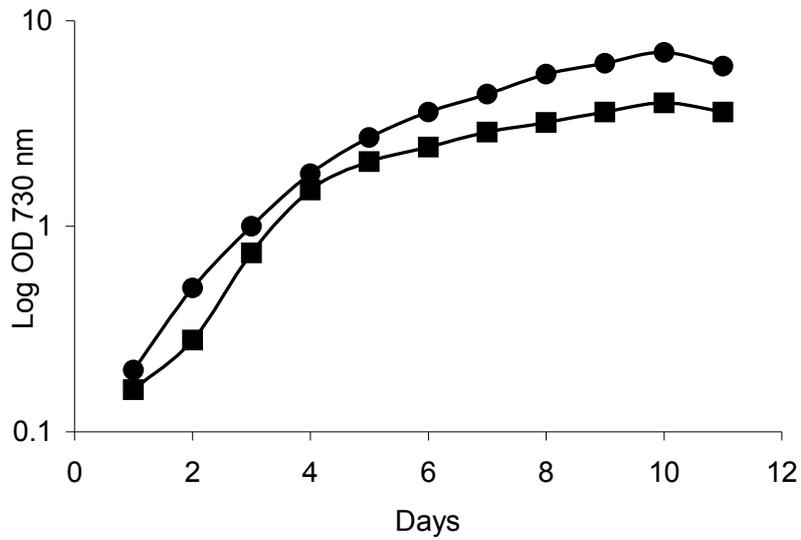


Figure 2: Growth profiles of *S. platensis* UMACC 161 (●) and *Synechocystis* sp. UNIWG (■). Cultivation was carried out at 25°C and continuously bubbled with filter-sterilized atmospheric air under continuous cool white light illumination (1020 lux).

Table 1: PHA accumulation by *S. platensis* UMACC 161 and *Synechocystis* sp. UNIWG in nitrogen free medium.

Carbon sources	P(3HB) content (wt% of CDW)	
	<i>S. platensis</i> UMACC 161	<i>Synechocystis</i> sp. UNIWG
Control (complete medium)	0	0
CO ₂ only (without reduced carbon source)	1	1
Sodium acetate and CO ₂	10	14

S. platensis UMACC 161 was cultured in Kosaric medium (pH 9) for 10 days and then transferred to nitrogen free medium (pH 9) containing 0.5% (w/v) sodium acetate in addition to carbon dioxide.

Synechocystis sp. UNIWG was cultured in BG-11 medium (pH 7.1) for 10 days and then transferred to nitrogen free medium (pH 9) containing 0.5% (w/v) sodium acetate in addition to carbon dioxide. P(3HB) content was determined by GC.

Table 2: Mobilization of P(3HB) synthesized by *S. platensis* UMACC 161 and *Synechocystis* sp. UNIWG.

Strains	P(3HB) content (wt% of CDW)	
	Before mobilization	After mobilization
<i>S. platensis</i> UMACC 161	10	2
<i>Synechocystis</i> sp. UNIWG	7	3

S. platensis UMACC 161 cells from the second stage (cultivated in the presence of acetate) were transferred to complete Kosaric medium to induce P(3HB) mobilization. Cells were incubated in the complete Kosaric medium for a period of 8 days.

Synechocystis sp. UNIWG cells from the second stage (cultivated in the presence of acetate) were transferred to complete BG-11 medium to induce P(3HB) mobilization. Cells were incubated in the complete BG-11 medium for a period of 10 days.

