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Poly(3-hydroxybutyrate) biosynthesis by *Cupriavidus necator*: a review on waste substrates utilization for a circular economy approach

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Abstract

Cupriavidus necator owes its popularity to the ability to produce large amounts of the polyester poly(3-hydroxybutyric acid), or PHB. During the last decades, *C. necator* has earned an increasing interest in the field of biopolymers and bioplastic production. This versatile bacterium can grow both autotrophically on a mixture of gases (e.g. hydrogen, oxygen, and carbon dioxide) and heterotrophically, on a wide range of organic substrates, including waste feedstocks. In anoxic conditions, it can also perform denitrification by using nitrate as the electron acceptor.

The goal of this review is to underline the versatility of *C. necator* and its ability to produce biopolymers on a wide variety of carbon sources, comparing pure and waste substrates, used in circular economy biobased PHA production. This study highlights the most suitable feeding strategies to reach the highest PHB accumulation, underlying the important role that *C. necator* could play for next generation biopolymers.

1.1 Introduction

The discovery of polyethylene in 1933 and the industrial economic boom led to the widespread use of these petrochemical plastics in the manufacturing industry. They were used for the production of several everyday goods, from kitchenware to cases for electronic devices, as well as for surgical applications and packaging. Conventional plastics have totally changed our World because they are cheap and durable, easy to process and highly resistant to chemical and biological degradation. In 2003, the annual production of thermoplastic resins (which includes poly(ethylene), poly(propylene), poly(styrene) and poly(vinyl) chloride)

27 was about 33 million metric tons in the United States, 30 million tons in the EU, and 35 million tons in China,
28 Japan and Korea. Incredibly these figures have continued to increase since 2003. However, this huge rise in
29 the use of plastic has resulted in a proportional increase of plastic pollution. Water and landfill pollution are
30 becoming an urgent daily issue that global politics and international agreements can no longer avoid. Ryberg
31 et al. (2019) reported that out of the 322 million tonnes (Mt) of plastics (excluding elastomers and synthetic
32 fibres, which would lead to 388 Mt of plastics) produced globally in 2015, around 6.2 (Mt) of macro-plastics,
33 which are larger than 5 mm, and 3 Mt of micro-plastics (smaller than 3 mm) were found in the environment
34 (Ryberg et al., 2019).

35 In this scenario, the urgent environmental need for a valid and sustainable substitute material is
36 evident. This need requires the investigation and development of harmless, economically competitive and
37 biodegradable polymers, which are open to production perspectives with more enthralling economic
38 principles and applications like those concerning circular economy approaches.

39 The currently available biodegradable biopolymers, also known as “green polymers”, are being
40 intensively studied as a replacement for conventional plastics. Three important examples of biopolymers are
41 the following: poly(hydroxyalkanoates) (PHAs), poly(lactic acid) (PLA) and poly(butylene succinate) (PBS).
42 Their wide potential is supported by their competitive physicochemical, thermal, and mechanical properties
43 which are similar to those of poly(propylene) (PP) and low-density poly(ethylene) (LDPE) (Singh Saharan et
44 al., 2014). The main difference between PLA and PBS production with respect to PHA synthesis is that they
45 can be produced upon synthetic and enzymatic polymerization of lactic and succinic acid respectively, while
46 PHA polymerization can be (and actually is) mainly performed by bioplastic producer bacteria and archaea
47 (Kourmentza et al., 2017b). Furthermore, PHA fermentation strategies aim to reduce environmental impact
48 and production costs, thus encouraging the development of fermentation lines based on wastes utilization,
49 in agreement with the circular economy perspective (Karan et al., 2019).

50 So far, a wide repertoire of both bacteria and archaea has been tested and will continue to be used for
51 PHA fermentative production. PHAs are synthesized by bacteria as storage material, preferably under
52 restricted growth conditions (mainly during a lack of nitrogen, phosphorous or oxygen and an excess of a
53 carbon source (Shang et al., 2003)). The highest performing PHA producing hosts are *Cupriavidus necator*,

54 *Pseudomonas* sp. (*Pseudomonas putida*), *Alcaligenes* sp. (mainly *Azohydromonas australica*) *Bacillus*
55 *megaterium*, *Aeromonas* sp. and *Burkholderia* sp. Among these, *Cupriavidus necator* and *Azohydromonas lata*
56 represent the most suitable PHA producing organisms studied so far, with the highest production capacity
57 (10,000 t/a of poly(3-hydroxybutyrate for *C. necator*) (Singh Saharan et al., 2014). *C. necator* can accumulate
58 up to 90% of PHB per cell dry weight guaranteeing high biomass yields at the same time. The resulting
59 polymer consists of short-chain-length (SCL) monomers only (3–5 carbon atoms) (Hanisch et al., 2006).
60 Furthermore, the *C. necator* genome has also been intensively studied to understand biopolymer
61 biosynthesis. The genes encoding the three-step biosynthetic enzymes were discovered in the late 1980s and
62 they were cloned from *C. necator* H16 (wild type strain), in three independent laboratories (Schubert et al.,
63 1988). These genes were able to codify three classes of enzymes: the first enzyme, acting in the polyesters
64 biosynthesis, is a 3-ketothiolase (PhaA), which condenses two molecules of acetyl-CoA to acetoacetyl-CoA,
65 followed by the NADPH dependent acetoacetyl-CoA reductase (PhaB1, PhaB2 and PhaB3), which reduces
66 acetoacetyl-CoA to R-(–)-3-hydroxybutyryl-CoA, and PHB synthase (PhaC1), aimed to the polymerization of
67 R-(–)-3-hydroxybutyryl-CoA (Schubert et al., 1988).

