Screening and selection of a cyanobacteria for production of poly-β-hydroxybutyrate in a closed photobioreactor

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Abstract

Polyhydroxybutyrate (PHB) is a melt-processable, semi-crystalline thermoplastics made by biological fermentation. A key feature of this plastic is its biodegradability.

PHB is currently produced by bacterial fermentation, and is constrained by its high production costs compared to the more conventional petroleum derived polymers with comparable properties. PHB is also produced within certain species of microalgae. These photosynthetic microoganisms use CO_2 as their sole carbon source and so offer a potentially cheaper method for producing PHB, as well as sequestering what would otherwise be a contributor to global warming. For this process to be successful it is necessary to find a species of cyanobacteria that has a high occurrence of PHB within the cells and is also suitable for commercial production. In this research, selection criteria were developed for the screening of microalgae for PHB accumulation and suitability for culture in a novel closed photobioreactor (CPBR), developed by CSIRO using a "top-down" approach. The selection criteria were developed, through a series of preliminary experiments, and economic and environmental considerations. Preliminary experiments were conducted in the CPBR using *Synechoccous* PCC7002, a species of cyanobacteria thought to produce PHB, to identify any system specific selection criteria. The experiments were conducted at several different light and temperature boundary conditions of the CPBR to determine the characteristics of the microalgae. From these experiments it was found that for the alga to be successfully cultivated in the CPBR it must be able to withstand bubbling aeration and not form microbial mats. From the economic considerations it was determined that high productivity, high final cell density, and high PHB content are desirable. Looking at the environmental considerations, it is also necessary that a native species of microalgae is used.

Following a literature review many species of microalgae that produce PHB were found however there was a paucity of literature concerning the potential of Australian species to produce PHB. The following six species of microalgae were identified as possible PHB producers, as they were similar to species already known to prduce PHB: *Anabaena flos-aquae, Anabaena solitaria, Nodularia spumigenia, Pseudanabaena, Microcystis aeruginosa,* and *Microcystis flos-aquae*. These species were tested against the selection criteria to choose a species for growth in the CPBR. Initial gross screening was conduced in aerated flasks to identify if the species were able to produce PHB and met the system specific selection criteria; rigorous screening was then conducted on the remaining species that passed the initial gross screening. Rigorous screening was conducted in flasks under different nutrient conditions to determine which species had the highest productivity, cell density, and PHB content. From these experiments a final candidate was selected, *Anabaena solitaria*. The methodology was then validated by cultivating the selected species in the CPBR; this was successful with *Anabaena* solitaria accumulating 8.5 mg L^{-1} PHB under the balanced conditions.

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Michael Roberts and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Chapter 1

1 Introduction

Each year large quantities of plastic are sent to landfill, with plastic bags constituting a significante portion (van Wegen 2000). In 2008, several governments in Australia recognised the impact plastic bags were having on the environment and passed legislation to minimise plastic bags, effective from 2009. Some states elected to place a price on all plastic carry bags, however South Australia decided on a complete ban (Vaughan 2008). The average life span of a polypropylene (PP) plastic bag is 10,000 years in landfill. Poly-β-hydroxybutyrate (PHB) is completely biodegradable and can be broken down in as little time as a few years (Anderson *et al.* 1995), because many common microorganisms in soil can metabolise PHB as their sole food source (Brandl *et al.* 1995). Microalgae, particularly cyanobacteria are native producers of PHB, and potentially provide a viable alternative to plastic bags from traditional petroleum derived polymers.

1.1 Microalgae

The term microalgae covers a large range of phytoplankton that exists as either prokaryotic or eukaryotic microorganisms. Within the prokaryota kingdom there are Cyanobacteria, in the eukaryota kingdom exist the Cryptophyta, Pyrrhophyta, Raphidoohyta, Chrysophyta, Euglrnophyta, and Chlorophyta. These organisms have adapted and evolved to suit almost any aquatic environment, from the freezing waters at the poles, to the volcanic vents and everywhere in between. They are the primary food source of many aquatic animals and as such form the base of the marine food chain. Microalgae are the primary producers of oxygen on the planet, generating 40–50% of the world's atmospheric oxygen (Anderson 2005). The ability to photosynthetically reduce CO_2 is one of the reasons that microalgae are the topic of a steady scientific interest. Many see the potential of a cheap substrate and the ability to sequester CO_2 as well as produce useful products, worthy of investigation. There are as many as 30,000 known species of microalgae and more are being discovered and categorised, however only a few hundred are actively being investigated and very few are produced industrially (Chaumont 1993).

Microalgae require a nutrient media, light, and a carbon source (CO_2). The key growth parameters for microalgal cell growth are light intensity, CO_2/O_2 ratio, pH, temperature, and nutrients.

1.1.1 The Role of Light

Light intensity is by far the most important growth parameter for photosynthetic organisms and is often the limiting factor in microalgal growth (Pulz 2001). Light is important to the growth of microalgae as, like for higher plants, it provides energy to the cell via photosynthesis. Once the nutritional requirements and temperature are optimised, the algae will be limited only by the light available. An understanding of photosynthesis is necessary to understand the importance of light to photosynthetic cultures (Richmond 1999).

Photosynthesis occurs in the chloroplasts of higher plants and algae, and the thylakoid membranes of cyanobacteria. The methods by which this occur are almost identical for all photosynthetic organisms, and so the discussions of the following sections are relevant to both eukaryotic (plants, algal) and prokaryotic (cyanobacterial) photosynthetic pathways (Steinback *et al.* 1985). Photosynthesis is made up of two sets of reactions: light reactions and dark reactions.

Light reactions of photosynthesis are so called because they require photons to carry out the chemical reactions. The first step of the light reactions is to capture and redirect photons towards a 'photochemical reaction centre', where the energy may be harnessed by the cell.

The photochemical reaction centre consists of two photosystems: photosystem I (PS-I) and photosystem II (PS-II). A series of processes occur from PS-I to PS-II in order to achieve the reduction of water (to oxygen) and the production of ATP and NADPH in the correct ratio for carbohydrate synthesis in the dark reactions. One process involves the transfer of electrons extracted during the reduction of water through a series of transfer proteins, by excitation from photon adsorption at the photosystems, as illustrated in Figure 1.1.

NOTE:
This figure is included on page 3 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.1: Non-cyclic photophosphorylation during photosynthesis (Tobin *et al.* 1997)

This process is able to utilise the energy of excited electrons to drive non-cyclic photophosphorylation (ATP production) and reduction (NADPH production), and utilises both PS-I and PS-II. However, this process alone does not generate sufficient ATP for carbohydrate synthesis, and so a second process, occurring only in PS-I is required to make up the shortfall. In this reaction (depicted in Figure 1.1), light absorbed by PS-I is used to excite an electron, which establishes a proton gradient to drive ATP synthesis upon return to the ground state. These reactions together constitute the method by which photosynthesis captures energy from light and reduces water to yield oxygen.

The next step in photosynthesis is the dark reaction, or Calvin-Benson cycle, where ATP and NADPH generated during the light reactions are used to reduce CO_2 into carbohydrate, Figure 1.2. The dark reaction is so named because it does not require photons, it may occur in both the presence and absence of light (Blankenship 2002). The Calvin-Benson cycle consists of a series of enzymes that, using energy from ATP and reducing power from NADPH, carry out the reduction of CO_2 to carbohydrate.

Too much light causes damage to the photosystems thereby reducing the effectiveness of the cells, this is called photoinhibition. Photoinhibition occurs when the PS-II mechanism becomes oversaturated, causing damage to the mechanism, inhibiting electron flow from water through

the reaction centre, and as such reduces the ability of the cells to photosynthesise (Jensen *et al.* 1993).

Figure 1.2: Cyclic PS-I pathway for ATP generation (Tobin *et al.* 1997)

If algae are exposed to high quantities of light over a prolonged period an excessive flow of electrons from the PS-II to the PS-I may result. This in turn can cause an electron jam that may lead to the generation of triplet chlorophyll and oxygen radicals, which can damage the PS-II, and other cellular components. The phenomenon is called photo-oxidation, and is observed as chlorophyll bleaching and a loss of floatation properties (Jensen *et al.* 1993).

Too little light equates to not enough energy reaching the cell, under this condition the organism is said to be photo-limited, limiting cellular growth (Jensen *et al.* 1993).

1.1.2 The Effect of Temperature

Culture temperature is very important, it is well known that changing the temperature of a bacterial culture by a single degree can double the growth rate of bacteria, the same can also be true for microalgae. Like bacteria the temperature range for microalgae can be expansive, from cryophilic (below 15°C) to mesophilic (moderate range, i.e. 20–40°C) and thermophilic (heat loving, above 40°C). All species currently grown industrially are mesophilic. The growth of microalgae is very dependant on temperature, the growth rate tends to increase exponentially

with increasing temperature until the optimal temperature is reached. Exceeding the optimal temperature by as little as 2–4°C can lead to a pronounced decrease in performance, furthermore at temperatures 15–20°C below the optimal temperature growth of microalgae may cease (Richmond 1999).

The control and optimisation of temperature is very important for algal growth, because temperature is also a significant factor in many other growth properties. Increased temperature decreases the solubility of both CO_2 and O_2. Dissolved oxygen is inhibitory to microalgae growth and so a high temperature can be desirable to assist in removing oxygen from the system, however a delicate balance is needed as otherwise the decrease in dissolved CO_2 may result in the algae being carbon limited. Jensen and Knutsen (1993) have shown that low temperatures and high light intensity can result in an increase in the amount of radicals formed and can result in photo-oxidation. Additionally, it was found that higher temperatures could reduce photoinhibition, because the destruction of the photosystems, particularly the PS-II components, are less temperature sensitive than the regeneration processes (Jensen *et al.* 1993).

1.1.3 Nutrients

Besides carbon, which accounts for 46–49% of cell composition, microalgae require several other macro nutrients (Reynolds 1984) that assist in cell growth and function. The main nutrients other than carbon that are required by the cell are nitrogen, usually in the form of nitrates (8–11% cell composition) and phosphorous, typically as phosphates (0.7–1.1% cell composition). Other nutrients may include sources of organic carbon, and include several micronutrients such as trace metals.

Microalgae can be grown in several modes, these are photo-autotrophically, mixotrophically, and finally heterotrophically. Photo-autotrophs use dissolved CO_2 or one of its hydrated forms for cell growth, the energy for their metabolism coming only from light (Richmond 1999). Heterotrophs gain all of their energy needs and carbon from organic sources. Mixotrophs exist between the two, being able to use inorganic carbon in the presence of light and organic carbon in the absence of light. Mixotrophs have an obvious advantage over photo-autotrophs and heterotrophs in that they are able to utilise both CO_2 and organic carbon sources meaning they are able to grow continuously (Richmond 1999).

Carbon as CO_2 is the most important nutrient for algal growth, CO_2 is necessary in photosynthesis, providing carbon to algae. As stated above, carbon can contribute up to 50% of the cellular mass, this means that approximately 1.8 kg of CO_2 is needed to produce 1 kg of algae (Becker 1994). For most algae CO_2 is absorbed in one of its hydrated forms, CO_2 can appear in water in a number of forms as bicarbonate, hydrogen carbonate ions, and carbonate ions (which state it is depends on the pH and temperature of the water). The equilibrium between CO_2 – H_2CO_3 – HCO_3^- – CO_3^{2-} is also an important buffer in water systems and for algal growth (Grobbelaar 2004). As algae grow they deplete the CO_2 from their surrounds and excrete OH^- resulting in an elevated pH, within high densities of algae it is not unusual to find pH as high as 11 (Grobbelaar 2004). In mass cultures the pH needs to be controlled by the addition of CO_2 in an optimum range to prevent carbon limitation (Becker 1994), however excessive CO_2 can be harmful to the algae.

The CO_2/O_2 balance is important for all microalgal culture. If the CO_2 concentration is high the culture may become too acidic, which may inhibit enzymes or deactivate proteins needed by the cells, and so can cause the cells to stop growing (Pulz 2001). Conversely O_2 concentration needs to be kept low as high concentrations can decrease photosynthesis rates and lead to photorespiration, furthermore an increased concentration of oxygen can contribute to photo-oxidation. One of the ways of maintaining the CO_2/O_2 balance is by stripping the oxygen with air enriched with CO_2, and making the culture more turbulent, another way is by pulsing CO_2 into the system to maintain pH (Jensen *et al.* 1993; Pulz 2001).

After carbon, nitrogen is the next most important nutrient for algal production, typically algae use nitrates and phosphates, some are capable of utilizing urea or ammonia as their nitrogen source (Becker 1994). Some cyanobacteria are capable of fixing nitrogen directly from the atmosphere (Grobbelaar 2004). Nitrate concentration is closely related to protein and carbohydrate production. When algal cells are nitrogen limited, the phycobilisomes degrade and carbon fixed during photosynthesis is switched from protein synthesis to carbohydrate production. Under nitrogen limitation photosynthesis continues at a reduced rate until nitrogen concentration drops below a threshold level, and then cell division ceases (Becker 1994; Hu 2004).

If nitrogen limitation occurs over a period of time lipids accumulate within the cell, as the enzymes needed for lipid manufacture are less susceptible to degradation than the enzymes needed for carbohydrate synthesis. This leads to most of the carbon being bound in lipids; this is very useful as most useful intracellular products from microalgae are lipids (Becker 1994). However, while some of the useful intracellular lipids are accumulated whilst nitrogen limited the overall productivity of the cell is reduced.

Phosphorous is a major macronutrient, important in the creation of many structural and functional molecules within the cell, for example the synthesis of nucleic acid (Becker 1994; Hu 2004). Within their natural milieu phosphorous is often a limiting nutrient, typically existing as orthophosphate and in organic compounds (Becker 1994). Phosphorous is often limiting as it binds easily to other ions (CO_2^{2-} and Fe^{2+}), this renders the phosphorous unavailable to the cell (Grobbelaar 2004).

Phosphate limitation causes similar effects to nitrogen deficiency, carbohydrate tends to increase and chlorophyll *a* decreases. However, unlike nitrogen deficiency, phycobilisome degradation is reduced (Hu 2004). Some intracellular compounds have been found to accumulate under phosphorous depleted conditions, primarily carbohydrates and lipids.

1.1.4 Common Culturing Conditions

Microalgae exist and thrive in a vast range of natural environments, and consequently have adapted to a vast range of growth conditions. As previously stated they can be cryophilic, mesophilic, and thermophilic, however in all of these conditions microalgae require CO_2 and light to grow photo-autotrophically. It is very difficult to pinpoint the optimal conditions for microalgae as interaction between the growth parameters is very high, as an example temperature affects CO_2 solubility and cellular metabolism. Determining the optimum growth conditions of microalgae are further complicated because the system in which they are grown may also have an effect on their growth, for example local shear rates may be detrimental to algal growth. Optimal conditions vary between genus of the same species. However most microalgae are mesophilic and a general range of operating conditions can be determined.

For most microalgae the optimum growth conditions are between 25–40^{o}C, a pH of 7–9 (Aiba 1982), the optimum incident light intensity varies widely between species and can be anywhere

between 20–1800 μmol m^{-2} s^{-1}. These provide a general set of boundary conditions for the growth of microalgae however these conditions interact and are species and system dependant. Because of this it is necessary to explore these ranges for any new system.

1.2 Products from Microalgae

Many products have been discovered from microalgae but the few that are cultured industrially consist of *Chlorella* (health food), *Spirulina* (health food), *Dunaliella* (β-carotene) and *Haematococcus* (astaxin) and a few others for the aquaculture industry as feed for fish. An indication of how rapidly demand for cultivated microalgae is growing is the production of *Spirulina* (Borowitzka 1999) shown in Figure 1.3.

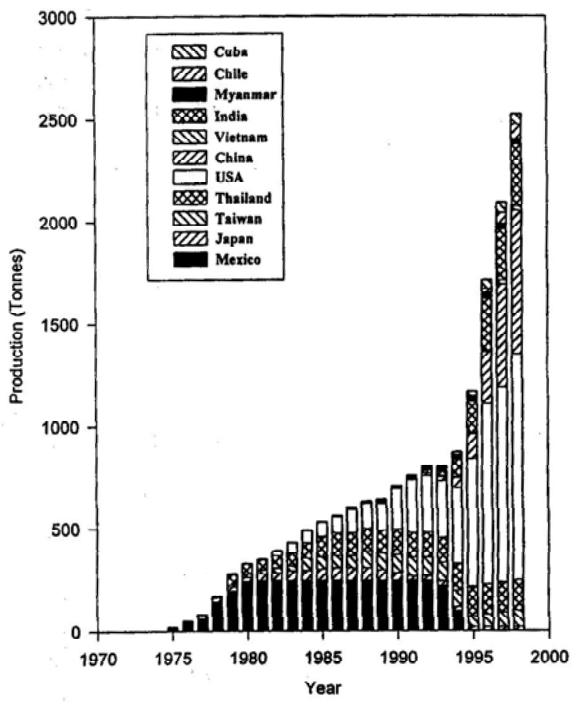

Figure 1.3: Production growth of *Spirulina* over the past 25 years, by country

Microalgae are also a known source of fatty acids (Reynolds 1984), which has evoked the interest of producing renewable fuels such as biodiesel from them. It is also well known that many cyanobacteria, given the right conditions, are capable of producing hydrogen as another renewable energy source. This has created great scientific interest as hydrogen is a clean

burning fuel, many studies have been conducted into biological hydrogen production (Howarth *et al.* 1985; Markov *et al.* 1995; Nakada *et al.* 1995; Benemann 1997; Eroglu *et al.* 1999; Miyake *et al.* 1999; Benemann 2000; Akkerman *et al.* 2002; Hallenbeck *et al.* 2002; Lopes Pinto *et al.* 2002; Melis 2002).

While not a product, the potential of microalgae to sequester atmospheric CO_2 has been the subject of great interest over the past few decades, with many studies looking at using microalgae solely as a CO_2 sink. There have been two distinct approaches to this problem using microalgae, the first is designing systems and selecting microalgae for the sole purpose of sequestering CO_2, either atmospheric or directly from the production source (Pirt *et al.* 1983; Suh *et al.* 1998; Sobczck *et al.* 2000; Stewart *et al.* 2005). The second is using the CO_2 to produce other products from microalgae such as *Chorella* (Hirata *et al.* 1996), glutamate (Matsunaga *et al.* 1991), or a biodegradable plastic, polyhydroxybutyrate (Ishizaki *et al.* 1991; Asada *et al.* 1999). While many other methods for sequestering CO_2 exist, such as geological sequestration and oceanic disposal (Adams *et al.* 1995; Hendriks *et al.* 1995), only microalgal sequestering completely removes the CO_2, storing it as biomass, without requiring the transport of CO_2 to another location as is the case for geological sequestration. Furthermore, microalgal sequestration offers near zero environmental impact (Stewart *et al.* 2005), as opposed to oceanic disposal, which has the potential to acidify oceans that would damage oceanic ecosystems. The potential for microalgae to sequester CO_2 and produce useful products is economically attractive because without these products the biomass must be produced a significantly lower cost.

A group of products from microalgae that are under investigation are biopolymers. Cyanobacteria are native producers of polyhydroxyalkanoates (PHA). PHAs are polyesters of various hydroxyalkanoates that are produced by many gram-positive and gram-negative bacteria. These polymers are accumulated intracellularly to levels as high as ninety percent of the cell dry weight under conditions of nutrient stress and act as a carbon source and energy reserve, PHAs have a sufficient high molecular mass (in the range of 50,000 to 1,000,000 Da (Reddy *et al.* 2003)) to have polymer characteristics that mimic conventional polymers such as polypropylene (Madison *et al.* 1999).

1.3 Poly-hydroxybutyrate

Polyhydroxybutyrate, PHB, belongs to the polyhydroxyalkanoate class of biodegradable plastics (Patwardhan *et al*. 2004). These classes of polymers are polyesters of various hydroxyalkanoate monomer units and can be synthesized by numerous kinds of microorganisms, such as bacterial strains *(Ralstonia Eutropha),* genetically modified bacteria strains for improved PHB yields *(Recombinant Escherichia Coli),* many cyanobacterial species, and the genetically engineered cyanobacterium (*i.e. Synechococcus PCC7942*) – a transformant with PHB synthesizing genes from *Ralstonia Eutropha*.

PHB is the most often occurrence of PHA and the general monomer unit and molecular structure of PHB is as shown in the Figure 1.4.

NOTE:
This figure is included on page 10 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.4: General monomer unit and molecular structure of PHB (van Wegen 2000)

The properties of PHB make it attractive as a future material, mostly due to its biodegradability and hydrophobicity. These properties, coupled with the ability to use non-petroleum derived feedstock make PHB a sustainable alternative to conventional plastics. PHB has an advantage over other biodegradable polymers in that it is fully biodegradable; soil microbes consume PHB completely breaking it down into CO_2 and water (Brandl *et al.* 1995). Other 'degradable' polymers rely on degradable cross linkages within the polymer structure; these cross linkages

are either photosensitive or biodegradable. If the cross linkages are photosensitive then the plastic needs to be exposed to light to degrade, as most plastics end up buried in landfill these do not degrade. Nevertheless as the cross linkages degrade the plastic is broken up into fragments that still will take thousands of years to disappear and in the ensuing time may still cause serious damage to wild life and the environment.

Biological PHB is a fully biocompatible bio-polymer that can be thermoformed to a vast range of products such as fibers, films and bottles in an analogous manner to that of the conventional petrochemical plastics. Table 1.1 shows some of the possible applications of PHB and other PHAs (Vincenzini *et al*. 1999).

Table 1.1: Uses of PHA's [adapted from Vincenzini, M. and R. De Philippis (1999)]

Application field	Uses	Properties useful for specific uses
Agriculture	Controlled release of pesticides, plant growth regulators, hericides, fertilizers, covering foils, seed encapsulation	Biodegradability, retarding properties
Chiral chromatography	Stationary phase for columns	Chiral properties
Chiral Synthesis	Sources of chiral precursors	Stereoregularity, chiral properties
Disposables	Razors, trays for food, utensils, ect.	Biodegradability, good mechanical properties
Hygiene Products	Diapers, feminine hygiene products	Biodegradability, moisture resistance, good water barrier
Medical	Absorbable sutures, surgical pins, staples, bone plates, films around bone fracture	Biocompatibility, biodegradability, piezoelectric properties
Miscellaneous	Autoseparative air filters, fibre-reinforced, biodegradable goods	Biodegradability, good mechanical properties
Packaging	Bottles, films for food packaging, paper coating	Biodegradability, moisture resistance, good water barrier, good mechanical properties, low O_2 permeability
Pharmaceutical	Retarded drug release, drug carrier	Biodegradability, moisture resistance, retarding properties

PHB in its pure state is completely crystalline and as a result it possesses a high level of strength. However pure PHB has a poor level of toughness and exhibits severe aging over several days (van Wegen 2000). This aging or brittle behaviour is due to the formation of many small cracks that join under stress and cause premature failure. This problem can be overcome by the implementation of annealing procedures (van Wegen 2000). The problem can also be tackled by blending the pure PHB with further addition of valerate units into the polymer. However, these issues are beyond the scope of this research.

PHB is a prime candidate for PP replacement because it has many of the same physical properties Table 1.2 (de Koning 1995; Poirier *et al.* 1995). The introduction of biodegradable plastics will reduce the volume of plastics in landfill.

Table 1.2: Physical properties of PHB, Values from Poirier *et al* are shown in brackets

	PHB	**Polypropylene**
Crystallinity mass fraction	0.6 (0.8)	0.65
Tg	-5°C – 5°C (15°C)	-15°C
Tm	175°C	174°C
Tensile modulus	3.5 GPa	
Tensile strength	40 MPa	(38 MPa)
Percent elongation to break	2-5% (30% with annealing at 150^0C)	(400%)

PHB was first detected among the family of PHAs by Lemoigne in 1926 as an element in the bacterium of *Bacillus Pseudomonas* (Patwardhan *et al.* 2004). However, not much research had been done with cyanobacteria despite their capability to accumulate PHB intracellularly, as demonstrated by Carr (1966). Many species of cyanobacteria that produce PHB have been documented (Stal 1992; Miyake *et al.* 1996; Vincenzini *et al.* 1999; Wu *et al.* 2001), Table 1.3. they may have been pure cultures and importantly for some species microscopic techniques were used to determine the presence of PHB and may have only identified inclusion bodies.

PHB accumulates as distinct inclusions body in the cells and is believed to be a carbon storage compound for the cell, so in times of high growth and/or low carbon supply it can be used for energy and growth (Stal 1992).

Other than the accumulation of reserve PHB compounds intracellularly, it has been reported that there is another accompanying accumulation product, glycogen (Wu *et al.* 2001). This glycogen product must therefore have certain relationships that affect the expression of cyanobacterial PHB. Cyanobacteria synthesize this glycogen product as the major carbohydrate reserve, resulting in direct competition to PHB (Wu *et al.* 2001). Because of this it is likely that it is possible to increase the accumulation of PHB in cyanobacteria by manipulating the pathway of cyanobacterial glycogen. (Carr 1966) showed that the PHB accumulation in cyanobacteria was strongly induced by the presence of acetate.

Table 1.3: Species of microalgae known to produce PHB (Stal 1992; Miyake *et al.* 1996; Vincenzini *et al.* 1999; Wu *et al.* 2001)

Alcaligenes eutrophus	*Gloeobacter violaceus ATCC 29082*	*Oscillatoria sp*
Anabaenopsis siamensis	*Gloeocapsa ATCC 27928*	*Phormidium sp.*
Anabanea cylindrica	*Gloeocapsa PCC 6501*	*Phoromidium borianum*
Anabanea cylindrica	*Gloeocapsa PCC 73106*	*Schizothrix calcicola*
Anabanea cylindrica 10 C	*Gloeocapsa sp. PCC7428*	*Scytonema sp.*
Anabanea cylindrica ATCC 27899	*Gloeothece PCC 6909*	*Spirulina jenneri*
Anabanea doliolum HN-085	*Gloeotrichia raciborskii HN-03*	*Spirulina jenneri NK1*
Anabanea hallensis HN-15	*Gloethece PCC6909*	*Spirulina laxissima*
Anabanea sp.	*Gloethece sp*	*Spirulina laxissima MG5*
Anabanea torulosa SAG B26.79	*Gloethece sp PCC6501*	*Spirulina maxima*
Anabanea variabilis	*Lyngbya aestuarii*	*Spirulina platensis*
Anacystis cyanea	*Mastigocladus laminosus*	*Spirulina subsalsa*
Aphanizomenom gracile SAG B31.79	*Microcoleus chthonoplastes*	*Spirulina subsalsa 85*
Aphanocapsa PCC6308	*Microcoleus chtonoplastes*	*Staieria sp PCC7437*
Aphanocapsa PCC6714	*Microcoleus sp*	*Synechoccus sp*
Aphanocapsa PCC6806	*Microcystis aeruginosa*	*Synechococcus MA19*
Aphanocapsa sp	*Microcystis aeruginosa*	*Synechococcus PCC 6301*
Calothrix thermalis SAG B37.79	*Nodularia haveyana SAG B50.79*	*Synechococcus PCC 6307*
Chlorogloea fritschii	*Nostoc commune HN-120*	*Synechococcus PCC 7002*
Chroococcus PCC 7946	*Nostoc commune SAG B1453-5*	*Synechococcus PCC 7942*
Crinalium epipsammum SAG B22.89	*Nostoc linckia*	*Synechocyitis PCC 6803*
Cyanospira rippkae ATCC 43194	*Nostoc muscorum*	*Synechocysitis PCC 6308*
Cyanothece PCC 7424	*Nostoc sp.*	*Synechocystis sp*
Cyanothece PCC7424	*Nostoc spp*	*Synechocystis sp PCC6803*
Cyanothece PCC8303	*Oscillatoria limnetica*	*Tolypothrix tenuis*
Fischerella SAG B.46.79	*Oscillatoria limosa*	*Trichodesmium thiebautii*
Gloeobacter PCC 7421	*Oscillatoria limosa 23*	

Cyanobacteria will generally produce PHB to around 5–6 wt% of the dry cell weight (DCW). The PHB accumulation in the cells of each of the listed cyanobacteria, Table 1.3, varied from trace amounts in the *Nostoc spp* species up to 20% of the cellular dry weight of *Synechocystis sp* under appropriate growth conditions (Miyake *et al.* 1996). Much work has been done on maximising PHB production; it has been found that PHB is expressed under nitrogen limitation and carbon surplus (Anderson *et al.* 1995). This is because PHA synthase is increased in nitrogen-starved conditions (Asada *et al.* 1999).

The biosynthesis and degradation route for the occurrence of PHB in *Alcaligens Eutrophus* can be represented by a cyclic process route as shown in the Figure 1.5 below.

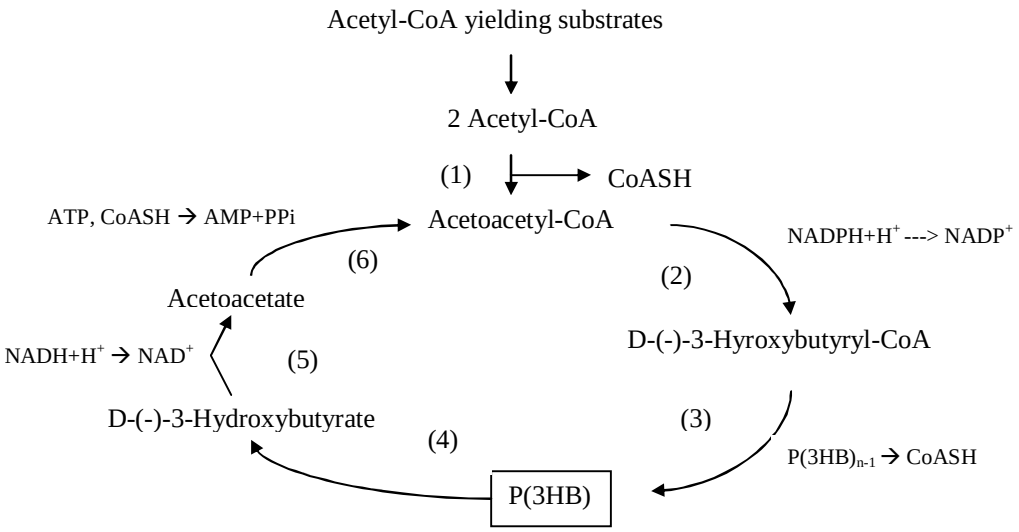

Figure 1.5: P(3HB) metabolism as it occurs in *Alcaligens eutrophus*. (Vincenzini *et al.* 1999)

From Figure 1.5, the route for the formation of PHB starts from the acetyl-CoA molecules and proceeds via three distinct sequential, enzyme mediated reactions as follows (Vincenzini *et al.* 1999);

1. A condensation of two acetyl-CoA molecules to yield acetoacetyl-CoA by the action of the enzyme of the 3-ketothiolase.
2. A reduction of acetoacetyl-CoA to D (-)-3-hydroxybutyryl-CoA catalyzed by an NADPH-dependent reductase.
3. A PHA synthase-catalysed polymerization of the 3-hydroxybutyrate monomer units.