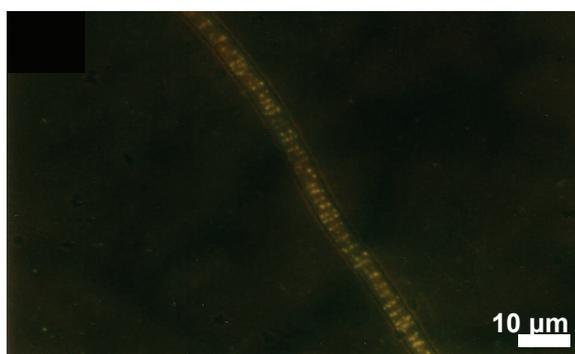
Nile blue A staining was also used to monitor qualitatively the biosynthesis and mobilization processes of PHAs. In *S. platensis*, the cells with high P(3HB) content (10 wt% of CDW) were filled with distinct fluorescing orange particles (Fig. 3A). The quantity and brightness of particles reduced daily until finally no fluorescence was detected after 10 days. Figure 3B shows the Nile blue A stained cells after 4 days of incubation in the complete Kosaric medium. The number of particles and the intensity of fluorescence were obviously lesser than before the onset of mobilization. However, this method could not monitor qualitatively the biosynthesis and mobilization processes in *Synechocystis* sp. because the accumulated P(3HB) granules to our surprise, cannot be stained by Nile blue A. Due to this, TEM was used to visualize the ultrastructure of the accumulated P(3HB) granules.

Electron Microscopy

To further understand the effect of P(3HB) accumulation on cyanobacterial cell morphology, TEM was carried out on both *S. platensis* and *Synechocystis* sp. osmium-fixed cells. In this study, we found that significantly large amounts of electron-transparent granules were present only in the nitrogen-starved *S. platensis* and *Synechocystis* sp. cells incubated for 15 and 21 days, respectively in the presence of 0.5% (w/v) sodium acetate (Fig. 4). By comparing the micrographs, it was found that the granules exist in both strains are sharing the same morphology. These granules appeared spherical and clear edged. However, the number and sizes of granules were varied in *S. platensis* and *Synechocystis* sp.



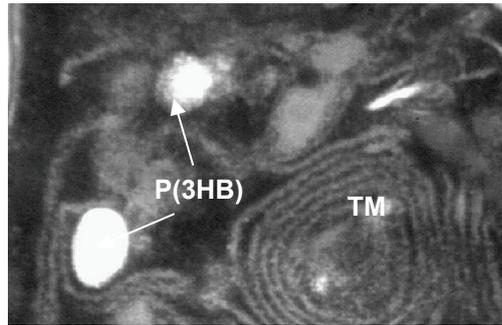
(a)



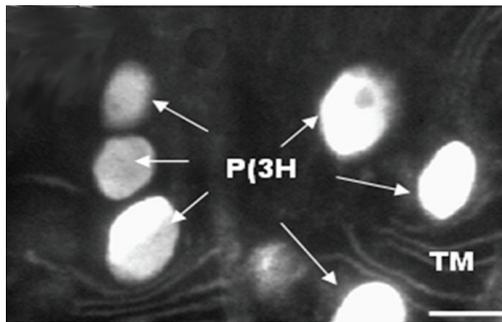
(b)

Figure 3: (a) Nile blue A stained *S. platensis* cells containing 10 wt% P(3HB) of the CDW. The arrow shows the P(3HB) granules that appear as bright orange particles in the cell cytoplasm when observed using a UV microscope. (b) Nile blue A stained *S. platensis* containing 2 wt% P(3HB) of the CDW after mobilization.

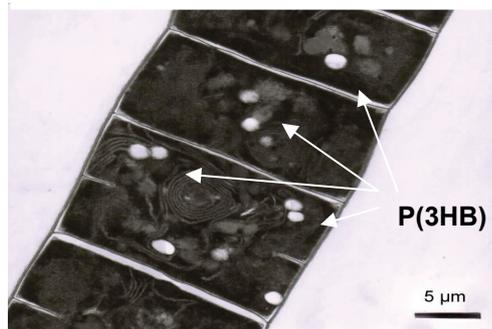
In *S. platensis*, the number of electron-transparent granules in each trichome ranged from 2 to 6 and with diameters ranging from 140–310 nm (Figs. 4A, B & C). From the micrograph, it was found that these granules were distributed randomly in the cell cytoplasm and were nearby the thylakoid membranes. For both vegetative and nitrogen-starved cells of *S. platensis*, distinct thylakoid membranes with no specific pattern of orientation can be seen (Figs. 4C & D). Figure 4D shows a cross-section of the vegetative cell of *S. platensis*. The arrow shows the presence of phycobilisomes attached to the external surface of the thylakoid membranes. Their distribution between two adjacent thylakoid membranes give a zipper-like-appearance when viewed in transversal sections. Under nitrogen-starved conditions, the presence of phycobilisomes on the surface of thylakoids was unclear, probably being degraded by cells as a nitrogen source to prolong survival.