68 *C. necator* as well as other microorganisms which naturally produce PHB, have a significant role to play
69 in the green economy and circular economy perspectives. Current PHA production is still based on refined
70 sugars, with an estimated production cost which is 3-4 times higher than PP and PE polymers (about US\$0.60–
71 0.87/lb) and this impairs their industrialization and commercialization (Kourmentza et al., 2017b). Therefore,
72 using *C. necator* fermentation to produce PHB may overcome the major issue of the overall production costs
73 when waste materials are used as the substrates.

74 In this review, we report the major advantages of the use of PHB as an optimal replacement for
75 conventional petrochemical polymers when its biosynthesis occurs through *C. necator* fermentation using
76 different substrates and very high cell densities (>200 g/L). Here we report the analysis and the comparison
77 of the biopolymer production yield and present the main waste substrates used so far for *C. necator*
78 fermentative PHB production, in order to highlight their potential large-scale applications in circular economy
79 strategies.

2.1 Overview on biopolymers industrial and economic trends

Sustainability of biopolymer production processes is one of the major advantages for their use, in particular for PHA production. However, PHA biosynthesis has some limits. One bottleneck is represented by the substrates used by the fermentative bacteria to obtain PHA. In fact, pure substrates, such as pure glucose, pure oils, ethanol, glycerol, may increase the overall production costs (Haas et al., 2008; Koller et al., 2017).

Purified sugar is the substrate of choice for fermentative bacteria: it is purified from plant sugar resources in the foodstuff industry (Kookos et al., 2019). It is quite expensive and inconvenient to use for these purposes: it derives from the food industry, which raises not only economic issues, but ethical concerns too (Kim et al., 2016). For these reasons, the inedible plant waste materials, such as agro or industrial by-products, would be perfect and convenient candidates for PHA production, from the economic, environmental, and ethical points of view. The availability of wastes for use as “renewable” materials and the increasing demand for the use of biodegradable polymers, as well as the favorable green international agreements and business policies, are expected to benefit PHA market growth (www.marketsandmarkets.com).

Nowadays, the bioplastics and biopolymers markets have a size projection of USD 27.9 billion by 2025. Of this huge investment value, the production of PHA via biotechnological routes is characterized by a compound annual growth rate (CARG) of 11.2%, and it will account for 98 million USD by 2024. Recently, as published in a report in 2017, the global PHA market, which was estimated at US \$73.6 million in 2016, is likely to reach US \$ 93.5 million by 2021, and it will be characterized by a compound annual growth rate (CAGR) of 4.88% (Kourmentza et al., 2017; www.marketsandmarkets.com).

In 2017, conventional petroleum based plastic prices ranged from 0.57 EUR/kg to 1.59 EUR/kg (Karan et al., 2019). The price of the widely marketed corn starch-based bioplastic would have a more competitive commercial value, because of the very attractive raw starting material commercial value (0.34 EUR/kg), but some other biopolymers still need to reach a competitive price, as reported below, including PHA.

2.2 PHA industrial applications

105 Nowadays, PHA are gaining a significant role in the packaging sector, but also in the food and beverage
106 area for bags, sheets and disposable cutlery, mainly supported by the increasing interest of Pacific Asia and
107 Latin America countries (www.marketsandmarkets.com). Despite some cases of PHA resin synthesis
108 obtained via enzymatic-based production, performed by pioneer U.S.A. companies (www.newlight.com), at
109 the pilot or industrial scale, bioplastic production is mainly carried on through sugar-based fermentations. In
110 this case, single or multiple mixed bacterial cultures convert different carbon sources (e.g. sucrose, fatty
111 acids, glycerol) into bioplastic polymers.

112 The selected carbon source is one of several factors affecting the overall production outcome for
113 bioplastic production, which could be generally estimated by analyzing the process from two different
114 perspectives: one is related to the all upstream processes, which regard the chosen settings of fermentation
115 (modes of fermentation to reach specific yield and rate of product, bioreactors, fermentation strategies such
116 as discontinuous batch and fed-batch cultivation modes, chosen substrates, polymers producing bacteria
117 fermentation costs and required conditions, etc.), and the other one concerns the downstream processes
118 costs, involving the purification procedures (solvents, time and labor required), analysis of the product, etc
119 (Fernández Dacosta, 2018; Koller et al., 2017; Kookos et al., 2019).

120 Currently, a lot of competitive companies are actively working on these topics worldwide. In order to
121 understand the variety of the factors which may change and affect biopolymer production, some of the major
122 PHA producing companies are compared below for product, substrate, biocatalyst and production capacity.

123 **2.3 Poly(3-hydroxybutyrate) and co-polymers: chemical and physical properties and industrial applications**

124 PHB is a biopolymer belonging to the PHA biopolymeric group, classified upon the structure of the
125 monomeric unit. PHA can be divided into short-chain length (scl), which consist of 3–5 carbon atoms, or
126 medium chain length (mcl), which consist of 6–14 carbon atoms. These structural arrangements can also
127 coexist in the same polymer, conferring specific chemical and physical properties to the biopolymer, such as
128 crystallinity, melting temperature, glass transition temperature, degree of polymerisation (Khanna and
129 Srivastava, 2005). PHB structure consists in 3-C carbon backbone hosting a methyl substituent in position 3.

