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Cyanobacterial Polyhydroxybutyrate for Sustainable Bioplastic Production: Critical Review and Perspectives

Authors

Shawn Price[1*], Unnikrishnan Kuzhiumparambil[1], Mathieu Pernice[1*] and Peter J. Ralph[1]

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

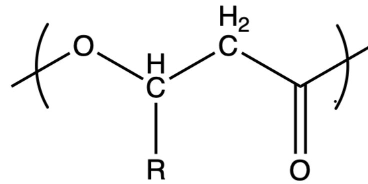
Plastics are now one of the most widely used materials worldwide. From applications in the automotive, construction and biomedical industries to agricultural films for farmers, disposable packaging to ensure food quality, and much more; it is undeniable that plastics enable modern society to function. 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

71 currently being investigated as third generation bioplastic technology and could offer a
72 solution to this growing problem.
73
74 Algae and cyanobacteria are among the oldest and most widespread life-forms on Earth. They
75 can be found in almost all freshwater, marine and brackish aquatic ecosystems, in addition to
76 various terrestrial environments (Mata et al. 2010). The term algae encompasses aquatic
77 photosynthetic organisms of great genetic diversity; from single cell microalgae to
78 multicellular species such as seaweed. Algae and cyanobacteria are well known for the many
79 advantages they hold as a sustainable source of biomass to multiple industries (Mata, Martins
80 & Caetano 2010). They can be cultivated in salt water or wastewater and do not necessarily
81 compete for freshwater resources. Nutrients do not need to come from synthetic fertiliser
82 and can instead be obtained from wastewater streams and some species are able to fix
83 nitrogen directly from the atmosphere. No arable land is required for cultivation facilities and
84 compared to terrestrial crops, algae and cyanobacteria can offer biomass productivities that
85 are tens to hundreds times higher than that of terrestrial plants and as a result require less
86 area to produce the same amount of biomass (Adeniyi, Azimov & Burluka 2018). In addition
87 to this, several species of cyanobacteria, the ancestors of all eukaryotic algae, are able to
88 naturally synthesise the biodegradable thermoplastic, poly-hydroxy-butyrate (PHB), within its
89 cells (Balaji, Gopi & Muthuvelan 2013; Singh & Mallick 2017).
90
91 PHAs are a class of condensation polymers that serve as energy storage compounds and are
92 present in many aerobic and anaerobic microorganisms. Over 150 different types of PHAs
93 have been identified (Balaji, Gopi & Muthuvelan 2013); however, PHB is by far the most
94 prevalent of the PHA biopolymers across different taxonomic groups including

photoautotrophic cyanobacteria. Figure 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: 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

114 runoff, increasing demand for freshwater and fertiliser and habitat destruction to clear area

115 for farmland.

116

117 ## 2. PHA/PHB in Cyanobacteria and Algae

118

119 Cyanobacteria are a group of oxygen-producing photosynthetic bacteria. They require simple

120 inorganic nutrients for growth including atmospheric carbon dioxide, nitrogen, phosphorous,

121 some trace metals and micronutrients. Some cyanobacteria are capable of mixotrophic

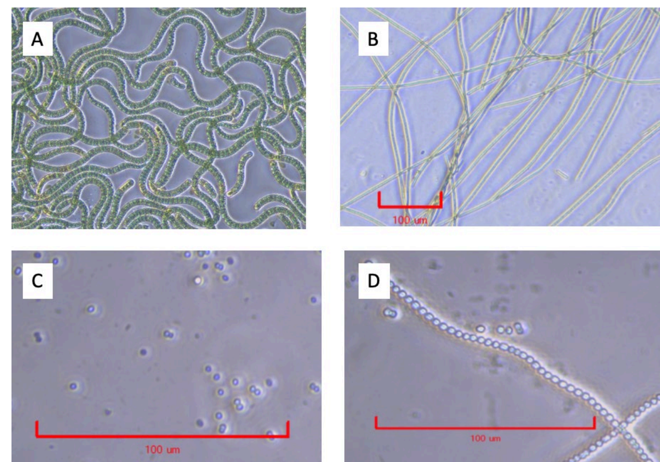
122 metabolism and can thus grow in the dark without photosynthesis, if an organic carbon

123 substrate is provided. As mentioned previously, some cyanobacteria can directly fix

124 atmospheric nitrogen, when nitrate sources are lacking in the environment. Figure 2 shows

125 some species of cyanobacteria that are capable of synthesising PHB.

126

127 

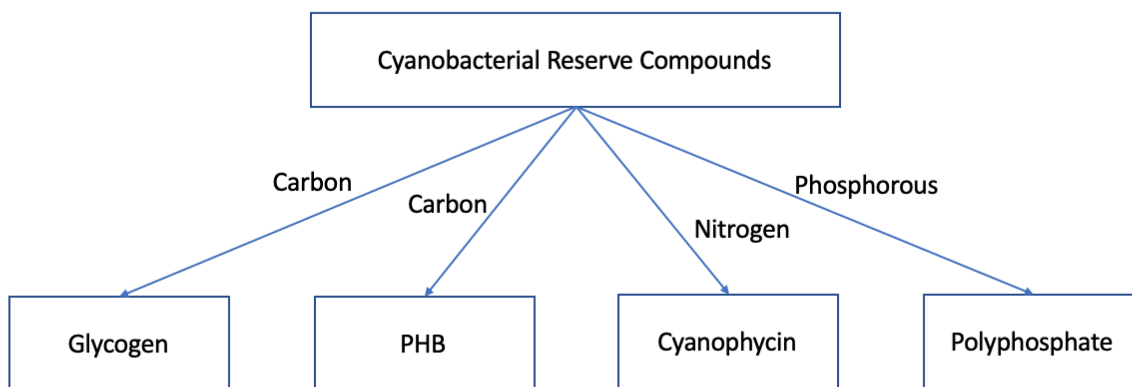
128 *Figure 2: Four species of algae capable of PHB production; (**A**) Athrospira maxima*

129 *(**B**) Oscillatoria jasorvensis (**C**) Synechocystis PCC6803 (**D**) Nostoc muscorum*

130

131 ### 2.1. WHY DO CYANOBACTERIAL CELLS ACCUMULATE PHB?

132 Being the first photosynthesisers on Earth, cyanobacteria are among the oldest known
133 organisms and have been exposed to a range of different environmental conditions to which
134 they have adapted. As a result they can produce an array of storage compounds, including
135 PHB, which assist in living in environments with fluctuating nutrients (Stal 1992). Figure 3
136 provides an overview of the major cyanobacterial storage compounds.
137

138 

139 *Figure 3: Major cyanobacterial storage polymers (information adapted from* (Flores &
140 Herrero 2014)*)*
141

142 These storage compounds include phosphate stored as polyphosphate granules (Thompson,
143 Oh, Rhee, 1994) and nitrogen in the form of cyanophycin (Esteves-Ferreira et al. 2018). These
144 compounds effectively uncouple the growth of cyanobacteria to the external concentrations
145 of phosphate or nitrogen and create a buffer to nutrient fluxes in dynamic environments by
146 having internal reserves accessible to support growth under limiting conditions.
147

148 Energy and carbon are also stored in a similar strategy. In times of surplus light and CO_2,
149 glycogen (a poly glucose) is synthesised with excess metabolic energy, especially when growth
150 is limited by other compounds such as nitrogen or phosphorous (Kaewbai-ngam,

151 Incharoensakdi & Monshupanee 2016; Singh et al. 2017). Glycogen is then oxidised during
152 the dark period as an energy source via the oxidative pentose phosphate pathway (Smith
153 1983). Similar to glycogen, PHA/PHB in cyanobacteria also utilise excess intracellular carbon
154 (in the form of Acetyl-CoA) and reduction equivalents (NADH) for their synthesis. Previous
155 researchers believed glycogen to be a more efficient energy storage compound relative to
156 PHA/PHB due to cyanobacteria having an incomplete tricarboxylic acid cycle (de Philippis et
157 al. 1992). However, recent research shows that the cyanobacterial tricarboxylic cycle is still
158 operational through the use of different enzymes (Steinhauser, Fernie & Araújo 2012; Zhang
159 & Bryant 2011). Despite this, the exact role of PHB in cyanobacterial metabolism is still
160 unclear, as glycogen occurs in significant amounts in most cyanobacteria and having multiple
161 carbon storage compounds for the same purpose is inefficient. In support of this, most
162 microorganisms produce either glycogen or PHB and not both (Damrow et al. 2016).
163 Although, it is possible that PHB could serve as a longer term carbon storage compound in
164 the cell compared to glycogen (Koller 2015). It has been proposed by Phillipis et al (1992) that
165 PHB's main role is to regulate excess reducing power and act as an electron sink (although
166 glycogen fulfils this role too). Hauf et al demonstrated that the intracellular redox state of the
167 cell is critical to producing PHB, with a high NADPH to NADP+ being a condition for PHB
168 accumulation (Hauf et al. 2013). This could be for protecting the organism against excess
169 charge or as storage for other electron demanding processes such as nitrogen fixation, of
170 which many species of cyanobacteria are capable (de Philippis et al. 1992). However, there
171 are many non-nitrogen fixing species of cyanobacteria that accumulate PHB. Another
172 proposed pathway is that the Acetyl-CoA derived from metabolising PHB can be used for
173 other biosynthetic purposes (Stal 1992) such as fatty acid biosynthesis and nitrogen
174 assimilation. Lastly, Sznajder et al (2015) have proposed that PHB could play a structural role

175 during the process of dividing nucleoids in future daughter cells.
176
177 Interestingly, in a study by Kucho et al (2005) on the model cyanobacteria *Synechocystis* sp.
178 PCC6803, it was determined that the expression of PHB synthesis-related genes were linked
179 to a circadian rhythm. Cyclic expression peaked at the transition from light to dark, along with
180 other genes related to respiration (Kucho et al. 2005). Based on this observation, the authors
181 suggested that PHB could play a role in supplying energy and carbon during the night;
182 however, the study did not examine glycogen expression at all. Köbler et al (2018) created
183 *Synechocystis* sp. PCC6803 mutants lacking a key circadian rhythm regulator, RpaA, which
184 induces the expression of 'dusk' genes to prepare the cell for the onset of the dark period. It
185 was found that in the RpaA deleted mutants, the expression of PHB synthesis genes (PhaE
186 and PhaC) were significantly reduced.
187
188 Although PHB accumulates to a lesser degree than glycogen in cyanobacteria, it has been
189 suggested that under temporarily unfavourable energetic conditions (such as at night)
190 cyanobacteria are more likely to consume glycogen over PHB (Drosg 2015). Koch et al (2019)
191 proved that the majority of PHB is indeed produced from existing intracellular glycogen stores
192 upon the prolonged exposure to nitrogen starvation, and not directly from atmospheric CO_2.
193 This was achieved by creating glycogen lacking mutants through knocking out glycogen
194 synthase and phosphorylase enzymes, in which an extreme reduction in PHB production was
195 observed. Acetyl-coA can be produced from glycogen through three different pathways in
196 *Synechocystis* sp. PCC6803; the Embden Meyerhof Parnas (EMP), Entner Doudoroff (ED) and
197 Oxidative Pentose Phosphate (OPP). Through blocking each pathway separately, it was shown

