Developing Next Generation Algae Bioplastic Technology

by Shawn Price

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

under the supervision of Peter Ralph, Mathieu Pernice & Unnikrishnan Kuzhiumparambil

University of Technology Sydney
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CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Shawn Price, declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctorate of Philosophy, in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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Preface

Thesis format

This thesis is written in the format of a thesis by compilation— a combination of published chapters and those unpublished but with the intention of publication in a peer reviewed scientific journal in the near future. Given that this thesis is presented as a series of ready to submit manuscripts, there is an element of repetition in the introduction of some chapters since they are each submitted as stand-alone manuscripts.

The first chapter is a literature review which introduces the field of cyanobacteria as a production platform for the bioplastic poly-hydroxy-butyrate (PHB). The second chapter explores random mutagenesis for the creation of novel cyanobacterial mutant strains with enhanced PHB productivities. The third chapter investigates the use of chemical modulators to elicit and inhibit PHB productivity in cyanobacteria. The fourth chapter explores the use of wastewater as a medium for cyanobacterial PHB production. The fifth chapter is a techno-economic assessment of the economic viability of industrial production of cyanobacterial PHB. The final chapter is a synthesis of the thesis with final perspectives on this exciting research area.

Publications

At the time of thesis submission, Chapter 1 and 5 have been published in the peer reviewed *Journal of Environmental Chemical Engineering* (IF 5.909). Chapters 2, 3 and 4 are currently in review in different peer reviewed journals and are expected to be published in 2023.

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Abstract

Plastics enable the modern world to function. They have applications in agriculture, medical biotechnology, consumer products, electronics, construction and much more. Unfortunately, their widespread use comes at the cost of the environment in the form of aquatic and terrestrial pollution. However, bioplastics made from renewable resources that can biodegrade in the environment offers a solution to this problem, but they are held back due to high substrate costs of sugar feedstock required for fermentation.

Cyanobacteria are microscopic photosynthetic organisms capable of converting atmospheric CO_2 into a widely used bioplastic, PHB (poly-hydroxy-butyrate). Although this feedstock is significantly cheaper compared to current bioplastic production, the industrial production of cyanobacterial PHB is still not economically viable due to lower PHB productivity rates and high cultivation equipment costs compared to fermentation. In order to progress towards economic viability, four areas were explored as separate data chapters in this thesis:

Chapter 2: Creation of novel mutant strains through random chemical mutagenesis with superior cyanobacterial PHB productivity

Targeted genetic engineering requires advanced technical manipulation and prior knowledge of which genes to target. However, random mutagenesis can create mutants with novel mutations which result in a desired phenotype that can be sequenced to learn about new PHB metabolic mechanisms. In this study, ethyl methane sulfonate (EMS) was used to create a mutant library which was screened using fluorescent activated cell sorting (FACS) to sort single cells with BODIPY 493/503 (a neutral lipid dye) into well plates. These mutants were then screened for growth rate before being tested for PHB productivity. Two mutant strains were created with enhanced PHB yields (29% and 26% higher than wild type), biomass densities (36% and 33% higher than wild type) and PHB volumetric densities (75% and 67% higher than wild type).

Chapter 3: Identifying chemical enhancers and inhibitors of cyanobacterial PHB metabolism

Chemical modulators which affect cyanobacterial metabolism can be used to increase PHB production at industrial scales. The mechanisms of enhancers and inhibitors of PHB production can also be studied to identify genetic and regulatory information on PHB metabolism. Thus, 10 different compounds (including oxidants, antioxidants, phytohormones) were screened at 3 concentrations (0.1 µM, 1 µM and 10 µM) to identify compounds which boosted and reduced PHB production. Two treatments, 0.1 µM IAA and 1 µM methyl jasmonate were found to increase PHB yield (55% and 19% compared to control). Two treatments, 10 µM allopurinol and 10 µM ethynylestradiol, were found to decrease biomass density, PHB yield and PHB density.

Chapter 4: Exploring municipal wastewater as a media for cyanobacterial PHB production

Using wastewater as a substrate not only reduces demand for fresh water, but also reduces production costs through not requiring synthetically made media. However, wastewater as a substrate introduces the possibility of culture contamination, presence of inhibitory pollutants and provided a unique nutrient profile. This study demonstrated the potential for primary domestic wastewater as a nutrient source of cyanobacterial biomass cultivation with no significant difference between biomass densities compared to the control culture. However, PHB yield was significantly inhibited (85% lower than control) which may have been linked to non-cyanobacterial biomass.

Chapter 5: Techno-economic modelling to identify key financial drivers of cyanobacterial PHB profitability

This techno-economic modelling study breaks down the key capital and operating costs and identifies the major financial barriers to profitability. For a base case scenario, a 10,000 tonnes of PHB bioplastic resin per year facility in Australia was used with breakeven and sensitivity analysis to assess economic viability. The financial model was then used to explore potential paths to financial viability such as examining the effect of the scale of production volume, additional revenue from utilising a biorefinery approach to cyanobacterial biomass, use of holding or ripening tanks to reduce cultivation costs and geographic location of the hypothetical production facility. The base case revealed that the

cost of production was $18.1k USD/tonne which is over four times the current market price of PHB. However, through the combination of several optimistic scenarios, the breakeven price could potentially reach $7.7k USD/tonne.

Chapter 1 - Cyanobacterial Polyhydroxybutyrate for Sustainable Bioplastic Production: Critical Review and Perspectives

This chapter has been published in the *Journal of Environmental Chemical Engineering*.

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Abstract

PHB is a promising bioplastic material that naturally accumulates in many strains of cyanobacteria. This comprehensive review covers recent advances in several topics including PHB metabolism, material properties, relevant extraction methods and protocols, industrial cultivation strategy, current economic assessment and much more. Ultimately, the profitability of cyanobacterial PHB production is controlled by low PHB productivity as well as expensive cultivation and harvesting equipment. Several research areas for improving viability of cyanobacterial PHB production have also been summarised and perspectives on future efforts suggested including; screening, genetic modification, wastewater cultivation and using chemical modulators among others.

Introduction

Plastics are now one of the most widely used materials worldwide. From applications in the automotive, construction and biomedical industries to agricultural films for farmers, disposable packaging to ensure food quality, and much more; it is undeniable that plastics enable modern society to function. Unfortunately, the economic and social benefits of plastics currently come at a cost to the environment. It is estimated that over 300 million tonnes of plastic are now produced annually (Plastics Europe, 2017). Approximately 9% of all plastics have been recycled, 12% incinerated and 79% has ended up either in landfill or polluting terrestrial and aquatic environments (Geyer, Jambeck & Law 2017), with over 10

million tonnes of plastic entering the ocean each year (Jambeck et al. 2015). The strong physical properties and chemically inert qualities that make plastics useful for their everyday applications also make these materials extremely difficult to breakdown and decompose in the environment (Shah et al. 2008). The disintegration of plastic items into smaller fragments over time can cause the formation of micro-plastics which are absorbed by organisms at the bottom of the food chain, leading to accumulation of plastic material up the food chain. Plastic pollution also allows organic pollutants to enter the food web as they are adsorbed onto plastic surfaces (Van et al. 2012). On top of the environmental damage that plastics causes, our current source of over 99% of plastics are finite reserves of petroleum. The production and consumption of plastic is only projected to increase with a growing world population and expanding consumer classes in many parts of the world. Currently, less than 1% of all plastics produced are biodegradable (Ashter 2016). Thus, there is a critical need to move towards bioplastics that are both sustainably sourced from renewable materials and that also biodegrade into harmless compounds that do not damage the environment.

The term bioplastics can refer to plastic materials that are either biodegradable, biobased or both (Rujnić-Sokele & Pilipović 2017). Biodegradability of plastics is affected by many environmental parameters including chemical, physical and biological (such as temperature, exposure to shear forces, UV radiation, microbial community) and properties of the polymer blend itself (such as functional groups present, molecular weight of polymer chains, crystallinity, tacticity and additives present) (Shah et al. 2008). 'Biobased' refers to sourcing the raw material from renewable biomass sources rather than petroleum reserves. It should be noted that a biodegradable plastic may not necessarily be biobased (such as polycaprolactone made from fossil fuels), and a biobased plastic compound may not necessarily be biodegradable (such as polyethylene produced from a biomass source). However, it is desirable for bioplastic in many applications, such as packaging, to be both biobased and biodegradable to ensure they are sustainably sourced and reduce environmental pollution.

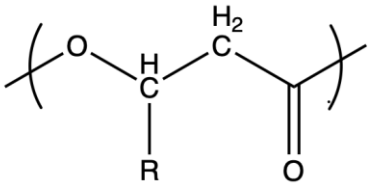
First generation bioplastics that are both biobased and biodegradable are sourced from terrestrial crop biomass and either use naturally occurring biopolymers such as starch and

cellulose or further bioprocessing to create plastics such as poly lactic acid (PLA) or poly-hydroxy-alkanoates (PHAs). The downside of such products included increasing competition for arable land, freshwater usage, fertiliser and raising food prices. Second generation bioplastic technology incorporated agricultural waste and discarded biomass for the production of bioplastics (Brodin et al. 2017). However, these waste streams alone are not large enough in volume and high enough in quality and consistency to provide sufficient biomass to replace global plastic demand with bioplastics. Algae and cyanobacteria are currently being investigated as third generation bioplastic technology and could offer a solution to this growing problem.

Algae and cyanobacteria are among the oldest and most widespread life-forms on Earth. They can be found in almost all freshwater, marine and brackish aquatic ecosystems, in addition to various terrestrial environments (Mata et al. 2010). The term algae encompasses aquatic photosynthetic organisms of great genetic diversity; from single cell microalgae to multicellular species such as seaweed. Algae and cyanobacteria are well known for the many advantages they hold as a sustainable source of biomass to multiple industries (Mata, Martins & Caetano 2010). They can be cultivated in salt water or wastewater and do not necessarily compete for freshwater resources. Nutrients do not need to come from synthetic fertiliser and can instead be obtained from wastewater streams and some species are able to fix nitrogen directly from the atmosphere. No arable land is required for cultivation facilities and compared to terrestrial crops, algae and cyanobacteria can offer biomass productivities that are tens to hundreds times higher than that of terrestrial plants and as a result require less area to produce the same amount of biomass (Adeniyi, Azimov & Burluka 2018). In addition to this, several species of cyanobacteria, the ancestors of all eukaryotic algae, are able to naturally synthesise the biodegradable thermoplastic, poly-hydroxy-butyrate (PHB) (Balaji, Gopi & Muthuvelan 2013; Singh & Mallick 2017) and co-polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) within its cells (Taepucharoen et al. 2017).

PHAs are a class of condensation polymers that serve as energy storage compounds and are present in many aerobic and anaerobic microorganisms. Over 150 different types of PHAs have been identified (Balaji, Gopi & Muthuvelan 2013); however, PHB is by far the most

prevalent of the PHA biopolymers across different taxonomic groups including photoautotrophic cyanobacteria. Figure 1-1 shows the general structure of the PHA class of molecules.



R = hydrogen	Poly(3-hydroxypropionate)
R = methyl	Poly(3-Hydroxybutyrate)
R = ethyl	Poly(3-hydroxyvalerate)
R = propyl	Poly(3-hydroxyhexanoate)
R = pentyl	Poly(3-hydroxyoctanoate)
R = nonyl	Poly(3-hydroxydodecanoate)

Figure 1-1: Molecular structure of PHAs with R representing possible aliphatic functional groups.

PHA and PHB plastic is industrially produced using fermentation with heterotrophic bacteria under aerobic conditions (Levett et al. 2016). Monomeric sugars used as the carbon substrate in fermentation are usually obtained from the hydrolysis of terrestrial biomass material which comes at a significant financial cost and is associated with multiple environmental impacts from agricultural practices (Levett et al. 2016).

There is also ongoing research to use wastewater from different industries as an alternate carbon source (Flavigny & Cord-Ruwisch 2015; Venkateswar Reddy et al. 2012). The main advantage of using cyanobacteria for bioplastic production is its ability to sequester carbon dioxide from the atmosphere and directly convert it into PHB (Singh et al. 2017). This has the potential to enable bioplastic production with a lower environmental footprint compared to fermenting crop biomass which has significant environmental issues including agricultural runoff, increasing demand for freshwater and fertiliser and habitat destruction to clear area for farmland.

PHA/PHB in Cyanobacteria and Algae

Cyanobacteria are a group of oxygen-producing photosynthetic bacteria. They require simple inorganic nutrients for growth including atmospheric carbon dioxide, nitrogen, phosphorous, some trace metals and micronutrients. Some cyanobacteria are capable of mixotrophic metabolism and can thus grow in the dark without photosynthesis, if an organic carbon substrate is provided. As mentioned previously, some cyanobacteria can directly fix atmospheric nitrogen, when nitrate sources are lacking in the environment. Figure 1-2 shows some species of cyanobacteria that are capable of synthesising PHB.

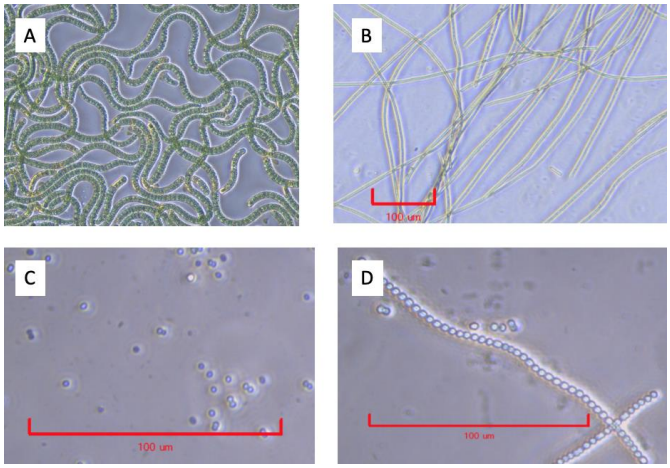


*Figure 1-2: Four species of algae capable of PHB production; (**A**) Athrospira maxima (**B**) Oscillatoria jasorvensis (**C**) Synechocystis PCC6803 (**D**) Nostoc muscorum.*

WHY DO CYANOBACTERIAL CELLS ACCUMULATE PHB?

Being the first photosynthesisers on Earth, cyanobacteria are among the oldest known organisms and have been exposed to a range of different environmental conditions to which they have adapted. As a result they can produce an array of storage compounds, including PHB, which assist in living in environments with fluctuating nutrients (Stal 1992). Figure 1-3 provides an overview of the major cyanobacterial storage compounds.

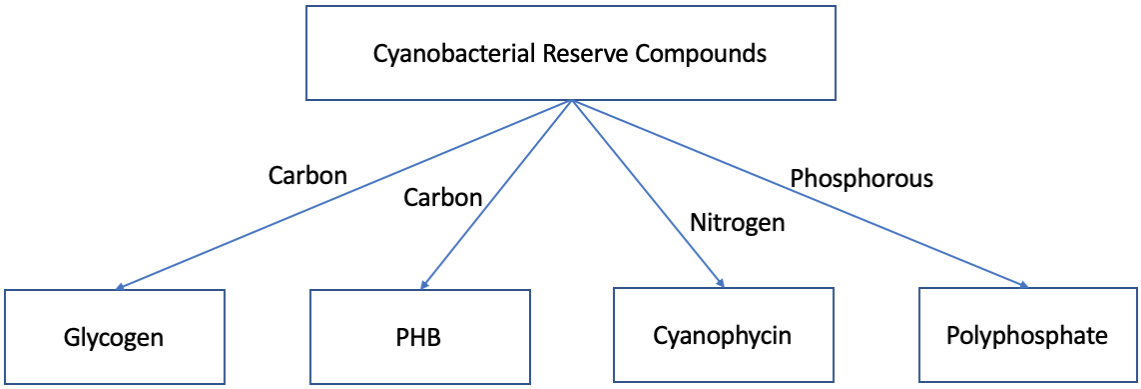


Figure 1-3: Major cyanobacterial storage polymers (information adapted from (Flores & Herrero 2014)).

These storage compounds include phosphate stored as polyphosphate granules (Thompson, Oh, Rhee, 1994) and nitrogen in the form of cyanophycin (Esteves-Ferreira et al. 2018). These compounds effectively uncouple the growth of cyanobacteria to the external concentrations of phosphate or nitrogen and create a buffer to nutrient fluxes in dynamic environments by having internal reserves accessible to support growth under limiting conditions.

Energy and carbon are also stored in a similar strategy. In times of surplus light and CO_2, glycogen (a poly glucose) is synthesised with excess metabolic energy, especially when growth is limited by other compounds such as nitrogen or phosphorous (Kaewbai-ngam, Incharoensakdi & Monshupanee 2016; Singh et al. 2017). Glycogen is then oxidised during the dark period as an energy source via the oxidative pentose phosphate pathway (Smith 1983). Similar to glycogen, PHA/PHB in cyanobacteria also utilise excess intracellular carbon (in the form of Acetyl-CoA) and reduction equivalents (NADH) for their synthesis. Previous researchers believed glycogen to be a more efficient energy storage compound relative to PHA/PHB due to cyanobacteria having an incomplete tricarboxylic acid cycle (de Philippis et al. 1992). However, recent research shows that the cyanobacterial tricarboxylic cycle is still operational through the use of different enzymes (Steinhauser, Fernie & Araújo 2012; Zhang & Bryant 2011). Despite this, the exact role of PHB in cyanobacterial metabolism is still unclear, as glycogen occurs in significant amounts in most cyanobacteria and having

multiple carbon storage compounds for the same purpose is inefficient. In support of this, most microorganisms produce either glycogen or PHB and not both (Damrow et al. 2016). Although, it is possible that PHB could serve as a longer term carbon storage compound in the cell compared to glycogen (Koller 2015). It has been proposed by Phillipis et al (1992) that PHB's main role is to regulate excess reducing power and act as an electron sink (although glycogen fulfils this role too). Hauf et al demonstrated that the intracellular redox state of the cell is critical to producing PHB, with a high NADPH to NADP+ being a condition for PHB accumulation (Hauf et al. 2013). This could be for protecting the organism against excess charge or as storage for other electron demanding processes such as nitrogen fixation, of which many species of cyanobacteria are capable (de Philippis et al. 1992). However, there are many non-nitrogen fixing species of cyanobacteria that accumulate PHB. Another proposed pathway is that the Acetyl-CoA derived from metabolising PHB can be used for other biosynthetic purposes (Stal 1992) such as fatty acid biosynthesis and nitrogen assimilation. Lastly, Sznajder et al (2015) have proposed that PHB could play a structural role during the process of dividing nucleoids in future daughter cells.

Interestingly, in a study by Kucho et al (2005) on the model cyanobacteria *Synechocystis* sp. PCC6803, it was determined that the expression of PHB synthesis-related genes were linked to a circadian rhythm. Cyclic expression peaked at the transition from light to dark, along with other genes related to respiration (Kucho et al. 2005). Based on this observation, the authors suggested that PHB could play a role in supplying energy and carbon during the night; however, the study did not examine glycogen expression at all. Köbler et al (2018) created *Synechocystis* sp. PCC6803 mutants lacking a key circadian rhythm regulator, RpaA, which induces the expression of 'dusk' genes to prepare the cell for the onset of the dark period. It was found that in the RpaA deleted mutants, the expression of PHB synthesis genes (PhaE and PhaC) were significantly reduced.

Although PHB accumulates to a lesser degree than glycogen in cyanobacteria, it has been suggested that under temporarily unfavourable energetic conditions (such as at night) cyanobacteria are more likely to consume glycogen over PHB (Drosg 2015). Koch et al (2019) proved that the majority of PHB is indeed produced from existing intracellular glycogen stores upon the prolonged exposure to nitrogen starvation, and not directly from

atmospheric CO_2. This was achieved by creating glycogen lacking mutants through knocking out glycogen synthase and phosphorylase enzymes, in which an extreme reduction in PHB production was observed. Acetyl-coA can be produced from glycogen through three different pathways in *Synechocystis* sp. PCC6803; the Embden Meyerhof Parnas (EMP), Entner Doudoroff (ED) and Oxidative Pentose Phosphate (OPP). Through blocking each pathway separately, it was shown that the EMP and OPP pathways were the most important for providing carbon for PHB production from internal glycogen stores (Koch et al. 2019).

Damrow et al (2016) also created several *Synechocystis* sp. PCC6803 mutants lacking glycogen synthesis and/or PHB synthesis capability to investigate the role of both carbon polymers in the response to stressful environmental conditions. Under low light and nitrogen stress conditions, the PHB-deficient mutant performed similarly to the wild type in growth and recovery capability. However, the glycogen-deficient mutant and double PHB glycogen-deficient mutant both showed significant decreases in viability and growth. It was concluded that glycogen plays a greater role in both energy storage and macronutrient acclimation responses compared to PHB.

In a study by Raberg et al (2014), the proteome of a mutant *Cupriavidus necator* (the model heterotrophic PHB organism previously known as *Ralstonia eutropha*) strain lacking PHB synthesis was investigated. To prevent over acidification of the cell from accumulating metabolites, such as pyruvate and Acetyl-CoA, these mutants excreted pyruvate and upregulated the synthesis of several proteins to convert pyruvate and Acetyl-CoA into other metabolites, with a large proportion of the excess Acetyl-CoA entering the tricarboxylic acid cycle (Raberg et al. 2014). Thus, in both heterotrophs and autotrophs, PHB seems to be a non-essential metabolite for survival; however the relevance of this study to phototrophic PHB production could be strengthened by comparing a PHB-deficient cyanobacterial mutant proteome to its wild type too. Hauf et al did perform metabolomics on a PHB deficient cyanobacterial mutant and found difference in amino acids, TCA intermediates and sugars compared to the wild type under nitrogen stress conditions. One of the key findings was an increase in sorbitol levels in the PHB-deficient mutant, which indicated a more oxidizing

intracellular state due to a lower NADPH to NADP ratio. This highlighted the importance of a highly reducing intracellular environment for PHB production.

To determine the exact role and function of PHB in cyanobacteria, future studies should focus on different types of PHB-deficient mutants grown in varied environments with a range of proteomic, metabolomics, transcriptomic and functional genomics approaches. It will be important to determine where the excess Acetyl-CoA sink occur in these PHB-deficient cyanobacteria. Future studies investigating the metabolic significance of PHB would also benefit from comparing the results between both heterotrophs and autotrophs. Differences in phenotypes could potentially identify whether PHB plays a similar or different metabolic role in different classes of organisms.

PHB CELLULAR METABOLISM

The conversion of atmospheric CO_2 into PHA begins with the Calvin Benson cycle, via glycolysis to pyruvate and then to Acetyl-CoA. The metabolic pathway for the biological synthesis of PHB, the most prevalent PHA, consists of three enzymatic reactions converting Acetyl-CoA to PHB. The first enzyme, PhaA (PHA specific ß-ketothiolase), combines two Acetyl-CoA molecules into Acetoacetyl-CoA. The second enzyme, PhaB (Acetoacetyl-CoA reductase), then reduces this compound leading to hydroxybutyryl-CoA. The final step involves PhaEC (PHB synthase) and the addition of the hydroxybutyryl-CoA fatty acid monomer to a growing PHB molecule via an ester bond (Balaji, Gopi & Muthuvelan 2013). The reaction scheme is shown in Figure 1-4. The general synthesis of PHB from heterotropic and phototrophic organisms are the same; however, the four genes of PhaA, PhaB and PhaEC are located on a single operon in heterotrophs (such as in *Cupriavidus nectator*), while in cyanobacteria (*Synechocystis* PCC6803) the genes are located in two separate operons approximately 500Kbp apart. The first loci contains the genes for PhaA and PhaB and are putatively co-expressed. The second loci contains the genes for PhaEC, a heterodimer consisting of PhaE and PhaC (Drosg 2015) (Hauf et al. 2015).

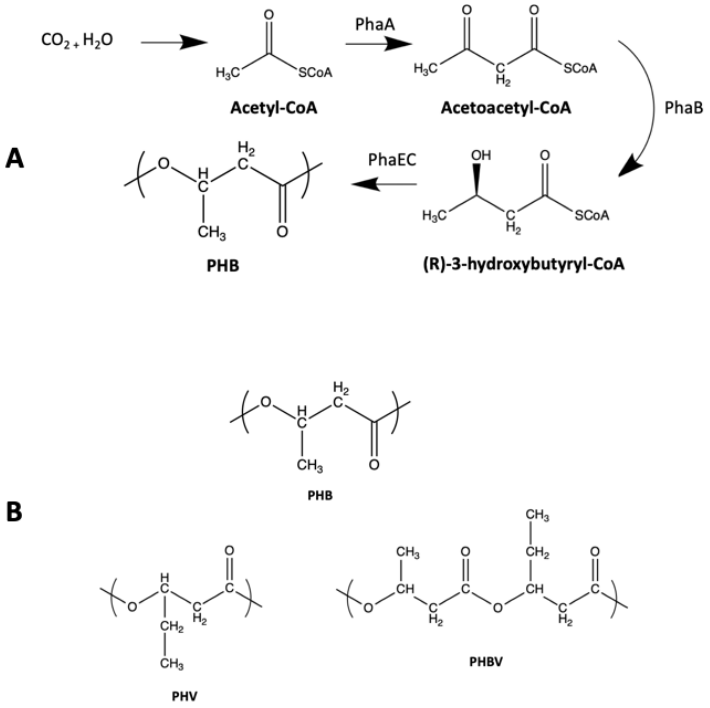


Figure 1-4: ***(A)*** *Metabolic pathway of PHB synthesis in Synechocystic PCC 6803* ***(B)*** *Molecular structures of PHB, PHV and PHBV.*

Apart from the three PhaA, PhaB and PhaEC enzymes not much more is known about the other PHB-related proteins in cyanobacteria in contrast to that of heterotrophs (Hauf et al. 2015). It is known that in *Cupriavidus nectator* the PHB granules exist in a protein complex. Some of these proteins, for example are transcriptional regulators (PhaR) and others are depolymerases (PhaZ) (Hauf et al. 2015). In total, approximately 8 different PHB-related proteins across different heterotrophic bacteria have been identified and their roles are summarised in Table 1. It is likely that similar proteins with similar roles would exist in cyanobacteria; however, this has yet to be confirmed. For example, the extra and intracellular depolymerases required for PHB degradation have not been identified in any cyanobacterial strains to date, but have been identified in many heterotrophic bacteria (Flores & Herrero 2014).

Table 1-1: Summary of different PHB related proteins and their roles

PHB Related Protein Name	Function
PhaA	Responsible for first step in PHB formation, converting 2 acetyl-coA molecules to acetoacetyl-coA.
PhaB	Responsible for second step in PHB formation, reduction of acetoacetyl-coA to form hydroxybutryl-coA.
PhaEC	Heterodimer of PhaE and PhaC which adds the hydroxybutyryl-coA monomer to growing PHB chain.
PhaR	Transcriptional regulator modulating synthesis of PHB. In *Ralstonia eutropha* PhaR binds upstream of PhaP1 gene in promoter region repressing its expression (Waltermann & Steinbuche 2005).
PhaZ	Depolymerase responsible for degradation and using stored PHB.
PhaM / PhaF	Mediate nucleoid attachment of PHB granule and thus provides a mechanism for equal distribution of PHB to daughter cells during cell division. In a study with this gene knocked out, PHB granules were unequally distributed to daughter cells (Pfeiffer, Wahl & Jendrossek 2011).
PhaP	Promotes stress resistance through chaperone activity and regulates the surface area to volume ratio of PHB granules in cytosol. In a study with this gene knocked out, cells had the same mass of PHB, but stored in fewer larger granules. Knockout mutants could only have half as many PHB granules in the cytosol (Hauf et al. 2015).
Pta	Phosphotransacetylase (Pta) catalyses the reversible conversion of Acetyl-CoA to Acetyl phosphate. It is likely that Pta activity may regulate the activity of the PHB synthesis pathway through the presence of Acetyl phosphate, as Acetyl phosphate may play a role in activating PHB synthase (Miyake et al. 2000).
sll0783	sll0783 is a protein of unknown function in a cluster of 7 genes that appears to be related to nitrogen starvation acclimatisation. A mutant Synechocystis PCC 6803 strain with sll0783 knocked out was unable to synthesise PHB, but still had similar concentrations of precursor Acetyl-CoA and similar expression of PhaA, PhaB and PhaEC. From metabolomic analysis, the mutant was shown to have a less

	reducing intracellular state compared to wild type. This suggests that sll0783 plays a role in regulating the redox potential (Hauf et al. 2013).
sll0461 (proA)	sll0461 encodes gamma-glutamyl phosphate reductase (proA). A disruption in this native gene from inverse metabolic engineering resulted in a higher PHB phenotype in *Synechocystis* PCC 6803 (Tyo et al. 2009).
sll0565	sll0565 encodes a hypothetical protein. A disruption in this native gene from inverse metabolic engineering resulted in a higher PHB phenotype in *Synechocystis* PCC 6803 (Tyo et al. 2009).

PHB is the only PHA produced photoautorophically. However, multiple PHA polymers including PHV (poly-hydroxy-valerate) can be produced by growing cyanobacteria under mixotrophic or heterotrophic conditions. Growth with different carbon substrates such as valerate instead of acetate can cause PHV to be accumulated instead. Alternative co-polymers of PHA can be produced when mixed substrates are used, such as valerate and acetate which results in 3 hydroxy-butyrate co 3 hydroxy-valerate. Figure 1-5 shows the co-polymer molecular structures in comparison to PHB. Such co-polymers tend to offer better material properties such as reduced brittleness and stiffness (Zhao & Turng 2015). Table 2 compares several physical properties of PHB, PHBV, some other PHAs and polypropylene.

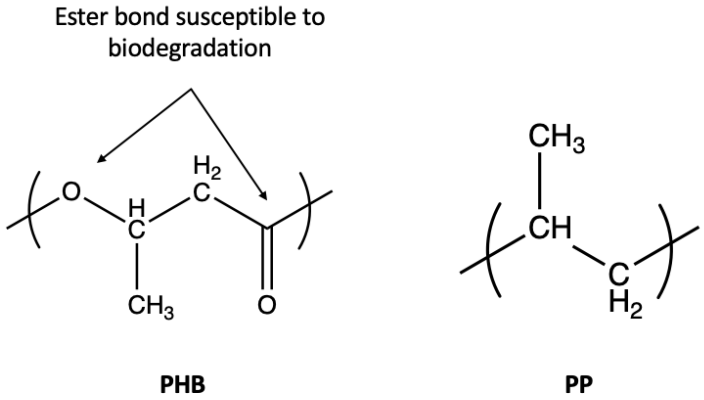


Figure 1-5: Molecular structure of PHB and polypropylene (PP).

Although not all cyanobacteria produce PHB, many species of cyanobacteria can. Most yields are negligible (at below 1%) and many yields are well under 5% by dry cell weight (dcw) in most studies using non-genetically modified strains grown under photoautotrophic conditions (Drosg 2015; Singh et al. 2017; Stal 1992; Troschl, Meixner & Drosg 2017). Noticeably, heterotrophic production has been able to double PHB yield in some species of cyanobacteria compared to phototrophic only production and peaked at around ~40% dcw (Singh et al. 2017; Singh & Mallick 2017b; Troschl, Meixner & Drosg 2017). A thermophilic strain of cyanobacteria isolated from a Japanese hot spring, *Synechococcus* MA19, was reported to achieve the highest reported phototrophic cyanobacterial PHB yield of 55% (Nishioka et al. 2001). However, the highest non thermophilic and non-genetically modified cyanobacteria yields are around 20-25% (Kaewbai-ngam, Incharoensakdi & Monshupanee 2016; Troschl, Meixner & Drosg 2017). Genetically engineered and recombinant cyanobacteria peaked at roughly 26% PHB yield (Carpine et al. 2017). Most recently, UV random mutagenesis was able to generate a mutant strain with 37% yield PHB (Kamravamanesh et al. 2018).

The polymeric lipid, PHB, has been claimed to be the storage compound exclusively for prokaryote, whereas eukaryotes instead accumulate lipid bodies in the form of triacylglycerols (Ansari & Fatma 2016; Waltermann & Steinbuche 2005). Surprisingly, two eukaryotic genus of algae, *Botryococcus* and *Chlorella*, have now recently been reported to naturally produce PHB. *Botryococcus braunii* was reported to achieve 10-16% yield PHB (Kavitha et al. 2016) and *Chlorella pyrenoidosa* and *Chlorella fusca* achieving yields of 17% and 27% (Cassuriaga et al. 2018; Das, Sathish & Stanley 2018).

The only other occasions where a eukaryotic algae produced PHB has been through genetic engineering efforts. The first was achieved by Chaongang et al (2010) who transformed *Chlamydomonas* reinhardtii by inserting only phaB and phaC genes, as phaA activity was claimed to be intrinsic. A yield of only 0.0006% PHB was achieved. Secondly, Hempel et al (2011) genetically modified *Phaeodactylum tricornutum* with PHA synthesis genes from *Cupriavidus nectator* with a yield of 10% PHB achieved.

The above points are highlights from the literature, however detailed tables with major PHB yields achieved have been summarised by Singh et al (2017) and Troschl et al (2017). It should be noted that heterotrophic bacteria have been shown to achieve yields as high as 80% PHB (Wang & Lee 1997) in conjunction with higher biomass productivities compared to cyanobacteria. The economic implications of heterotrophy vs autotrophy for industrial production will be discussed later.

MAXIMISING PHOTOAUTOTROPHIC PHB YIELDS

From reviewing the available PHB cyanobacteria literature, the most common inducer of high PHB production has been a limitation of nitrogen in a carbon excess environment (Campbell, Stevens, Jr., & Balckwill, 1982; Kaewbai-ngam et al., 2016; Keith E. Tyo, Hang Zhou, 2006; Panda, Sharma, & Mallick, 2005;). However, some cyanobacteria have shown optimal PHB yields either under a combination, or complete substitution of nitrogen for other nutrient limitations. For example, in the study by Kaewbai et al (2016), 137 strains of cyanobacteria were screened for PHB yields under a range of different nutrient limitations including nitrogen, phosphorous, potassium and different combinations of the three. Over 50% of the screened cyanobacterial strains showed a significant increase in PHB yield under nitrogen limitation conditions. Of these, 75% had peak PHB yields when nitrogen was limited (either by itself or in combination with other nutrients). One species however, *Mastigocladopsis* sp., had peak PHB yield of 7% under nutrient balanced conditions and showed decreases in PHB yield under all nutrient deprivation environments. Of interest is that most (over 75%) of the cyanobacterial species only accumulated significant amounts of PHB under only one of the above nutrient conditions (nitrogen, potassium, phosphate or combination), as opposed to a few strains accumulating significant amounts of PHB across a range of nutrient limitations. This suggests that high PHB accumulation for different species or strains are environmentally specific, which has implications for future screening efforts.