130 PHB is a stable thermoplastic and a solid and rigid polymer. Its melting point ranges between 168-182
131 °C, the glass transition temperature around 2-4°C, and the elongation to break between 5-8%. PHB is also
132 characterized by a density of 1.18 – 1.26 g cm⁻³ (Georgios et al., 2016). Thanks to these features it can be
133 easily used in several sectors. In particular, PHB is already diffused in the packaging sector, where PHB is
134 employed to make bioplastics bags, sheets and disposable cutlery. It is also used to produce moulded goods,
135 paper coatings, non-woven fabrics, adhesives, films and performance additives, as well as for biomedical
136 purposes (Bugnicourt et al., 2014).

137 However, despite these interesting properties, PHB is also known to have high degree of crystallinity
138 and to be brittle, stiff and possess (Sudesh et al., 2000). Therefore, this biopolymer can also be used in a
139 blend with conventional petroleum derived plastic or with natural or inorganic fibres. Usually, PHA
140 copolyesters consisting of PHB and medium-chain-length-3-hydroxyalkanoates have been expected to be
141 flexible and practical, unlike PHB homopolymer; this is due to their similar properties to several common
142 petroleum-based plastics (Insomphun et al., 2015). Still, PHB can also be chemically modified by different
143 synthetic functional groups, resulting in a material with a new range of properties, in order to expand the
144 properties and potential applications (Yeo et al., 2018).

145 Several studies have been focused on the development of new monomers and new polymer
146 compositions. The aim is to obtain a more commerciable copolymer, and this has been done, for instance,
147 by adding some SCL or MCL monomers to PHB. Among these comonomers that have been incorporated into
148 PHB, there are: 4-hydroxybutyrate (4HB) and 3-hydroxyvalerate (3HV), and MCL monomers such as 3-
149 hydroxyhexanoate (3HHx), and also 3-hydroxy-4-methylvalerate (3H4MV) (Doi et al., 1995). This last MCL has
150 been studied in a recent study, in which a novel PHA containing 3-hydroxy-4-methylvalerate (3H4MV) has
151 been synthesized using the transformed strain of *Cupriavidus necator* PHB4. This strain harbors the PHA
152 synthase gene of *Chromobacterium* sp. USM2 (phaCCs). (Chia et al., 2010). Researchers obtained a novel PHA
153 short chain length and medium chain length (SCLeMCL) polymer, which contains four different monomers
154 (3HB, 3HV, 3H4MV and 3HHx). It was discovered that films made with this novel SCLeMCL polymer showed
155 interesting elasticity and flexibility properties, since it was able to contract after being released from

156 stretching. Moreover, the soil surface degradation of this novel polymer film was found to be faster than PHB
157 polymer film (Chia et al., 2010).

158 In another study, an improved artificial pathway of *C. necator* for the biosynthesis of P(3HB-co-3HHx)
159 with high 3HHx composition has been established (Insomphun et al., 2015). An effective deletion of *phaB1*
160 gene, which causes the depression of (R)-specific reduction of acetoacetyl-CoA, led to the formation of the
161 C6–monomer unit from fructose driven by crotonyl-CoA carboxylase/reductase (Ccr). Furthermore, the co-
162 overexpression of *phaJ4a* gene, which encodes medium-chain-length (R)-enoyl-CoA hydratase, with *ccr*
163 promoted the incorporation of both 3HB and 3HHx units into the polymer (Insomphun et al., 2015). Further
164 introduction of *emdMm* synthetic gene, encoding for an ethylmalonyl-CoA decarboxylase derived from
165 mouse, shown to be remarkably effective for P(3HB-co-3HHx) biosynthesis, probably by converting
166 ethylmalonyl-CoA into butyryl-CoA. Finally, a remarkable cellular content of P(3HB-co-3HHx) composed of 22
167 mol% 3HHx has been synthesized starting from fructose using this engineered strain of *C. necator* with
168 Δ phaB1 genotype expressing *ccr*, *phaJ4a*, and *emd* (Insomphun et al., 2015).

169 The biosynthesis of PHA is directly dependent on the type of carbon sources supplied, but carbon
170 sources can also affect the type of synthesized polymer, which may lead to two different groups of carbon
171 sinks: the structurally related carbon sources and the structurally unrelated carbon sources (Murugan et al.,
172 2017; Wang et al., 2013). Therefore, beside genetic engineering, also the combination of different substrates
173 can be used to induce different copolymers production. In one study, different combinations of palm olein
174 (POI) and fructose was furnished as carbon sinks to recombinant *C. necator* Re2058/pCB113 for the
175 biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) [P(3HB-co-3HHx)] (Murugan et al., 2017). A
176 cell dry weight (CDW) of 5.13 g/L, 67% PHA/CDW and accumulated a copolymer containing 27 mol% 3HHx in
177 shake flask cultures have been obtained in cultures supplied with 5 g/L PO alone as carbon source. When 5
178 g/L fructose was supplied alone as the carbon sink the cultures produced CDW of 2.32 g/L, 11% PHA/CDW
179 and accumulated only PHB homopolymer. Then, using a co-culturing combination of 5 g/L POI and 7 g/L fructose,
180 CDW of 7.41 g/L and 80% PHA/CDW was obtained with 17 mol% 3HHx monomer fraction. (Murugan et al.,
181 2017).

