Cyanobacterial Polyhydroxybutyrate for Sustainable Bioplastic Production: Critical Review and Perspectives

Authors
Shawn Price1*, Unnikrishnan Kuzhiumparambil1, Mathieu Pernice1* and Peter J. Ralph1

Affiliations
Climate Change Cluster, Faculty of Science, The University of Technology, Sydney, Australia

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.

Keywords
PHB, cyanobacteria, bioplastic, biopolymer, biodegradable, algae, sustainability

Corresponding Authors:

Email address:
Mathieu.Pernice@uts.edu.au
Shawn.A.Price@student.uts.edu.au
1. 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 tons 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), within its cells (Balaji, Gopi & Muthuvelan 2013; Singh & Mallick 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 shows the general structure of the PHA class of molecules.

![Molecular structure of PHAs with R representing possible aliphatic functional groups](image)

**Figure 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.

2. 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 2 shows some species of cyanobacteria that are capable of synthesising PHB.

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

2.1. 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 3 provides an overview of the major cyanobacterial storage compounds.

![Cyanobacterial Reserve Compounds](image)

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,
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 CO₂. 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.

2.2. PHB CELLULAR METABOLISM

The conversion of atmospheric CO₂ 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 β-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 4. The general synthesis of PHB from heterotrophic 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 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: Summary of different PHB related proteins and their roles

<table>
<thead>
<tr>
<th>PHB Related Protein</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>PhaA</td>
<td>Responsible for first step in PHB formation, converting 2 acetyl-coA molecules to acetoacetyl-coA.</td>
</tr>
<tr>
<td>PhaB</td>
<td>Responsible for second step in PHB formation, reduction of acetoacetyl-coA to form hydroxybutyl-coA.</td>
</tr>
<tr>
<td>PhaEC</td>
<td>Heterodimer of PhaE and PhaC which adds the hydroxybutyryl-coA monomer to growing PHB chain.</td>
</tr>
<tr>
<td>PhaR</td>
<td>Transcriptional regulator modulating synthesis of PHB. In <em>Ralstonia eutropha</em> PhaR binds upstream of PhaP1 gene in promoter region repressing its expression (Waltermann &amp; Steinbuche 2005).</td>
</tr>
<tr>
<td>PhaZ</td>
<td>Depolymerase responsible for degradation and using stored PHB.</td>
</tr>
<tr>
<td>PhaM / PhaF</td>
<td>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 &amp; Jendrossek 2011).</td>
</tr>
<tr>
<td>Gene</td>
<td>Function and Characteristics</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>PhaP</td>
<td>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).</td>
</tr>
<tr>
<td>Pta</td>
<td>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).</td>
</tr>
<tr>
<td>sll0783</td>
<td>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).</td>
</tr>
<tr>
<td>sll0461 (proA)</td>
<td>sll0461 encodes gamma-glutamyl phosphate reductase (proA). A disruption in this native gene from inverse metabolic engineering resulted in a higher PHB phenotype in <em>Synechocystis</em> PCC 6803 (Tyo et al. 2009).</td>
</tr>
<tr>
<td>sll0565</td>
<td>sll0565 encodes a hypothetical protein. A disruption in this native gene from inverse metabolic engineering resulted in a higher PHB phenotype in <em>Synechocystis</em> PCC 6803 (Tyo et al. 2009).</td>
</tr>
</tbody>
</table>

PHB is the only PHA produced photoautotrophically. 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 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.

2.3. SPECIES OF PHB-PRODUCING CYANOBACTERIA AND ALGAE

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.

2.4. 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.

2.5. 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 focusing 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 been 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).

3. 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 metabolism 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 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))

<table>
<thead>
<tr>
<th>Properties</th>
<th>PHB</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting temperature (Celsius)</td>
<td>177</td>
<td>176</td>
</tr>
</tbody>
</table>
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).

4. 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.

4.1. 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.
4.2. CULTIVATION PARAMETER CONSIDERATIONS FOR MAXIMUM CELL DENSITY

**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 CO₂ 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 *Aethrospira 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 CO₂ 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 CO₂ 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.

4.3. 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.
5. 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 3: Global manufacturers and production volumes of PHA plastic (data adapted from (Singh et al. 2017))

<table>
<thead>
<tr>
<th>Company</th>
<th>Location</th>
<th>Bioplastic Brand Name</th>
<th>Production / Planned Capacity (kt / year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-on</td>
<td>Italy</td>
<td>Minerv</td>
<td>10</td>
</tr>
<tr>
<td>Kaneka</td>
<td>Singapore</td>
<td>Mirel</td>
<td>10</td>
</tr>
<tr>
<td>Meredian</td>
<td>USA</td>
<td>-</td>
<td>13.5</td>
</tr>
<tr>
<td>Metabolix</td>
<td>USA</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>Mitsubishi Gas</td>
<td>Japan</td>
<td>Biogreen</td>
<td>0.05</td>
</tr>
<tr>
<td>Chemicals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHB Industrial S/A</td>
<td>Brazil</td>
<td>Biocycle</td>
<td>0.05</td>
</tr>
<tr>
<td>Shenzhen O’Bioer</td>
<td>China</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TEPHA</td>
<td>USA</td>
<td>ThephaFLEX/ThephELAST</td>
<td>-</td>
</tr>
</tbody>
</table>
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; Kaewbainangam, 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.

6. 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 CO₂ 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 CO₂, 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 consortia 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 muscorum* was grown on waste poultry litter and achieved a 65% yield dw 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). 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 533.4 mg/L 3HB were obtained. Most recently, 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 $6 \times 10^{-4}$ % 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.

7. 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.