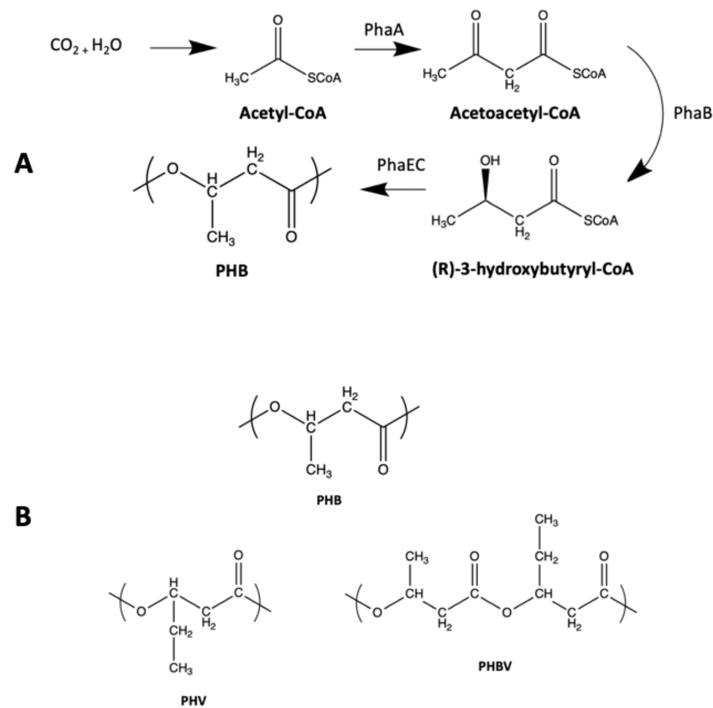
198 that the EMP and OPP pathways were the most important for providing carbon for PHB
199 production from internal glycogen stores (Koch et al. 2019).
200
201 Damrow et al (2016) also created several *Synechocystis* sp. PCC6803 mutants lacking glycogen
202 synthesis and/or PHB synthesis capability to investigate the role of both carbon polymers in
203 the response to stressful environmental conditions. Under low light and nitrogen stress
204 conditions, the PHB-deficient mutant performed similarly to the wild type in growth and
205 recovery capability. However, the glycogen-deficient mutant and double PHB glycogen-
206 deficient mutant both showed significant decreases in viability and growth. It was concluded
207 that glycogen plays a greater role in both energy storage and macronutrient acclimation
208 responses compared to PHB.
209
210 In a study by Raberg et al (2014), the proteome of a mutant *Cupriavidus necator* (the model
211 heterotrophic PHB organism previously known as *Ralstonia eutropha*) strain lacking PHB
212 synthesis was investigated. To prevent over acidification of the cell from accumulating
213 metabolites, such as pyruvate and Acetyl-CoA, these mutants excreted pyruvate and
214 upregulated the synthesis of several proteins to convert pyruvate and Acetyl-CoA into other
215 metabolites, with a large proportion of the excess Acetyl-CoA entering the tricarboxylic acid
216 cycle (Raberg et al. 2014). Thus, in both heterotrophs and autotrophs, PHB seems to be a non-
217 essential metabolite for survival; however the relevance of this study to phototrophic PHB
218 production could be strengthened by comparing a PHB-deficient cyanobacterial mutant
219 proteome to its wild type too. Hauf et al did perform metabolomics on a PHB deficient
220 cyanobacterial mutant and found difference in amino acids, TCA intermediates and sugars
221 compared to the wild type under nitrogen stress conditions. One of the key findings was an

222 increase in sorbitol levels in the PHB-deficient mutant, which indicated a more oxidizing
223 intracellular state due to a lower NADPH to NADP ratio. This highlighted the importance of a
224 highly reducing intracellular environment for PHB production.
225
226 To determine the exact role and function of PHB in cyanobacteria, future studies should focus
227 on different types of PHB-deficient mutants grown in varied environments with a range of
228 proteomic, metabolomics, transcriptomic and functional genomics approaches. It will be
229 important to determine where the excess Acetyl-CoA sink occur in these PHB-deficient
230 cyanobacteria. Future studies investigating the metabolic significance of PHB would also
231 benefit from comparing the results between both heterotrophs and autotrophs. Differences
232 in phenotypes could potentially identify whether PHB plays a similar or different metabolic
233 role in different classes of organisms.
234

235 ## 2.2. PHB CELLULAR METABOLISM

236 The conversion of atmospheric CO_2 into PHA begins with the Calvin Benson cycle, via
237 glycolysis to pyruvate and then to Acetyl-CoA. The metabolic pathway for the biological
238 synthesis of PHB, the most prevalent PHA, consists of three enzymatic reactions converting
239 Acetyl-CoA to PHB. The first enzyme, PhaA (PHA specific ß-ketothiolase), combines two
240 Acetyl-CoA molecules into Acetoacetyl-CoA. The second enzyme, PhaB (Acetoacetyl-CoA
241 reductase), then reduces this compound leading to hydroxybutyryl-CoA. The final step
242 involves PhaEC (PHB synthase) and the addition of the hydroxybutyryl-CoA fatty acid
243 monomer to a growing PHB molecule via an ester bond (Balaji, Gopi & Muthuvelan 2013). The
244 reaction scheme is shown in Figure 4. The general synthesis of PHB from heterotropic and
245 phototrophic organisms are the same; however, the four genes of PhaA, PhaB and PhaEC are

246 located on a single operon in heterotrophs (such as in *Cupriavidus nectator*), while in
247 cyanobacteria (*Synechocystis* PCC6803) the genes are located in two separate operons
248 approximately 500Kbp apart. The first loci contains the genes for PhaA and PhaB and are
249 putatively co-expressed. The second loci contains the genes for PhaEC, a heterodimer
250 consisting of PhaE and PhaC (Drosg 2015) (Hauf et al. 2015).



251

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

254

255 Apart from the three PhaA, PhaB and PhaEC enzymes not much more is known about the
256 other PHB-related proteins in cyanobacteria in contrast to that of heterotrophs (Hauf et al.
257 2015). It is known that in *Cupriavidus nectator* the PHB granules exist in a protein complex.
258 Some of these proteins, for example are transcriptional regulators (PhaR) and others are
259 depolymerases (PhaZ) (Hauf et al. 2015). In total, approximately 8 different PHB-related
260 proteins across different heterotrophic bacteria have been identified and their roles are

261 summarised in Table 1. It is likely that similar proteins with similar roles would exist in
262 cyanobacteria; however, this has yet to be confirmed. For example, the extra and intracellular
263 depolymerases required for PHB degradation have not been identified in any cyanobacterial
264 strains to date, but have been identified in many heterotrophic bacteria (Flores & Herrero
265 2014).
266
267 *Table 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).

268

269 PHB is the only PHA produced photoautorophically. However, multiple PHA polymers

270 including PHV (poly-hydroxy-valerate) can be produced by growing cyanobacteria under

271 mixotrophic or heterotrophic conditions. Growth with different carbon substrates such as
272 valerate instead of acetate can cause PHV to be accumulated instead. Alternative co-polymers
273 of PHA can be produced when mixed substrates are used, such as valerate and acetate which
274 results in 3 hydroxy-butyrate co 3 hydroxy-valerate. Figure 5 shows the co-polymer molecular
275 structures in comparison to PHB. Such co-polymers tend to offer better material properties
276 such as reduced brittleness and stiffness (Zhao & Turng 2015). Table 2 compares several
277 physical properties of PHB, PHBV, some other PHAs and polypropylene.
278

279 ### 2.3. SPECIES OF PHB-PRODUCING CYANOBACTERIA AND ALGAE

280 Although not all cyanobacteria produce PHB, many species of cyanobacteria can. Most yields
281 are negligible (at below 1%) and many yields are well under 5% by dry cell weight (dcw) in
282 most studies using non-genetically modified strains grown under photoautotrophic
283 conditions (Drosg 2015; Singh et al. 2017; Stal 1992; Troschl, Meixner & Drosg 2017).
284 Noticeably, heterotrophic production has been able to double PHB yield in some species of
285 cyanobacteria compared to phototrophic only production and peaked at around ~40% dcw
286 (Singh et al. 2017; Singh & Mallick 2017b; Troschl, Meixner & Drosg 2017). A thermophilic
287 strain of cyanobacteria isolated from a Japanese hot spring, *Synechococcus* MA19, was
288 reported to achieve the highest reported phototrophic cyanobacterial PHB yield of 55%
289 (Nishioka et al. 2001). However, the highest non thermophilic and non-genetically modified
290 cyanobacteria yields are around 20-25% (Kaewbai-ngam, Incharoensakdi & Monshupanee
291 2016; Troschl, Meixner & Drosg 2017). Genetically engineered and recombinant
292 cyanobacteria peaked at roughly 26% PHB yield (Carpine et al. 2017). Most recently, UV
293 random mutagenesis was able to generate a mutant strain with 37% yield PHB
294 (Kamravamanesh et al. 2018).

295

296 The polymeric lipid, PHB, has been claimed to be the storage compound exclusively for
297 prokaryote, whereas eukaryotes instead accumulate lipid bodies in the form of
298 triacylglycerols (Ansari & Fatma 2016; Waltermann & Steinbuche 2005). Surprisingly, two
299 eukaryotic genus of algae, *Botryococcus* and *Chlorella*, have now recently been reported to
300 naturally produce PHB. *Botryococcus braunii* was reported to achieve 10-16% yield PHB
301 (Kavitha et al. 2016) and *Chlorella pyrenoidosa* and *Chlorella fusca* achieving yields of 17% and
302 27% (Cassuriaga et al. 2018; Das, Sathish & Stanley 2018).