182 In this review, substrates and waste substrates for PHB biosynthesis are the main topics. Therefore,
183 the reader is invited to consider this section about copolymers as an appendix of the review which might be
184 useful to make evaluations on the type of polymers (homopolymer or copolymer) which could be produced,
185 considering both the final products aim, but also the type of substrates or waste substrates reported in this
186 review which can be used to accumulate bioplastics in *C. necator* and which may affect the final polymer
187 properties.

188 **2.4. Biological PHA production**

189 Commonly, the PHA produced through fermentation by naturally producing bioplastic organisms are
190 accumulated in the form of granules named “*carbonosomes*”. These granules vary in number and size
191 depending on the bacterium species, but usually have a diameter of about 500 nm (Jendrossek, 2009). In
192 microorganisms (e.g. *C. necator*), mobilization of PHB granules mainly occurs during carbon starvation and it
193 requires specific enzymes for the depolymerization of PHB, which is used as carbon sources (Jendrossek,
194 2009).

195 So far, thanks to improvements in fermentation techniques and genetic manipulation approaches, a
196 wide repertoire of microorganisms has been investigated for PHA natural occurring biosynthesis. The
197 knowledge of these bacterial genomes and their biosynthetic pathways can help to perform genetic
198 engineering manipulation in order to increase the biopolymer yield and rate of biosynthesis.

199 In particular, the PHA biosynthetic pathway is commonly linked to all the bacterium’s central metabolic
200 pathways, such as glycolysis, as well as Krebs Cycle, Calvin Cycle, β -oxidation, de novo fatty acids synthesis,
201 amino acid catabolism and serine pathways (Lu et al., 2009; Yamane, 1993). In fact, several intermediates of
202 these main metabolic pathways are shared with PHA biosynthesis. The most important shared intermediate
203 is an acetyl-CoA molecule, that is the staple unit for the biopolymer synthesis.

204 For the majority and most utilized biopolymer producing strains, the main metabolic flux for PHA
205 synthesis, which could be called the “classical biosynthetic pathway”, starts from sugar derived acetyl-CoA
206 to form PHA (Pohlmann et al., 2006). As many others, this flux is strongly dependent on the nutrient

207 conditions: under nutrient-rich conditions the coenzyme A, produced from the Krebs Cycle, has high content
208 and acts as an inhibitor for the 3-ketothiolase enzyme (PhaA). This is the first enzyme acting in the PHA
209 metabolic flux and causes the channelling of acetyl-CoA towards PHB biosynthesis. On the contrary, under
210 nutrient-shortage conditions, i.e. when the excess of carbon sources coexist with limited concentrations of
211 essential nutrients, such as phosphorus and nitrogen, the low coenzyme A concentration does not impair the
212 activity of the PhaA enzyme, thus allowing acetyl-CoA to be directed towards the PHAs synthetic pathway.

213 The development of competitive bioprocesses for engineering biocatalysts, such as high producing
214 PHA bacteria, are potentially important potential on industrial scale, as they can supply the production of
215 large amounts of biopolymers, especially PHAs, at low prices (Chen, 2012). Also *Haloarchaea* have recently
216 risen special interest due to the capability of some species to produce significant concentrations of
217 polyhydroxyalkanoate (PHA), polyhydroxybutyrate (PHB), and polyhydroxyvalerate (PHV), with some
218 advantages with respect mesophilic bacteria (e.g. these are vulnerable to contamination compared to
219 extremophiles) (Simó-Cabrera et al., 2021). Genereally, industrial biotechnology applications require non-
220 pathogenic, fast growing bacteria that do not produce toxins and have genomes that can be easily
221 manipulated, if needed. The attempt to reduce PHA production costs mainly concerns the use of engineering
222 strains that show higher PHA production efficiency, especially those growing and fermenting on raw and/or
223 waste material, which may further minimize the costs, and therefore also requiring also less energy
224 consumption during the PHA biosynthesis and accumulation processes. It is important to remember that
225 each of the microorganisms fermentation steps and conditions must be studied and evaluated in order to
226 minimize the costs and optimize the performance (Wang et al., 2014) and this is why downstream processing
227 simplification may affect the overall costs and yield too.

228 Engineering strategies for PHAs accumulation more often may involve genes related to the oxygen
229 uptake, since oxygen limitation may occur after obtaining high cell densities when PHB production initiate,
230 but also in genes related to PHAs biosynthetic pathways: these and other modifications may enhance PHAs
231 production (Wang et al., 2014). For instance, as reported in a relevant and recent study, PHB pathway
232 optimization has been also investigated in *E. coli* by modifying the expression levels of the three genes *phbC*,
233 *phbA*, and *phbB*, from *phbCAB* operon, which was cloned from the PHAs producing strain *C. necator*:

234 researchers created several bacterial strains, including those accumulating cellular contents of 0 to 92%
235 gPHB/gCDW (Li et al., 2016). This study is considered a pioneer in the demonstration of this semi-rational
236 approach, because it combines library design, construction, and screening as an efficient tool aimed to
237 optimize PHB production (Li et al., 2016).