(a)

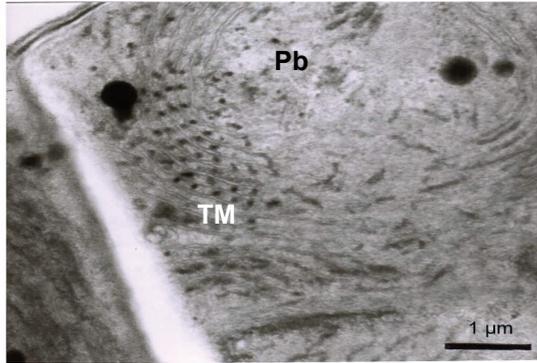


(b)

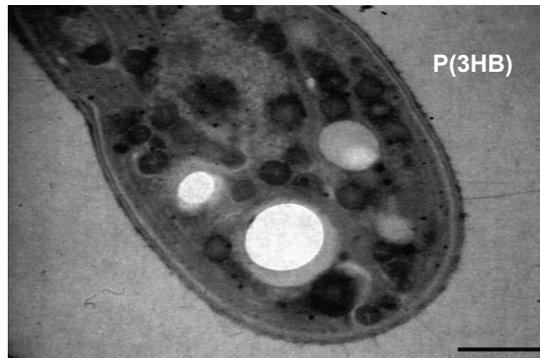


(c)

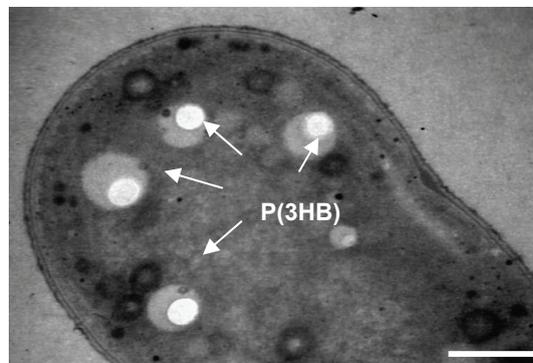
Figure 4: (a), (b) and (c) Transmission electron micrographs of *S. platensis* UMACC 161 cells from nitrogen-free culture supplemented with 0.5% (w/v) sodium acetate showing the presence of electron-transparent P(3HB) granules (~10 wt% of CDW). TM – Thylakoid membranes. Bars 200 nm; (d) Transmission electron micrograph of *S. platensis* UMACC 161 vegetative cell. Pb – Phycobilisomes; (e) and (f) Transmission electron micrographs of *Synechocystis* sp. UNIWG from nitrogen-free culture supplemented with 0.5% (w/v) sodium acetate showing the presence of electron-transparent P(3HB) granules (~14 wt% of CDW). Bars 500 nm (*continued on next page*).



(d)



(e)



(f)

Figure 4: (continued)

For comparison purposes we also investigated the number and sizes of PHA granules in *Synechocystis* sp. The amount of granules accumulated in *Synechocystis* sp. was much higher (10–17 granules) but were smaller in size (100–250 nm in diameter) (Figs. 4E & F). In *Synechocystis* sp., the granules seem to be located in the cell periphery near the cell membrane rather than in the center of the cytoplasmic region. The thylakoid membranes in *Synechocystis* sp. cells were peripheral as in most unicellular cyanobacteria. Besides the P(3HB) granules, some electron-dense inclusions were also found in the cell cytoplasm of *Synechocystis* sp. (Fig. 4E, short arrows). The presence of these inclusions contributed to an additional peak in the GC (results not shown). As revealed by the GC analysis, the amount of these unidentified inclusions increased with prolonged cultivation under nitrogen-starved conditions. These unidentified inclusions may be a form of storage material as they were only present in nitrogen-starved cells.

TEM also revealed the presence of spicules on the cell surface of vegetative cells of *Synechocystis* sp. (Fig. 5A). The spicules appeared rigid and straight and were found distributed all over the surface of the cells. The length of the spicules was ranging from 150–175 nm. Our findings found that under nitrogen-starved conditions, the number of these spicules was reduced (Fig. 5B). This observation indicated that the spicules are either degraded or not synthesized under nitrogen-starvation conditions.

