# STUDIES ON THE STARCH-LIKE GRANULES CO-ACCUMULATED WITH POLYHYDROXYBUTYRATE IN *SPIRULINA PLATENSIS*

**by**

**YEW SAW PENG**

**Thesis submitted in fulfillment of the requirements for the degree of**

**Master of Science**

**February 2007**

## ACKNOWLEDGEMENTS

During the course of this project, many people have made invaluable contributions towards the work in creating an environment. I would here like to take the opportunity to express my sincere gratitude to all those people:-

I would like to first thank my supervisor Dr. K. Sudesh Kumar and co-supervisor Dr. Razip Samian for their invaluable support and advice throughout this journey.

Also not forgetting all the lab members and non-lab members who have contributed directly and indirectly to the accomplishment of my master degree study.

My sincere appreciation to my family for their emotional and financial support.

I would also like to express my sincere gratitude to the Ministry of Science, Technology and Innovation (MOSTI) for the financial support under the National Science Fellowship (NSF) scheme.

Finally, I thank the Toray Science Foundation, Japan for funding this research with a Science and Technology research grant that had enabled me to have full concentration on the research study.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURE

**2.0 LITERATURE REVIEW** **PAGE**



**4.0 Results**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| DCW | Dry Cell Weight |
| EDX | Energy Dispersive X-ray |
| GC | Gas Chromatography |
| GC-MS | Gas Chromatography-Mass Spectrometry |
| gDNA | Genomic DNA |
| PHA | Polyhydroxyalkanoate |
| PHB | Polyhydroxybutyrate |
| SEM | Scanning Electron Microscopy |
| TEM | Transmission Electron Microscopy |

**STUDIES ON THE STARCH-LIKE GRANULES CO-ACCUMULATED WITH POLYHYDROXYBUTYRATE IN *SPIRULINA PLATENSIS***

## ABSTRACT

*Spirulina platensis* is capable of synthesizing small amount of polyhydroxyalkanoate (PHA) under nitrogen starvation condition. Gas chromatography (GC) analysis revealed that about 10 % PHA of the cell dry weight (CDW) was accumulated under mixotrophic culture condition containing 0.5% (w/v) of sodium acetate. However, Nile blue A stained cells showed the presence of large quantities of granules in the cell cytoplasm when viewed under fluorescent microscope. The qualitative observation was in contrast to the quantitative GC analysis which suggested that not all the fluorescent granules are PHAs. It was hypothesized that *S. platensis* co-accumulated other storage products that were stained with Nile blue A along with PHA under nitrogen starvation condition. Closer observation by transmission electron microscopy (TEM) revealed the presence of starch-like granules along with PHA granules. Analysis of several types of commercial starches confirmed that starch granules could be stained by Nile blue A although the intensity of the fluorescence was lower than for PHA granules. In order to further characterize the starch-like granules, large genomic DNA fragments of *S. platensis* were randomly cloned into *E. coli* S17-1 and resulting recombinants were screened for Nile blue A positive characteristic. One such recombinant *E. coli* was able to accumulate morphologically similar granules. GC and PHA synthase activity analyses

confirmed that these are not PHA granules. GC-MS analysis revealed that the recombinant *E. coli* accumulated starch-like compound consisting of glucose monomers. The granules exhibited birefringence under polarized light, which is a characteristic of semi-crystalline starch granules. This study showed for the first time that *S. platensis* co-accumulated PHA and starch-like granules during nitrogen starvation. Suppression of the starch-like granule biogenesis might produce a higher PHA accumulation in *S. platensis*.

**KAJIAN KE ATAS GRANUL**

**MENYERUPAI KANJI YANG DIHASILKAN BERSAMA DENGAN**

**PENGHASILAN POLIHIDROKSIBUTIRAT OLEH *SPIRULINA PLATENSIS***

## ABSTRAK

Di bawah keadaan nitrogen terhad, *Spirulina platensis* berkeupayaan mensintesis polihidroksialkanoat (PHA) dalam kuantiti yang sendikit. Analisis gas kromatografi (GC) pula menunjukkan bahawa kira-kira 10% PHA daripada berat kering sel dihasilkan di bawah keadaan miksotrophik dengan penambahan 0.5 %(w/v) natrium asetat. Namun, sel yang diwarnakan dengan Nile blue A menunjukkan bahawa PHA granul dihasilkan dalam kuantiti yang tinggi di dalam sel sitoplasma di bawah pemerhatian mikroskop pendarflour. Pemerhatian kualitatif adalah bertentangan dengan analisis kuantitatif GC. Ini mencadangkan bahawa bukan kesemua granul yang berfluoresing adalah PHA. Ini juga menghipotesiskan bahawa di bawah keadaan nitrogen penghad, bahan simpanan lain yang mampu diwarnakan oleh Nile blue A juga dihasilkan bersama dengan penghasilan PHA. Pemerhatian yang lebih terperinci melalui TEM pula menunjukkan granul yang menyerupai kanji hadir bersama dengan granul PHA. Analisis terhadap kanji komersil menyesahkan bahawa granul kanji ini boleh diwarnakan oleh Nile blue A walaupun intensiti pendarflour adalah lebih rendah jika dibandingkan dengan granul PHA. Untuk mengkaji granul menyerupai kanji ini dengan lebih lanjut lagi, fragment gDNA *S. platensis* yang besar telah diklonkan secara rawak ke dalam *E. coli* S17-1 dan perumah