The first enzyme in this sequence, 3-ketothiolase, is a significant controlling parameter in the expression of PHB in microbial cells. It controls the synthesis of PHB by utilizing free CoA molecules as key regulatory molecules to inhibit/promote the accumulation PHB in the cells. The route for the PHB as shown in Figure 1.5 may vary from one microbial species to another; minor modifications to this biosynthesis pathway are necessary for different species. An example of this is *Rhodospirillum rubrum*, it requires the additional action of two enoyl-CoA

hyratases to form D(-)-3-hydroxybutyryl-CoA from acetoacetyl-CoA without changing the basic biosynthesis principles (Vincenzini *et al.* 1999).

As for cyanobacteria, the biosynthesis pathway for different genera or species of cyanobacteria may differ and further modifications on the pathway will be needed. Only a hypothesized regulation in the PHB mechanism for cyanobacteria of the species *Synechococcus sp. MA19* was found (shown in Figure 1.6).

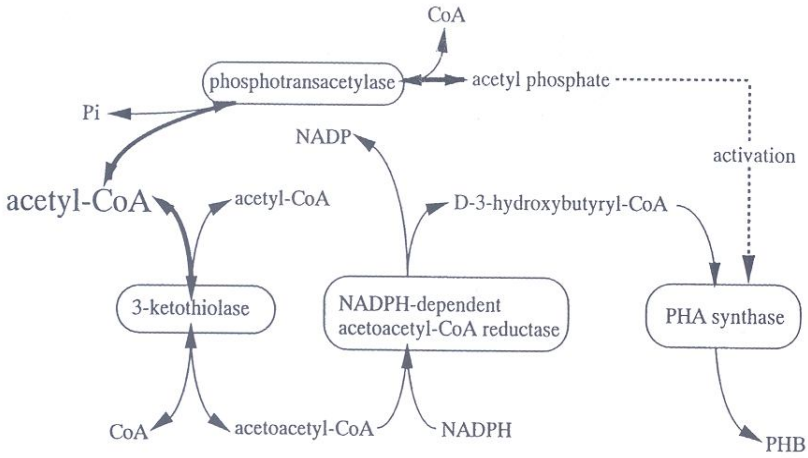

Figure 1.6: Hypothesized regulation in PHB metabolism by Synechococcus sp. MA19 (Asada *et al.* 1999)

Studies conducted into the economics of PHB production from bacteria have shown that in some cases the economics are comparable to traditional petroleum-based polymers. Poirier *et al* (1995) showed the cost of PHB from bacteria to be approximately 15 times the cost of traditional products. Studies done by van Wegen (2000) contrasted with the findings of Poirier *et al* (1995) showing the cost for PHB to be similar to traditional petroleum-derived polymers, this was mainly due to the cost effectiveness of using waste water products for the growth media. Nevertheless these studies compare the economics of bacterial PHB and not PHB derived from cyanobacteria. PHB from cyanobacteria have the potential to reduce costs substantially because of the less expensive or complex substrates required for their growth, as well as the potential to sequester harmful CO_2. Because of the ability of these microalgae to produce PHB while photosynthetically reducing CO_2 they are highly important for our future. It is worth noting there is a paucity of completed research on PHB from cyanobacteria, given

that as photosynthetic microorganisms they could lead to the inexpensive production of PHB from CO_2 and solar energy.

Effective harnessing of the potential of photosynthetic microorganisms for the production of PHB or other products requires the careful selection of species of microalgae and pairing it with an appropriate method of mass production, as levels found naturally would be insufficient for commercial production. The potential of microalgae as commercial sources of PHB has not yet been fully explored, and while many cyanobacteria have been identified that produce PHB no mass culture has been attempted. Systems for the mass culture of microalgae are called photobioreactors (PBR).

1.4 Methods for producing microalgae and cyanobacteria

Cyanobacteria and other microalgae are typically cultivated in photobioreactors (PBR), which can be divided into two main categories: open and closed systems. Many reviews have been published on the benefits or otherwise of each of these systems (Chaumont 1993; Borowitzka 1999; Lee 2001; Pulz 2001), however the central argument has remained the same. That is, open PBR are cheaper to build and operate but have poorer performance, and closed systems allow greater control and more species to be cultivated but are more costly to operate.

Open systems are, as the name suggests, open to external un-controlled influences, whereas closed systems are isolated from these external influences. Open systems include raceway channels and ponds, and are the current preferred method of producing microalgae. Open systems require a species of cyanobacteria or microalga that is robust and grows under selective pressures such as high salinity or high/low pH, these requirements exist so that a monoculture can be maintained and this excludes many species from being cultured.

Closed PBR can be further divided into separate categories, air lift, bubble column, internal illumination, external illumination, tubular, and flat plate; the literature contains abundant examples of these (Pirt *et al.* 1983; Lee *et al.* 1990; Javanmardian *et al.* 1991; Matsunaga *et al.* 1991; Iain *et al.* 1992; Ratchford *et al.* 1992; Hu *et al.* 1996; Fernandez *et al.* 1998; Camacho *et al.* 1999; Miron *et al.* 2000; Suh *et al.* 2003). Also included in this list are the bag reactors favoured by the aquaculture industry. The aquaculture industry use closed systems to cultivate

microalgae as feed for abalone and fish, and the main reason for bag reactors is the species used are unsuited to mass culture in an open system and the industry requires a better quality product that is free of contamination. However, these systems are very inefficient. Closed systems allow milder culture conditions and so a wider variety of species can be cultured.

The purpose of a closed PBR is to achieve better control of the growth parameters, given that light is most often the limiting growth requirement (these systems must allow light to permeate the entire culture). To achieve this all these systems have a high illuminated surface to volume ratio. Tubular designs are favoured because of the high surface to volume ratio, however the only possible means to scale up these reactors is to increase the length of the PBR as increasing the diameter means that not all the cells would receive sufficient light. This has the possibility of leading to inhibitory build up of oxygen in the system after a certain length. On the other hand flat plate systems maintain the width of the vessel and so are scalable in two dimensions.

As identified by Pulz (2001) the most important parameters to control are temperature and pH. pH is relatively simple to control by adjusting the CO_2/air ratio, or pulsing the CO_2 into the reactor. Temperature control for the growth of microalgae is very important, as reviewed previously, temperature control of PBR is very primitive and not appropriate in all conditions. In open systems it is not possible to control the temperature, however in closed systems it seems not to have been included in the design stage. Typical temperature control of PBR can be sorted into three types: environment control, evaporation, and immersion. Environment control is used in labs and in some cases for growing algae in bags, this method relies on controlling the environment in which the PBR is contained This method is usually manifested as a temperature controlled room or any other indoor environment that has smaller extremes than the outdoor environment but this method is potentially very costly. Evaporation is a typical method for closed outdoor systems, the PBR is sprayed with water and the evaporation cools the PBR, an example of this is the Biocoil™ developed by Borowitzka (Borowitzka 1999). This method is most effective in arid areas, however it is very wasteful of water and inefficient. The immersion method relies on submerging the PBR in temperature controlled water bath, which still allows light to be transmitted. Immersion is commonly used on flat plate and on some serpentine[1] PBR, it is efficient but has the drawbacks of reducing the light available (light is attenuated

[1] Serpentine = long bent tubular air lift

through the water) and water is lost to evaporation (Tredici 2004). However while the reviewed methods for the control of temperature are satisfactory they would be expensive and inefficient.

1.4.1 Comparison of open and closed systems

Open systems resemble the natural environment of the microalgae, and as the name suggests are open to outside influences and conditions, and are the most commonly used primarily because an open culture is easier to build and operate and it is thought that closed PBR are more costly as cooling systems are required (Tredici *et al.* 1992). Closed systems come in many different configurations but the most common are tubular and flat panel. Pulz (2001) developed a comparison between the two systems and this is shown below.

Table 1.4: Comparison of PBR systems

Parameter	Open Systems	Closed Systems
Contamination risk	Extremely high	Low
Footprint	High	Low
Water losses	Extremely high	Almost none
CO_2 losses	High	Almost none
Biomass quality	Not susceptible	Susceptible
Variability as to cultivate species	Limited to few species that will grow in selective environment	Nearly all species may be cultivated
Reproducibility of production	Dependent on exterior conditions	Possible within certain tolerances
Process Control	Not possible	Given
Standardisation	Not possible	Possible
Weather dependence	Absolute	Insignificant
Time to max cell density	6 – 8 weeks	2 – 4 weeks
Efficiency of treatment process	Low	High

It is generally agreed that the key difference between the two cultivation systems is that more control of the main biological parameters given above is possible with a closed system (Chaumont 1993; Lee 2001; Pulz 2001). Furthermore, in a closed system it is easier to maintain an algal monoculture meaning that a greater number of species can now be cultivated. Because of this it is now possible to exploit the microalgae to produce products cheaply that were previously uneconomical or impossible to produce. Previously it was only possible to cultivate

Chlorella, *Spirulina*, and *Dunaliella* because they grow in extreme conditions that are unfavourable to other algae species. One of the disadvantages of closed PBR is that they are difficult to scale up. Currently only open systems are used to produce large quantities of algae. The reason for this seems to be because closed systems do not scale very well, and are more expensive to build, maintain, and run. However, because fewer species can be successfully cultured in open systems, there is still a great interest in closed PBR. Whilst closed systems have a more relaxed selection criteria it is still necessary to select species that will thrive in these systems, as such it is necessary to determine the constraints of these systems and to identify a set of criteria that a species must possess to be cultivated effectively.

Table 1.5: Comparison of open and closed PBR (adapted from (Lee 2001))

PBR	ID (cm)	Location	Highest Productivity ($g\ L^{-1}\ d^{-1}$)	alga
Tubular	12.3	Italy	0.25	*Spirulina maxima*
	2.6	Italy		*Spirulina* sp.
	2.5	Israel	1.60	*Spirulina plantensis*
	2.6	Spain	0.32	*Isochrsis galbana*
	6.0	Spain	2.02	*Phaeodactylum*
	3.0	Spain	2.76	*Phaeodactylum*
	6.0	France	0.36	*Porphyridium cruentum*
	2.5	Singapore	2.90	*Chlorealla pyrenoidosa*
	1.2	Singapore	3.64	*Chlorealla pyrenoidosa*
Coil	2.4	Australia	1.20	*Tetraselmis chuii*
Column	20.0	Spain	0.69	*Phaeodactylum*
	2.6	Israel	1.60	*Isochrsis galbana*
Flat plate	10.4	Israel	0.30	*Spirulina plantensis*
	1.3	Israel	4.30	*Spirulina plantensis*
	3.2	Italy	0.80	*Spirulina plantensis*

It is very difficult to compare productivities of different PBR because of the different geographic locations, culture modes and algal strains used (Chaumont 1993), nevertheless these productivities provide insight into the performance of the PBR. Open ponds and raceway PBR typically reach cell concentrations within the range 0.1–0.5 $g\ L^{-1}$ with productivities between 0.18 $g\ L^{-1}\ d^{-1}$ and 2.5 $g\ L^{-1}\ d^{-1}$ (Borowitzka 1999; Lee 2001). Many closed PBR have been developed, generally variations of flat plate or tubular PBR, typically these PBR have a narrow light path averaging 2.5 cm, and cell densities of up to 20 $g\ L^{-1}$(Lee 2001). Productivities of

closed PBR vary between 0.25 – 3.5 g L^{-1} d^{-1}. Table 1.5 above shows a comparison of open and closed PBR adapted from Lee (2001).

1.5 PBR developed by CSIRO

A flat plate bubble column PBR was developed at CSIRO Manufacturing and Infrastructure Technology (MIT) by Dr Dilip Desai. The vessel design is based on other published designs, but has a longer light path than is conventional (5 cm), while still maintaining a high surface to volume ratio. Adding to this, the PBR is also equipped with a cooling/heating coil making temperature control easy and efficient, unlike any of the PBRs reviewed above. Originally the PBR was designed to be the centre of a carbon sequestering system, taking CO_2 produced by power generation or fermentation and converting it to biomass and useful products such as PHB, as shown below in Figure 1.7.

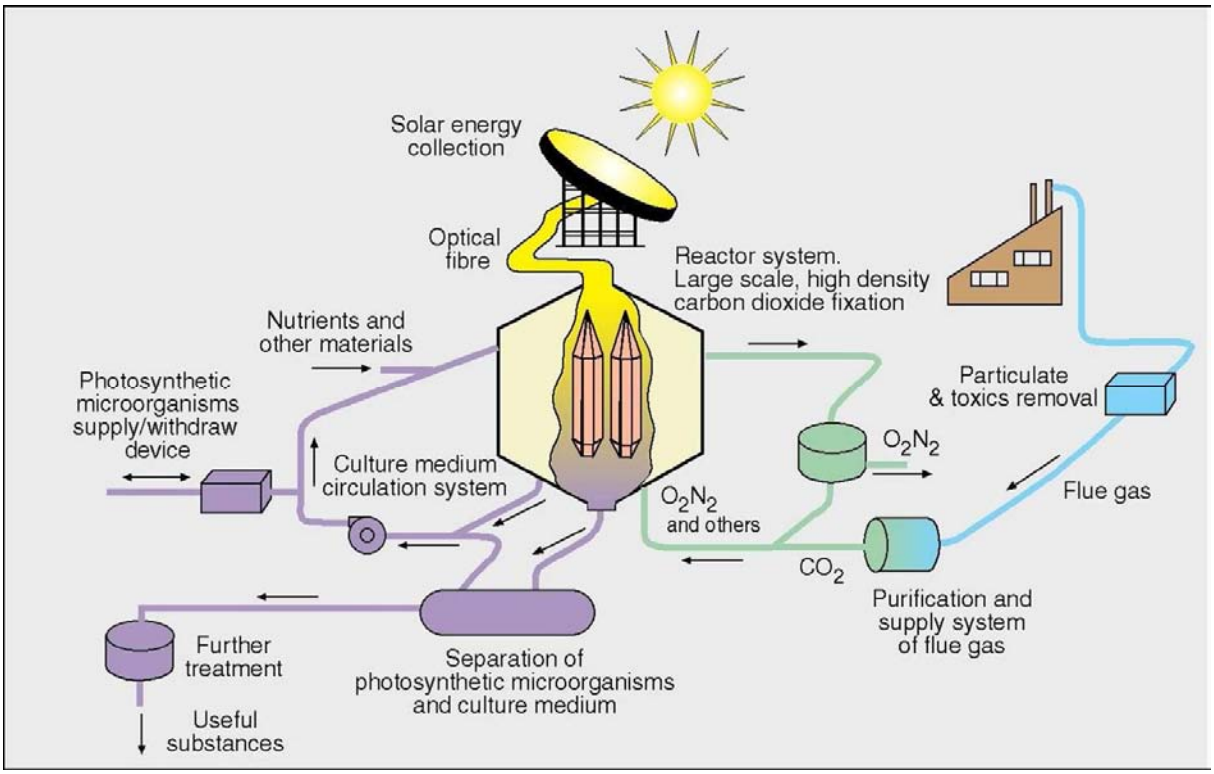

Figure 1.7: Incorporation of the PBR to sequester CO_2 and produce products

The ability of this PBR to grow microalgae is untested and as such, species of microalgae that are suitable for growth in this PBR are unknown. Preliminary studies need to be conducted to

identify the constraints within which the PBR operates. These conditions can be used to develop a set of key requirements that a microalga must possess, to be cultivated in the PBR. These requirements can be then used as screening criteria to select a species of microalgae.

1.6 Selection Criteria

Aside from the PBR specific selection criteria there are many general criteria that make a process successful. As shown in Table 1.3 many species of microalgae are suspected or have been confirmed of being able to produce PHB, what often is unknown is the cellular content of PHB produced, also there is a dearth of information regarding the capacity of Australian species of microalgae to produce PHB. It is vitally important at all stages of designing a process to consider the environmental effects, in the event of a critical failure of the PBR a non-indigenous species could make its way into the eco-system causing unknowable amount of havoc. The advantage of using Australian species of cyanobacteria is that Australian isolates are more readily available, AQIS certification is not necessary, and if the process is found to be commercially acceptable then in the event of a failure of the PBR and any containment structures the environment is less likely to suffer in the long term. Provided the species of cyanobacteria is also non-toxic the damage can be further reduced, and a potential hazard to plant personnel is removed.

The selection of an appropriate microalga is dependant on the system in which it is to be cultivated, as previously mentioned the requirements for the successful cultivation of algae in an open pond differ dramatically from the requirements of a closed photobioreactor system such as we are using. An open system requires a species of cyanobacteria that is more robust and grows under selective pressure such as high salinity, a closed system has other requirements; many reviews have been published on the benefits or otherwise of each of these systems (Borowitzka 1999; Lee 2001; Pulz 2001).

There are other desirable characteristics microalgae need possess that should be considered; a high growth rate is always desirable, as well as a high maximum cell density, a moderate temperature and light requirement can help reduce operating costs, yet we cannot forget that a high intracellular concentration of PHB is especially important for a successful product.

1.7 Scope of Research

The aim of this research is to screen and select a species of cyanobacteria that produces PHB for production in the novel PBR developed by CSIRO. To achieve this a "top down" method will be used, this approach is novel in that traditionally a species of microalgae is first chosen then a PBR is selected for it, this is called a "bottom up" approach. Initial experiments are needed to define the key characteristics that a microalgae will require to be successfully cultivated in the PBR. Further experiments screen and select an Australian species of microalgae that produces PHB and possesses the characteristics needed to be successfully cultivated in the CSIRO PBR. Finally the selected microalgae is cultivated in the PBR to validate its growth conditions.

Chapter 2

2 Preliminary Studies

2.1 Summary

Preliminary experiments were conducted in the CSIRO PBR (CPBR), to evaluate its potential for cultivating microalgae and the properties that a microalga need have to be successfully cultivated in the CPBR. Four experiments were conducted at the operating limits of the CPBR but still cultivate the mesophilic cyanobacteria *Synechoccous PCC 7002*. This cyanobacteria was used because of its robust nature and availability, and because it is thought to produce PHB. The experiments showed that the CPBR was able to cultivate the cyanobacteria, producing a maximum productivity of 0.210 g L^{-1} d^{-1} at 603 μmol m^{-2} s^{-1} and 29°C. Furthermore it was discovered that *Synechoccous PCC 7002* was unable to produce PHB, however a set of selection criteria were determined for the CPBR so that a cyanobacteria could be screened for PHB production in the CPBR.

2.2 Goal of the Preliminary Reactor Studies

Preliminary studies are needed to determine the key aspects necessary for selecting a cyanobacterium for growth in the CPBR. As mentioned previously, the ability of the PBR to grow microalgae is unknown. It is necessary to evaluate the performance of the CPBR and compare it to other photobioreactors. These preliminary studies were also used to define the system constraints; this is needed to develop a set of selection criteria such that a selected species of algae will be able to be grown in the CPBR.

This is different from the traditional approach in that it is a top-down design method, as opposed to a bottom-up methodology. Traditionally, researchers have identified a species of microalgae that produces a useful product and then have selected/designed a reactor system to cultivate it, however with this approach the reactor system is not considered until the end and so it is possible that the selected species may not be able to be effectively grown in any available PBR. In top-down design first the PBR is selected and then a species of microalgae is selected to be cultivated using that system. With this approach the system constraints of the PBR are used as

selection criteria, ensuring that the species will grow effectively within the PBR. However so that the system constraints can be determined, a series of preliminary experiments need to be conducted using a species of microalgae, for these preliminary experiments the cyanobacterium *Synechoccous* PCC 7002 was used.

There has been some interest in the cyanobacterium *Synechoccous* PCC 7002 in recent times as a candidate for growth in photobioreactors. *Synechoccous* PCC 7002 possesses favourable characteristics for growth under artificially imposed conditions typical of PBR (tolerant of high temperature, salinity, carbon dioxide concentrations, relatively small generation time), it also may be engineered to produce useful products, (Asada *et al.* 1999; Benemann 2000) due to its natural competence and lack of interfering restriction enzymes (Steinback *et al.* 1985), this was backed up by Dr Dean Price of The Australian National University (ANU) who supplied the algae. It is also thought that this cyanobacteria is able to produce PHB (Vincenzini *et al.* 1999), as granular inclusions have been observed within the cell, however this does not guarantee the presence of PHB, other qualitative methods are needed to confirm this. Furthermore, *Synechoccous* PCC7002 was already being cultivated in the lab, and subsequently was selected for the preliminary studies as it was available and had desirable characteristics.

2.3 Experimental Design

2.3.1 Experimental Methodology

There are several parameters are important for the growth of cyanobacteria, specifically temperature, incident light intensity, and the media pH were found to be the most significant. For the production of PHB it is necessary to maintain a carbon surplus, as the only carbon source is CO_2 this means the pH levels need to be low, however not so low as to be detrimental to cell growth or function. Typical ranges for these parameters were found to be between 25–40^{o}C, a pH of 7–9, and light intensities of 20–1800 μmol m^{-2} s^{-1}. Values were selected that explored these ranges. It stands to reason that the algae would be most difficult to cultivate at the maximum and minimum of these bounds, where they are stressed producing the least desirable growth characteristics that can then be used as selection criteria, also the maximum and minimum control bounds of the CPBR are unknown and need be explored.

Several experiments were conducted with a combination of high/low light and high/low temperatures with pH maintained constant, to determine the selection criteria necessary for successful cultivation of the microalgae, as imposed by the system. For each of these experiments the cells were tested for PHB so as to confirm the presence of PHB in *Synechoccous* PCC7002.

2.3.2 Materials and Methods

SW-BTP media was used in the preliminary reactor studies for growing *Synechoccous* PCC7002. SW-BTP has the following composition: 50 mg L^{-1} K_2HPO_4, 5 g L^{-1} $MgSO_4.7H_2O$, 36 mg L^{-1} $CaCl_2.2H_2O$, 1.5 g L^{-1} $NaNO_3$, 0.6 g L^{-1} KCl, 18 g L^{-1} NaCl, 2.86 mg L^{-1} H_3BO_3, 1.81 mg L^{-1} $MnCl_2.4H_2O$, 0.222 mg L^{-1} $ZnSO_4.7H_2O$, 0.390 mg L^{-1} $Na_2MoO_4.2H_2O$, 0.079 mg L^{-1} $CuSO_4.5H_2O$, 0.049 mg L^{-1} $Co(NO_3)_2.6H_2O$, 6.7 mg L^{-1} ferric citrate, and 14.9 mg L^{-1} Na_2EDTA. The media also contained 4 mg L^{-1} biotin and 4mg L^{-1} B_{12}. The final pH of the media was found to be 8.5.

The preliminary studies were conducted at CSIRO Division of Manufacturing and Infrastructure Technology at Highett, Victoria. Seed cultures were grown in 1 L Schott bottles under natural illumination and continuously bubbled with air supplied from one 950 L h^{-1} fish tank pump. The bubbling rate was controlled via a valve placed in line with the air to the bottle. These bottles were used to maintain stock culture and grow reactor inoculates. The bottles were heat treated at 280ºC for 6–12 hours to inactivate any organisms. SW-BTP was filter sterilised into the bottles, and 10% by volume of stock culture was added. After three days the cultures were in the exponential growth phase and could be used as PBR inoculates.

The principles of operation of the PBR are relatively straightforward. The CPBR is air-lift and externally illuminated, this means that air is used to mix the culture broth unlike in a mechanically mixed PBR. The reactor vessel was initially filled with 8.5 L of sterilised media, the head unit was then screwed on and the light intensity set, while the reactor was sparged with air. The temperature and pH were allowed to reach the desired set points and equilibrate, then the 1 L inoculate was added and the experiment started. A 500W Halogen lamp was used to illuminate the reactor surface, this light provided the light energy for the algae to photosynthesise as well as thermal energy. Air, at a rate of 2.5 L h^{-1} (corresponding to 80% of the rotameter reading), enriched with CO_2 was supplied to the system to provide the air-lift and

as the carbon source. Samples were taken at regular intervals three times a day to determine growth rates and dry cell weight, a further sample was taken every 24 hours for PHB analysis. The nominal volume of the reactor is 9.5 L. The PBR setup allows for the control of three parameters, light, pH, and temperature. The control of these parameters is discussed further below. Reactor volume was maintained by topping up the PBR with fresh media, this was necessary because volume was constantly decreasing from evaporation, and sampling.

Light was added to the culture via one 500W halogen lamp to one side of the reactor, the amount of light incident on the vessel surface was adjusted by moving the lights closer or further from the PBR. The halogen globe was changed at the beginning of each experiment to ensure that the light condition was the same for each experiment. The incident and exit light intensity was measured at nine points across the glass surface; the average of these nine points was used as the incident and exit intensity. The incident intensity was measured periodically throughout the experiment to make certain that it was still at the set point, small adjustments were made as necessary. The intensity was controlled with an accuracy of ±50 μmol m^{-2} s^{-1}, this represents between 6–25% of the set point value. The main source of this error was depreciation in the light source, and other uncontrolled external lights. To control the latter error source, the room lights were left on continuously and external windows were blacked out.

pH was controlled by sparging the reactor vessel with CO_2, the purpose of which was twofold, firstly to control pH and secondly and most importantly to provide the algae with a carbon food source. For these preliminary studies the pH was controlled manually, a 0–300 mL s^{-1} flow meter was used to control the rate at which CO_2 was passed through the reactor; a Hanna pH electrode was inserted into the reactor head unit to display pH. CO_2 was manually adjusted to maintain the pH set point.

Temperature was controlled using a refrigerated recirculated bath. A T-type thermocouple, inserted into the reactor head unit, was used to monitor temperature. The inlet and outlet on the head unit were connected to the recirculated bath, and the reservoir in the bath was filled with distilled water, the thermocouple probe was inserted into the reactor vessel and attached to the reactor head unit. The temperature set-point was set by adjusting the bath temperature on the

control unit and using the thermocouple to achieve the desired media temperature. Once set very little drift was observed. The setup of the reactor can be seen in Figure 2.1 below.

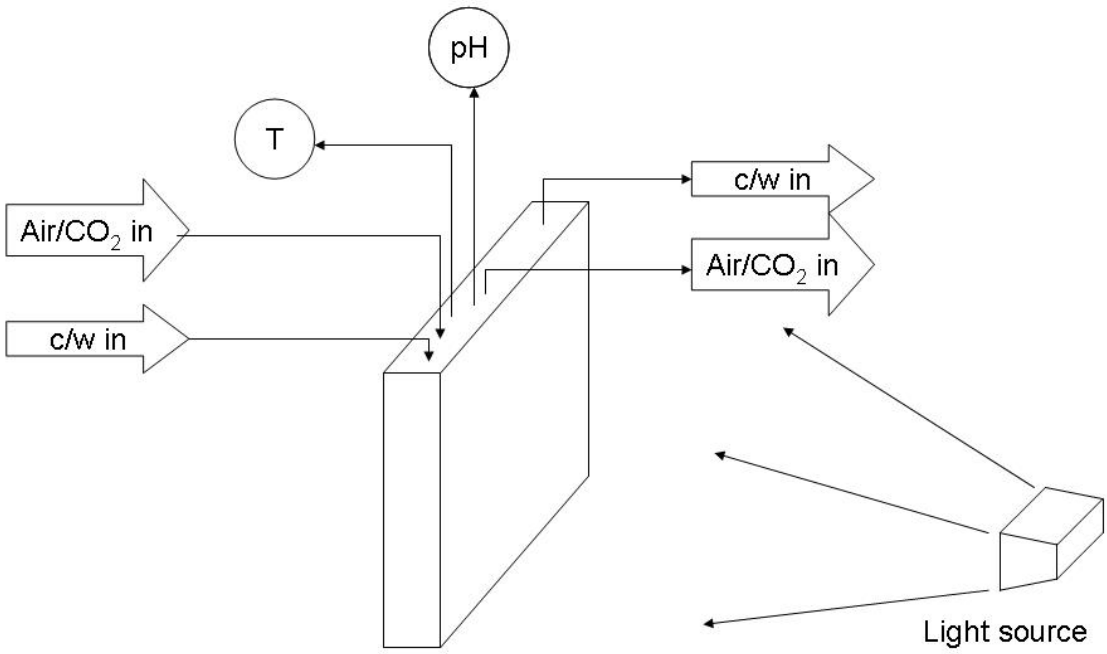

Figure 2.1: Reactor setup

Direct method of measuring dry cell mass (DCM) was used. 10 mL samples of culture broth were collected in pre-dried and weighed tubes, these samples were centrifuged. The supernatant was removed; the pellet was then washed with 10 mL of RO water, centrifuged again, and the supernatant deducted. The tube with algae pellet was dried at 80ºC until constant mass was reached. This typically took 1–3 days, once dried the tubes were re-weighed; the difference in mass was the dry cell weight.

2.4 Analysis of Preliminary Study

The aims of the preliminary study as stated previously were to determine a set of selection criteria that a microalga need possess such that it could be successfully cultivated in the CPBR. Furthermore the capacity of the CPBR to cultivate microalgae needed to be determined as well as the limits at which it can operate. High and low values were selected for CPBR temperature and incident light intensity; combinations of these were used to grow the cyanobacteria *Synechoccous* PCC7002, a summary of these combinations is shown in Table 2.1.