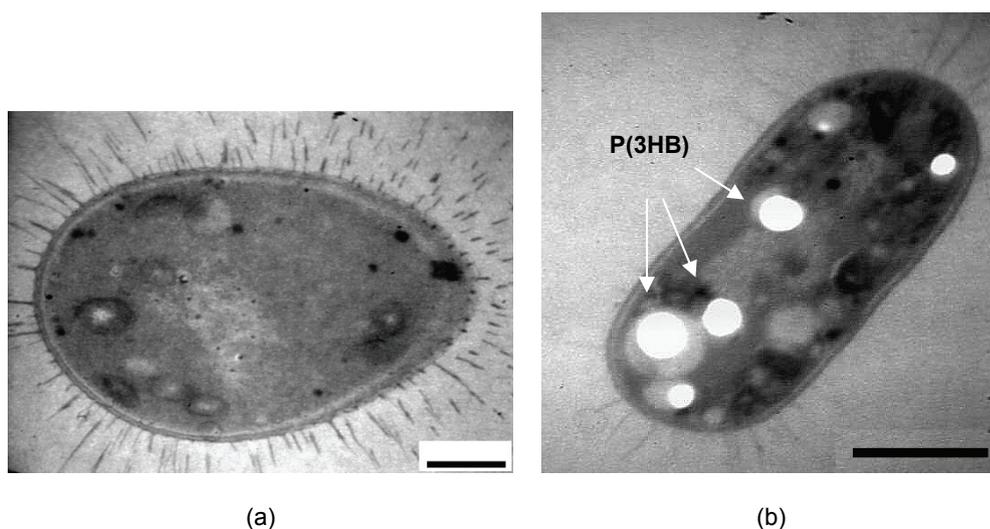
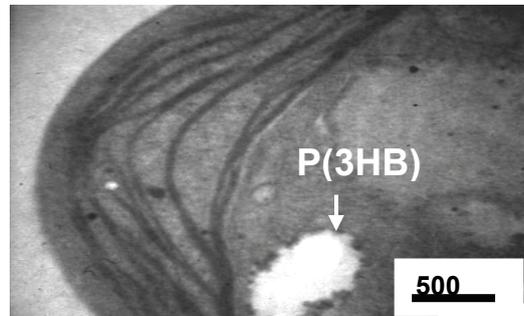


Figure 5: Transmission electron micrographs of *Synechocystis* sp. UNIWG (a) from balanced culture with spicules distributed all over the cell surface. Bar 500 nm; (b) Cells from nitrogen free culture with the absence of most of the spicules.

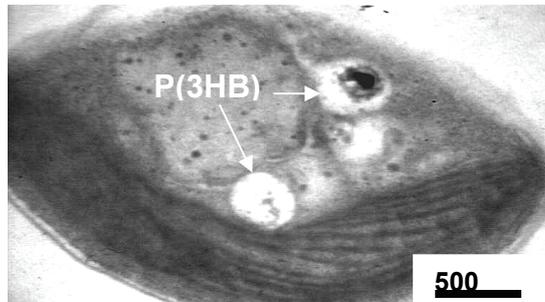
Note: (a) Bar 500 nm
(b) Bar 1 μ m

The mobilization process was observed in detail using TEM. Figures 6A and 6B show that the mobilization of P(3HB) in *S. platensis* resulted in smaller granules and the quantity of granules in each trichome was reduced to less than two within 8 days. The boundary region of the P(3HB) granules has also become less defined following mobilization. Thylakoids were arranged on one side of the cell with no specific pattern of orientation.

Under TEM observation, the mobilization process in *Synechocystis* sp. was not significant as in *S. platensis*. There was no significant reduction in the sizes and the number of P(3HB) granules in the cells (Figs. 6C & D). Interestingly, the unidentified electron-dense inclusions which were previously found in the nitrogen-starved cells were absent with the restoration of nitrogen source. The mobilization of the unidentified storage granules was a rapid process compared to P(3HB) mobilization. Besides that, the number of spicules was also recovered when the cells were transferred into the complete medium. This suggested that the spicules may play a role as cellular appendages for the attachment or anchoring of *Synechocystis* sp. cells to environments with sufficient nitrogen source.