Many strains of cyanobacteria in this screening study were capable of atmospheric nitrogen fixation, and showed the greatest PHB yield under nitrate-limited conditions. This was the case for several *Calothrix* species (Zehr 2011) which showed high yields of PHB (17-25% dcw) which was only induced under nitrogen limitation. Other nutrient limitation conditions

such as phosphorous (including combined with nitrogen limitation) resulted in significantly less PHB accumulation. This supports the earlier statement that PHB could serve as a reservoir for excess reducing potential for electron demanding processes such as nitrogen fixation (de Philippis et al. 1992). Future studies between PHB and specifically nitrogen-fixing strains could provide additional insights to these unanswered questions.

Most cyanobacterial studies to date focus on nitrogen deprivation only for optimising PHB yield, with some including phosphorous and combinations of the two. In heterotrophic bacteria, other nutrient limitations such as oxygen and sulphur in conjunction with a carbon excess are used to induce PHB production (Raberg et al. 2014). Thus, combinations of nitrogen, phosphorous, potassium, sulphur and potentially oxygen deprivation during the dark period could be explored for optimising PHB yields and gaining a greater understanding of the role PHB metabolism in cyanobacteria.

DETECTION METHODS AND ANALYSIS TECHNIQUES OF PHB

For the purpose of quantifying PHB during an experiment or screening for the presence of PHB in untested strains of cyanobacteria, several techniques are available. Sudan Black B has been used as a stain for microscopic visualisation (Balaji, Gopi & Muthuvelan 2013). After heat fixing to glass slides, a Sudan Black B staining solution of 0.3% in 70% ethanol is added to the slides and left to incubate for 5-10 minutes. After this, slides are then placed in xylene solution until decolourised and then counterstained with 0.5% safranine solution in water (Wei et al. 2011). There are several other dyes that have higher staining efficiencies such as Nile Red, Nile Blue, and BODIPY; however these techniques stain all neutral lipid compounds and are not PHB specific (Rumin et al. 2015).

Nile Blue A is an oxazine dye that has been used extensively in this field of research for microscopic visualisation and in some instances the fluorescent quantification of PHB. The method was first developed by Ostle in 1982 and involved immersing heat fixed samples on glass slides into a 1% Nile Blue A solution for 10 minutes at 55˚C. Following a wash with acetic acid and water to remove excess dye, the cells were excited at 460 nm (Ostle 1982). The incubation time, temperature and dye concentration have been modified in different

methods over the past four decades. Notably, Oshiki et al (2011) were able to develop at high throughput rapid quantification for PHB for heterotrophic bacteria from wastewater sludge. The method involved a 0.02% Nile Blue A solution which was added in a 1:1 ratio to cell culture and incubated at room temperature for 3 minutes. A fluorescent plate reader was used with a 490 nm excitation and 590 nm emission wavelength setting.

It is likely that during incubation, the Nile Blue A oxazone dye molecules convert to the oxazine version of the dye which is also known as Nile Red. This is further confirmed by the fluorescent peak of 550-590 nm observed with Nile Blue (Oshiki, Satoh & Mino 2011) which is the same as the Nile Red fluorescent peak for non-polar lipids (Rumin et al. 2015). The oxazone Nile Red molecule is non-polar and can bind to the non-polar PHB granule better than the oxazine Nile Blue A molecule which has a polar imminium functional group. For this reason, it is likely that Nile Red is the superior dye as a higher overall staining efficiency can be achieved without an additional oxazine to oxazone conversion step. Recently, Nile Red has become more widely used since the initial method paper by Ostle et al (1982) was published and has been used more frequently than Nile Blue in cyanobacterial PHB detection. Several different methods of Nile Red staining are available and involve different solvents (such as DMSO or ethanol), in the presence of different incubation times, the presence of glycerol to facilitate permeation of cell membrane, cell concentration, vortexing and agitation (Khetkorn et al. 2016; Morschett, Wiechert & Oldiges 2016; Shrivastav, Mishra & Mishra 2010). In addition to fluorescent microscopy and microplate reader, flow cytometry has also been used with this method focussing on the quantification of PHB (Tyo & Hang Zhou, 2006). The Nile Red PHB staining protocol must be optimised for different cyanobacterial species due to differences in cell physiology and the cells must first have sufficient yield of PHB before a signal can be measured. This threshold is different for each cell; however, a yield of at least greater than 1% dcw is recommended. Lastly, the autofluorescence of cyanobacteria often overlap with the Nile Red and Blue fluorescent peaks (Luimstra et al. 2018; Schubert, Schiewer & Tschirner 1989; Schulze et al. 2011) which interferes with detecting PHB granules.

BODIPY (boron-dipyrromethene) fluorescent dyes have also be used for microalgae lipid studies and PHA in heterotrophic bacteria (Biernacki et al. 2017; Rumin et al. 2015). BODIPY

has been claimed to have less background staining, a higher specificity and sensitivity to PHB compared to Nile Red (Kacmar et al. 2006). In addition, BODIPY fluorescent dyes can be chosen with different fluorescent peaks that do not correspond with cyanobacteria autofluorescence to achieve higher signal clarity. Despite these advantages, BODIPY has not been used for PHB quantification in cyanobacteria as of yet.

The dyes and protocols mentioned above stain for total lipids, including PHB. However, for PHB quantification, the total fluorescence must be correlated to the amount of PHB present in the cell culture. For this purpose, chemical analysis methods are required to create a standard calibration curve to relate dye fluorescence to the amount of PHB. Gas chromatography (GC) and high performance liquid chromatography (HPLC) are the two most commonly used methods (Ansari & Fatma 2016; Wu, Shen & Wu 2002). It is unknown whether the PHB to lipid ratio and ultimately PHB to total dye fluorescence signal would change over time, at different cell growth stages or under different nutrient deprivations. Thus, it is recommended that future protocols create these calibration curves under different conditions to either ensure an accurate calibration curve for that environment or to confirm that the PHB to dye fluorescence signal remains constant.

A standard PHB GC protocol begins with harvesting biomass through centrifugation, washing and drying. 2 mL 1,2-dichloroethane, 2 mL of hydrochloric acid propanol mixture (1:5 ratio) and 200 µL of standard solution (2 g benzoic acid and 50 mL propanol) are added to the dried biomass and incubated at 100˚C for 2 hours (Riis & Mai 1988). Water is added after cooling and the heavy phase can be injected into the chromatograph. Quantification is achieved by measuring the peak area and comparing against a hydroxy butyric chemical standard (Riis, & Mai, 1988). For HPLC, cell biomass is harvested, washed and dried. Following this the dried pellet is digested in concentrated sulfuric acid for one hour which converts the PHB polymer chain into crotonic acid monomers. The sample is then diluted to prevent damage to column from the sulfuric acid and then run in the column against a crotonic acid standard for quantification and to determine reference elution time (Koller & Rodríguez-Contreras 2015). A PHB standard curve can also be run in parallel to determine the PHB to crotonic acid digestion efficiency.

Law et al (1961) created a UV spectroscopy protocol for quantifying PHB. After processing PHB to crotonic acid by using sulfuric acid similar to above, crotonic acid abundance can be determined by measuring the absorbance at 235 nm wavelength. Pre-processing the biomass sample with chloroform, acetone or dichloromethane to isolate PHB is recommended due to the possible signal interference from other chemical compounds in the biomass mixture (Law & Slepecky 1960). Alternatively, this method can be used on eluted compounds from a chromatography column when using HPLC to confirm the eluted material is crotonic acid (Hauf et al. 2015).

PHB Material Properties

PHB is similar to polypropylene in terms of molecular structure. Both polymers have similar sized monomers with a single methyl side pendant group resulting in comparable physical properties such as being water-resistant and very close melting temperatures. However, the greatest difference is that the PHB monomers are joined via ester bonds which allows for biodegradation when exposed to a microbial community in the environment (Shah et al. 2008). Microorganisms can release enzymes which cleaves these ester bonds and degrade the polymer into oligomers and monomers, which is followed by uptake into the cytosol and metabolisation to either carbon dioxide or methane under aerobic or anaerobic conditions, respectively. In contrast, polypropylene, and most conventional petrochemical plastics, are impervious to microorganism enzymes and biodegradation due to their carbon-carbon bonds. Although polypropylene can be physically disintegrated into smaller pieces, its molecular structure makes it far more chemically inert than PHB causing their accumulation in the environment resulting in plastic pollution and the subsequent production of microplastics.

Table 2 compares some of the physical properties of PHB, polypropylene and some other PHAs. PHB and isotactic polypropylene (PP) have extremely similar melting temperatures, crystallinity and tensile strength. However, PHB is somewhat more brittle (unable to accommodate a different shape before shattering or breaking under an applied force) as can be seen by the extension or elongation of the material at breaking point. Despite this difference, PHB is still a highly valued candidate for PP replacement in many applications. It

should be noted that after polyethylene, PP is the second most prevalent petrochemical plastic used across the world (Geyer, Jambeck & Law 2017).

Table 1-2: Comparison of physical properties between PHB and polypropylene (PP) (data adapted from (Anbukarasu, Sauvageau & Elias 2015; Balaji, Gopi & Muthuvelan 2013; Samper et al. 2018)).

Properties	**PHB**	**PP**
Melting temperature (Celsius)	177	176
Glass transition temperature (Celsius)	2	-10
Initial degradation temperature (Celsius)	220	357
Crystallinity (%)	60	50-70
Tensile strength (MPa)	43	38
Extension to break (%)	5	400

The melting temperature and glass transition temperature are two critical thermal properties that determine how a plastic polymer can be used for a particular purpose. The melting temperature determines what temperature the resin must be heated to before processing, for example by injection moulding. Because PHB's melt temperature is close to its degradation temperature, processing results in thermal instability and can cause a reduction in PHB molecular weight and physical properties (El-hadi et al. 2002). The glass transition temperature is the temperature at which a thermoplastic polymer becomes less glassy and brittle and shifts towards a less rigid and rubbery state as the polymer molecules have more energy to overcome a crystalline and ordered structure and begin to orientate themselves randomly (Stevens, 2016). Because PHB's glass transition temperature is close to room temperature, this results in a growing amorphous phase over time, whereby the polymer chains gain higher degrees of freedom and lose structure. In addition to these two problems, PHB has a low nucleation density. Nucleation density is the number of phase change spherulites per unit volume that form when the polymer melt shifts from liquid to solid as it cools (Fraser, Keller, Odell, & Wills, 1978). Because there are less nucleation

points, the spherulite crystals are bigger which can result in cracks and splits (contributing to brittleness) adversely affecting mechanical characteristics. Despite this, research is being carried out to blend PHB with other polymers and additives to improve processability and mechanical properties (Armentano et al. 2015; Fonseca, Souza & Or 2017; Ni & Woo 2013). Additionally, a select few species of cyanobacteria have been shown to produce the co-polymer PHBV which can have some superior material qualities to PHB such as improved elongation (Tarawat, Incharoensakdi & Monshupanee 2020). However, the exact material properties depend on co-polymer factors such as the exact ratio of butyrate and valerate monomers.

Industrial Cyanobacterial PHB Production

The industrial cultivation options and considerations for cyanobacterial PHB production are similar to those already reviewed for the commercial cultivation for algae biofuels and other commodity (low value high volume) algal products (Adeniyi, Azimov & Burluka 2018; Mata, Martins & Caetano 2010). The two main options are open or closed cultivation systems which each have their own advantages and disadvantages.

CULTIVATION SYSTEM

Open Raceway Ponds (ORPs) are large shallow ponds approximately 40 cm deep, generally mixed by a rotating paddle wheel (Richardson et al. 2014). The ponds are filled with media and often have carbon dioxide and air sparging systems to provide the culture with required gases during the day and night, respectively. ORPs are harder to monitor and control often because the culture is not fully mixed and there can be significant variability throughout system such as for pH, temperature and cell density. Because of reduced mixing, more cell settling and less sunlight is distributed across the cyanobacterial population, resulting in less than optimal biomass productivity. In ORPs, there is also significantly lower carbon dioxide utilisation due to open nature of the pond which allows for degassing. ORPs also suffer from high amounts of evaporation which will vary depending on site location. If marine strains are cultured then concentration of salts over time will require significantly higher amounts of media bleed and replacement. Greenhouses covering raceway ponds are an option to reduce evaporation; however, the added cost often does not cover the loss in water

financially, despite being the environmentally superior option. Cultivation in open systems results in extreme seasonal variability and a lack of reproducibility, although similar problems are experienced by terrestrial crop cultivation too. Lastly, ORPs are open to many vectors for both contamination of the media and for cultivation algae or cyanobacteria to enter surrounding ecosystems. These vectors include abiotic factors such as rain or wind and biotic factors such as birds, insects or microbial predators. Culture crashes can occur if other microalgal species or cyanobacterial predators such as viruses or protozoa enter the cultivation system. Escaped cultivation strains into surrounding ecosystems can also disrupt and alter food chains (Henley et al. 2013).

Closed Photo Bioreactors (PBRs) on the other hand are sealed systems with a transparent material such as glass or plastic which allow for light to penetrate. Many designs for closed PBRs exist such as flat panel, hanging plastic bag, or tubular. PBRs are able to achieve far higher amounts of mixing and control over cultivation parameters such as temperature and pH. There is a higher surface area to volume of culture too, which allows for a greater light distribution across the cultivation culture, and the combination of these two factors results in far higher biomass productivities and maximum cell densities. These systems are also more resilient to culture crashes and exposing cultivation strains to the environment; however, the gas venting and media introduction ports still confer routes for contamination and can never remain completely axenic. Greater carbon dioxide utilisation can be achieved in PBRs due to longer retention times and lower evaporation of water is also a benefit. However, these systems are significantly more expensive to build and operate.

Figure 1-6: Methods of cultivating algae. ***(A)*** *Bubbled column PBR* ***(B)*** *closed horizontal tubular PBR with pump for mixing* ***(C)*** *Open raceway pond.*

Lighting and gas exchange – Photoautotrophic production requires light in the photosynthetically active radiation range (typically between 400-700 nm). Light must be supplied such that it is not the limiting factor for growth. Effective mixing is required to reduce settling and ensure movement of cells in the media and maximise the exposure of the average cell population to sufficient light. The light intensity must not be too high, as this will result in photoinhibition whereby cellular photosynthetic machinery becomes damaged. Artificial lighting may be economically feasible only for high-value algae products such as nutraceutical or pharmaceutical compounds, whereas it is cost prohibitive for commodity products of lower unit value such as bioplastics and biofuels. Thus, growth with sunlight is the most viable option. While carbon dioxide must be supplied during the light period for photosynthetic metabolism, oxygen may need to be supplied during the dark period for respiration depending various cultivation factors. To maximise gas exchange, for both carbon dioxide and oxygen, smaller bubbles which maximise surface area and a longer bubble retention time in the liquid phase are required.

Nutrient source – For photoautotrophic production, the main nutrient requirements for cyanobacteria are nitrogen, phosphorous, and carbon dioxide with small amounts of minerals and trace metals. The bulk nitrogen and phosphorous can be supplied in industrial forms using commodity chemicals such as urea and diammonium phosphate. Concentrated carbon dioxide sources will be discussed later. Although heterotrophic production of PHB results in higher PHB yields, using organic substrates in an open cultivation facility will drastically increase the likelihood of culture crashes (Troschl, Meixner & Drosg 2017) as observed in pilot production (200L) scale cultures in Austria (Troschl et al. 2017). However, cyanobacteria under phototrophic production will begin to release organic carbon through excretion or cell lysis upon death which can become an energy source for heterotrophic bacteria leading to a crash. If media is reused between cultures then total organic carbon (TOC) should be closely monitored and kept low to prevent culture crashes. Under nitrogen and/or phosphorous limited conditions for PHB production, cyanobacteria cells will have a lower fitness and be more prone to culture crashes too.

Another potential source of nutrients is wastewater – whether it is integrated into an existing wastewater treatment plant scheme or the run off from agriculture – wastewater is

able to provide much of the bulk nutrients and most of the trace minerals and metals in excess. However, growing PHB in wastewater would add complexity in inducing nutrient limitation and reduce the ability to control nutrient stoichiometry for optimal cyanobacterial growth and PHB production. However, dilution and addition of other nutrients would potentially be a viable option to control the nutrient ratios in the wastewater. Another consideration are the increased contaminants (both biotic and abiotic) such as competing microbes, pollutants, or toxic heavy metals that could adversely affect the cyanobacterial growth and PHB accumulation. In addition to adversely affecting production economics, contaminants (such as lipophilic compounds) may affect the final product quality.

Concentrated CO_2 sources - Cyanobacteria typically use atmospheric carbon dioxide for their growth in the natural environment. However, due to low levels of carbon dioxide in atmospheric air, concentrated sources of carbon dioxide offer the advantage of higher growth rates, PHB yields and thus higher PHB productivities. Potential high concentration carbon dioxide sources include combustion flue gas from energy generation or waste 'off gases' from chemical processes such as cement production or fermentation industries. Such sources of carbon dioxide may have to be first treated by processes such as scrubbing to remove chemical contaminants before used in the culture.

pH – Most cyanobacteria are alkalophiles and grow between pH ranges of 7-9 (Robert, 2005). An alkaline pH allows for an increased efficiency in intracellular carbon concentration machinery (Mangan et al. 2016) and ultimately assists with energy production through photosynthesis. The pH of a cultivation system will not remain static over time due to the large uptake of ions linked to the higher amounts of biomass, thus there is a need for pH control. For example, carbonate and nitrate ion absorption will tend to raise the pH of the media over time (Mattson 2009). The pH can be lowered and thus controlled by bubbling carbon dioxide into the culture via a feedback response system (Robert, 2005). This simultaneously keeps pH at the optimum range for the strain being grown, while ensuring an excess of carbon for PHB production and biomass growth.

Temperature – The optimum temperature for most cyanobacteria is approximately 25˚C or slightly warmer (Coles & Jones 2000; Robarts & Zohary 1987). However, psychrophile

cyanobacteria grow in Arctic and Antarctic climates (Martineau et al. 2013; Nadeau, Milbrandt & Castenholz 2001) and thermophilic cyanobacteria such as those from hot springs thrive in temperatures over 50˚C (Steunou et al. 2006). Although temperature extremes may provide a selective environment for cultivation and help reduce culture crashes, large scale heating or cooling of industrial ponds or photo bioreactors are unlikely to be economically feasible.

Selective cultivation conditions – Other than thermophilic cyanobacteria, there are other species that prefer extreme environments such halophiles or alkalophiles like *Athrospira platensis* (Shiraishi 2016) and *Synechocystis* DUN52 (Mohammad, Reed & Stewart 1983). Such strains can be kept in their preferred chemical conditions which are unfavourable for other contaminating microorganisms. PBRs are somewhat difficult to clean and sterilise between runs and ORPs are completely unfeasible to sterilise. Thus, another method to reduce culture crashes is potentially switching between fresh and salt water cyanobacteria species between runs, as this extreme change in environment will likely destroy most contaminating microbes.

Final strain choice – Several factors must be taken into account when choosing the cyanobacterial strain for PHB production. Ultimately, PHB productivity (which is a combination of PHB yield and biomass productivity) will be one of the most important parameters. However, resilience to culture crashes and fluctuations in environmental conditions such as temperature and light are also extremely important. It may be worthwhile having multiple strains or species at a single production site throughout the year based on climatic variations in temperature and light availability. In addition to this, it is important to choose non-harmful bloom-forming species or species that do not release toxic compounds (Henley et al. 2013). Ideally, a PHB production strain would have traits that make it superior for PHB production in a controlled growth facility, but traits that cause it to be less competitive and fit in the wild to reduce impact upon surrounding environmental ecosystems.

Cultivation Strategy – Because PHB yields tend to be low under balanced nutrient conditions, continuous culture and harvesting of cyanobacteria is unlikely to be industrially

viable. Thus, the overall strategy for industrial PHB accumulation is similar in essence to that at bench scale. The initial phase is optimised for biomass which is followed by holding the culture at nutrient deprivation with excess carbon present. However, most studies involve growing the culture in balanced media and re-suspending biomass into a nutrient-limited media (normally lacking nitrogen) and then measuring the accumulation of PHB over time. In practice, it will not be cost effective to harvest all algae and re-suspend it in new nutrient-limited media during commercial operation. The most realistic solution is to grow it under batch conditions with continuous excess of carbon dioxide, and wait for nitrogen or the nutrient of limitation for the particular cultivation strain to deplete. The optimal ratios of nutrients must be determined in advance and media will be made to this composition before inoculation. For example, Carpine et al determined that BG-11 with 50% of the nitrate concentration was optimal for PHB accumulation in laboratory scale *Synechocystis* PCC6803 cultures without resuspending cultures in new nutrient deplete media (Carpine et al. 2019). Troschl et al ran pilot plant scale cyanobacterial production runs with approximately 33% nitrate concentration of the original BG-11 formula for a 200 L tubular PBR with *Synechocystis salina* (Troschl, Meixner & Drosg 2017). A key difference to laboratory culturing for PHB is that the nutrient(s) of limitation will run out slowly, as opposed to a sudden flux to zero from re-suspension. As a result, the effect of the nutrient limitation imposed by a slow depletion with constant carbon dioxide similar to industrial cultivation must be investigated before a strain is chosen for industrial cultivation. Such study should determine the optimal nutrient stoichiometry to include in initial media for optimising batch cyanobacterial PHB cultivation. Alternatively or in combination with the above, non-limiting nutrients could be topped up during cultivation or during the PHB accumulation phase. In a recent study by Troschl et al, the 200 L PBR was run for 75 days in which four production cycles were ran with intracellular PHB and glycogen bring monitored. Three distinct phases were observed; the first was phototrophic biomass production with fresh nutrients and distinct green culture (5-6 days) followed by the second stage of lower biomass production, but PHB and glycogen accumulation as nutrients depleted (6-8 days) with a distinct yellow culture observed due to chlorosis. The final phase of the production cycle (6-8 days) showed decreased CO_2 consumption with intracellular glycogen decreasing and PHB increasing. Thus a novel idea was conceived of adding a PHB ripening phase at the end of production runs in a stirred tank (as opposed to a PBR or ORP) as this phase was not

light dependant as evidenced from the low CO_2 consumption (Troschl et al. 2018). The advantage of this tactic is a lower area footprint of a production facility as the PBRs or ORPs would have a shorter batch time, as cultures can be moved to high volume tanks (with a small footprint) for approximately a quarter of the cultivation time. It is likely these holding tanks would also have lower operating and capital costs than an equivalent volume PBR or ORP. Additionally, photoautotrophy growth can be followed by heterotrophy growth in two stage cultivation. This can be combined with the above 'PHB ripening phase' described above through adding an organic carbon substrate to maximise PHB productivity (Itthirit, Incharoensakdi & Monshupanee 2021).

HARVESTING AND DOWNSTREAM PROCESSING

Harvesting and dewatering – Several techniques for harvesting and dewatering cyanobacterial biomass exist and nothing in particular is unique about this step for PHB production compared to previous work done on eukaryotic algae harvesting for other applications such as biofuels (Fasaei et al. 2018). It should be noted that cyanobacteria have smaller cell sizes than most eukaryotic algae (ranging from 0.5-40 micrometers, but generally less than 10). Despite this, process equipment such as filtration, centrifugation flocculation and gravity settling are still applicable for harvesting. For dewatering, vacuum drum dryers, spray drying or rotary drum dryers can all be used (Fasaei et al. 2018). It is important for downstream processing steps, such as solvent extraction, that most of the water content from the biomass is removed. A final water content of 5% or less is recommended as this decreases the polymer molecular weight reduction during processing which can adversely affect material properties (Kosseva & Rusbandi 2018).

Downstream processing – The existing technology for processing and separating PHB from heterotrophic bacterial biomass is also applicable to cyanobacterial biomass (Koller 2015). The two most prevalent options are solvent extraction and biomass digestion (Kosseva & Rusbandi 2018). The principle of solvent extraction relies upon using a chemical compound that selectively dissolves the PHB (often with heating and mixing applied), while leaving residual biomass undissolved. This is then followed by a liquid solid phase separation step

such as filtration or centrifugation. The PHB is then precipitated out of solution upon the addition of a second miscible solvent which forces the polymers out of the first solvent. After this step, another liquid-solid phase separation step is carried out to separate the PHA crystals. Commonly used solvents include acetone, chloroform and dichloromethane (Levett et al. 2016). The second form of PHA purification is biomass digestion, whereby an enzyme or harsh alkali / acidic compound selectively dissolves biomass while leaving the PHA polymer undissolved. Sulfuric acid or hypochlorite are examples of such compounds; however, such systems tend to degrade the molecular weight of the polymer molecules and as a result reduce physical and chemical properties of the final product (Fei et al. 2016). Benefits of using enzymes such as lysozymes, nucleases and proteases are their mild operating conditions, selective ability to hydrolyse cell walls and ability to leave PHA polymers undegraded (Kapritchkoff et al. 2006). Current research is also being carried out using supercritical carbon dioxide for PHA separation from biomass (Gumel, Annuar & Chisti 2013); however, this technology comes at higher capital and operational costs (Kosseva & Rusbandi 2018).

Two key differences exist between heterotrophic bacteria and cyanobacteria PHA processing. Firstly, the yields of cyanobacteria are much lower than that in heterotrophic bacteria (Singh & Mallick 2017). As a result, larger downstream processing equipment to handle larger biomass volumes would be required to obtain the same amount of PHA production capacity than from heterotrophic bacteria. However, this excess PHA extracted biomass also presents on opportunity to be used to create co-products such as food, feed, other plastics or energy production in a biorefinery approach. Secondly, the presence of chlorophyll and pigments in cyanobacteria are potential impurities that could make it through the solvent extraction process. However, purification steps such as washing the PHA crystals with acetone, hypochlorite or using ozone can reduce this impact. Alternatively, a pre-extraction step for pigments and chlorophyll could be used to remove these contaminants and obtain higher value products such as nutraceuticals.

PHB Economic Assessment

The bioplastics market was valued to be over $17 billion USD in 2017, and projected to more than double by 2022 to over $43 billion USD. Bioplastics make up roughly 1% of the total plastic market, and of the bioplastics market PHA products make up roughly 1% (Ashter 2016). Currently, only PHA produced through heterotrophic bacteria is commercially available. Table 3 shows some of the current global PHA manufacturers. The price of PHA ranges between $2-16 USD per kg or $2,000 - $16,000 USD per tonne (Kosseva & Rusbandi 2018; Levett et al. 2016; Reddy et al. 2003) most likely depending on the quality of the final polymer blend.

Table 1-3: Global manufacturers and production volumes of PHA plastic (data adapted from (Singh et al. 2017)).

Company	Location	Bioplastic Brand Name	Production / Planned Capacity (kt / year)
Bio-on	Italy	Minerv	10
Kaneka	Singapore	Mirel	10
Meredian	USA	-	13.5
Metabolix	USA	-	50
Mitsubishi Gas Chemicals	Japan	Biogreen	0.05
PHB Industrial S/A	Brazil	Biocycle	0.05
Shenzen O'Bioer	China	-	-
TEPHA	USA	ThephaFLEX/ThephELAST	-
Tianan Biological Materials	China	Enmat	2
Tianjin Green Biosciences	China	Green Bio	10
Tianjin Northern Food	China	-	-
Yikeman Shandong	China	-	3

Currently, the cost of producing algae biomass has been estimated by several techno economic analysis studies to be in the realm of roughly $500 - $1,200 USD per tonne of biomass (undried post centrifuge in ~20% solid slurry) using different cultivation systems (Dutta, Neto & Coelho 2016; Hoffman et al. 2017). However, other studies concluded significantly higher costs of production of $2,800 - $9,500 USD per tonne of biomass (~20% solid) (Banerjee & Ramaswamy 2019) and $10,000 – $36,000 USD per tonne of biomass (~20% solid) (Banerjee & Ramaswamy 2019). Fasaei et al estimated the cost of drying biomass from slurry to powder form to be roughly $350 - $760 USD per tonne of dry biomass.

If a baseline PHB yield of 10% is assumed, then roughly 10 tonnes of dried biomass is required for PHB extraction to produce 1 tonne of PHB resin. Therefore, the dried biomass raw material for downstream processing and extracting PHB is already in the realm of $8500 - $19,600 USD per tonne of PHB with optimistic biomass costs, and over $176,000 - $367,600 USD per tonne of PHB with conservative biomass costs. These costs do not yet account for capital and operating costs of PHB extraction from biomass. Thus, compared to the current market price of $2,000 - $16,000 USD per tonne of heterotrophically produced PHB, cyanobacterial is not yet cost competitive. There is also the potential of using residual biomass for other revenue generating applications; however, more detailed cost estimations are required for predicting profitability.

Panuschka et al were the first to release a techno-economic analysis of cyanobacterial PHB production. Their study used PHB yields of 15% and 60% with a TLS (thin layer system) or tubular PBR in their different scenarios. Additionally, the effect of climate was evaluated by modelling production in a southern Europe and central Europe site. The plant used waste biomass for biogas generation with digestate being recycled for nutrients. In summary, the cheapest cost of PHB resin was $26.4 USD per kg ($26, 400 USD per tonne) assuming a 60% yield using a TLS in southern Europe. However, if yields of 15% are assumed, the price of PHB increases to $103.5 USD per kg ($103,500 USD per tonne) (Panuschka et al. 2019). It should be noted that 60% of total costs for all scenarios was attributed to biomass cultivation and harvesting costs.

There are several reasons why cyanobacterial PHB production is too expensive and has not yet been commercialised. The cost of cultivating algae or cyanobacterial biomass is more expensive than obtaining biomass from terrestrial crop sources. Despite lower biomass productivities from terrestrial crops, the cost of building and operating ORPs and PBRs is higher than traditional terrestrial agricultural crop production, and this outweighs the economic benefit of a smaller sized algae farm. Secondly, terrestrial crops are easier and cheaper to harvest as the biomass is far more concentrated than that of algae and cyanobacteria in a liquid media (which is approximately 0.1% solid from an ORP). The capital and operational cost of running liquid solid phase extraction process equipment such as centrifuges or filters to harvest biomass of a few grams per litre is a significant expenditure (Fasaei et al. 2018).

In addition to this, both PHB product and biomass productivity is quite low compared to heterotrophic processes (Singh et al. 2017). Most algae cultures only reach a maximum biomass concentration of a few grams per litre, which is 10-fold to 100-fold less than the concentration that can be achieved with heterotrophic strains. For PHB specifically, one of the highest reported cyanobacterial productivities obtained from genetically modified *Synechocystis* PCC6803 was 7.3 mg/L/day (Carpine et al. 2017). This is in the realm of over ten thousand times lower than heterotrophic bacteria which have PHB productivities reported at over 75,000 – 120,000mg/L/day (Ryu et al. 1997; Wang & Lee 1997). This is due to two reasons; firstly heterotrophic cell yields of PHB are higher (highest reported is over 80% dcw (Wang & Lee 1997)). Secondly, heterotrophic biomass productivity is also significantly higher than photoautotrophic growth.

In addition their lower volumetric productivity, cyanobacterial PHB production would also require more surface area of land per volume of production as culture must be exposed to light. Because of these two reasons, the area required for a cyanobacterial PHB production facility would be in the magnitude of hundreds to thousands times larger compared to the footprint of a heterotrophic PHB production plant of the same capacity. However, it is important to note that this does not include the area required to grow terrestrial crops to create the organic carbon feedstock required for heterotopic production. It has been reported that roughly 25-50% of heterotrophic PHA production costs come from the cost of

the carbon substrate (Halami 2008; Kosseva & Rusbandi 2018). Thus, one of the areas where cyanobacteria are economically superior is that they can be produce PHB from carbon dioxide which can be ideally procured for free with the correct site location and a suitable carbon dioxide emitting source. Potentially, a carbon capture revenue stream could be incorporated into the business model.

Cyanobacteria also suffer from low PHB yields which are almost always below 10% in wild types, and in most cases well below 2% (Ansari & Fatma 2016; Bhati et al. 2010; Kaewbai-ngam, Incharoensakdi & Monshupanee 2016; Troschl, Meixner & Drosg 2017; Vincenzini et al. 1990). Genetic engineering has not been very successful in increasing PHB yields in cyanobacteria (Troschl, Meixner & Drosg 2017). Low yields also increase the costs of downstream processing, because there is a higher waste product to desired product ratio. It is important that such a facility has an economic use of the residual biomass. Anaerobic digestion offers an opportunity to create renewable energy and recycle nutrients from the digestate; however, the revenue generated is quite small. Other uses for the biomass, such as potential conversion to PLA plastic or bio stimulants for agriculture could be explored.

Although there are currently significant technical and economic disadvantages to photoautotrophic PHB production using cyanobacteria compared to heterotrophic production, there is a need to overcome these challenges due to the environmental benefits of shifting bioplastics production from terrestrial agriculture. Ultimately, the key driver to achieving financial viability is increasing PHB productivity. This will reduce the capital and operating cost by reducing the area of cultivation required to meet production volumes. This also reduces downstream processing costs with a lower amount of non-PHB ‘waste biomass’ produced and reducing total biomass entering the downstream processing steps. However, there is also a need for more studies to report on PHB productivity (mass per volume and time) and not just the final yield achieved. Although some studies present yield and time required, without the amount of biomass reported, the PHB productivity cannot be calculated. Ultimately, the most important key to assessing financial viability will be the average PHB productivity of a strain, as this will dictate the required area of cultivation land.

Improving Viability of PHB Production from Cyanobacteria

From the economic assessment above, improving cyanobacterial PHB viability shares many similarities with general eukaryotic algae cultivation improvements such as;

- Need for lower cultivation and harvesting costs
- Increased cell densities and higher productivities
- Better light utilisation
- Resistance to culture crashes
- Recycling of nutrients
- Improved CO_2 absorption

However, for the specific purpose of improving PHB viability from cyanobacteria, the following research areas are suggested:

Screening Studies – Screening studies involve the testing of newly bioprospected strains of cyanobacteria for the presence and yields of PHB in the hopes of discovering elite strains with elevated productivities. Most screening papers investigate one or two cyanobacteria. However, Ansari et al (2016) conducted a screen of 23 strains of cyanobacteria while Kaewbai et al (2016) screened 137 strains for their PHB yields under nutrient limitation environments. Such efforts are important for finding high PHB yielding cyanobacteria for the purpose of cultivation and for the further investigation in understanding the variability in PHB production between different strains and species.