303

304 The only other occasions where a eukaryotic algae produced PHB has been through genetic
305 engineering efforts. The first was achieved by Chaongang et al (2010) who transformed
306 *Chlamydomonas* reinhardtii by inserting only phaB and phaC genes, as phaA activity was
307 claimed to be intrinsic. A yield of only 0.0006% PHB was achieved. Secondly, Hempel et al
308 (2011) genetically modified *Phaeodactylum tricornutum* with PHA synthesis genes from
309 *Cupriavidus nectator* with a yield of 10% PHB achieved.

310

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

317

318 ## 2.4. MAXIMISING PHOTOAUTOTROPHIC PHB YIELDS

319 From reviewing the available PHB cyanobacteria literature, the most common inducer of high
320 PHB production has been a limitation of nitrogen in a carbon excess environment (Campbell,
321 Stevens, Jr., & Balckwill, 1982; Kaewbai-ngam et al., 2016; Keith E. Tyo, Hang Zhou, 2006;
322 Panda, Sharma, & Mallick, 2005;). However, some cyanobacteria have shown optimal PHB
323 yields either under a combination, or complete substitution of nitrogen for other nutrient
324 limitations. For example, in the study by Kaewbai et al (2016), 137 strains of cyanobacteria
325 were screened for PHB yields under a range of different nutrient limitations including
326 nitrogen, phosphorous, potassium and different combinations of the three. Over 50% of the
327 screened cyanobacterial strains showed a significant increase in PHB yield under nitrogen
328 limitation conditions. Of these, 75% had peak PHB yields when nitrogen was limited (either
329 by itself or in combination with other nutrients). One species however, *Mastigocladopsis* sp.,
330 had peak PHB yield of 7% under nutrient balanced conditions and showed decreases in PHB
331 yield under all nutrient deprivation environments. Of interest is that most (over 75%) of the
332 cyanobacterial species only accumulated significant amounts of PHB under only one of the
333 above nutrient conditions (nitrogen, potassium, phosphate or combination), as opposed to a
334 few strains accumulating significant amounts of PHB across a range of nutrient limitations.
335 This suggests that high PHB accumulation for different species or strains are environmentally
336 specific, which has implications for future screening efforts.
337
338 Many strains of cyanobacteria in this screening study were capable of atmospheric nitrogen
339 fixation, and showed the greatest PHB yield under nitrate-limited conditions. This was the
340 case for several *Calothrix* species (Zehr 2011) which showed high yields of PHB (17-25% dcw)
341 which was only induced under nitrogen limitation. Other nutrient limitation conditions such
342 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).

367 Nile Blue A is an oxazine dye that has been used extensively in this field of research for
368 microscopic visualisation and in some instances the fluorescent quantification of PHB. The
369 method was first developed by Ostle in 1982 and involved immersing heat fixed samples on
370 glass slides into a 1% Nile Blue A solution for 10 minutes at 55˚C. Following a wash with acetic
371 acid and water to remove excess dye, the cells were excited at 460 nm (Ostle 1982). The
372 incubation time, temperature and dye concentration have been modified in different
373 methods over the past four decades. Notably, Oshiki et al (2011) were able to develop at high
374 throughput rapid quantification for PHB for heterotrophic bacteria from wastewater sludge.
375 The method involved a 0.02% Nile Blue A solution which was added in a 1:1 ratio to cell culture
376 and incubated at room temperature for 3 minutes. A fluorescent plate reader was used with
377 a 490 nm excitation and 590 nm emission wavelength setting.
378
379 It is likely that during incubation, the Nile Blue A oxazone dye molecules convert to the
380 oxazine version of the dye which is also known as Nile Red. This is further confirmed by the
381 fluorescent peak of 550-590 nm observed with Nile Blue (Oshiki, Satoh & Mino 2011) which
382 is the same as the Nile Red fluorescent peak for non-polar lipids (Rumin et al. 2015). The
383 oxazone Nile Red molecule is non-polar and can bind to the non-polar PHB granule better
384 than the oxazine Nile Blue A molecule which has a polar imminium functional group. For this
385 reason, it is likely that Nile Red is the superior dye as a higher overall staining efficiency can
386 be achieved without an additional oxazine to oxazone conversion step. Recently, Nile Red has
387 become more widely used since the initial method paper by Ostle et al (1982) was published
388 and has been used more frequently than Nile Blue in cyanobacterial PHB detection. Several
389 different methods of Nile Red staining are available and involve different solvents (such as
390 DMSO or ethanol), in the presence of different incubation times, the presence of glycerol to

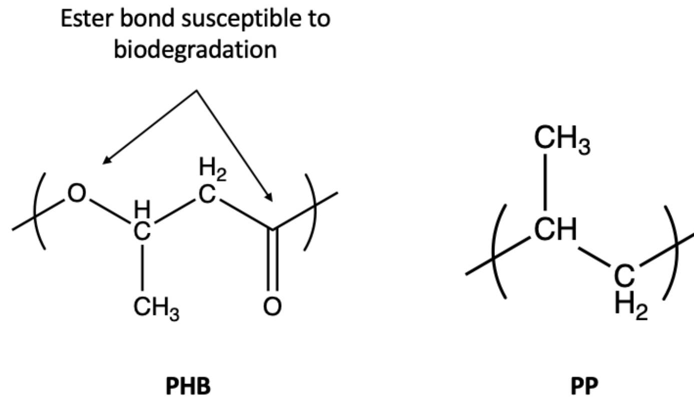
391 facilitate permeation of cell membrane, cell concentration, vortexing and agitation (Khetkorn
392 et al. 2016; Morschett, Wiechert & Oldiges 2016; Shrivastav, Mishra & Mishra 2010). In
393 addition to fluorescent microscopy and microplate reader, flow cytometry has also been used
394 with this method focussing on the quantification of PHB (Tyo & Hang Zhou, 2006). The Nile
395 Red PHB staining protocol must be optimised for different cyanobacterial species due to
396 differences in cell physiology and the cells must first have sufficient yield of PHB before a
397 signal can be measured. This threshold is different for each cell; however, a yield of at least
398 greater than 1% dcw is recommended. Lastly, the autofluorescence of cyanobacteria often
399 overlap with the Nile Red and Blue fluorescent peaks (Luimstra et al. 2018; Schubert, Schiewer
400 & Tschirner 1989; Schulze et al. 2011) which interferes with detecting PHB granules.
401
402 BODIPY (boron-dipyrromethene) fluorescent dyes have also be used for microalgae lipid
403 studies and PHA in heterotrophic bacteria (Biernacki et al. 2017; Rumin et al. 2015). BODIPY
404 has been claimed to have less background staining, a higher specificity and sensitivity to PHB
405 compared to Nile Red (Kacmar et al. 2006). In addition, BODIPY fluorescent dyes can be
406 chosen with different fluorescent peaks that do not correspond with cyanobacteria
407 autofluorescence to achieve higher signal clarity. Despite these advantages, BODIPY has not
408 been used for PHB quantification in cyanobacteria as of yet.
409
410 The dyes and protocols mentioned above stain for total lipids, including PHB. However, for
411 PHB quantification, the total fluorescence must be correlated to the amount of PHB present
412 in the cell culture. For this purpose, chemical analysis methods are required to create a
413 standard calibration curve to relate dye fluorescence to the amount of PHB. Gas
414 chromatography (GC) and high performance liquid chromatography (HPLC) are the two most

415 commonly used methods (Ansari & Fatma 2016; Wu, Shen & Wu 2002). It is unknown whether
416 the PHB to lipid ratio and ultimately PHB to total dye fluorescence signal would change over
417 time, at different cell growth stages or under different nutrient deprivations. Thus, it is
418 recommended that future protocols create these calibration curves under different
419 conditions to either ensure an accurate calibration curve for that environment or to confirm
420 that the PHB to dye fluorescence signal remains constant.
421
422 A standard PHB GC protocol begins with harvesting biomass through centrifugation, washing
423 and drying. 2 mL 1,2-dichloroethane, 2 mL of hydrochloric acid propanol mixture (1:5 ratio)
424 and 200 µL of standard solution (2 g benzoic acid and 50 mL propanol) are added to the dried
425 biomass and incubated at 100˚C for 2 hours (Riis & Mai 1988). Water is added after cooling
426 and the heavy phase can be injected into the chromatograph. Quantification is achieved by
427 measuring the peak area and comparing against a hydroxy butyric chemical standard (Riis, &
428 Mai, 1988). For HPLC, cell biomass is harvested, washed and dried. Following this the dried
429 pellet is digested in concentrated sulfuric acid for one hour which converts the PHB polymer
430 chain into crotonic acid monomers. The sample is then diluted to prevent damage to column
431 from the sulfuric acid and then run in the column against a crotonic acid standard for
432 quantification and to determine reference elution time (Koller & Rodríguez-Contreras 2015).
433 A PHB standard curve can also be run in parallel to determine the PHB to crotonic acid
434 digestion efficiency.
435
436 Law et al (1961) created a UV spectroscopy protocol for quantifying PHB. After processing
437 PHB to crotonic acid by using sulfuric acid similar to above, crotonic acid abundance can be
438 determined by measuring the absorbance at 235 nm wavelength. Pre-processing the biomass

439 sample with chloroform, acetone or dichloromethane to isolate PHB is recommended due to
440 the possible signal interference from other chemical compounds in the biomass mixture (Law
441 & Slepecky 1960). Alternatively, this method can be used on eluted compounds from a
442 chromatography column when using HPLC to confirm the eluted material is crotonic acid
443 (Hauf et al. 2015).
444

445 3. PHB Material Properties

446 PHB is similar to polypropylene in terms of molecular structure. Both polymers have similar
447 sized monomers with a single methyl side pendant group resulting in comparable physical
448 properties such as being water-resistant and very close melting temperatures. However, the
449 greatest difference is that the PHB monomers are joined via ester bonds which allows for
450 biodegradation when exposed to a microbial community in the environment (Shah et al.
451 2008). Microorganisms can release enzymes which cleaves these ester bonds and degrade
452 the polymer into oligomers and monomers, which is followed by uptake into the cytosol and
453 metabolisation to either carbon dioxide or methane under aerobic or anaerobic conditions,
454 respectively. In contrast, polypropylene, and most conventional petrochemical plastics, are
455 impervious to microorganism enzymes and biodegradation due to their carbon-carbon bonds.
456 Although polypropylene can be physically disintegrated into smaller pieces, its molecular
457 structure makes it far more chemically inert than PHB causing their accumulation in the
458 environment resulting in plastic pollution and the subsequent production of microplastics.