recombinan disaring melalui pencirian Nile blue A positif. Salah satu recombinan *E. coli* berupaya menghasil granul yang mofologi serupa. GC dan PHA sintase aktiviti menyesahkan bahawa ini bukan granul PHA. Analisis yang lebih lanjut melalui GC-MS pula menunjukkan bahawa sebatian yang disintesis oleh *E. coli* recombinan ini berasal daripada monomer glucose. Granul ini memberikan ciri 'birefringence' di bawah cahaya yang berkutub dan ini merupakan salah satu pencirian untuk granul kanji yang semi-kristal. Kajian ini menunjukkan buat kali pertama bahawa *S. platensis* PHA dan granul yang menyerupai kanji ini dihasilkan bersama di bawah keadaan nitrogen penghad. Penghentian biogenesis granul yang menyerupai kanji ini akan meningkatkan penghasilan PHA dalam *S. platensis*.

## 1.0 INTRODUCTION

The exponential growth of the human population has led to the accumulation of huge amounts of non-degradable waste materials across our planet. Living conditions in the biosphere are therefore changing dramatically, in such a way that the presence of non-degradable residues is affecting the potential survival of many species. For this reason, many countries have prompted special programmes directed towards the discovery of new commonly used materials that can be readily eliminated from the biosphere, and have designed novel strategies aimed at facilitating the transformation of contaminants.

Polyhydroxyalkanoate (PHA) is a natural product that is synthesized and catabolised by different microorganisms and that have potential broad biotechnological applications. Devices made from PHA are designed to have controlled degradation rates, preferably less than one year under physiological conditions. Examples of such devices include sutures, suture fasteners, meniscus repair devices, rivets, tacks, staples, screws (including interference screws), bone plates and bone plating systems, surgical mesh, repair patches, slings, cardiovascular patches and orthopedic pins (Williams *et al*., 2005).

They can be assimilated by many species (biodegradable) (Yew *et al.*, 2006) and do not cause toxic effects in the host (biocompatible) (Sudesh *et al.*, 2000; Zinn *et al.*, 2001)， conferring them over conventional synthetic products.

Currently, the main limitations for the bulk production of PHA is its high production and recovery costs (Anderson and Dawes, 1990; Madison and Huisman, 1999; Witholt and Kessler, 1999). In that sense, the ultimate cost effective

producer of PHA would be photosynthetic organisms such as transgenic plants (Poirier *et al.*, 1992) and cyanobacteria (Asada *et al.*, 1999). Research over the last decade has resulted in the development of transgenic plants carrying the PHA biosynthetic genes of bacteria. Like higher plants, cyanobacteria are also oxygen-evolving photoautotrophs with the added advantage that some of them naturally possess the key enzyme in PHA biosynthesis. In addition, due to their minimal nutrient requirement, cyanobacteria are viewed as attractive hosts for the production of PHA (Asada *et al.*, 1999). However, in comparison with other heterotrophic bacterial strains, it is generally thought that cyanobacteria can only produce very small amounts of PHA (~ 5 % of the CDW). Unlike conventional bacteria like *Pseudomonas aeruginosa* accumulates PHA up to 36% of CDW (Haba *et al*., 2007), while moderate halophile *Halomonas boliviensis*, isolated from a Bolivian saline soil sample, was able to accumulate up to 88% of CDW of PHB when grown under conditions of nutrient limitation and excess carbon source (Quillaguamán *et al*., 2006).

The use of recombinant microorganisms to efficiently produce PHA has been described extensively. The main disadvantage of this production route is its high cost. Plants obviously represent an attractive alternative for polyhydroxybutyrate (PHB) production. Pioneering work performed by Poirier *et al* (1995) in *Arabidopsis* shows that PHB synthesis is feasible in plants. A dramatic increase in the amount of PHB produced by *Arabidopsis* sp. (Stanier and Cohen-Bazire, 1977) plants was achieved when PHB synthesis was engineered in chloroplasts. Surprisingly, only a few reports of PHB production in crop plants have been published, and those that have been reported showed very low amounts of

PHB accumulation. The only exception is the work resulting in transgenic *Brassica napus* plants in which the seeds expressed up to 7% PHB of the dry weight. Like higher plants, cyanobacteria are probably the largest, most diverse, and most widely distributed group of photosynthetic prokaryotes.

However there are many shortcomings for plants and cyanobacteria to produce PHB intensively. Functional expression of PHB biosynthesis genes in transgenic plants, the inhibition of normal growth of these transgenic plants and the expression of these genes in relevant agricultural crops are just some of the problems faced. There is an additional requirement that ideally PHB be accumulated in these plants in similar amounts to their endogenous storage compounds, such as sucrose, starch or triacylglycerols (Poirier *et al.*, 1995). Like many other microorganisms, cyanobacteria produce intracellular polysaccharides with many of the features of a carbon and energy reserve material.