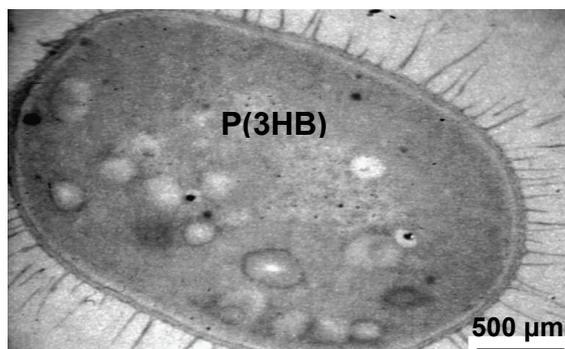


(a)

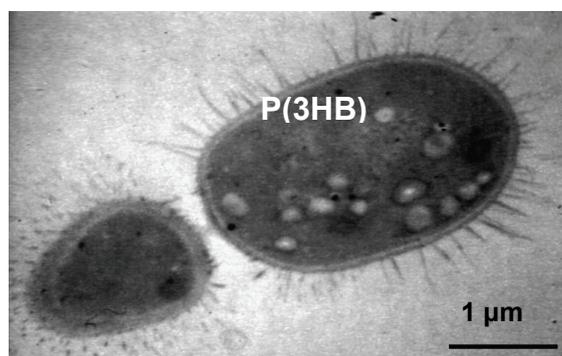


(b)

Figure 6: Transmission electron micrograph of (a, b) *S. platensis* UMACC 161 cells and (c, d) *Synechocystis* sp. UNIWG containing mobilized PHA granules (continued on next page).



(c)



(d)

Figure 6: (continued)

DISCUSSION

In this study, we demonstrated that *S. platensis* UMACC 161 and *Synechocystis* sp. UNIWG accumulated P(3HB) granules in the cell cytoplasm when nitrogen source was depleted in the medium. It is known that P(3HB) is accumulated by a wide variety of prokaryotes under nutrient imbalance conditions such as depletion of nitrogen sources. Under such conditions, cells cannot multiply and therefore carbon sources are stored in the form of water insoluble P(3HB) granules in the cell cytoplasm. P(3HB) plays the role as specialized energy and carbon reserve compound in cells under stress conditions (Senior & Dawes 1973).

The amount of P(3HB) accumulated by *S. platensis* seems lower (10 wt% of CDW) than in *Synechocystis* sp. (14 wt% of CDW). This might be due to the larger size and mass of *S. platensis* compared to *Synechocystis* sp. Therefore, the P(3HB) content in wt% of the CDW as determined by GC would be lower. Electron microscopy also revealed that the P(3HB) granules accumulated by *S. platensis* were larger in size than those in *Synechocystis* sp.

In this study, we found that Nile blue A staining of *Synechocystis* sp. could not stain the accumulated P(3HB) granules. This was surprising as Nile blue A can be used to stain and visualize PHA granules in bacteria as well as in plant cells with thicker cell wall (Poirier 2001). Previous study done by Sudesh *et al.* (2002) showed that PHA granules in *Synechocystis* sp. PCC 6803 can be detected by using the Nile blue A staining technique. The *Synechocystis* sp. UNIWG strain used in this study is a brackish strain while the *Synechocystis* sp. PCC 6803 is a freshwater strain. Otherwise, both strains share similar characteristics. In fact *Synechocystis* sp. UNIWG was morphologically and phylogenetically similar to the freshwater *Synechocystis* sp. PCC 6803 with 100% 16S rRNA gene sequence similarity (results not shown).

S. platensis and *Synechocystis* sp. are non-diazotrophic cyanobacteria that assimilate combined nitrogen sources from the surrounding environment. Nitrogen limitation of cyanobacterial cells has been reported to induce a well-characterized set of cellular responses. These include: visible chlorosis or "yellowing" (Allen & Smith 1969), degradation of phycobiliproteins (Steven *et al.* 1981; Collier & Grossman 1992), alteration of the ratio of phycocyanin to allophycocyanin (Yamanaka & Glazer 1980), degradation of thylakoid membranes (Steven *et al.* 1981), a decrease in chlorophyll content, an increase in carotenoid content or carotenoid/chlorophyll ratio, and an increase in glycogen content (Steven *et al.* 1981).