459

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

461

462 Table 2 compares some of the physical properties of PHB, polypropylene and some other
463 PHAs. PHB and isotactic polypropylene (PP) have extremely similar melting temperatures,
464 crystallinity and tensile strength. However, PHB is somewhat more brittle (unable to
465 accommodate a different shape before shattering or breaking under an applied force) as can
466 be seen by the extension or elongation of the material at breaking point. Despite this
467 difference, PHB is still a highly valued candidate for PP replacement in many applications. It
468 should be noted that after polyethylene, PP is the second most prevalent petrochemical
469 plastic used across the world (Geyer, Jambeck & Law 2017).

470

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

474

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

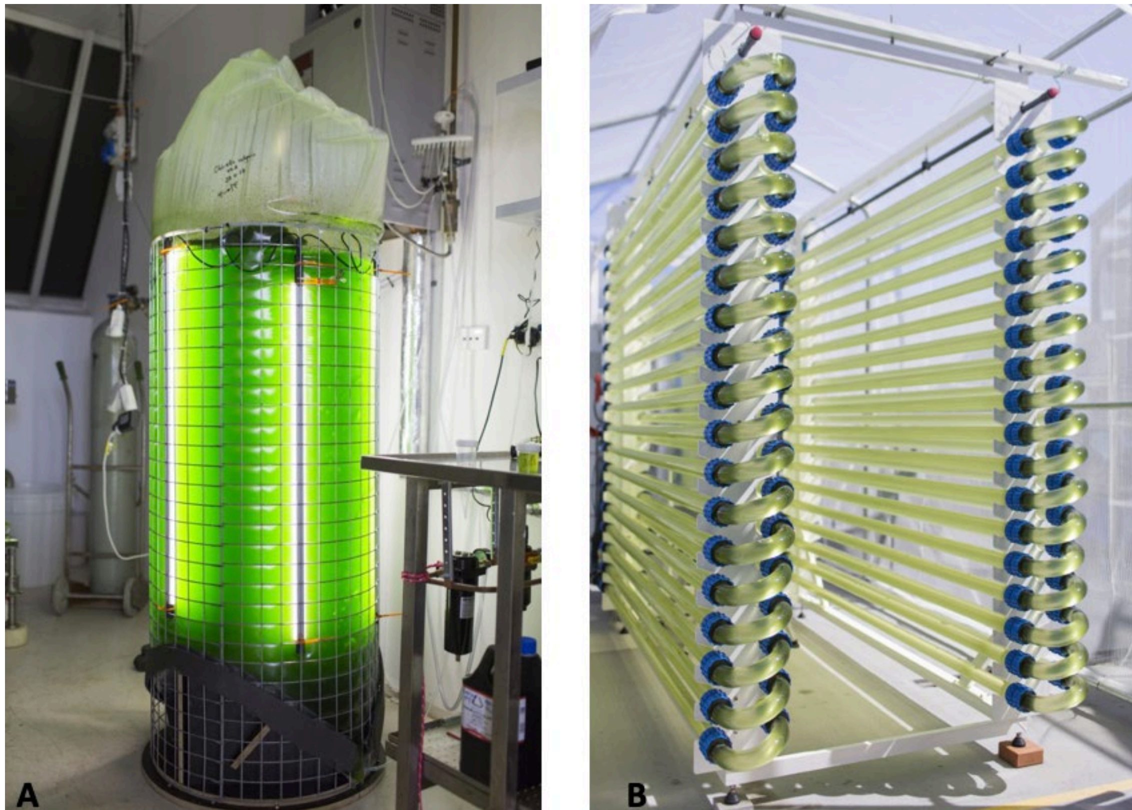
475

476 The melting temperature and glass transition temperature are two critical thermal properties
477 that determine how a plastic polymer can be used for a particular purpose. The melting
478 temperature determines what temperature the resin must be heated to before processing,
479 for example by injection moulding. Because PHB's melt temperature is close to its
480 degradation temperature, processing results in thermal instability and can cause a reduction
481 in PHB molecular weight and physical properties (El-hadi et al. 2002). The glass transition
482 temperature is the temperature at which a thermoplastic polymer becomes less glassy and
483 brittle and shifts towards a less rigid and rubbery state as the polymer molecules have more
484 energy to overcome a crystalline and ordered structure and begin to orientate themselves
485 randomly (Stevens, 2016). Because PHB's glass transition temperature is close to room
486 temperature, this results in a growing amorphous phase over time, whereby the polymer
487 chains gain higher degrees of freedom and lose structure. In addition to these two problems,
488 PHB has a low nucleation density. Nucleation density is the number of phase change
489 spherulites per unit volume that form when the polymer melt shifts from liquid to solid as it
490 cools (Fraser, Keller, Odell, & Wills, 1978). Because there are less nucleation points, the
491 spherulite crystals are bigger which can result in cracks and splits (contributing to brittleness)
492 adversely affecting mechanical characteristics. Despite this, research is being carried out to

493 blend PHB with other polymers and additives to improve processability and mechanical
494 properties (Armentano et al. 2015; Fonseca, Souza & Or 2017; Ni & Woo 2013).
495
496 ## 4. Industrial Cyanobacterial PHB Production
497 The industrial cultivation options and considerations for cyanobacterial PHB production are
498 similar to those already reviewed for the commercial cultivation for algae biofuels and other
499 commodity (low value high volume) algal products (Adeniyi, Azimov & Burluka 2018; Mata,
500 Martins & Caetano 2010). The two main options are open or closed cultivation systems which
501 each have their own advantages and disadvantages.
502
503 ### 4.1. CULTIVATION SYSTEM
504 Open Raceway Ponds (ORPs) are large shallow ponds approximately 40 cm deep, generally
505 mixed by a rotating paddle wheel (Richardson et al. 2014). The ponds are filled with media
506 and often have carbon dioxide and air sparging systems to provide the culture with required
507 gases during the day and night, respectively. ORPs are harder to monitor and control often
508 because the culture is not fully mixed and there can be significant variability throughout
509 system such as for pH, temperature and cell density. Because of reduced mixing, more cell
510 settling and less sunlight is distributed across the cyanobacterial population, resulting in less
511 than optimal biomass productivity. In ORPs, there is also significantly lower carbon dioxide
512 utilisation due to open nature of the pond which allows for degassing. ORPs also suffer from
513 high amounts of evaporation which will vary depending on site location. If marine strains are
514 cultured then concentration of salts over time will require significantly higher amounts of
515 media bleed and replacement. Greenhouses covering raceway ponds are an option to reduce

516 evaporation; however, the added cost often does not cover the loss in water financially,
517 despite being the environmentally superior option. Cultivation in open systems results in
518 extreme seasonal variability and a lack of reproducibility, although similar problems are
519 experienced by terrestrial crop cultivation too. Lastly, ORPs are open to many vectors for both
520 contamination of the media and for cultivation algae or cyanobacteria to enter surrounding
521 ecosystems. These vectors include abiotic factors such as rain or wind and biotic factors such
522 as birds, insects or microbial predators. Culture crashes can occur if other microalgal species
523 or cyanobacterial predators such as viruses or protozoa enter the cultivation system. Escaped
524 cultivation strains into surrounding ecosystems can also disrupt and alter food chains (Henley
525 et al. 2013).
526
527 Closed Photo Bioreactors (PBRs) on the other hand are sealed systems with a transparent
528 material such as glass or plastic which allow for light to penetrate. Many designs for closed
529 PBRs exist such as flat panel, hanging plastic bag, or tubular. PBRs are able to achieve far
530 higher amounts of mixing and control over cultivation parameters such as temperature and
531 pH. There is a higher surface area to volume of culture too, which allows for a greater light
532 distribution across the cultivation culture, and the combination of these two factors results
533 in far higher biomass productivities and maximum cell densities. These systems are also more
534 resilient to culture crashes and exposing cultivation strains to the environment; however, the
535 gas venting and media introduction ports still confer routes for contamination and can never
536 remain completely axenic. Greater carbon dioxide utilisation can be achieved in PBRs due to
537 longer retention times and lower evaporation of water is also a benefit. However, these
538 systems are significantly more expensive to build and operate.
539

540



541 *Figure 6: Methods of cultivating algae.* ***(A)*** *Bubbled column PBR* ***(B)*** *closed horizontal tubular*

542 *PBR with pump for mixing*

543

544 ## 4.2. CULTIVATION PARAMETER CONSIDERATIONS FOR MAXIMUM CELL DENSITY

545 **Lighting and gas exchange** – Photoautotrophic production requires light in the
546 photosynthetically active radiation range (typically between 400-700 nm). Light must be
547 supplied such that it is not the limiting factor for growth. Effective mixing is required to reduce
548 settling and ensure movement of cells in the media and maximise the exposure of the average
549 cell population to sufficient light. The light intensity must not be too high, as this will result in
550 photoinhibition whereby cellular photosynthetic machinery becomes damaged. Artificial
551 lighting may be economically feasible only for high-value algae products such as nutraceutical
552 or pharmaceutical compounds, whereas it is cost prohibitive for commodity products of lower
553 unit value such as bioplastics and biofuels. Thus, growth with sunlight is the most viable

554 option. While carbon dioxide must be supplied during the light period for photosynthetic
555 metabolism, oxygen may need to be supplied during the dark period for respiration
556 depending various cultivation factors. To maximise gas exchange, for both carbon dioxide and
557 oxygen, smaller bubbles which maximise surface area and a longer bubble retention time in
558 the liquid phase are required.
559

560 **Nutrient source** – For photoautotrophic production, the main nutrient requirements for
561 cyanobacteria are nitrogen, phosphorous, and carbon dioxide with small amounts of minerals
562 and trace metals. The bulk nitrogen and phosphorous can be supplied in industrial forms using
563 commodity chemicals such as urea and diammonium phosphate. Concentrated carbon
564 dioxide sources will be discussed later. Although heterotrophic production of PHB results in
565 higher PHB yields, using organic substrates in an open cultivation facility will drastically
566 increase the likelihood of culture crashes (Troschl, Meixner & Drosg 2017) as observed in pilot
567 production (200L) scale cultures in Austria (Troschl et al. 2017). However, cyanobacteria
568 under phototrophic production will begin to release organic carbon through excretion or cell
569 lysis upon death which can become an energy source for heterotrophic bacteria leading to a
570 crash. If media is reused between cultures then total organic carbon (TOC) should be closely
571 monitored and kept low to prevent culture crashes. Under nitrogen and/or phosphorous
572 limited conditions for PHB production, cyanobacteria cells will have a lower fitness and be
573 more prone to culture crashes too.
574