Recently, Jau *et al.* (2005) reported three strains of *Spirulina platensis* that were isolated from different locations which could synthesize PHB under nitrogen-starved condition with a maximum accumulation of 10% of CDW under mixotrophic culture conditions. The present study is a further attempt to improve PHB biosynthesis in *S. platensis*. To achieve this goal, it is essential to understand why the PHA content is low even when additional carbon sources are present in the culture medium. Intriguingly, it was observed that although the PHB content was low (<10% of the CDW), there were many PHB-looking granules in the cytoplasm of *S. platensis* based on Nile blue A staining results. Closer inspection showed that not all the PHB granules that were stained with Nile blue A fluoresced bright orange in color when viewed under a fluorescent microscope. Normally fluorescent

orange indicates the presence of PHB granules within the cell. Some of the granules were slightly yellowish. This observation led to the hypothesis that some of the granules stained by Nile blue A might not be PHB. During conditions that favor PHB synthesis, *S. platensis* may also synthesize another kind of storage product. If this hypothesis is true, then it would become clear why the PHB content is low. It would also enable the development of strategies to improve PHB synthesis in *S. platensis*, i.e, by inhibiting the carbon flux to the other storage products. In consideration of the importance of improving PHB biosynthesis in *S. platensis*, this study focuses on the identification and characterization of the other storage granules that are co-accumulated with PHB in *S. platensis*.

**RESEARCH OBJECTIVES:**

The objectives of this study are:

1) To identify and characterize the starch-like granules co-accumulated with PHB by *S. platensis* during nitrogen starvation condition.
2) Cloning of the biosynthetic genes involved in the formation of starch-like granules in *S. platensis*.
3) Expression of the biosynthetic genes for starch-like granules in *E. coli*.

# 2.0 LITERATURE REVIEW OF THIS STUDY

## 2.1 Overview of Polyhydroxyalkanoate (PHA)

### 2.1.1 History of PHA

In the early 1920s, a microbiologist at Pasteur Institute in Paris isolated a polymer from *Bacillus megaterium* by chloroform extraction and demonstrated that it was a polyester of 3-hydroxybutyric acid, polyhydroxybutyric (PHB) (Lemoigne, 1926). Since Lemoigne discovered PHB, the polymer has presented many challenges to microbiologists and biochemists who are interested in its physiological functions and metabolism. The general knowledge of microbial PHB was first summarized in a comprehensive review by Dawes and Senior (1973).

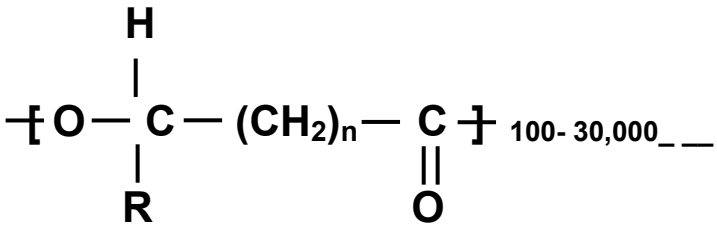
Later, it was found that PHB is only one type in a huge family of polymers collectively known as polyhydroxyalkanoate (PHA). In 1971, Wallen and Rohwedder isolated PHA by chloroform extraction of activated sludge (Wallen and Rohwedder, 1971). The monomers that were detected in chloroform extracts of activated sewage sludge are 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx) as the major and minor constituents, respectively. About a decade later following the identification of heteropolymers, analysis of marine sediments by capillary gas chromatography revealed the presence of 3HB and 3HV as the predominant components among 11 other short-chain 3-hydroxyalkanoate monomers (Findlay and White, 1983).

### 2.1.2 Chemical structure and monomer constituents of PHA

The many different PHAs that have been identified to date are primarily linear; head-to-tail polyesters composed of 3-hydroxy fatty acid monomers. In these polymers, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer (Figure 2.1).

Historically, PHB has been studied most extensively and has triggered commercial interest in this class of polymers. PHB is the most common type of PHA.

Copolymers of PHB can be formed by cofeeding of substrates and may result in the formation of polymers containing 3HV or 4-hydroxybutyrate (4HB) monomers. Together, polymers containing such monomers form a class of PHAs typically referred to as short-chain length (SCL), which consist of 3-5 carbon atoms, and medium-chain length (MCL), which consist of 6-14 carbon atoms (Anderson and Dawes, 1990). These PHAs are synthesized from fatty acids or other aliphatic carbon sources, and, typically the composition of the resulting PHA depends on the growth substrate used (Brandl *et al.*, 1988; Huisman *et al.*, 1989). The PHA synthase of *Wautersia eutropha* can polymerise 3HAs consisting of 3-5 carbon atoms whereas that present in *Pseudomonas oleovorans* can only accumulate 3HAs of 6-14 carbon atoms.