In depth Growth & PHB Productivity Optimisation Experiments – Growing strains with promising initial yields of PHB after screening under a range of different environments is also important. PHB synthesis can be affected by pH, temperature, light conditions and mixing. Understanding which strains produce more or less PHB under different light and temperature could assist with finding different cultivation strains for different climates, locations or seasons throughout the year at the same site. As mentioned previously, growing cyanobacteria for PHB production without resuspension in new media is important for understanding what nutrient composition the initial media should contain.

Secretion of PHB – Secretion of PHB would assist with reducing the costs of downstream processing as separation from residual biomass with steps such as solvent extraction would no longer be needed. Diatoms have been previously been exposed to different stimuli to increase cell wall permeability and secretion of certain metabolites (Vinayak et al. 2015). Exposing cyanobacteria to similar stimuli such as electric fields, ultrasound or certain chemicals could result in secretion of PHB. However there is difficulty in scaling up electric fields or ultrasound technology to industrial scales. Genetic engineering of PHB secretion has been achieved in *E. coli* (Rahman et al. 2013) where phasin proteins bound to PHB granules were secreted through the use of a HlyA signal peptide. Similar approaches could be established in cyanobacteria. Furthermore, Rahman's paper includes a protocol for detecting secreted PHB which is applicable to the chemical and physical stimuli mentioned above.

Processing Cyanobacterial Biomass & PHB Biorefinery – While there have been several research efforts into the optimisation of extracting PHB from heterotrophic biomass (Fiorese et al. 2009; López-Abelairas et al. 2015), there is a need for investigating the separation of PHB from cyanobacterial biomass. This is significant because of the differences in biochemical composition between cyanobacteria and heterotrophic biomass. Due to lower PHB yields in cyanobacteria, there is a greater amount of proteins, carbohydrates and other lipids. In addition, the pigments used for light harvesting in cyanobacteria tend to be soluble in organic solvents, and will degrade the final PHB material properties in a tradition solvent extraction process flow without a pigment removal stage. Meixner et al were the first to investigate a cyanobacterial PHB biorefinery approach and processing optimisation using *Synechocystis salina* biomass. It was found that cell disruption and pigment removal produced PHB with superior material properties. The methane production potential for biogas generation was also evaluated. PHB yields of 6-7% were obtained and with additional pigment product yields of total solids (TS) being chlorophylls 0.27–1.98 mg/g TS, carotenoids 0.21–1.51 mg/g TS, phycocyanin 0–127 mg/g TS (Meixner et al. 2018). However, there is still a need to test other processing methods such as acid or alkaline digestion, supercritical CO_2, enzymatic digestion, and also investigate other uses of residual biomass.

Growth in presence chemical compounds – In algal biofuel and high value product research, chemicals compounds that affect the carbon metabolism of microalgae have been investigated for their effect on boosting certain metabolite yields (Commault et al. 2019; Franz et al. 2013). These compounds can act as phytohormones (plant signalling hormones), regulate cyanobacterial metabolic pathways, induce oxidative stress responses or act as direct metabolic precursors (Yu, Chen & Zhang 2015). By growing cyanobacterial cultures in microplates or flasks that is exposed to a chemical library, the effect on PHB productivity can be determined through one of the detection methods mentioned previously in the review such as dye staining or chromatography. Further downstream work such as metabolomics, transcriptomics or proteomics can then elucidate the exact mechanism behind increased PHB productivity and provide further knowledge towards relevant pathways and genes.

Mixed culture (consortium) growth – The vast majority of cyanobacterial PHB papers have used *supposedly* single species cultures. However, PHB production could potentially be altered in the presence of multiple strains of cyanobacteria and other microorganisms. Not only are culture contaminations impossible to realistically avoid at industrial scale PHB cultivation, but mixed microbial communities replicate natural environmental conditions and such consortiums are more resistant to culture crashes too (Lian et al. 2018). A synergistic relationship can occur between oxygen producing / carbon dioxide utilising phototrophs and oxygen respiring / carbon dioxide producing heterotrophs that provide carbon and oxygen to each other (Sutherland et al. 2016). In addition to this, phototrophs and heterotrophs can benefit each other's growth rates through other mechanisms such as the sharing of nutrients, vitamins, hormones and other compounds (Croft et al. 2005; Kazamia et al. 2012; Ramanan et al. 2016) which occurs extensively in natural systems such as microbial mats (Hoschek et al. 2019).

Arias et al (2018) obtained a mixed cyanobacterial culture from wastewater and exposed it to N and P limitation and different photoperiods, with a maximum PHB concentration of 104mg/L and 6.5% dcw yield. PHA yields of 20% were achieved with a mixed photosynthetic consortium exposed to a feast-famine regime, however this was under mixotrophic conditions (Fradinho et al. 2013). In an alternate approach, Löwe et al (2017) and Weiss et al

(2017) both used engineered sucrose secreting strains of *Synechococcus elongatus* with *Pseudomonas putida* and *Halomonas boliviensis* respectively. In both of these studies, the phototrophic fixation of carbon dioxide into sucrose was fed to the PHB producing heterotrophic bacteria with PHB productivities of around 25 mg/L/day (Löwe et al. 2017; Weiss, Young & Ducat 2017).

In addition, the potential for cyanobacterial PHB production in mixotrophic biofilms has yet to be explored. Most recently, a capillary reactor utilising a mixotrophic biofilm consortia of *Synechocystis* PCC 6803 and *Pseudomonas sp. VLB120* achieved extremely high maximum biomass concentrations of around 50g/L dcw (Heuschkel et al. 2019; Hoschek et al. 2019), although PHB yield was not tested.

Further work on mixed purely photosynthetic cultures for PHB production and growth optimisation of consortiums with different combinations of microorganisms presents much potential for future investigation. However, it is unlikely that fluorescent techniques could be used to quantify PHB accumulation due to the different cell physiologies and dynamic populations over time which would result in a changing fluorescent signal to PHB correlation; however, chemical analysis techniques are still applicable.

PHB productivity under different wavelengths of light – The yield of certain algal compounds under cultivation of different wavelengths of light has been investigated by several researchers (Mohsenpour & Willoughby 2013; Teo et al. 2014). For example, green light was found to enhance chlorophyll production in *Chlorella vulgaris,* whereas red light enhanced phycobilin proteins in *Gloethece membranacea.* Although some studies have been carried out investigating the effect of partial light spectra on cyanobacteria (Luimstra et al. 2018), at this time there are no current studies into how different wavelengths of light would affect PHB yields. It should be noted that for industrial cultivation, using certain wavelengths of light suggests the use of artificial lighting which is most likely economically unfeasible. However, this research could still increase understanding of PHB and its role in cyanobacteria.

Wastewater PHB algae production – Wastewater has been used as a substrate for photosynthetic cyanobacteria and heterotrophic bacteria. *Nostoc muscurom* was grown on waste poultry litter and achieved a 65% yield dcw of PHBV under mixotrophic conditions (Bhati & Mallick 2016). Integrating cyanobacterial cultivation for PHB production into a wastewater treatment scheme has the potential to add an extra revenue line to a potential business model, in addition to remediating water.

Random Mutagenesis – Current approaches to metabolic engineering for a desired phenotype are limited by the requirement for prior knowledge of kinetics, proteomics, transcriptomics, genomics, availability of molecular tools and much more to achieve an effective outcome. However, inducing completely random mutations into a cyanobacterial culture and screening individuals for a desired phenotype (in this case higher PHB productivity and yield through fluorescent activated cell sorting (FACS)) allows for the bypassing of all the previously mentioned limitations of genetic engineering. Keith et al (2006) have developed a methodology for using Nile Red staining to stain *Synechocystis* PCC 6803 mutant libraries for increased PHB yields. Because *Synechocystis* PCC 6803 only has a yield of ~5-10% PHB under phototrophic conditions, the potential for creating mutants of other cyanobacterial species (such as *Calothrix* sp.) with higher naturally occurring PHB yields provides a higher baseline for desirable mutations to occur. Recently, Kamravanesh et al used UV radiation to increase yields of PHB from 16% to 37% in *Synechocystis* PCC 6714. The mutant strain with this high yield was shown to have a single amino acid missense mutation in an ABC phosphate transporter. This was hypothesised to cause a cascade of signalling and regulation changes compared to the wild type under phosphate-limited conditions, resulting in the higher PHB yield phenotype. Further mutagenesis work can still create other increased PHB yielding mutants with novel genetic mutations which can be further investigated as candidates for targeted genetic engineering.

Targeted Genetic Engineering - The cyanobacterial model organism *Synechocystis* PC 6803 is widely used for photosynthetic studies and has had its genome sequenced for several decades now with mutant variants readily available (Anderson & McIntosh 1991; Kaneko & Tabata 1997). It has been successfully transformed in a number of studies and has been

used for the investigation of producing PHB as well as biohydrogen, isoprene and other chemical commodities (Touloupakis et al. 2016).

The highest report of PHB contents in genetically engineered cyanobacteria was 63% dcw by Koch et al (2020). In this study, a *Synechocystis* PCC 6803 strain was created that lacked the regulatory protein PirC and had PhaA and PhaB synthases introduced from *Cupriavidus necator.* Over-expression of natural PHB genes led to an increase from 10% PHB yield to 26% yield in *Synechocystis* PCC6803 (Khetkorn et al. 2016). The promoter of the Rubisco gene has been successfully used as a promoter for PHA expression systems in *Synechocystis* PCC6803. The rationale behind this being that Rubisco activity is strongly upregulated in the presence of increased carbon dioxide and regulation of PHA synthesis could be achieved with varying carbon dioxide concentration (Miyasaka et al. 2013).

In another study, *Synechococcus* sp. PCC7942, which cannot naturally synthesise PHA, was genetically engineered with introduced additional *Cupriavidus nectator* PHA synthesis genes which improved yields from 3% to 25% dcw under phototrophic and heterotrophic conditions, respectively (Takahashi et al. 1998). *Synechococcus* PCC7002 was used by Akiyama et al (2011) to develop a non-antibiotic plasmid expression system where the recA gene (encoding for an essential DNA repair enzyme) was included in a plasmid cassette and introduced into a recA deficient mutant cyanobacteria. This allowed for greater plasmid stability as the recA gene is required for survival. Antibiotic resistance genes used for screening in traditional plasmid expression systems tend to be excluded from the cell in the absence of antibiotics, and industrial cultivation with antibiotics is not an economically nor environmentally feasible option. The PHA genes used were from *Cupriavidus nectator* and a 52% PHB yield was obtained; however this was under heterotrophic conditions. A photoautotrophic only PHB yield was not reported (Akiyama et al. 2011).

Wang et al (2013) engineered a *Synechocystis* PCC 6803 strain optimised for producing the PHB monomer hydroxybutyrate (both (S)- and (R)-3-hydroxybutyrate (3HB)) through inactivation of PHB polymerase). Two additional pathways for producing the monomers from Acetyl-CoA were also introduced. The monomers were readily secreted without changing transporter expression and titres of 533.4mg/L 3HB were obtained. Carpine et al

engineered *Synechocystis* PCC 6803 focussing on central carbon metabolism as opposed to over-expression or introducing PHB synthesis genes. Three different target areas including deletions of phosphotransacetylase (Pta) and acetyl-CoA hydrolase (Ach) and the expression of a heterologous phosphoketolase (XfpK) from *Bifidobacterium breve* produced 12% PHB yield, titre of 232 mg/L and a productivity of 7.3 mg/L/day which was reported to be the highest to date. Lastly, the eukaryotic algae *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum* were genetically modified to express introduced PHA synthesis genes and achieved yields of 6 x 10^{-4} % and 10.6% PHB respectively (Chaogang et al. 2010; Hempel et al. 2011).

Future work in this area could include screening other species of eukaryotic algae with high pools of Acetyl-CoA for introduction of PHB synthesis genes. Hempel et al were able to achieve a 10% yield without any further optimisation such as plastid targeting, codon optimisation, nuclear integration or using a non-inducible promoter. Suppression of glycogen or other lipid metabolite biosynthetic pathways to increase the carbon flow to the PHB pathway is also promising. Lastly, creating a PHB de-polymerase knockout cyanobacteria that is unable to metabolise stored intracellular PHB could result in higher yields and PHB productivity.

Conclusion

For a sustainable future, we must shift to using biodegradable and renewably sourced bioplastic materials. However, high raw materials costs for heterotrophically produced bioplastic and environmental impacts from terrestrial agriculture mean that PHB production from atmospheric carbon dioxide using cyanobacteria provides a promising path forward. This review has covered various topics from the central PHB metabolism, material properties and associated methods and protocols. While there is still uncertainty on the exact role of PHB in the cell, it is likely it is used as an energy storage compound and regulator of excess reducing charge. Industrial cultivation issues of cyanobacterial PHB and economic factors regarding why industrial commercialisation is not yet economically viable have also been examined. Ultimately, the two key factors to achieving profitability are higher PHB productivity and cheaper cyanobacterial cultivation equipment. Several

promising research areas for improving cyanobacterial PHB viability have been reviewed including screening, genetic modification, wastewater cultivation, downstream processing and growth optimisation.

Chapter 2 - Random Chemical Mutagenesis Followed By FACS to Enhance Cyanobacterial PHB Bioplastic Production

Abstract

Poly-hydroxy-butyrate (PHB) bioplastic resin can be made directly from atmospheric CO_2 using cyanobacteria. However, higher PHB productivities are required before large-scale production is economically viable. Random mutagenesis offers a way to create new production strains with increased PHB yields and increased biomass densities without complex technical manipulation associated with genetically modified organisms. This study used staining with lipid fluorescent dye (BODIPY 493/593) and fluorescence-activated cell sorting (FACS) to select high lipid content mutants and followed this with a well plate growth screen. Thirteen mutants were selected for flask cultivation and two strains produced significantly higher PHB yields (29% and 26% higher than wild type), biomass accumulation (36% and 33% higher than wild type) and volumetric PHB density (75% and 67% higher than wild type). The maximum PHB yielding strain (% dcw) was 12.0%, which was 43% higher than the wild type (8.3% in this study). The highest volumetric PHB density was 18.8 mg PHB / L compared to 10.7 mg PHB / L by the wild type. To develop cyanobacterial strain with higher PHB productivities, the combination of random chemical mutagenesis and FACS holds great potential to promote cyanobacteria bioplastic production becoming economically viable.

Introduction

Plastics play a central role in modern society with applications in almost every major industry including agriculture, medicine, consumer products, electronics, construction and much more. However, their widespread use has resulted in pollution of terrestrial and aquatic environments as well as increased carbon emissions through their production and incineration of petrochemical plastic waste (Nielsen et al. 2020). Bioplastics, such as poly-hydroxy-butyrate (PHB), can be produced from renewable biomass sources, are carbon

neutral and biodegradable (Bharti & Swetha 2016). PHB in particular can be used in applications such as food packaging and is a promising replacement for polypropylene (Markl 2018) which is one of the most widely used plastics, globally (Maddah, 2016).

Cyanobacteria are single-celled photosynthetic bio-factories capable of converting atmospheric CO_2 and simple inorganic nutrients into a diverse portfolio of bio-products such as biofuels, human food, animal feed, pharmaceuticals (Khan, Shin & Kim 2018) and bioplastics (Troschl, Meixner & Drosg 2017). PHB is an carbon storage metabolite natively produced by several species of cyanobacteria, but is yet to be commercialised due to high production costs (Price et al. 2020). Increasing PHB productivity is a key driver in reducing the cost per tonne of cyanobacterial PHB to economically viable levels. One strategy to enhance PHB production in cyanobacteria is through genetic modification.

Efforts of targeted genetic engineering to increase cyanobacterial PHB production include introduction of exogenous or extra PHA synthesis genes (Hondo et al. 2015; Takahashi et al. 1998), increasing central carbon metabolism (through deleting phosphotransacetylase, acetyl- CoA hydrolase and the expression of a heterologous phosphoke-tolase) (Carpine et al. 2017), secretion of the monomer 3-hydroxy-butyrate (Wang et al. 2013), altering carbon partitioning through deleting the ADP-glucose pyrophosphorylase gene (Wu, Shen & Wu 2002) and deletion of the regulatory PirC gene (Koch et al. 2020). While these efforts have had varying degrees of success, a major downside of targeted genetic engineering work is the requirement for prior specific knowledge of genetic constructs (such as which genes to targets, which metabolic pathways to block, which regulatory proteins to delete etc).
In contrast to this, random mutagenesis has the potential to enhance a desired phenotype through a completely unknown, unexpected and novel modification. Therefore, this has the potential to increase our knowledge of new regulatory aspects of a phenotype, if the mutant strains which over express or under express this specific phenotype trait are also sequenced (Kamravamanesh et al. 2018). Lastly, random mutagenesis also requires minimum technical manipulation compared to targeted genetic engineering approaches (Rowlands 1984).

Mutagenesis can be divided into two main categories: physical (using high energy radiation such as UV, gamma and X-rays) and chemical (generally using mutagens such as EMS (ethyl methane sulfonate) and nitrosomethyl guanidine (NTG)). EMS has already been used in previous studies to produce mutant microalgae strains which over produce certain metabolites such as eicosapentaenoic acid (EPA) (Chaturvedi & Fujita 2006), pigments (Huesemann et al. 2009), hydrogen (Flynn, Ghirardi & Seibert 2002) and lipids (Doan & Obbard 2012). EMS has also been used to enhance the PHB production in heterotrophic bacterial strains (Adwitiya et al. 2009; Bashir et al. 2014; Obruca et al. 2013). Thus, because of the proven success of EMS in microalgae and bacterial PHB studies, it was selected for this study to produce cyanobacterial mutants with increased PHB production.

In a recent study involving the cyanobacteria *Synechocystis PCC 6714*, Kamravamanesh et al. (2018) used UV radiation to increase yields of PHB from 16% to 37%. This mutant strain was found to have a single amino acid missense mutation in an ABC phosphate transporter gene. This was predicted to cause a regulation change compared to the wild type under nutrient limitation conditions, resulting in higher PHB yields. There are no known studies using chemical mutagenesis on cyanobacteria for increased PHB production. In addition, most studies described in this short literature review use plated colonies to isolate mutants. Instead, this study used a lipid fluorescent dye with high PHB affinity (Kacmar et al. 2006), BODIPY 493/593, and fluorescent activated cell sorting (FACS) to select only high lipid content mutants for the first screen when isolating strains.

Materials and Methods

Experimental Overview

Figure 2-1 shows the overall workflow and stages for this experiment. 3 weeks after treatment with EMS at various concentrations (0.5- 4.0M), single cells in the top 1% population of BODIPY 493/593 (a neutral lipid dye) fluorescence were sorted into individual wells in 96 well plates. 192 surviving strains were then grown in a 48 well plates and mutant strains evaluated, based on maximum optical density as a proxy for growth. The top 13

strains were then selected for flask cultivation which allowed for sufficient dry biomass production and PHB quantification.

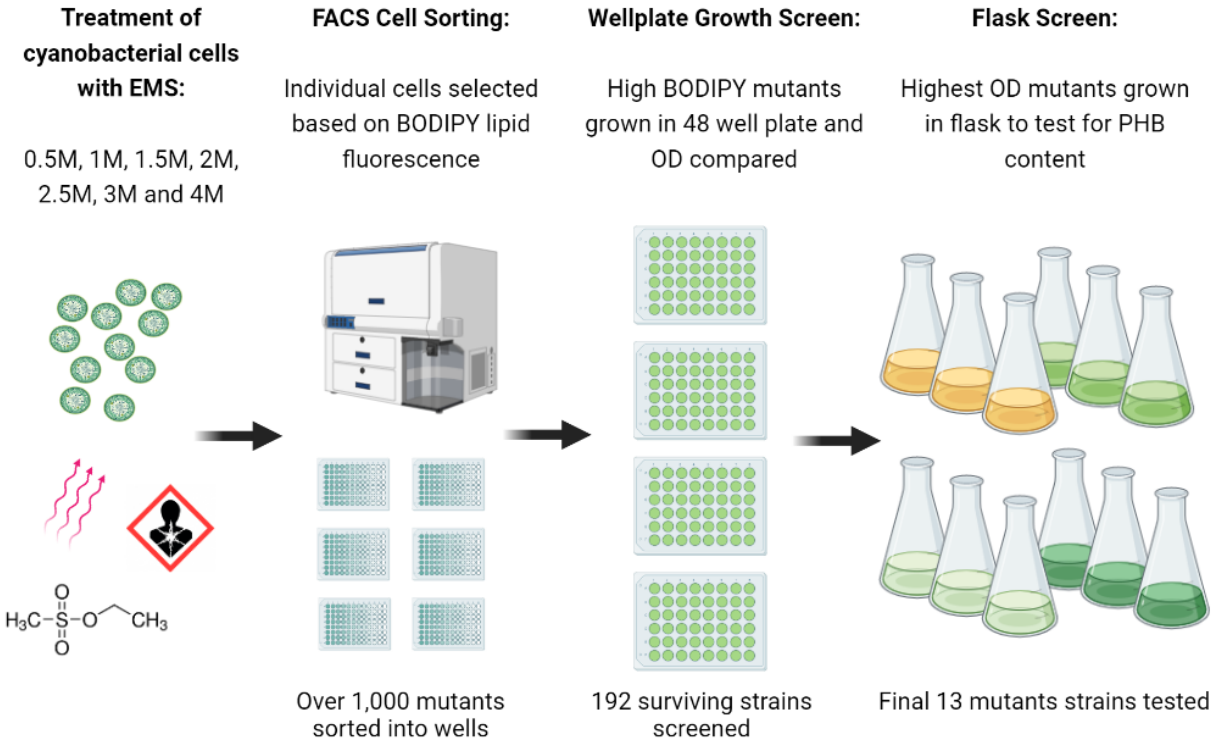


Figure 2-1: High level overview of experimental procedure.

Cyanobacterial Strain and Cultivation Conditions

A brackish cyanobacterial strain *Synechocystis cf. salina Wislouch (No. 192)* from the Culture Collection of Autotrophic Organisms (CCALA) was maintained at 25 °C, 50 µmol photons m^{-2} s^{-1} PAR light, on a 16:8 day-night cycle in standard BG11 medium (Rippka, Deruelles & Waterbury 1979). The same conditions were used for all well plate cultivation and mutant creation steps of this experiment.

For the flask screening stage of the experiment, the strains were inoculated at a cell density of 2 x 10^6 cells mL^{-1} in a modified BG11 medium (BG11M) (Meixner et al. 2016), and 20 mM HEPES buffer. The main differences between BG11M to BG11 included, approximately 250% phosphate and 40% nitrate, 6 g L^{-1} of NaCl and other slight trace metal modifications. 50 mL of culture volume was grown in 250 mL glass flasks. Cultures were grown under 100 µmol photons m^{-2} s^{-1} light (increased from 50 µmol photons m-2 s-1 PAR light for flasks due to the

different light distribution compared to cultivation in well plates), on a 16:8 day-night cycle, with 3% atmospheric CO_2 and 95 rpm orbital shaking in an incubator (Climo-shaker ISF1-X Kuhner Shaker). All cultures were grown in triplicate.

Chemical Mutagenesis Treatment and Kill Curve

Cells were initially inoculated at 2 x 10^6 cells mL^{-1}, and upon day 7 of cultivation, exponential growth phase cells were exposed to 0.5M, 1M, 1.5M, 2M, 2.5M, 3M and 4M EMS (Sigma Aldrich) for 1 hour in darkness, at 25 ^oC and 300 rpm agitation (Doan & Obbard 2012). After incubation, cultures were centrifuged at 3000 rcf for 8 minutes and supernatant discarded. Cell pellets were washed three times in a 10% (w / v) solution of sodium thiosulphate to remove any leftover residue of EMS. Cultures were resuspended in BG11 and left overnight in darkness to prevent photoreactivation repair of damaged DNA. Cultures were then grown in conditions described above until stationary phase. By examining the viability of cultures exposed to varying EMS concentrations, a kill curve was established. All treatments of EMS concentrations were conducted in triplicate.

Fluorescence-Activated Cell Sorting (FACS) of Mutant Cultures

On day 21 following EMS treatment, cell cultures were subjected to fluorescence-activated cell sorting (FACS) on a flow cytometer (BD Influx). Cultures were first stained with BODIPY, a fluorescent dye that detects neutral lipid bodies. 100 µL of a 0.01% (w / v) solution of BODIPY 493/593 (Sigma Aldrich) was added to 1 mL of treated culture, vortexed for 10 seconds and then incubated in dark for 5 minutes (Rumin et al. 2015).

Cells were then loaded into the flow cytometer and initially detected by their chlorophyll signals, excited by a 488 nm laser and detected in 692±40 nm channel. Single-cell gating to gate out cell aggregates was achieved through area versus pulse width signals on trigger channel. Single cells were then displayed in a FACS plot as chlorophyll signal versus BODIPY 493/593 signal, excited by 488 nm laser and detected in 530±40 nm channel. Undyed wild type cells were used as negative control to set BODIPY positive gate. Cells that fell into the BODIPY positive gate and were in the top 1% in BODIPY fluorescence signal were selected and sorted one cell per well using single cell sort mode into in a 96 well plate containing 200 µL of BG11 medium per well, and in conditions described above for 5 weeks. 192 of the

surviving cultures were transferred to 48 well plates for the growth screen of the experiment.

OD Measurements

For the 48 well plate growth screen, cultures were analysed directly for optical density upon inoculation and every 3 days after. For the flask screen, upon inoculation and every 3 days after, 200 µL of each culture was transferred into a 96 well plate in a biosafety cabinet. A plate reader (Tecan Infinite M1000Pro) was used to measure optical density at 750 nm for cyanobacterial biomass accumulation.

Dry Biomass and PHB Quantification

Flask cultures were harvested on day 28 of cultivation by centrifugation (at 3000 rcf for 8 minutes), by which time the cultures were close to chlorosis (visual inspection). Pellets were washed with deionised water and then freeze-dried for 24 hours at -80 ^{o}C (Christ Freeze Dryer, Scitek) at 0.1 mPa. Samples were weighed to determine dry biomass mass density and then digested in concentrated 98% sulfuric acid for 2 hours at 90 ^{o}C. This process converted PHB to crotonic acid, which was quantified through HPLC analysis. An organic acid Aminex HPX-87H column was used with an UHPLC machine (Agilent Technologies 1290 Infinity). Elution time and UV absorbance spectrum of a digested PHB standard (Sigma Aldrich) and crotonic acid (Sigma Aldrich) were used to confirm digestion efficiency and the accuracy of crotonic acid measurements in samples. A calibration curve relating peak area and crotonic acid concentration was used to quantify the PHB content in the acid digested samples.

Determining Photosynthetic Health

Fluorescence measurements were performed using an imaging system (MAXI PAM Walz, Effeltrich, Germany). 200 µL of culture were added in 42 wells of a PCR 96 microwell plate (Bio-Rad HSP9655) which was dark-adapted for 10 minutes in the MAXI PAM enclosure. A standard rapid light curve (RLC) measurement protocol was used to determine relative

electron transfer rates (rETR). Samples were exposed to 10 seconds of each light intensity, which covered a range from 16 to 1256 µmol photons m^{-2} s^{-1} actinic blue light. Maximum quantum yield of Photosystem II (Fv/Fm) was determined prior to measuring the RLC.

Results and Discussion

Kill Curve

In multiple mutagenesis studies, a kill curve is generated to determine the maximum degree of mutagenic treatment (by exposure time, concentration of chemical mutagen or intensity of radiation) that a culture can take before complete death of cell population. This ensures that a sufficiently harsh treatment causing genetic damage will also induce novel mutations, while still retaining a surviving sub-population. In this study, the viability of cultures surviving 21 days after exposure to mutagenic treatment was used because this incubation period (before FACS of mutants) would ensure cells would also be slightly nutrient starved and therefore have elevated neutral lipid and PHB content.

Figure 2-2 shows the viability of cells 21 days after EMS treatment. 2.5 M of EMS was the highest treatment with all three triplicate cultures surviving, whereas 3 M and 4 M only had one of the three triplicates show viability (confirmed through microscopy). Mutants from 2 M, 2.5M, 3 M and 4 M were chosen for sorting into 96 well plates based on BODIPY fluorescence.

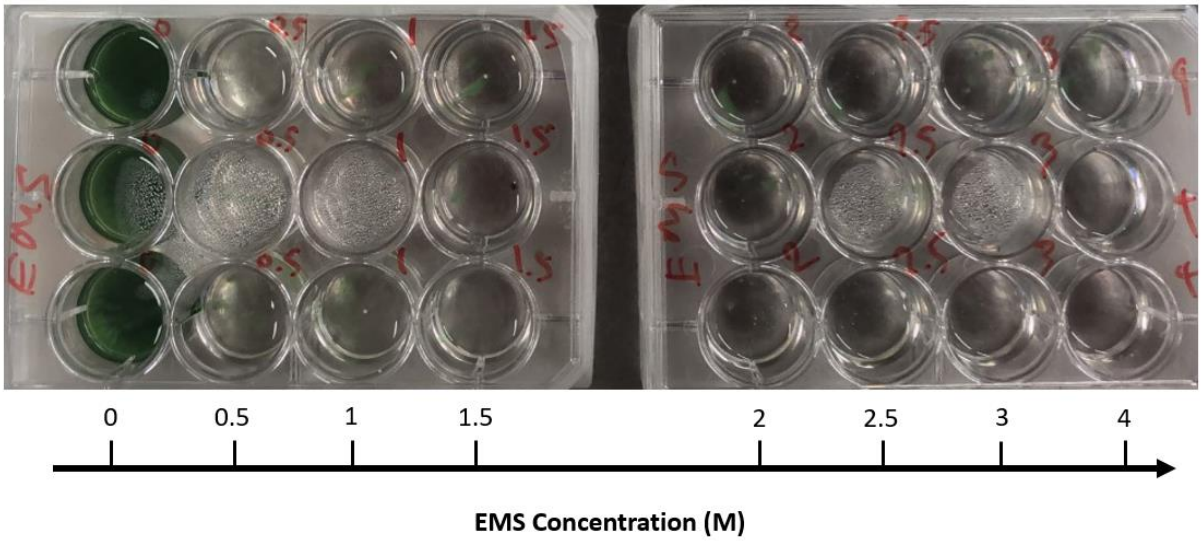


Figure 2-2: Kill curve showing viability of cultures 21 days after treatment with varying EMS concentrations, with each concentration in triplicate by column.

48 well plate growth screen: OD

5 weeks after single cell sorting, 192 of the surviving strains were inoculated into 48 well plates. Of these, only 13 mutants in total from both the 2.5 M and 4 M treatment reached higher OD's or grew comparatively faster than that of the wild type. These 13 mutants were selected for scale up to 250 mL flask cultivation to subsequently gravimetrically assess biomass productivity and to quantify PHB yield through HPLC analysis.

Flask Screen of final 13 Mutant Strains: Biomass Density, PHB Yield and PHB Volumetric Density

Three of the 13 selected mutant strains achieved significantly higher PHB than the wild type (Figure 2-3A). The wild type achieved a PHB yield of 8.3% with Strain 1 achieving 10.8% (29% increase), Strain 3 achieving 10.5% (26% increase) and Strain 11 achieving 12% (43% increase) in PHB yield. Of these, only Strain 1 and 3 had significantly higher biomass density (1.75 g / L and 1.71 g / L) compared to the wild type (1.29 g / L). This amounted to a 35% and 32% increase in biomass, respectively. Strain 1 and 3 also had superior volumetric PHB densities at 18.8 mg PHB / L and 18.0 mg PHB / L, compared to the wild type which achieved only 10.7 mg PHB / L. This was a 75% and 67% increase, respectively.

Only one strain showed a significant reduction in PHB yield, Strain 6 which resulted in 3.1% PHB yield (63% reduction) compared to wild type. This strain also showed a significant reduction in biomass density (43% lower), compared to wild type. It is likely that this strain had mutations which impaired its general fitness and ability to grow, and accumulate biomass. As a flow on effect, a reduced amount of carbon was available for PHB formation too.

Two strains showed significantly less biomass density (Strain 6 and Strain 7), despite having higher optical densities than the wild type in the previous phase of the experiment. This could potentially be due to the instability of the mutations over time, or if some of the mutations in these strains negatively affected the long-term viability of the strain. A similar

occurrence also arised in one mutant strain in another study where after multiple cultivations the strain showed decreased biomass growth and PHB yield (Kamravamanesh et al. 2018). Alternatively, the mutations in these strains may allow them to grow more efficiently under the specific environmental conditions of the first growth stage of the experiment (48 well plate) and not the second (flask cultivation).