Table 2.1: Conditions for preliminary experiments

Experiment	Temperature (ºC)	Incident Light Intensity ($\mu mol\ m^{-2}\ s^{-1}$)
1	22	192
2	42	936
3	22	939
4	29	603

Experiments 1–3 were used to determine the limits of the system, the results for these experiments are shown in Figure 2.2. It was found that the CPBR was capable of achieving a minimum temperature of 22ºC, to achieve lower temperatures especially at higher light intensities (hence higher heat flux into the system) the cooling unit needed to be set at close to 0ºC. This could have lead to ice forming in the unit, and resulted in damage to the cooling system. The maximum temperature was set at 42ºC because *Synechoccous* PCC7002 is mesophilic, and so above 42ºC limited growth would be expected. The high and low light set points were limited by the distance at which the light source could be set, at high intensity the light source was 10 cm away from the surface of the CPBR, any closer risked thermally cracking the glass and also gave an uneven light distribution across the surface of the glass. The minimum incident light intensity was the furthest distance the light could safely be set from the CPBR surface. A summary of the experiments is shown in Table 2.2.

Table 2.2: Summary of preliminary experiments growth data

Experiment	Maximum productivity ($g\ L^{-1}\ d^{-1}$)	Maximum DCM ($g\ L^{-1}$)
1	0.088	2.17
2	0.223	1.22
3	0.098	3.34
4	0.210	4.79

The results show that the CPBR is capable of producing microalgae however the conditions used were far from the optimum. Experiment 1 was conduced at low light and low temperature and as such exhibited a low productivity of 0.088 $g\ L^{-1}\ d^{-1}$ and low final cell density (2.17 $g\ L^{-1}$), compared to the other experiments. This is because the algae were photolimited, this is observed in Figure 2.1 (a) as the average light intensity levels out very quickly resulting in a corresponding decrease in productivity at the 7 day mark, until the stationary phase is reached. During the experiment it was observed that some algae attached to surfaces in clumps forming biofilms. Increasing the flowrate of air to the maximum achievable flow rate of 3 $L\ h^{-1}$, did not

prevent the formation of these biofilms, also it was thought that increasing the flowrate of air beyond a threshold level may be detrimental to cellular growth as the local shear rates around the rising bubble and resulting recirculation of fluid in the reactor could damage the cells.

Experiment 2 was conducted at high light intensity and high temperature, the growth curve for this experiment is shown in Figure 2.2 (b). The growth curve appears noisy, from the findings of Jensen and Knutsen (1993) it is likely that the culture was photoinhibited, the temperature was high and most likely inhibited enzyme function resulting in poor growth. This can be observed by the long lag phase and low maximum DCM. The maximum productivity was observed to be 0.223 g L^{-1} d^{-1}, however this misleading as it was taken during the exponential growth phase, within the 6–9 days region, and would not be able to be maintained at these conditions. It was observed, at this condition, that the microalgae formed a biofilm on the cooling coil, fouling the heat exchange surface and reducing the rate of heat transfer.

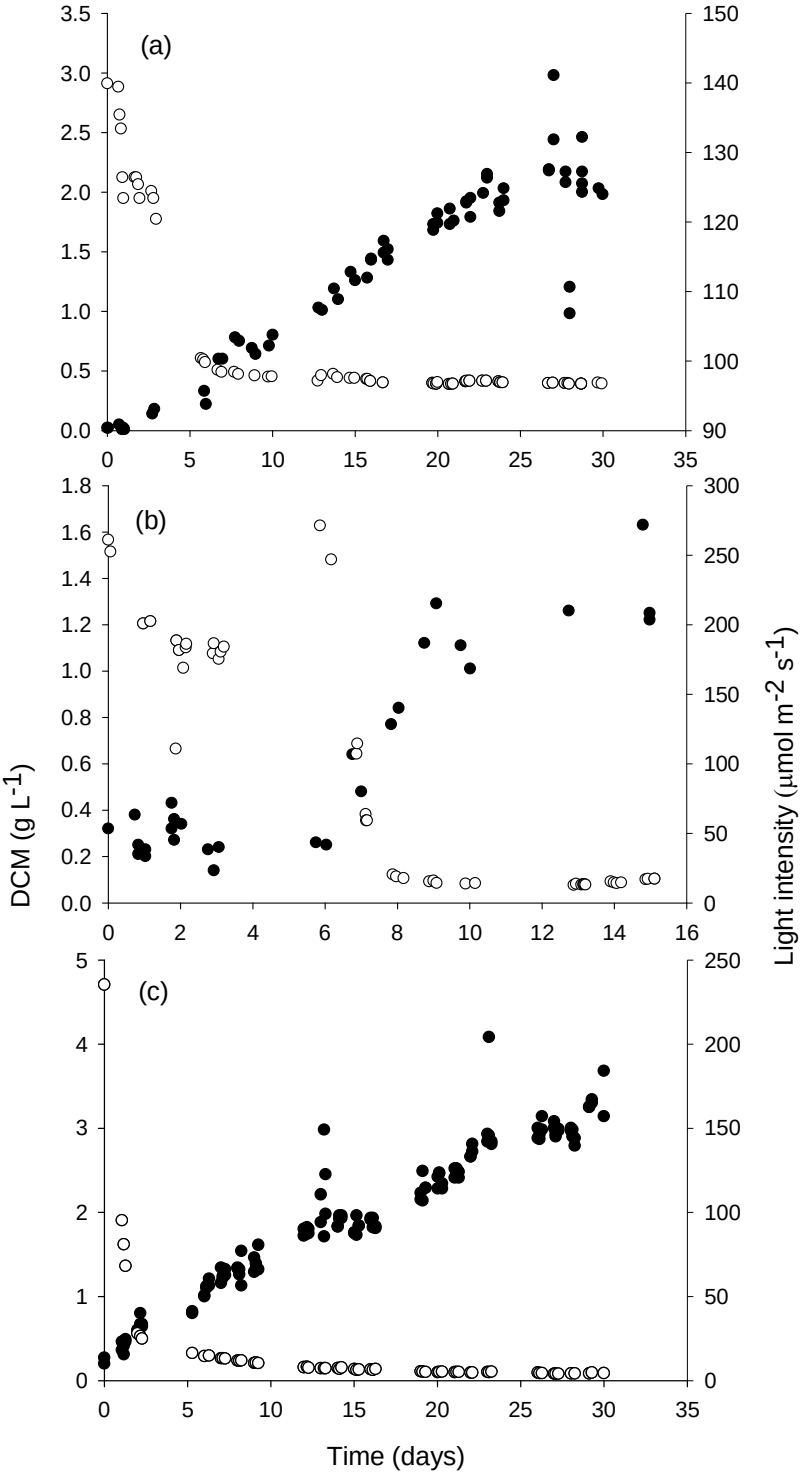

Figure 2.2: Growth and average intensity for (a) experiment 1, (b) experiment 2, and (c) experiment 3, see Table 2.1 for conditions of each experiment. Note the different ordinate and abscissa scales in each graph. Key: ● DCM vs time, ○ Exit Intensity vs time.

The biofilm of algae formed on the illuminated surface of the CPBR; consequently the culture became photolimited leading to stationary phase growth.

Figure 2.2 (c) shows the results of experiment 3, which was conducted at low temperature and high light intensity. The growth rate at these conditions was higher than experiment 1, low temperature and low light, as the culture was not photolimited. What was observed was photo-oxidation, see §1.1.1, as a result of photoinhibition. Briefly the excess light caused the generation of oxygen radicals that bleached the chlorophyll in the algae, this was observed as some loss of colour from the typical blue-green to a straw colour. Furthermore, the algae formed a biofilm around the edges of the illuminated surface. This resulted in a low growth rate when compared to those presented in Table 1.5, however it must be stressed that the values presented in Table 1.5 are the maximum productivities produced under optimum conditions. Despite this, a high cell density was produced, partly because the temperature was low and so the cellular repair mechanisms, which are sensitive to higher temperatures, were not effected as was observed in experiment 2 (Jensen *et al.* 1993). As microalgae grow the culture becomes more optically dense, allowing less light to penetrate the culture, leading to photolimitation and resultant low cell growth and eventually zero growth (stationary phase) where there is only enough light to maintain the population (Suh *et al.* 2001), as was observed in experiment 1. In experiment 3 the initial culture was photoinhibited however as time progressed the culture became photolimited, as can be observed in Figure 2.2 (c), as the exiting light intensity approaches a limiting value. The cell density was higher than that observed for experiment 1 because there was more light available to support a larger population. This is typical of most cultures, where available light is the most common limiting factor (Pulz 2001). The growth curve exhibits 'bumpy' growth, due to a loss of control. The experimental setup had manual pH control, which was achieved by varying the flow of carbon dioxide though a rotameter, this was incredibly difficult to control. Adding to this was the fact that access to the reactor was limited, the plateaus in growth occur on and after weekends which are shown as large gaps in the data, these gaps are also present in the other experiments.

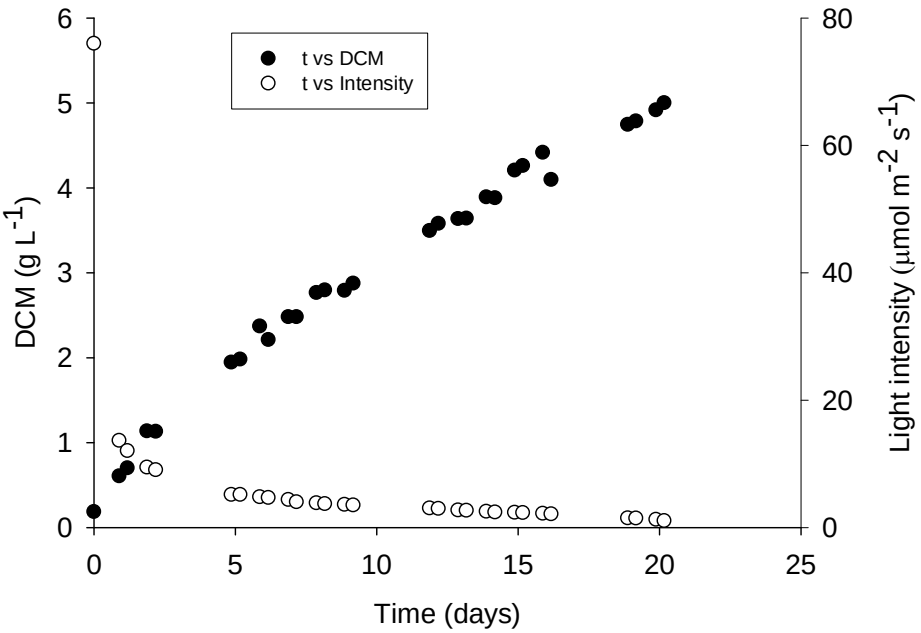

Figure 2.3: Experiment 4, T = 29ºC, I = 603 µmol m^2 s^{-1}

It is necessary to reiterate that experiments 1–3 were conducted at sub–optimal conditions; it was the purpose of these experiments to determine the range of operation of the reactor and determine any reactor specific selection criteria for a microalgae. It is also necessary to reiterate that it is very difficult to compare different PBR and different microalgae, in this case especially so because the conditions were sub–optimal, however an attempt was made. The optimal conditions for growing *Synechoccous* PCC7002 are unknown; however experiments 1–3 did determine the boundary conditions beyond which growth would be unsatisfactory. Experiment 1 determined the lower boundary, experiment 2 the upper boundary, and experiment 3 (a combination of experiments 1 and 2), determined that the optimum condition is not trivial as the factors that affect growth are highly interactive and system dependant making it impossible to predict the optimal growth condition. Because of the paucity of information available for the growth of *Synechoccous* PCC7002 a mid point between the upper and lower boundaries was used for experiment 4, the conditions for which are shown in Table 2.1 and the results in Figure 2.3. Despite sub–optimal conditions the productivity was 0.210 g L^{-1} d^{-1}, and the maximum cell density achieved was 4.79 g L^{-1}. The productivity, while low, is comparable to many of the PBR shown in Table 1.5, particularly to the flat plate PBR of 10cm light path. The performance of

the CPBR is however better than most open systems, having a higher productivity and having a cell density up to 9.5× higher (Lee 2001).

Samples were taken throughout each experiment to test for the presence of PHB. Analysis of these samples revealed that no PHB was being produced. The presence of PHB was expected as granular inclusions had been observed in *Synechoccous* PCC7002 (Vincenzini *et al.* 1999). A partial genetic database existed for *Synechoccous* PCC7002 on Cyanobase. Using *Synechocystis* sp PCC6803, a species conclusively know to produce PHB (Wu *et al.* 2001), with a full genetic sequence avaliable as a template, it was found that for a cyanobacterium to produce PHB it needs the genes phaA, phaB, and phaC (Hai *et al.* 2001). Using these genes as a comparison, it was discovered that *Synechoccous* PCC7002 did not possess the required genes to produce PHB; therefore it was highly unlikely that it would be able to produce PHB, and if it did so, it accomplished it in an unknown manner. No analysis was done to test for the presence of glycogen, a necessary material for the production of PHB, or was the possibility of genetic manipulation explored as this is beyond the scope of this research. Analysis of *Synechoccous* PCC7002 in nutrient limited cultures, to enhance the expression of PHB (§1.3), was not conducted as this was deemed pointless, because it does not possess the requisite genes necessary to produce PHB under any conditions.

2.5 Outcomes

The purpose of the preliminary studies was to (i) define a set of reactor specific selection criteria for a microalgae so that it can be grown in the CPBR, (ii) evaluate the capacity of the CPBR to produce microalgae, and (iii) determine if *Synechoccous* PCC7002 is able to produce PHB.

The performance of CPBR was found to be comparable to other closed PBR even at sub–optimal conditions. The maximum productivity achieved during these studies was 0.210 g L^{-1} d^{-1}. It was also found that *Synechoccous* PCC7002 did not produce PHB as it lacks the necessary genes.

Two reactor specific selection criteria were identified from these studies. Firstly, for a species to be optimally cultivated in the CPBR it is necessary that it not form microbial mats, some species of microalgae attach to surfaces to grow (Richmond 2004), this is undesirable within a closed system for a couple of reasons. The primary reason is that they tend to attach to the

illuminated surface, which is understandably problematic. If they attach to the illuminated surface it will greatly reduce the light available to the rest of the culture reducing product yield, this could make the difference between a successful and unsuccessful process. The other reason is that it can make the product difficult to harvest especially in a continuous culture; it could also result in longer turnaround times in batch culture, which in turn would result in a loss of productivity, or a larger capital outlay for more reactors. Another reason is for a closed system, specifically an air-lift PBR like the CPBR, to be successful the species of cyanobacteria needs to be able to withstand the bubbling agitation. This will eliminate most filamentous species and species that have flagella as they may be damaged by the bubbling and circulating action. These selection criteria were used to select a species of microalgae to be cultivated in the CPBR.

Chapter 3

3 Species Screening and Selection

3.1 Summary

Six selected species of cyanobacteria were subjected to a gross screening process, to determine if they (i) produced PHB and (ii) were able to be cultured effectively. Three species, *Anabaena solitaria*, *Microcystis aeruginosa* and *Microcystis flos-aquae*, survived this screening process. They were then further screened under various growth conditions for maximal growth rate and PHB concentration. Each species was grown phototrophically, mixotrophically, and in nitrate and phosphate deficient conditions. *Anabaena solitaria* was identified as the best species for growth in the closed flat-plate PBR, accumulating 1.3 mg L^{-1} under phototrophic conditions.

3.2 Selection Criteria

Two CPBR specific criteria were discovered along with the general criteria identified in section 1.6. The selection criteria are:

- Native strain of microalgae
- High growth rate
- High maximum cell density
- Moderate temperature requirement
- Moderate light requirement
- Able to withstand bubbling aeration
- Not form microbial mats

Because there is a paucity of information about the occurrence of PHB in Australian cyanobacteria it is necessary to test and screen a wide selection of cyanobacteria. To this end six species of Australian cyanobacteria were obtained from the AWQC (Australian Water Quality Centre). The species were selected because they were similar to species that were known to produce PHB, that is they were the same species or genus.

The selected species detailed in Table 3.1 were initially screened for their ability to produce PHB, and for their growth characteristics, such as being able to withstand aeration, and not form microbial mats. Any species that remain after the gross screening were subjected to further

rigorous screening to determine the highest PHB concentration with best growth characteristics. When screening species it is necessary to rank these requirements to facilitate decision making. The most important trait is that the species not form microbial mats so that it can be effectively grown in the CPBR. This is followed by a high growth rate and cell density, as traditionally downstream processing accounts for up to 70%–80% of overall operating costs so any reduction here is preferred. Next, a high amount of product should be produced, in this case PHB. Using these criteria a species will be selected for growth in the PBR.

Table 3.1: Selected Australian cyanobacteria to be screened for PHB

Name	ID No.	Source
Anabaena flos-aquae	187	Mungindi Border, NSW
Anabaena solitaria	177C	Collarenebri, NSW
Nodularia spumigenia	NOD 001	Lake Alexandrina, Milang, SA
Pseudanabaena	012C	Nyabing Dam, WA
Microcystis aeruginosa	046E	Heidelberg, VIC
Microcystis flos-aquae	052	Barossa Reservoir, SA

3.3 Materials and Methods

All cultures of algae were initially prepared in the same manner. Initial cultures were obtained from the AWQC in ASM-1 media and tissue culture flasks, containing 5 mL of algae culture. 1mL of these cultures was then used to inoculate 50 mL Erlenmeyer flasks containing 10 mL of ASM-1 media, which contained: 40.62 mg L^{-1} $MgCl_2.6H_2O$, 49.33 mg L^{-1} $MgSO_4.7H_2O$, 29.4 mg L^{-1} $CaCl_2.2H_2O$, 1.08 mg L^{-1} $FeCl_3.6H_2O$, 2.47 mg L^{-1} H_3BO_3, 1.37 mg L^{-1} $MnCl_2.4H_2O$, 0.44 mg L^{-1} $ZnCl_2$, 6.64 mg L^{-1} Na_2EDTA, 0.0216 mg L^{-1} $CoSO_4.7H_2O$, 0.00013 mg L^{-1} $CuCl.2H_2O$, 17.4 mg L^{-1} K_2HPO_4, 14.2 mg L^{-1} Na_2HPO_4, 170 mg L^{-1} $NaNO_3$. The pH of ASM-1 was measured to be 7.5. These new cultures were grown using an illuminated shaker table under 100 μmol m^{-2} s^{-1} of light. However the cultures performed poorly in this media, possibly because of the low phosphate and nitrate compositions in ASM-1, BG-11 on the other hand contained higher levels of phosphate, nitrate, and also more sulphurous compounds. The cultures were moved to BG-11 media where they immediately performed better. The composition of the modified BG-11 is as follows: 30.5 mg L^{-1} K_2HPO_4, 75 mg L^{-1} $MgSO_4.7H_2O$, 56 mg L^{-1} $CaCl_2.2H_2O$, 1.5 g L^{-1} $NaNO_3$, 2.86 mg L^{-1} H_3BO_3, 1.81 mg L^{-1} $MnCl_2.4H_2O$, 0.222 mg L^{-1} $ZnSO_4.7H_2O$, 0.390 mg L^{-1} $Na_2MoO_4.2H_2O$, 0.079 mg L^{-1} $CuSO_4.5H_2O$, 0.049 mg L^{-1} $Co(NO_3)_2.6H_2O$, 6.7 mg L^{-1} Ferric

citrate, and 14.9 mg L^{-1} Na_2EDTA. The pH was measured to be approximately 8. Culture broth taken from these flasks was then used to create further 50 mL flasks, this was done many times so that the cultures could adapt to the new environment. After the cultures had established themselves they were used to inoculate 250 mL flasks containing 150 mL of media, these flasks became the stock flasks. Each inoculate was 10% by final volume of algal culture, and Stock Culture flasks were created fortnightly from the previous stock culture, this was done to ensure the best culture health.

For the cultures grown in nitrogen-starved (-N) or phosphate-starved (-P) conditions the $NaNO_3$ or K_2HPO_4, respectively was omitted from the media. Cultures grown under mixotrophic conditions were grown in BG-11 media with 10 mmol of sodium acetate added.

100 mL of stock culture was used to create 1 L flasks that were bubbled with air enriched with CO_2 in a specially designed growth cabinet shown in Figure 3.1. Cultures were incubated under a constant illumination of 200 μmol m^{-2} s^{-1}. Temperature varied between 25–35°C. When cultures were bubbled with air enriched with CO_2, (~2%) the pH was monitored and controlled manually to between 8–8.5 units, by varying the CO_2 input.

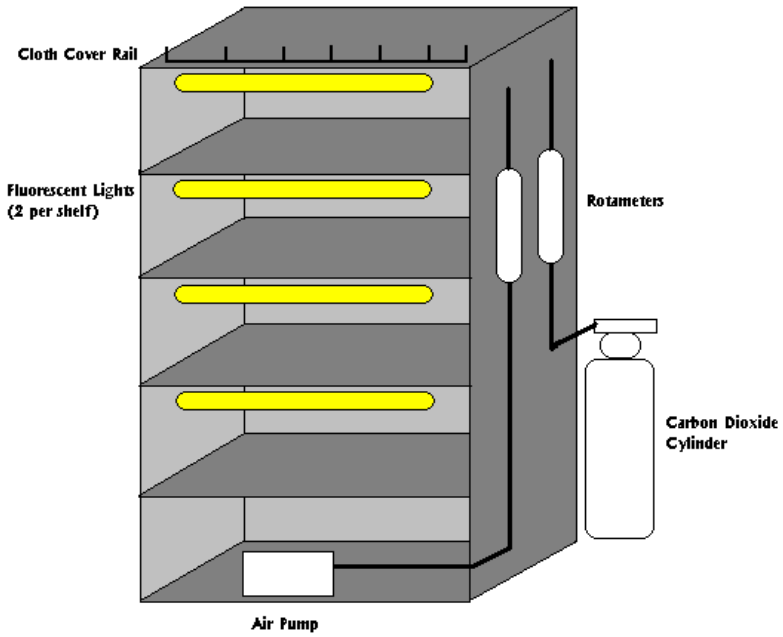

Figure 3.1: Schematic of the growth cabinet used to grow cultures

To determine if the methods being used were accurate a species of cyanobacteria, *Synechocytis* PCC6803, well known to produce PHB, was acquired and used as a benchmark. However this species of cyanobacteria was unsuitable for reactor studies because migrated to dark corners of the culture vessel. This would result in poor mixing and possibly lower cell densities. There are many reasons for this one such common reason is that it was dark adapted.

All the experiments were carried out in duplicate. Two independent cultures were grown under identical conditions at the same time. Growth rate and PHB accumulation were measured for both. This was done to minimise experimental error and as additional confirmation of findings.

Two methods were used to determine the dry cell concentration of microalgae. Initially the direct method of measuring dry cell weight was used. 5–10 mL samples of culture broth were collected in pre-dried tubes, centrifuged, the supernatant removed, and then dried at 80°C until constant mass was reached. However this method was inaccurate for cultures with very low cell density, often returning low and sometimes negative results. The reason is that the difference in mass between the heavy tube and the algae was within the error limits of the scales. This method is much more accurate at larger cell densities where the difference in mass is larger. More accuracy can be obtained by collecting larger amounts of culture broth but this was unacceptable for small volume cultures, and was not sustainable for larger volumes as addition of large amounts of media required to make up for the loss by sampling affected the growth. One of the consequences was that frequent sampling (small time intervals) was inadvisable because of the need to keep adding media to the culture to maintain nutrient levels and constant volume, which affected the growth. Furthermore, because the samples took time to centrifuge and to dry, (up to 3 days) the turnaround time for the data was large.

Many researchers have used light absorbance to determine the culture density (Campbell *et al.* 1982). Light absorbance is directly related to cell concentration, this relationship is known as the Beer-Lambert law, shown as Equation 3.1. The Beer-Lambert law indicates that the change in absorbance at a particular wavelength will be logarithmically linear with concentration; however this law does not take into account the effect of light scattering that occurs at higher cell densities. Unlike absorbance, where the light is absorbed by the cell, scattering is where the light is reflected from the cell and scattered in random directions, which affects the reading of

exit light intensity, I_1. To use this method it is necessary to keep the cell density low enough so that scattering is not significant. In high cell density cultures this means that the samples need to be diluted so that this does not occur, the dilution factor is then used to re-calculate the actual cell mass. The selection of the wavelength used to measure the absorbance is very important; at certain wavelengths of light the chlorophylls will absorb light, along with other substances within the cell. This is useful for finding the concentrations of these compounds but is detrimental to determining dry cell weight, because these chemical compounds can change with cell health, growth phase, and growth conditions. A wavelength of 730 nm was selected because it was found that no compounds absorbed at this wavelength, thus it was only the presence of the cells that was changing the absorbance. It was found that at this wavelength scattering occurred at absorbances higher than 0.8.

$$A = \alpha lc = -\log_{10}\left(\frac{I_1}{I_o}\right) \qquad \text{Eq. 3.1}$$

1 mL of culture was pipetted into a cuvette and its absorbance measured and recorded at 730 nm throughout the growth of the microalgae. Once the absorbance levelled out indicating the stationary phase had been reached samples for traditional dry cell weight determination were taken. Another sample was taken and a series of dilutions were performed from 15%–4% culture, absorbances of these were measured and recorded. From these a calibration curve was fitted, a log-linear model was used (predicted by the Beer Lambert Law), this calibration can be seen in the Appendix (§Calibration Data). The trend goes though the origin and can be described by Equation 3.2, $R^2 = 0.9586$, confirming a good fit. This calibration was performed after each experiment.

$$\text{Dry Cell Weight (g/L)} = 0.1775 \times \text{Absorbance}(730nm) \qquad \text{Eq. 3 2}$$

4 mL of sample was taken to be tested for PHB. Cells were first centrifuged and the supernatant removed, the cells were then dried and subjected to methanolysis in the presence of 3% v/v sulphuric acid in accordance with Braunegg *et al* (1978), with 200 μL of methyl benzoate as an internal standard. 2 μL of the resulting organic layer was analysed on a Shimadzu GC-17A gas chromatograph using an Econowax EC-1000 capillary column (15 m by 0.54 mm; Alltech).

Hydrogen was used as the carrier gas (17 mL min^{-1}). The temperature of the injector and the detector were 230°C and 275°C respectively. The column oven temperature program was the same as used by Brandl *et al* (1988). Standard solutions of PHB methyl ester were eluted onto the column and the retention time was found to be approximately 6.2 minutes. The PHB content is calculated using the ratio of the area of the PHB peak with the internal standard, this calibration is shown in the Appendix (§Calibration Data).

3.4 Gross Screening

The six Australian species shown in Table 3.1 were initially grown under phototrophic and nutrient balanced conditions as described above, and the growth rates and PHB content were determined. During the gross screening process the cultures were then grown in phosphate and nitrate starved conditions, and once again the growth rates and PHB content were determined. More focus was placed on confirming the presence, or absence, of PHB as well as determining if the cultures were suitable to grow in the CPBR, that is, they are able to withstand bubbling agitation and not form microbial mats. The species that met these simple requirements were then subjected to a more rigorous screening process.

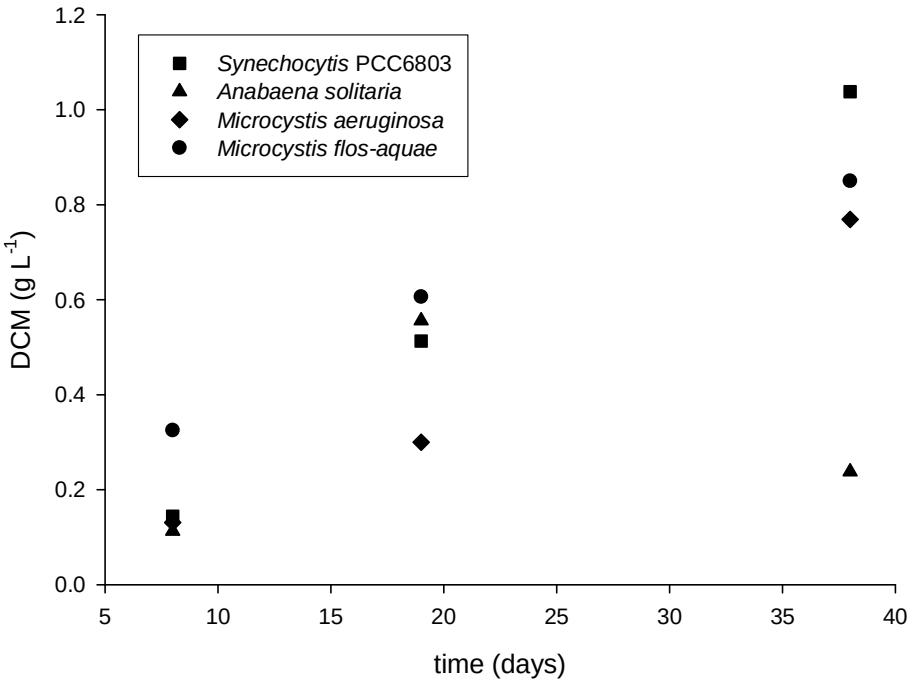

Figure 3.2: Cell Growth of viable cultures compared to 6803

From inoculation in balanced media it was immediately noticeable that all the strains behaved differently. With air enriched with CO_2 the *Anabaena flos-aquae* and *Nodularia spuigenia*, crashed soon after inoculation, gradually turning brown and ceasing to grow, despite the pH remaining within 8–8.5 pH units. *Pseudanabaena* formed biofilms on any available surface making it impossible to determine dry cell weights, however analysis was done to determine the presence of PHB.

Anabaena flos-aquae and *Nodularia spumigenia* did not grow well under the given conditions. The *Pseudanabaena* started to grow well after a short period of time but grew in large clumps, which is not a desirable property of the required cyanobacteria. Due to the temperamental nature of these species and their unwillingness to grow in these conditions, similar to those of a PBR, they were removed from the screening process.