| | | |
|---|---|---|
| n = 1 | R = hydrogen | 3-hydroxypropionate |
| | R = methyl | 3-hydroxybutyrate |
| | R = ethyl | 3-hydroxyvalerate |
| | R = propyl | 3-hydroxyhexanoate |
| | R = pentyl | 3-hydroxyoctanoate |
| | R = nonyl | 3-hydroxydodecanoate |
| n = 2 | R = hydrogen | 4-hydroxybutyrate |
| | R = methyl | 4-hydroxyvalerate |
| n = 3 | R = hydrogen | 5-hydroxyvalerate |
| | R = methyl | 5-hydroxyhexanoate |
| n = 4 | R = hexyl | 6-hydroxydodecanoate |

Figure 2.1 General structure of PHA and some of its monomers (Doi, 1990).

### 2.1.3 Conditions favoring PHA accumulation

The effect of the growth conditions on PHA metabolism was first studied by Macrae and Wilkinson (1958) for an asporogenous strain of *Bacillus megaterium*. They made the important observation that the quantity of PHA accumulated increased as the carbon to nitrogen ratio increased. Their results suggested that, like polyphosphate and carbohydrate reserves, PHA accumulation occurred in response to an imbalance in growth brought about by nutrient limitations. This significant observation began the investigation into the physiological role of PHA. It was understood later that bacteria make and store PHA when they lack the complete range of nutrients required for cell division but have generous supplies of carbon source (Doi, 1990; Sudesh *et al.*, 2000). The biosynthesis of PHA was shown to be initiated by a magnesium or sulfate deficiency, as well as by nitrogen, phosphate (Dawes and Senior, 1973) and/or oxygen limitations (Ward *et al.*, 1977).

A glucose-utilizing bacteria *Alcaligenes eutrophus* accumulates up to 80% PHB of CDW with glucose as the carbon source (Holmes,1985). In year 1983, De Smet et al. observed the presence of intracellular granules consisting of PHO in Pseudomonas oleovarans ATCC 29347 grown in two-phase medium containing 50% (v/v) octane.

### 2.1.4 Number of PHA granules per cell

The role of PHA as a carbon and energy reserve material in bacteria has been previously reviewed by Dawes and Senior (1973). PHA granules exist as discrete inclusion that are typically 0.2-0.5 μm in diameter, localized in the cell

cytoplasm and may be visualized quite clearly with a phase contrast light microscope due to their high refractivity (Dawes and Senior, 1973). When thin sections of PHA-containing bacteria are observed by transmission electron microscopy, the PHA inclusions appear as electron-dense bodies (Yew *et al.*, 2005).

### 2.1.5 Detection and analysis of PHB

Microbiologists have traditionally detected the presence of PHB granules in bacterial cells by staining with Sudan Black B. Native PHA granules can be stained with Sudan black B (Burdon, 1946) indicating that they are of a lipid nature (Kallio and Harrington, 1960; Williamson and Wilkinson, 1958). However, Ostle and Holt (1982) advocated the use of Nile blue A, a water-soluble basic oxazine dye that has a greater affinity and higher specificity that Sudan Black for PHB, and that gives a bright orange fluorescence at a wavelength of 460 nm. Other inclusion bodies, such as glycogen and polyphosphate, do not stain with Nile blue A, emphasizing its usefulness. Recently, it was demonstrated that Nile blue A and its fluorescent oxazone form, Nile Red can be used directly to detect PHA in growing bacterial colonies on agar plate (Rehm *et al.*, 2002).

### 2.1.6 Biological considerations

The diversity of different monomers that can be incorporated into PHAs, combined with a biological polymerization system that generated high-molecular weight materials is an area in which there is much to study (Kusaka *et al.*, 1999). In fact, it presents an enormous challenge to our scientific discipline to fully explore

this biology to ensure that environmentally obtainable polyesters are available for generations to come.

#### 2.1.6.1 Biodegradability

An important characteristic of PHA is their biodegradability. In nature, a vast consortium of microorganisms are able to degrade PHA by using secreted PHA hydrolases and PHA depolymerases (Jendrossek *et al.*, 1996). The activities of these enzymes may vary and depend on the composition of the polymer, its physical form (amorphous or crystalline) and most importantly, the environmental conditions. The degradation rate of a piece of P(3HB) typically ranged from a few months (in anaerobic sewage) to years (in seawater) (Jendrossek *et al.*, 1996; Mergaert *et al.*, 1993). In a recent innovative application of PHA, nanosized titanium dioxide was immobilized onto PHB film in order to create a photocatalytically active biodegradable material (Yew *et al.*, 2006).