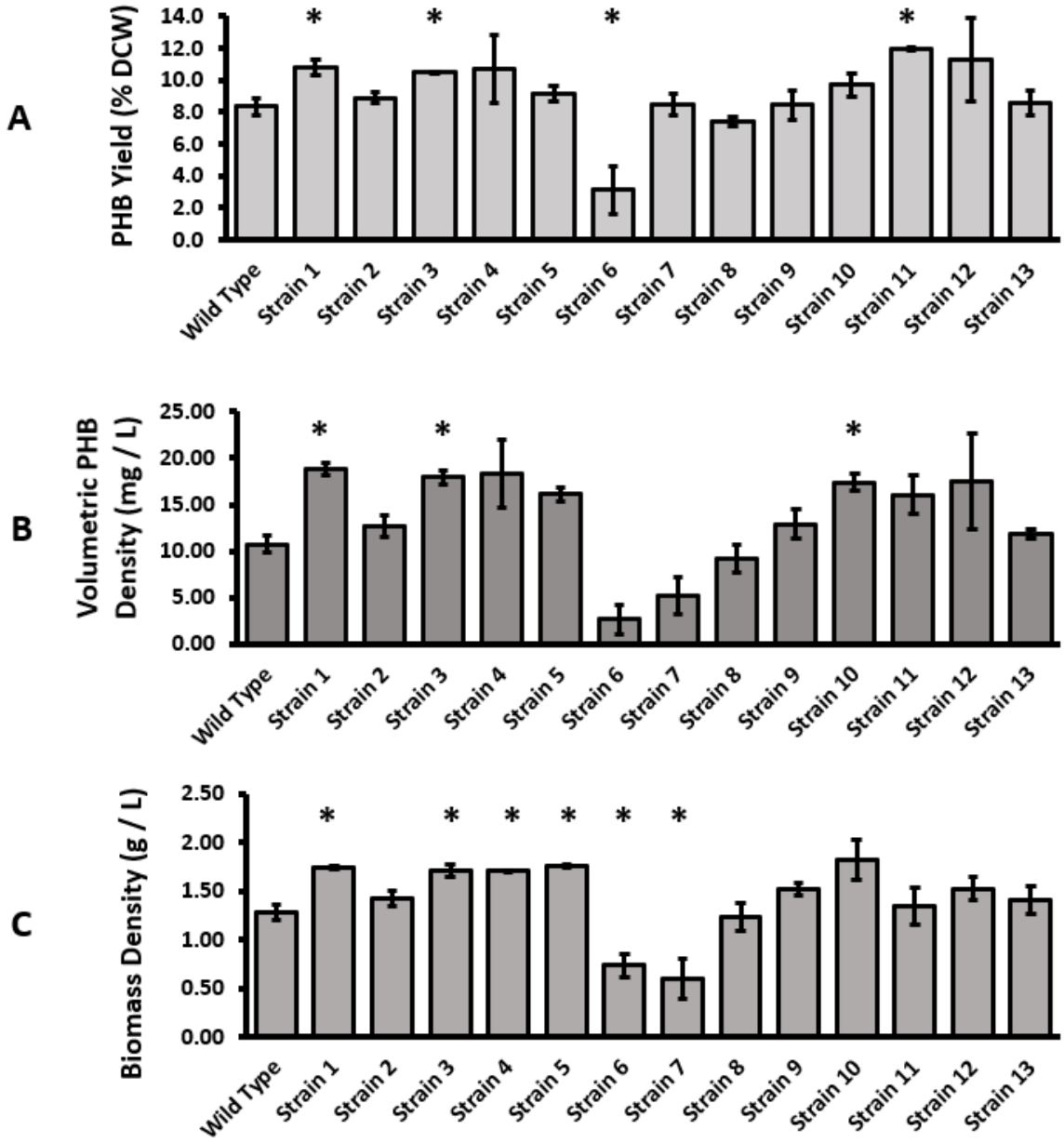


Figure 2-3: (A) PHB Yield % Dry Cell Weight, (B) Volumetric PHB Density and (C) Biomass Density achieved by wild type and 13 mutant strains by day 28 of culturing.

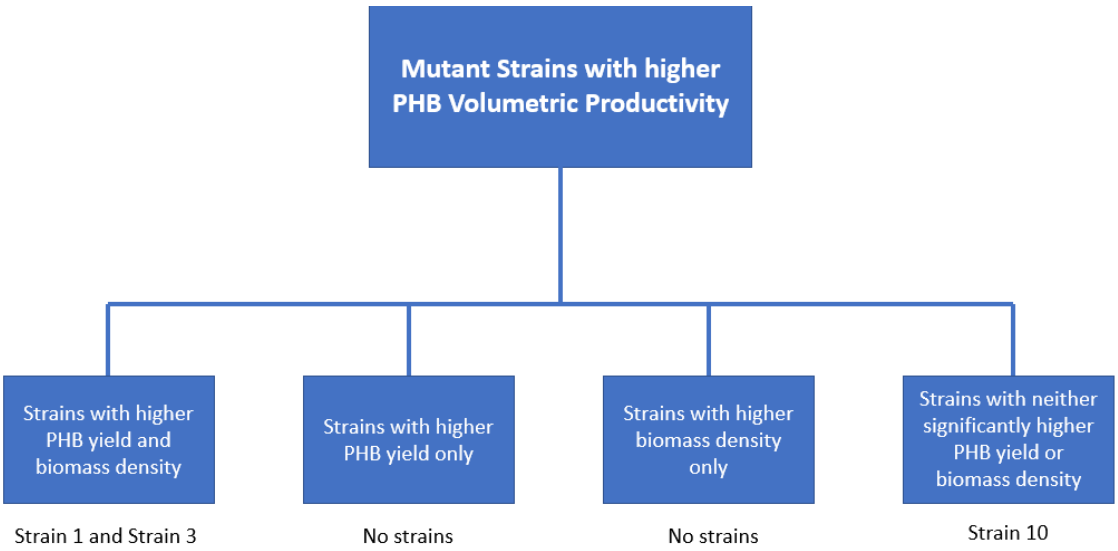


Figure 2-4: Conceptual categories of mutants produced.

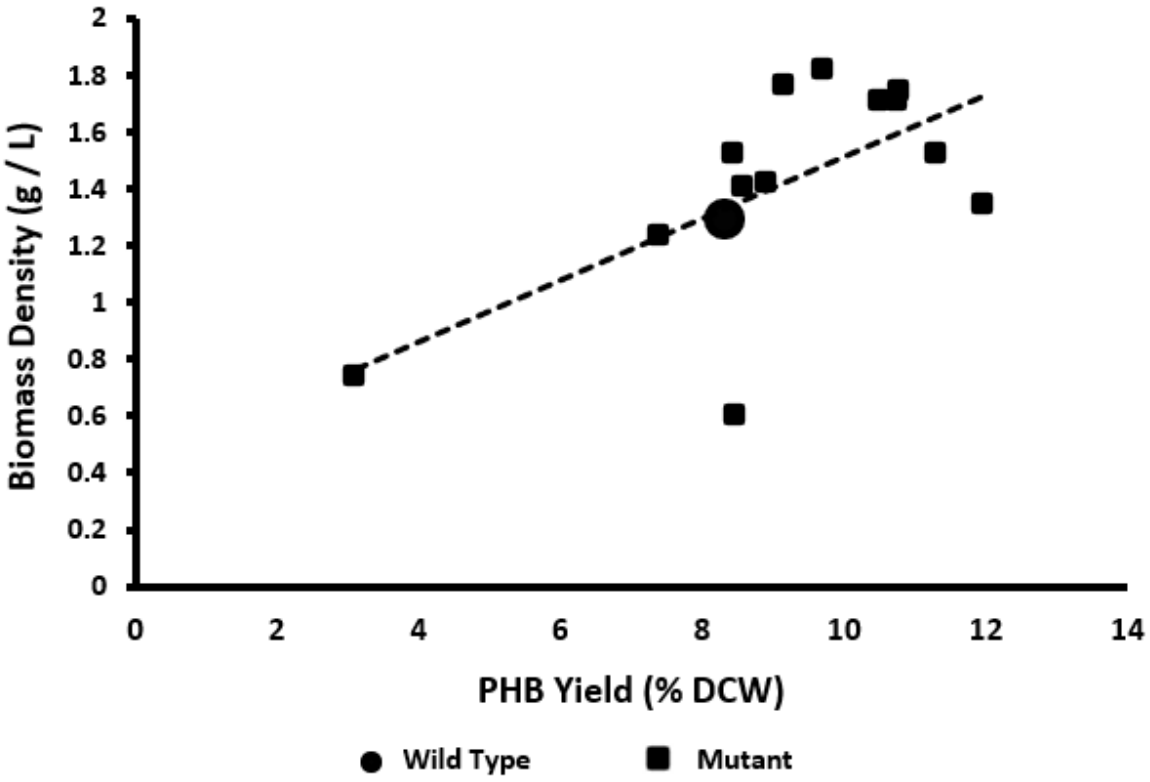


Figure 2-5: Scatter plot of biomass density and PHB yield of characterised 13 EMS mutants.

Figure 2-5 suggests a positive correlation between biomass density and PHB yield in the mutants created in this study. This means that the overall driver for increased or decreased PHB yields may be linked to mutations affecting biomass density and growth efficiency, rather than specific PHB regulatory mutations, such as enhancing the effectiveness of PHB synthesis proteins or providing more carbon substrate for PHB synthesis through blocking competing pathways. Indeed, the two best strains with the highest PHB volumetric density (Strain 1 and Strain 3) also had a higher biomass density increases relative to wild type (36% and 33%) compared to higher PHB yields to wild type (29% and 26%).

An alternate explanation for this positive correlation could be related to the choice of screens when selecting which mutants to progress through the study. The first of the two screens was the use of a neutral lipid fluorescent dye (BODIPY 493/593) which has previously been used to quantify PHB content (Kacmar et al. 2006). This inherently selected cells with a higher lipid content and most likely some with higher PHB content. The second screen was a growth screen based on OD, which also inherently allowed cells with faster biomass efficiencies to pass through to the final stage. Thus, the increase in PHB yield and increased biomass densities may still be independent of one another. In support of this, in a study screening higher lipid producing mutant microalgae cells (*Nanochloropsis sp*.), no significant difference in biomass density was found in the final mutants containing higher lipid content despite also selecting for increased OD in previous stages (Doan & Obbard 2012).

Flask Screen of final 13 Mutant Strains: OD & Photosynthetic Activity

Figure 2-6 shows the optical density of all 13 mutant strains, split into three separate sections ((A) highest 5, (B) middle 4 and (C) bottom 4 strains by PHB yield). Strain 1 and 3 show superior OD on all days of the experiment after inoculation, as did all other stains in the highest 5 and middle 4. Overall, the OD align with the dry biomass density measurements taken on the final day of culturing.

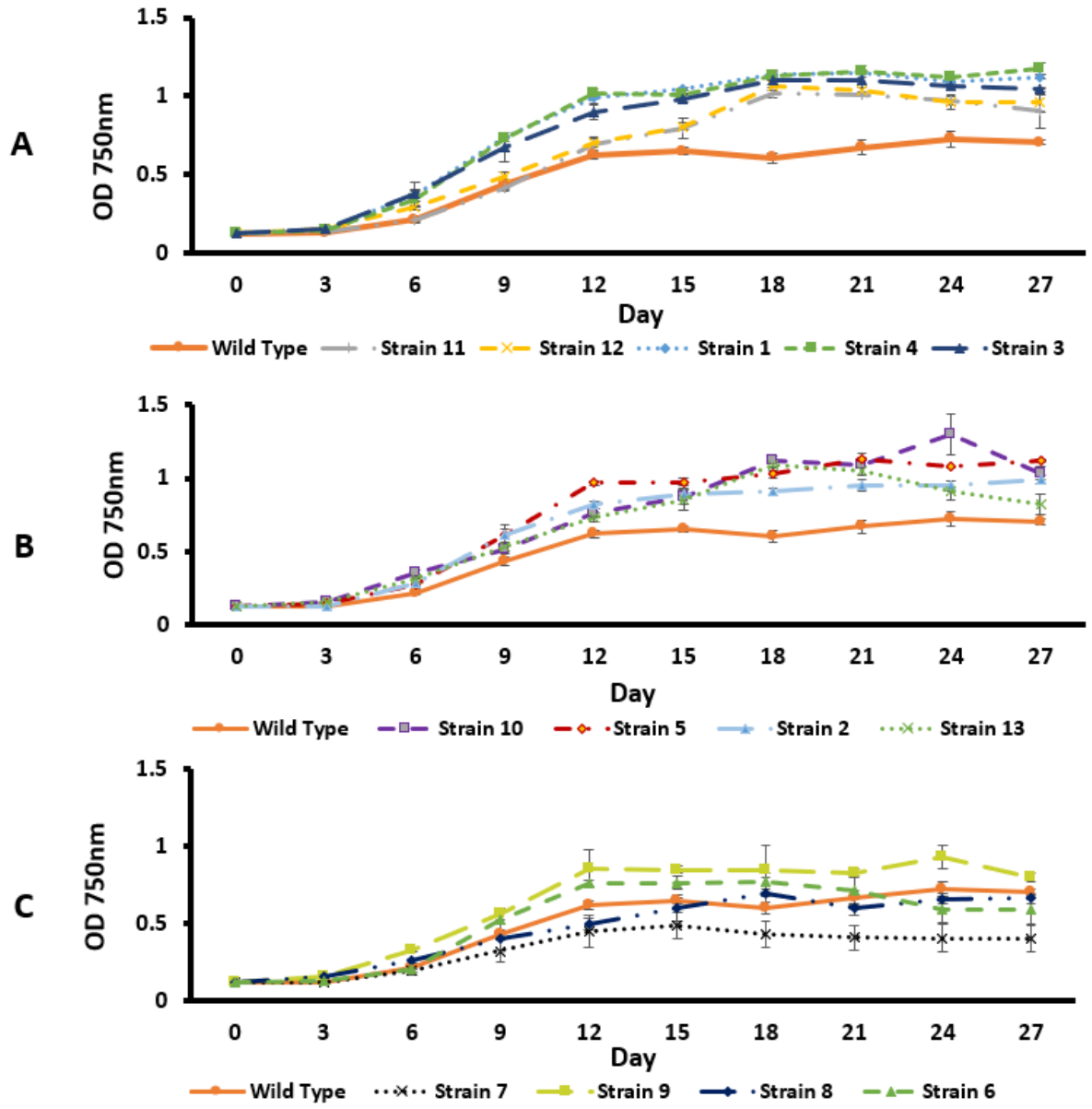
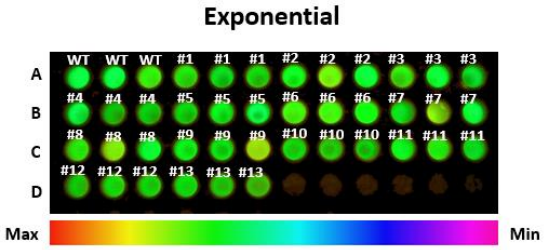
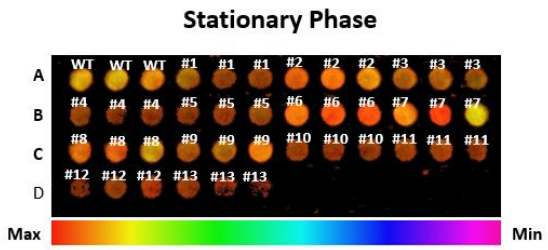


Figure 2-6: Optical density (750nm) of top 5 strains by PHB yield (A), middle 4 strains by PHB yield (B) and bottom 4 strains by PHB yield (C).

Figure 2-7 shows the maximum quantum yield of PSII (F_v / F_m) and maximum electron transport rate of the 13 mutant strains compared to the wild type at both exponential and stationary phases of the culture. There was no statistically significant difference in quantum yield or maximum electron transport rate at the exponential timepoint. At stationary phase, all mutants had either significantly lower quantum yields and maximum electron transport rates, or had no significant difference to the wild type. Despite this, several strains were able to attain far higher biomass densities, such as Strain 1 and 3, which both had

significantly lower photosynthetic parameters. This suggests that no photosynthetic processes are driving the increased biomass and PHB phenotypes behind the mutants. In the top UV mutant cyanobacterial strain in another study, the strain produced over 40% higher biomass compared to the wild type, with only a single mutation in a phosphate transporter gene (Kamravamanesh et al. 2018). Even though this mutation has no correlation to photosynthesis directly, this strain was able to uptake ~30% more CO_2 during cultivation and attain higher biomass and PHB yields. Thus, there is evidence to support that the mutations for increased cyanobacterial PHB and biomass accumulation don't necessarily require direct increases in photosynthetic activity, and instead can be driven by other downstream metabolic processes.





Exponential	F_v / F_m	±	p value	$rETR_{max}$	±	p value
Wild Type	0.37	0.04	-	58.57	14.59	-
Strain #1	0.33	0.02	0.31	51.80	4.19	0.48
Strain #2	0.34	0.06	0.52	42.13	9.43	0.18
Strain #3	0.35	0.03	0.63	48.13	3.52	0.29
Strain #4	0.35	0.03	0.65	59.20	4.23	0.95
Strain #5	0.36	0.02	0.79	53.73	1.58	0.60
Strain #6	0.33	0.02	0.23	39.50	1.57	0.09
Strain #7	0.33	0.07	0.42	41.67	18.42	0.28
Strain #8	0.32	0.06	0.33	44.47	12.68	0.28
Strain #9	0.31	0.07	0.32	45.53	11.28	0.29
Strain #10	0.32	0.01	0.19	47.73	2.59	0.27
Strain #11	0.35	0.02	0.48	50.97	5.64	0.45
Strain #12	0.33	0.01	0.23	53.33	2.63	0.57
Strain #13	0.32	0.03	0.19	52.73	2.51	0.53

Stationary	F_v / F_m	±	p value	$rETR_{max}$	±	p value
Wild Type	0.12	0.01	-	14.73	1.77	-
Strain #1	0.08	0.02	**0.03**	5.33	4.47	**0.03**
Strain #2	0.09	0.02	0.07	9.43	3.40	0.07
Strain #3	0.09	0.01	**0.02**	4.40	1.87	**0.00**
Strain #4	0.07	0.01	**0.00**	2.40	2.33	**0.00**
Strain #5	0.07	0.01	**0.01**	3.17	1.16	**0.00**
Strain #6	0.07	0.01	**0.01**	3.50	2.52	**0.00**
Strain #7	0.10	0.05	0.60	5.83	5.40	0.05
Strain #8	0.09	0.03	0.23	7.17	2.11	**0.01**
Strain #9	0.09	0.01	**0.03**	8.77	2.41	**0.03**
Strain #10	0.06	0.00	**0.00**	1.77	2.02	**0.00**
Strain #11	0.07	0.00	**0.00**	2.13	2.87	**0.00**
Strain #12	0.04	0.03	**0.02**	1.63	2.33	**0.00**
Strain #13	0.04	0.03	**0.02**	0.37	0.64	**0.00**

Figure 2-7: Maximum quantum yield and maximum electron transport rate of wild type and 13 mutant strains during exponential and stationary growth phases.

Figure 2-8A and 2-8B show that both final PHB yield and biomass density correlate positively with PSII maximum electron transport rate at the exponential phase of the culture. Meanwhile, Figure 2-8C and 2-8D show the opposite, with a negative correlation between PSII maximum electron transport rate. Together, this could potentially suggest that the cultures with the highest final biomass densities and PHB yields carried out more photosynthesis earlier in the culture and then switched to a more passive secondary

metabolism, with less active photosynthetic activity, relative to the other mutants. Since cyanobacterial PHB is formed from the conversion of already stored intracellular glycogen accumulated earlier (Koch et al. 2019), it is likely that these more efficient mutants are fixing more carbon earlier in the culture and storing it as glycogen. During the stationary phase, these mutants then perform less electron transport and utilise these larger stores of intracellular glycogen for PHB production. However, this would need to be confirmed by future studies through sequencing of mutants to determine the molecular mechanism driving the altered metabolism.

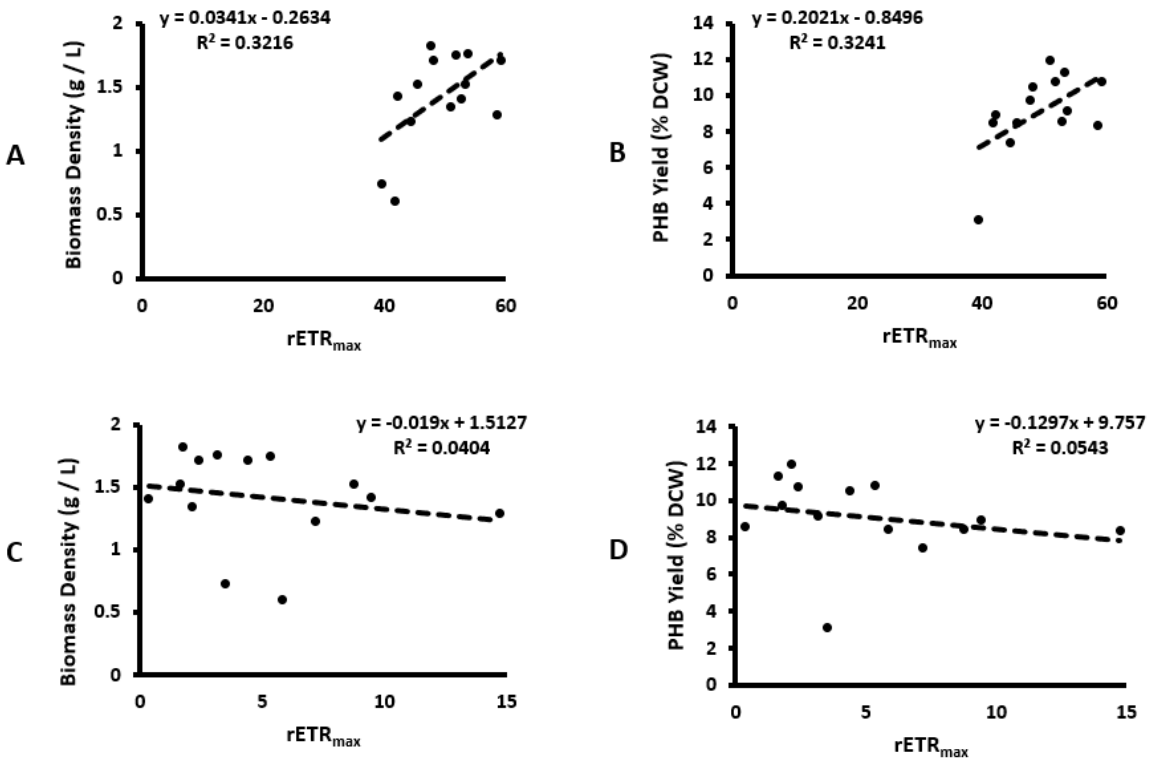


Figure 2-8: Exponential growth maximum transport rate correlated with (A) final biomass density and (B) final PHB Yield. Stationary growth maximum transport rate correlated with (C) final biomass density and (D) final PHB Yield.

Conclusion

This study demonstrates the potential of combining random chemical mutagenesis and FACS, with a final step of growth comparison using optical density in well plate to create a

cyanobacterial mutant library for improved PHB productivity. Of the final 13 mutant strains tested in flask cultivation, two strains produced significantly higher PHB yields (29% and 26% higher than wild type), biomass accumulation (36% and 33% higher than wild type) and volumetric PHB density (75% and 67% higher than wild type). The maximum PHB yielding strain (% dcw) was 12.0%, which was 43% higher than the wild type which achieved 8.3% in this study. The highest volumetric PHB density was 18.8 mg PHB / L compared to 10.7 mg PHB / L by the wild type. To increase yields further, multiple rounds of mutagenesis could be used to achieve even higher PHB production strains. Future omics studies using approaches such as transcriptomic or proteomic analysis are still required to elucidate the molecular mechanisms driving the improved phenotype identified in this study. Additional work could also include testing of the stability of mutant phenotypes over time.

Chapter 3 - Chemical Elicitors and Inhibitors of Cyanobacterial PHB and Biomass

Abstract

While cyanobacteria can produce the bioplastic poly-hydroxy-butyrate (PHB), current yields and productivity are too low for this to be economically viable. Chemical compounds such as phytohormones have the potential to directly trigger regulatory pathways driving PHB synthesis. In this study a range of 10 different chemicals (including oxidants, antioxidants, sterol inhibitors, phytohormones and more) were screened for their ability to both increase and inhibit PHB yield, biomass density and PHB volumetric productivity. Two phytohormone treatments, 1 µM methyl jasmonate and 0.1 µM IAA were found to elicit increased PHB production while 10 µM allopurinol and 10 µM ethynylestradiol were shown to result in lower PHB yields. Potential mechanisms for each of the elicitors and inhibitors are suggested and further discussed.

Introduction

Cyanobacteria are photosynthetic microorganisms that require simple inorganic nutrients for growth as well as atmospheric carbon dioxide. They are a sustainable source of biomass as they can be grown in photobioreactors or ponds on non-arable land using marine or wastewater (Badger et al. 2006; Rueda et al. 2020) and have superior biomass productivities to terrestrial crops (Chisti 2007). The biomass can be used as a sustainable resource for the production of a range of commodity products such as bioplastics, animal feed stock, biofuels, biofertilizer, aquaculture, nutraceuticals and platform chemicals (Price et al. 2020; Priyadarshani & Rath 2012; Wang et al. 2015).

Several species of cyanobacteria natively produce a bioplastic compound, poly-hydroxy-butyrate (PHB) (Kaewbai-ngam, Incharoensakdi & Monshupanee 2016), with the highest yield generally occurring under imbalanced nutrient stresses such as nitrogen deprivation

(Balaji, Gopi & Muthuvelan 2013; Singh & Mallick 2017). Once separated from the biomass, this PHB bioplastic resin can be used in applications such as food packaging and is considered as a biodegradable alternative to polypropylene (Markl 2018). Cyanobacterial PHB production is not yet economically feasible due to the high costs associated with cultivation and harvesting equipment as well as low PHB yields (Panuschka et al. 2019; Price et al. 2020).

PHB productivity in cyanobacteria may be increased through the use of various chemical triggers which have the potential to improve biomass growth and alter cyanobacterial biochemical composition (Price et al. 2020). In order to understand regulatory pathways driving PHB synthesis and ultimately to better control cyanobacterial PHB production, it is important to identify chemical compounds that can directly elicit or inhibit PHB synthesis in cyanobacteria. Screening a chemical library of chemical compounds requires no prior knowledge of metabolic pathways or their regulation, whilst this can uncover the regulation mechanism behind PHB productivity (and other phenotypes).

While inhibitors of PHB production are only useful for understanding PHB regulation and metabolic processes, chemical elicitors of PHB could potentially be utilised at industrial scale production if dosed into growth media. With the final application for using these PHB eliciting chemical compounds at industrial scales, most likely as a media component, these chemical compounds would ideally have the following traits:

- High efficacy / low dosage requirement for biomass and PHB yield increase;
- Low cost for the dosage;
- Minimal to no environmental impact if released into environment;
- Non-hazardous for handling;
- No negative impact on the PHB plastic product;
- Ability to be separated from the media after use (potentially in a wastewater treatment step), through downstream biomass processing or is directly consumed during growth.

Yu et al (2015) proposed four mechanisms by which metabolite productivity can be altered in cyanobacteria by chemical triggers:

1. Chemicals which regulate a discrete metabolic pathway
2. Chemicals which regulate multiple aspects of cyanobacterial metabolism such as phytohormones
3. Chemicals which induce oxidative stress responses
4. Chemicals which act as precursors for certain metabolites

Since one environmental benefit of cyanobacterial PHB production is to avoid using organic carbon compounds as a substrate and instead use atmospheric CO_2, the use of PHB precursors such as acetate was not considered as a trigger for increasing PHB productivity. The following is a brief review of the use of chemical triggers in eukaryotic microalgae, cyanobacteria and increasing PHB yields in heterotrophic bacteria.

Non-Cyanobacteria (Eukaryotic Algae) Chemical Triggers Studies

While there are a significant list of studies investigating the use of chemical triggers to affect the growth of eukaryotic algae (see Table 1, Yu, Chen & Zhang 2015), the following review focusses on two studies which explored lipid productivity. This is because PHB is a lipid polymer metabolite, and thus using chemicals proven to modulate lipid metabolism may affect PHB productivity.

The first study by Franz et al (2013) screened 54 chemicals for their effect on lipid production in *Nannochloropsis* sp., *Nannochloris* sp. and *Phaedactylum tricornutum*. Nile Red in well plates was used to initially screen for increased lipid levels with shortlisted compounds being used with larger culture volumes. Several compounds were found to increase lipid productivity by >200% without compromising biomass productivity. Concentrations between 20 µM to 200 nM were explored. The top compounds for increasing intracellular lipids were epigallocatechin gallate (EGCG), CDK2 inhibitor 2 and cycloheximide (200-400% increase in Nile Red). Propyl gallate and butylated hydroxyanisole (BHA) could be used in large-scale applications considering the increased lipid content and low cost of these chemicals.

The second study by Conte et al (2018) screened a library of 1200 chemicals for their effect on *Phaedactylum tricornutum*. Five compounds (nocodazole, antimycin A, ethynylestradiol, mevastatin and allopurinol) were shortlisted based on three criteria: increasing Nile Red fluorescence, low toxicity (effect on viability) and difference of mechanisms. For example, ethynylestradiol is a sterol metabolism inhibitor, while allopurinol is a cyclic nucleotide signalling / xanthine antioxidant. One of the most effective class of compounds in increasing TAGs in diatoms were the sterol inhibitors which shuffled carbon away from competing sterol sinks. However, cyanobacteria lack the ability to synthesise sterols (Von Elert, Martin-Creuzburg & Le Coz 2003) and the effect of these compounds on their growth has not been previously reported.

There are significant metabolic differences between cyanobacteria and eukaryotic microalgae. For example, eukaryotic microalgae can produce sterols and longer chain unsaturated fatty acids, whereas prokaryotic cyanobacteria tend to produce shorter chain saturated fatty acids. Furthermore, PHB is a prokaryotic energy storage compound (Waltermann & Steinbuche 2005). This means that chemical triggers may have completely different metabolic effects on eukaryotic microalgae compared to cyanobacteria, and compounds which enhance lipid production in eukaryotic microalgae may not necessarily increase PHB productivity in cyanobacteria.

Cyanobacteria Chemical Triggers Studies

Because PHB productivity is a combination of both biomass growth and PHB yield of biomass, the following studies focus on chemical triggers that were proven to increase cyanobacterial biomass growth rates and lipid yields.

Three studies proved the effectiveness of using plant growth promoting hormones (phytohormones) in achieving superior cyanobacterial biomass productivities. In the first study, Suzuki et al (2004) grew *Synechococcus leopoliensis* in the presence of eight different compounds. Five were synthetic local anaesthetics (procaine, procainamide, tetracaine, lidocaine and dibucaine) and three were phytohormones (indole-3-acetic acid (IAA), 2,4,-

dichlorophenoxyacetic acid (2,4-D) and kinetin). Concentrations used were 0.1 µM, 1 µM and 10 µM. Anaesthetic compounds have channel blocking activities and have been shown to affect prokaryotes including cyanobacteria. Lidocaine at a concentration of 1 µM had the most significant increase of biomass with treated cultures growing 80-100% faster and denser. Procaine at 1 µM showed a ~30% increase in growth rate, which matched previous literature achieved in other species of cyanobacteria; however higher concentrations (> 10 µM) resulted in growth inhibition. Of the phytohormones, IAA showed the greatest increase at 100%, kinetin second at 50% and 2,4-D at 30% at 0.1-1 µM concentration.

In the second study, Vikas et al (2014) grew the model organism *Synechocystis* PCC 6803 in the presence of Calliterpenone (a growth promoter from the plant *Callicarpa marcophylla*. 15-50 µL of 0.01 mM solution was added to 100 mL of BG11 with 15 µL giving a 316% increase in biomass, 140% increase in carbohydrates and 130% increase in lipids (protein not reported). Lastly, *Spirulina platensis* was grown in the presence of the phytohormone 24-epibrassinolide (24-epiBL), in addition to salinity stress (NaCl). Increased soluble protein, higher salinity stress resistance and increased biomass productivity (~15% higher than without phytohormone treatment) was reported in the 24-epiBL cultures (Saygideger & Deniz 2008). In a third cyanobacterial PHB study, Mallick et al (2007) foundt 3-(3,4-dichlorophe-nyl)-1,1-dimethylurea (DCMU) reduced PHB content in *Nostoc muscorum.* *C*arbonylcyanide m-chlorophenylhydrazone (CCCP), dicyclohexylcarbodiimide (DCCD) treatment, L-methionine-DL-sulfoximine (MSX) and azaserine were found to increase PHB yields. Many of the antibiotic chemicals which induced higher PHB yields targeted protein synthesis.

Lipid metabolism was investigated by Cheng et al (2012) where metabolomic analysis of two cyanobacteria (*Synechocystis* PCC 6803 & *Anabaena* PCC 7120) and a microalga *Scenedesmus obliquus* at stationary phase was performed. The metabolite ethanolamine was found to correlate with higher lipid levels in all strains, and the researchers then examined the effect of growing *Scenedesmus obliquus* in the presence of exogenous ethanolamine as it had the highest lipid yield. 0.5 mM and 2 mM concentrations of ethanolamine were used which had a negative impact on biomass production. 2 mM

ethanolamine roughly doubled the lipid yield from 12% to 22%, while 0.5 mM had no effect on lipid yield. However, this study did not use ethanolamine on the cyanobacterial strains.

PHB in cyanobacteria has also been proposed to serve an electron sink role (Vincenzini et al. 1990), and therefore may be affected through changes in oxidative pressure. Abd et al grew *Spirulina* platensis in the presence of hydrogen peroxide to induce oxidant stress with the aim of increasing the yields of antioxidant carotenoid compounds. Optimal hydrogen peroxide dosages did increase pigment yields; however, dry biomass decreased with increasing hydrogen peroxide exposure (Abd El-Baky, El Baz & El-Baroty 2009).

Thus, there are a range of studies which have demonstrated the ability for chemical compounds to effect both biomass productivity and biomass composition in cyanobacteria. However, no studies to date have explored the effect upon PHB composition or productivity in cyanobacteria.

Heterotrophic PHB Chemical Triggers Studies

Two studies were found where external chemical stimulants were used to affect the productivity of PHB in heterotrophic culture. Jung and Lee (2000) investigated the effect of increased oxidative conditions on *Ralstonia eutropha* using hydrogen peroxide and methyl viologen in the 1-10 µM concentration range. Lower oxidant concentrations (1-4 µM) increased both biomass accumulation and PHB content, while higher dosages (10 µM) caused a decrease in both biomass growth rate and PHB concentration.

The maximum PHB content was observed on cells treated with 4 µM of either oxidant which achieved 40% PHB yield with methyl viologen and 38% PHB yield with hydrogen peroxide compared to the untreated control with only 28% yield PHB. Jung et al also recommended dosing the oxidant after cell density had already been accumulated, to prevent negative effects on cell culture health during the growth phase. It was also observed that the degradation of PHB occurred more slowly under high chemical oxidative pressure. In another study, IAA at 0.05 – 2.0 mM concentrations was used on the bacterium *Sinorhizobium meliloti* resulting in an increased PHB content. It was proposed that the chemical affected the regulation of enzymes in the tricarboxylic acid cycle resulting in higher

amounts of Acetyl-CoA which is a direct precursor to the PHB biosynthesis pathway (Imperlini et al. 2009).

While there are previous studies on altering cyanobacterial metabolism (for biomass productivity or other metabolites such as pigments) as well as increasing PHB yields in heterotrophic bacteria – there have been no studies testing the effect of chemical compounds on PHB productivity in cyanobacteria. Hence, 10 shortlisted chemicals (Table 3-1), which have demonstrated effects on the biomass productivities and composition in eukaryotic algae or cyanobacteria and proven effects on PHB in heterotrophic bacteria based on the literature review above, were investigated for their effect on cyanobacterial PHB productivity in this study. The chemicals were also chosen based on their cost and availability.