This left the *Anabaena solitaria*, *Microcystis aeruginosa* and *Microcystis flos-aquae* as the remaining cyanobacteria that grew well under the given conditions, as seen in Figure 3.2, and as such were the main focus of the PHB testing.

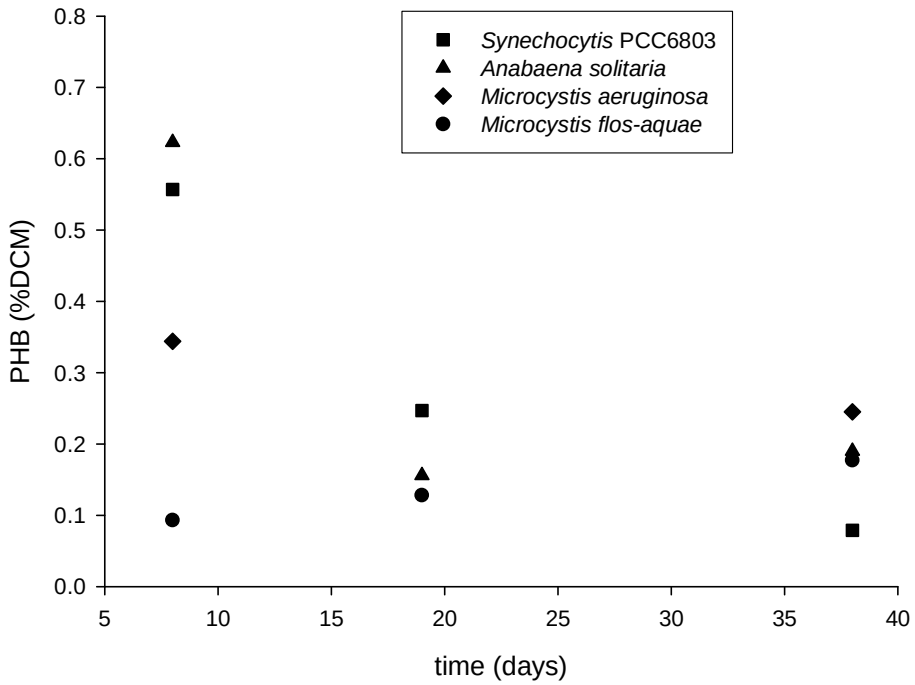

Figure 3.3: PHB content as percentage of DCM

It is shown in Figure 3.3 that in early growth conditions the amount of PHB produced in cells remained quite similar with the exception of *Microcystis aeruginosa*, which produced a considerably larger amount of PHB. The *Microcystis aeruginosa* continued to show good PHB production in cells, however it did not respond to the nitrogen deficient media as well as the 6803. The *Microcystis flos-aquae* consistently showed the least amount of PHB grown in cells, with the *Anabaena solitaria* being the second best producer. The *Synechocytis* PCC6803 performed as expected from the literature (Asada *et al.* 1999), hence validating the methods used.

Of the six species tested, three appear appropriate for studying growth in a CPBR; *Microcystis aeruginosa*, *Microcystis flos-aquae*, and *Anabaena solitaria*. These three remaining species were then rigorously screened to select the best candidate for the reactor studies.

3.5 Rigorous Selection

The three species identified as possible candidates for growth in the CPBR, *Microcystis aeruginosa*, *Microcystis flos-aquae*, and *Anabaena solitaria* were subjected to more rigorous screening procedures. Each strain was cultured in a balanced media, mixotrophic media, and both nitrogen and phosphate deficient media. The growth and PHB accumulation of each species were closely monitored.

3.5.1 Balanced Conditions

Figure 3.4 show the balanced growth of all three species. It can be seen from these graphs that the initial concentration of PHB for all three cultures is initially high and then decreases in the lag phase and exponential phase before increasing in the stationary phase. This was to be expected as PHB is thought to be a carbon storage compound within the cell, therefore during growth stages the PHB is being utilised by the cell for growth, and it is not until the stationary phase that the PHB accumulates, this is in agreement with the literature and can be observed in the works of other authors (Campbell *et al.* 1982; Ishizaki *et al.* 1991). The initial concentration of PHB was high because the seed cultures were under the stationary phase, during which PHB was stored. In the exponential growth phase, the PHB concentration decreased because of the utilisation of PHB for cell growth, and the PHB concentration at the late exponential phase started to increase slightly because PHB was stored again as the growth reduces.

Under the balanced conditions, the accumulation of PHB seems to be similar. *Anabaena solitaria* was able to accumulate PHB up to around 1.3 mg L^{-1} by the end of the experiment. The *Microcystis flos-aque* culture contained 1.9 mg L^{-1} by the end of the experiment, and the *Microcystis aeruginosa* contained 1.2 mg L^{-1} by the end of the experiment. These results are all very similar, however the weight percent of PHB within the cell is very small, well below what is normally reported in the literature. *Anabaena solitaria* and *Microcystis flos-aque* grew very fast with productivities of 0.0041 g L^{-1} d^{-1} and 0.0035 g L^{-1} d^{-1}. The productivity of *Anabaena solitaria* was greater, but the final cell densities were very similar. *Microcystis aeruginosa* was the poorest performer of all three with a lower final cell density, poorer productivity, and low PHB concentration. Based on these experiments alone it would be difficult to choose between *Anabaena solitaria* and *Microcystis flos-aque*, however for balanced conditions, because growth rate is ranked higher in the selection criteria *Anabaena solitaria* would be the best choice for growth in the CPBR.

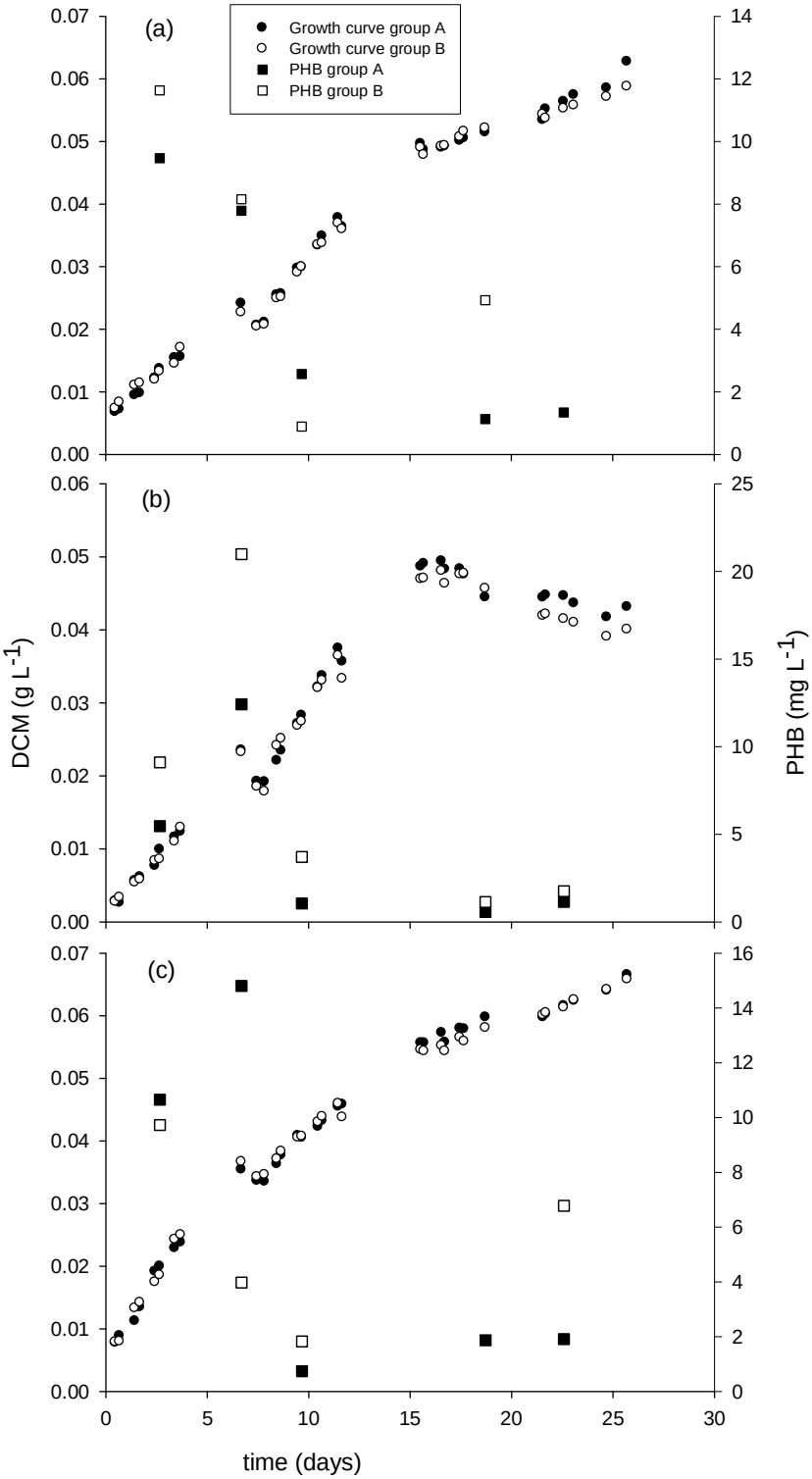

Figure 3.4: Growth and PHB accumulation in nutrient balanced conditions, (a) *Anabaena solitaria*, (b) *Microcystis aeruginosa*, and (c) *Microcystis flos-aque*. Note the different ordinate scales.

3.5.2 Mixotrophic Conditions

Mixotrophic growth conditions is where an extraneous amount of carbon substrate or any other chemical is added with the aim to stimulate the cell growth or to trigger production of valuable intracellular chemicals. Addition of this acetate source may provides more acetyl moieties to form more free acetyl-CoA molecules and hence, the accumulation of PHB in the cells will directly be enhanced, if compared to photoautotrophic growth using only carbon dioxide gas as the sole carbon source (Wu *et al.* 2001).

Figure 3.5 shows the results obtained from when the cultures were grown mixotrophically. It can be seen that the final cell density of the three cultures are higher by at least 5× the final cell density under photoautotrophic conditions. This was expected as the exogenous carbon source acetate will promote growth of cells. The productivities of each of the species was also higher, *Anabaena solitaria* had the highest productivity, almost 2× *Microcystis aeruginosa*, and *Microcystis flos-aque* respectively. A summary of the results is shown in Table 3.2.

Table 3.2: Summary results of mixotrophic growth

Species	Max Productivity ($g\ L^{-1}\ d^{-1}$)	Max cell density ($g\ L^{-1}$)	Max final PHB ($mg\ PHB\ L^{-1}$)
Anabaena solitaria	0.032	0.60	1.8
Microcystis aeruginosa	0.017	0.35	1.5
Microcystis flos-aque	0.018	0.38	1.8

From Figure 3.5 it can be seen that as observed for balanced conditions

the initial concentration of PHB decreased in the case of *Anabaena solitaria* and *Microcystis flos-aque*, however for *Microcystis aeruginosa* the concentration appears to remain constant but this is misleading as there are very few data points available corresponding to the detection of PHB. This does not mean there was no PHB present, just that it was below the detection limit of the GC. The final PHB concentration for *Anabaena solitaria* was not significantly higher than was observed for balanced conditions, showing a gradual increase in PHB content as the growth enters the stationary phase, in agreement with the observations in the literature for PHB accumulation. *Microcystis flos-aque* showed no improved accumulation of PHB, in agreement with the literature (Wu *et al.* 2001); the exogenous carbon source (glucose) was mainly used to synthesise glycogen after being absorbed by cell and did not improve the PHB content. In this research glycogen concentration in the cells was not studied, it would be sensible to hypothesise

that the exogenous carbon source (acetate) added to the culture was used to produce glycogen and did not increase the content of PHB in the culture. However this result raises the possibility that PHB accumulation could be induced in the culture once a high cell density is achieved. Further studies need to be conducted to determine if this is valid.

From this experiment it can be seen, for mixotrophic conditions, that *Anabaena solitaria* is the best choice for production in the CPBR, as it has the highest growth rate and DCM.

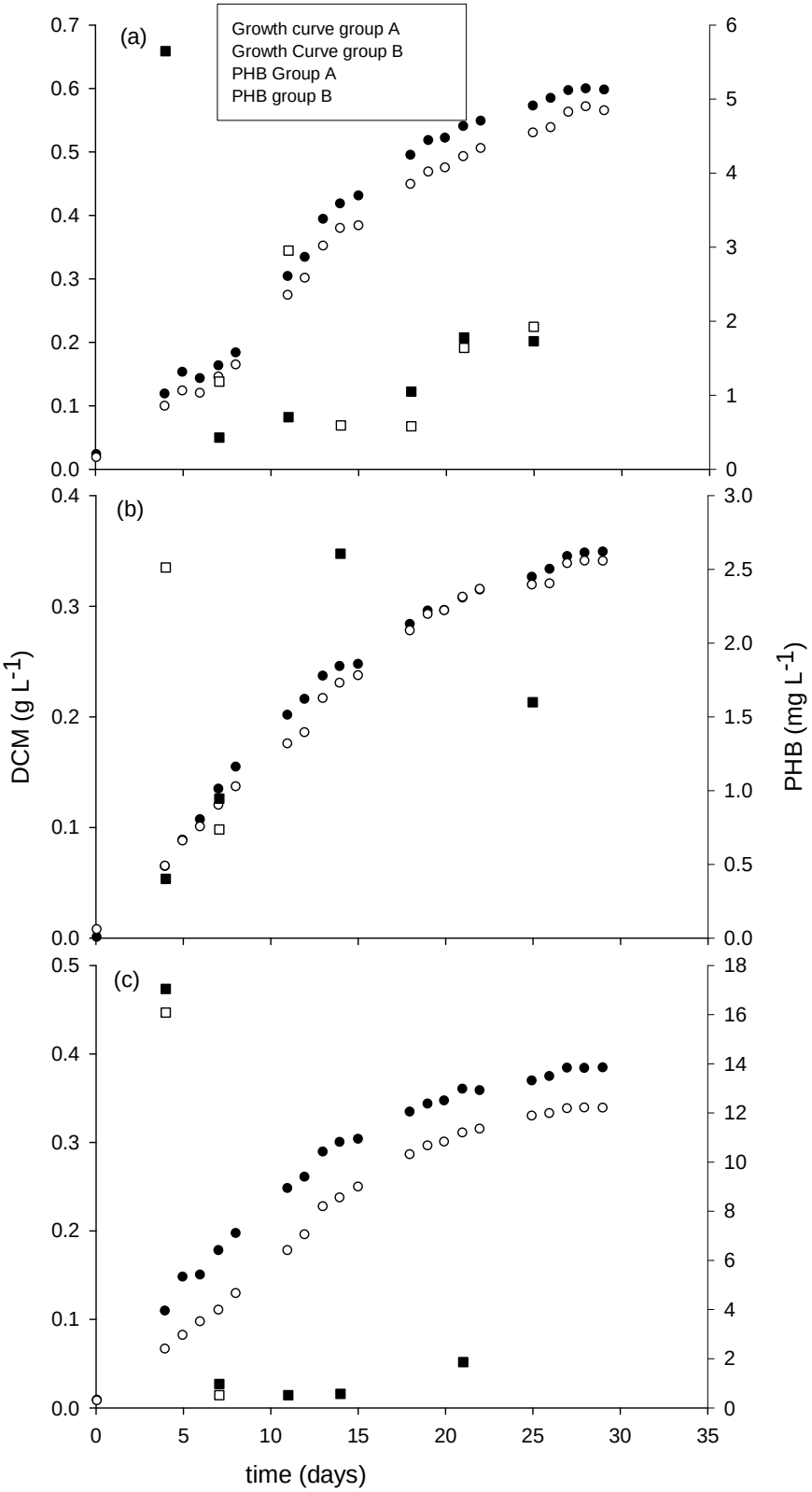

Figure 3.5: Growth and PHB accumulation in mixotrophic conditions, (a) *Anabaena solitaria*, (b) *Microcystis aeruginosa*, and (c) *Microcystis flos-aque*. Note the different ordinate scales.

3.5.3 Nitrogen Deficient Conditions

From the literature it was discovered that nitrogen deficient media will promote the production of PHB within the cell (Asada *et al.* 1999). In this study it was found that under nitrogen sufficient conditions little PHB accumulation occurred, however under nitrogen deficient conditions PHB was accumulated up to 30% of the DCM. Nitrogen limited conditions are expected to produce an increase in PHB concentration as nitrogen limitation switches protein production to carbohydrate production. Figure 3.6 shows the results of growing the cultures under nitrogen limited conditions. The final cell densities of the three cultures were lower than the mixotrophic conditions, and the growth rate was slower. This was expected as under these conditions the rate of photosynthesis decreases until there is not sufficient nitrogen present for cell division as is observed for *Microcystis aeruginosa* in Figure 3.6 (b) after 15 days of cultivation.

Table 3.3: Summary results of nitrogen limited growth

Species	Max Productivity ($g\ L^{-1}\ d^{-1}$)	Max cell density ($g\ L^{-1}$)	Max final PHB ($mg\ PHB\ L^{-1}$)
Anabaena solitaria	0.012	0.15	15.7
Microcystis aeruginosa	0.010	0.14	1.6
Microcystis flos-aque	0.011	0.15	48.7

It is worth noting that the final cell densities were higher than for the balanced conditions due to the health of the inoculate and initial concentration of cells. The two *Microcystis* species reach stationary phase very quickly because they are unable to fix nitrogen from the air, whereas *Anabaena solitaria* has a long lag phase followed by rapid growth, the lag phase was probably due to activation of the heterocysts to fix nitrogen from the air. The *Anabaena solitaria* was able to achieve a higher productivity after the lag phase, which enables the culture to reach a similar cell density with the *Microcystis* cultures. Furthermore, all the cultures changed colour from blue-green to brown and all except *Anabaena solitaria* lost their floating capability, settling on the bottom of the flask despite the aeration. The PHB concentration was significantly greater than was observed under balanced or mixotropic growth. Both *Anabaena solitaria* and *Microcystis flos-aque* performed well under nitrogen deficient conditions accumulating approximately 16 mg L^{-1} and 49 mg L^{-1} of PHB respectively, however because the *Microcystis*

lost its floating properties *Anabaena solitaria* would be better suited to the CPBR under these conditions.

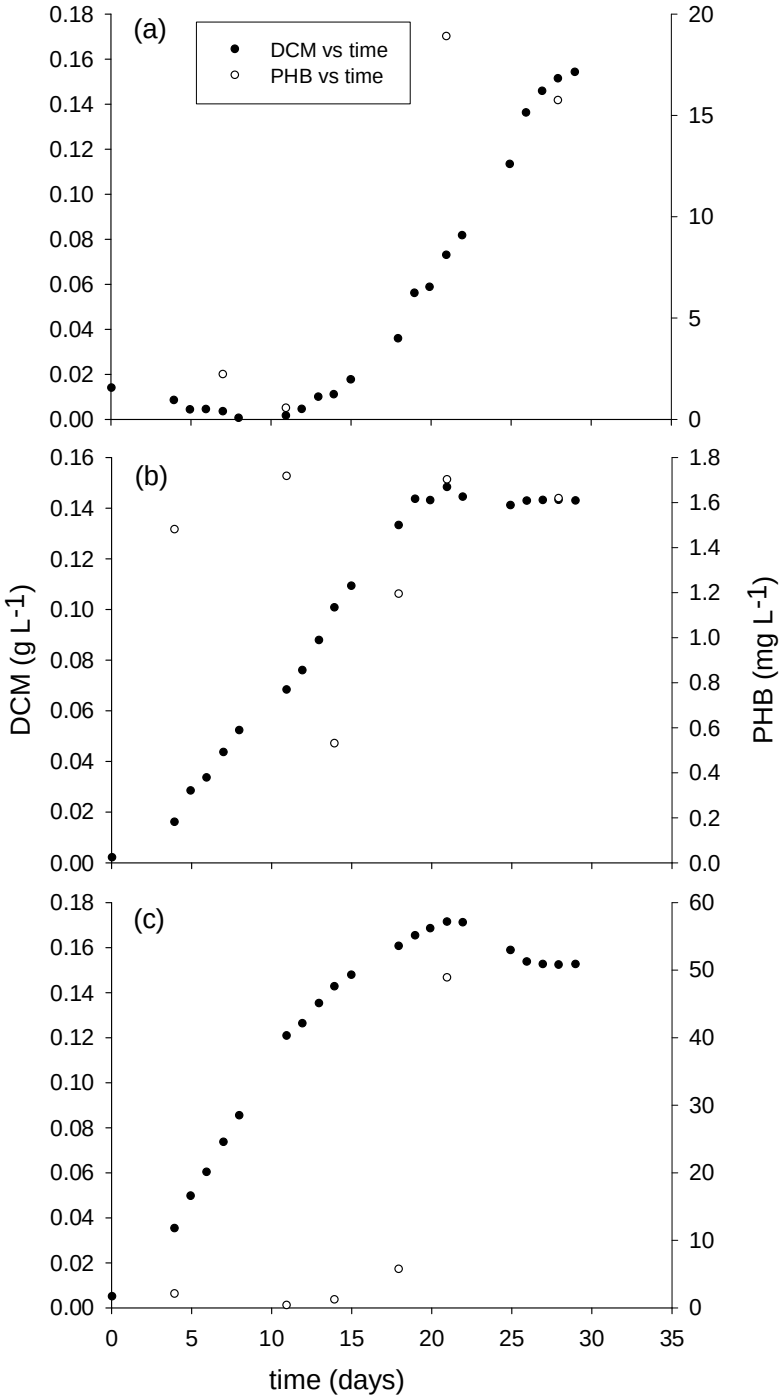

Figure 3.6: Growth and PHB accumulation in nitrogen limited conditions, (a) *Anabaena solitaria*, (b) *Microcystis aeruginosa*, and (c) *Microcystis flos-aque*. Note the different ordinate scales.

3.5.4 Phosphate Deficient Conditions

Like nitrate deprivation, phosphate deprivation has been shown to promote PHB accumulation (Wu *et al.* 2001). Figure 3.7 shows the growth of the cultures when phosphate limited, furthermore all the cultures changed colour from blue-green to pale yellow, and settled on the bottom of the flask, which was similar to what has been encountered by others (Wu *et al.* 2001). From the graphs, it is immediately noticeable that growth curves of all three species of cyanobacteria are very noisy, also the growth rates are very low, in fact only *Anabaena solitaria* exhibited any positive growth.

The reason behind the reduced growth rates is because phosphorus is needed for the synthesis of chlorophyll *a*, and this inhibits the growth of the culture as chlorophyll is needed for photosynthesis. All species did not produce any meaningful PHB, and any PHB that was present in the cells was quickly consumed, perhaps due to adaption to the phosphorus deficient environment, which required the use of the energy in PHB. Overall, phosphate deficient conditions are not suitable for the production of PHB in a PBR, the cultures formed flocs and a biofilm, which are to be avoided for reasons described above.

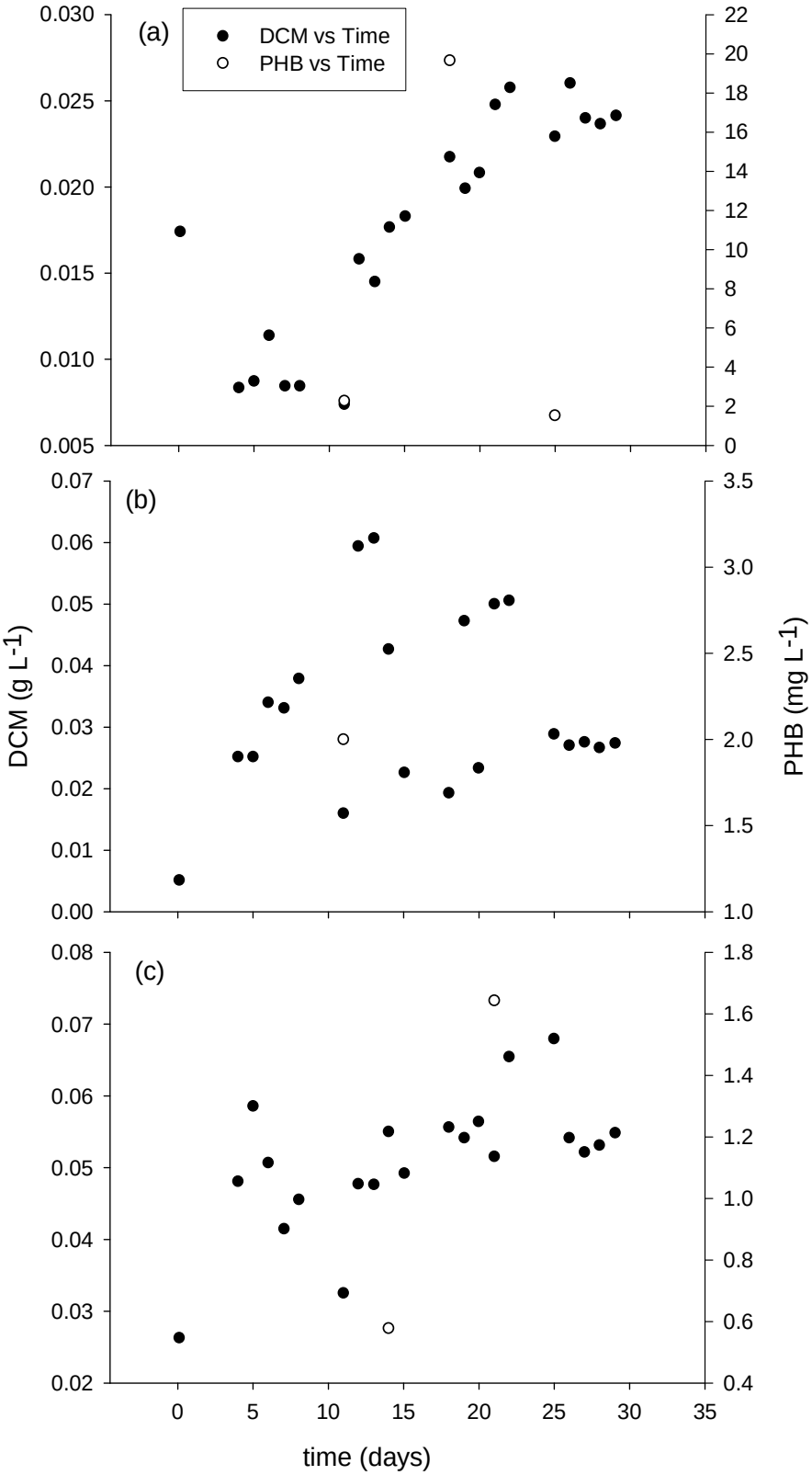

Figure 3.7: Growth and PHB accumulation in phosphate limited conditions, (a) *Anabaena solitaria*, (b) *Microcystis aeruginosa*, and (c) *Microcystis flos-aque*. Note the different ordinate scales.

3.6 Final Candidate and Recommendations

The overall screening of the six Australian cyanobacteria initially yielded three potential candidates for PHB production in the CPBR and of these three species two consistently had both comparable productivies and PHB concentration. It is intended that the PBR be operated under normal balanced conditions. Because of this the balance experiments were considered above the other experiments, and applying the selection criteria it was concluded that *Anabaena solitaria* be used in the PBR because of its higher growth rate and good PHB production, and that it met the CPBR specific selection criteria. After analysing the mixotrophic, nitrogen and phosphate limited experiments it seems that *Anabaena solitaria* was the best all round performer. While *Anabaena solitaria* does not have the highest concentration of PHB under all circumstances it did have the highest productivites of all the tested species, and had consistently high cell densities. It is more important that the higher cell densities are achieved than high concentrations of PHB. The reasoning behind this is that a species of cyanobacteria that produces 50% (w/w) PHB but only grows to 0.5 g L^{-1} (that is 0.25 g L^{-1} PHB) is just as good as a species of cyanobacteria that produces 5% (w/w) PHB and grows to 5 g L^{-1}, however because the latter is less dilute the downstream processing costs will be greatly reduced. This is another reason for choosing *Anabaena solitaria*.

The weight percentages of PHB found in these species is much lower than expected or is usually reported in the literature. Nitrogen limited cultures did show greater PHB accumulation but at the expense of high growth. While beyond the scope of the current research it is recommended that a fed batch experiment be done to determine if it is possible to obtain high cell densities and then induce PHB production.

Six species of cyanobacteria is a very small sample size from which to choose. However screening takes a significant amount of time and there are 30,000+ species of cyanobacteria and microalgae so it simply was not feasible to screen more species with the limitations of this project. The results give confidence that with further screening using the developed methodology and criteria a more suitable species of cyanobacteria can be found that has higher PHB concentrations and higher cell densities.

Chapter 4

4 Production of Selected Species in CPBR

4.1 Summary

Anabaena solitaria was cultivated in the CPBR. While it met the necessary selection criteria, it is not always possible to exactly match laboratory conditions to actual conditions. Because of this it is necessary to validate the growth of *Anabaena solitaria* in the CPBR to determine if it will be a successful candidate for production of PHB in the CPBR. Experiments similar to the preliminary studies were conducted and it was found that *Anabaena solitaria* was able to be successfully grown in the CPBR in a range of conditions.

4.2 Purpose of Reactor Studies

The purpose of these reactor studies was to validate the determined selection criteria. It is not always possible to fully replicate reactor conditions such as temperature, pH, and mixing. These variables can have a significant effect on the growth and physiology of the microalgae, for example at high temperatures the algae may form a biofilm as was observed in the preliminary studies which would make the species not viable for industrial growth in the CPBR. While every effort has been made to select a species with desirable characteristics the performance in the CPBR may differ from that in the laboratory flask experiments, and as such it is necessary to validate the selected species in the CPBR. It is especially important to determine if *Anabaena solitaria* meets the reactor specific selection criteria as these are most difficult for which to screen. To accomplish this experiments similar to those for the preliminary studies were conducted to determine if *Anabaena solitaria* has the desired criteria of a high productivity, PHB production, high cell density, moderate light requirement, and the reactor specific criteria of not forming microbial mats and withstanding bubbling aeration while being cultivated in the CPBR.