#### 2.1.6.2 Renewable nature

As important as the biological characteristics and biodegradability of PHA is the fact that their production is based on renewable resources (Doi, 1990). Fermentative production of PHA is based on agricultural products such as sugar (Reddy *et al.*, 2002), carbon dioxide (Asada *et al.*, 1999; Miyake *et al.*, 2000) and fatty acids (Fuchtenbusch *et al.*, 2000; Fukui and Doi, 1998; Solaiman *et al.*, 2000) as carbon and energy sources. This agricultural feedstock is derived from $CO_2$ and water, and after their conversion to biodegradable PHA, the breakdown products

are again $CO_2$ and water. PHA receives widespread attention because they are based on renewable compounds instead of diminishing fossil fuel stockpiles (Sudesh *et al.*, 2000).

### 2.1.7 Potential applications of biodegradable plastics produced from PHA

PHAs are partially crystalline polymers with a degree of crystallinity ranging from 60-80% (Sudesh *et al.*, 2000). The family of PHAs exhibits a wide variety of mechanical properties from hard crystalline to elastic, depending on composition of monomer units which broadens its application area.

The possible application of bacterial PHA is directly connected with their properties such as biological degradability and thermoplastics characteristics. The applications of bacterial PHA is mainly focused on 3 principal areas which are medical and pharmaceutical (Smits and Williams, 1999; Sodian *et al.*, 2000), coating (van der Walle *et al.*, 1999) and commodity packaging (Holmes, 1985). The most advanced development of bacterial PHA is in the medical field, especially pharmaceutical applications. However they have a considerable potential as consumer goods products (Sudesh, 2004; Zinn *et al.*, 2001).

## 2.2 Cyanobacteria as potential PHA producer

### 2.2.1 Introduction to cyanobacteria

Cyanobacteria (blue-green algae, blue-green bacteria, cyanophytes) are probably the largest, most diverse, and most widely distributed group of photosynthetic prokaryotes (Stanier and Cohen-Bazire, 1977). Cyanobacteria are prokaryotic organisms which can carry out oxygenic photosynthesis with a

generation time of 13 h under photoautotrophic growth (Vermaas, 1996). Unlike other photosynthetic bacteria, the cyanobacteria have chlorophyll *a.* Their ecological importance has long been recognized in the process of eutrophication and in their ability to survive and emerge in varying stressful habitats.

Cyanobacteria are oxygenic, photoautotrophic prokaryotes which possess two photosystems (PSI and PSII) that can release electrons from water and fix carbon dioxide via the Calvin-Benson-Bassham pathway (Stanier and Cohen-Bazire, 1977). The fixed carbon is partially deposited in the form of intracellular polymers such as glycogen, other polyglucans and cyanophycin, which serve as carbon and energy reserves (Dionisi *et al.*, 2005). Carr (1966) reported the presence of PHA inclusion bodies in cyanobacteria following extraction of P(3HB) from *Chloroglea fritschii*.

### 2.2.2 Reserve polymers of cyanobacteria

Cyanobacteria are able to accumulate a variety of putative reserve materials. These include cyanophycin, polyphosphate, polysaccharide and PHB. Polysaccharide and PHB are usually considered to be carbon and energy reserves, polyphosphate is a potential energy or phosphate reserve and the polypeptide cyanophycin is, in principle, a nitrogen reserve with a possible additional minor role of supplying limited amounts of carbon and energy (Dionisi *et al.*, 2005).

#### 2.2.2.1 Cyanophycin

Cyanophycin is a trivial name given to the major component of the structured granules which are visible in thin sections of cyanobacteria. It is unique in cyanobacteria and occurs in most but not all species of these oxygenic photosynthetic bacteria (Lawry and Simon, 1982; Mittendorf *et al.*, 1999). Cyanophycin functions as a temporary nitrogen reserve. It accumulates nitrogen during the transition from the exponential to stationary phase and disappears when balanced growth is restored (Mackerras *et al.*, 1990). Cyanophycin is deposited in the cytoplasm as membrane less granules (Lawry and Simon, 1982). The unicellular *Synechocystis* sp. strain PCC6803 synthesizes the polymer to a maximum amount of 16% of the cell dry mass under conditions stimulating cyanophycin synthesis, i.e., when light intensity and temperature are reduced and when arginine and aspartic acids are depleted (Mittendorf *et al.*, 1999).

#### 2.2.2.2 Polyphosphate

Many microorganisms, including cyanobacteria, contains granules of polyphosphate which may function as a source of phosphate for the synthesis of nucleic acids and phospolipid or as a source of energy for ATP formation (Merrick, 1979). Polyphosphate like cyanophycin is commonly accumulated by cyanobacteria during stationary phase of growth or when phosphate is added to a culture of starved organisms (Sutherland *et al.*, 1979). Such reserves disappear during phosphate starvation and are able to support a limited amount of growth (Sutherland *et al.*, 1979).

#### 2.2.2.3 Polyhydroxyalkanoate (PHA)

Cyanobacteria produce PHA, a class of biodegradable polyesters that are synthesized by many genera of eubacteria as well as some of the representatives of the archaebacteria (Doi, 1990). The well-studied type PHA is PHB. The presence of PHA inclusion bodies in cyanobacteria was first reported by Carr (1966). He observed the inclusion bodies in *Chloroglea fritschii* (Dionisi *et al.*, 2005). PHA is carbon and energy storage compounds that are synthesized and deposited in the cytoplasm as insoluble inclusions. Cyanobacteria can obtain their precursors for production of PHA from $CO_2$ which is assimilated through photosynthesis rather than more complex organic carbon sources (Asada *et al.*, 1999).