Acknowledgements

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Shiraishi, H. 2016, ‘Cryopreservation of the edible alkalophilic cyanobacterium Arthrospira platensis’,


Zhao, H. & Turng, L.. 2015, Mechanical performance of microcellular injection molded biocomposites from
FigureCaptions

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

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

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

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

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

Figure 6: Methods of cultivating algae. (A) Bubbled column PBR (B) closed horizontal tubular PBR with pump for mixing
Tables and Figures

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

<table>
<thead>
<tr>
<th>PHB Related Protein Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhaA</td>
<td>Responsible for first step in PHB formation, converting 2 acetyl-coA molecules to acetoacetyl-coA.</td>
</tr>
<tr>
<td>PhaB</td>
<td>Responsible for second step in PHB formation, reduction of acetoacetyl-coA to form hydroxybutryl-coA.</td>
</tr>
<tr>
<td>PhaEC</td>
<td>Heterodimer of PhaE and PhaC which adds the hydroxybutyryl-coA monomer to growing PHB chain.</td>
</tr>
<tr>
<td>PhaR</td>
<td>Transcriptional regulator modulating synthesis of PHB. In <em>Ralstonia eutropha</em> PhaR binds upstream of PhaP1 gene in promoter region repressing its expression (Waltermann &amp; Steinbuche 2005).</td>
</tr>
<tr>
<td>PhaZ</td>
<td>Depolymerase responsible for degradation and using stored PHB.</td>
</tr>
<tr>
<td>PhaM / PhaF</td>
<td>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 &amp; Jendrossek 2011).</td>
</tr>
<tr>
<td><strong>PhaP</strong></td>
<td>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).</td>
</tr>
<tr>
<td><strong>Pta</strong></td>
<td>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).</td>
</tr>
<tr>
<td><strong>sll0783</strong></td>
<td>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).</td>
</tr>
<tr>
<td>sll0461 (proA)</td>
<td>sll0461 encodes gamma-glutamyl phosphate reductase (proA). A disruption in this native gene from inverse metabolic engineering resulted in a higher PHB phenotype in <em>Synechocystis</em> PCC 6803 (Tyo et al. 2009).</td>
</tr>
<tr>
<td>sll0565</td>
<td>sll0565 encodes a hypothetical protein. A disruption in this native gene from inverse metabolic engineering resulted in a higher PHB phenotype in <em>Synechocystis</em> PCC 6803 (Tyo et al. 2009).</td>
</tr>
</tbody>
</table>
Table 2: Comparison of physical properties between PHB, polypropylene (PP) and other PHAs (data adapted from (Anbukarasu, Sauvageau & Elias 2015; Balaji, Gopi & Muthuvelan 2013; Samper et al. 2018))

<table>
<thead>
<tr>
<th>Properties</th>
<th>PHB</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting temperature (Celsius)</td>
<td>177</td>
<td>176</td>
</tr>
<tr>
<td>Glass transition temperature (Celsius)</td>
<td>2</td>
<td>-10</td>
</tr>
<tr>
<td>Initial degradation temperature (Celsius)</td>
<td>220</td>
<td>357</td>
</tr>
<tr>
<td>Crystallinity (%)</td>
<td>60</td>
<td>50-70</td>
</tr>
<tr>
<td>Tensile strength (MPa)</td>
<td>43</td>
<td>38</td>
</tr>
<tr>
<td>Extension to break (%)</td>
<td>5</td>
<td>400</td>
</tr>
</tbody>
</table>
Table 3: Global manufacturers and production volumes of PHA plastic (data adapted from (Singh et al. 2017))

<table>
<thead>
<tr>
<th>Company</th>
<th>Location</th>
<th>Bioplastic Brand Name</th>
<th>Production / Planned Capacity (kt / year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-on</td>
<td>Italy</td>
<td>Minerv</td>
<td>10</td>
</tr>
<tr>
<td>Kaneka</td>
<td>Singapore</td>
<td>Mirel</td>
<td>10</td>
</tr>
<tr>
<td>Meredian</td>
<td>USA</td>
<td>-</td>
<td>13.5</td>
</tr>
<tr>
<td>Metabolix</td>
<td>USA</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>Mitsubishi Gas Chemicals</td>
<td>Japan</td>
<td>Biogreen</td>
<td>0.05</td>
</tr>
<tr>
<td>PHB Industrial S/A</td>
<td>Brazil</td>
<td>Biocycle</td>
<td>0.05</td>
</tr>
<tr>
<td>Shenzen O’Bioer</td>
<td>China</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TEPHA</td>
<td>USA</td>
<td>ThephaFLEX/ThephELAST</td>
<td>-</td>
</tr>
<tr>
<td>Tianan Biological Materials</td>
<td>China</td>
<td>Enmat</td>
<td>2</td>
</tr>
<tr>
<td>Tianjin Green Biosciences</td>
<td>China</td>
<td>Green Bio</td>
<td>10</td>
</tr>
<tr>
<td>Tianjin Northern Food</td>
<td>China</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yikeman Shandong</td>
<td>China</td>
<td>-</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 1: Molecular structure of PHAs with R representing possible aliphatic functional groups

$\begin{align*}
R = \text{hydrogen} & \quad \text{Poly(3-hydroxypropionate)} \\
R = \text{methyl} & \quad \text{Poly(3-Hydroxybutyrate)} \\
R = \text{ethyl} & \quad \text{Poly(3-hydroxyvalerate)} \\
R = \text{propyl} & \quad \text{Poly(3-hydroxyhexanoate)} \\
R = \text{pentyl} & \quad \text{Poly(3-hydroxyoctanoate)} \\
R = \text{nonyl} & \quad \text{Poly(3-hydroxydodecanoate)}
\end{align*}$
Figure 2: Four species of algae capable of PHB production; (A) Athrospira maxima (B) Oscillatoria jasorvensis (C) Synechocystis PCC6803 (D) Nostoc muscorum
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