4.3 Materials and Methods

The experimental plan for these experiments was initially the same as the preliminary studies; however there were a few differences, as the CPBR was moved from CSIRO to The University

of Adelaide. This required that the CPBR be decommissioned at CSIRO and recommissioned in Adelaide, also not all of the required support equipment was included in the transfer most importantly the refrigerated bath for temperature control and the light source. The lack of this equipment resulted in some experimental changes. Temperature was controlled by manually adjusting the cooling water flow rate.

These studies cultivated *Anabaena solitaria* using BG-11 media. Inoculates were prepared in the same manner as the preliminary studies. Dry cell weight (DCW) was determined in the same manner as for the screening and selection studies. The calibration between optical density at 730nm and DCW was done at the end of each experiment. The calibration is shown in the Appendix (§Calibration Data). PHB content was also determined in the same manner as for the screening and selection studies.

An automatic pH controller was obtained from CSIRO. pH was controlled (Hanna HI8711E pH controller) by pulsing the broth with CO_2, which also as supplies carbon to the culture. The probe for the controller was attached to the head unit of the CPBR, and inserted into the broth. The target pH is set on the controller unit. The CO_2 was supplied to the unit from a G-size food grade CO_2 bottle, the gas flows to the controller, when the pH rises above the set point the CO_2 flows though a rotameter that is used to control the flow, and is then mixed with the air entering the system. The pH is controlled to within ±0.05 units; this error is due to the hysteresis of the controller. The pH was controlled at 7.5 for all of these experiments.

Light was added to the culture via two 500 W halogen lights positioned to one side of the reactor. The amount of light incident on the vessel surface was adjusted by moving the lights closer or further from the CPBR. The halogen globes were changed at the beginning of each experiment to ensure that the light condition was the same for each experiment. The incident and exit light intensity was measured at nine points across the glass surface; the average of these nine points was used as the incident and exit intensity. The incident intensity was measured periodically throughout the experiment to make certain it was still at the set point, making small adjustments as necessary. The intensity was controlled to an accuracy of ±50 $\mu mol\ m^{-2}\ s^{-1}$, the main sources of error is depreciation in the light source and other uncontrolled external lights. To minimise the latter error source the same measures were taken as in the preliminary studies.

The lights provided not only the light energy for photosynthesis but also a considerable amount of thermal energy. Sufficient thermal energy was radiated from the light source such that only cooling was needed to achieve the target temperatures. Temperature was measured using a K-type thermocouple and its control was achieved, as described above, by manually adjusting the flow of water to the cooling coil inside the CPBR. The deviation from the target temperature was ±0.75°C; this error is generated from the manual control. The setup of the CPBR shown in Figure 2.1 was the same.

4.4 Growth and PHB production of Anabaena solitaria in a closed PBR

Table 4.1: Configurations of experiments conducted

	Temperature (°C)	Intensity (μmol m^{-2} s^{-1})
Experiment 1	28	500
Experiment 2	40	500
Experiment 3	38	500
Experiment 4	38	900

Experiments similar to those conducted in the preliminary studies were performed; however because of the relocation of the CPBR the experimental setup was different, and therefore the conditions were also different. A different light source was used, which affected the minimum and maximum incident light achievable. The CPBR was operating in a different environment, which meant the background light and the ambient temperature were different, thus affecting the light to the culture and also the effectiveness of the cooling. As stated in the materials and methods, the temperature was controlled manually with mains cooling water. The lights, which are the major contributor to the thermal energy input to the system were different, and the ambient temperature in the lab was typically 3–5°C cooler than at CSIRO, so the temperatures achievable in the CPBR were different. Furthermore, because of pH control the pH was different for these studies. All differences make it very difficult to compare these experiments with the preliminary studies, nevertheless experiments were conducted at the boundary limits of the system to determine if any detrimental effects could be observed. Table 4.1 shows the configuration of the experiments conducted.

Figures 4.1, 4.2, 4.3, and 4.4 show the growth curves and PHB production for *Anabaena solitaria* for each experimental run. Table 4.2 shows the maximum productivities for each experiment.

Table 4.2: Comparison of productivity for each experiment

Experiment	Final PHB (mg L^{-1})	Maximum Productivity (g L^{-1} d^{-1})
1	5.2	0.271
2	8.5	0.191
3	1.5	0.338
4	N/A	0.214

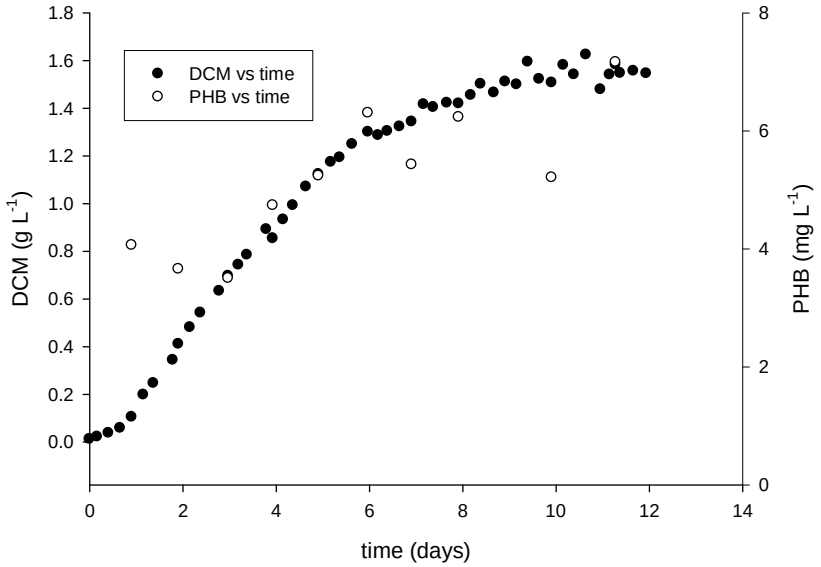

Figure 4.1: Experiment 1

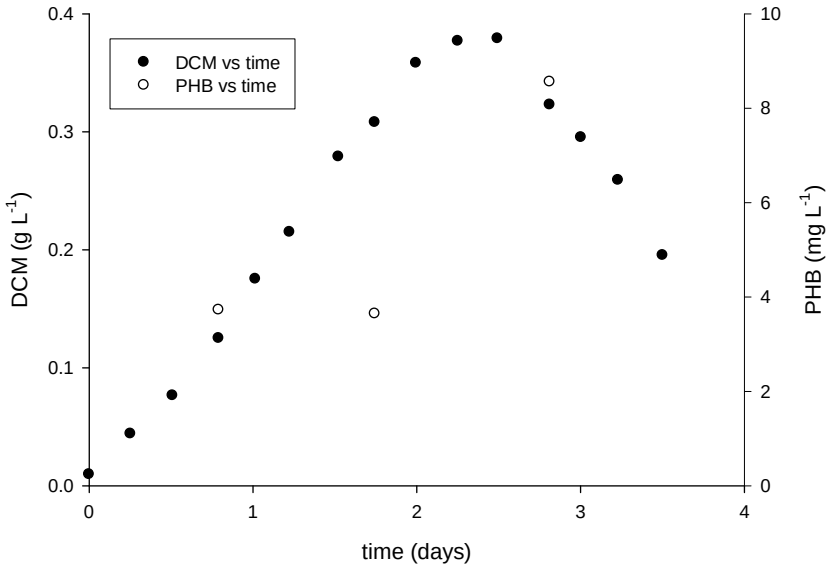

Figure 4.2: Experiment 2

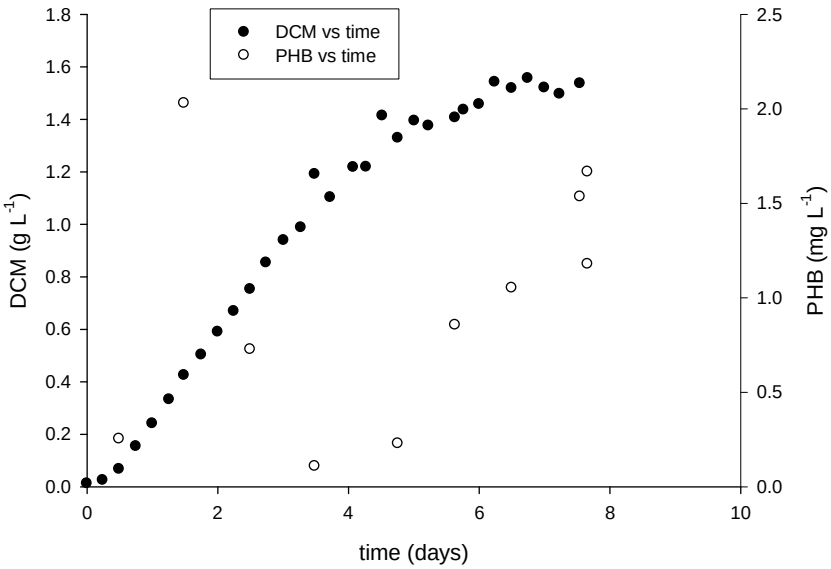

Figure 4.3: Experiment 3

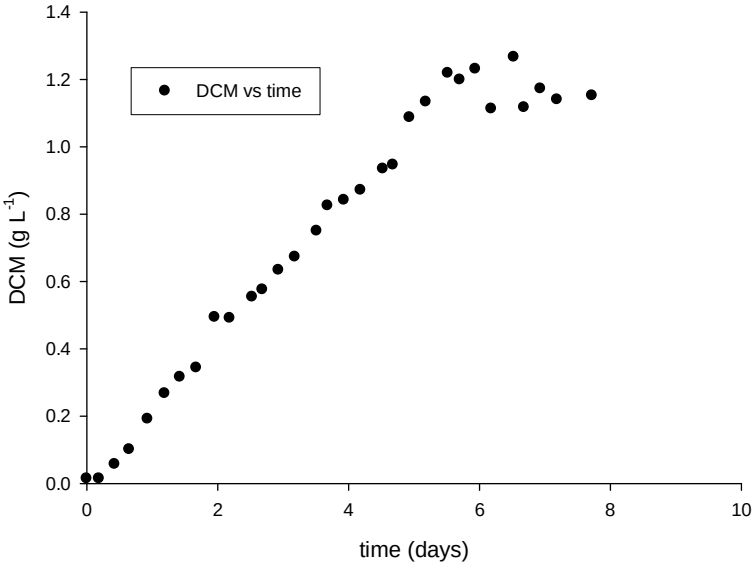

Figure 4.4: Experiment 4

The results reveal very different behaviour between the CPBR and the lab scale cultures. In the lab scale experiments PHB initially decreased (thought to be because PHB is a carbon storage compound and as such was not being accumulated because it was being consumed as a growth substrate) whereas in the CPBR the PHB content remains constant. This difference may be because the pH, hence the carbon supply, within the reactor is controlled and so there is an

abundance of readily available CO_2 for growth, thus the organism does not need to expend energy to metabolise the intracellular PHB, and as such the PHB content remains stable. It can also be seen that the PHB content increases once the culture enters the decline phase, indicative of a secondary metabolite. There is some noise in the PHB data for all the experiments; this was most likely caused in the sample preparation. When samples were esterified at 300°C some of the seals on the reaction tubes failed, releasing a small amount of volatile sample, which changed the concentrations. This was most likely due to repeated exposure to hot chloroform and methanol (the seals were inspected before each reaction but some leakage still occurred). This leakage may have resulted in the PHB content becoming slightly concentrated, as the solvent is more volatile than the ester.

Experiment 1 shown in Figure 4.1, was conducted at conditions similar to experiment 4 of the preliminary studies. These results for *Anabaena solitaria* are similar to those achieved for *Synechoccous* PCC7002, achieving a productivity of 0.271 g L^{-1} d^{-1} compared to 0.210 g L^{-1} d^{-1}, however the maximum cell density was lower at only 1.6 g L^{-1}. This difference is because they are different species and *Anabaena solitaria* has increased metabolic burden because it is producing PHB. As with experiment 4 in the preliminary studies no biofilm was observed, however it is important to note that the culture withstood the bubbling agitation of the CPBR producing a good productivity when compared to *Synechoccous* PCC7002 in the preliminary studies and other PBR given that the experiment was conducted at sub-optimal conditions for the growth of *Anabaena solitaria*. Under the experimental conditions the average final PHB concentration was 5 mg L^{-1}.

Figure 4.2 shows experiment 2, which was conducted at high temperature and moderate light. Table 4.1 shows the growth conditions for this experiment. This experiment shows an initial growth phase for the first two days, followed by a sharp decline in cell mass for the remaining culture time. After the initial inoculation the culture quickly turned brown, and flocs of dead algae could be seen in the broth. While flocs of dead algae did form, no biofilms formed on any of the heat exchange surfaces or illuminated surface, also the flocs stayed in suspension. The broth turned from the typical blue green, to grey green, and finally brown. The reason for this is that the temperature was too high for sustained growth. The CPBR was inoculated with a healthy inoculate in the exponential growth phase, as with all experiments, and the light

condition was the same for both experiments so the only conclusion that can be drawn is that the temperature is beyond the optimal range of the organism. It is interesting to note the PHB content increased during the decline of the algae, however because so few data points were collected it is statistically insignificant and therefore inconclusive.

Experiment 4, Figure 4.4, was conducted at high light intensity and moderate temperature. At these conditions in the preliminary studies photoinhibition was observed, and resulted in low productivities. In the CPBR *Anabaena solitaria* did not show any of the characteristics of photoinhibition, there was no observed change in colour, and only a decrease in productivity compared to Experiments 1 and 3. Furthermore, no biofilm formed on the illuminated surface or heat exchange surfaces, validating that *Anabaena solitaria* meets the selection criteria for growth in the CPBR.

Figure 4.5 shows the results normalised against the initial cell density to make comparisons easier. It is obvious that experiment 2 was outside of the organisms preferred operating conditions, and that a temperature of 38°C is better for growth than 28°C. Further evidence of this is that the maximum growth rate for experiment 3 was 3.92×10^{-5} s^{-1} compared with 2.84×10^{-5} s^{-1} for experiment 1, whereas the growth rate for experiment 2 was 2.09×10^{-5} s^{-1}. From Figure 4.3, it is very interesting to note that the PHB production of experiment 3 is much lower than experiment 1 while the productivity of experiment 3 is higher than experiment 1, suggesting that lower temperatures favour PHB production over growth.

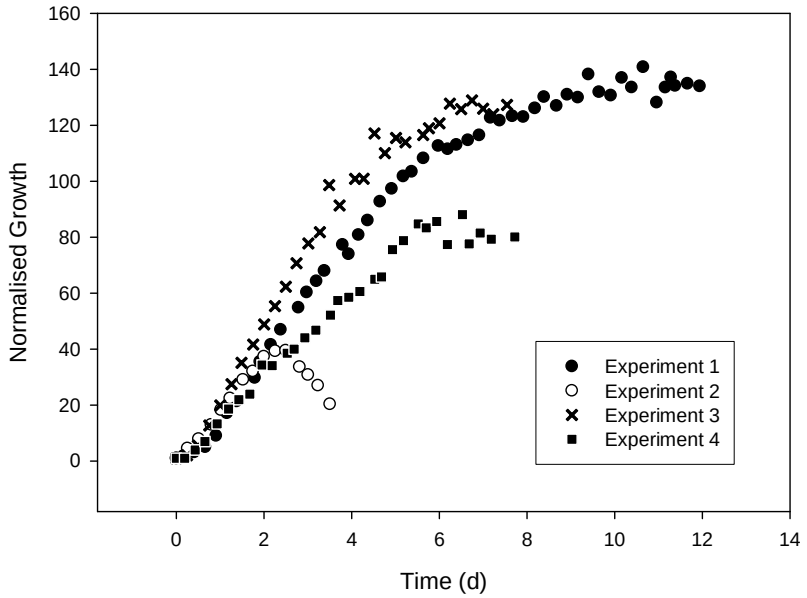

Figure 4.5: Comparison of all Experiments

From Figure 4.5, a comparison of experiments 3 and 4 shows that increasing the incident light intensity decreased the growth rate. No PHB data exist for experiment 4 because the GC was out of commission, and the capillary column had degraded.

4.5 Outcomes of Reactor Studies

These experiments show a successful validation of the selection criteria, and show that *Anabaena solitaria* is a suitable candidate for growth in the CPBR. *Anabaena solitaria* had productivities comparable with the preliminary studies and other PBR, reasonable cell densities were achieved, and PHB levels consistent with the literature were produced. It is necessary to reiterate that the CPBR was operating at suboptimal conditions, and so the highest yield achievable was not reached. Greater productivity could be achieved with optimisation. Importantly the reactor specific selection criteria have been met. *Synechoccous* PCC7002, when stressed with either high temperature or incident light intensity produced a biofilm that fouled the illuminated surfaces and heat exchange surfaces of the CPBR, but *Anabaena solitaria* did not exhibit such behaviour.

Chapter 5

5 Conclusions and Recommendations

5.1 Conclusions

The aim of this study was to screen and select a suitable species of microalgae that produces PHB for cultivation in the CPBR. The following set of generic selection criteria were initially proposed:

- High growth rate
- High final cell density
- Moderate temperature and light requirement
- High PHB content
- Australian native species

It was not known if there were any reactor specific selection criteria. The CPBR specific criteria were determined by conducting preliminary experiments with *Synechoccous* PCC7002. These studies revealed the reactor specific criteria of:

- Must withstand bubbling agitation
- Must not form biofilms

Furthermore, it was thought that *Synechoccous* PCC7002 would produce PHB (Vincenzini *et al.* 1999), however it was found that it did not produce PHB as it did not possess the necessary genes.

Using the above criteria a rigorous screening methodology was developed and applied to several species of Australian cyanobacteria identified to be potential PHB producers. From this process *Anabaena solitaria* was found to be suitable for growth in the CPBR.

The selection criteria and methodology were further validated by cultivating *Anabaena solitaria* in the CPBR. *Anabaena solitaria* performed as required achieving a maximum productivity of 0.338 g L^{-1} d^{-1} and a maximum PHB concentration of 8.5 mg L^{-1}, in separate experiments.

Because of this *Anabaena solitaria* should be considered as a potential candidate for the production of PHB.

5.2 Recommendations

Further work is needed, as the scope of these studies was limited to suboptimal conditions. It is therefore recommended that optimisation and scale up studies be conducted so that an accurate economic analysis can be conducted to fully evaluate the potential for microalgae to produce PHB. The number of species screened was limited and, given the vast number of microalgal species it is recommended that more species be screened using the developed selection criteria and methodology, to find a better candidate for cultivation in the CPBR. It is also recommended that further closed PBR be investigated, as they may provided a better platform for the production of PHB from cyanobacteria, however new selection criteria would need to be developed for any new system.

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Appendix

Standard Operating Procedures

Algal Growth Media Preparation

For these experiments several growth media will be used these are modified BG-11 for both freshwater and sea water, SW-BTP media which is only used for sea water.

A common component of all the media is the metals mix, the procedure for making this is detailed below

Metals Mix

The Metals mix is made up of two components and is used in all the media. The first compontent is the FeEDTA mix and the second component is the trace metals mix.

Part One - FeEDTA mix.

- 6.7 g Ferric citrate]
- 14.9 g Na_2EDTA] 500 mL with H_20 after boiling/pH adjust.

1. Add above component to 500mL of RO water. Adjust pH to 7.0 and boil to dissolve

(NB: requires a lot of 5N NaOH and colour darkens at around pH 7.0. Check pH after boiling and readjust to ~ 7 if necessary. Reboil if necessary.

2. Mix with trace metals mix when complete.

Part Two - Trace metals mix.

- H_3BO_3 2.86 g
- $MnCl_2.4H_2O$ 1.81 g
- $ZnSO_4.7H_2O$ 0.222 g
- $Na_2MoO4.2H_2O$ 0.390 g
- $CuSO_4.5H_2O$ 0.079 g
- $Co(NO_3)_2.6H_2O$ 0.049 g

1. Mix above components in 500 mL of RO water.

2. Mix together with FeEDTA mix to give a 1 litre Metals Stock.

3. Store in bottles in the fridge. Use 1 mL per litre for 1 X JDM media..

BG-11 media

To prepare BG-11 media add the salts below to RO water. For a sea water media supplement this with 18g/dm^3 (1X) sodium chloride.

	MW	**for 8dm^3 (2X)**	**for 1dm^3 (2X)**	**for 1dm^3(1X)**
K_2HPO_4	174.2	0.488 g	61 mg	30.5 mg
$MgSO_4$.7H2O	246.7	1.20	150 mg	75 mg
$CaCl_2.2H_2O$	147.02	0.576	72 mg	56 mg
$NaNO_3$	84.99	24.0	3.0 g	1.5 g
Metals mix	--	16 mL	2 mL	1 ml

Too reduce error 100X stock solutions of the first three salts need to be prepared and stored. The recipes for these are shown below.

K_2HPO_4	3.05 g / 100 ml
$MgSO_4$.7H2O	7.5 g / 100 ml
$CaCl_2.2H_2O$	5.6 g / 100 ml

Then add 1ml of the above solutions to the media, instead of weighing out small amounts of salts.

Sterilize the media in the Autoclave following the Autoclave SOP.

Once sterile aseptically add 100 nm^3 (μL) of Biotin (vitamin H) and B12 just prior to use.

Important: autoclave immediately to stop fine precipatate forming (autoclaving next day does not work). Do not leave old JDM media in the large 8 L mixing bottle. 2 X JDM without buffer is not stable after auotclave (ppt forms) but 1X JDM is OK.
For plates, 2 X JDM is buffered with 100 mM TES-KOH (for 50 mM final). The 1 M Tes-KOH pH 8 stock must be autoclaved to stop fungal growth and then kept in the fridge.
For BTP liquid media, add 10 mL of 1M BTP-NaOH (pH 8.0) per litre of 1 X JDM for 10 mM final concentration. Keep 1 M stock of buffer in fridge, preferably autoclaved.

SW-BTP media

The preparation of SW-BTP media is similar to the preparation of BG-11 media. Add the salts below in the quantity listed to RO water in the order listed.

per litre

K_2HPO_4	50 mg
$CaCl_2$	36 mg
$MgSO_4.7H_2O$	5 g
$NaNO_3$	1.5 g
KCl	0.6 g
NaCl	18 g
JDM metals mix	1 mL
1M BTP-HCl (pH 8.0)	10 mL

Autoclave and store at RT (fast cool to reduce precipitates). Add 1 mL of biotin (see above) just prior to use of the liquid media.

These modicfications based on A+ from Stevens etal (1973) J Phycol. **9**; 427-430. Essential features of SW-BTP were (compared to A+) to reduce $CaCl_2$ by 7.5 times and replace 4 mM Tris with 10 mM BTP; also a 50% increase in nitrate and generally less of the trace metals. NB: Original A+ contains 1 mL of 4 mg/L Biotin per litre of media. (Note that Vicki/Don claim that biotin can inhibit transformation in 7002.

Procedure for growing of algal cultures

Preparation of cultures in flasks

1. sterilze appropriate Elinmier flask.
2. aseptically add sterile media to the flask.
3. inoculate with 10%(v/v) algal culture.
4. replace plug in the top of the flsak and leave sitting.
5. swirl occasionally to remove dissolved oxygen, and alloew gas exchange.

Preperation of cultures for gowth cabinet

1. Perform steps 1 – 3 of method above.
2. Cut a length of the small diameter tubing so that it will reach from the nozzle into the bottom of the flask.
3. Connect the cut length to the nozzle and feed the tubing into the flask.
4. Replace plug in the top of the flask.
5. Turn on the nozzle untill a decent amount of bubbles are issueing from the tube mouth into the flask.

Dry cell mass determination

By centrifugation

1. Weigh a pre-dried 10mL skirted sample tube, and lable it.
2. Aseptically take 8mL of culture into a the sample tube.
3. Centrifuge the tube minus the lid at 5000rpm for 10 minutes using the Sorval RC-5 centrifuge with the HB-6 rotor and the rubber inserts.

NB: use the centrifuge according to the centrifuges SOP.

4. Remove the supernatant from the tube and dispose of it.
5. Place the tubes minus with the lid on loosly in an oven at 60^0C.
6. Leave in oven until the tube has reaced constant weight.
7. Record the weight.
8. The dry cell concentration is; the weight from setp 7 minus the weight from step 1 divded by the sample volume (8mL), times 1000. (g/L).

By filtration

1. Place a sigle piece of the 70mm GA55 filter paper into a empty and clean agar plate with several holes drilled into it.
2. Put the agar plate in the oven at 50-60^0C and dry it for 3-4 hours (overnight is best).
3. Weigh the agar plate and filter paper.
4. Put the agar plate and the filter paper into the vacuum desiccator and leave it for 1 hour.
5. Weigh the plate and paper.
6. Take out filer paper and place in 70mm Buchner funnel, apply vacuum and wet the filter paper so it lays flat.
7. Carfully filter 10mL of sample through the filter paper, being particulary carfull not to get any sample on the edges of the paper. *The best way to do this is to add the sample drop by drop in concentric circles or as a spira (strting from the center)l, if you get too close to the edge start from the center again.*
8. Wash the sample with 20mL of 0.5M Ammoniumbicarbonate.
9. Carfully remove the paper so that it does not rip.
10. Place it in the same agar plate and place it in the oven till dry, again overnight is best.
11. Remove the agar plate and weight it and the paper.
12. Place them in the desiccator again and leave for 1-2 hours.
13. Weight them again.
14. The dry weight is the weight from step 13 subtact the weight from step 5.

PHB determination by GC

Standards preparation.

Acidified Methanol Solution Preparation

1. Accurately measure 50mL of Methanol into a beaker.

2. Slowly add 1.5mL of Sulphuric Acid to the beaker.
3. The solution is now a 3%(v/v) Acidified Methanol Solution.

Internal Standard Preparation

1. Accurately measure 20.14mL of Methanol.
2. Add 80μL of Methyl Benzoate to the Methanol.
3. The solution is now a 5.5g/Kg internal standard.

PHB standard preparation

1. Place 25mL container on scales and zero it.
2. Accurately weigh 0.8g of PHB into it.
3. Remove from scales, and in a fume hood add 10mL of Chloroform and 10mL of Acidified Methanol. This is now the stock solution.
4. To 8mL reaction tubes add the stock solution in the following amounts, in a fume hood.

Final conc^{n} necessary (mg)	Amount stock sol^{n} added to tube (μL)	Vol. Chloroform added to tube (mL)	Vol. A/Methanol added to the tube (mL)
4	100	1.95	1.950
3	75	1.963	1.963
2	50	1.975	1.975
1	25	1.989	1.989

5. To each tube add 200 μL of internal standard.
6. Tightly cap the tubes and place in baking box.
7. Bake at 100^{0}C for at least 3.5 hours (210 min) shaking occasionally. (*NB. The longer in the oven the better, allowing for more total reaction*).

Sample Preparation

1. Using the centrifuge the tubes at 1100g (3000 rpm) for 10 minutes.
2. Remove the supernatant using an 11cm needle, taking care to avoid dislodging the pellet.
3. Repeat steps 1 to 6 for all tubes.
4. Dry the samples in the oven at 40^{0}C for 30 – 60 min.
5. Make up some standards as described in the standards preparation section section below.
6. Dry the tubes in the oven for 20 minutes at 40^{o}C, then cool and dry in a vaccume dessicator to 1 hour.
7. Preheat the oven to 100^{o}C for step 14.
8. This step must be performed in a fume hood. To each GC tube, add 2 cm^{3} chloroform, 2mL acidified methanol and 200 nm^{3} of internal standard. See below for reagent preparation.
9. Seal each GC tube TIGHTLY with a cap. DO NOT USE CAPS WHICH ARE VISIBLY WEAKENED. During the baking procedure, the internal pressure of the tubes will reach 3-4 atmospheres. Beware of some older caps which have rubber seals instead of the necessary teflon.

10. Mix well by shaking vigorously.
11. Place the GC tubes (broth samples and standard solutions) into the plastic reaction box and tape the lid securely shut. NB the plastic box will not withstand extended exposure to chloroform. Avoid spilling chloroform on it.
12. Place the box into an oven at 100^{o}C for at least three and a half hours. Shake the box two or three times during the incubation.
13. At the end of this time, remove the box and allow it (and the GC tubes) to cool to room temperature (an hour or so). DO NOT OPEN THE BOX BEFORE IT HAS COOLED.
14. Once cooled, remove the GC tubes from the box. Add 1 mL of high purity reagent water ("Milli-Q") into each tube and agitate it with the vortex mixer for thirty seconds or so. Two phases should form after some settling time, with cell debris accumulating at the interface. Generally overnight is long enough for this to occur. The lower phase contains the PHB.
15. Remove the top phase from the tube and discard, it is ok if some of the lower phase is removed here as long as the entire top phase is removed.
16. Add ½ grain of anhydrous sodium carbonate (Na_2CO_3). This is done to scavenge any remaining water in the organic phase.

GC analysis

1. Start up the GC, following the SOP. If a piercing whistle sounds when the FID is lit, don't worry it disappears after a few minutes when the assembly warms up.
2. Load the appropriate method (eg PHA.met)
3. For each standard solution, inject exactly 2 nm^3 of the bottom phase. Try to avoid drawing any cell material into the syringe.
4. When the run is finished, view the chromatogram using "Post-Run Analysis" - "Single".
5. Calculate the ratio of butyrate peak area to internal std (methyl benzoate) peak area. Occasionally the GC software may have trouble integrating the peaks, you can use the 'manipulate' option to exert greater control over its behaviour.
6. Plot the standards' results to obtain a calibration curve.
7. For the other samples, inject exactly 2 nm^3 of the bottom phase and find the peak area. Use the calibration curve to calculate the mass of PHB present in the sample.