#### 2.2.2.4 Polysaccharide

Like many other microorganisms, cyanobacteria produce an intracellular polysaccharide with many features of a carbon and energy reserve material. In the thin section of these organisms, polysaccharide exists as discrete granules or rods and are located between the thylakoids (Ris and Singh, 1961). This cell constituent has been isolated from several cyanobacteria and characterized in reasonable detail (Chao and Bowen, 1971). These preparations, which were contaminated with a small amount of protein, produced only D-glucose when hydrolysed with acid, while α-amylase treatment yielded maltose and a α-macrodextrin accounting for 11-13% of the polysaccharide. The average chain length of α (1-4) linked units was 13 and is within the range (10-14) quoted for bacterial glycogens (Merrick, 1979). In contrast the size range of α (1-4) polyglucose molecule released by

treatment with iso-amylase was comparable to that for phytoglycogen but distinct from that of other microbial glycogen (Weber and Wöber, 1975).

### 2.2.3 Isolation and properties of cyanobacteria that are highly capable of accumulating PHA

Photosynthetic production of PHA may be one of the most promising ways to produce biodegradable plastics because of its environmentally acceptable characteristics. There are three groups of PHA accumulating organisms. These include organisms that accumulate PHA from carbon dioxide; chemoautotrophic bacteria such as hydrogen-oxidizing bacteria, genetically engineered higher plants and cyanobacteria (Asada *et al.*, 1999). One of the promising topics in PHA production is the probable use of genetically-engineered higher plants (Wanner *et al.*, 1986). Cyanobacteria, however, are indigenously the sole organisms of PHA accumulation by oxygenic photosynthesis.

Among the various cyanobacteria that are capable of synthesizing PHA, *Spirulina platensis* is the subject of interest in this study due to its natural ability to accumulate PHA as a storage product of $CO_2$ fixation and because large scale production of *S.platensis* cell biomass has been shown to be flexible (Vonshak, 1997). *S. platensis* is not toxic and being produced in large scales for use as health food. Its safety for human consumption has since been established through various toxicological studies sponsored by the United Nations Industrial Development Organization (Chamorro-Cevalos, 1980). In addition, *S. platensis* is an aquatic microorganism; therefore, it does not require fertile land and hence does not cause

soil erosion or groundwater contamination. *S. platensis* is also biotechnologically important due to its high nutritional value (Ciferri and Tiboni, 1985).

As shown in Table 1, it is already known that some cyanobacteria can accumulate PHA (PHB is mainly accruing), the storage materials abundant in prokaryotes under photoautotrophic or mixotrophic growth conditions with acetate. However, PHA content is generally about several percent of cell dry weight and this limits the progress of not only applied research but also of basic science on cyanobacterial PHA. There is little information on the metabolism and biochemistry of PHB biosynthesis by cyanobacteria although their physiological role has been estimated to serve as energy storage compound (Shewmaker and Stalker, 1992).

Table 2.1 PHA content in cyanobacteria

| Cyanobacteria | Polymer | Carbon source | Content (w/w) | Reference |
|---|---|---|---|---|
| *Chlorogloea fritschii* | PHB | $CO_2$ | N.D. | (Christiansen and Jensen, 1972) |
| *Chlorogloea fritschii* | PHB | Acetate | N.D. | (Christiansen and Jensen, 1972) |
| *Spirulina platensis* | PHB | $CO_2$ | 6% | (Campbell *et al.*, 1997) |
| *Spirulina platensis* | PHB | Acetate | 10% | (Jau *et al*., 2005) |
| *Spirulina maxima* | PHB | $CO_2$ | 0.7% | (De Philippis *et al.*, 1992) |
| *Spirulina maxima* | PHB | Acetate | 2% | (De Philippis *et al.*, 1992) |
| *Gloeothece sp.* | PHB | $CO_2$/acetate | 6% | (Shewmaker and Stalker, 1992) |
| *Oscillatoria limosa* | PHV | $CO_2$/acetate | 6% | (Shewmaker and Stalker, 1992) |
| *Gloeothece* PCC6909 | PHB | $CO_2$ | N.D. | (Arino *et al.*, 1995) |
| *Synechococcus* MA19 | PHB | $CO_2$ | 27.5% | (Suzuki *et al.*, 1996) |

ND (Not detected); PHB: poly(3-hydroxybutyrate; PHV: poly(3-hydroxyvalerate)

### 2.2.4 Genetically engineered cyanobacteria and transgenic plants that can accumulate PHB

#### 2.2.4.1 Cyanobacteria

The first example of genetically engineered cyanobacteria that accumulate PHB was *Synechococcus* sp. PCC7942. It did not accumulate PHB naturally but was transformed with the genes encoding for PHB-synthesis (3-ketothiolase, acetoacetyl-CoA reductase and PHB synthase) from *W. eutropha* (Suzuki *et al.*, 1996). The transformant accumulated about 1% PHB of cell dry weight under photoautotrophic and nitrogen starved conditions, but the wild type did not. A new transformant was made by using an improved vector system with a strong promoter (Nakamura *et al.*, 2005), but the PHB content was about the same (about 2-3% difference). Addition of acetate drastically enhanced the PHB content to about 10% of CDW under nitrogen supplemented conditions and to 25% of CDW under nitrogen starved conditions.