575 Another potential source of nutrients is wastewater – whether it is integrated into an existing
576 wastewater treatment plant scheme or the run off from agriculture – wastewater is able to
577 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

602 media over time (Mattson 2009). The pH can be lowered and thus controlled by bubbling
603 carbon dioxide into the culture via a feedback response system (Robert, 2005). This
604 simultaneously keeps pH at the optimum range for the strain being grown, while ensuring an
605 excess of carbon for PHB production and biomass growth.
606
607 **Temperature –** The optimum temperature for most cyanobacteria is approximately 25˚C or
608 slightly warmer (Coles & Jones 2000; Robarts & Zohary 1987). However, psychrophile
609 cyanobacteria grow in Arctic and Antarctic climates (Martineau et al. 2013; Nadeau,
610 Milbrandt & Castenholz 2001) and thermophilic cyanobacteria such as those from hot springs
611 thrive in temperatures over 50˚C (Steunou et al. 2006). Although temperature extremes may
612 provide a selective environment for cultivation and help reduce culture crashes, large scale
613 heating or cooling of industrial ponds or photo bioreactors are unlikely to be economically
614 feasible.
615
616 **Selective cultivation conditions –** Other than thermophilic cyanobacteria, there are other
617 species that prefer extreme environments such halophiles or alkalophiles like *Athrospira*
618 *platensis* (Shiraishi 2016) and *Synechocystis* DUN52 (Mohammad, Reed & Stewart 1983). Such
619 strains can be kept in their preferred chemical conditions which are unfavourable for other
620 contaminating microorganisms. PBRs are somewhat difficult to clean and sterilise between
621 runs and ORPs are completely unfeasible to sterilise. Thus, another method to reduce culture
622 crashes is potentially switching between fresh and salt water cyanobacteria species between
623 runs, as this extreme change in environment will likely destroy most contaminating microbes.
624

625 **Final strain choice** – Several factors must be taken into account when choosing the
626 cyanobacterial strain for PHB production. Ultimately, PHB productivity (which is a
627 combination of PHB yield and biomass productivity) will be one of the most important
628 parameters. However, resilience to culture crashes and fluctuations in environmental
629 conditions such as temperature and light are also extremely important. It may be worthwhile
630 having multiple strains or species at a single production site throughout the year based on
631 climatic variations in temperature and light availability. In addition to this, it is important to
632 choose non-harmful bloom-forming species or species that do not release toxic compounds
633 (Henley et al. 2013). Ideally, a PHB production strain would have traits that make it superior
634 for PHB production in a controlled growth facility, but traits that cause it to be less
635 competitive and fit in the wild to reduce impact upon surrounding environmental ecosystems.
636
637 **Cultivation Strategy** – Because PHB yields tend to be low under balanced nutrient conditions,
638 continuous culture and harvesting of cyanobacteria is unlikely to be industrially viable. Thus,
639 the overall strategy for industrial PHB accumulation is similar in essence to that at bench scale.
640 The initial phase is optimised for biomass which is followed by holding the culture at nutrient
641 deprivation with excess carbon present. However, most studies involve growing the culture
642 in balanced media and re-suspending biomass into a nutrient-limited media (normally lacking
643 nitrogen) and then measuring the accumulation of PHB over time. In practice, it will not be
644 cost effective to harvest all algae and re-suspend it in new nutrient-limited media during
645 commercial operation. The most realistic solution is to grow it under batch conditions with
646 continuous excess of carbon dioxide, and wait for nitrogen or the nutrient of limitation for
647 the particular cultivation strain to deplete. The optimal ratios of nutrients must be
648 determined in advance and media will be made to this composition before inoculation. For

649 example, Carpine et al determined that BG-11 with 50% of the nitrate concentration was
650 optimal for PHB accumulation in laboratory scale *Synechocystis* PCC6803 cultures without
651 resuspending cultures in new nutrient deplete media (Carpine et al. 2019). Troschl et al ran
652 pilot plant scale cyanobacterial production runs with approximately 33% nitrate
653 concentration of the original BG-11 formula for a 200 L tubular PBR with *Synechocystis salina*
654 (Troschl, Meixner & Drosg 2017). A key difference to laboratory culturing for PHB is that the
655 nutrient(s) of limitation will run out slowly, as opposed to a sudden flux to zero from re-
656 suspension. As a result, the effect of the nutrient limitation imposed by a slow depletion with
657 constant carbon dioxide similar to industrial cultivation must be investigated before a strain
658 is chosen for industrial cultivation. Such study should determine the optimal nutrient
659 stoichiometry to include in initial media for optimising batch cyanobacterial PHB cultivation.
660 Alternatively or in combination with the above, non-limiting nutrients could be topped up
661 during cultivation or during the PHB accumulation phase. In a recent study by Troschl et al,
662 the 200 L PBR was run for 75 days in which four production cycles were ran with intracellular
663 PHB and glycogen bring monitored. Three distinct phases were observed; the first was
664 phototrophic biomass production with fresh nutrients and distinct green culture (5-6 days)
665 followed by the second stage of lower biomass production, but PHB and glycogen
666 accumulation as nutrients depleted (6-8 days) with a distinct yellow culture observed due to
667 chlorosis. The final phase of the production cycle (6-8 days) showed decreased CO_2
668 consumption with intracellular glycogen decreasing and PHB increasing. Thus a novel idea
669 was conceived of adding a PHB ripening phase at the end of production runs in a stirred tank
670 (as opposed to a PBR or ORP) as this phase was not light dependant as evidenced from the
671 low CO_2 consumption (Troschl et al. 2018). The advantage of this tactic is a lower area
672 footprint of a production facility as the PBRs or ORPs would have a shorter batch time, as

673 cultures can be moved to high volume tanks (with a small footprint) for approximately a
674 quarter of the cultivation time. It is likely these holding tanks would also have lower operating
675 and capital costs than an equivalent volume PBR or ORP.
676
677

678 ### 4.3. HARVESTING AND DOWNSTREAM PROCESSING

679 **Harvesting and dewatering –** Several techniques for harvesting and dewatering
680 cyanobacterial biomass exist and nothing in particular is unique about this step for PHB
681 production compared to previous work done on eukaryotic algae harvesting for other
682 applications such as biofuels (Fasaei et al. 2018). It should be noted that cyanobacteria have
683 smaller cell sizes than most eukaryotic algae (ranging from 0.5-40 micrometers, but generally
684 less than 10). Despite this, process equipment such as filtration, centrifugation flocculation
685 and gravity settling are still applicable for harvesting. For dewatering, vacuum drum dryers,
686 spray drying or rotary drum dryers can all be used (Fasaei et al. 2018). It is important for
687 downstream processing steps, such as solvent extraction, that most of the water content from
688 the biomass is removed. A final water content of 5% or less is recommended as this decreases
689 the polymer molecular weight reduction during processing which can adversely affect
690 material properties (Kosseva & Rusbandi 2018).
691

692 **Downstream processing** – The existing technology for processing and separating PHB from
693 heterotrophic bacterial biomass is also applicable to cyanobacterial biomass (Koller 2015).
694 The two most prevalent options are solvent extraction and biomass digestion (Kosseva &
695 Rusbandi 2018). The principle of solvent extraction relies upon using a chemical compound
696 that selectively dissolves the PHB (often with heating and mixing applied), while leaving

697 residual biomass undissolved. This is then followed by a liquid solid phase separation step
698 such as filtration or centrifugation. The PHB is then precipitated out of solution upon the
699 addition of a second miscible solvent which forces the polymers out of the first solvent. After
700 this step, another liquid-solid phase separation step is carried out to separate the PHA
701 crystals. Commonly used solvents include acetone, chloroform and dichloromethane (Levett
702 et al. 2016). The second form of PHA purification is biomass digestion, whereby an enzyme or
703 harsh alkali / acidic compound selectively dissolves biomass while leaving the PHA polymer
704 undissolved. Sulfuric acid or hypochlorite are examples of such compounds; however, such
705 systems tend to degrade the molecular weight of the polymer molecules and as a result
706 reduce physical and chemical properties of the final product (Fei et al. 2016). Benefits of using
707 enzymes such as lysozymes, nucleases and proteases are their mild operating conditions,
708 selective ability to hydrolyse cell walls and ability to leave PHA polymers undegraded
709 (Kapritchkoff et al. 2006). Current research is also being carried out using supercritical carbon
710 dioxide for PHA separation from biomass (Gumel, Annuar & Chisti 2013); however, this
711 technology comes at higher capital and operational costs (Kosseva & Rusbandi 2018).
712
713 Two key differences exist between heterotrophic bacteria and cyanobacteria PHA processing.
714 Firstly, the yields of cyanobacteria are much lower than that in heterotrophic bacteria (Singh
715 & Mallick 2017). As a result, larger downstream processing equipment to handle larger
716 biomass volumes would be required to obtain the same amount of PHA production capacity
717 than from heterotrophic bacteria. However, this excess PHA extracted biomass also presents
718 on opportunity to be used to create co-products such as food, feed, other plastics or energy
719 production in a biorefinery approach. Secondly, the presence of chlorophyll and pigments in
720 cyanobacteria are potential impurities that could make it through the solvent extraction

721 process. However, purification steps such as washing the PHA crystals with acetone,
722 hypochlorite or using ozone can reduce this impact. Alternatively, a pre-extraction step for
723 pigments and chlorophyll could be used to remove these contaminants and obtain higher
724 value products such as nutraceuticals.

725 ## 5. PHB Economic Assessment

726

727 The bioplastics market was valued to be over $17 billion USD in 2017, and projected to more
728 than double by 2022 to over $43 billion USD. Bioplastics make up roughly 1% of the total
729 plastic market, and of the bioplastics market PHA products make up roughly 1% (Ashter 2016).
730 Currently, only PHA produced through heterotrophic bacteria is commercially available. Table
731 3 shows some of the current global PHA manufacturers. The price of PHA ranges between $2-
732 16 USD per kg or $2,000 - $16,000 USD per tonne (Kosseva & Rusbandi 2018; Levett et al.
733 2016; Reddy et al. 2003) most likely depending on the quality of the final polymer blend.