Reactor Operation

Cleaning

1. Make sure the power is turned off.
2. Disconnect hoses from the head unit.
3. Unscrew and remove head unit from reactor.
4. Detach the sprager from the head unit and place in a bucket of water. Blow air though the sprager to clear it out.
5. Dissasemble the sprager and using a toothbrush and 70% EtOH clean out the sprager.
6. Rinse sprager with RO water.
7. Syphon out the broth from the reactor into a suitable receptacle:

a. Fill a length of hose with water, making sure to remove all air bubbles. Cap both ends of the hose to seal the liquid in.
b. Place one end in the reactor and one end in the receptacle, make sure the receiving vessel is lower than the reactor.
c. Unseal both ends, the fluid should flow from the reactor to the receiving vessel.
d. Treat the effulent with lugols.

8. Remove the sealing foam from the head unit and discard
9. Clean the head unit with 70% EtOH and a tooth brush, rinse with RO water.
10. Remove and discard any sealing foam attached to the reactor.
11. Rinse the reactor vessel with RO water, and scrub with a long handled scrubbing brush.
12. Syphon out any water into the waste vessel (also called receiving vessel), as described above.
13. Repeat step 13 till reactor is clean
14. Rinse reactor with 70% EtOH, invert the vessel on a draining board and let dry
15. Reactor is now clean and ready for sterilising.

pH Controller Calibration

1. See operaton manual for Hanna HI8711E pH controller.

Operation

Sterilising Reactor

1. Screw head unit onto reactor vessel.
2. Cover any openings with alfoil.
3. Place in oven at 200^{o}C for three (3) hours.
4. Remove and wait till cool before using.

Preparing Reactor

1. Sterilise reactor.
2. Place reactor in the catchment tray, square to the light source.
3. Set incident intensity.
4. Stick closed cell PVC foam to the under side of the head unit, inside of the screws.
5. Thread K-type thermocouple wire though the thermocouple hole.
6. Attach the sprager to the underside of the unit.
7. Fill reactor , set temperature.
8. Place head unit on reactor, so that the sprager drops into the center of the vessel.
9. Allow the temperature to satablise then pour in 1dm^3 of culture though the sample port.
10. Screw down the reactor tighly.
11. Place pH probe in the correct position, and seal with Bluetack™.
12. Attach cooling hoses and air/CO_2 line to the reactor.
13. Seal any other openings with stoppers/Bluetack™.
14. Reactor vessel is prepared and ready to use.

Setting Incident Intensity

1. Adjust the light postion (either forewards or backwards).
2. Measure the light intensity using the light meter (1000 lux = 20μE/m^2.s), in the nine postions marked on the surface of the glass.
3. Average the result, this is the incident intensity.
4. If the intensity is too high, move the lights back and re-measure.
5. If the intensity is too low move the lights forward and remeasure.
6. Repeat streps 4 and 5 untill at the required light intensity.

Filling

1. Sterlise 8.5dm^3 of BG-11 media.
2. Pour media into reactor before attaching the head unit.
3. Attach head unit.
4. Allow temperature to stabilise.
5. Reactor is now filled.

Temperature and Cooling

1. Turn on lights.
2. Allow the temperature to rise to the desired level.
3. Turn on cooling water and adjust flow to maintain temperature.

Airflow

1. Turn on pumps.
2. Adjust rotameter so to the desired flow.

Data Collection

Cell Density

1. Collect 1cm^3 of sample in an curvette.
2. Measure the optical density at 730nm, zeroed against media.
3. Determine the cell density from the calibration chart.

Constructing a Calibration chart

1. Take a DCW sample from the reactor.
2. Take a 10cm^3 sample from the reactor and measure its optical density at 730nm
3. Dilute the sample by 1:5 and record the optical density.
4. Take the dilution from step 3 and dilute 1:5 and record optical density.
5. Repeat steps 4, each time diluting from the previous sample until there are 20 points.
6. Work out the dilutions for the DCW.
7. Plot DCW versus OD.
8. This is the calibration chart.
9. Re-calibrate at the end of each experiment.

Attenuation

1. Measure the intensiy of light leaving the reactor at the five points marked on the back.

2. Average the result
3. This is the exit intensity.

PHB

1. Collect a 4cm^3 sample from the reactor and test for PHB.

Shut Down

2. Turn off the pH controller, and pumps.
3. Remove all tubing from the reactor head unit.
4. Clean the reactor.

Preliminary Experiments – Raw Data

NB: All flowrates are given as the rotameter reading (cm). Light intensity readings were taken in mV using a calibrated photodiode. 1mV = 1000 lux.

Table III: Experiment 1 - Raw data

Date & Time	I (mV)		Flowrates (cm)			pH	T (C)	dry wt (g)	DCM (g/l)
	FO	BO	air	CO2	exhaust				
17/06/2003 15:39	-	-	23	5	-	8.6	21	0.0002	0.02
17/06/2003 17:00	9.64	4.35	24	1	2	8.3	23.8	0.0002	0.02
18/06/03 09:00	8.5	4.3	23	1	2	8.5	28.4	0.0005	0.05
18/06/2003 11:00	9.4	3.9	21	4.5	2.5	9.4	10.8	-0.0004	-0.04
18/06/03 13:00	9.7	3.7	23	1	2.5	8.7	21.2	1E-04	0.01
18/06/03 15:00	9.8	3	22	2	2.8	8.5	21.6	0.0002	0.02
18/06/03 16:20	9.4	2.7	22	1	2.8	8.5	22	1E-04	0.01
19/06/03 08:56	9.5	3	21	0.5	2.8	7.8	21.8	-0.001	-0.1
19/06/03 11:08		3	22.8	0	2.8	8.15	21.9	-0.0004	-0.0392157
19/06/03 14:00		2.9	22.8	0	2.8	8.39	22	-0.0006	-0.06
19/06/03 16:00		2.7	23	0	2.8	8.43	22	-0.0008	-0.08
20/06/03 09:01		2.8	23.4	5.75	3	8.53	21.9	0.0014	0.14
20/06/03 12:00		2.7	23	0	2.4	8.45	21.9	0.0018	0.18
20/06/03 16:00		2.4	23.2	0.5	3	8.55	22.1	0.0005	0.05
23/06/03 09:03		0.4	22.2	3	2.5	9.09	21.6	-0.0351	-3.9
23/06/2003 12:27		0.38	22.2	2	2.8	8.33	21.9	0.0033	0.33
23/06/03 15:00		0.34	23.2	1.5	3.5	8.4	22.1	0.0022	0.22
24/06/03 09:15		0.23	22.8	1	3.6	8.46	21.5	0.006	0.6
24/06/03 15:00		0.2	22.8	1.2	3.6	8.43	21.6	0.006	0.6
25/06/03 09:22		0.2	23.4	2.5	3.7	8.96	21.3	0.0078	0.78
25/06/03 15:14		0.17	23.2	1.5	3.5	8.27	22.4	0.0075	0.75
26/06/03 09:49			21	1.5	-	8.56	21.7	0.0069	0.69
26/06/03 15:00		0.15	21.2	1	6.8	8.5	22	0.0064	0.64
27/06/03 10:40		0.131	21.2	0	6.2	8.36	35.1	0.0071	0.71
27/06/03 15:55		0.137	21.2	0	6.3	8.23	21.4	0.008	0.8
30/06/03 10:32		0.075	25.3	0	7	10.04	22.2	0.0103	1.03
30/06/03 15:35		0.152	25.2	0.5	6.4	8.31	19.1	0.0101	1.01
01/07/03 09:00		0.172	23.8	0	7	9	21.7	0.0119	1.19
01/07/03 15:00		0.122	22.8	1	6.5	8.52	22.1	0.011	1.1
02/07/03 09:13		0.113	23	1	7	8.09	22.7	0.0133	1.33
02/07/03 15:46		0.113	23	0	7	8.5	22.1	0.0126	1.26
03/07/03 09:14		0.101	22.6	0	6.5	9.02	22.1	0.0128	1.28
3/07/2003 10:52		0.101		1.5		8.5	22.1		
3/07/2003 15:00		0.07	22.7	0	6.5	8.56	22.1	0.0143	1.43
3/07/2003 15:00		0.07	22.7	0	6.5	8.56	22.1	0.0144	1.44

Continued on next page…

Date & Time	I (mV)		Flowrates (cm)			pH	T (C)	dry wt (g)	DCM (g/l)
	FO	BO	air	CO2	exhaust				
4/07/2003 9:19		0.047	21.2	0	6.5	8.91	22.4	0.0149	1.49
4/07/2003 9:19		0.047	21.2	0	6.5	8.91	22.4	0.0159	1.59
4/07/2003 10:00			22	1		8.9			
4/07/2003 12:15				0		8.36			
4/07/2003 15:10			21.8	0.5	6.4	8.47	22.3	0.0143	1.43
4/07/03 15:10			21.8	0.5	6.4	8.47	22.3	0.0152	1.52
07/07/03 09:12		0.042	20.8	0.5	6.5	9.14	22.8	0.0168	1.68
07/07/03 09:12		0.042	20.8	0.5	6.5	9.14	22.8	0.0173	1.73
7/07/2003 9:15				1		9.14			
7/07/2003 9:50			22.7	1	6.8	8.96	22.8		
7/07/2003 11:07		0.032	21	0.5	6.8	8.5	22.7		
7/07/2003 14:47		0.033	21	0	6.4	8.38	22.8		
7/07/2003 15:13		0.031	21	0.5	5.8	8.44	22.8	0.0174	1.74
7/07/2003 15:13		0.031	21	0.5	5.8	8.44	22.8	0.0182	1.82
7/07/2003 16:33		0.055	21	0.5	6	8.51	22.7		
8/07/2003 9:08		0.027	21	0.5	6.2	8.57	22.7	0.0173	1.73
8/07/2003 9:08		0.027	21	0.5	6.2	8.57	22.7	0.0186	1.86
8/07/2003 12:22		0.03	20	0.5	5.8	8.57	22.8		
8/07/2003 15:00		0.032	20.6	0.5	6.4	8.52	22.8	-0.0165	-1.6666667
8/07/2003 15:00		0.032	20.6	0.5	6.4	8.52	22.8	0.0174	1.75757576
9/07/2003 9:12		0.067	20.7	0.5	6.2	8.77	22.9	0.0191	1.91
9/07/2003 9:12		0.067	20.7	0.5	6.2	8.77	22.9	0.0192	1.92
9/07/2003 9:22		0.067	20.7	1	6.2	8.77	23		
9/07/2003 10:43		0.074	23	0.5	6.5	8.54	22.2		
9/07/2003 15:06		0.072	22.5	0.5	6.4	8.6	22.2	0.0179	1.79
9/07/2003 15:06		0.072	22.5	0.5	6.4	8.6	22.2	0.0195	1.95
10/07/2003 9:35		0.074	21.5	0	6.5	8.39	21.8	0.0566	5.66
10/07/2003 9:35		0.074	21.5	0	6.5	8.39	21.8	0.0199	1.99
10/07/2003 15:26		0.073	22.6	0.5	6.7	8.48	21.8	0.0215	2.15
10/07/2003 15:26		0.073	22.6	0.5	6.7	8.48	21.8	0.0216	2.11764706
11/07/2003 9:18		0.066	21.2	0.5	6.8	8.45	21.8	0.0184	1.84
11/07/2003 9:18		0.066	21.2	0.5	6.8	8.45	21.8	0.0191	1.91
11/07/2003 11:28		0.05	0	0	0	8.51	22.3		
11/07/2003 11:57		0.05	22	0.5	6.4	8.55	23.8		
11/07/2003 15:21		0.051	22	0.5	6	8.49	21.7	0.0203	2.03
11/07/2003 15:21		0.051	22	0.5	6	8.49	21.7	0.0193	1.93
14/07/2003 9:05		0.039	19	0.5	6	8.51	22.2	0.0219	2.19
14/07/2003 9:05		0.039	19	0.5	6	8.51	22.2	0.0218	2.18
14/07/2003 16:00		0.043	21	0.5	5.2	8.5	22.3	0.0244	2.44
14/07/2003 16:00		0.043	21	0.5	5.2	8.5	22.3	0.0298	2.98
15/07/2003 9:30		0.039	20.5	0.5	6.4	8.81	22.6	0.0217	2.17
15/07/2003 9:30		0.039	20.5	0.5	6.4	8.81	22.6	0.0208	2.08
15/07/2003 13:27		0.039	21	0.5	5.9	8.48	22.3		
15/07/2003 15:28		0.033	21	0.5	5.9	8.5	21.2	0.0119	1.2020202

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Date & Time	I (mV)		Flowrates (cm)			pH	T (C)	dry wt (g)	DCM (g/l)
	FO	BO	air	CO2	exhaust				
15/07/2003 15:28		0.033	21	0.5	5.9	8.5	21.2	0.0098	0.98
16/07/2003 9:25		0.032	20.2	1	6.4	8.64	22.4	0.0207	2.07
16/07/2003 9:25		0.032	20.2	1	6.4	8.64	22.4	0.0246	2.46
16/07/2003 9:25		0.032	20.2	1	6.4	8.64	22.4	0.0217	2.17
16/07/2003 9:25		0.032	20.2	1	6.4	8.64	22.4	0.02	2
17/07/2003 9:20		0.045	20.6	0	6.3	8.46	22.1	0.0203	2.03
17/07/2003 15:03		0.034	20.8	0	6.3	8.48	21.8	0.0198	1.98

Table IV Experiment 2 - raw data

Date & Time	sample	I (mV) BO					air	CO_2	exh		
		1	3	5	7	9	(cm)	(cm)	(cm)	pH	T (C)
09/09/03 11:44		12.1	15.6	12.5	11.6	13.4	18.2	1.5		8.58	30.1
09/09/03 11:58							22	0		8.28	34.1
09/09/03 13:17		13.9	15.2	9.8	13.4	10.8	21.8	0		8.46	44.2
09/09/03 15:00	2.1						22.4	0.5		8.52	41.8
09/09/03 15:28							22.7	1		8.55	41.8
09/09/03 16:53							22.7	0.5		8.46	42.2
10/09/03 08:40	2.2						22	0		9.84	41.7
10/09/03 08:40							22	2.5		9.84	41.7
10/09/03 09:16							22	0		8.37	41.7
10/09/03 09:50							24	0		8.41	41.7
10/09/03 10:59	2.3	10.3	12.7	9.9	8.8	8.5	21.4	1		8.53	41.7
10/09/03 10:59	2.4	10.3	12.7	9.9	8.8	8.5	21.4	1		8.53	41.7
10/09/03 11:41							23	1		8.5	41.9
10/09/03 11:51							22.8	0		8.37	41.9
10/09/03 15:44	2.5	10.2	11.2	9.8	10.5	8.9	20	0	4.8	8.39	41.9
10/09/03 15:44	2.6	10.2	11.2	9.8	10.5	8.9	20	0	4.8	8.39	41.9
10/09/03 16:10							20	0.5	5	8.45	41.9
11/09/03 08:36		4.5	6.5	8.6	4	4.1	22	0	7	7.71	41.7
11/09/03 09:09	2.7	9.6	11.2	10	8.3	8	22.8	0	5.4	8.19	41.7
11/09/03 09:09	2.8	9.6	11.2	10	8.3	8	22.8	0	5.4	8.19	41.7
11/09/03 10:47	2.9	8.9	8.6	9.8	8.2	9.9	22.8	0	5.8	8.49	41.8
11/09/03 10:47	2.10	8.9	8.6	9.8	8.2	9.9	22.8	0	5.8	8.49	41.8
11/09/03 11:03							21	0	5.4	8.43	41.8
11/09/03 11:29							23.5	0.9	6.4	8.49	42.2
11/09/03 11:52							22.7	0	6.2	8.44	41.9
11/09/03 12:05							22.4	0	3	8.43	41.9
11/09/03 12:24							22	0	2.8	8.49	42.2
11/09/03 13:00							24	low	3	8.54	42.2
11/09/03 13:37		8.5	9.4	8.4	8.7	7.2	23.3	0.5	2.8	8.53	42.2
11/09/03 13:51							23	1	2.4	8.53	42.3
11/09/03 14:27							21.8	0	5.3	8.47	41.9
11/09/03 15:14		9.3	11.3	10.5	7.9	6.9	21.9	0.5	5.3	8.51	41.9
11/09/03 15:14		9.3	11.3	10.5	7.9	6.9	23.9	0.5	5.3	8.51	41.9
11/09/03 15:37	2.11	8.7	9.5	9.5	8.6	10.2	23.5	0	6	8.45	41.8
12/09/03 08:35		8.7	8.7	8.6	7.8	8.9	23	0	8	8.3	41.7
12/09/03 09:17	2.12	9.3	9.1	9.7	9.3	7.4	25	1	5.3	8.48	41.7
12/09/03 09:51		9.9	9.3	9.1	8.7	9.6	25	0.5	5.3	8.43	41.7
12/09/03 10:57							25	0	5.3	8.26	41.8
12/09/03 13:10	2.13	9	9.7	8.8	8.1	8.2	25	low	6.8	8.48	41.8
12/09/03 14:26		9.2	10	9.8	9.1	7	24	0.5	6	8.56	41.9
12/09/03 16:31	2.14	8.7	10.3	9.6	9	8.4	25	low	6.5	8.48	42
15/09/03 08:23		13.5	16	12.3	12.5	13.5	23.2	low	water filled	8.7	43.9
15/09/03 08:55	2.15										
15/09/03 10:39							23	0	2	8.55	41.5
15/903 15:50	2.16	12.1	12.1	13.6	11.5	12.4	25	0	3	8.13	41.4

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Date & Time	sample	I (mV) BO					air	CO_2	exh		
		1	3	5	7	9	(cm)	(cm)	(cm)	pH	T (C)
16/09/03 08:35		5.7	5.5	5.2	5.4	5	25	1.5		9.92	42.3
16/09/03 09:10	2.17	5.7	6.4	5.5	5	6	23.5	0		8.46	42.3
16/09/03 12:24							25	1		8.54	42.2
16/09/03 14:38		3.2	3.6	3.5	2.6	3	24.8	0.5		8.51	42.3
16/09/03 15:02	2.18	2.8	2.8	3.4	2.8	3	24	1		8.53	42.4
16/09/03 15:37		2.8	3.6	3	2.5	2.9	23.7	1		8.55	41.2
17/09/03 08:39		0.96	1.18	1.3	0.8	0.86	25	1	6.2	8.79	41.9
17/09/03 08:45							24.5	4	6.2	8.78	41.9
17/09/03 09:33							24.3	0	6	7.85	41.9
17/09/03 10:54	2.19	0.9	1	1	0.87	0.95	24.3	1	6	8.51	41.9
17/09/03 12:04		0.85	1	1.2	0.8	0.9	23.5	1	6	8.44	42.1
17/09/03 15:51	2.20	0.75	1	1.1	0.75	0.8	25	0.5	6	8.54	41.4
18/09/03 08:37		0.79	0.76	0.91	0.7	0.73	25	0.5		8.5	42.4
18/09/03 08:56	2.21	0.72	0.74	1	0.68	0.7	25	0.5		8.49	42.4
18/09/03 11:42		0.65	0.84	1	0.69	0.76	25	0.5	6	8.46	41.6
18/09/03 13:45		0.67	0.6	0.84	0.61	0.82	23.5	1	5.5	8.45	41.6
18/09/03 15:22		0	0	0	0	0	25	0	5.5	8.51	41.7
18/09/03 16:49	2.22	0	0	0	0	0	24.5	0.5	5.5	8.58	41.7
19/09/03 09:00	2.23	0.58	0.76	0.93	0.59	0.57	25	0	5.5	8.51	42.6
19/09/03 15:20	2.24						24.8	0.5	5.5	8.47	42.7
19/09/03 15:25		0.7	0.7	0.9	0.5	0.7	25	low	5.5	8.56	41.6
22/09/03 08:50	2.25	0.65	0.67	0.73	0.52	0.61	25	0	5.8	8.48	42.4
22/09/03 10:21		0.64	0.61	0.88	0.64	0.64	25	0.5	5.8	8.55	42.2
22/09/03 13:54		0.67	0.66	0.81	0.5	0.6	24.45	0.5	5.6	8.58	41.2
22/09/03 15:11		0.58	0.64	0.89	0.57	0.63	24	0	5.24	8.47	41.3
22/09/03 15:29		0.68	0.74	0.77	0.59	0.56	25	0.5	5.6	8.54	41.3
22/09/03 15:57		0.68	0.74	0.77	0.59	0.56	0.25	0.5	5.6	8.54	41.3
22/09/03 16:38	2.26	0.65	0.63	0.83	0.58	0.55	25	0.5	5.8	8.47	41.3
23/09/03 09:22		0.73	0.8	1	0.65	0.66	24	0	5.6	8.61	42.3
23/09/03 09:31	2.27						23.8	1	5.6	8.57	42.3
23/09/03 11:27		0.67	0.74	0.89	0.64	0.71	24	0.5	5.6	5.58	42.4
23/09/03 12:26							25	0	6	8.49	42.6
23/09/03 13:20		0.72	0.78	0.84	0.6	0.55	25	0.5	5.6	8.58	42.5
23/09/03 16:18	2.28	0.7	0.82	0.85	0.6	0.67	25	0	6	8.52	41.5
24/09/03 08:30		0.83	0.81	1	0.76	0.81	25	0	6	8.26	42.1
24/09/03 09:58	2.29	0.8	0.95	0.94	0.82	0.77	25	0	6	8.51	42.1
24/09/03 14:29	2.30	0.8	0.95	1.02	0.79	0.76	25	low	6	8.56	40.9
24/09/03 14:29	2.31	0.8	0.95	1.02	0.79	0.76	25	low	6	8.56	40.9
24/09/03 16:42		0.76	0.85	1.24	0.84	0.68	24	low	5.4	8.53	41.6
25/09/03 08:35		0.95	0.94	1.16	0.86	0.91	25	1.5	6	8.02	41.9
25/09/03 09:42	2.32	0.97	0.96	1.17	0.84	0.92	25	low	5.6	8.54	41.8
25/09/03 09:45	2.33	0.97	0.96	1.17	0.84	0.92	25	low	5.6	8.54	41.8
25/09/03 14:14		1.05	1.16	1.62	1.11	1.33	25	low	5.8	8.54	42.5
25/09/03 17:41	2.34						25	low	6	8.5	43.8
25/09/03 17:41	2.35						25	low	6	8.5	43.8

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Date & Time	sample	I (mV) BO					air	CO_2	exh		
		1	3	5	7	9	(cm)	(cm)	(cm)	pH	T (C)
26/09/03 09:56	2.36	1.22	1.44	1.59	0.95	1.24	25	low	6	8.52	42.9
26/09/03 09:56	2.37	1.22	1.44	1.59	0.95	1.24	25	low	6	8.52	42.9
26/09/03 13:37		1.21	1.4	1.9	1.27	1.24	25	low	6	8.57	40.3
29/09/03 09:39		1.01	1.17	1.51	1.04	1.17	25	low	6	8.88	43.2
29/09/03 09:56	2.40	1.12	1.2	1.62	1.11	1.2	25	low	6	8.8	42.7
29/09/03 09:56	2.41	1.12	1.2	1.62	1.11	1.2	25	low	6	8.8	42.7
29/09/03 11:12		1.01	1.18	1.54	1.1	1.22	24.4	1.5	6	8.79	41.8
29/09/03 12:06								1			
29/09/03 12:16		0.98	1.16	1.54	1.04	1.17	25	1	5.8	8.53	41.9
29/09/03 12:55		0.89	1.83	1.16	0.86	0.85	25	0.5	6	8.46	41.8
29/09/03 13:46		0.99	1.07	1.27	0.94	0.79	25	low	6	8.46	41.8
29/09/03 15:18		0.92	1.14	1.27	0.83	0.79	25	low	6	8.52	42.3
29/09/03 16:07		0.87	0.96	1.26	0.88	1.11	25	0.5	5.4	8.54	41.8
29/09/03 16:50	2.42	0.98	1.14	1.56	1.01	0.99	25	0.5	5.6	8.53	41.9
29/09/03 16:50	2.43	0.98	1.14	1.56	1.01	0.99	25	0.5	5.6	8.53	41.9
29/09/03 17:13		1.02	1.17	1.51	0.92	1.03	25	0.5	5.8	8.52	41.9
30/09/03 09:59	2.44	0.83	0.95	1.23	0.85	0.84	24	low	5.4	8.39	41.2
30/09/03 09:59	2.45	0.83	0.95	1.23	0.85	0.84	24	low	5.4	8.39	41.2
30/09/03 11:27		0.84	0.98	1.3	0.89	0.92	25	low	5.6	8.55	41.6
30/09/03 12:30		0.73	0.87	1.06	0.76	0.66	25	low	5.6	8.51	42.2
30/09/03 13:04		0.81	0.96	1.09	0.7	0.76	25	low	5.6	8.5	42.3
30/09/03 14:05		0.84	0.93	1.17	0.77	0.87	25	low	5.6	8.5	42.4
30/09/03 17:07	2.46	1.01	0.85	1.23	0.87	0.91	25	low	5.6	8.5	42.4
30/09/03 17:07	2.47	1.01	0.85	1.23	0.87	0.91	25	low	5.6	8.5	42.4
01/10/03 14:00		0.96	1.11	1.27	0.86	0.88	25	low	5.6	8.52	42.5
01/10/03 15:50	2.48	0.84	1.18	1.52	1.27	1.12	24	low	5.6	8.5	42.5
01/10/03 15:50	2.49	0.84	1.18	1.52	1.27	1.12	24	low	5.6	8.5	42.5
02/10/03 09:15	2.5	0.74	0.8	1	0.59	0.63	25	low		8.58	42.2
02/10/03 10:29		0.86	0.73	1.22	0.67	0.81	25	0.5		8.6	40.9
02/10/03 11:58		0.84	0.89	1.28	0.86	0.96	25	low	6	8.49	42.4
02/10/03 13:26		0.8	0.91	1.24	0.84	0.95	25	1.5	6	8.64	42.2
02/10/03 16:20		0.77	0.87	1.16	0.83	0.88	25	0	6	8.5	42.7
02/10/03 16:25		0.82	0.9	1.25	0.74	0.83	25	low	6	8.51	42.6
03/10/03 10:36	2.52						25	0	5	8.79	43.3
	2.53						25	0	5	8.55	42.1
06/10/03 09:30		1.17	1.15	1.74	1.28	1.11	24	1	4	9.18	
06/10/03 12:10	2.56	1.13	1.25	1.5	1.05	0.9	25	1	4	8.71	

Table V: Experiment 2 - growth data

date/time	Cell mass (g)	DCM (g/L)
09/09/03 15:00	0	0.32
10/09/03 08:40	0.736111111	0.38
10/09/03 10:59	0.832638889	0.21
10/09/03 10:59	0.832638889	0.25
10/09/03 15:44	1.030555556	0.23
10/09/03 15:44	1.030555556	0.2
11/09/03 09:09	1.75625	0.43
11/09/03 09:09	1.75625	0.32
11/09/03 10:47	1.824305556	0.27
11/09/03 10:47	1.824305556	0.36
11/09/03 15:37	2.025694444	0.34
12/09/03 09:17	2.761805556	0.23
12/09/03 13:10	2.923611111	0.14
12/09/03 16:31	3.063194444	0.24
15/09/03 08:55	5.746527778	0.26
15/09/03 15:50	6.034722222	0.25
16/09/03 09:10	6.756944444	0.64
16/09/03 15:02	7.001388889	0.48
17/09/03 10:54	7.829166667	0.77
17/09/03 15:51	8.035416667	0.84
18/09/03 08:56	8.747222222	1.12
18/09/03 16:49	9.075694444	1.29
19/09/03 09:00	9.75	1.11
19/09/03 15:20	10.01388889	1.01
22/09/03 08:50	12.74305556	1.26
24/09/03 09:58	14.79027778	1.63
24/09/03 14:29	14.97847222	1.22
24/09/03 14:29	14.97847222	1.25