Materials & Methods

An overview of the total experimental design is provided in Figure 3-1. As a preliminary screen, ten shortlisted compounds (Table 3-1) were tested at 0.1 µM, 1 µM and 10 µM concentrations (chosen from the concentration ranges used in similar studies) for their effect on cyanobacterial PHB yield and biomass productivity. Molecules with highest efficacy were selected based on the preliminary screen results and further experiments were performed.

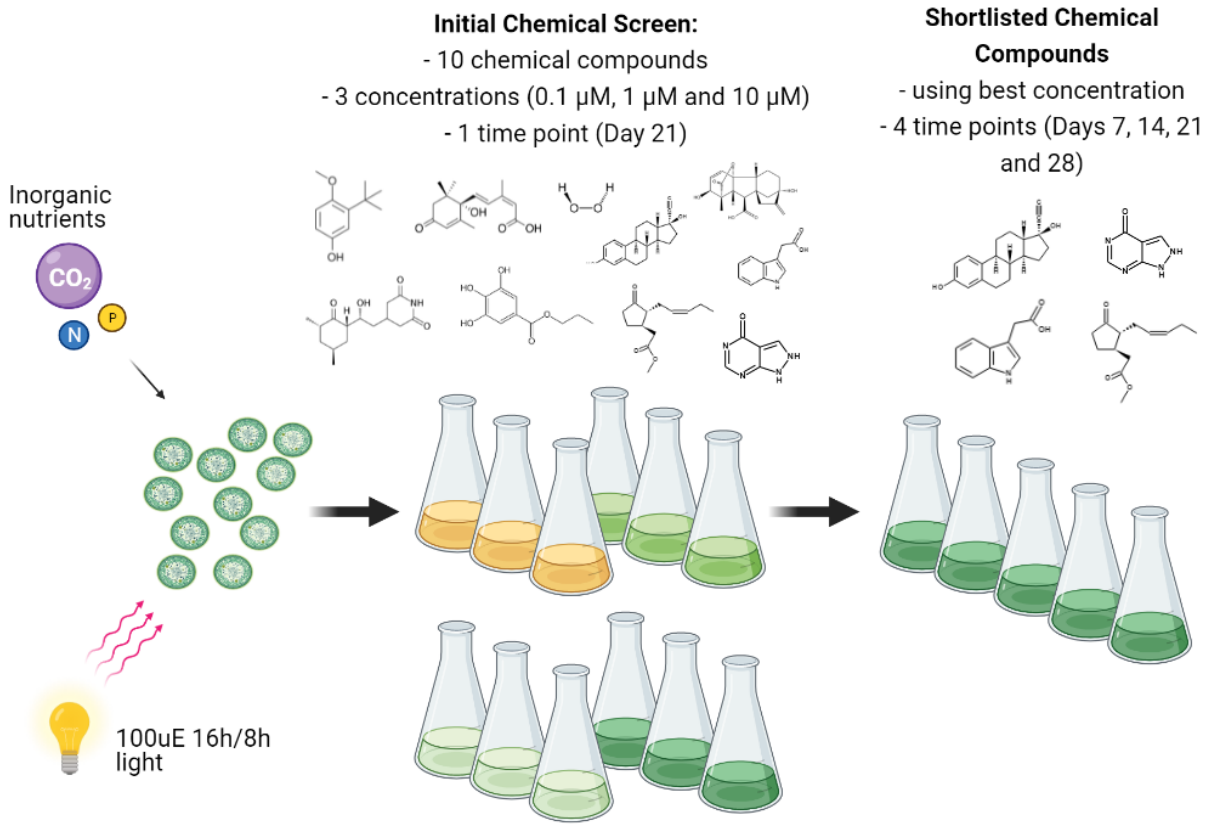


Figure 3-1: Experimental workflow overview including an initial screen of all 10 chemicals at 3 concentrations and a further test with more time points for the top performing chemical triggers

Table 3-1: List of 10 chemical compounds screened for their effect on cyanobacterial PHB yield and biomass productivity. Chemicals were dosed at 0.1 µM, 1 µM and 10 µM.

Chemical Name	**Effect**	**Potential effect on PHB synthesis**
Gibberellic Acid	Phytohormone	Phytohormones may increase PHB synthesis through multiple mechanisms including regulation of carbon partitioning and increasing rates of photosynthesis.
Indole-3-acetic Acid (IAA)		
Abscisic Acid		
Methyl Jasmonate		
Propyl gallate	Antioxidant	Changing the balance of oxidative stress may affect PHB synthesis through regulation of enzymes involved in the
Butylated Hydroxyanisole (BHA)		

		tricarboxylic acid cycle (Imperlini et al. 2009).
Ethynylestradiol	Sterol metabolism inhibitor	May redirect carbon / Acetyl-CoA towards PHB synthesis.
Hydrogen peroxide	Induces oxidative stress	Changing the balance of oxidative stress may affect PHB synthesis through regulation of enzymes involved in the tricarboxylic acid cycle (Imperlini et al. 2009).
Cycloheximide	Interferes with protein synthesis	Proven to increase lipid synthesis in eukaryotic algae (Franz et al, 2013) and thus may redirect carbon / Acetyl-CoA towards PHB synthesis.
Allopurinol	Cyclic nucleotide signalling / xanthine antioxidant	Proven to increase lipid synthesis in eukaryotic algae (Conte et al, 2018) and thus may redirect carbon / Acetyl-CoA towards PHB synthesis.

Cyanobacterial Strain and Cultivation Conditions

A brackish cyanobacterial strain *Synechocystis cf. salina Wislouch (No. 192)* from the Culture Collection of Autotrophic Organisms (CCALA) was maintained at 25 ^{o}C, 50 μmol photons m^{-2} s^{-1} PAR light, on a 16:8 day-night cycle in standard BG11 medium (Rippka, Deruelles & Waterbury 1979). For all cultures, the strain was inoculated at a cell density of 2 x 10^6 cells mL^{-1} in modified BG11 medium (BG11M) (Meixner et al. 2016), with the further addition of 20 mM HEPES buffer. The modified BG11 media used includes increased phosphate (250%) and nitrate (40%) content as well as the addition of NaCl (6 g L^{-1}). 100 mL of culture volume was grown in 250 mL glass flasks. Cultures were grown under 100 μmol photons m^{-2} s^{-1} light, on a 16:8 day-night cycle, with 3% atmospheric CO_2 and 95 RPM orbital shaking incubator (Climo-shaker ISF1-X Kuhner Shaker). All cultures were grown in triplicates.

OD Measurements

Upon inoculation 200 µL of each culture was transferred into a 96 well plate in a biosafety cabinet at a 3 days interval. A Tecan plate reader (Infinite M1000Pro) was used to measure optical density at 650 nm, 700 nm and 750 nm for cyanobacterial biomass accumulation.

Dry Biomass and PHB Quantification

100 mL of culture was harvested every 5 days through centrifugation (3000 rcf, 8 minutes). Pellets were washed with deionised water and freeze-dried overnight at -80 ^{o}C (Christ Freeze Dryer, Scitek) and 0.1 mPa. Samples were then weighed to determine dry biomass mass and then digested in concentrated 98% sulfuric acid for 2 hours at 90 ^{o}C, to convert PHB to crotonic acid before HPLC analysis (Karr, Waters & Emerich 1983). An organic acid Aminex HPX-87H column was used with a UHPLC machine (Agilent Technologies 1290 Infinity). A calibration graph was prepared by plotting peak area against the standard crotonic concentration. Standard PHB was also processed along with biomass to assess the efficiency of PHB recovery.

Statistical Analysis

For the screening experiment of all ten chemical compounds at 0.1 µM, 1 µM and 10 µM, a t-test was used (after confirming normality and equal variance) to compare the statistical significance of treatments to that of the control for PHB yield, PHB density and biomass density. For the shortlisted chemical compound experiments, a semi-parametric permutational analysis of variance (PERMANOVA) was used due to a non-normal distribution of data and presence of two independent variables (day and chemical compound treatment). A pairwise Adonis posthoc test was carried out to determine the significance of individual treatments.

Results & Discussion

Figures 3-2, 3-3 and 3-4 show the PHB yield, biomass density and PHB volumetric density from the preliminary screening experiment. Among the tested compounds IAA (0.1 µM) and methyl jasmonate (1 µM) induced higher PHB content in the biomass in comparison to the

control ($p<0.05$). IAA 0.1 µM resulted in a 29% PHB yield increase and methyl jasmonate 1 µM treatment resulted in a 46% PHB yield ($p<0.05$). These two treatments were shortlisted as the best PHB inducers for further investigation. Both IAA 0.1 µM and methyl jasmonate 1 µM had significantly positive effects on both biomass density (49% and 78% increase respectively) and PHB volumetric density (87% and 154% increase, respectively) ($p<0.05$). Interestingly, IAA showed an inhibitory effect upon both PHB yield and PHB volumetric density at 10 µM, which demonstrates the importance of determining the optimal dosages of chemical triggers.

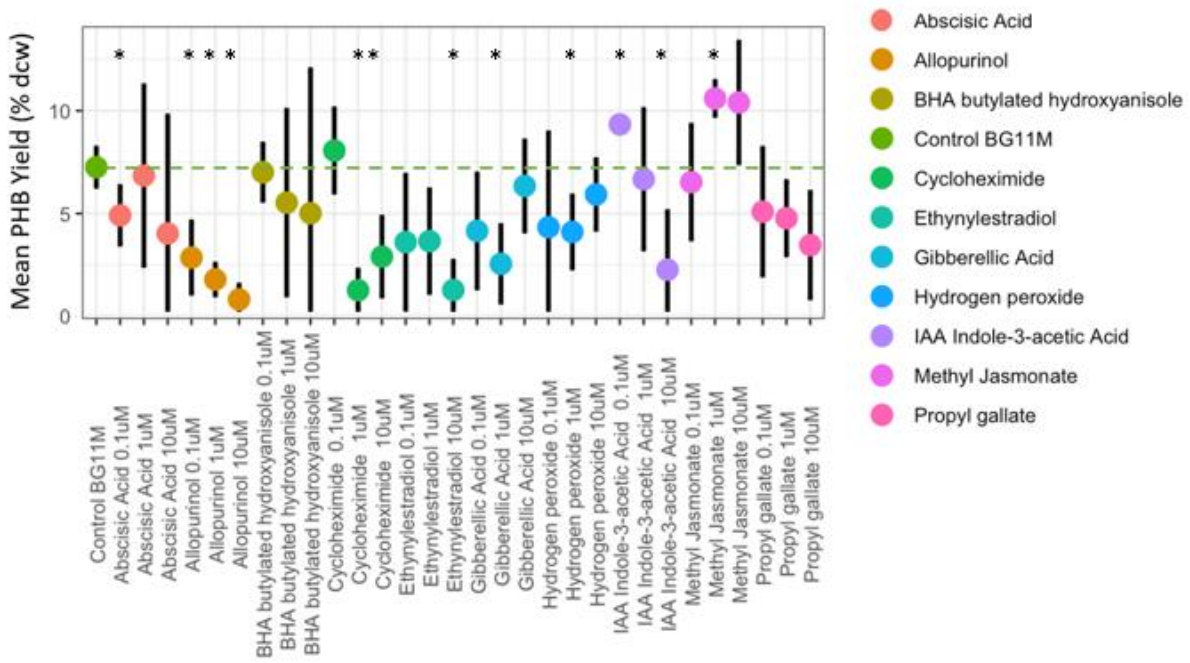


*Figure 3-2: PHB Yield of the cultures grown with 10 different chemicals at 0.1 µM, 1 µM and 10 µM (mean ± standard error, n=3). * denotes statistical significant from control ($p<0.05$).*

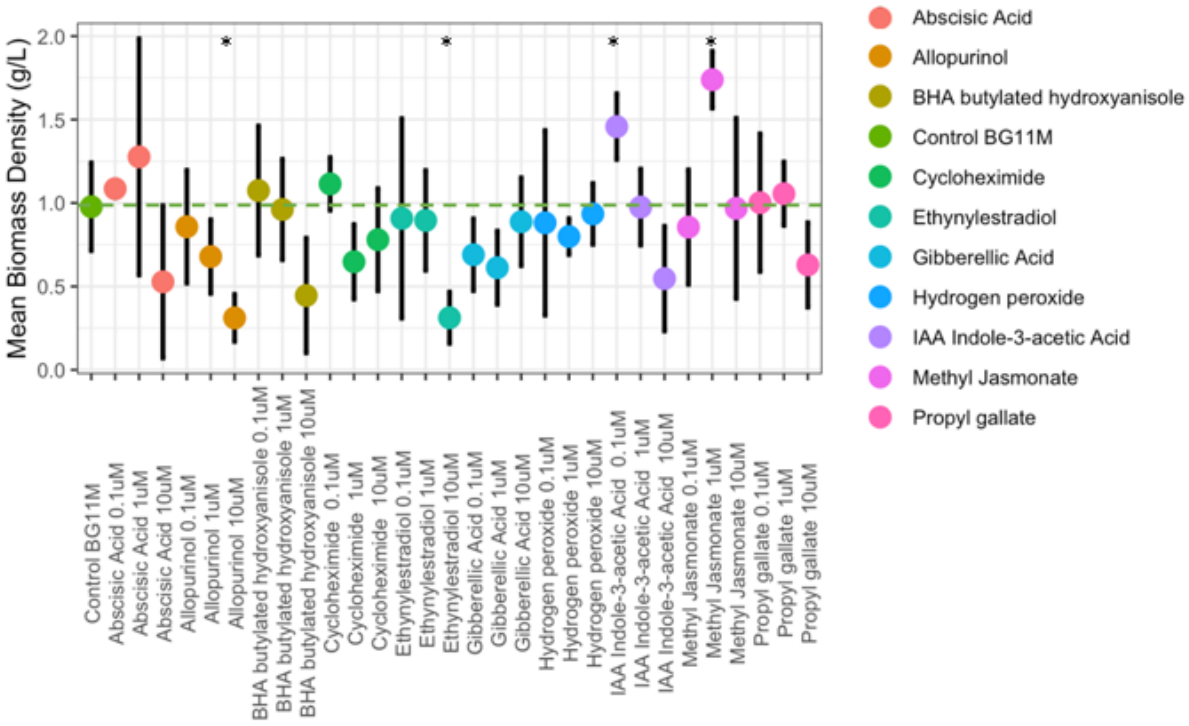


*Figure 3-3: Biomass density of the cultures grown with 10 different chemicals at 0.1 µM, 1 µM and 10 µM (mean ± standard error, n=3). * denotes statistical significant from control (p<0.05).*

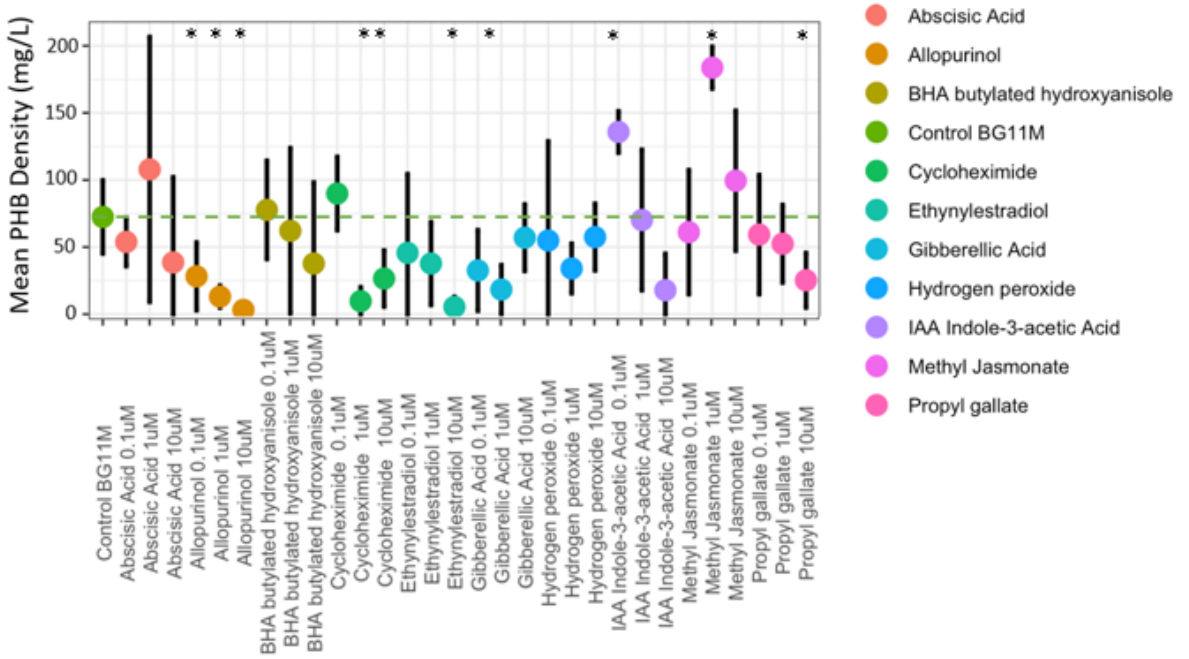


*Figure 3-4: PHB volumetric density of the cultures grown with 10 different chemicals at 0.1 µM, 1 µM and 10 µM (mean ± standard error, n=3). * denotes statistical significant from control (p<0.05).*

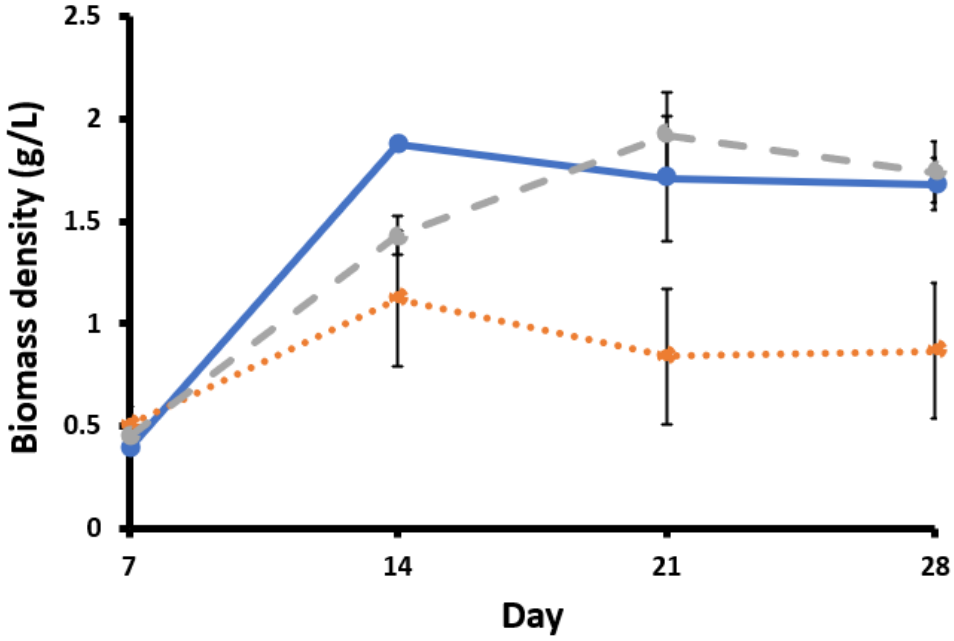
An inhibitory effect was observed at all three dosages of the compounds ethynylestradiol and allopurinol. PHB yields were lowest at 10 µM concentration compared to the control (82% and 88% PHB yield reduction, $p<0.05$). Ethynylestradiol 10 µM and allopurinol 10 µM also had significantly negative effects ($p<0.05$) on biomass density (both 88% reduction) and PHB volumetric density (92% and 96%, respectively).

The four shortlisted chemicals, IAA, methyl jasmonate, ethynylestradiol and allopurinol, are shown in Figure 3-5 which includes all tested concentrations of these compound during the initial chemical screen. However, only the most significant concentration for each chemical was used for further investigation.

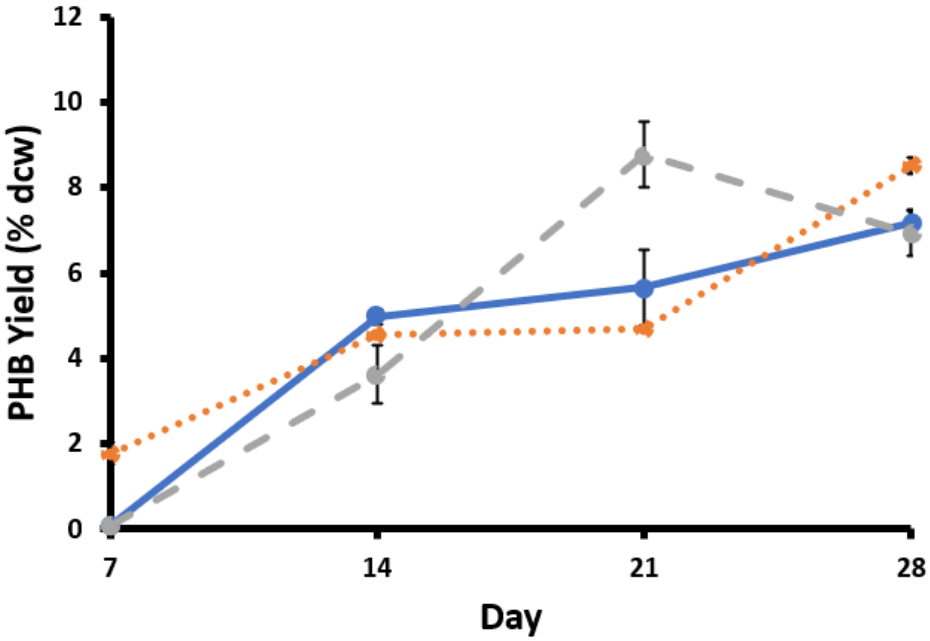
IAA: Chemical Elicitor of Cyanobacterial PHB

Figure 3-6 shows the effect of the IAA 0.1 µM upon the PHB yield, biomass density and PHB volumetric density of the cyanobacterial cultures. On day 21 of culturing, the IAA 0.1 µM treatment increased PHB yield by 55% ($p<0.05$). There was no significant difference in biomass density between the control and treatments in this part of the study. Due to the increase in PHB yield and similar biomass densities, the PHB productivity on day 21 was 68% higher ($p<0.05$) than that of the control. Not only did the 0.1 µM IAA culture achieve higher maximum PHB yield of 8.8% than the control's 7.1%, but it also achieved it in 75% of the culture time (7 days earlier).

(A)


Biomass density (g/L)
2.5
2
1.5
1
0.5
0
7
14
21
28
Day

(B)


PHB Yield (% dcw)
12
10
8
6
4
2
0
7
14
21
28
Day

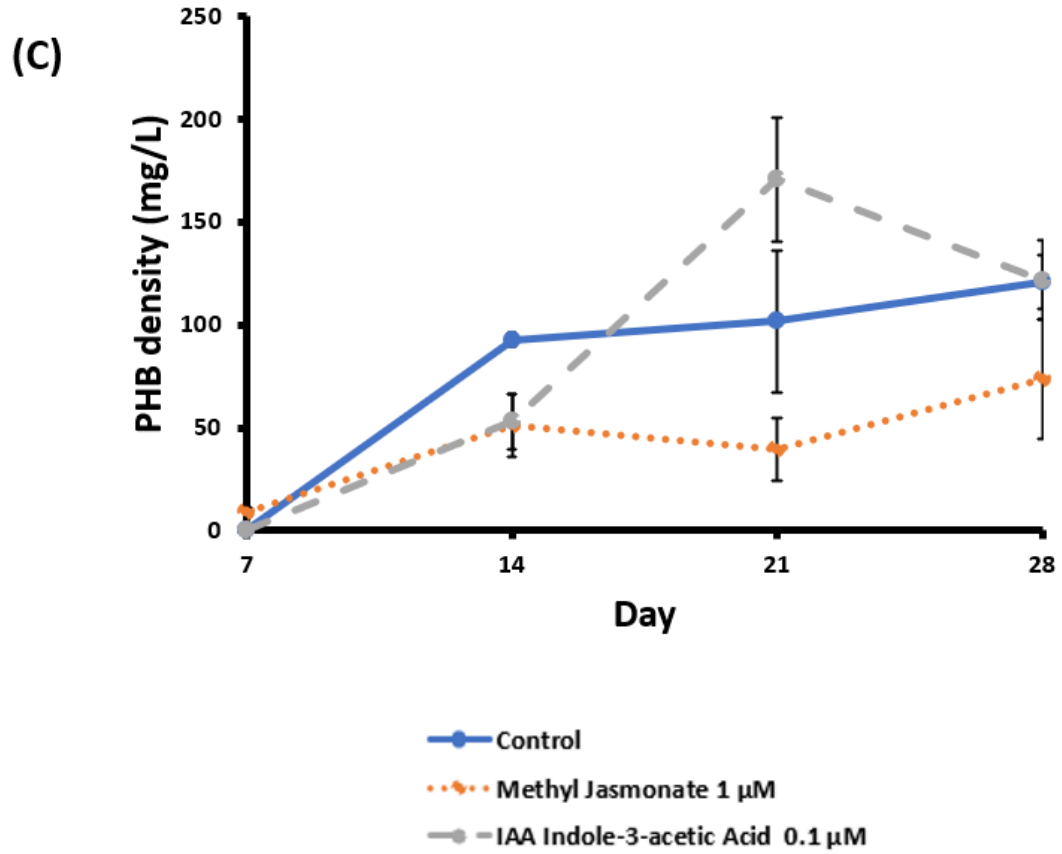


Figure 3-5: (A) Biomass density, (B) PHB Yield and (C) PHB volumetric density of IAA 0.1 µM and methyl jasmonate 1 µM cultures (mean ± standard error, n=3).

Without a central nervous system, plants are almost entirely dependent upon hormone based mechanisms to coordinate growth and development (Letham 1969). Phytohormones have shown to have effects on cyanobacterial growth as well (Saygideger & Deniz 2008; Suzuki et al. 2004). IAA is a phytohormone from the auxin family which regulates most major growth responses through different plant stages of growth (Li et al. 2016) such as root growth, stem elongation and leaf orientation.

The results obtained in this study are consistent to other studies using IAA on cyanobacteria and microalgae. IAA has been used to gain a 100% increase in biomass from the cyanobacteria *Synechococcus leopoliensis* (Suzuki et al. 2004) and was shown to increase the amount of chlorophyl pigment by 100% in *Chlorella vulgaris* cultures treated with 50 µM IAA (Bajguz & Piotrowska-Niczyporuk 2013), which contributed to an 83% increase in cell

number. IAA has been shown to increase photosynthetic capacity in higher plants leading to improved carbohydrate metabolism (Li et al. 2019). Thus, the increase of PHB yield and productivity observed in this study could be partially due to the increased photosynthesis and carbon fixation which resulted in faster growth, higher biomass and more carbon which could enter the PHB biosynthesis pathway. However, Bajguz et al (2013) also concluded from their study that auxins such as IAA could be involved in regulating other phytohormones such as brassinosteroids, thus the link between IAA and increased cyanobacterial PHB may involve more regulated pathways.

IAA was also directly shown to increase the PHB content in the bacterium *Sinorhizobium meliloti* through regulation of various enzymes associated with the tricarboxylic acid resulting in higher amount of Acetyl-CoA (Imperlini et al. 2009), a direct precursor to the PHB biosynthesis pathway. It is likely the same mechanism applies directly to the cyanobacteria *Synechocystis salina* used in this study as alterations of the tricarboxylic acid pathway (deletion of an associated protein) have been shown to result in a 150% higher PHB yield in *Synechocystis* PCC6803 (Monshupanee, Cha irattanawat & Incharoensakdi 2019).

Methyl Jasmonate: Chemical Elicitor of Cyanobacterial PHB

Figure 3-6 shows the effect of 1 µM methyl jasmonate upon the PHB yield, biomass density and PHB volumetric density of the cyanobacterial cultures. On day 28, 1 µM methyl jasmonate increased PHB yield by 19% ($p<0.05$). The methyl jasmonate 1 µM treatment had a lower average biomass density and this lower biomass density resulted in a lower PHB volumetric density ($p>0.05$), despite having a higher PHB yield per cell.

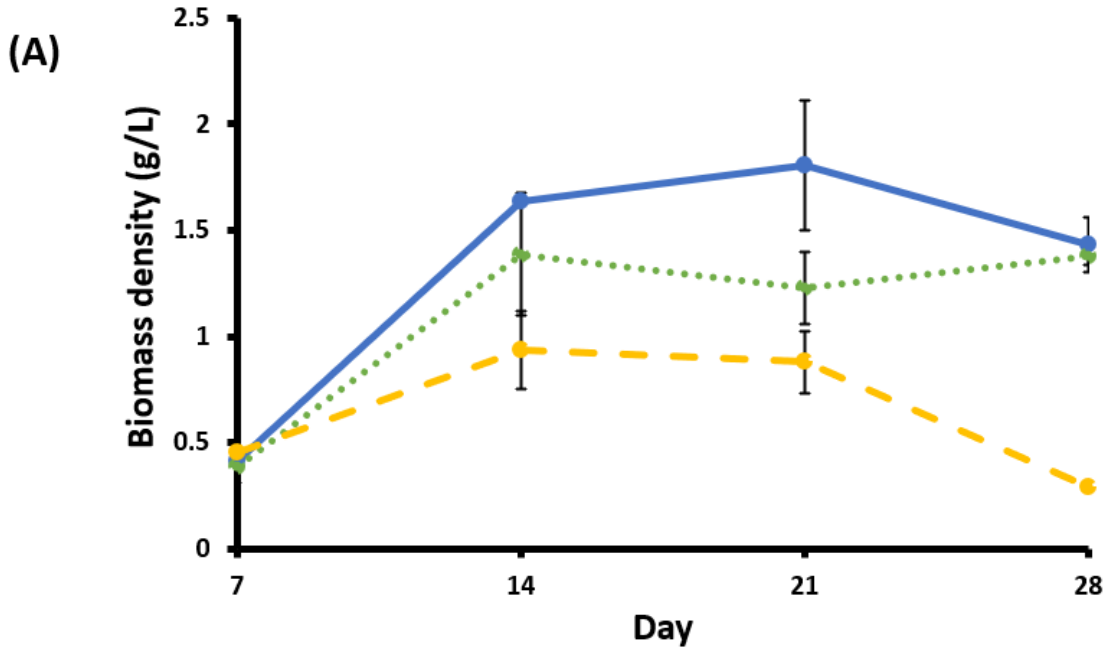
Methyl jasmonate has been shown to increase cell growth rate, protein content and fatty acid synthesis in the green algae *Scenedesmus* (Christov et al. 2001). Terpenoid synthesis was also increased in *Chlamydomonas reinhardtii* using methyl jasmonate (Commault et al. 2019). It was found that the enzymes in the MEP pathway (a competing shunt for carbon

which uses pyruvate as the first substrate) were upregulated. Since pyruvate and Acetyl-CoA pools are directly linked through a single enzyme (Xue & He 2015), the increased use of pyruvate in the MEP pathway should result in a lower PHB production in cyanobacteria, as less carbon is available in the form of Acetyl-CoA, the substrate for PHB synthesis. Another study using methyl jasmonate on *Chlamydomonas reinhardtii* resulted in increased tricarboxylic pathway intermediates, increased pyruvate (up to 100 fold to the control) and lower saturated fatty acid synthesis (Lee et al. 2016). In both studies, 1 mM methyl jasmonate treatments resulted in arrested cell growth – which was also observed on *Synechocystis salina* in this study, despite using a 1000 fold lower concentration of 1 µM. Methyl jasmonate has been shown to divert carbon from primary metabolic pathways to secondary metabolic pathways in higher plants (Pauwels, Inzé & Goossens 2009) which often results in decreased cell growth (Patil et al. 2014). Thus, it is likely that the methyl jasmonate treatment in this study resulted in more carbon for multiple secondary metabolite pathways, such as MEP and PHB synthesis (instead of MEP competing with PHB pathway for carbon), while less carbon is directed to primary pathways such as the tricarboxylic acid cycle (as observed by higher tricarboxylic acid cycle intermediates in the studies previously discussed).

Chemicals Inhibitors of Cyanobacterial PHB: Ethynylestradiol and Allopurinol

Figure 3-7 shows the effect of the 10 µM ethynylestradiol and 10 µM allopurinol treatments upon the PHB yield, biomass density and PHB volumetric density of the cyanobacterial cultures. Ethynylestradiol resulted in total cell culture death by day 28 of the experiment with negligible PHB yield or biomass density, while the control had over 1.5 g / L of dry biomass. While algae and cyanobacteria lack an endocrine system, endocrine disruptors, such as ethynylestradiol, have been studied for their effect on aquatic food webs due to societal pollution. It has been shown that endocrine disruptors reduced cell growth due to reduced PSII energy fluxes in cyanobacteria (Perron & Juneau 2011), *Chlorella vulgaris* (Pocock & Falk 2014) and diatoms (Liu et al. 2010). It is likely that the reduced PHB yield and biomass density was due to inefficient photosynthesis which resulted in less carbon for primary metabolism (for cell growth) and secondary metabolites (such as PHB) .

Allopurinol showed a significant reduction in PHB yield (32% decrease), biomass density (31% decrease) and PHB volumetric density (54% decrease) on day 21 of culturing ($p<0.05$). However, on days 7, 14 and by day 28 the allopurinol culture showed no significant difference in PHB yield or PHB volumetric density. This could suggest a transient effect on a later stage of the cyanobacterial cell culture. Allopurinol is a xanthine oxidise inhibitor and involved in purine catabolism, it was found to increase lipid content in diatoms (Conte et al. 2018). It was also found to affect the circadian rhythm sensing blue-light pathway of the dinoflagellate *Gonyaulax polyedra* (Deng & Roenneberg 1997) which would make for less efficient photosynthesis. However, this mechanism would have occurred throughout the entire cell culture period and does not explain the temporary decrease that was only observed on day 21.