238 As already mentioned, one of the most promising and well-known bacteria for PHAs production, but
239 especially for PHB biosynthesis, is *Cupriavidus necator*. Wild type *C. necator*, as well as several wild type and
240 mutated strains, have been studied so far for biopolymer accumulation, in order to find the most suitable
241 growth substrate and feeding fermentation strategy. For this reason, here we report the general features of
242 this bacterium and we analyzed a wide repertoire of substrates used so far to improve its PHB biosynthesis
243 and accumulation.

244 **3.1 *Cupriavidus necator***

245 The Gram-negative bacterium *Cupriavidus necator* has changed many names since its isolation from a
246 spring near Göttingen in 1960's (Bowien and Schlegel, 1981) because of its affiliation to different taxa, and
247 different names are still used. The wild type strain H16 was isolated as a member of the genus
248 *Hydrogenomonas*, but in 1969 it was transferred to the genus *Alcaligenes*, then in 1995 the name was
249 changed to *Rashtonia eutropha* H16 (Yabuuchi* et al., 1995), which was valid until 2004, when it became first
250 *Wautersia eutropha* and in the same year changed to *Cupriavidus necator* (Vandamme and Coenye, 2004).
251 Any case, the established name *C. necator* is still the most frequently used in literature, reported as a
252 facultative chemolithoautotrophic β -proteobacterium. It is important to remind that the wild type strain of
253 *C. necator* has several names: H16 (the most representative one), ATCC 17699, DSM 428, KCTC 22496, NCIMB
254 10442, Stanier 337 (Pohlmann et al., 2006; Schwartz et al., 2003).

255 This bacterium has the very favorable capability to perform both in autotrophic and heterotrophic
256 growth. When organic compounds are not available, microbial growth rate during lithoautotrophic growth
257 condition of *C. necator* is regulated by the supplying rate of the gaseous substrates, thus the bacterium
258 activates autotrophic CO₂ fixation via Calvin–Benson–Bassham (CBB) cycle and NiFe hydrogenases to oxidize
259 H₂ (Schwartz et al., 2003). *C. necator* has the ability to grow also in anoxic conditions, during which

260 denitrification is performed by using nitrate as electron acceptor. Alternatively, the organo-autotrophic
261 growth is carried on using formate as an energy source (Friedrich et al., 1979).

262 *C. necator* can metabolize several organic carbon sources, including sugar acids, amino acids, fatty
263 acids, alcohols, and aromatic compounds, TCA cycle intermediates, during heterotrophic growth,. This wide
264 repertoire and *C. necator* versatility enhance its industrial applications. For the wild type strain, sugars
265 utilization is mainly limited to fructose and N-acetylglucosamine, which are exclusively catabolized via the
266 Entner–Doudoroff pathway by its key enzyme 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC
267 4.1.2.14) (Johnson and Stanier, 1971; Pohlmann et al., 2006).

268 This ‘Knallgas’ bacterium became very popular thanks to its ability to accumulate large amounts of the
269 polyester PHB (about 90 %, wt/wt of cell dry weight nutrient shortage conditions) (Priefert et al., 1991),
270 providing the basis for renewable resource-based biodegradable plastic materials.

271 *C. necator* H16 genome has been recently sequenced and published (Pohlmann et al., 2006), allowing
272 the increase of genetic and metabolic studies, allowing several genetic modifications and encouraging
273 different industrial applications of this very versatile microorganism. *C. necator* genome comprises 7,416,678
274 bp, of which 6,626 coding sequences (CDS) were identified. Almost all essential genes for the metabolism
275 and for other essential cell functions are on Chromosome 1 (4,052,032 bp, 3,651 CDS), while a megaplasmid
276 and chromosome 2 (2,912,490 bp, 2,555 CDS) encode a repertoire of genes related to the metabolism of a
277 wide range of substrates and terminal electron acceptors, involved in CO₂ fixation, H₂ oxidation,
278 denitrification, etc., and contain only very few essential genes (Pohlmann et al., 2006). The presence of these
279 genes, encoding for enzymes involved in organic acid metabolism, allow the heterotrophic growth of *C.*
280 *necator*; these all genes are located on chromosome 1 (Pohlmann et al., 2006).

281 **3.2 *C. necator* industrial applications**

282 Although *C. necator* has the great potential to be easily engineered to produce valuable compounds
283 directly from CO₂, a lot of effort still must be done to develop genetic libraries to enable such attempts in an
284 optimal way. A variety of transcriptome, proteome and metabolome studies occurred after the complete
285 genome sequence of *C. necator* H16, making available valuable data, leading to elucidations about its

286 metabolism and allowing a systematic biology approach for the study of this bacterium (Volodina et al.,
287 2016).

288 Beside PHAs production, some novel biopolymers have recently enlarged the repertoire of products
289 which are known to be synthesized by *C. necator*, such as 3-mercaptoalkanoates and cyanophycin, low-
290 molecular weight compounds and alcohols, such as isopropanol (Grousseau et al., 2014). In one study, Bi
291 (2013) obtained a 6-fold titer improvement in hydrocarbon production in *C. necator* through the
292 development of a toolbox for metabolic engineering of this strain (Bi et al., 2013).