Table VI: Experiment 3 - raw data

date	time	sample	Flows (cm)			Temp	pH	I (mV)				
			air	CO2	exh	(C)		1	3	5	7	9
19/11/03	09:23	3-17	24.5	0.5	7.0	22.3	8.50	12.20	7.80	15.70	11.30	11.80
	13:28	3-19	23.8	0.5	7.0	22.6	8.68	11.10	7.80	13.50	11.90	7.50
20/11/03	13:55	3-21	24.0	1.5	7.0	22.3	9.16	4.40	3.20	5.80	5.80	4.60
	13:36	3-23	24.0	0.5	7.0	22.3	8.48	4.20	2.50	6.10	4.40	3.00
	16:06	3-25	2.4	0.5	7.0	22.3	8.63	2.90	2.90	5.10	3.60	2.50
21/11/03	09:26	3-27	23.8	0.0	7.0	21.8	8.49	1.40	1.20	1.60	1.50	1.30
	13:23	3-29	23.8	0.5	7.0	21.9	8.60	1.20	1.10	1.80	1.30	1.20
	15:48	3-31	23.8	0.0	7.0	22.0	8.52	1.03	0.92	1.75	1.42	1.09
24/11/03	16:12	3-33	25.0	0.5	7.0	21.8	8.55	0.68	0.55	1.20	0.85	0.79
25/11/03	09:35	3-35	22.6	0.5	6.5	21.9	8.57	0.60	0.52	1.05	0.85	0.59
	12:38	3-37	22.5	0.5	6.5	22.2	8.58					
	16:33	3-39	22.8	0.5	6.5	23.0	8.49	0.55	0.64	1.03	0.80	0.68
26/11/03	09:38	3-41	24.0	0.0	7.0	21.4	8.27	0.49	0.52	0.93	0.68	0.67
	12:16	3-43	13.8	0.5	7.0	21.5	5.54	0.49	0.44	1.01	0.73	0.63
	15:43	3-45	23.8	0.5	7.0	23.6	8.65	0.47	0.51	0.91	0.70	0.64
27/11/03	09:32	3-47	24.0	0.5	7.0	22.7	8.55	0.49	0.43	0.85	0.63	0.53
	12:00	3-49	24.0	0.5	7.0	21.5	8.53	0.43	0.40	0.84	0.65	0.59
	15:00	3-51	24.5	0.5	7.0	21.8	8.50	0.46	0.46	0.88	0.57	0.59
28/11/03	09:32	3-53	24.2	0.0	7.0	21.9	8.51	0.40	0.37	0.79	0.56	0.48
	12:08	3-55	24.0	>0.5	7.0	22.2	8.50	0.40	0.41	0.78	0.55	0.53
	15:32	3-57	25.0	>0.5	7.0	22.6	8.47	0.39	0.39	0.77	0.52	0.49
1/12/03	09:05	3-59	25.0	0.0	7.0	22.2	8.46	0.31	0.33	0.62	0.37	0.32
	13:28	3-61	25.0	1.5	7.0	22.1	8.65	0.30	0.30	0.58	0.40	0.42
	15:48	3-63	25.0	>0.5	7.0	22.2	8.65	0.29	0.29	0.57	0.37	0.37
2/12/03	09:31	3-65	25.0	1.0	7.5	22.2	8.70	0.28	0.29	0.53	0.37	0.34
	14:12	3-67	25.0	>0.5	7.0	22.4	8.56	0.29	0.28	0.59	0.34	0.32
	16:12	3-69	25.0	>0.5	7.0	22.6	8.44	0.27	0.26	0.55	0.36	0.38
3/12/03	09:57	3-71	23.7	0.0	7.3	22.6	8.47	0.29	0.31	0.61	0.34	0.30
	12:14	3-73	23.9	0.0	7.3	22.6	8.40	0.26	0.25	0.53	0.33	0.34
	15:25	3-75	23.7	0.0	7.2	22.4	8.38	0.26	0.29	0.62	0.38	0.35
4/12/03	09:47	3-77	22.9	0.0	6.5	N/A	8.47	0.25	0.25	0.56	0.32	0.31
	12:59	3-79	25.0	>0.5	7.0	21.8	8.54	0.26	0.24	0.50	0.24	0.32
	16:21	3-81	25.0	>0.5	7.0	N/A	8.61	0.27	0.22	0.51	0.32	0.30
5/12/03	09:14	3-83	23.6	<0.5	7.0	N/A	8.44	0.25	0.22	0.55	0.32	0.31
	12:26	3-85	23.6	0.0	7.0	N/A	8.45	0.27	0.21	0.55	0.29	0.25
	16:26	3-87	23.4	0.0	7.0	N/A	8.58	0.30	0.24	0.60	0.31	0.27
8/12/03	09:21	3-89	21.0	<0.5	6.5	N/A	8.61	0.22	0.18	0.49	0.23	0.23
	12:12	3-91	25.0	0.0	7.1	N/A	8.49	0.21	0.21	0.39	0.24	0.23
	16:22	3-93	24.5	3.2	7.4	N/A	8.69	0.21	0.18	0.40	0.24	0.23
9/12/03	10:00	3-95	22.5	0.0	6.7	N/A	8.50	0.19	0.19	0.40	0.23	0.19
	12:11	3-97	22.5	0.0	6.7	N/A	8.53	0.21	0.16	0.42	0.25	0.22
	16:13	3-99	22.4	<0.5	6.6	N/A	8.64	0.21	0.16	0.45	0.25	0.22

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date	time	sample	Flows (cm)			Temp	pH	I				
			air	CO2	exh	(C)		1	3	5	7	9
10/12/03	10:26	3-101	22.0	0.0	6.5	N/A	8.45	0.21	0.17	0.38	0.24	0.20
	13:14	3-103	24.1	<0.5	7.4	N/A	8.55	0.21	0.19	0.43	0.23	0.21
	16:12	3-105	24.2	0.0	7.4	N/A	8.52	0.21	0.19	0.38	0.23	0.23
11/12/03	09:12	3-107	24.5	<0.5	7.4	N/A	8.61	0.20	0.16	0.39	0.22	0.19
	11:41	3-109	25.0	0.0	7.4	N/A	8.51	0.20	0.16	0.35	0.22	0.22
12/12/03	09:30	3-111	25.0	0.0	7.8	N/A	8.56	0.21	0.14	0.44	0.23	0.23
	11:50	3-113	24.8	0.0	7.6	N/A	8.53	0.20	0.16	0.43	0.20	0.20
	15:30	3-115	24.6	0.0	8.5	N/A	8.52	0.22	0.17	0.45	0.22	0.23
15/12/03	10:05	3-117	24.6	0.0	7.5	N/A	8.55	0.19	0.19	0.35	0.21	0.21
	12:35	3-119	24.5	0.0	7.5	N/A	8.52	0.19	0.15	0.37	0.20	0.19
	16:08	3-121	24.4	0.0	7.4	N/A	8.50	0.18	0.17	0.36	0.20	0.19
16/12/03	09:40	3-123	24.2	0.0	7.4	N/A	8.55	0.18	0.15	0.33	0.20	0.16
	12:02	3-125	24.2	<0.5	7.3	22.6	8.55	0.18	0.14	0.31	0.20	0.16
	15:55	3-127	24.1	<0.5	7.3	22.8	8.42	0.18	0.15	0.31	0.20	0.16
17/12/03	09:57	3-129	24.2	<0.5	7.3	23.2	8.52	0.17	0.15	0.34	0.20	0.18
	12:14	3-131	N/A	N/A	N/A	28.7	8.41	N/A	N/A	N/A	N/A	N/A
	15:28	3-133	20.8	2.0	6.5	23.2	8.54	0.18	0.16	0.35	0.19	0.16
18/12/03	12:10	3-135	22.2	0.0	6.5	22.4	8.47	0.18	0.14	0.36	0.19	0.17
	16:05	3-137	22.8	<0.5	6.8	22.6	8.47	0.20	0.17	0.35	0.21	0.20
19/12/03	09:24	3-139	22.8	<0.5	6.9	22.9	8.43	0.19	0.16	0.36	0.21	0.19

Table VII: Experiment 3 growth data

Date & Time	Dcm (g/L)	time	Dcm (g/L)	time	Dcm (g/L)
19/11/03 09:23	0.2000	1/12/03 15:48	1.75	11/12/03 11:41	2.8100
19/11/03 09:23	0.2700	2/12/03 09:31	2.21	11/12/03 11:41	2.7200
20/11/03 10:58	0.4600	2/12/03 09:31	1.88	12/12/03 09:30	2.9300
20/11/03 10:58	0.3600	2/12/03 14:12	1.71	12/12/03 09:30	2.8400
20/11/03 13:36	0.4100	2/12/03 14:12	2.98	12/12/03 11:50	2.9100
20/11/03 13:36	0.3100	2/12/03 16:12	2.45	12/12/03 11:50	4.0800
20/11/03 16:06	0.4900	2/12/03 16:12	1.98	12/12/03 15:30	2.8400
20/11/03 16:06	0.4600	3/12/03 09:57	1.8300	12/12/03 15:30	2.8100
21/11/03 09:26	0.5900	3/12/03 09:57	1.8300	15/12/03 10:05	3.0000
21/11/03 09:26	0.6000	3/12/03 12:14	1.9600	15/12/03 10:05	2.8800
21/11/03 13:23	0.8000	3/12/03 12:14	1.9200	15/12/03 12:35	2.9200
21/11/03 13:23	0.6700	3/12/03 15:25	1.9300	15/12/03 12:35	2.8700
21/11/03 15:48	0.6700	3/12/03 15:25	1.9600	15/12/03 16:08	3.1400
21/11/03 15:48	0.6400	4/12/03 09:47	1.7600	15/12/03 16:08	2.9800
24/11/03 16:12	0.8000	4/12/03 09:47	1.7500	16/12/03 09:40	3.0800
24/11/03 16:12	0.8200	4/12/03 12:59	1.7300	16/12/03 09:40	3.0000
25/11/03 09:35	1.0000	4/12/03 12:59	1.9600	16/12/03 12:02	2.9300
25/11/03 09:35	1.0100	4/12/03 16:21	1.8400	16/12/03 12:02	2.9000
25/11/03 12:38	1.1000	4/12/03 16:21	1.8400	16/12/03 15:55	2.9700
25/11/03 12:38	1.1200	5/12/03 09:14	1.9100	16/12/03 15:55	2.9900
25/11/03 16:33	1.1300	5/12/03 09:14	1.9300	17/12/03 09:57	2.9700
25/11/03 16:33	1.2100	5/12/03 12:26	1.8200	17/12/03 09:57	3.0000
26/11/03 09:38	1.3400	5/12/03 12:26	1.9300	17/12/03 12:14	2.9000
26/11/03 09:38	1.1600	5/12/03 16:26	1.8100	17/12/03 12:14	2.9800
26/11/03 12:16	1.2400	5/12/03 16:26	1.8300	17/12/03 15:28	2.7900
26/11/03 12:16	1.2200	8/12/03 09:21	2.1500	17/12/03 15:28	2.8800
26/11/03 15:43	1.2500	8/12/03 09:21	2.2300	18/12/03 12:10	3.2500
26/11/03 15:43	1.3200	8/12/03 12:12	2.4900	18/12/03 12:10	3.2500
27/11/03 09:32	1.3400	8/12/03 12:12	2.1400	18/12/03 16:05	3.3000
27/11/03 09:32	1.3300	8/12/03 16:22	2.2900	18/12/03 16:05	3.3400
27/11/03 12:00	1.3200	8/12/03 16:22	2.2900	19/12/03 09:24	3.6800
27/11/03 12:00	1.2600	9/12/03 10:00	2.2800	19/12/03 09:24	3.1400
27/11/03 15:00	1.5400	9/12/03 10:00	2.4200		
27/11/03 15:00	1.1300	9/12/03 12:11	2.4700		
28/11/03 09:32	1.29	9/12/03 12:11	2.4600		
28/11/03 09:32	1.46	9/12/03 16:13	2.3400		
28/11/03 12:08	1.32	9/12/03 16:13	2.2800		
28/11/03 12:08	1.39	10/12/03 10:26	2.4100		
28/11/03 15:32	1.32	10/12/03 10:26	2.5200		
28/11/03 15:32	1.61	10/12/03 13:14	2.5200		
1/12/03 09:05	1.72	10/12/03 13:14	2.4800		
1/12/03 09:05	1.8	10/12/03 16:12	2.4800		
1/12/03 13:28	1.82	10/12/03 16:12	2.4100		
1/12/03 13:28	1.74	11/12/03 09:12	2.6600		
1/12/03 15:48	1.8	11/12/03 09:12	2.6600		

Table VIII: Experiment 4 - raw data

Date & Time	sample	temp (C)	air (cm)	pH	I (mV)					DCM (g/L)
					1	3	5	7	9	
3/02/2004		27.5	5	8.54	6.6	6.5	13.4	8.1	7.2	
4/02/2004 12:00	4.1 & 4.2	28.2	5	8.87	3.6	3.2	5.4	3.6	3.2	0.185
5/02/2004 9:30	4.3 & 4.4	29	5	8.66	0.6	0.53	1	0.66	0.62	0.605
5/02/2004 12:00		29.3	5	8.54	0.59	0.53	0.97	0.63	0.57	
5/02/2004 16:30	4.5 & 4.6	29.7	5	8.9	0.54	0.47	0.9	0.58	0.52	0.7
6/02/2004 9:00	4.7 & 4.8	27.6	5	8.74	0.42	0.39	0.67	0.45	0.43	1.135
6/02/2004 12:00		27.6	5	8.58	0.41	0.36	0.67	0.45	0.42	
6/02/2004 16:30	4.9 & 4.10	29.3	5	8.52	0.4	0.37	0.67	0.42	0.4	1.13
9/02/2004 8:39	4.11 & 4.12	29.3	5	8.9	0.21	0.21	0.38	0.25	0.24	1.945
12/02/2004 12:15		29.1	5	8.43	0.22	0.22	0.37	0.25	0.24	
9/02/2004 16:11	4.13 & 4.14	29.2	5	8.5	0.23	0.19	0.38	0.25	0.24	1.98
10/02/2004 8:45	4.15 & 4.16	28.8	5	8.63	0.21	0.19	0.34	0.24	0.22	2.37
10/02/2004 11:52		28.7	5	8.62	0.2	0.19	0.33	0.23	0.22	
10/02/2004 16:04	4.17 & 4.18	28.8	5	8.59	0.2	0.18	0.35	0.22	0.22	2.21
11/02/2004 9:00	4.19 & 4.20	28.5	5	8.49	0.19	0.16	0.33	0.21	0.2	2.48
11/02/2004 16:00	4.21 & 4.22	28.6	5	8.59	0.17	0.15	0.3	0.19	0.19	2.48
12/02/2004 8:50	4.23 & 4.24	28.8	5	9.06	0.17	0.15	0.28	0.18	0.18	2.765
12/02/2004 11:56		28.9	5	8.42	0.16	0.16	0.28	0.19	0.18	
12/02/2004 16:01	4.25 & 4.26	29.1	5	8.41	0.16	0.15	0.27	0.18	0.17	2.795
13/02/2004 8:43	4.27 & 4.28	29.2	5	8.89	0.15	0.15	0.26	0.17	0.17	2.79
13/02/2004 12:13		29.1	5	8.47	0.15	0.13	0.26	0.17	0.16	
13/02/2004 16:03	4.29 & 4.30	29.3	5	8.46	0.15	0.14	0.26	0.17	0.16	2.875
16/02/2004 8:54	4.31 & 4.32	29.7	5	8.61	0.13	0.12	0.22	0.15	0.14	3.495
16/02/2004 12:00		29.8	5	8.55	0.12	0.13	0.22	0.14	0.14	
16/02/2004 16:16	4.33 & 4.34	29.8	5	8.56	0.12	0.13	0.22	0.13	0.14	3.58
17/02/2004 9:03	4.35 & 4.36	29.6	5	8.36	0.11	0.11	0.2	0.13	0.13	3.635
17/02/2004 12:06		29.6	5	8.57	0.12	0.12	0.2	0.13	0.12	
17/02/2004 16:06	4.37 & 4.38	29.7	5	8.59	0.11	0.11	0.19	0.13	0.13	3.64
18/02/2004 9:00	4.39 & 4.40	29.5	5	8.82	0.1	0.1	0.19	0.12	0.12	3.89
18/02/2004 11:46		29.4	5	8.49	0.1	0.1	0.18	0.11	0.11	
18/02/2004 16:25	4.41 & 4.42	29.7	5	8.52	0.1	0.1	0.18	0.11	0.11	3.88
19/02/2004 9:01	4.43 & 4.44	29.5	5	8.49	0.1	0.09	0.18	0.11	0.11	4.205
19/02/2004 12:25		29.5	5	8.49	0.1	0.1	0.17	0.12	0.11	
19/02/2004 16:00	4.45 & 4.46	29.7	5	8.49	0.1	0.09	0.17	0.11	0.11	4.26
20/02/2004 9:00	4.47 & 4.48	29.7	5	8.66	0.1	0.08	0.16	0.11	0.1	4.415
20/02/2004 12:00		29.9	5	8.49	0.1	0.09	0.16	0.11	0.1	
20/02/2004 16:04	4.49 & 4.50	30.1	5	8.57	0.09	0.08	0.16	0.1	0.1	4.095
23/02/2004 9:00	4.51 & 4.52	29.3	5	8.33	0.06	0.05	0.12	0.07	0.07	4.745
23/02/2004 12:00		29.3	5	8.6	0.052	0.046	0.113	0.064	0.057	
23/02/2004 16:01	4.53 & 4.54	29.5	5	8.56	0.064	0.05	0.116	0.07	0.062	4.785
24/02/2004 9:00	4.55 & 4.56	28.7	5	8.46	0.05	0.04	0.11	0.061	0.053	4.915
24/02/2004 11:56		29.1	5	8.47	0.038	0.033	0.099	0.051	0.044	
24/02/2004 16:00	4.57 &4.58	29.1	5	8.46	0.041	0.031	0.096	0.05	0.044	5
25/02/2004 9:00	4.59 & 4.60	29.4	5	8.57	0.05	0.045	0.108	0.062	0.054	5.12

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Date & Time	sample	temp (C)	air (cm)	pH	I (mV)					DCM (g/L)
					1	3	5	7	9	
25/02/2004 12:00		29.6	5	8.55	0.082	0.072	0.14	0.095	0.085	
25/02/2004 16:00	4.61 & 4.62	29.3	5	8.51	0.08	0.072	0.133	0.088	0.084	5.13
26/02/2004 8:58	4.63 & 4.64	29.8	5	8.63	0.074	0.069	0.133	0.087	0.083	
26/02/2004 13:25		29.5	5	8.4	0.068	0.066	0.11	0.082	0.075	
26/02/2004 16:00	4.65 & 4.66	29.6	5	8.54	0.056	0.5	0.11	0.81	0.75	

Screening – Raw Data

Table IX: *Anabaena solitaria* phototrophic rigorous screening - collected data

Time(Days)	DCW (g/L)		PHB (mgPHB/L)		Concentration (%PHB)	
	A	B	A	B	A	B
0.469	0.007	0.007				
0.688	0.007	0.008				
1.434	0.009	0.011				
1.691	0.010	0.011				
2.431	0.012	0.012				
2.667	0.014	0.013	9.46810	11.63438	69.25258	87.86444
3.406	0.015	0.015				
3.698	0.016	0.017				
6.688	0.024	0.023	7.78467	8.15598	32.27377	35.99195
7.458	0.021	0.020				
7.833	0.021	0.021				
8.444	0.025	0.025				
8.656	0.026	0.025				
9.469	0.030	0.029				
9.667	0.030	0.030	2.56812	0.88861	8.58933	2.96771
10.469	0.033	0.033				
10.677	0.035	0.034				
11.469	0.038	0.037				
11.667	0.036	0.036				
15.535	0.050	0.049				
15.688	0.049	0.048				
16.561	0.049	0.049				
16.719	0.049	0.049				
17.465	0.050	0.051				
17.667	0.051	0.052				
18.719	0.051	0.052	1.13019	4.92967	2.19712	9.45894
21.556	0.053	0.054				
21.698	0.055	0.054				
22.583	0.056	0.055	1.33954		2.37703	
23.090	0.057	0.056				
24.708	0.059	0.057				
25.708	0.063	0.059				

Table X: *Microcystis aeruginosa* phototrophic rigorous screening - collected data

Time(Days)	DCW(g/L)		PHB (mgPHB/L)		Concentration (mgPHB/gDCW)	
	A	B	A	B	A	B
0.469	0.003	0.003				
0.688	0.003	0.003				
1.434	0.006	0.005				
1.691	0.006	0.006				
2.431	0.008	0.008				
2.667	0.010	0.009	5.46665	9.10325	55.15696	106.29283
3.406	0.012	0.011				
3.698	0.012	0.013				
6.688	0.024	0.023	12.42039	20.98307	52.83837	90.32119
7.458	0.019	0.018				
7.833	0.019	0.018				
8.444	0.022	0.024				
8.656	0.023	0.025				
9.469	0.027	0.027				
9.667	0.028	0.027	1.04787	1.05625	3.70761	3.84846
10.469	0.032	0.032				
10.677	0.034	0.033				
11.469	0.037	0.036				
11.667	0.036	0.033				
15.535	0.049	0.047				
15.688	0.049	0.047				
16.561	0.049	0.048				
16.719	0.048	0.046				
17.465	0.048	0.048				
17.667	0.048	0.048				
18.719	0.044	0.046	0.50557	0.57289	1.13766	1.25559
21.556	0.044	0.042				
21.698	0.045	0.042				
22.583	0.045	0.041	0.78276	1.14588	1.75363	2.76283
23.090	0.044	0.041				
24.708	0.042	0.039				

Table XI: *Microcystis flosaquae* phototrophic rigorous screening - collected data

Time(Days)	DCW(g/L)		PHB		Concentration	
	Through O	Through O	mgPHB/L		(mgPHB/gDCW)	
	A	B	A	B	A	B
0.469	0.008	0.008				
0.688	0.009	0.008				
1.434	0.011	0.013				
1.691	0.013	0.014				
2.431	0.019	0.017				
2.667	0.020	0.019	10.65175	9.72454	53.30283	52.44480
3.406	0.023	0.024				
3.698	0.024	0.025				
6.688	0.035	0.037	14.80647	3.97530	41.79852	10.84162
7.458	0.034	0.034				
7.833	0.033	0.035				
8.444	0.036	0.037				
8.656	0.038	0.038				
9.469	0.041	0.041				
9.667	0.040	0.041	0.73980	0.58787	1.82667	1.44414
10.469	0.042	0.043				
10.677	0.043	0.044				
11.469	0.045	0.046				
11.667	0.046	0.044				
15.535	0.056	0.055				
15.688	0.056	0.054				
16.561	0.057	0.055				
16.719	0.056	0.054				
17.465	0.058	0.057				
17.667	0.058	0.056				
18.719	0.060	0.058	1.86754		3.12492	
21.556	0.060	0.060				
21.698	0.060	0.060				
22.583	0.062	0.061	1.90945	6.77949	3.10082	11.05610
23.090	0.062	0.063				
24.708	0.064	0.064				
25.708	0.067	0.066				

Table XII: *Anabaena solitaria* mixothrophic rigorous screening - collected data

Time (Days)	DCW (g/L)		PHB (mg/L)		Conc. (mg/g)	
	A	B	A	B	A	B
0.094	0.02230	0.01743				
3.993	0.11782	0.09849	5.648028	491.8069	4.793909	499.3696
5.000	0.15213	0.12255				
6.000	0.14208	0.11889				
7.056	0.16217	0.14457	0.428581	1.183838	0.264271	0.81887
8.042	0.18265	0.16356				
11.000	0.30305	0.27347	0.703298	2.95479	0.232073	1.08049
11.979	0.33315	0.30012				
13.021	0.39308	0.35093				
13.990	0.41748	0.37865	14.00478	0.591862	3.354561	0.156309
15.042	0.42998	0.38286				
18.000	0.49398	0.44810	1.04827	0.579982	0.212208	0.12943
19.021	0.51725	0.46764				
19.969	0.52080	0.47406				
21.010	0.53944	0.49205	1.779593	1.639513	0.329894	0.333202
22.000	0.54773	0.50475				
24.979	0.57158	0.52919	1.730678	1.924525	0.302787	0.363673
25.990	0.58373	0.53748				
27.000	0.59595	0.56188				
27.990	0.59878	0.57037	373.108		62.31095	
29.042	0.59682	0.56419				

Table XIII: *Microcystis aeruginosa* mixothrophic rigorous screening - collected data

	DCW (g/L)		PHB(mg/L)		Conc. (mg/g)	
Time (Days)	A	B	A	B	A	B
0.094	0.00028	0.00723				
3.993	0.06424	0.06463	0.401946	2.512417	0.625696	3.887346
5.000	0.08810	0.08734				
6.000	0.10664	0.10002				
7.056	0.13421	0.11964	0.945499	0.736746	0.704486	0.615783
8.042	0.15410	0.13636				
11.000	0.20101	0.17505		35.86711		20.4899
11.979	0.21534	0.18521				
13.021	0.23627	0.21614				
13.990	0.24504	0.22993				
15.042	0.24691	0.23670				
18.000	0.28301	0.27737				
19.021	0.29517	0.29206				
19.969	0.29571	0.29541				
21.010	0.30702	0.30761				
22.000	0.31414	0.31482				
24.979	0.32569	0.31872	1.599049		0.490967	
25.990	0.33281	0.31977				
27.000	0.34447	0.33802				
27.990	0.34762	0.34037	122773.5	4072.817	35318.24	1196.587
29.042	0.34851	0.34011				

Table XIV: *Microcystis flosaquae* mixothrophic rigorous screening - collected data

	DCW (g/L)		PHB (mg/L)		Conc. (mg/g)	
Time (Days)	A	B	A	B	A	B
0.094	0.00805	0.00735				
3.993	0.10878	0.06588	17.04738	16.08159	1.567156	2.441009
5.000	0.14727	0.08143				
6.000	0.14950	0.09668				
7.056	0.17726	0.10989	0.969768	0.518721	0.547092	0.47205
8.042	0.19658	0.12865				
11.000	0.24731	0.17719	0.518531		2.096649	
11.979	0.26012	0.19511				
13.021	0.28855	0.22678				
13.990	0.29948	0.23679	0.569261	969.8544	0.190084	409.5805
15.042	0.30301	0.24895				
18.000	0.33371	0.28555				
19.021	0.34291	0.29549				
19.969	0.34623	0.29975				
21.010	0.35963	0.31011	1.859466		0.517051	
22.000	0.35801	0.31439				
24.979	0.36894	0.32923				
25.990	0.37392	0.33202				
27.000	0.38327	0.33752				
27.990	0.38323	0.33833	1135.658		296.3387	
29.042	0.38366	0.33822				

Table XV: *Anabaena solitaria* nitrogen deficient conditions rigorous screening - collected data

Time (Days)	DCW (g/L)	PHB(mg/L)	Conc. (mg/g)
0.094	0.01360		
3.993	0.00811	1038.99455	7640.429877
5.000	0.00391		
6.000	0.00405		
7.056	0.00315	2.17431916	69.13266679
8.042	0.00016		
11.000	0.00120	0.51313746	42.76573167
11.979	0.00413		
13.021	0.00954		
13.990	0.01067		
15.042	0.01722		
18.000	0.03547		
19.021	0.05572		
19.969	0.05834		
21.010	0.07259	18.858205	25.9780868
22.000	0.08125		
24.979	0.11295	1.50446718	1.331948661
25.990	0.13582		
27.000	0.14540		
27.990	0.15098	15.6970959	10.39680478
29.042	0.15379		

Table XVI: *Microcystis aeruginosa* nitrogen deficient conditions rigorous screening - collected data

Time (Days)	DCW (g/L)	PHB(mg/L)	Conc. (mg/g)
0.094	0.00171		
3.993	0.01571	1.47687366	86.41741707
5.000	0.02808		
6.000	0.03326		
7.056	0.04325	145.199975	335.6853088
8.042	0.05192		
11.000	0.06793	1.71327385	2.522014562
11.979	0.07564		
13.021	0.08747		
13.990	0.10039	0.52640286	0.524375312
15.042	0.10890		
18.000	0.13287	1.18997305	0.895559955
19.021	0.14321		
19.969	0.14272		
21.010	0.14790	1.69792992	1.148050012
22.000	0.14409		
24.979	0.14075		
25.990	0.14255		
27.000	0.14280		
27.990	0.14287	1.61428424	1.129878299
29.042	0.14260		

Table XVII: *Microcystis flosaquae* nitrogen deficient conditions rigorous screening - collected data

Time (Days)	DCW (g/L)	PHB (mg/L)	Conc. (mg/g)
0.094	0.00465		
3.993	0.03494	1.95507934	42.01760886
5.000	0.04935		
6.000	0.05988		
7.056	0.07325		
8.042	0.08503		
11.000	0.12050	0.26131646	0.21686519
11.979	0.12591		
13.021	0.13484		
13.990	0.14234	1.09078842	0.766351461
15.042	0.14745		
18.000	0.16023	5.60979756	3.501007411
19.021	0.16493		
19.969	0.16807		
21.010	0.17106	48.7483195	28.49782493
22.000	0.17073		
24.979	0.15845		
25.990	0.15333		
27.000	0.15222		
27.990	0.15201	9087.35797	5977.993647
29.042	0.15218		

Table XVIII: *Anabaena solitaria* phosphate deficient conditions rigorous screening - collected data

Time (Days)	DCW (g/L)	PHB (mg/L)	Conc. (mg/g)
0.094	0.01742		
3.993	0.00836	44.0252456	252.7258395
5.000	0.00874		
6.000	0.01139		
7.056	0.00846	67.6271784	799.6705449
8.042	0.00846		
11.000	0.00739	2.28180588	30.86257391
11.979	0.01583		
13.021	0.01451		
13.990	0.01767	1148.16193	6496.568457
15.042	0.01831		
18.000	0.02175	19.6662831	90.42019125
19.021	0.01993		
19.969	0.02084		
21.010	0.02479	1228.85702	4957.411418
22.000	0.02578		
24.979	0.02294	1.54061005	6.715847516
25.990	0.02603		
27.000	0.02400		
27.990	0.02367	198.638161	839.0490951
29.042	0.02416		

Table XIX: *Microcystis aeruginosa* phosphate deficient conditions rigorous screening - collected data

Time (Days)	DCW (g/L)	PHB (mg/L)	Conc. (mg/g)
0.094	0.00515		
3.993	0.02521		
5.000	0.02521		
6.000	0.03404		
7.056	0.03312	159.07597	480.2757399
8.042	0.03791		
11.000	0.01601	2.00142182	12.5019556
11.979	0.05944		
13.021	0.06072		
13.990	0.04269	1429.176545	3347.776603
15.042	0.02263		
18.000	0.01932		
19.021	0.04729		
19.969	0.02337		
21.010	0.05005	1920.28838	3836.684828
22.000	0.05060		
24.979	0.02889	1726.238393	5975.299713
25.990	0.02705		
27.000	0.02760		
27.990	0.02668		
29.042	0.02742		

Table XX: *Microcystis flosaquae* phosphate deficient conditions rigorous screening - collected data

Time (Days)	DCW (g/L)	PHB(mg/L)	Conc. (mg/g)
0.094	0.02631		
3.993	0.04810	190.38637	723.724781
5.000	0.05860		
6.000	0.05070		
7.056	0.04150		
8.042	0.04558		
11.000	0.03256		
11.979	0.04775		
13.021	0.04766		
13.990	0.05504	0.578340033	1.050689074
15.042	0.04923		
18.000	0.05565		
19.021	0.05418		
19.969	0.05643		
21.010	0.05157	1.643613063	3.187082882
22.000	0.06546		
24.979	0.06798	2483.726393	3653.610181
25.990	0.05418		
27.000	0.05218		
27.990	0.05313		
29.042	0.05487		