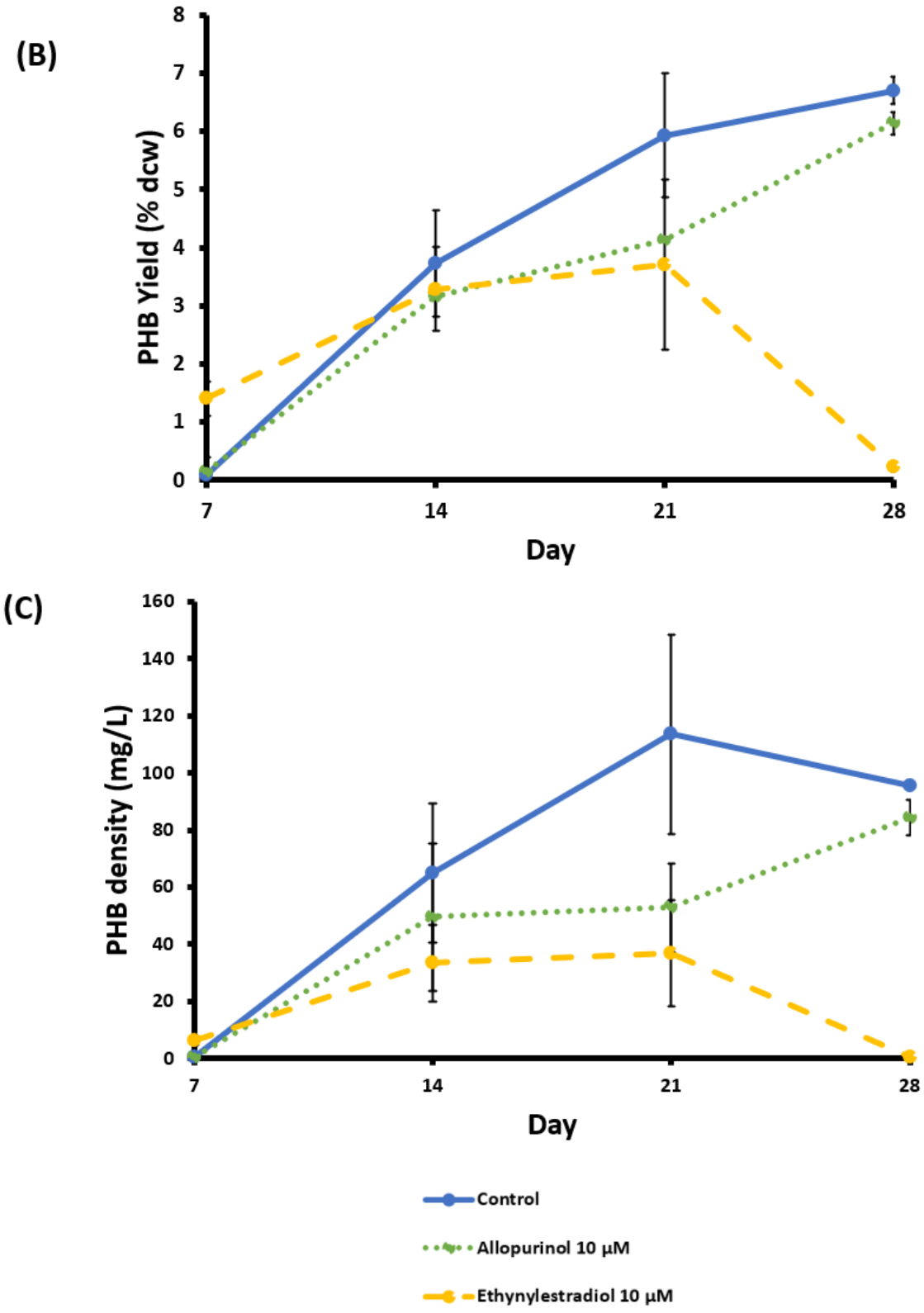


Figure 3-6: (A) Biomass density, (B) PHB Yield and (C) PHB volumetric density of ethynylestradiol 10 µM and allopurinol 10 µM cultures (mean ± standard error, n=3).

Conclusion

From the 10 initial chemicals screened, 1 µM methyl jasmonate and 0.1 µM IAA (both phytohormones) were found to have positive effects on PHB yield, biomass accumulation and PHB density, while 10 µM ethynylestradiol and 10 µM allopurinol were found to negatively affect these phenotypes. Methyl jasmonate has been shown to redistribute carbon away from primary metabolism and cell growth towards secondary metabolic pathways in both higher plants and microalgae. The higher PHB yield coupled with lower biomass density observed from this time series experiment to support the proposed mechanism of action. IAA has been shown to increase PHB content in heterotrophic bacteria through regulating the enzymes of the tricarboxylic cycle and providing more Acetyl-CoA for the PHB synthesis pathway, and a similar mechanism likely occurs in cyanobacteria as supported from the increased PHB yield in this study. Ethynylestradiol (an endocrine disruptor) and allopurinol (a xanthine oxidise inhibitor) likely decreased PHB yield and biomass accumulation through negatively affecting photosynthetic efficiency. While these are proposed mechanisms of action, the actual genes and proteins of interest which are up or down-regulated in response to the chemical trigger can be identified through transcriptomic and proteomic techniques. Additional further investigation could include testing these shortlisted chemical compounds for their effect on other species of cyanobacteria or under different growth conditions (limited nitrogen or phosphorous). In conclusion, both elicitors and inhibitors of cyanobacterial PHB production are of research interest as they can provide targets for genetic engineering and knowledge on PHB regulation. By identifying chemical compounds triggering regulation in PHB synthesis in cyanobacteria, this study paves the way for further work investigating molecular mechanisms driving PHB synthesis with the ultimate goal to improve PHB production in cyanobacteria and make it economically viable.

Chapter 4 - Assessing the suitability of domestic wastewater as a medium for cyanobacterial PHB Bioplastic Production

Abstract

Cyanobacteria can be used as an alternative platform to produce poly-hydroxy-butyrate (PHB) bioplastic resin from atmospheric CO_2; however, production costs still need to be reduced for commercialisation to be viable. Using wastewater as a source of nutrients for cyanobacterial cultivation can significantly help reducing production costs. In this study, domestic wastewater was assessed as a medium for cyanobacterial growth and PHB production. Primary domestic wastewater produced 1.3 g L^{-1} cyanobacterial biomass whereas the control culture in synthetic modified BG11 media grew to 1.6 g L^{-1} of biomass. PHB productivities differed greatly between the primary wastewater culture (0.9% w/w yield) and control BG11M media (6% w/w/ yield) despite the similar biomass accumulation and timing of nitrogen limitation on day 10 of culturing. A spike of non-cyanobacterial biomass during the PHB accumulation phase was observed and this may have contributed to the lower PHB yield obtained in the wastewater cultured biomass.

Introduction

Plastics play a central role in modern society, from their use in agriculture, medical implants, consumer products, electronic devices and more. With over 300 million tonnes of petrochemical plastics produced globally each year (Plastics Europe, 2017), it is estimated that 9 % are recycled and 12 % incinerated, with the remaining 79 % disposed in landfill or, discarded into the environment (Geyer, Jambeck & Law 2017). There has been a growing awareness of the negative environmental effects associated with plastic pollution, as it accumulates in both terrestrial and aquatic environments. This has resulted in a recent shift by governments, businesses and individuals towards the use of more sustainable materials that minimise pollution throughout its lifecycle. Petrochemical plastic production is estimated to account for 8-9% of global oil and gas consumption in either raw input

materials or energy used in processing (Nielsen et al. 2020). Biobased and biodegradable bioplastics are a fast-growing alternative to petrochemical plastic materials due to their superior sustainable qualities, such as being produced from a renewable biomass source and ability to degrade into harmless compounds in the environment after they are discarded (Shah et al. 2008). However, the majority of current bioplastic production uses terrestrial crop biomass as the feedstock (Zeller et al. 2013), which must compete with food crops for natural resources, including freshwater and arable land. Over 33 % of ice free global land use is taken up by agriculture (Ramankutty et al. 2018), and 70 % of global freshwater is used for agriculture and irrigation (Sato et al. 2013). Replacing petrochemical plastics with crop-based bioplastics will increase the demand for the remaining arable land and freshwater resources, as well as compete with food crops.

Cyanobacteria are photosynthetic prokaryotes that require simple inorganic nutrients for growth including carbon dioxide, nitrogen, phosphorous, some trace metals and micronutrients. Cyanobacteria can be grown in photobioreactors and ponds without the need for arable land and can be grown in a range of water qualities, including brackish, marine and wastewaters. Several species of cyanobacteria naturally produce poly-hydroxy-butyrate (PHB), an energy storage metabolite that is commonly known to be produced under certain nutrient limitation conditions, such as nitrogen deprivation (Balaji, Gopi & Muthuvelan 2013; Singh & Mallick 2017). PHB is a condensation polyester that is used as a resin in bioplastic manufacturing for applications such as food packaging and is considered as a potential biodegradable substitute for polypropylene (Markl 2018). However, cyanobacterial PHB production is not yet economically feasible, due to the high costs associated with the cultivation equipment and current low PHB yield (Price et al. 2020). One option to reduce operational costs is the use of wastewater as a source of nutrient-media for the cyanobacteria.

Existing research on cyanobacterial PHB production in wastewater has focused mainly on aquaculture wastewater (Krasaesueb, Incharoensakdi & Khetkorn 2019; Samantaray, Nayak & Mallick 2011) or centrate, the liquid wastewater from anaerobic digestion (Arias, Fradinho, et al. 2018; Meixner et al. 2016). To date, no published research has investigated the potential of domestic wastewater for cyanobacterial PHB production. The goal of this

research was to investigate the suitability of primary domestic wastewater as a nutrient-rich media for the cultivation of cyanobacterial biomass and assess PHB productivity potential. Primary wastewater, which is raw wastewater with only rough initial screening to remove large particles, was chosen as it would have more nutrients to support growth as opposed to secondary wastewater (after oxidation) or tertiary wastewater (after nitrate and phosphate removal).

Materials and Methods

Figure 4-1 provides an overview of the experimental procedure used for this study.

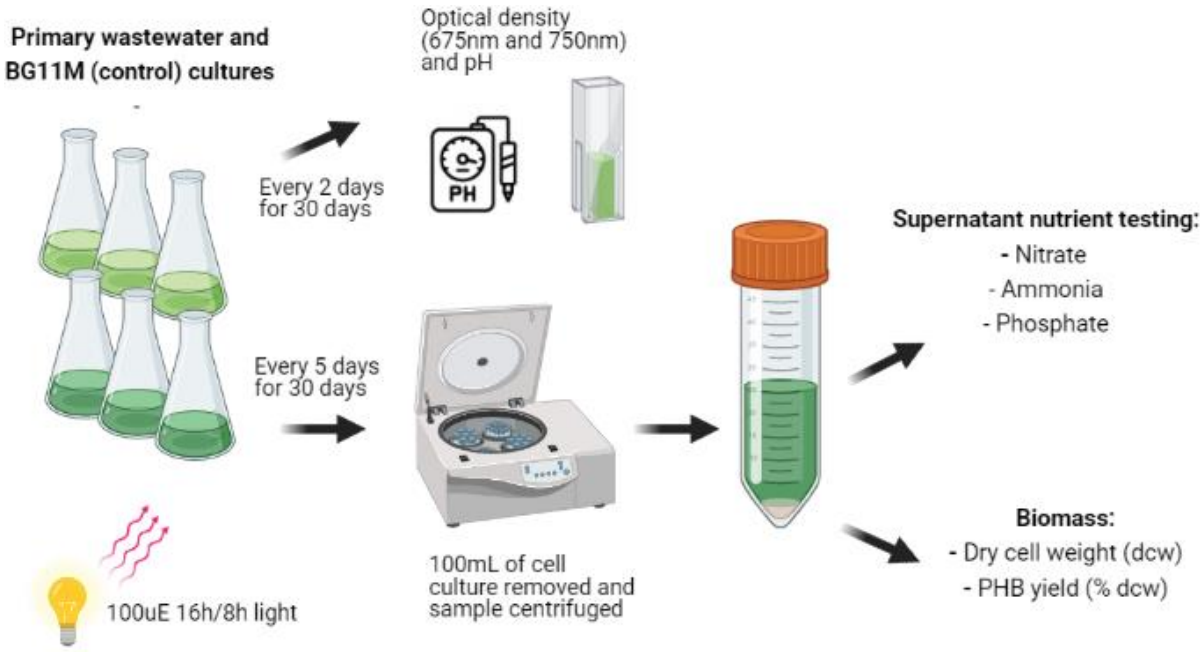


Figure 4-1: Overview of the experimental procedure.

Wastewater Collection

Domestic wastewater from a combined residential and shopping complex was collected from the Central Park Mall Wastewater Treatment Plant, Sydney, Australia. Primary wastewater was collected from an outlet after only rough initial screening.

Cyanobacterial Strain and Cultivation Conditions

The brackish cyanobacterial strain *Synechocystis cf. salina Wislouch (No. 192)* was obtained from the Culture Collection of Autotrophic Organisms (CCALA) and maintained at 25 ^{o}C, 50 μmol photons m^{-2} s^{-1} PAR light, on a 16:8 day-night cycle in standard BG11 medium (Rippka, Deruelles & Waterbury 1979). For this study, the strain was inoculated at a cell density of 2 x 10^{6} cells mL^{-1} into either primary wastewater or modified BG11 medium (BG11M) (Meixner et al. 2016), with the further addition of 20 mM HEPES buffer. The main modifications of BG11M to BG11 included, approximately 250 % phosphate and 40 % nitrate, in addition to 6 g L^{-1} of NaCl and other slight modifications. 400 mL of culture volume was grown in 1 L glass flasks. Cultures were grown under 100 μmol photons m^{-2} s^{-1} light, on a 16:8 day-night cycle, with 3% CO_2 and 95 RPM orbital shaking (Climo-shaker ISF1-X Kuhner Shaker). All cultures were grown in triplicate.

pH and OD Measurements

Upon inoculation and every 2 days after, 200 μL of each culture was transferred into a 96 well plate in a biosafety cabinet. Optical density was measured using a (Tecan) plate reader (Infinite M1000Pro) at 675 nm for cyanobacterial biomass accumulation and 750 nm as a proxy for total biomass accumulation. pH was measured using a micro pH probe (Sigma Aldrich) attached to a reading unit (TPS WP-81 Rowe Scientific) and calibrated against laboratory standards.

Dry Biomass and PHB Quantification

50 mL of culture was harvested every 5 days through centrifugation (3,000 rcf 8 minutes). Pellets were washed with deionised water and freeze-dried overnight at -80 ^{o}C (Christ Freeze Dryer, Scitek) and 0.1 mPa. Samples were then weighed to determine dry biomass mass and then digested in concentrated 98% sulfuric acid for 2 hours at 90 ^{o}C, to convert PHB to crotonic acid before HPLC analysis. The column used was an organic acid Aminex HPX-87H with an Agilent Technologies 1290 Infinity HPLC machine. Elution time and UV absorbance spectrum of a digested PHB standard (Sigma Aldrich) and crotonic acid (Sigma Aldrich) confirmed effectiveness of digestion and the presence of crotonic acid in samples. A calibration curve relating peak area and crotonic acid concentration was used to quantify the PHB content in the acid digested samples.

Dissolved Nutrient Analysis

Supernatant was collected from media that was centrifuged at the same time as biomass collection. All samples were syringe-filtered through a 0.2 µm membrane before nutrient analysis. Organic carbon and inorganic carbon were measured on a Total Organic Carbon Analyser (Shimadzu TOC-L) with the sum of these measurements being the total carbon. Nitrate (NO_3-N) was determined colour metrically, following reduction in a Vanadium (III) column and corrected for the presence of nitrite, in non-reduced samples (ThermoFisher Scientific 2015c). Ammonium (NH_4-N) was measured colourmetrically at 660 nm, following reaction sodium dichloroisocyanurate (ThermoFisher Scientific 2015a). Phosphate(PO_4-P) was measured colourmetrically at 880 nm, following reaction with ammonium molybdate and antimony potassium tartrate in the presence of ascorbic acid (ThermoFisher Scientific 2015b). All nutrient measurements were undertaken on a spectrophotometer (Thermo Scientific Gallery Plus) and calibration curves developed from standards provided by ThermoFisher Scientific.

Statistical Analysis

A semi-parametric permutational analysis of variance (PERMA-NOVA) using Primer software was carried out to test the Euclidean dissimilarities between different measurements across time treatments in a two-factor design (Achlatis et al. 2018, 2019). The first factor was time and the second being treatment. The pseudo-F statistic and $P_{(perm)}$ value were obtained using a full model type III sums of squares based on dissimilarity matrices and 4,999 permutations. Significant interaction effects of treatment were assessed using the pairwise permutational pseudo-T post hoc tests. Three levels of significance were set a $P_{(perm)} < 0.05$, 0.005 and 0.0005.

Results & Discussion

Biomass Accumulation, Optical Density, and PHB yield

The maximum biomass density obtained by the BG11M and primary wastewater cultures was 1.6 g L^{-1} and 1.3 g L^{-1} , respectively on day 30 of culturing (Figure 4-2). Both cultures

reached stationary phase by day 15 at which the biomass densities were 1.4 g L^{-1} and 1.2 g L^{-1}, respectively. Cultures were kept past stationary phase to expose them to nutrient limitation which is known to induce PHB accumulation (Troschl et al. 2018) (Figure 4-3). There was no statistically significant difference in the biomass concentration for all measurements across the entire 30 days of culturing between the primary wastewater and BG11M cultures ($p>0.05$ for all time points Figure 4-2). This shows the promise of using wastewater as a medium for cyanobacterial production, since similar biomass can be obtained without incurring the cost of manufacturing synthetic growth media.

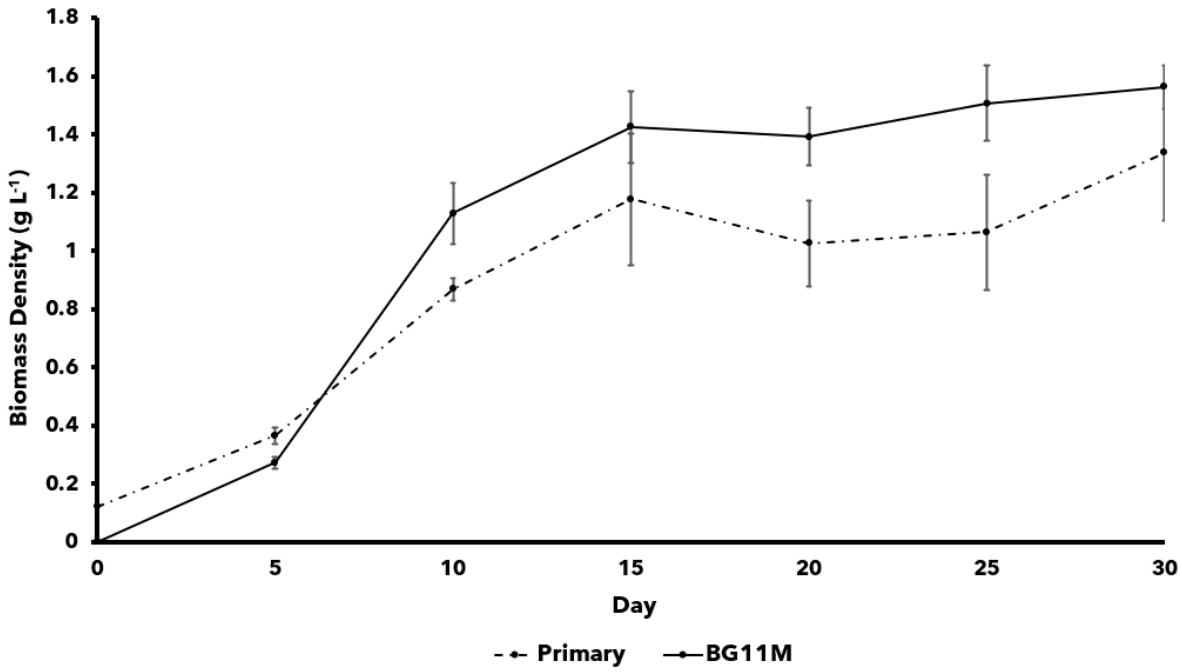


Figure 4-2: Biomass accumulation measured gravimetrically every 5 days for primary wastewater culture (blue line) and BG11M culture (yellow line). There was no statistically significant difference in the biomass accumulation across the entire 30 days of culturing between the primary wastewater and BG11M culture ($p>0.05$). All measurements were in triplicate with standard error of the mean shown by error bars.

The maximum PHB yield (6.1%) occurred in BG11M culture on day 25 of culturing (Figure 4-3). A maximum of 0.93% PHB yield was also measured in the primary wastewater culture on day 25 (Figure 4-3). Meixner et al. (2016, 2018) obtained similar PHB yields of ~6% for

Synechocystis salina grown in BG11M, under similar growth conditions (Meixner et al. 2016, 2018). Previous work using a consortia of cyanobacteria, algae, bacteria and protozoa to treat a mixture of anaerobic centrate and secondary wastewater, was able to achieve PHB yields of < 0.5% (Arias, Uggetti, et al. 2018) which was similar to the value achieved in this study.

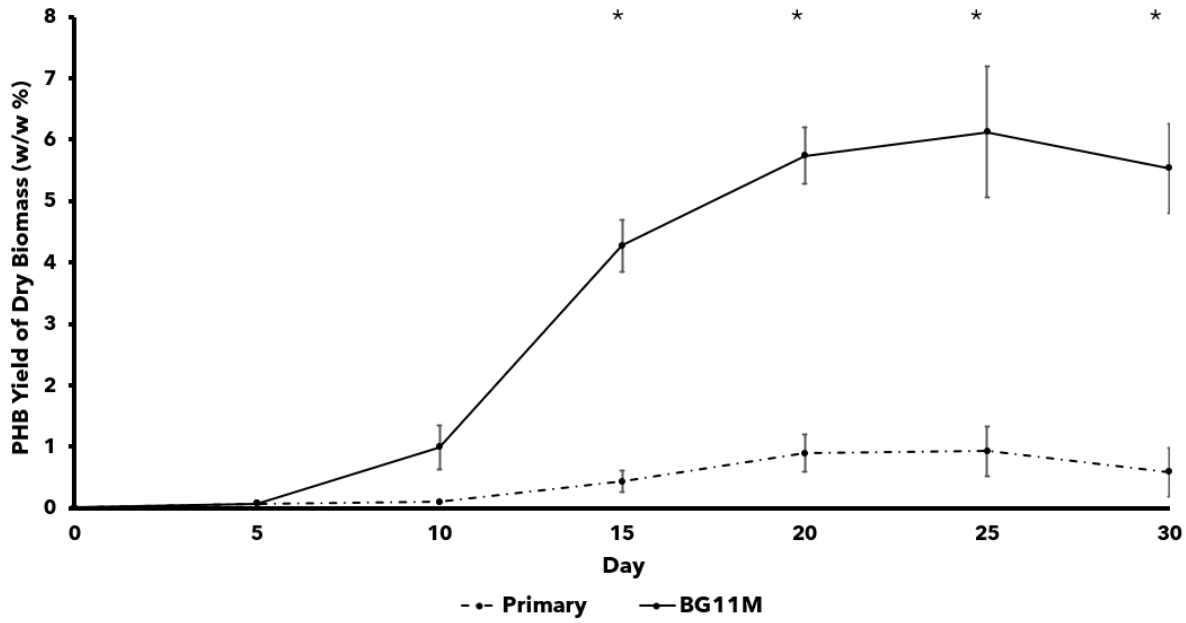


*Figure 4-3: PHB % yield normalised to dry biomass determined by HPLC every 5 days for primary wastewater culture and BG11M culture. * denotes a statistically significant difference (p<0.05). All measurements were in triplicate with standard error of the mean shown by error bars.*

Despite both BG11M and primary wastewater cultures achieving similar biomass, their PHB yield, normalised to biomass concentration, differed significantly ($p<0.01$) with the primary wastewater culture having significantly less PHB yield for all days after measurable accumulation began on day 10. Both the BG11M and primary wastewater cultures followed a similar pattern of PHB accumulation starting on day 10 and a peak on day 25 followed by a small drop on day 30. The presence of bacterial biomass in the primary wastewater culture may have contributed to the lower PHB yields observed. Cyanobacterial biomass was measured at an optical density of 675 nm and corrected for turbidity by subtracting optical density at 750 nm. Figure 4-4 shows that both the primary wastewater culture and the

BG11M culture followed a similar trajectory for the first 16 days, after which the primary wastewater had a drop in cyanobacterial biomass. From Figure 4-2, it can be seen that the total biomass in the primary wastewater culture remained mostly constant over this time. This suggests that the cyanobacterial biomass was replaced by non-cyanobacterial biomass during this time. Interestingly, the majority of PHB accumulation in primary wastewater took place during this time and thus the rise in non-cyanobacterial biomass could be the reason for the lower PHB yield observed, compared to the BG11M culture. While some bacteria have been reported to produce PHB, the lower PHB yield observed in the primary wastewater suggests that the bacterial community associated with the wastewater in this study may have significantly diminished the PHB measured.

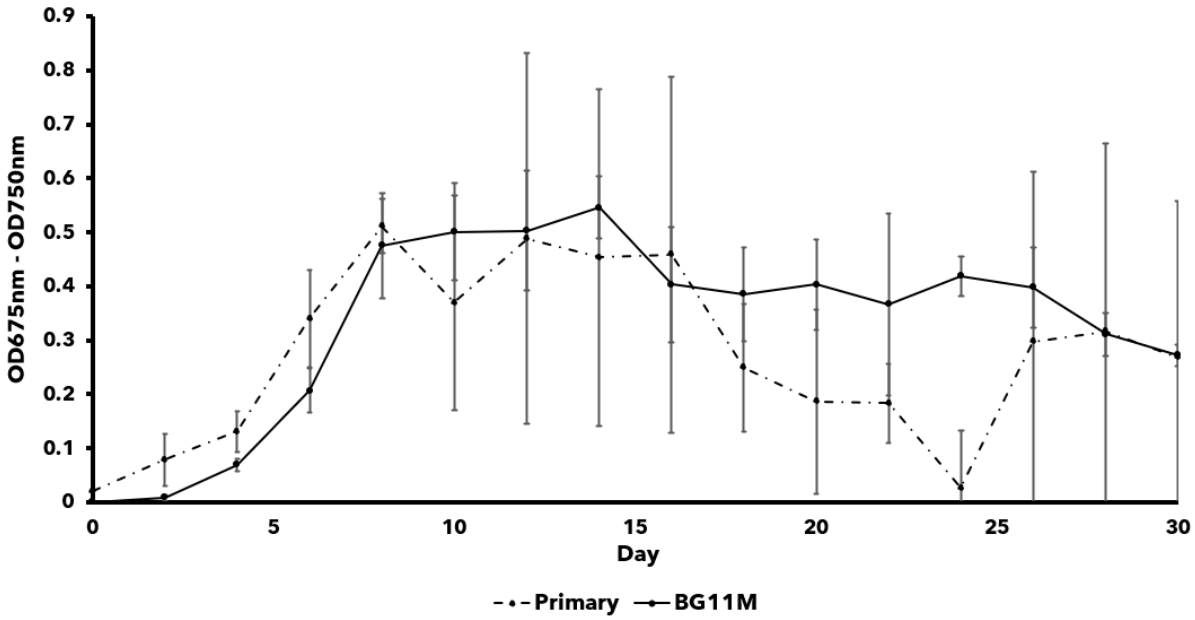


Figure 4-4: Optical density measurements at 675nm corrected for turbidity (by subtracting optical density at 750nm) taken every 2 days of primary wastewater cultures and BG11M culture (mean ± SEM).

Nutrients: Phosphate, Ammonium and Nitrate

The primary wastewater cultures used all 8.4 mg P L^{-1} of phosphate by day 10, showing promise for economically efficient nutrient removal (Figure 4-5). The BG11M cultures had only utilised 6.7 mg P L^{-1} of phosphate in the culture by the same time (initially 12.6 mg P L^{-1}). Phosphate utilisation slowed down for the BG11M culture and plateaued by day 20 with a final utilisation of 9.5 mg P L^{-1} and leaving behind a residual 3 mg P L^{-1} in the media. Similar phosphate use rates were achieved by cyanobacteria in another study (Chevalier et al. 2000). A potential reason for phosphate uptake differences between the two media, was the presence of bacteria in the wastewater that most likely competed with the cyanobacterium for phosphate.

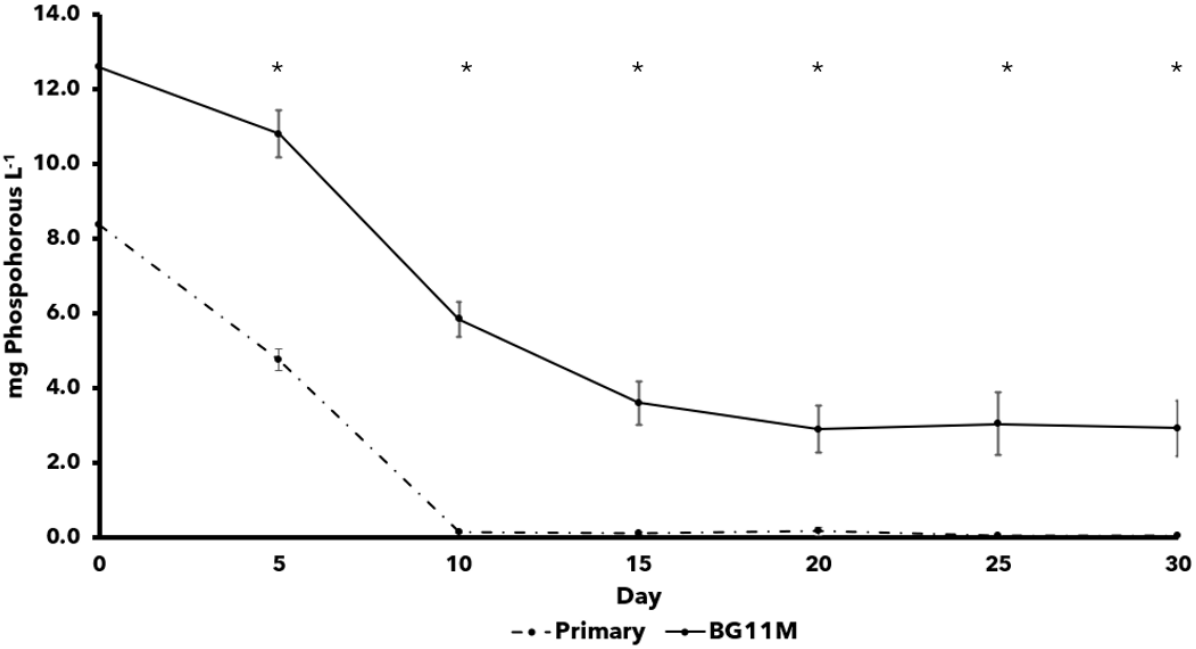


*Figure 4-5: Phosphate of primary wastewater culture and BG11M culture measured every 5 days. * denotes a statistically significant difference (p<0.05). All measurements were in triplicate with standard error of the mean shown by error bars.*

For primary wastewater, the majority of dissolved inorganic nitrogen (DIN) was in the form of ammonium-nitrogen. In contrast, DIN was in the form of nitrate-nitrogen for BG11M. Once again, the primary wastewater was able to use all DIN (58 mg N L^{-1}) within 10 days (Figure 4-6), similar to phosphate. In contrast, the BG11M culture used all of its DIN (84 mg N L^{-1}) by day 10.

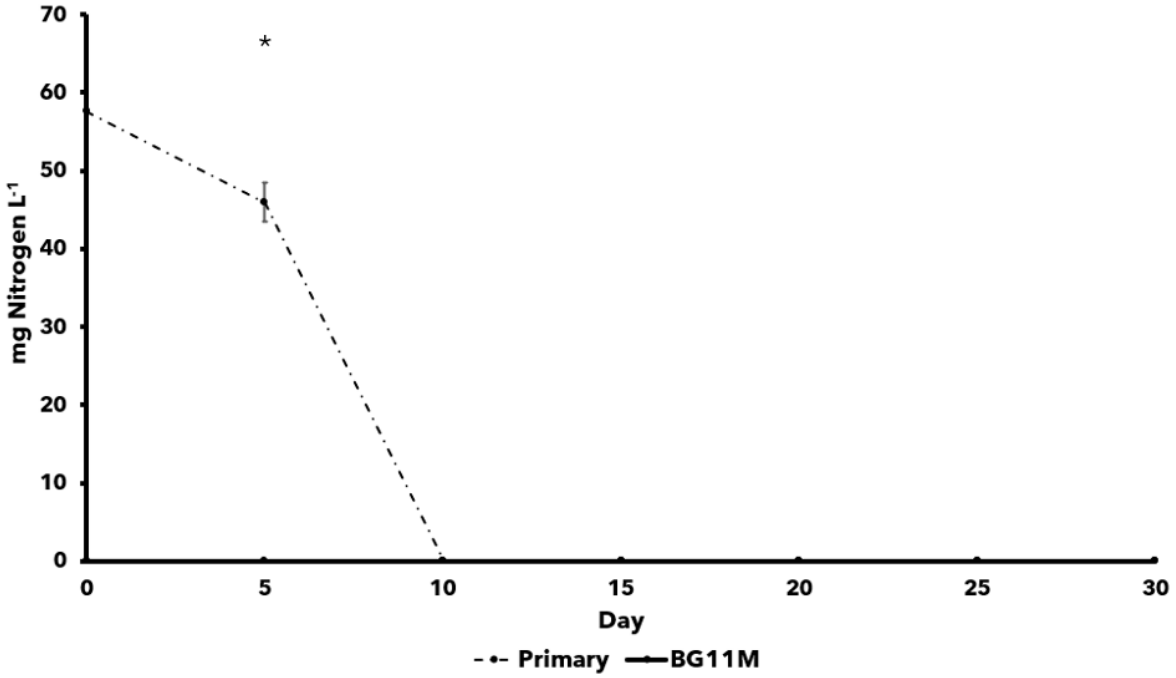


*Figure 4-6: Ammonium of primary wastewater culture and BG11M culture measured every 5 days. * denotes a statistically significant difference (p<0.05). All measurements were in triplicate with standard error of the mean shown by error bars.*

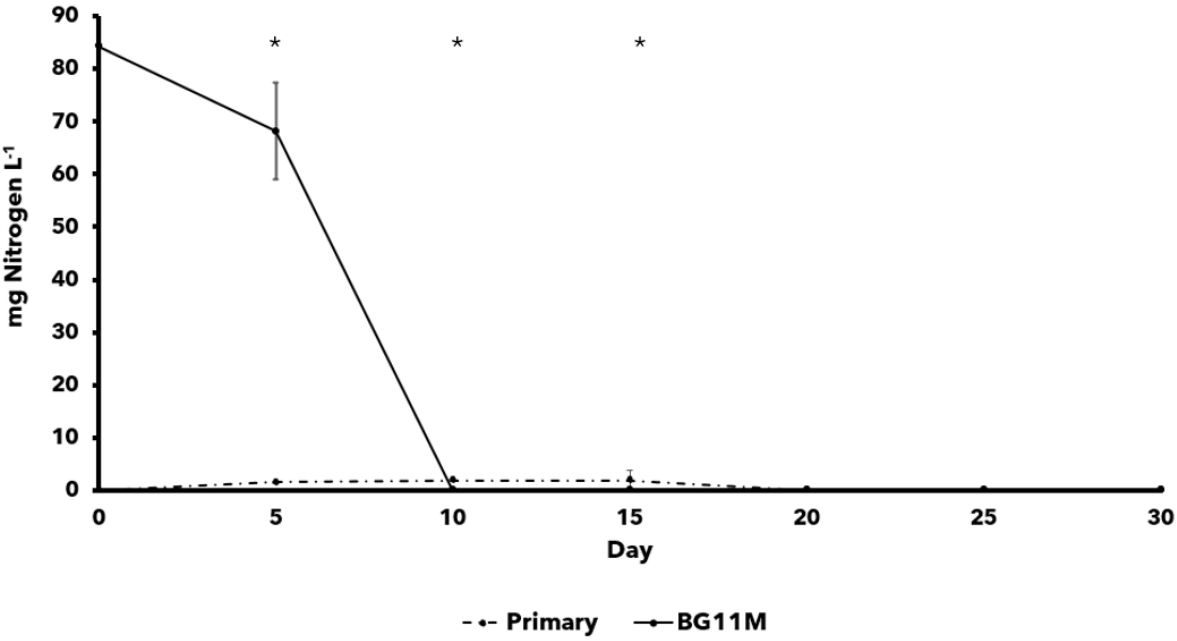


*Figure 4-7: Nitrate of primary wastewater culture and BG11M culture measured every 5 days. * denotes a statistically significant difference ($p<0.05$). All measurements were in triplicate with standard error of the mean shown by error bars.*

The timing of nitrogen limitation on day 10 for both primary (ammonium) and BG11M (nitrate) corresponds with the onset of PHB accumulation (Figure 4-6 and 4-7). This suggests that PHB accumulation accelerated once all nitrogen in the growth medium was depleted. On day 10, the primary wastewater culture became phosphate-deplete, while net phosphate removal in the BG11M cultures stabilised on day 15 (Figure 4-5). In a study that screened 134 PHB-producing strains of cyanobacteria for PHB production under different nutrient limitations (-N, -P, -K, -P & -K and a combination of all 3), the majority of the strains accumulated the most PHB under nitrogen only limitation – including *Synechocystis* sp. PCC6803 (10.7%). Under both nitrogen and phosphorus limitation, the yield was only 4.7% (Kaewbai-ngam, Incharoensakdi & Monshupanee 2016).