The "yellowing" effect or chlorosis induced by nitrogen deficiency was more significant in *S. platensis* as the blue-green cultures gradually bleached into yellowish-green after 5–7 days of cultivation under nitrogen-starved conditions. However, this phenomenon was not significant in *Synechocystis* sp. The bleaching was due to the degradation of phycobiliprotein, which is a nitrogen storage compound in cyanobacterial cells with deep blue color (Allen 1984). The amino acids released by proteolytic degradation of phycobiliprotein can further be used to synthesize new materials to maintain cell growth. Sauer *et al.* (1999) demonstrated that nitrogen chlorosis in non-diazotrophic cyanobacterium is a specific acclimation process enabling the cells to survive prolonged periods of nitrogen depletion (Sauer *et al.* 1999).

Steven *et al.* (1981) demonstrated that nitrogen depletion also results in the degradation of thylakoid membranes in the cyanobacterium, *Agmenellum quadruplicatum*. The degradation of thylakoid membranes was also observed in nitrogen depleted cells of both *S. platensis* and *Synechocystis* sp. although not completely. Figure 4A shows that the P(3HB) granules appeared to be localized near the thylakoid membranes in *S. platensis*. Thylakoid membranes are energy generating sites of photosynthesis. P(3HB) formation in cells provide a mechanism for the removal of excess reducing equivalents resulting from a disruption of the balanced formation of ATP and NADPH from photosynthesis. This may be the reason why P(3HB) granules in cyanobacteria are frequently located near thylakoid membranes (Sudesh *et al.* 2002).

The presence of the unknown electron-dense inclusions in nitrogen-starved cells of *Synechocystis* sp. was observed frequently through electron microscopy. Such inclusions existed abundantly in nitrogen-starved cells of *Synechocystis* sp. and were degraded with the restoration of nitrogen source into

the medium. Therefore we think that these inclusions may play the same role as P(3HB) granules as a storage material in *Synechocystis* sp. These unknown inclusions seem to share some similarities in morphology with plant starch granules. Further studies to identify and characterize these inclusions are currently in progress in our lab.

Pili-like structures have been observed on the surface of certain unicellular and filamentous cyanobacteria (Dick & Stewart 1980; Yew *et al.* 2005). However, no specific functions have been attributed to these appendages and there is no understanding of the cellular mechanisms or environmental factors that control their biosynthesis. In this study, TEM revealed the presence of rigid spicules on the surface of vegetative cells of *Synechocystis* sp. (Fig. 5A). Under nitrogen-starved conditions, the number of these spicules was reduced (Fig. 5B). This observation indicates that spicules are either degraded and used as a nitrogen source, or not synthesized under nitrogen-starvation conditions. As reported by Allen (1984), cyanobacteria are known to degrade cellular components during nutrient deficiency in order to prolong their survival.

Many microorganisms synthesize and accumulate PHA in the form of water insoluble granules (Anderson & Dawes 1990; Doi 1990). The accumulated PHA can be used as carbon and energy sources if they are mobilized by the intracellular depolymerase (Choi *et al.* 1999; Handrick *et al.* 2000; Saegusa *et al.* 2001). The mobilization of P(3HB) in *S. platensis* appeared to be relatively fast compared to its biosynthesis process. In contrast, the mobilization of P(3HB) granules by *Synechocystis* sp. was relatively slower. Under TEM observation, the mobilization process in *Synechocystis* sp. was not as significant as in *S. platensis*. There was no significant reduction in the number and sizes of P(3HB) granules (Figs. 6C & D) as compared to that in *S. platensis*. Mobilization of the accumulated P(3HB) was characterized by changes in the granule size, numbers and morphology. The boundary region of the granules became less defined probably because of changes in the surface protein layer (Sudesh *et al.* 2004) which might result in the release of P(3HB) from the granule. Interestingly, the rigid spicules reappeared when the cells were transferred into the complete medium. The spicules may therefore function as cellular appendages for attaching the cells to environments that are conducive for survival.

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