734

735 *Table 3: Global manufacturers and production volumes of PHA plastic (data adapted from*
736 (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

737

738 Currently, the cost of producing algae biomass has been estimated by several techno
739 economic analysis studies to be in the realm of roughly $500 - $1,200 USD per tonne of
740 biomass (undried post centrifuge in ~20% solid slurry) using different cultivation systems
741 (Dutta, Neto & Coelho 2016; Hoffman et al. 2017). However, other studies concluded
742 significantly higher costs of production of $2,800 - $9,500 USD per tonne of biomass (~20%
743 solid) (Banerjee & Ramaswamy 2019) and $10,000 – $36,000 USD per tonne of biomass (~20%
744 solid) (Banerjee & Ramaswamy 2019). Fasaei et al estimated the cost of drying biomass from
745 slurry to powder form to be roughly $350 - $760 USD per tonne of dry biomass.
746
747 If a baseline PHB yield of 10% is assumed, then roughly 10 tonnes of dried biomass is required
748 for PHB extraction to produce 1 tonne of PHB resin. Therefore, the dried biomass raw material
749 for downstream processing and extracting PHB is already in the realm of $8500 - $19,600 USD
750 per tonne of PHB with optimistic biomass costs, and over $176,000 - $367,600 USD per tonne
751 of PHB with conservative biomass costs. These costs do not yet account for capital and
752 operating costs of PHB extraction from biomass. Thus, compared to the current market price
753 of $2,000 - $16,000 USD per tonne of heterotrophically produced PHB, cyanobacterial is not
754 yet cost competitive. There is also the potential of using residual biomass for other revenue

755 generating applications; however, more detailed cost estimations are required for predicting
756 profitability.
757

758 Panuschka et al were the first to release a techno-economic analysis of cyanobacterial PHB
759 production. Their study used PHB yields of 15% and 60% with a TLS (thin layer system) or
760 tubular PBR in their different scenarios. Additionally, the effect of climate was evaluated by
761 modelling production in a southern Europe and central Europe site. The plant used waste
762 biomass for biogas generation with digestate being recycled for nutrients. In summary, the
763 cheapest cost of PHB resin was $26.4 USD per kg ($26, 400 USD per tonne) assuming a 60%
764 yield using a TLS in southern Europe. However, if yields of 15% are assumed, the price of PHB
765 increases to $103.5 USD per kg ($103,500 USD per tonne) (Panuschka et al. 2019). It should
766 be noted that 60% of total costs for all scenarios was attributed to biomass cultivation and
767 harvesting costs.
768

769 There are several reasons why cyanobacterial PHB production is too expensive and has not
770 yet been commercialised. The cost of cultivating algae or cyanobacterial biomass is more
771 expensive than obtaining biomass from terrestrial crop sources. Despite lower biomass
772 productivities from terrestrial crops, the cost of building and operating ORPs and PBRs is
773 higher than traditional terrestrial agricultural crop production, and this outweighs the
774 economic benefit of a smaller sized algae farm. Secondly, terrestrial crops are easier and
775 cheaper to harvest as the biomass is far more concentrated than that of algae and
776 cyanobacteria in a liquid media (which is approximately 0.1% solid from an ORP). The capital
777 and operational cost of running liquid solid phase extraction process equipment such as

778 centrifuges or filters to harvest biomass of a few grams per litre is a significant expenditure
779 (Fasaei et al. 2018).
780
781 In addition to this, both PHB product and biomass productivity is quite low compared to
782 heterotrophic processes (Singh et al. 2017). Most algae cultures only reach a maximum
783 biomass concentration of a few grams per litre, which is 10-fold to 100-fold less than the
784 concentration that can be achieved with heterotrophic strains. For PHB specifically, one of
785 the highest reported cyanobacterial productivities obtained from genetically modified
786 *Synechocystis* PCC6803 was 7.3 mg/L/day (Carpine et al. 2017). This is in the realm of over ten
787 thousand times lower than heterotrophic bacteria which have PHB productivities reported at
788 over 75,000 – 120,000mg/L/day (Ryu et al. 1997; Wang & Lee 1997). This is due to two
789 reasons; firstly heterotrophic cell yields of PHB are higher (highest reported is over 80% dcw
790 (Wang & Lee 1997)). Secondly, heterotrophic biomass productivity is also significantly higher
791 than photoautotrophic growth.
792
793 In addition their lower volumetric productivity, cyanobacterial PHB production would also
794 require more surface area of land per volume of production as culture must be exposed to
795 light. Because of these two reasons, the area required for a cyanobacterial PHB production
796 facility would be in the magnitude of hundreds to thousands times larger compared to the
797 footprint of a heterotrophic PHB production plant of the same capacity. However, it is
798 important to note that this does not include the area required to grow terrestrial crops to
799 create the organic carbon feedstock required for heterotopic production. It has been
800 reported that roughly 25-50% of heterotrophic PHA production costs come from the cost of
801 the carbon substrate (Halami 2008; Kosseva & Rusbandi 2018). Thus, one of the areas where

802 cyanobacteria are economically superior is that they can be produce PHB from carbon dioxide
803 which can be ideally procured for free with the correct site location and a suitable carbon
804 dioxide emitting source. Potentially, a carbon capture revenue stream could be incorporated
805 into the business model.
806
807 Cyanobacteria also suffer from low PHB yields which are almost always below 10% in wild
808 types, and in most cases well below 2% (Ansari & Fatma 2016; Bhati et al. 2010; Kaewbai-
809 ngam, Incharoensakdi & Monshupanee 2016; Troschl, Meixner & Drosg 2017; Vincenzini et
810 al. 1990). Genetic engineering has not been very successful in increasing PHB yields in
811 cyanobacteria (Troschl, Meixner & Drosg 2017). Low yields also increase the costs of
812 downstream processing, because there is a higher waste product to desired product ratio. It
813 is important that such a facility has an economic use of the residual biomass. Anaerobic
814 digestion offers an opportunity to create renewable energy and recycle nutrients from the
815 digestate; however, the revenue generated is quite small. Other uses for the biomass, such
816 as potential conversion to PLA plastic or bio stimulants for agriculture could be explored.
817
818 Although there are currently significant technical and economic disadvantages to
819 photoautotrophic PHB production using cyanobacteria compared to heterotrophic
820 production, there is a need to overcome these challenges due to the environmental benefits
821 of shifting bioplastics production from terrestrial agriculture. Ultimately, the key driver to
822 achieving financial viability is increasing PHB productivity. This will reduce the capital and
823 operating cost by reducing the area of cultivation required to meet production volumes. This
824 also reduces downstream processing costs with a lower amount of non-PHB 'waste biomass'
825 produced and reducing total biomass entering the downstream processing steps. However,

826 there is also a need for more studies to report on PHB productivity (mass per volume and
827 time) and not just the final yield achieved. Although some studies present yield and time
828 required, without the amount of biomass reported, the PHB productivity cannot be
829 calculated. Ultimately, the most important key to assessing financial viability will be the
830 average PHB productivity of a strain, as this will dictate the required area of cultivation land.
831

832 6. Improving Viability of PHB Production from Cyanobacteria

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

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

841

842 However, for the specific purpose of improving PHB viability from cyanobacteria, the
843 following research areas are suggested:
844

845 **Screening Studies –** Screening studies involve the testing of newly bioprospected strains of
846 cyanobacteria for the presence and yields of PHB in the hopes of discovering elite strains with
847 elevated productivities. Most screening papers investigate one or two cyanobacteria.
848 However, Ansari et al (2016) conducted a screen of 23 strains of cyanobacteria while Kaewbai
849 et al (2016) screened 137 strains for their PHB yields under nutrient limitation environments.

850 Such efforts are important for finding high PHB yielding cyanobacteria for the purpose of
851 cultivation and for the further investigation in understanding the variability in PHB production
852 between different strains and species.
853
854 **In depth Growth & PHB Productivity Optimisation Experiments –** Growing strains with
855 promising initial yields of PHB after screening under a range of different environments is also
856 important. PHB synthesis can be affected by pH, temperature, light conditions and mixing.
857 Understanding which strains produce more or less PHB under different light and temperature
858 could assist with finding different cultivation strains for different climates, locations or
859 seasons throughout the year at the same site. As mentioned previously, growing
860 cyanobacteria for PHB production without resuspension in new media is important for
861 understanding what nutrient composition the initial media should contain.
862
863 **Secretion of PHB –** Secretion of PHB would assist with reducing the costs of downstream
864 processing as separation from residual biomass with steps such as solvent extraction would
865 no longer be needed. Diatoms have been previously been exposed to different stimuli to
866 increase cell wall permeability and secretion of certain metabolites (Vinayak et al. 2015).
867 Exposing cyanobacteria to similar stimuli such as electric fields, ultrasound or certain
868 chemicals could result in secretion of PHB. However there is difficulty in scaling up electric
869 fields or ultrasound technology to industrial scales. Genetic engineering of PHB secretion has
870 been achieved in *E. coli* (Rahman et al. 2013) where phasin proteins bound to PHB granules
871 were secreted through the use of a HlyA signal peptide. Similar approaches could be
872 established in cyanobacteria. Furthermore, Rahman's paper includes a protocol for detecting
873 secreted PHB which is applicable to the chemical and physical stimuli mentioned above.