While the influence of the type of nitrogen source on PHB production has not been reported in cyanobacteria, there is some literature on the effect of nitrogen source on heterotrophic PHB production. Two methanotrophs, *Methylosinus trichosporium OB3b* and *Methylocystis parvus OBBP,* were grown with nitrate and ammonium as their nitrogen source. It was shown that *Methylosinus trichosporium OB3b* produced more PHB using nitrate than ammonium and *Methylocystis parvus OBBP* produced more PHB using ammonium than nitrate. Ammonium is the preferred nitrogen source for many cyanobacteria as it can be incorporated almost directly into the amino acid production pathways, whereas nitrate must be reduced to nitrite and then ammonia before use (Sanz-Luque et al. 2015). Theoretically, the use of nitrate as a nitrogen source is an electron demanding process and will compete with reducing power used in the biosynthetic pathway of PHB production (Vincenzini et al. 1990). However, the BG11M media with nitrate resulted in higher PHB productivity.

Beyond the tested carbon, nitrogen and phosphorus sources, other differences in chemical composition between the primary wastewater and BG11M, may have influenced PHB

production. For example, another difference is the presence of salt in the BG11M media, which has been shown to influence PHB production in some heterotrophic organisms (Cui et al. 2017; Obruca et al. 2017). However, in a recent study it was shown that PHB conferred no advantage to salinity stress adaption in *Synechocystis* sp. PCC6803 (Koch, Berendzen & Forchhammer 2020).

Conclusion

This study shows that primary domestic wastewater can be used as a source of nutrient-rich media to produce similar amounts of biomass to that achieved in the synthetic growth media. *Synechocystis salina* was able to efficiently utilise all ammonium and phosphorus in the primary wastewater and produced 1.3 g L^{-1} biomass, whereas the control culture grew to 1.6 g L^{-1} of biomass. However, despite the similar biomass accumulation and timing of nitrogen limitation on day 10 of culturing, PHB productivities differed greatly between the primary wastewater culture (0.9%) and control BG11M media (6%). The exact cause for the difference in biomass composition was unknown, but may have been due to differences in salinity, different nitrogen sources, microbial community interactions, or the presence of other, unknown chemical compounds inhibiting PHB production in the primary wastewater compared to BG11M media. Further studies investigating these specific factors (e.g. using a modified control media with similar nitrogen and phosphorous concentrations to the wastewater) would help reveal more about underlying PHB regulation in cyanobacteria. Other further research could involve optimising wastewater through addition of nutrients, two stage cultivation (optimising for biomass in wastewater and then PHB accumulation separately) and testing other species of cyanobacteria (e.g. with nitrogen fixation ability).

Chapter 5 - Techno-Economic Analysis of Cyanobacterial PHB Bioplastic Production

Abstract

Poly-hydroxy-butyrate (PHB) is a promising bioplastic compound which is produced by photosynthetic cyanobacteria. The production of cyanobacterial PHB has the potential to reduce traditional PHB production costs through using atmospheric CO_2 as a carbon substrate, as opposed to using expensive organic carbon substrates needed for heterotrophic PHB production. This techno-economic analysis explores the financial profitability of using open raceway ponds to cultivate the cyanobacteria and solvent extraction on biomass to produce 10,000 tonnes of PHB per year. With a 10% PHB yield used, the capital cost for the plant was $193.5M USD and the operating cost was $147.3M USD per year. At $4,000 per tonne for PHB, the revenue of this plant was only $40M USD per year, resulting in a 20-year Net Present Value (NPV) of -$1.8B USD. The breakeven minimum PHB selling price (for a zero 20-year NPV) was $18,339 USD per tonne. From the sensitivity analysis it was revealed that the key driver behind profitability of cyanobacterial PHB production is PHB yield. Lastly, seven different hypothetical scenarios are explored to increase the economic viability of the cyanobacterial production. These strategies ranged from additional revenue streams from pigment extraction, treatment of wastewater, to decreasing capital costs by using deep 'PHB ripening ponds'. Operational costs were reduced through use of flocculants, solar power purchase agreements and an on-site anaerobic digester to reduce both energy and nutrient costs through digestate use as pond media. The optimistic combination of all these factors resulted in a final minimum PHB selling price of $7,704 USD per tonne; still roughly twice the current market value of PHB.

Introduction

Due to the versatile physical properties of plastics, these materials have become widely used in modern society, with applications in virtually every industry. Unfortunately, the disposal of this

material is causing a growing environmental problem as only 9% of all plastics have been recycled, 12% incinerated and the remaining 79% is either disposed in landfill or has become a form of pollution in terrestrial and aquatic environments (Geyer, Jambeck & Law 2017). In addition to this, petrochemical plastics are derived from finite fossil fuel resources. Bioplastics solve both problems as they can biodegrade into harmless compounds in the environment (Shah et al. 2008) and are made from renewable biomass sources that use atmospheric CO_2 for growth (Rujnić-Sokele & Pilipović 2017).

Unfortunately, bioplastics struggle to compete with petrochemical plastics economically (Ezgi Bezirhan Arikan & Havva Duygu Ozsoy 2015) due to their higher cost of production. Poly-hydroxy-butryate (PHB) is a condensation polyester that is considered as a potential biodegradable substitute for polypropylene (Markl 2018). Current industrial PHB production involves heterotrophic fermentation of an organic carbon substrate, which can contribute to roughly 50% of all production costs (Raza, Abid & Banat 2018). While there is current research on using various waste streams as a substrate for PHB production such as wastewater sludge, whey and agricultural crop residues (Nikodinovic-Runic et al. 2013), these feedstocks are limited in abundance and decentralised in nature (Levett et al. 2016). However, cyanobacteria have the potential to disrupt market this by using atmospheric CO_2 as their carbon substrate for PHB production.

Cyanobacteria are photosynthetic microorganisms that require simple inorganic nutrients for growth including carbon dioxide, nitrogen, phosphorous, some trace metals and micronutrients. In contrast to terrestrial plant crops, which are used to create the organic carbon substrate needed for PHB production, cyanobacteria can be grown in photobioreactors (PBRs) or open raceway ponds (ORPs) without using arable land (Robert A 2005). In addition to this, cyanobacteria do not require freshwater and can be grown in a range of water qualities, including brackish, marine and wastewaters. Unfortunately, the industrial production of cyanobacterial PHB is not yet economically feasible with optimistic cost estimates in the realm of $8,500 - $19,600 USD per tonne and conservative cost estimates as high as $176,000 -

$367,000 USD per tonne (Price et al. 2020); compared to current PHB market prices which are approximately $4,000 USD per tonne (Kosseva & Rusbandi 2018). At best, cyanobacterial PHB is at least twice as expensive and at worst is off by two magnitudes of order. Because of this wide range of production costs, there is a need for more accurate cost estimation of cyanobacterial PHB production.

Panuschka et al. (2019) released the first (and only other published) techno-economic analysis of cyanobacterial PHB production. Their study assumed PHB yields of a conservative 15 % and optimistic 60 % scenario using two different cultivation systems. The first was a TLS (thin layer system) and the second being a tubular PBRs. Two climate scenarios were used to model production at different site locations of southern Europe and central Europe. Alkaline digestion of biomass was used to extract PHB and waste biomass was used for biogas generation with nutrients recycled for cyanobacterial cultivation through the use of digestate to make growth media. In summary, the cheapest cost of PHB resin was $26.4 USD per kg ($26,400 USD per tonne) assuming a 60 % yield using a TLS in southern Europe. However, if yields of 15 % are assumed, the price of PHB increased to $103.5 USD per kg ($103,500 USD per tonne).

This techno-economic analysis study explores the use of different technology choices such as using ORPs for cultivation and solvent extraction for downstream processing of cyanobacterial biomass. In addition to this, several scenarios are investigated in this study to understand their effect on financial viability including the use of:

- solar energy for plant operation to reduce electricity costs
- wastewater as a media for cyanobacterial growth to reduce nutrient cost and provide an additional revenue stream
- deep ‘PHB ripening ponds’ to reduce ORP capital and operating costs
- biogas production to reduce electricity costs and nutrient costs
- pigment production using a biorefinery approach to provide an additional revenue stream
- flocculation to decrease harvesting costs

- CO_2 supply at a cost and carbon credits

Approach, Rationale and Key Assumptions

Summary of Production Process, Plant Scale and Location

Current PHB production facilities using heterotrophic fermentation technology range from capacities of 50 tonnes per year (pilot scale) to up to 50,000 tonnes per year (Metabolix plant in the USA) (Singh et al. 2017). However, the majority of PHB facilities are sized to produce approximately 10,000 tonnes per year (Bio-on, Tianjin and Metabolix) (Levett et al. 2016). Thus, the production scale of 10,000 tonnes per year was also chosen to leverage proven economies of scale for this cyanobacterial PHB production plant. All material flows were then based around this annual production volume. The plant is located in Australia and thus Australian pricings for land, water and electricity are used, but all figures are reported in USD.

A process flow diagram for the hypothetical production plant is shown in Figure 5-1. ORPs are used for cultivation of cyanobacteria and are harvested at a biomass density of 0.5 g dcw (dry cell weight) / L using centrifuges. The algae slurry (~20% solid w/w%) is then dried in a drum-dryer producing an algae powder which is ~80-90% solid w/w%. The powder is then loaded into a solvent extraction vessel (continuous stirred tank reactor), where acetone is added and the mixture heated to lyse cells and dissolve PHB. Following centrifugation to remove the PHB extracted biomass, the acetone-PHB mixture is added to a precipitation vessel where water is introduced, and the mixture cooled resulting in the precipitation of PHB (Levett et al. 2016). Finally, a vacuum filter drum-dryer with scraper is used to separate the PHB resin. This process flow represents the base case scenario for techno-economic analysis, and additional scenarios (such as including the cost of CO_2 and utilisation non PHB cyanobacterial biomass) are assessed, separately.

Equipment Costing Methodology

Pricing for process equipment was obtained from a variety of sources including direct quotation with vendors, searching online marketplaces, supplier catalogues, consultation with subject matter experts and recent literature values from other techno-economic analysis studies.

Where possible, the cost of process equipment matched the capacity required for the hypothetical production plant. In the case this information was not available, a standard scaling equation was applied below (Equation 1) where Cost(A) and Cost(B) are the price of the process equipment at Size(A) and Size(B), respectively. The scaling factor *n* are derived from engineering databases, supplier catalogues or literature. In this case, the majority of process equipment had a scale factor of 0.6 (Fasaei et al. 2018).

Equation 1: $$\frac{Cost(A)}{Cost(B)} = \left(\frac{Size(A)}{Size(B)}\right)^{n}$$

To determine the full capital cost of installation, commissioning and associated work (e.g. miscellaneous piping, mechanical or electrical work) a Lang Factor of 3.5 (common for liquid solid separation plants such as in the milk industry) was applied (Fasaei et al. 2018).

Cultivation

A 10% PHB yield (dcw %) was assumed, as this yield has been demonstrated at pilot scale in multiple studies (Troschl et al. 2018; Troschl, Meixner & Drosg 2017). Thus, for a production volume of 10,000 tonnes of PHB per year, at least 100,000 tonnes of cyanobacterial biomass must be successfully processed. An overall ~85% efficiency for harvesting and downstream processing was assumed, resulting in a harvest flowrate of ~120,000 tonnes per year of dry biomass.

Open raceway ponds were selected as they represented one of the most cost-effective microalgae biomass production systems for low value bioproducts. The cultivation strategy involved two types of ORPs. The first was a biomass accumulation pond which would be kept at optimal nutrient levels and biomass concentration ranges (semi-continuous). A combination of urea and diammonium phosphate was used to provide nitrogen and phosphorous. Ponds would be mixed with paddles wheels and CO_2 bubbled to maintain pH. Periodically, media containing biomass from these biomass accumulation ponds would be pumped to semi-batch PHB accumulation ponds where nutrient levels would not be replenished, allowing for the nutrient starvation environment as required for PHB accumulation. A final harvesting biomass density of 0.5 g / L from the PHB accumulation ponds was assumed (Fasaei et al. 2018) and an areal productivity of 15 g / m^2 / day was used (Rogers et al. 2014) for the biomass accumulation

ponds. A media mixing system and smaller seed ORPs to inoculate new biomass accumulation ponds after culture crashes or complete media refreshes were also costed into the techno-economic analysis model. It was assumed that 80% of CO_2 dosed into the ponds would be absorbed by cyanobacteria (Davis et al. 2016).

Harvesting and Drying

Centrifugation was chosen for harvesting as it is a mature and low risk technology proven in multiple industries that require solid-liquid separation. An energy expenditure of 1.94 kWh / kg dry cyanobacterial biomass was used (from a study using an inlet biomass density of 0.5 g / L and an outlet concentration of 15-20% solids) (Fasaei et al. 2018). For final drying, a drum-dryer was chosen over a spray-dryer due to lower overall costs (considering both CAPEX and OPEX) (Fasaei et al. 2018). This process operation would then dry the biomass to 80-90% solid powder, ready for downstream processing.

Downstream Processing of PHB from Biomass

There are multiple processing technologies that can extract PHB from biomass including acid or alkali digestion of non-PHB biomass, supercritical CO_2 and enzyme assisted digestion. However, solvent extraction seems to be the most mature and widely used technology due to its high efficiency, negligible PHB deterioration as well as allowing solvent recovery through distillation (Kunasundari & Sudesh 2011). While traditional organic solvents such as dichloromethane can be used to extract PHB, acetone was selected as the solvent of choice, being a more environmentally benign alternative (Levett et al. 2016). To separate the PHB content from the dried cyanobacterial biomass, acetone would be added in a 9:1 ratio of acetone to cyanobacteria biomass by mass in continuous stirred tank reactor. The mixture would be heated to 90 degrees Celsius at 2 bar for two hours, with a total batch time of 3.5 hours assumed for loading, cleaning and other downtime. Under these conditions, PHB should solubilise (Levett et al. 2016) and can be separated from residual cyanobacterial biomass through centrifugation. Three water washes of the residual biomass cake are then used to remove any remaining acetone. The acetone-water mixture and acetone-PHB mixture are

combined in a precipitation vessel (another continuous stirred tank reactor) where the temperature is reduced to 40 degrees Celsius and a final ratio of 2:1 acetone to water for 1 hour. Under these conditions, PHB will precipitate and can be filtered using a vacuum filter drum-dryer. A distillation column is also included to recover and separate the acetone and water which can be reused for multiple batches (Levett et al. 2016). A 2% make-up volume per year is assumed to prevent the accumulation and build-up of contaminants such as salts (Levett et al. 2016).

Scenario Analysis

Seven additional hypothetical scenarios, in addition to a combination of all seven scenarios, are considered as deviations from the base case outlined above. These are outlined in Table 1 below.

Table 5-1: Hypothetical scenarios explored for their impact on the base business case.

Scenario No.	Scenario Name	Description and Key Assumptions
1	Solar energy supply	The base model assumed an electricity price of $0.19 USD / kWh (as per current industrial electricity market prices); however, if a solar power purchase agreement is executed, the cost of electricity can be reduced to $0.04 USD / kWh according to forecasted renewable energy prices (Graham et al. 2020).
2	Wastewater treatment	The base model assumed all nutrients are bought at a cost. This scenario assumes that ~17% reduction in nutrient costs can be avoided through the use of wastewater centrate (Xin et al. 2016). In addition to this, a wastewater treatment revenue is also assumed at a rate of 80% of a municipal wastewater treatment cost of $0.095 USD / kL (Xin et al. 2016) of wastewater. Media is assumed to be 50% wastewater by volume in this scenario.

3	Deep PHB Ripening Ponds	After biomass accumulation in balanced nutrient conditions, the PHB accumulation process uses intracellular glycogen for its metabolic production. During this stage, there is little to no photosynthesis and it has been proposed that this final phase can be completed in dark ripening tanks (Troschl et al. 2018). Thus, deep PHB accumulation ponds (double depth) are assumed in this scenario to reduce the land required and both capital and total operating costs of open raceway ponds.
4	Biorefinery (pigments)	In the base case, the only revenue stream comes from the sale of PHB resin; however, in this scenario, pigments are also produced. While nutrient (in particular nitrogen) starvation results in a reduction in pigment content of cyanobacteria, the addition of nitrogen to chlorotic cyanobacterial cells triggers pigment recovery within 24 hours. During this window, studies have shown the PHB is not degraded during this window (Klotz et al. 2016). An approximate 5 mg / g (or 0.5% dcw) pigment yield (chlorophyll, carotenoids and phycobiliproteins) is assumed (Meixner et al. 2018). Additional pigment downstream processing costs are also incorporated into this scenario.
5	Flocculation	In the base case, centrifuge of biomass at 0.5 g / L is carried out for the dewatering step. However, flocculation has the ability to concentrate biomass to significantly reduce both harvesting capital and operating costs. In a study involving PHB producing cyanobacteria, a cost effective polyacrylamide polymer (at a cost of $0.03 USD / kL treated) successfully concentrated biomass with the recycled dosed media showing no negative impact on further cultivation (Labeeuw et al. 2021). In this scenario, the flocculant is an additional operating expense, and a sedimentation pond is

		also costed; however, the harvesting costs are reduced as a harvest density of 10 g / L is assumed.
6	Anaerobic Digestion	In the base case, the remaining 90% of non-PHB biomass is assumed to be discarded at no additional cost. In this scenario, this residual biomass is utilised in an anaerobic digestor to produce electricity for use on-site. The literature value used for the biomethane yield of PHB extracted cyanobacterial biomass was 348 m^3 methane / tonne VS (volatile solids) (Meixner et al. 2018). In addition to this, digestate has been proven as an effective media for cyanobacterial PHB production (without any further nutrient addition) (Meixner et al. 2016). However, in this scenario a 75% reduction in nutrient cost is applied to allow for media optimisation.
7	CO_2 at Cost and Carbon Credits (applied with anaerobic digestion)	In the base case, CO_2 is supplied at cost neutrality under the assumption it would be supplied by an external party aiming to reduce their emissions at their cost. In this scenario, all CO_2 transport and processing required for use in ORPs is costed. CO_2 from anaerobic digestion co-generation plant is also assumed to feed the ORPs. Finally, a carbon credit pricing of $13.5 USD / tonne CO_2 abated is assumed for the anaerobic digestion electricity generated and carbon in PHB resin produced.
8	Combination of all above scenarios	An ideal scenario that includes the success of all the above factors by combining renewable electricity price, wastewater treatment, deep PHB ripening ponds, pigment production, flocculation, anaerobic digestion and carbon credits.

Financial Parameters

A 20-year Net Present Value (NPV) is used to assess the economic viability of the plant when selling PHB at current PHB market prices of $4,000 USD / ton. Breakeven selling prices of PHB resin are based on a zero 20-year NPV. A sensitivity analysis is also carried out to determine the key economic drivers affecting the financial viability of the project. The plant was assumed to be financed completely by debt (no equity) at an interest rate of 8%. A discount rate of 10% was used for the discounted cash flow analysis and a corporate tax of 25% (current in Australia as of 2021) is assumed for years where the project makes a profit. Construction and commissioning was assumed to take two years with full scale production and revenue generation occurring in the third year. All monetary values reported in this paper are in USD.

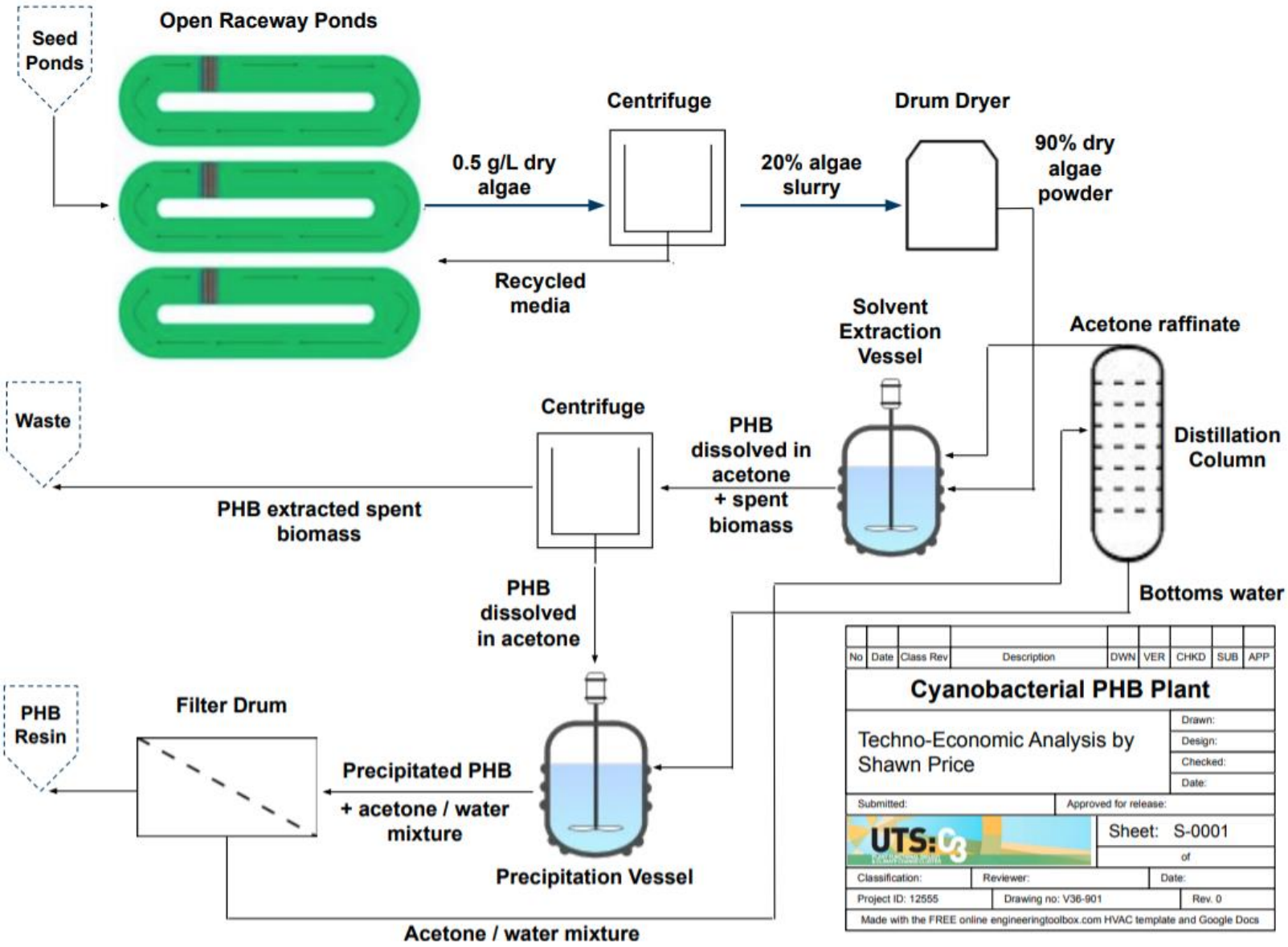


Figure 5-1: Process flow diagram for cyanobacterial PHB production plant.

Results and Discussion

The total capital cost for the plant was $193.5M USD (see Figure 5-2) with the majority of this being from cultivation. The ORP capital cost alone was $136.3M USD making up over 70% of total capital costs, with the second highest item being $25.0M USD in indirect costs (including project management, engineering design, development approval etc). The third and fourth most expensive items were land purchase at $12.8M USD (6.6% of capital cost) and centrifuge system for ORP pond harvesting at $10.4M USD (5.4% of capital cost). A breakdown of all capital cost items can be found in Appendix A. Other techno-economic analysis studies on production plants for manufacturing algae bioproducts such as astaxanthin, biofuels and bioplastics also identified the cost of cultivation systems to make up the majority of the total capital cost (Panis & Carreon 2016; Panuschka et al. 2019; Xin et al. 2016), which aligns with the results of this study. Dewatering and drying accounts for 6.6% of total capital costs (at $12.8M USD) and downstream processing accounts for only 2.5% of total capital costs (at $5.0M USD).

The model predicts that 69,960 ML of water will be used each year from a combination of media refreshes to prevent build-up of contamination (four times a year following Sapphire Energy's best operating practices), replacement of water lost to evaporation and the requirement of fresh media after culture crashes. Of these three uses of water, over 98% of the water demand is attributed to the media refreshes once in every three months.

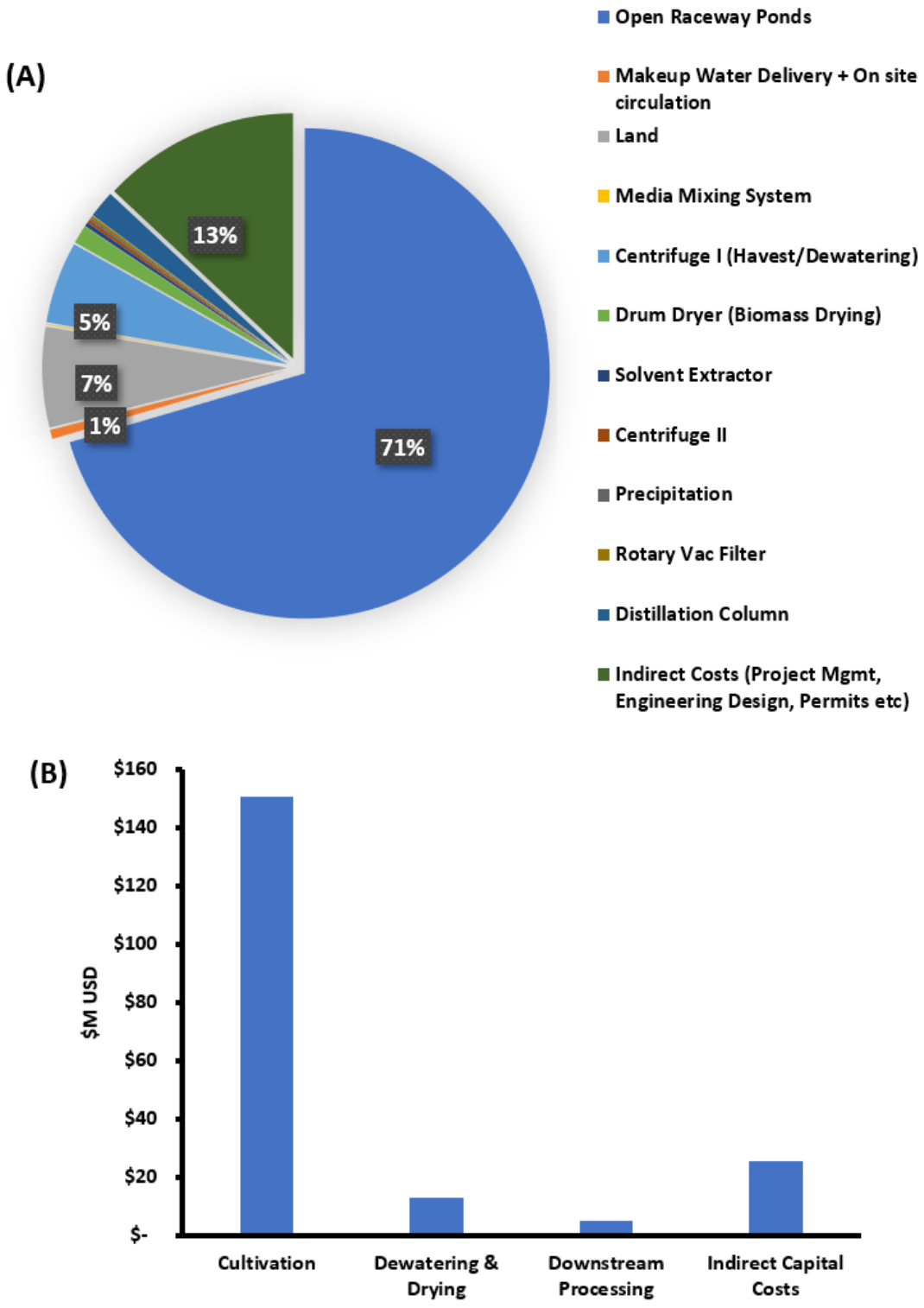
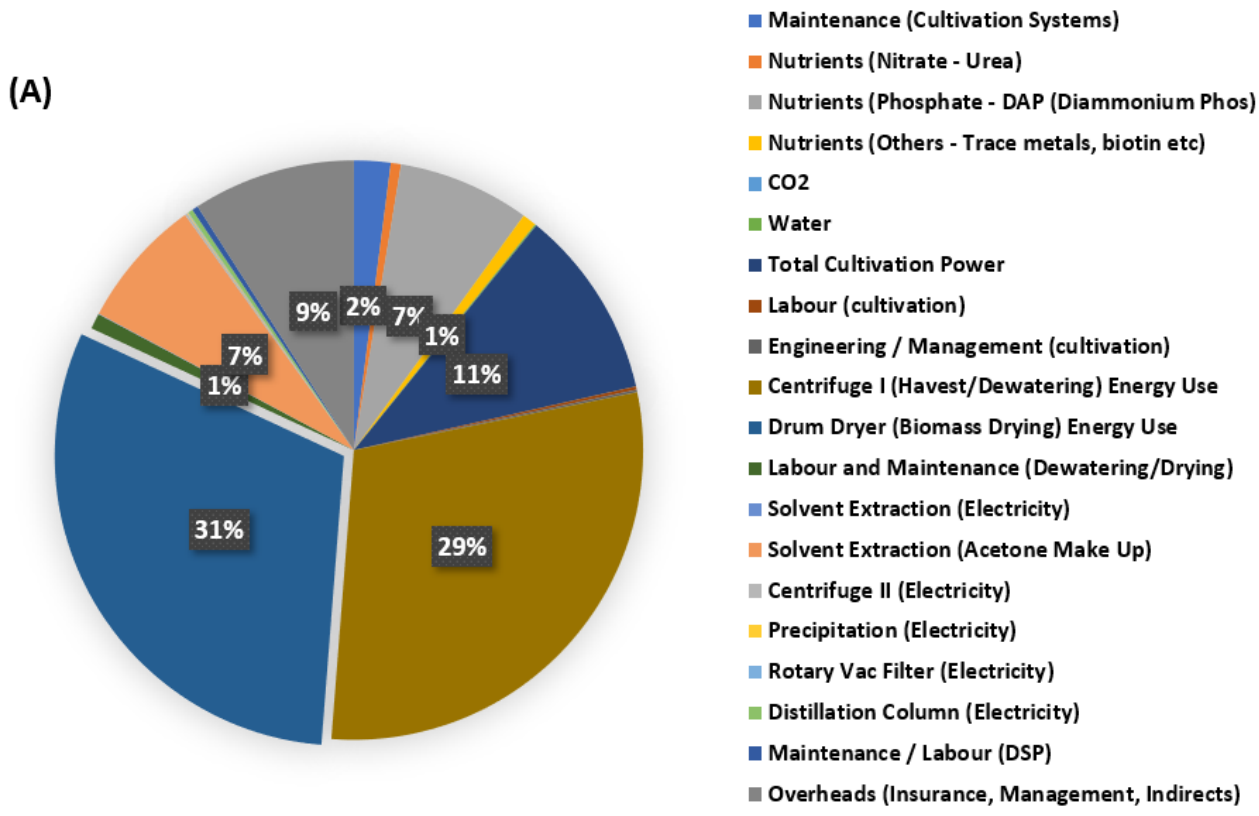


Figure 5-2: Capital cost breakdown (total $193.5M USD), (A) by individual items and (B) by production section.

The total annual operating costs for the plant were $147.3M USD per year (see Figure 5-3 below) with most of this cost attributed to dewatering and drying. The energy costs for

dewatering and drying accounted for $88.5M USD per year, consisting of 60% of the total operating cost alone. The process of separating cyanobacterial biomass from extremely dilute pond media (0.5 g / L equivalent to 0.05% solid) and creating a biomass powder of over 80%+ solid for solvent extraction is extremely energy intensive. The second highest operating costs come from the power required to mix the ORPs using paddlewheels and pump pond media which accounts for $15.6M USD per year. The next highest operating cost, which also comes under cultivation, is $12.9M in nutrients required for growth of the cyanobacteria. Lastly, the most significant downstream processing cost comes from the acetone make up which amounts to $10.7M USD per year. A breakdown of all line operating cost items can be found in Appendix B.