Reactor Studies

Table XXI: *Anabaena solitaria* CPBR experiment 1

date/time	culture time (d)	O.D. (10%)	Temperature (°C)	pH	Intensity (,000Lux) 1	3	5	7	9	Cell density (g/L)
4/11/2005 14:12	0.00	0.0065	28.1	7	10.1	10.4	10.2	9.8	12.2	0.0120185
4/11/2005 18:13	0.17	0.0122	28.2	8.18	10.8	11.5	12.1	10.4	12.6	0.0225578
5/11/2005 0:00	0.41	0.0208	27.6	7.42	9.1	9	9.5	8.6	10.6	0.0384592
5/11/2005 6:00	0.66	0.0327	28.1	7.58	7.6	7.1	8.2	7.2	8.3	0.0604623
5/11/2005 11:55	0.90	0.0587	28.3	7.42	4.1	4.1	4.8	4.1	4.8	0.1085363
5/11/2005 17:57	1.16	0.1113	27.8	7.44	1.2	1.2	1.4	1.2	1.5	0.2057937
5/11/2005 23:08	1.37	0.1388	28	7.45	0.57	0.58	0.65	0.54	0.64	0.2566412
6/11/2005 9:08	1.79	0.1935	28.1	7.45	0.19	0.18	0.23	0.17	0.21	0.3577815
6/11/2005 12:00	1.91	0.2311	27.9	7.46	0.15	0.14	0.18	0.14	0.15	0.4273039
6/11/2005 17:55	2.15	0.2706	28.1	7.5	0.11	0.1	0.14	0.09	0.11	0.5003394
6/11/2005 23:19	2.38	0.3053	27.8	7.46	0.1	0.1	0.13	0.09	0.1	0.5644997
7/11/2005 9:02	2.78	0.3566	27.8	7.45	0.093	0.089	0.115	0.089	0.09	0.6593534
7/11/2005 13:35	2.97	0.392	27.9	7.55	0.085	0.084	0.113	0.078	0.085	0.724808
7/11/2005 18:52	3.19	0.4182	28	7.55	0.079	0.079	0.099	0.075	0.08	0.7732518
7/11/2005 23:15	3.38	0.4419	27.9	7.52	0.074	0.077	0.097	0.07	0.076	0.8170731
8/11/2005 9:14	3.79	0.5024	28	7.51	0.052	0.067	0.088	0.058	0.066	0.9289376
8/11/2005 12:30	3.93	0.4808	28.3	7.47	0.07	0.074	0.089	0.067	0.068	0.8889992
8/11/2005 17:55	4.15	0.5254	28.3	7.45	0.064	0.067	0.088	0.06	0.066	0.9714646
8/11/2005 22:55	4.36	0.5591	28	7.46	0.06	0.068	0.078	0.055	0.066	1.0337759
9/11/2005 5:40	4.64	0.603	27.8	7.45	0.056	0.057	0.071	0.051	0.058	1.114947
9/11/2005 12:07	4.91	0.6326	28.2	7.47	0.059	0.049	0.065	0.054	0.057	1.1696774
9/11/2005 18:25	5.18	0.6614	28	7.54	0.051	0.056	0.081	0.045	0.06	1.2229286
9/11/2005 23:00	5.37	0.672	27.7	7.55	0.052	0.055	0.067	0.044	0.055	1.242528
10/11/2005 5:24	5.63	0.7035	27.9	7.57	0.047	0.053	0.063	0.042	0.048	1.3007715
10/11/2005 13:35	5.97	0.7323	28.2	7.46	0.046	0.047	0.063	0.045	0.044	1.3540227
10/11/2005 18:45	6.19	0.7246	27.8	7.55	0.052	0.051	0.061	0.041	0.055	1.3397854
10/11/2005 23:30	6.39	0.7344	28.3	7.5	0.044	0.049	0.064	0.049	0.046	1.3579056
11/11/2005 5:50	6.65	0.7452	27.4	7.45	0.04	0.047	0.058	0.038	0.045	1.3778748
11/11/2005 12:00	6.91	0.7566	28	7.46	0.046	0.043	0.059	0.037	0.049	1.3989534
11/11/2005 18:10	7.17	0.7975	27.9	7.52	0.046	0.043	0.057	0.049	0.046	1.4745775
11/11/2005 23:10	7.37	0.7909	28.2	7.52	0.042	0.044	0.058	0.044	0.043	1.4623741
12/11/2005 6:09	7.66	0.8011	27.6	7.45	0.042	0.042	0.057	0.05	0.044	1.4812339
12/11/2005 12:10	7.92	0.7996	28	7.47	0.044	0.042	0.057	0.044	0.043	1.4784604
12/11/2005 18:30	8.18	0.8193	28	8.42	0.043	0.041	0.056	0.047	0.045	1.5148857
12/11/2005 23:30	8.39	0.8457	28.1	7.58	0.041	0.043	0.055	0.044	0.043	1.5636993
13/11/2005 6:20	8.67	0.8254	28.1	7.5	0.041	0.043	0.054	0.044	0.042	1.5261646
13/11/2005 12:10	8.92	0.851	28.4	7.5	0.041	0.041	0.051	0.044	0.04	1.573499
13/11/2005 18:00	9.16	0.8446	28.2	7.57	0.047	0.042	0.055	0.049	0.044	1.5616654
13/11/2005 23:47	9.40	0.8982	27.9	7.46	0.043	0.044	0.051	0.044	0.035	1.6607718
14/11/2005 5:39	9.64	0.8574	28	7.52	0.038	0.036	0.048	0.039	0.035	1.5853326
14/11/2005 12:05	9.91	0.8491	28.4	7.54	0.044	0.04	0.047	0.038	0.034	1.5699859
14/11/2005 18:05	10.16	0.8904	28.1	7.51	0.042	0.038	0.05	0.04	0.037	1.6463496

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date/time	culture time (d)	O.D. (10%)	Temperature (°C)	pH	Intensity (,000Lux) 1	3	5	7	9	Cell density (g/L)
14/11/2005 23:30	10.39	0.8682	27.8	7.53	0.038	0.034	0.049	0.04	0.035	1.6053018
15/11/2005 5:46	10.65	0.915	27.7	7.5	0.043	0.038	0.051	0.038	0.035	1.691835
15/11/2005 13:10	10.96	0.8329	28.3	7.45	0.044	0.04	0.053	0.041	0.034	1.5400321
15/11/2005 17:55	11.15	0.8677	27.9	7.52	0.048	0.04	0.058	0.05	0.045	1.6043773
15/11/2005 20:57	11.28	0.8916	27.3	7.52	0.045	0.04	0.054	0.047	0.042	1.6485684
15/11/2005 23:17	11.38	0.8717	27.9	7.54	0.04	0.038	0.049	0.038	0.035	1.6117733
16/11/2005 6:05	11.66	0.8765	27.9	7.45	0.042	0.038	0.051	0.043	0.035	1.6206485
16/11/2005 12:45	11.94	0.8708	28.4	7.46	0.041	0.036	0.048	0.038	0.033	1.6101092

Table XXII: *Anabaena solitaria* CPBR experiment 1 PHB concentration

#	time (d)	DCW (g/L)	PHB (mg/ml)	PHB (mg/L)	PHB (mg/g)
1	0.90	0.11	0.00406	4.060	37.407
2	1.91	0.43	0.00366	3.657	8.559
3	2.97	0.72	0.00350	3.501	4.830
4	3.93	0.89	0.00474	4.737	5.329
5	4.91	1.17	0.00524	5.237	4.478
6	5.97	1.35	0.00630	6.304	4.656
7	6.91	1.40	0.00543	5.428	3.880
8	7.92	1.48	0.00623	6.232	4.215
9	8.92	1.57			
10	9.91	1.57	0.00521	5.209	3.318
11	11.28	1.65	0.00716	7.164	4.346
12	11.94	1.61			

Table XXIII: *Anabaena solitaria* CPBR experiment 2

date/time	culture time (d)	O.D . (10%)	Temperature (°C)	pH	Intensity (,000Lux) 1	3	5	7	9	Cell density (g/L)
25/11/2005 18:00	0.00	0.0054	40.1	7.53	10.4	10.1	13	12.1	11.6	0.0094608
26/11/2005 0:05	0.25	0.0248	40	7.49	8.7	7.8	10.6	10.4	9.4	0.0434496
26/11/2005 6:15	0.51	0.043	41.3	7.5	7.6	6.9	9.2	8.9	8.4	0.075336
26/11/2005 13:00	0.79	0.0704	40	7.49	3.7	3.5	4.6	4.7	4.3	0.1233408
26/11/2005 18:22	1.02	0.0987	40.2	7.53	2.8	2.8	3.6	3.6	3.5	0.1729224
26/11/2005 23:23	1.22	0.1211	39.8	7.55	1.6	1.6	2.1	3.1	1.9	0.2121672
27/11/2005 6:33	1.52	0.1571	39.1	7.57	1.13	1.03	1.34	1.36	1.22	0.2752392
27/11/2005 11:52	1.74	0.1735	40.3	7.45	0.8	0.78	1.02	1.02	0.9	0.303972
27/11/2005 17:55	2.00	0.2018	39.9	7.45	0.7	0.67	0.87	0.84	0.8	0.3535536
28/11/2005 0:03	2.25	0.2123	40.2	7.53	0.63	0.61	0.78	0.74	0.7	0.3719496
28/11/2005 5:52	2.49	0.2135	37.2	7.54	0.6	0.6	0.76	0.7	0.67	0.374052
28/11/2005 13:31	2.81	0.1819	40.2	7.53	0.7	0.68	0.89	0.88	0.77	0.3186888
28/11/2005 18:06	3.00	0.1663	41.1	7.54	0.87	0.86	1.11	1.11	0.98	0.2913576
28/11/2005 23:31	3.23	0.1459	40.4	7.46	1.15	1.09	1.44	1.39	1.34	0.2556168
29/11/2005 6:03	3.50	0.11	39.3	7.53	1.78	1.67	2.18	2.17	1.89	0.19272

Table XXIV: *Anabaena solitaria* CPBR experiment 2 PHB concentration

#	time (d)	DCW (g/L)	PHB (mg/ml)	PHB (mg/L)	PHB (mg/g)
1	0.79	0.123341	0.00373	3.72547	30.20470
2	1.74	0.303972	0.00364	3.64332	11.98570
3	2.81	0.318689	0.00856	8.55572	26.84662

Table XXV: *Anabaena solitaria* CPBR experiment 3

					Intensity (,000Lux)					
date/time	culture time (d)	O.D. (10%)	Temperature (°C)	pH	1	3	5	7	9	Cell density (g/L)
13/02/2006 17:56	0.00	0.0068	38.2	7.51	12.3	12.7	11.7	14.8	12.9	0.0122808
13/02/2006 23:47	0.24	0.014	37.6	7.52	11.4	11.7	11.2	13.8	12.3	0.025284
14/02/2006 05:45	0.49	0.0377	38.2	7.56	6.8	6.7	6.8	8.3	7.4	0.0680862
14/02/2006 11:58	0.75	0.0865	38.1	7.58	2.41	2.35	2.44	2.92	2.56	0.156219
14/02/2006 17:58	1.00	0.1355	38.4	7.48	0.823	0.766	0.836	0.975	0.831	0.244713
15/02/2006 00:08	1.26	0.1871	37.6	7.56	0.361	0.36	0.394	0.421	0.375	0.3379026
15/02/2006 05:36	1.49	0.239	38.3	7.54	0.217	0.22	0.249	0.262	0.226	0.431634
15/02/2006 11:58	1.75	0.2832	38.4	7.49	0.152	0.16	0.176	0.178	0.158	0.5114592
15/02/2006 18:00	2.00	0.3322	37.9	7.51	0.134	0.134	0.156	0.152	0.139	0.5999532
15/02/2006 23:55	2.25	0.3766	38	7.54	0.111	0.112	0.135	0.132	0.115	0.6801396
16/02/2006 05:54	2.50	0.4238	38.2	7.55	0.0934	0.0995	0.123	0.119	0.103	0.7653828
16/02/2006 11:46	2.74	0.4807	38.2	7.56	0.083	0.086	0.105	0.103	0.095	0.8681442
16/02/2006 18:11	3.01	0.5289	38.1	7.59	0.082	0.085	0.101	0.1	0.045	0.9551934
17/02/2006 00:32	3.28	0.5562	37.7	7.67	0.087	0.076	0.093	0.089	0.08	1.0044972
17/02/2006 05:37	3.49	0.6707	37.8	7.45	0.066	0.067	0.082	0.08	0.069	1.2112842
17/02/2006 11:17	3.72	0.621	37.8	7.55	0.07	0.065	0.08	0.076	0.074	1.121526
17/02/2006 19:46	4.08	0.6857	39	7.45	0.067	0.06	0.074	0.072	0.068	1.2383742
18/02/2006 00:28	4.27	0.6862	38	7.57	0.059	0.062	0.074	0.069	0.063	1.2392772
18/02/2006 06:25	4.52	0.7961	37.7	7.45	0.06	0.057	0.069	0.067	0.064	1.4377566
18/02/2006 12:08	4.76	0.7483	38	7.51	0.05	0.054	0.067	0.062	0.059	1.3514298
18/02/2006 18:11	5.01	0.7853	38.1	7.46	0.062	0.053	0.07	0.065	0.062	1.4182518
18/02/2006 23:22	5.23	0.7747	38	7.52	0.053	0.052	0.064	0.062	0.058	1.3991082
19/02/2006 9:07	5.63	0.7922	37.9	7.49	0.047	0.048	0.063	0.058	0.053	1.4307132
19/02/2006 12:16	5.76	0.8086	37.8	7.47	0.048	0.049	0.061	0.053	0.056	1.4603316
19/02/2006 17:59	6.00	0.8208	38	7.46	0.049	0.05	0.062	0.058	0.055	1.4823648
19/02/2006 23:42	6.24	0.8685	38.1	7.52	0.051	0.048	0.06	0.059	0.056	1.568511
20/02/2006 5:55	6.50	0.8551	37.9	7.49	0.049	0.045	0.054	0.052	0.048	1.5443106
20/02/2006 11:51	6.75	0.8765	38.3	7.51	0.042	0.042	0.055	0.052	0.05	1.582959
20/02/2006 17:55	7.00	0.8563	38.3	7.5	0.042	0.05	0.058	0.055	0.054	1.5464778
20/02/2006 23:29	7.23	0.8428	38	7.54	0.048	0.045	0.059	0.055	0.051	1.5220968
21/02/2006 7:00	7.54	0.8654	37.7	7.5	0.048	0.046	0.057	0.053	0.048	1.5629124
21/02/2006 9:45	7.66	0.8964	37.7	7.52	0.046	0.045	0.058	0.056	0.048	1.6188984

Table XXVI: *Anabaena solitaria* CPBR experiment 3 PHB concentration

#	time (d)	DCW (g/L)	PHB (mg/ml)	PHB (mg/L)	PHB (mg/g)
1	0.492	0.068086	0.00025	0.2528436	3.71358072
2	1.486	0.431634	0.00203	2.02888405	4.70047321
3	2.499	0.765383	0.00073	0.72646897	0.94915769
4	3.487	1.211284	0.00011	0.10847772	0.08955596
5	4.758	1.35143	0.00023	0.22846166	0.16905181
6	5.633	1.430713	0.00086	0.85565418	0.59806129
7	6.499	1.544311	0.00105	1.05178003	0.68106768
8	7.544	1.562912	0.00153	1.53418218	0.98161751
9	7.659	1.618898	0.00167	1.66626468	1.02925834
10	7.659	1.618898	0.00118	1.17806552	0.72769577

Table XXVII: *Anabaena solitaria* CPBR experiment 4

date/time	culture time (d)	O.D. (10%)	Temperature (°C)	pH	Intensity (,000Lux)					Cell density (g/L)
					1	3	5	7	9	
5/07/2006 19:38	0.00	0.0081	38.1	7.53	21.3	21.9	26.8	22.4	22.7	0.0143937
6/07/2006 00:10	0.19	0.0082	37.8	7.39	18.3	18.2	25.3	20.6	21.8	0.0145714
6/07/2006 05:55	0.43	0.0321	37.8	7.4	15.5	13.8	20.7	17.3	16.1	0.0570417
6/07/2006 11:15	0.65	0.0568	38.2	7.41	9.2	9.3	12.9	10.3	10.1	0.1009336
6/07/2006 18:00	0.93	0.1078	38.2	7.52	3.6	3.6	5.2	4.3	4.3	0.1915606
7/07/2006 00:15	1.19	0.1506	37.7	7.55	1.5	1.4	2	1.7	1.7	0.2676162
7/07/2006 05:50	1.42	0.1779	37.7	7.48	0.775	0.794	1.18	0.912	0.892	0.3161283
7/07/2006 11:52	1.68	0.1936	38.3	7.44	0.549	0.598	0.847	0.635	0.605	0.3440272
7/07/2006 18:35	1.96	0.2781	38.1	7.45	0.395	0.481	0.605	0.452	0.435	0.4941837
8/07/2006 00:05	2.19	0.2765	37.4	7.5	0.322	0.335	0.5	0.363	0.345	0.4913405
8/07/2006 08:20	2.53	0.3121	38	7.51	0.24	0.254	0.388	0.287	0.272	0.5546017
8/07/2006 12:05	2.69	0.3242	38.2	7.52	0.228	0.246	0.364	0.264	0.26	0.5761034
8/07/2006 18:00	2.93	0.3569	38	7.56	0.2	0.222	0.322	0.239	0.221	0.6342113
9/07/2006 00:00	3.18	0.3789	37.6	7.49	0.184	0.198	0.304	0.222	0.209	0.6733053
9/07/2006 08:00	3.52	0.4225	37.5	7.56	0.158	0.168	0.262	0.194	0.188	0.7507825
9/07/2006 12:00	3.68	0.4647	39.2	7.46	0.141	0.172	0.278	0.182	0.17	0.8257719
9/07/2006 18:00	3.93	0.4741	38	7.56	0.134	0.155	0.258	0.159	0.157	0.8424757
10/07/2006 00:06	4.19	0.4908	38.3	7.51	0.123	0.141	0.253	0.157	0.158	0.8721516
10/07/2006 08:16	4.53	0.5264	37.6	7.56	0.113	0.132	0.225	0.142	0.151	0.9354128
10/07/2006 12:00	4.68	0.5331	38.4	7.46	0.107	0.137	0.222	0.132	0.14	0.9473187
10/07/2006 18:00	4.93	0.6122	38.1	7.51	0.109	0.125	0.213	0.126	0.129	1.0878794
11/07/2006 0:00	5.18	0.6383	37.8	7.56	0.104	0.127	0.215	0.128	0.137	1.1342591
11/07/2006 8:05	5.52	0.6864	37.8	7.53	0.102	0.12	0.206	0.129	0.136	1.2197328
11/07/2006 12:28	5.70	0.6754	38.5	7.53	0.0894	0.117	0.196	0.118	0.125	1.2001858
11/07/2006 18:10	5.94	0.6934	38.6	7.47	0.0923	0.116	0.195	0.124	0.123	1.2321718
12/07/2006 0:00	6.18	0.6267	37.4	7.56	0.092	0.108	0.198	0.119	0.135	1.1136459
12/07/2006 8:15	6.53	0.7134	37.5	7.46	0.09	0.113	0.194	0.127	0.123	1.2677118
12/07/2006 12:00	6.68	0.6289	38.1	7.5	0.088	0.111	0.192	0.122	0.125	1.1175553
12/07/2006 18:00	6.93	0.6603	38.3	7.42	0.091	0.117	0.197	0.127	0.126	1.1733531
13/07/2006 0:06	7.19	0.6421	37.1	7.57	0.095	0.123	0.2	0.118	0.137	1.1410117
13/07/2006 12:55	7.72	0.6489	38.8	7.48	0.106	0.121	0.2	0.123	0.135	1.1530953

Calibration Data

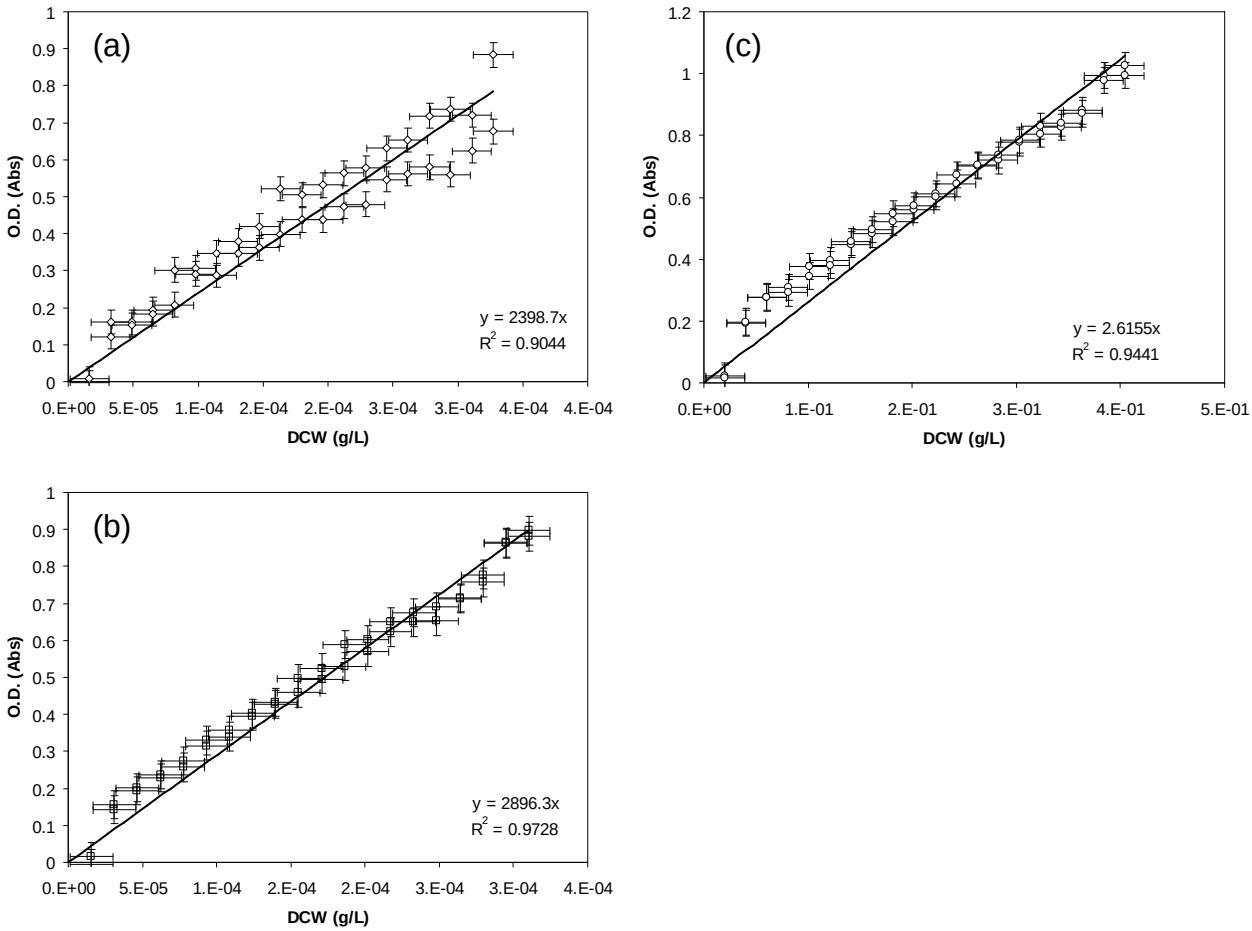
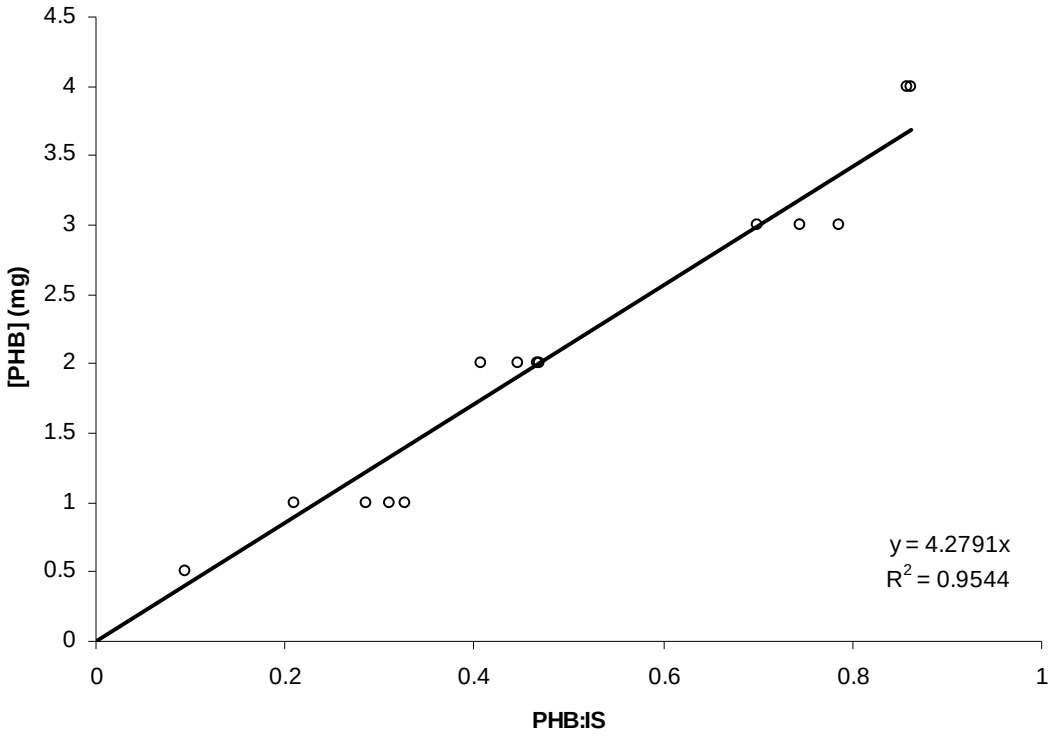


Figure A.1: Calibration of optical density @730nm against dry cell mass; (a) Anabaena solitaria, (b) Microcystis aeruginosa, (c) Microcystis flos-aquae for rigorous screening



FigureA.2: Calibration of peak area ratio of Internal Standard to PHB against mass of PHB

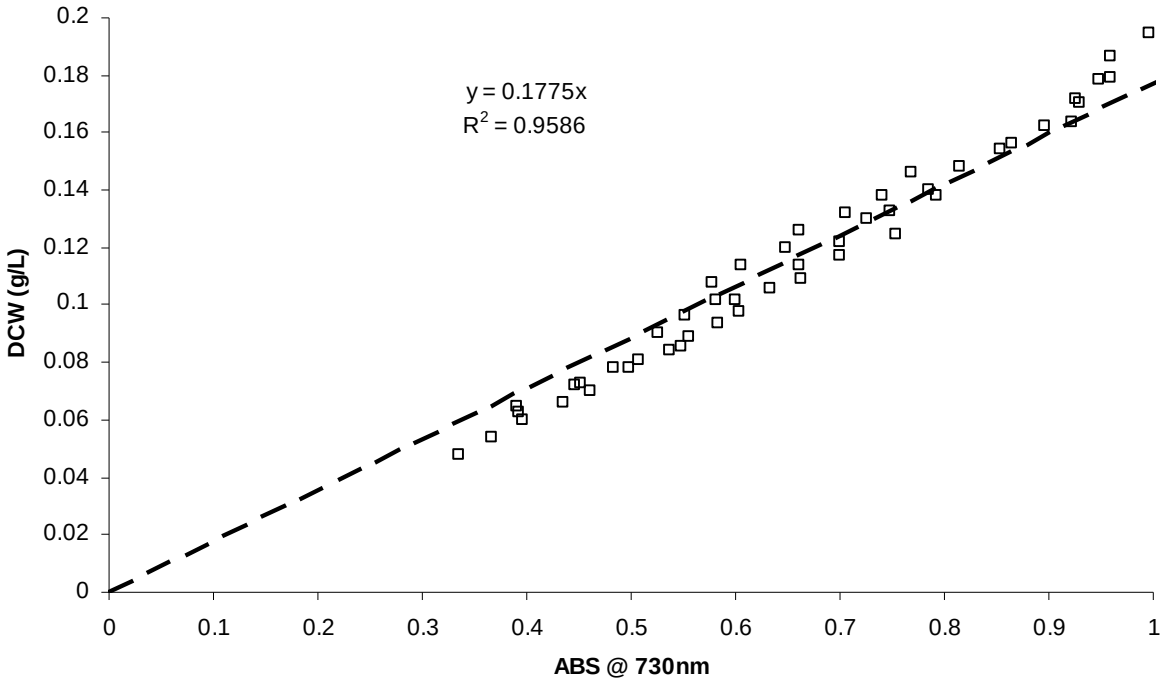


Figure A.3: Calibration of Absorbance against Dry Cell Weight

Published Papers

Desai, D. K., M. D. Roberts, K. D. King and D. Price (2003). A Mathematical Model for an Internally Radiating Air-Lift Photobioreactor. Chemeca 2003, Adelaide.

A MATHEMATICAL MODEL FOR AN INTERNALLY RADIATING AIR-LIFT PHOTOBIOREACTOR

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Desai, D.K., Roberts, M.D., King, K.D. & Price, D. (2003) A mathematical model for an internally radiating air-lift photobioreactor.
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Roberts, M. D., D. K. Desai, K. D. King and D. Price (2004). Further development of a mathematical model for an internally radiating air-lift photobioreactor. Chemeca 2004, Sydney.

FURTHER DEVELOPMENT OF A MATHEMATICAL MODEL FOR AN INTERNALLY RADIATING AIR-LIFT PHOTOBIOREACTOR

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Roberts, M.D., Desai, D.K., King, K.D. & Price, D. (2004) Further development of a mathematical model for an internally radiating air-lift photobioreactor.
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Determination of Polyhydroxybutyrate (PHB) content for some Australian Cyanobacteria

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Roberts, M.D., Telford, S.J., King, K.D., Desai, D.K., & Price, D. (2005) Determination of polyhydroxybutyrate (PHB) content for some Australian cyanobacteria.
ISAP-10, the 10th International Conference on Applied Phycology, Kunming, China.

Roberts, M. and K. D. King (2008). "Heat capacity of cyanobacterial slurries." Journal of Biotechnology **136S**: S558.

HEAT CAPACITY OF CYANOBACTERIAL SLURRIES

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Roberts, M.D.& King, K.D. (2008) Heat capacity of cyanobacterial slurries. *Journal of Biotechnology, v. 136 (Supplement 1), pp. S558.*