874

875 **Processing Cyanobacterial Biomass & PHB Biorefinery –** While there have been several
876 research efforts into the optimisation of extracting PHB from heterotrophic biomass (Fiorese
877 et al. 2009; López-Abelairas et al. 2015), there is a need for investigating the separation of
878 PHB from cyanobacterial biomass. This is significant because of the differences in biochemical
879 composition between cyanobacteria and heterotrophic biomass. Due to lower PHB yields in
880 cyanobacteria, there is a greater amount of proteins, carbohydrates and other lipids. In
881 addition, the pigments used for light harvesting in cyanobacteria tend to be soluble in organic
882 solvents, and will degrade the final PHB material properties in a tradition solvent extraction
883 process flow without a pigment removal stage. Meixner et al were the first to investigate a
884 cyanobacterial PHB biorefinery approach and processing optimisation using *Synechocystis*
885 *salina* biomass. It was found that cell disruption and pigment removal produced PHB with
886 superior material properties. The methane production potential for biogas generation was
887 also evaluated. PHB yields of 6-7% were obtained and with additional pigment product yields
888 of total solids (TS) being chlorophylls 0.27–1.98 mg/g TS, carotenoids 0.21–1.51 mg/g TS,
889 phycocyanin 0–127 mg/g TS (Meixner et al. 2018). However, there is still a need to test other
890 processing methods such as acid or alkaline digestion, supercritical CO_2, enzymatic digestion,
891 and also investigate other uses of residual biomass.
892

893 **Growth in presence chemical compounds –** In algal biofuel and high value product research,
894 chemicals compounds that affect the carbon metabolism of microalgae have been
895 investigated for their effect on boosting certain metabolite yields (Commault et al. 2019;
896 Franz et al. 2013). These compounds can act as phytohormones (plant signalling hormones),
897 regulate cyanobacterial metabolic pathways, induce oxidative stress responses or act as

898 direct metabolic precursors (Yu, Chen & Zhang 2015). By growing cyanobacterial cultures in
899 microplates or flasks that is exposed to a chemical library, the effect on PHB productivity can
900 be determined through one of the detection methods mentioned previously in the review
901 such as dye staining or chromatography. Further downstream work such as metabolomics,
902 transcriptomics or proteomics can then elucidate the exact mechanism behind increased PHB
903 productivity and provide further knowledge towards relevant pathways and genes.
904
905 **Mixed culture (consortium) growth –** The vast majority of cyanobacterial PHB papers have
906 used *supposedly* single species cultures. However, PHB production could potentially be
907 altered in the presence of multiple strains of cyanobacteria and other microorganisms. Not
908 only are culture contaminations impossible to realistically avoid at industrial scale PHB
909 cultivation, but mixed microbial communities replicate natural environmental conditions and
910 such consortiums are more resistant to culture crashes too (Lian et al. 2018). A synergistic
911 relationship can occur between oxygen producing / carbon dioxide utilising phototrophs and
912 oxygen respiring / carbon dioxide producing heterotrophs that provide carbon and oxygen to
913 each other (Sutherland et al. 2016). In addition to this, phototrophs and heterotrophs can
914 benefit each other's growth rates through other mechanisms such as the sharing of nutrients,
915 vitamins, hormones and other compounds (Croft et al. 2005; Kazamia et al. 2012; Ramanan
916 et al. 2016) which occurs extensively in natural systems such as microbial mats (Hoschek et
917 al. 2019).
918
919 Arias et al (2018) obtained a mixed cyanobacterial culture from wastewater and exposed it to
920 N and P limitation and different photoperiods, with a maximum PHB concentration of
921 104mg/L and 6.5% dcw yield. PHA yields of 20% were achieved with a mixed photosynthetic

922 consortium exposed to a feast-famine regime, however this was under mixotrophic
923 conditions (Fradinho et al. 2013). In an alternate approach, Löwe et al (2017) and Weiss et al
924 (2017) both used engineered sucrose secreting strains of *Synechococcus elongatus* with
925 *Pseudomonas putida* and *Halomonas boliviensis* respectively. In both of these studies, the
926 phototrophic fixation of carbon dioxide into sucrose was fed to the PHB producing
927 heterotrophic bacteria with PHB productivities of around 25 mg/L/day (Löwe et al. 2017;
928 Weiss, Young & Ducat 2017).
929
930 In addition, the potential for cyanobacterial PHB production in mixotrophic biofilms has yet
931 to be explored. Most recently, a capillary reactor utilising a mixotrophic biofilm consortia of
932 *Synechocystis* PCC 6803 and *Pseudomonas sp. VLB120* achieved extremely high maximum
933 biomass concentrations of around 50g/L dcw (Heuschkel et al. 2019; Hoschek et al. 2019),
934 although PHB yield was not tested.
935
936 Further work on mixed purely photosynthetic cultures for PHB production and growth
937 optimisation of consortiums with different combinations of microorganisms presents much
938 potential for future investigation. However, it is unlikely that fluorescent techniques could be
939 used to quantify PHB accumulation due to the different cell physiologies and dynamic
940 populations over time which would result in a changing fluorescent signal to PHB correlation;
941 however, chemical analysis techniques are still applicable.
942
943 **PHB productivity under different wavelengths of light –** The yield of certain algal compounds
944 under cultivation of different wavelengths of light has been investigated by several
945 researchers (Mohsenpour & Willoughby 2013; Teo et al. 2014). For example, green light was

946 found to enhance chlorophyll production in *Chlorella vulgaris,* whereas red light enhanced
947 phycobilin proteins in *Gloethece membranacea.* Although some studies have been carried out
948 investigating the effect of partial light spectra on cyanobacteria (Luimstra et al. 2018), at this
949 time there are no current studies into how different wavelengths of light would affect PHB
950 yields. It should be noted that for industrial cultivation, using certain wavelengths of light
951 suggests the use of artificial lighting which is most likely economically unfeasible. However,
952 this research could still increase understanding of PHB and its role in cyanobacteria.
953

954 **Wastewater PHB algae production –** Wastewater has been used as a substrate for
955 photosynthetic cyanobacteria and heterotrophic bacteria. *Nostoc muscurom* was grown on
956 waste poultry litter and achieved a 65% yield dcw of PHBV under mixotrophic conditions
957 (Bhati & Mallick 2016). Integrating cyanobacterial cultivation for PHB production into a
958 wastewater treatment scheme has the potential to add an extra revenue line to a potential
959 business model, in addition to remediating water.
960

961 **Random Mutagenesis –** Current approaches to metabolic engineering for a desired
962 phenotype are limited by the requirement for prior knowledge of kinetics, proteomics,
963 transcriptomics, genomics, availability of molecular tools and much more to achieve an
964 effective outcome. However, inducing completely random mutations into a cyanobacterial
965 culture and screening individuals for a desired phenotype (in this case higher PHB productivity
966 and yield through fluorescent activated cell sorting (FACS)) allows for the bypassing of all the
967 previously mentioned limitations of genetic engineering. Keith et al (2006) have developed a
968 methodology for using Nile Red staining to stain *Synechocystis* PCC 6803 mutant libraries for
969 increased PHB yields. Because *Synechocystis* PCC 6803 only has a yield of ~5-10% PHB under

970 phototrophic conditions, the potential for creating mutants of other cyanobacterial species
971 (such as *Calothrix* sp.) with higher naturally occurring PHB yields provides a higher baseline
972 for desirable mutations to occur. Recently, Kamravanesh et al used UV radiation to increase
973 yields of PHB from 16% to 37% in *Synechocystis* PCC 6714. The mutant strain with this high
974 yield was shown to have a single amino acid missense mutation in an ABC phosphate
975 transporter. This was hypothesised to cause a cascade of signalling and regulation changes
976 compared to the wild type under phosphate-limited conditions, resulting in the higher PHB
977 yield phenotype. Further mutagenesis work can still create other increased PHB yielding
978 mutants with novel genetic mutations which can be further investigated as candidates for
979 targeted genetic engineering.
980
981 **Targeted Genetic Engineering -** The cyanobacterial model organism *Synechocystis* PC 6803 is
982 widely used for photosynthetic studies and has had its genome sequenced for several decades
983 now with mutant variants readily available (Anderson & McIntosh 1991; Kaneko & Tabata
984 1997). It has been successfully transformed in a number of studies and has been used for the
985 investigation of producing PHB as well as biohydrogen, isoprene and other chemical
986 commodities (Touloupakis et al. 2016). Over-expression of natural PHB genes led to an
987 increase from 10% PHB yield to 26% yield in *Synechocystis* PCC6803 (Khetkorn et al. 2016).
988 The promoter of the Rubisco gene has been successfully used as a promoter for PHA
989 expression systems in *Synechocystis* PCC6803. The rationale behind this being that Rubisco
990 activity is strongly upregulated in the presence of increased carbon dioxide and regulation of
991 PHA synthesis could be achieved with varying carbon dioxide concentration (Miyasaka et al.
992 2013).
993

994 In another study, *Synechococcus* sp. PCC7942, which cannot naturally synthesise PHA, was
995 genetically engineered with introduced additional *Cupriavidus nectator* PHA synthesis genes
996 which improved yields from 3% to 25% dcw under phototrophic and heterotrophic conditions,
997 respectively (Takahashi et al. 1998). *Synechococcus* PCC7002 was used by Akiyama et al (2011)
998 to develop a non-antibiotic plasmid expression system where the recA gene (encoding for an
999 essential DNA repair enzyme) was included in a plasmid cassette and introduced into a recA
1000 deficient mutant cyanobacteria. This allowed for greater plasmid stability as the recA gene is
1001 required for survival. Antibiotic resistance genes used for screening in traditional plasmid
1002 expression systems tend to be excluded from the cell in the absence of antibiotics, and
1003 industrial cultivation with antibiotics is not an economically nor environmentally feasible
1004 option. The PHA genes used were from *Cupriavidus nectator* and a 52% PHB yield was
1005 obtained; however this was under heterotrophic conditions. A photoautotrophic only PHB
1006 yield was not reported (Akiyama et al. 2011).
1007
1008 Wang et al (2013) engineered a *Synechocystis* PCC 6803 strain optimised for producing the
1009 PHB monomer hydroxybutyrate (both (S)- and (R)-3-hydroxybutyrate (3HB)) through
1010 inactivation of PHB polymerase). Two additional pathways for producing the monomers from
1011 Acetyl-CoA were also introduced. The monomers were readily secreted without changing
1012 transporter expression and titres of 533.4mg/L 3HB were obtained. Most recently, Carpine et
1013 al engineered *Synechocystis* PCC 6803 focussing on central carbon metabolism as opposed to
1014 over-expression or introducing PHB synthesis genes. Three different target areas including
1015 deletions of phosphotransacetylase (Pta) and acetyl-CoA hydrolase (Ach) and the expression
1016 of a heterologous phosphoketolase (XfpK) from *Bifidobacterium breve* produced 12% PHB
1017 yield, titre of 232 mg/L and a productivity of 7.3 mg/L/day which was reported to be the