293 It is possible to use isolates of several microorganisms, as well as for *C. necator*, *Clostridium* species
294 and other naturally propanol producers strain, to improve their 2-propanol titer or even make 2-propanol as
295 the main product by genetic engineering: by deleting the side reactions (such as ethanol and butanol
296 pathways), or by identifying the more efficient 2-propanol producing pathways of these 2-propanol. (Ng et
297 al., 2013; Osburn et al., 1937). *Clostridium beijerinckii* strains are among those strains which have been
298 reported for 2-propanol production (Ng et al., 2013; Osburn et al., 1937). The greatest limitations to
299 biological production of 2-propanol are the very low 2-propanol titer and productivity of the
300 existing *Clostridium* species, since the reported maximum 2-propanol concentration achieved by
301 natural *Clostridium* strains was 36 mM (Andreesen et al., 1989). In fact, the 2-propanol fermentation process
302 must be economically feasible before it can replace the petrochemical processes, and the existing 2-propanol
303 fermentation technologies using natural or engineered strains still could not achieve this goal.

304 Another *C. necator* application is the microbial production of solvents, including acetone and butanol,
305 which are important feedstocks for the chemical and biofuel industry (Bi et al., 2013; Chakravarty and
306 Brigham, 2018). For what concerns butanol, oil-derived *n*-butanol has been historically used in industrial
307 chemical markets and in cosmetics and it will be used for sugar-derived biofuels due to its high energy
308 content, low miscibility with water and low corrosion properties. (Jurgens et al., 2012). 1-Butanol is obtained
309 mainly from oil by propylene oxo synthesis, but can be also produced by fermentation using different
310 microorganism, including *C. necator*, and some *Clostridium* species (wild, mutant or modified) or various
311 genetically modified (GM) microorganisms with a cloned 1-butanol metabolic pathway (Patakova et al.,
312 2013). The synthesis isobutanol (isomer of 1-butanol) can occur in *C. necator* and other microorganisms

313 which use branched-chain amino acid biosynthesis pathways, in particular through the valine synthesis
314 pathway (Lu et al., 2012, Lu et al., 2016). Isobutanol is an alcohol biofuel with > 90% of the energy density of
315 petroleum-based gasoline; it can be used to run vehicles without gasoline blending, since it is totally
316 compatible with the current fuel distribution infrastructure of most countries (Chakravarty and Brigham,
317 2018; Lu et al., 2012). A quite recent study (Lu et al., 2012) reported the production of branched-chain
318 alcohols initiated during nitrogen or phosphorus shortage in engineered strains of *C. necator*: good yield of
319 branched chain alcohols (over 180 mg/L) in flask culture were obtained using a mutant strain, which also
320 resulted to be more tolerant to isobutanol toxicity than the wild-type *C. necator*. After the deletion of the
321 genes encoding three potential carbon sinks (*ilvE*, *bkdAB*, and *aceE*) in this chosen strain, the production titer
322 improved to 270 mg/L of isobutanol and 40 mg/L of 3-methyl-1butanol (Chakravarty and Brigham, 2018). A
323 “rival” for *C. necator* for microbial production of 1-butanol are *Clostridium* species, which can convert a wide
324 range of substrates to produce it, such as glycerol, lactose, inulin, acetic and butyric acid (Patakova et al.,
325 2013). In order to use better understood, faster growing, more 1-butanol tolerant or aerobic
326 microorganisms, the 1-butanol metabolic pathway has been transferred into like *Escherichia*
327 *coli*, *Lactobacillus* or *Saccharomyces cerevisiae*. Although some 1-butanol production by these GM organisms
328 was demonstrated, clostridia still shows the greatest potential for efficient 1-butanol production (Kharkwal
329 et al., 2009; Patakova et al., 2013). However, the limitations for large industrial scale butanol production
330 through *Clostridium* species are low productivity and yield and cost effective downstream processing.
331 (Jurgens et al., 2012).

332 Thanks to its ability to use various carbon sources, *C. necator* became an interesting candidate host to
333 investigate for synthesis of renewable biofuel and other solvents production (Bi et al., 2013; Chakravarty and
334 Brigham, 2018). In particular, metabolic engineering of *C. necator* has been performed for the production of
335 terpens, alcohols, alka(e)nes and methyl ketones using various resources. This has been possible thanks to
336 the highly performing research activity focused on *C. necator* genetic engineering, aimed to reach a truly
337 sustainable and economical solvent production.

338 An example of this application is the genetic engineered *C. necator* strain for ethanol biosynthesis. Two
339 different pathways have been designed for ethanol biosynthesis: i) in one pathway acetate is the main carbon

340 source; in this case the key enzyme are the native acetyl-CoA synthase enzyme and a heterologously
341 expressed bi-functional acetaldehyde-CoA dehydrogenase and alcohol dehydrogenase enzyme (Chakravarty
342 and Brigham, 2018; Lee et al., 2016). ii) In the second pathway pyruvate is converted to acetaldehyde using
343 a heterologously expressed pyruvate decarboxylase enzyme and acetaldehyde is then converted to ethanol
344 by a heterologously expressed alcohol dehydrogenase (Jeon et al., 2013). The good results obtained provided
345 a strategy for biofuel production from a cheap carbon source, such as food waste (Lee et al. 2016).