Later, PHA synthase activity in *Synechocystis* sp. PCC6803 was increased two-fold by introducing the PHA biosynthetic genes of *W. eutropha* (Sudesh *et al*., 2002). Both the wild-type and recombinant *Synechocystis* sp. PCC6803 were subjected to a 2-stage cultivation whereby the second stage nitrogen-limited medium contained various carbon sources (sodium acetate, 4-hydroxybutyric acid, 1,4-butanediol, γ-butyrolactone, pentanoic acid and propionic acid) in addition to $CO_2$. The ability of other carbon sources to promote PHA biosynthesis and to incorporate other hydroxyalkanoate monomer such as 3HP, 3HV and 4HB were tested. Both the wild-type and recombinant *Synechocystis* sp. PCC6803 cells were

only able to accumulate PHAs containing 3HB as the sole monomer. Increased PHA synthase activity in the recombinant resulted in only a marginal increase of PHB accumulation when cultivated in the presence of acetate.

#### 2.2.4.2 Transgenic plants

Synthesis of PHA in plants was first demonstrated in 1992 by the accumulation of PHB in the cell cytoplasm of *Arabidopsis thaliana* (Poirier *et al*., 1992). Since then, a range of different PHAs have been synthesized in various species through the creation of novel metabolic pathways either in the cytoplasm , plastid (Arai *et al.*, 2001; Hahn *et al.*, 1999) or peroxisome (Arai *et al.*, 2002). However, the initial driving force behind the synthesis of PHA in plants has recently emerged as a useful and novel tool to study fundamental aspects of plant metabolism.

Nevertheless, a major problem in the production of PHA in plants is the adverse effect of the expression of the *phb* genes on plant growth. Expression of high amounts acetoacetyl-CoA reductase in transgenic plants caused a significant reduction in growth and seed production relative to wild-type. The apparent problems associated with the accumulation of PHB granules in various organelles and the reduced growth of transgenic plants may be alleviated by regulating the tissue specificity, timing of expression, and cellular localization of the enzymes involved in PHB synthesis.

PHB produced in plants would be a renewable resource and would have prices comparable to that of non-biodegradable plastics produced from oil. There is a complete range of genes available from various microorganisms that can be

used for metabolic engineering of plants. All of these achievements indicate that it is possible to direct the synthesis of PHA to a specified location in transgenic plants.

### 2.2.5 The importance of transgenic plants and cyanobacteria in PHA production

Cost effectiveness is one of the main factors that have hampered the usage of PHA as commodity plastics. In that sense, the ultimate cost effective producer of PHA would of course be transgenic plants (Pool, 1989). Several initial attempts using transgenic *A. thaliana* were successful in producing small amounts of PHB (Pool, 1989). However, the host plants were adversely affected. This could probably be due to depletion of one or more essential substrates for growth. This problem was then eliminated by transgenic manipulations and PHB was produced more effectively in the transgenic plant plastids (Pool, 1989). There was also an attempt to produce PHB within cotton fiber lumens, thus modifying the chemical and thermal properties of the fiber (John and Keller, 1996).

In the near future, PHA production using transgenic plants is expected to reduce the cost to an economically acceptable level (Poirier *et al.*, 1995). Considering the soaring world population and the increased demand for food, fertile land may have to be used efficiently for food production. Microalgae have many uses, freshwater microalga *Chlorella vulgaris*, an important organism in tertiary wastewater treatment (Gonzalez *et al*., 1997; Lau *et al*., 1997). In such a situation, the utilization of recombinant cyanobacteria and/or microalgae for PHA production may be another option. Large-scale production of *Spirulina* for

consumption as dietary and food supplements, animal feed, and also as biofertilizer seems to be feasible (Allnutt, 1996; Patterson, 1996). An ideal process would be when PHA can be harvested as a byproduct during bioremediation process employing cyanobacteria. Cyanobacteria are potential microorganisms for the development of a sustainable process by converting $CO_2$ and reduced carbon sources such as sago and tapioca starch to PHB (Yew *et al*., 2004).