1018 highest to date. Lastly, the eukaryotic algae *Chlamydomonas reinhardtii* and *Phaeodactylum*
1019 *tricornutum* were genetically modified to express introduced PHA synthesis genes and
1020 achieved yields of 6 x 10^{-4} % and 10.6% PHB respectively (Chaogang et al. 2010; Hempel et al.
1021 2011).
1022
1023 Future work in this area could include screening other species of eukaryotic algae with high
1024 pools of Acetyl-CoA for introduction of PHB synthesis genes. Hempel et al were able to
1025 achieve a 10% yield without any further optimisation such as plastid targeting, codon
1026 optimisation, nuclear integration or using a non-inducible promoter. Suppression of glycogen
1027 or other lipid metabolite biosynthetic pathways to increase the carbon flow to the PHB
1028 pathway is also promising. Lastly, creating a PHB de-polymerase knockout cyanobacteria that
1029 is unable to metabolise stored intracellular PHB could result in higher yields and PHB
1030 productivity.
1031

1032 7. Conclusion

1033 For a sustainable future, we must shift to using biodegradable and renewably sourced
1034 bioplastic materials. However, high raw materials costs for heterotrophically produced
1035 bioplastic and environmental impacts from terrestrial agriculture mean that PHB production
1036 from atmospheric carbon dioxide using cyanobacteria provides a promising path forward.
1037 This review has covered various topics from the central PHB metabolism, material properties
1038 and associated methods and protocols. While there is still uncertainty on the exact role of
1039 PHB in the cell, it is likely it is used as an energy storage compound and regulator of excess
1040 reducing charge. Industrial cultivation issues of cyanobacterial PHB and economic factors
1041 regarding why industrial commercialisation is not yet economically viable have also been

1042 examined. Ultimately, the two key factors to achieving profitability are higher PHB
1043 productivity and cheaper cyanobacterial cultivation equipment. Several promising research
1044 areas for improving cyanobacterial PHB viability have been reviewed including screening,
1045 genetic modification, wastewater cultivation, downstream processing and growth
1046 optimisation.

1047

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1392 *green plastics: PLA and PHBV*, Woodhead Publishing Series in Composites Science and Engineering, pp.
1393 141–60.
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1395 ## Figure Captions

1396 *Figure 1: Molecular structure of PHAs with R representing possible aliphatic functional*

1397 *groups*

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1399 *Figure 2: Four species of algae capable of PHB production; (**A**) Athrospira maxima*

1400 *(**B**) Oscillatoria jasorvensis (**C**) Synechocystis PCC6803 (**D**) Nostoc muscorum*

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1402 *Figure 3: Major cyanobacterial storage polymers (information adapted from* (Flores &

1403 Herrero 2014)*)*

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1405 *Figure 4: **(A)** Metabolic pathway of PHB synthesis in* Synechocystic *PCC 6803 **(B)***

1406 *Molecular structures of PHB, PHV and PHBV*

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1408 *Figure 5: Molecular structure of PHB and polypropylene (PP)*

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1411 *tubular PBR with pump for mixing*

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1415 ## Tables and Figures

1416 *Table 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).

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1419 *Table 2: Comparison of physical properties between PHB, polypropylene (PP) and other*

1420 *PHAs (data adapted from* (Anbukarasu, Sauvageau & Elias 2015; Balaji, Gopi &

1421 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

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1424 *Table 3: Global manufacturers and production volumes of PHA plastic (data adapted*

1425 *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

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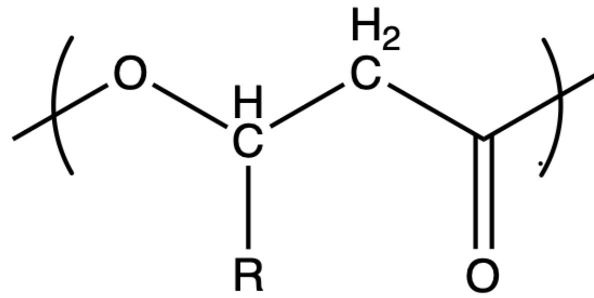

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R = hydrogen	Poly(3-hydroxypropionate)
R = methyl	Poly(3-Hydroxybutyrate)
R = ethyl	Poly(3-hydroxyvalerate)
R = propyl	Poly(3-hydroxyhexanoate)
R = pentyl	Poly(3-hydroxyoctanoate)
R = nonyl	Poly(3-hydroxydodecanoate)

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1432 *groups*

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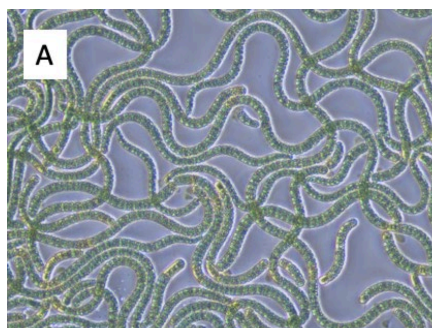

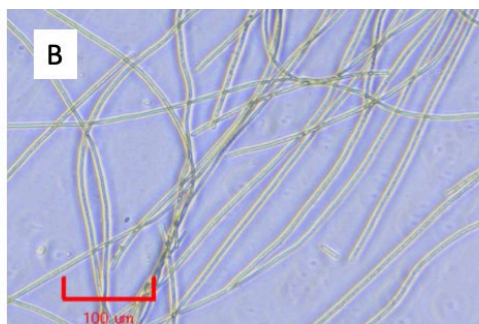

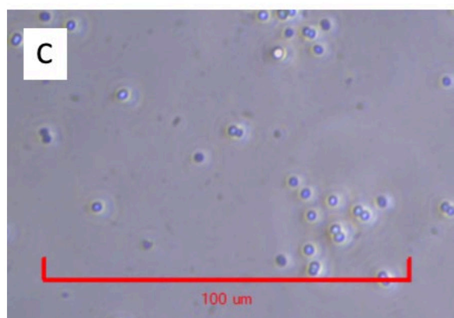

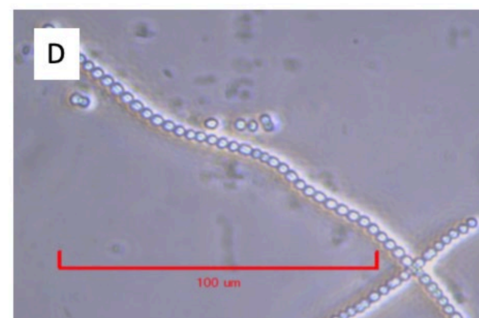

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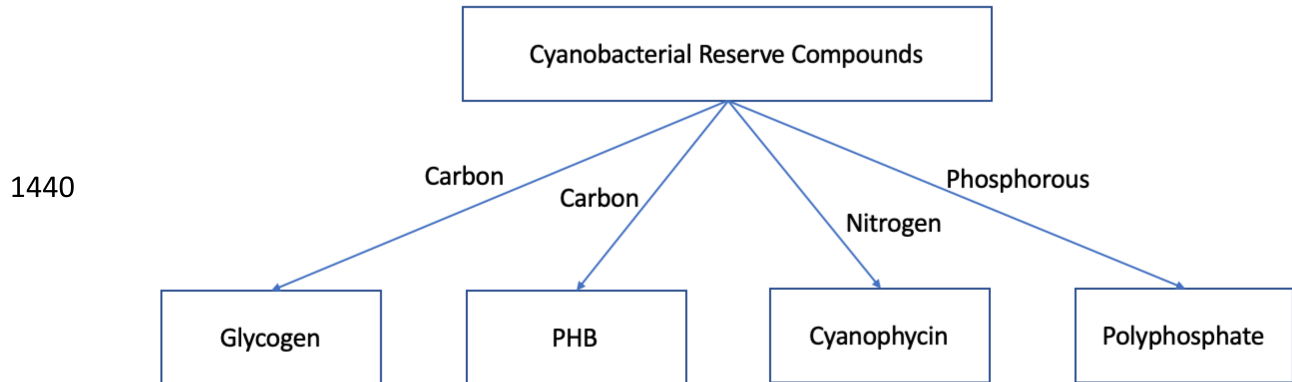

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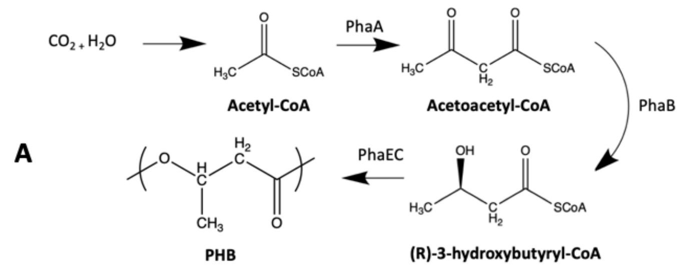

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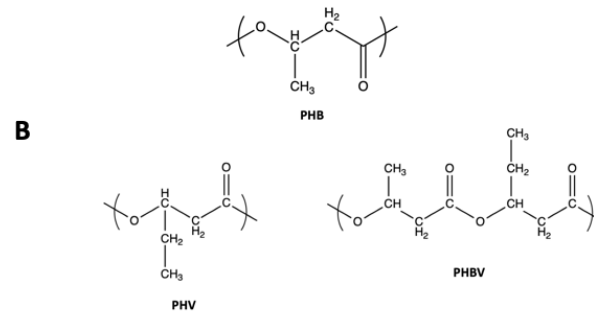

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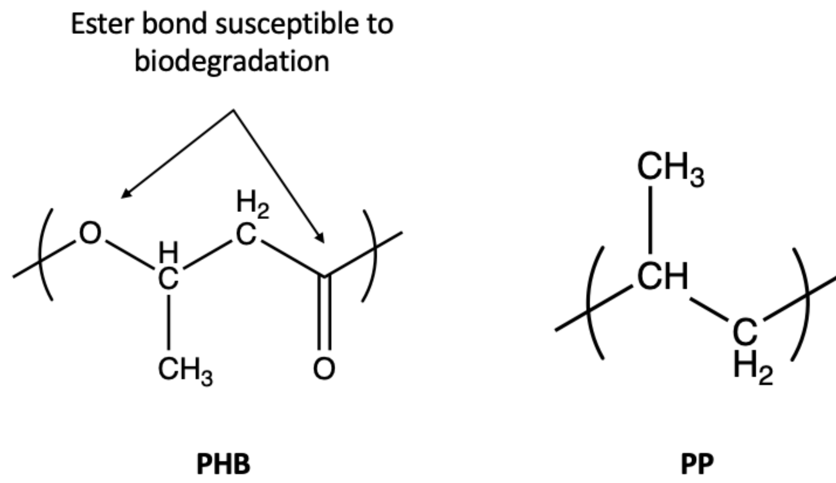

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