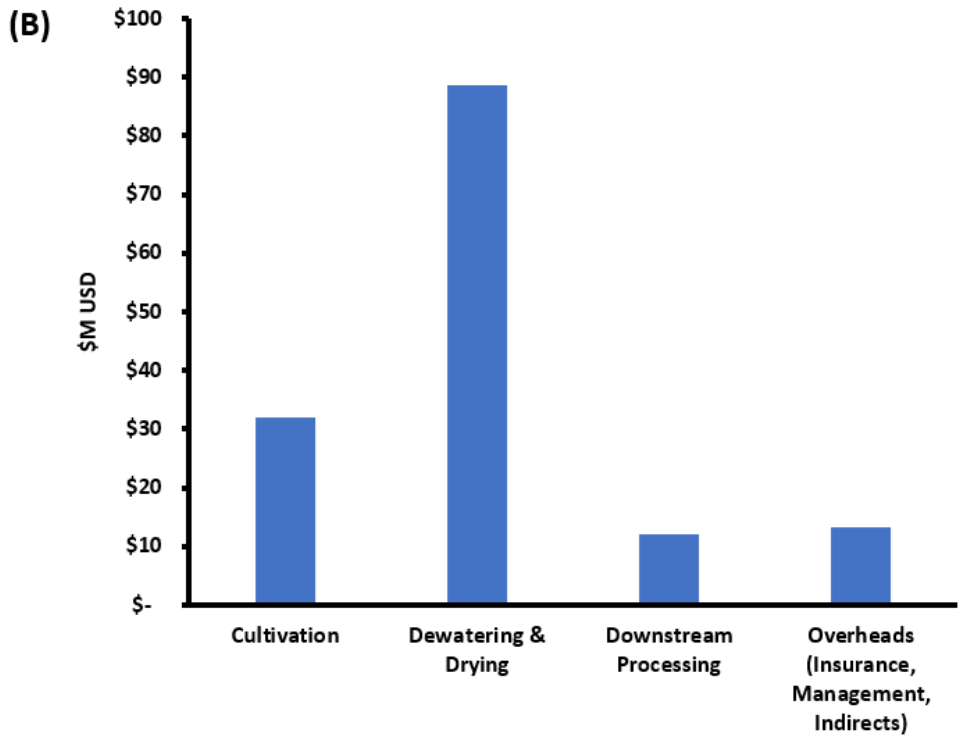


Figure 5-3: Annual operating cost breakdown (total 147.3M USD per annum), (A) by individual items and (B) by production section.

The overall metric used for assessing the economics of the project was a 20-year NPV. For the base case scenario considered a plant selling 10,000 tons of cyanobacterial PHB priced at $4,000 USD / ton, the 20-year NPV was -$1,842,451,195 USD (-$1.8B USD). One solution to achieving profitability for this hypothetical plant is increasing the selling price until a desirable NPV is achieved. Figure 5-4 below shows that the breakeven sell price for cyanobacterial PHB resin, which results in a zero 20-year NPV, is $18,399 USD / ton. This breakeven price is over four times as expensive as the current average market price for PHB resin. This result is significantly cheaper than the results obtained in Panuschka et al's (2019) study, where the cost of production alone were $111,080 – $356,860 USD per tonne assuming a 15% PHB yield. It should be noted that the costing for this system was for a plant capacity of only 39.9 tons per year which is over 250 times lower than the 10,000 tons per year capacity in this study. This undoubtedly affects the economies of scale and increases both the capital and operating costs of production per unit.

Figure 5-4: B*reakeven analysis to determine minimum PHB sell price of $18,399 USD / tonne for a zero 20-year Net Present Value (NPV).*

A sensitivity analysis was carried out to determine the strongest economic factors behind the profitability of cyanobacterial PHB production (see Figure 5-5 below). The capital cost and operating cost of cultivation, dewatering / drying and downstream processing (DSP) were increased and decreased by 30% to observe the effect on the minimum PHB selling price (for a zero 20-year NPV). By far the most important parameter behind profitability of cyanobacterial PHB production is the PHB yield with a 7% PHB yield (30% decrease in PHB yield) increasing the minimum PHB selling price to $26,172 USD per tonne and a 13% PHB yield (30% increase in PHB yield) decreasing the minimum PHB selling price to $14,206 USD per tonne. PHB yield is the key driving factor behind the financial performance of this plant as it impacts the costs of basically all unit operations. With a higher PHB yield, less ORPs ponds are required as less biomass needs to be grown to obtain the same amount of PHB. Additionally, less biomass means less energy required to dewater and dry the biomass. All downstream processing equipment will also have lower capital and operating costs as they can be sized smaller to handle less non-PHB biomass. Hence, the economics of this plant is far more sensitive to PHB yield than to biomass productivity, as biomass productivity would only reduce the capital cost and operating cost of cultivation.

The cyanobacterial PHB economics is also sensitive to the dewatering / drying operating cost and cultivation capital cost, whereas downstream processing has little effect on the minimum PHB selling price. Because the dewater / drying operating cost is largely made up of electricity costs to operate the pond harvesting centrifuges and biomass drying tunnel, electricity price was also included in the sensitivity analysis. A 30% increase in electricity cost resulted in a minimum biomass sell price of $20,354 USD per tonne, while a 30% decrease in electricity cost resulted in a minimum biomass sell price of $16,444 USD per tonne.

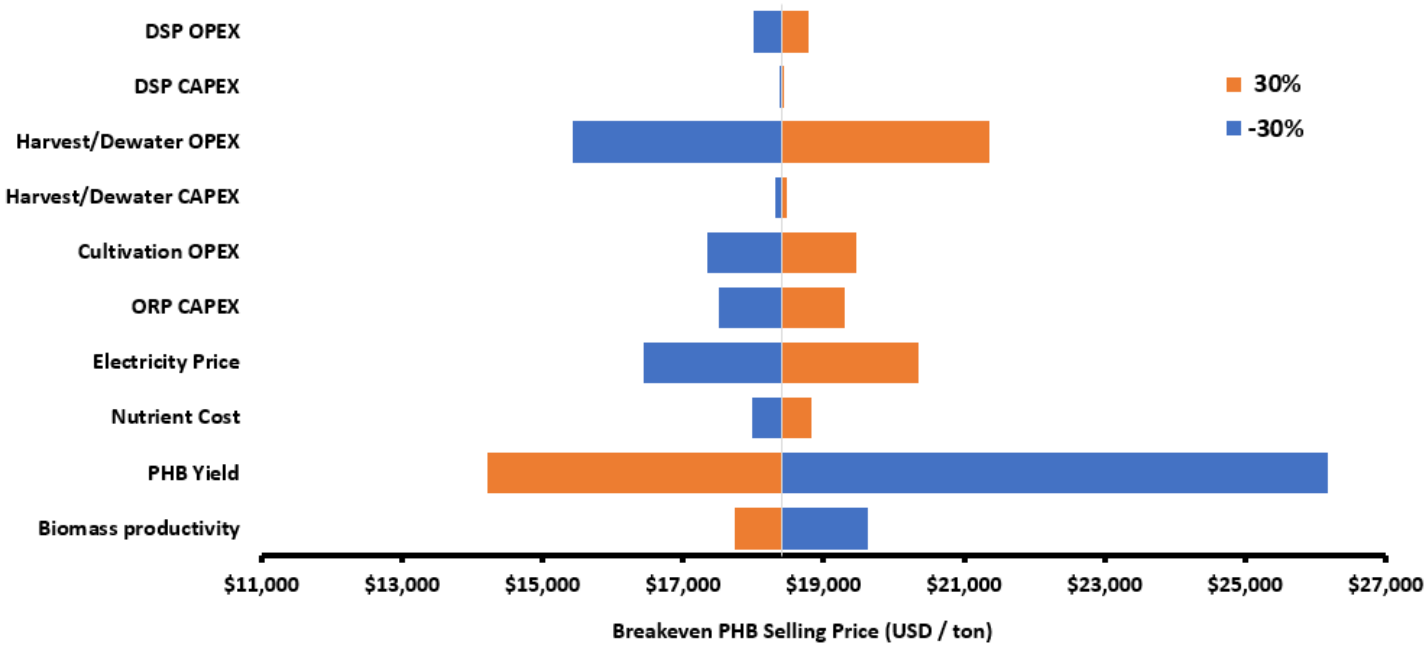


Figure 5-5: *Sensitive analysis on factors affecting breakeven PHB sell price*.

The effect of varying PHB yield from 5 - 50% on the minimum PHB selling price can be seen in Figure 5-6 below. At 5% PHB, the minimum selling price for PHB is $36,518 per ton, whereas a PHB yield of 20% (double that of the base case) results in a minimum selling price of $9,305 per tonne. The curve is extremely sharp towards the side of smaller PHB yields, as incremental jumps in PHB yield result in large reduction in capital and operating costs for all stages of the process (e.g. going from 2.5% to 5% PHB yield is a doubling which results in

halving many costs). Conversely, the curve approaches a point of diminishing returns because at high PHB yields, further increases in PHB yield don't decrease capital and operating costs as much. Eventually, an asymptote is reached where even if the biomass is 100% PHB, there are unavoidable operating and capital expenses which cannot be reduced or avoided. To achieve a breakeven selling price of $4,000 per tonne (current PHB market price), a PHB yield of ~47% is required. Cyanobacterial phototrophic PHB yields of up to 15% at pilot scale (200 L tubular PBR) have been demonstrated (Troschl et al. 2018); however some wild type cyanobacterial species have the ability to produce up to 25% PHB in laboratory conditions (Kaewbai-ngam, Incharoensakdi & Monshupanee 2016). While a mutant cyanobacterial strain has achieved 37% PHB yield and a genetically modified strain achieving 63% PHB yield, these strains are yet to be proven at scale and represent optimistic scenarios which are most likely currently unachievable at industrial scale.

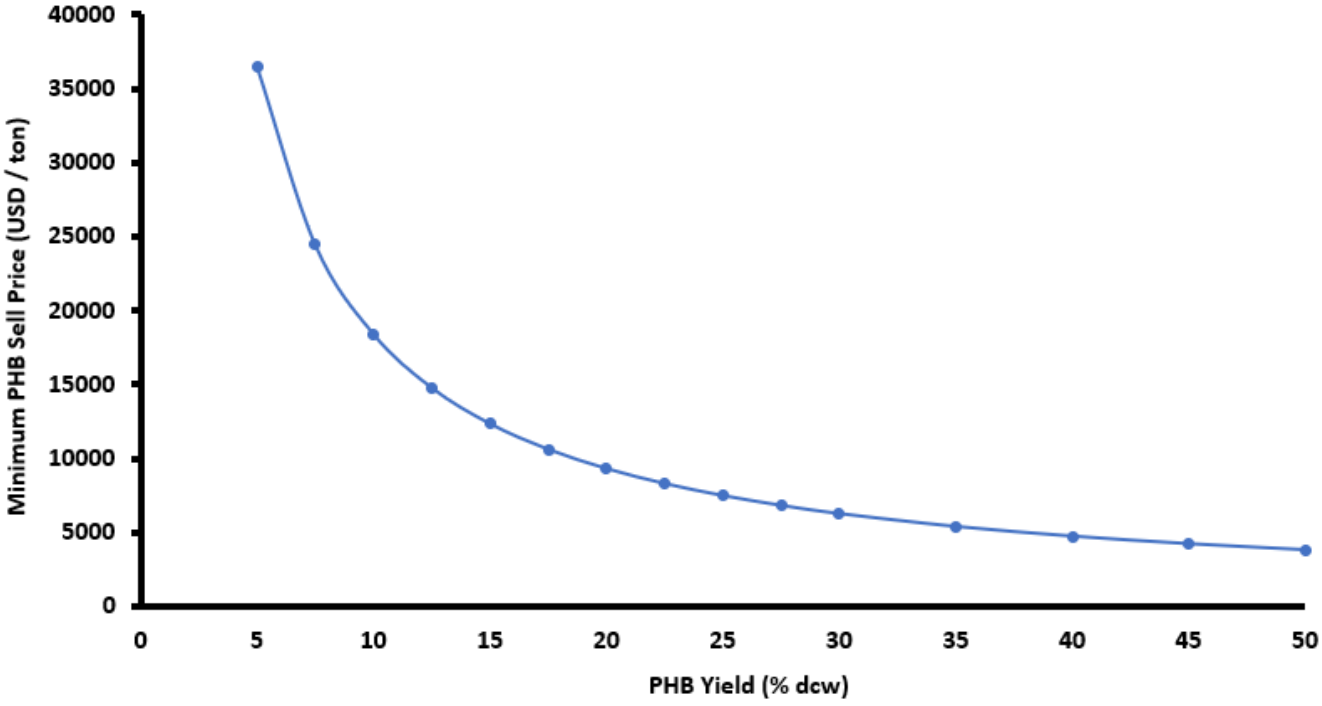


Figure 5-6: Effect of PHB yield on minimum PHB sell price.

Increasing the selling price of PHB resin and increasing PHB yield are not the only paths to economic viability. An additional seven scenarios were considered to analyse the impact on economic viability of cyanobacterial production. In these scenarios, reducing operating costs through renewable energy power purchase agreements, using nutrients from wastewater

and digestate and using flocculation for harvesting are explored. The addition of new revenue streams such as wastewater treatment and sale of pigments and carbon credits are also investigated as potential paths to economic viability. An overview of the seven scenarios and their key assumptions can be found in Table 1.

Scenario 1 assumed a reduction in electricity price from renewable solar energy and achieves the lowest minimum biomass selling price of all individual scenarios at $13,254 USD per tonne. This is not surprising as the sensitivity analysis revealed that electricity price is a key driver of the financials of this project. In total, 556 GWh are used in the base case and this scenario (with 83% of electricity use coming from dewatering and drying). With an electricity cost of $0.19 USD per kWh for the base scenario, total electricity costs amount to $105.6M USD per year of the operating costs (total operating cost $147M USD per year). A reduction in electricity price achieved through solar energy to $0.04 USD per kWh (Graham et al. 2020)reduces electricity costs to $22.2M USD per year with an annual savings of $83.4M USD per year.

In scenario 2, a wastewater treatment revenue of $0.095 USD per kL treated and a total of 115,891 ML of wastewater is treated each year with the assumption that media is mixed in a 1:1 ratio with wastewater and topped up with additional nutrients where required. This results in modest additional revenue of $8.8M USD per year (compared to $40.0M USD per year revenue for $4,000 USD per tonne and 10,000 tonnes PHB per year) and nutrients costs decreased from $12.9M USD per year in the base case to $10.6M USD per year in this scenario. As a result, the minimum PHB selling price decreased to $17,268 USD per tonne.

Scenario 3 utilises deep ripening ponds which hold a greater volume of media and therefore cyanobacterial biomass. Although this results in lower light distribution across the culture, the PHB ripening phase occurs with minimal to no photosynthesis (Troschl et al. 2018). The biomass accumulation ponds which are not nutrient starved are of standard depth as these cells are actively photosynthesising and require efficient light distribution. In this scenario, the ORP capital cost drops from the base case $136.3M USD to $105.2M USD and the minimum PHB selling price decreased to $17, 138 USD per tonne.

In Scenario 4, pigments were introduced as a co-product to bring in an additional revenue stream. At a 0.5% recoverable pigment yield, the production volume was 588 tons per year. This resulted in a revenue of $58.8M USD per year (assuming a conservative average sell price of $100 USD per kg (Alibaba, 2021)). This additional revenue is more than the $40.0M

USD base revenue from PHB resin sold at market price. The additional downstream processing costs increase both the capital and operating costs by roughly double, but as revealed earlier by the sensitivity analysis, the breakeven minimum PHB selling price is not sensitive to downstream processing capital or operating cost changes. Thus, the more than doubling in revenue results in a decreased minimum PHB selling price of $13,945 USD per tonne.

The use of a polyamide flocculant was studied in scenario 5. This flocculant increased the harvest density from 0.5 g / L to 10 g / L which resulted in significant capital and operating cost savings. The base case capital cost for the pond harvesting centrifuges was originally $10.4M USD which was reduced to $1.7M USD in this scenario. The centrifuge operating baseline cost was originally $43.3M USD which was reduced to $2.2M USD per year. These capital and operating cost savings more than compensated for the additional $7.0M USD in flocculant cost per year and $1.2M USD for the flocculation tank.

Scenario 6 incorporates an anaerobic digestion plant which uses the other 90% non-PHB cyanobacterial biomass to produce biogas and digestate. The biogas is fired in a co-generation plant to produce heat energy (used for drying) and electricity (used for all processes) and the digestate is used for media preparation to recycle nutrients. The capital cost for the anaerobic digestor was $35.2M USD, but due to the reduction in nutrient costs ($9.6M reduction per year) and electricity produced (84.5 GWh per year valued at $16M USD per year at $0.19 USD per kWh), the minimum PHB selling price was reduced to $16,458 USD per tonne.

Scenario 7 incorporated the anaerobic digestion system in scenario 6 in addition to the cost to supply CO_2 to the ORPs and carbon credits from the operating. The CO_2 from the anaerobic digestor was used on-site to offset the CO_2 required from external sources. In this scenario, the costs of purifying, compressing and transportation costs for the CO_2 amounted to an additional operating cost of $20.3M USD per year. The carbon credit revenue was only $1.1M USD per year, of which over 95% came from the anaerobic digestion carbon abatement through using biogas and the remaining carbon credits were assumed to be from the CO_2 in the 10,000 tons of PHB per year. In this scenario, the minimum biomass selling price increased to $18,538 USD per tonne above the base case.

Finally, all seven scenarios were combined resulting in the lowest minimum biomass selling price obtained so far, at $7,704 USD per tonne. While some of these hypothetical scenarios are closer to industrial realisation than others, it is promising to see how these potential pathways to bringing down the cost of cyanobacterial PHB. An overview of all the scenario breakeven PHB selling prices are shown in Figure 5-7 below, compared to the base case. More detail on the justifications for assumptions used in the scenario modelling can be found in Table 1.

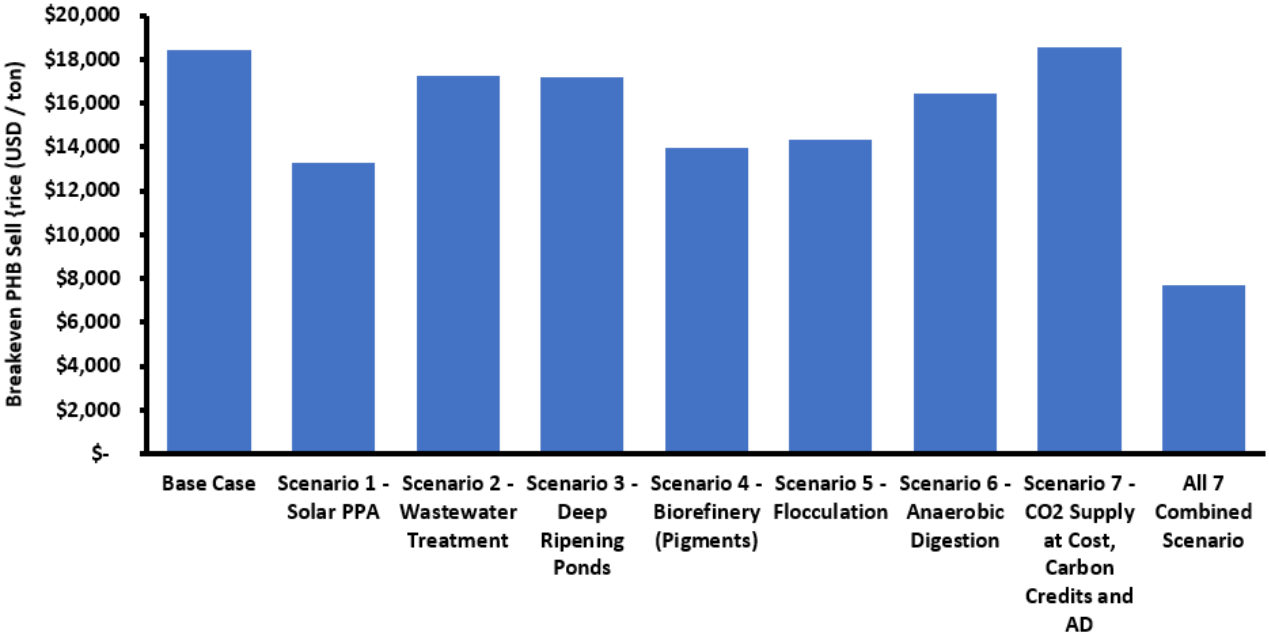


Figure 5-7: Breakeven PHB sell price of different scenarios explored.

Conclusion

Cyanobacterial PHB offers a promising pathway towards the production of cheaper bioplastic through using atmospheric CO_2 as a direct metabolic substrate. However, there are still significant technical and economic barriers to be solved before production can be economically viable. This study took the current state of technology to explore the profitability of a facility with a production capacity of 10,000 tons of cyanobacterial PHB per year. The 20-year NPV was found to be -$1.8B USD if the PHB is sold at current market prices, and a minimum PHB selling price of $18,339 USD per tonne to breakeven (zero 20-year NPV), which is over four times the current market price. Seven different scenarios were

then explored to determine pathways to financial viability including additional revenue streams (pigment production, wastewater treatment and carbon credits), reducing capital costs (deep PHB ripening ponds) and reducing operational costs (anaerobic digestion for nutrients and on-site electricity, flocculation and a renewable energy power purchase agreement). The optimistic combination of all scenario's led to a final biomass selling price of $7,704 USD per tonne.

Synthesis

Summary of cyanobacterial PHB

Cyanobacterial PHB has the potential to be an environmentally and economically superior option for producing PHB as opposed to heterotrophic production. This is because cyanobacterial PHB cultivation uses sunlight and atmospheric CO_2 as a substrate as opposed to an expensive organic carbon source which requires terrestrial crop production (Troschl, Meixner & Drosg 2017). However, the high capital and operating costs of cyanobacterial PHB cultivation hold back the commercially viable industrial deployment of this technology (see Chapter 4 on Techno Economic Modelling). By increasing the PHB productivity (yield of PHB per cell dry weight and increased biomass productivity), both the capital and operating costs can be reduced. Another potential way to increase profitability of cyanobacterial cultivation for PHB production is using wastewater as a media to provide cheap (or potentially cost free) nutrients.

Thus, with this context, this thesis aimed to convert cyanobacteria into a more economic production platform for PHB through: (i) increasing the PHB productivity of cyanobacteria through the creation of novel mutant strains (Chapter 2), (ii) identifying chemical compounds capable of modulating cyanobacterial PHB productivity (Chapter 3), (iii) assessing the viability of using wastewater as a media and cheap nutrient source (Chapter 4) and (iv) determining the current economic viability of a cyanobacterial PHB production facility with current state of technology and scenario analysis to understand the effect of potential pathways to profitability (Chapter 5).

Thesis outcomes, reflections and next steps

Mutagenesis (Chapter 2) can be used to achieve moderate increases in PHB yields

Mutant libraries were created using the chemical mutagen EMS at different concentrations (0.5 – 4M). A novel screening technique was utilised to individually screen cells based on BODIPY 493/593 (lipid dye) fluorescence with fluorescent activated cell sorting (FACS). After screening the surviving mutant strains for growth in well plates, the fastest growing mutants were selected for flask cultivation and subsequent PHB quantification. Two mutant strains

were created which produced significantly higher PHB yields compared to the wild type (29% and 26% increase relative to wild type). The maximum PHB yield achieved was 12% compared to the wild type 8.3% (43% increase in yield).

To continue this work, several areas should be explored including:

- Mutation stability - the PHB enhanced mutants should be tested for the stability of their mutations over time. The ability to retain their superior PHB production phenotype must be confirmed for any mutant if they are to be used for commercial production.
- Repeated generations of mutagenesis – while a modes incremental gain of PHB productivity was achieved in just one generation, there is potential to produce extremely high yield PHB strains if subsequent rounds of mutagenesis is then carried out on best performing mutants strains.
- Omics interrogation – the gene regulation and expression of the novel mutants should be interrogated via genomics, transcriptomics and/or proteomics to understand the molecular mechanism driving the enhanced PHB productivities. This will reveal targets for genetic engineering and the potential to combine mutations from different PHB-enhanced mutants.

Chemical elicitors (Chapter 3) can be used to achieve moderate increases in PHB yields

Ten different chemical compounds (including oxidants, antioxidants, phytohormones and others) at 0.1-10 µM were screened for their ability to both elicit and inhibit cyanobacterial PHB production. Two phytohormones (IAA and methyl jasmonate) were identified to increase PHB productivity in cyanobacteria, while two compounds (allopurinol and ethynylestradiol) were found to decrease PHB productivity in cyanobacteria. Similar to the mutagenesis efforts in Chapter 2, only incremental PHB gains were achieved by the elicitors (maximum of 8.8% and 8.3% PHB yield by IAA and methyl jasmonate respectively, compared to 7.1% PHB yield from the control).

To continue this work, several areas should be explored including:

- Omics interrogation – similar to understanding the enhanced PHB phenotype effect in mutagenesis, the gene regulation and expression of the PHB eliciting and inhibiting chemical treatments should be interrogated via genomics, transcriptomics and/or proteomics to understand the molecular mechanisms driving the enhanced and supressed PHB productivities. This will reveal targets for genetic engineering and the potential to combine mutations from different PHB enhanced mutants.
- Combining mechanisms - there is potential to combine the genetic and regulatory mechanisms of action from both the chemical compounds (Chapter 3) and mutagenesis (Chapter 2) to further enhance the PHB production phenotype.

Wastewater (Chapter 4) can be used as a nutrient source for cyanobacterial biomass, but not for PHB production

Domestic primary wastewater was shown to be a viable media and nutrient source to produce similar biomass quantities compared to synthetic media. While this is promising for producing cyanobacterial biomass for other applications, the PHB yields were significantly lower (0.9% in primary wastewater compared to 6% in the control BG11M media). There are several key differences between the BG11M media and primary wastewater that could be responsible for the lower PHB yield observed, including: the presence of bacteria in wastewater, difference in dominant nitrogen source (nitrate in BG11M and ammonia in wastewater) as well as different levels of other nutrients (such as 50% less phosphate and untested trace metals). Despite not identifying the exact cause of reduced PHB, the cyanobacteria were able to remove all nitrogen and phosphate from the primary wastewater, demonstrating their ability for effective nutrient removal.

To continue this work, several areas should be explored including:

- Identifying the mechanisms causing reduced PHB accumulation – similar to understanding the decreased PHB accumulation in the chemical inhibitors in Chapter 3, a factor (or combination of factors) in the wastewater was responsible for reducing PHB production. However, while the majority of PHB inhibitors in Chapter 3 also significantly reduced biomass productivity (with PHB yields declining probably due to an overall lack of cellular fitness and uptake of carbon), the biomass

productivity was not significantly reduced when grown on wastewater. This suggests a different mechanism of action reducing PHB, which should be explored. Potential mechanisms could be:

    - *Specific PHB enzyme inhibition* causing less Acetyl-CoA to enter the PHB synthesis pathway. Acetyl-CoA can still be used for other metabolic purposes and hence growth unaffected.
    - *Increased requirement of fatty acid biosynthesis* which could reduce PHB levels. Fatty acid biosynthesis pathways directly compete for Acetyl-CoA and hence upregulation of enzymes related to these pathway would decrease PHB cellular content.
    - *Increased energetic demand* of the cells could also explain the observed PHB yield as Acetyl-CoA may be entering other pathways such as the tricarboxylic acid cycle.

- Testing other wastewater as a media for cyanobacterial PHB production – while this particular primary wastewater source resulted in PHB inhibition, other sources of wastewater (such as from food production or agricultural runoff) may make for non-PHB inhibiting nutrient sources.
- Combination with chemical elicitors and mutagenesis – the increased cyanobacterial biomass productivities from phytohormone treatment or from mutagenesis could be applied to remove larger quantities of nitrogen and phosphorous from wastewater.

Techno Economic Analysis (Chapter 5) revealed that cyanobacterial PHB is over four times as expensive as heterotrophic PHB

From detailed financial modelling of a production facility producing 10,000 tonnes per year of PHB resin from cyanobacteria, the breakeven price (for a zero-dollar 20 year NPV) was $18.3k USD per tonne assuming a 10% PHB yield. Compared to the average market price of $4k USD per tonne, this represents a greater than fourfold higher cost per tonne. Through scenario modelling to increase revenue and reduce costs (including solar energy PPAs, wastewater treatment revenue, deep ripening ponds, pigment co-production, flocculation and onsite anaerobic digestion) it was revealed at an optimistic PHB production cost of $7.7k USD per tonne could be achieved.

From the cost curve of PHB resin (Chapter 5) the effect of mutagenesis (Chapter 2) and chemical elicitors (Chapter 3) can be estimated into the costs of production. PHB yields of ~10% have been demonstrated at pilot scale using cyanobacteria under phototrophic conditions grown in a tubular PBR (Troschl et al. 2018). If a modest boost in the range of 25-50% were to be achieved from mutagenesis and chemical elicitation efforts, this would put the final PHB yield at around 15%. According to the cost curve in Figure 1, this would result in a minimum PHB selling price of ~$12.5k USD (disregarding the cost of PHB eliciting phytohormones) which is still over three times as expensive as current market prices. While the use of mutants is cost negligible, the use of phytohormones to dose industrial sized raceway ponds is unlikely to be economically viable (for example, 25mL of methyl jasmonate costs ~200 USD (Sigma Aldrich)). Thus, even with mutagenesis and chemical elicitation efforts, industrial cultivation is still not yet viable.

While wastewater treatment and nutrient cost offsetting was accounted for as a scenario in this chapter, the experimental data from using primary wastewater (Chapter 4) as a media source resulted in extreme PHB inhibition. This was not accounted for in the financial modelling and thus this scenario is currently further away from realisation than the other scenario, unless an alternative wastewater nutrient source is proven to not reduce PHB yields.

Final perspectives on the future commercial viability of cyanobacterial PHB

Cyanobacterial PHB production is still far from being cost competitive with heterotrophic PHB production. While increased PHB yields are key to reducing capital costs (less pond area required for the same amount of PHB) and operating costs (less biomass to be harvested and dried for the same amount of PHB) – increasing PHB yields alone will not lead to commercial viability. From closer examination of Figure 1, the declining minimum selling price for PHB approaches an asymptote as there are unavoidable costs even if the cyanobacterial cells were >99% PHB (such as minimum raceway pond and harvesting costs). The asymptotic value is still around 4k USD per tonne which is more than the current market value for PHB currently. For comparison, PHB yields of >20% have been achieved in some wild type cyanobacterial strains (Kaewbai-ngam, Incharoensakdi & Monshupanee

2016), greater than 30% in UV cyanobacteria mutants (Kamravamanesh et al. 2018) and greater than 60% in genetically engineered strains although these remain unproven in industrial scale cultivation (i.e. in an outdoor raceway pond without the resuspension in nutrient deplete media).

While the combined scenario modelling (Chapter 4) resulted in a minimum selling price of $7.7k USD per tonne, this assumed a 10% PHB yield. If the scenarios were realised along with a greater PHB yield, there is potential for cyanobacterial PHB production to reach <$4k USD per tonne and be cheaper than heterotrophic PHB. Consequently, the best paths to commercial viability are those that not only increase the PHB yield, but also introduce new revenue streams (e.g. biorefinery production of co products such as biogas and pigments, or wastewater treatment revenue) or reduce costs in other ways (such as deep PHB ripening ponds or flocculation).

Appendix

Chapter 5 Appendix

Appendix 5A – Capital cost breakdown for base case scenario.

Item	Value (USD)	Cost Allocation
Open Raceway Ponds	$ 136,344,161.55	Cultivation
Makeup Water Delivery + On site circulation	$ 1,184,664.91	Cultivation
Land	$ 12,794,381.04	Cultivation
Media Mixing System	$ 106,200.00	Cultivation
Centrifuge I (Harvest/Dewatering)	$ 10,439,613.00	Dewatering & Drying
Drum Dryer (Biomass Drying)	$ 2,355,651.84	Dewatering & Drying
Solvent Extractor	$ 546,368.89	Downstream Processing
Centrifuge II	$ 398,173.83	Downstream Processing
Precipitation	$ 267,300.00	Downstream Processing
Rotary Vac Filter	$ 337,689.55	Downstream Processing
Distillation Column	$ 3,480,487.15	Downstream Processing
Indirect Costs (Project Management, Engineering Design, Permits…etc)	$ 25,238,203.76	Overall
Total CAPEX	**$ 193,492,895.51**	

Appendix 5B – Operating cost breakdown for base case scenario.

Item	Value	Cost Allocation
Maintenance (Cultivation Systems)	$ 3,008,588.15	Cultivation
Nutrients (Nitrate - Urea)	$ 845,800.51	Cultivation
Nutrients (Phosphate - DAP (Diammonium Phos)	$ 10,858,522.85	Cultivation
Nutrients (Others - Trace metals, biotin etc)	$ 1,170,432.34	Cultivation
CO_2	$ - *	Cultivation
Water	$ 117,043.23	Cultivation
Total Cultivation Power	$ 15,616,801.52	Cultivation
Labour (cultivation)	$ 275,000.00	Cultivation
Engineering / Management (cultivation)	$ 210,000.00	Cultivation
Centrifuge I (Harvest/Dewatering) Energy Use	$ 43,364,705.88	Dewatering & Drying
Drum Dryer (Biomass Drying) Energy Use	$ 45,160,332.54	Dewatering & Drying
Labour and Maintenance (Dewatering/Drying)	$ 1,279,526.48	Dewatering & Drying
Solvent Extraction (Electricity)	$ 48,368.57	Downstream Processing
Solvent Extraction (Acetone Make Up)	$ 10,714,713.94	Downstream Processing
Centrifuge II (Electricity)	$ 207,620.18	Downstream Processing
Precipitation (Electricity)	$ 66,547.38	Downstream Processing
Rotary Vac Filter (Electricity)	$ 60,498.96	Downstream Processing
Distillation Column (Electricity)	$ 381,817.67	Downstream Processing
Maintenance / Labour (DSP)	$ 503,001.94	Downstream Processing
Overheads (Insurance, Management, Indirects)	$ 13,388,932.21	Overall

*CO_2 supply is assumed cost neutral in base case scenario. CO_2 supply at a cost is considered in scenario analysis.

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