### 2.2.6 Shortcoming using recombinant cyanobacteria or transgenic plants for production of PHA

Effort has been devoted in recent years to increasing PHA yields and productivity. Achieving production costs that are in the same range as those of chemically synthesized plastics may be feasible, given the recent creation of PHA-producing transgenic plants (Poirier *et al.*, 1995). Pioneering work performed by Poirier *et al* (1995) in *Arabidopsis* showed that PHB synthesis is feasible in plants. A dramatic increase in PHB amounts were achieved by *Arabidopsis* when PHB synthesis was engineered in chloroplasts. Surprisingly, few reports of PHB production in crop plants have appeared, and in those that have been reported only very low amounts of PHB were produced. The only exception is the work resulting in transgenic *Brassica napus* plants with seeds accumulating 7% (w/w) PHB by weight (Houmiel *et al.*, 1999).

## 2.3 Starch biosynthesis

Starch in plants accumulate either in the chloroplast of the leaf cell during photosynthesis (photosynthetic starch) or in the amyloplast of non-photosynthetic storage organs (storage starch) (Müller-Röber and Kossmann, 1994; Preiss, 1991; Preiss and Sivak, 1996). Starch is only found in photosynthetic eukaryotes or their nonphotosynthetic derivatives (such as apicomplexa parasites or nonphotosynthetic dinoflagellates) (Ball and Morell, 2003). Most living cells contain intracellular storage α-glucans that are deposited either as soluble or insoluble polymers. The most common storage α-glucans are glycogen and starch. Glycogen, which is a polymer formed by α-1,4-linked D-glucose residues with numerous α-1-6-glucosidic branch points. Glycogen is usually found in the form of amorphous granules in the cell cytoplasm of most bacteria, yeast , fungi and animal cells (Ball and Morell, 2003). Starch is an extremely valuable polymer both nutritionally and as an industrial raw material. It is stored in photosynthetically active leaf chloroplasts as transient starch and in seeds tubers of economically important crops such as maize (*Zea mays*), rice (*Oryza sativa*), and potatoes (*Solanum tuberosum*) as storage starch.

An excellent example of the power of unicellular algae is the use of *Chlamydomonas reinhardtii* to understand starch metabolism, which resulted in the discovery of new functions even in enzymes that were well characterized (Delrue *et al.*, 1992). *C. reinhardtii* produces starch granules that are similar to those in other plants morphologically as well as composition and fine structure (Buléon *et al.*, 1997).

### 2.3.1 The structure of starch

Starch is found as semi-crystalline granules in the chloroplasts of green algae and higher plants. These starch granules are anhydrous structures that are formed by a mixture of an essentially unbranched α-1,4-linked D-glucose polymer (amylose) and a larger polymer (amylopectin) with the same basic structure and more α-1,6 branch points (Buléon *et al.*, 1997).

Amylose is usually branched at a lower level (approximately one branch per 1000 residues) by α(1-6) linkages and makes up ~30% of starch (Martin and Smith, 1995). This proportion, however, may vary considerably with in different plant species (a range of 11 to 35% was found in a survey of 51 species) (Detherage *et al.*, 1995) and also the type of plant organ, the developmental age of that organ and the growth conditions of the plant (Shannon and Garwood, 1984).

Amylopectin, which consists of highly branched glucan chains, makes up ~70% of starch and is predominantly high-molecular weight polymer (Martin and Smith, 1995). Chains of roughly 20 α(1-4)-linked glucose residues are joined by α(1-6) linkages to other branches. An average amylopectin molecule is 200 to 400 nm long(20 to 40 clusters) and ~15 nm in width (Kainuma, 1988) .

### 2.3.2 The starch granule

Within the starch granules, which vary from <1 μm to >100 μm in size, the amylopectin molecules are arranged radially. The adjacent branches within the branch clusters may form double helices that can be packed regularly, and gives a crystalline which is determined in part by the branch lengths in amylopectin. Closely packed, highly crystalline structures are characteristics of wheat and barley

endosperms which are associated with the average branch lengths of about 20 residues. More open structures which contain more water are found in potato tuber and are associated with longer branch lengths of about 22 glucan residues (Martin and Smith, 1995). The degree of branching and consequently the crystallinity of starch granules may vary considerably, even between different organs of the same plant. However, the starch granule is not uniformly crystalline and contains relatively amorphous regions. Amylose molecules form single helical structures and are thought to be packed into these amorphous regions, which are present throughout the granule (Martin and Smith, 1995).

Granules from storage organs and leafs have rather different macrostructures. Starch granules from storage organs show internal semicrystalline growth rings that are differentially sensitive to chemical and enzymatic attack (Martin and Smith, 1995). The denser, more resistant layer may be regions of closer packing of branches with in clusters of parallel amylopectin molecules. The formation of these rings may be the result of periodic differences in the rate of starch synthesis. For example, in wheat endosperm, the growth rings follow clear diurnal patterns and are lost in plants grown under continuous illumination (Buttrose, 1962). However, growth rings are not universally responsive to illumination and may result from other periodic fluctuations in biosynthesis.

Starch granules in leaves are generally smaller than those in storage organs and have a distinct macrostructure. They are thought to have a crystalline core with an amorphous outer mantle that consists of less highly branched glucan polymers (Steup *et al.*, 1983). Most of the turnover in starch during day/night cycles involves the amorphous mantle of the granules (Steup *et al.*, 1983).