346 Beside *C. necator*, there are several other interesting microorganisms for ethanol production, also from
347 watses, such as yeasts. One brilliant example of this application is the case of Brazilian distilleries: recent
348 scientific and technological advances (e.g. agricultural and fermentation process management and
349 engineering and the use of sugar cane varieties) have led to increased efficiency in bioethanol production by
350 *Saccharomyces cerevisiae* strains and a very high cell densities inside the fermenter (10–17% w/v, wet basis),
351 in a very short fermentation time (Basso et al., 2008). Unfortunately, with respect to *C. necator* fermentation,
352 one of the cons working with most of the yeast strains are some undesirable fermentation features, such as
353 high sedimentation rate (even during fermentation), excessive foam formation, or longer fermentation time
354 and high residual sugar after fermentation (Basso et al., 2008). Moreover, ethanol toxic effect to yeast cells
355 when in high concentration must be considered, even if its effect varies in different strains (Zhao and Bai,
356 2009).

357 To conclude this excursion on industrial application of *C. necator*, these are several examples of
358 different genetic engineering approaches acting on metabolic genes of this bacterium, which is easy to
359 engineer thanks to a wide repertoire of molecular biology genetic tools. A bottleneck to overcome for
360 putative industrial applications, like for other microorganisms, is the high production costs during large-scale
361 production of biomass, but also the economic challenge for biotechnologically valuable by-products
362 (Kourmentza et al., 2017a). One possible solution would be the use of inexpensive raw substrates, which may
363 significantly reduce the costs. This is the reason of several improvement of *C. necator* cultivation conditions,
364 mutagenesis and metabolic engineering, all having the aim to optimize the conversion of diverse substrates
365 to PHAs or other products of interest (Kourmentza et al., 2017b).

366 Among these different waste substrates, it is possible to find a wide repertoire of industrial by-
367 products, which are the most promising renewable substrates, and residual compounds like glycerol, raw
368 sugar-rich materials like molasses, starch and lignocellulose, but also other high carbon content substrates
369 like soybean, palm and corn oils. During nutrient shortage growth, *C. necator* directs most of its carbon flux
370 to the synthesis of PHB, while under autotrophic growth conditions with H₂/CO₂ as nutrient sources, *C.*
371 *necator* can synthesize 61 g/L of PHB (representing ~70% of total cell weight) in 40 h (Jiang et al., 2016).

372 After a brief analysis of *C. necator* industrial application, we focus this review on PHAs biosynthesis,
373 reporting here both main substrates used so far and waste substrates which have been studied so far and
374 that can help the future performance of *C. necator* biopolymer industrial production.

375 **4.1 Substrates for PHAs biosynthesis through *C. necator* fermentation**

376 Sugars, fatty acids, alcohols and gaseous substrates are the main used by *C. necator* to grow and
377 produce PHB. *C. necator* shows different uptake rates and variation in metabolism for each one of these
378 groups of substrates, with the consequent fluctuation in product yield and efficiency of biosynthesis.

379 In this review, the wide repertoire of substrates for *C. necator* PHB biosynthesis is summarized and
380 divided into two analytical groups: the first one concerns the so called “synthetic” substrates, as well as
381 refined and purified carbon sinks. The advantage of using these commercialized substrates is the knowledge
382 of metabolism of *C. necator* growing and fermenting using these as carbon sinks, which are known to lead to
383 high yield and product efficiency; the disadvantage is the enhanced overall production cost for PHB.

384 The second group of substrates concerns the so called “waste substrates”, which may be raw materials
385 or purified carbon sinks from a waste material, depending on the way they are produced and available. This
386 group includes the byproducts of any production process, as resources to produce valuable compounds by
387 entering in a second process and avoiding to be wasted. Their application would represent a big step forward,
388 both from environmental and economic point of view, and their use relies on the circular economy ideal of
389 the avoidance of waste production, since each waste could be the source for other processes for valuable
390 product of interest.

391 Here, both these groups are reported using as examples some of the results got so far for *C. necator*
392 biopolymer biosynthesis and accumulation.

393 4.1.1 General sugars utilization features in *C. necator*

394 Approximately 50% of PHAs production processes account on carbon sources used (Khosravi-Darani et
395 al., 2013), considering simple sugars and triacylglycerols, which may come from carbohydrates and from
396 plants oils and animals fats, respectively, as the main carbon sinks.

397 There are some differences reflected in PHAs bioaccumulation, which depend on the carbon source
398 used and on the carbon chain length: first of all, monosaccharides and disaccharides can be easily and
399 directly fermented to produce PHAs, with respect to polysaccharides which should be first hydrolysed before
400 being fermentable. Therefore, the rate of availability of the carbon sink inevitably affects the production rate.
401 Here we report the main sugars used as substrate for *C. necator* fermentation and PHB biosynthesis,
402 underlying the differences with respect the growth, amount of polymer accumulated and the production
403 rate, basing on *C. necator* metabolism.

404 It is important to underline that not only *C. necator* wild strain H16 has been considered in the
405 following paragraphs, but different *C. necator* strains have been reported because of the differences in
406 substrate selection. In fact, despite the assimilation of some limited simple sugars such as fructose and
407 gluconate, the wild type strain does not assimilate glucose. The only glucose-utilizing *C. necator* strains are
408 mutant strains, which have been isolated by spontaneous mutagenesis and UV (Raberg et al., 2011) or
409 constructed by targeted genetic engineering (Poirier et al., 1995).

410 4.1.2 Fructose uptake and utilization

411 Fructose and gluconate are catabolized by *C. necator* exclusively via the Entner–Doudoroff pathway
412 (ED pathway), whose key enzyme is 2-keto-3- deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14)
413 (Sichwart et al., 2011). Entner-Doudoroff (ED) pathway is mostly activated for the metabolism of hexoses
414 (e.g. fructose), rather than the Embden-Meyerhoff Parnas pathway (EMP pathway or glycolysis), and this
415 explains why the majority of fluxes in *C. necator* pass through 6-phosphogluconate and undergo the glycolytic

416 pathway at glyceraldehyde-3-phosphate and pyruvate nodes (Franz et al., 2012). Despite *C. necator* central
417 carbon metabolism is already well known, the carbohydrate uptake and transport remain instead almost
418 unknown in this challenging bacterium.

419 Most probably, fructose is imported by a CUT2 family ATP binding cassette (ABC)-type transporter,
420 encoded by *frcACB* genes, which are located on chromosome 2 of *C. necator* H16 (Priefert et al., 1991).
421 Interestingly, a wide set of genes located on chromosome 1 determines the amino sugar N-acetylglucosamine
422 utilization, like for those genes codifying for enzymes anabolically operating in the EMP pathway; fructose
423 uptake is presumably operated by a phosphotransferase-type transport system (H16_A0311-0316)
424 (Pohlmann et al., 2006). For what concern fructose degradation via the ED pathway, this concerns the
425 formation of the intermediate glucose-6-phosphate; in fact, fructose-grown cells of strain H16 exhibit
426 hexokinase activity, phosphorylating not only fructose but also mannose and glucose (Gottschalk, G. 1964)
427 and intracellular glucose can be oxidized by this bacterium, even if *C. necator* H16 lacks a system to transport
428 glucose into the cells.

429 4.1.3 Gluconate uptake and utilization

430 Gluconate is transported via a gluconate-H symporter catalyzed by an enzyme, a gluconate permease
431 (GntP), and by a transporter and (GntT). In addition to this, *C. necator* has another import system, the so-
432 called “PEP-PTS”, which precisely stands for “functional phosphoenolpyruvate-carbohydrate
433 phosphotransferase system”; this is specific for the uptake of a derivative of glucose the amino sugar N-
434 acetylglucosamine.

435 The PEP-PTS is important for optimal utilization of carbohydrates and derivatives compounds in
436 complex environments. It is involved in the catalyzation of the translocation and phosphorylation cascade,
437 which occurs soon after the uptake of N-acetylglucosamine (Barabote and Saier, 2005). In *C. necator* it is also
438 possible to find two homologous genes the N-acetylglucosamine-specific IABC (*nagF* and *nagE*) (Kaddor and
439 Steinbüchel, 2011; Orita et al., 2012).

440 4.1.4 Glucose uptake and utilization

441 The genome sequence of *C. necator* H16 availability allowed elucidation regarding glucose uptake.
442 Glucose and its derived polymers are the most abundant carbon sources in nature, with the consequent great
443 interest in extending utilization range of *C. necator* to these carbon sources for polyester production.
444 However, with respect to glucose uptake, this bacterium does not possess homologous and specific proteins
445 related to transport systems which are energy-dependent specific carriers, belonging either to
446 phosphoenolpyruvate-dependent phosphotransferase system (PTS) transporters like for amino sugar N-
447 acetylglucosamine, or cation-linked permeases, or ABC-type transporters like for fructose, or energy
448 independent facilitator-type transporters. Therefore, glucokinase (Glk) or hexokinase may acts on formation
449 of Glucose-6-phosphate from the imported glucose besides transportation by a phosphotransferase system
450 (PTS) which mediates a 6-phosphorylation-associated transportation (Orita et al., 2012).

451 Notwithstanding the still unclear uptake and transport mechanisms, spontaneously or UV mutated
452 strain of *C. necator* have been obtained and then studied to better understand these systems and the related
453 metabolic pathways involved in PHAs and PHB biosynthesis.

454 In the late of 1980s, such mutants have been used in pioneer pilot-scale for industrial production of
455 PHB starting from mixed carbon sources of glucose and propionate (Orita et al., 2012). One of the most
456 interesting strain is the glucose utilizing mutant *C. necator* NCIMB 11599, which turned out to be mutated in
457 a region of genes for putative N-acetylglucosamine-specific phosphoenolpyruvate-dependent PTS (PEP-PTS)
458 and its upstream region (Orita et al., 2012). It is also important to underline the marginal role of the PEP-PTS
459 for optimal utilization of carbohydrates and related compounds in complex environments, so much so that
460 some bacteria developed this system not only for sugar import but mostly for regulatory functions of carbon
461 metabolism (Kotrba et al., 2001). Most probably, the phosphorylation state of PEP-PTS proteins in *C. necator*
462 affects basic cellular functions (Krauß et al., 2009); for instance, the PEP-PTS represents a linkage between
463 nitrogen and carbon metabolism, which are the main energy source for bacteria.

464 4.1.5 Mannose

465 *C. necator* H16 lacks mannose specific transporters, but mannose phosphorylation can still occur
466 thanks to a native hexokinase (a mannofructokinase encoded by *mak* gene), which activity, however, was
467 found to be less efficient than phosphorylation of fructose and glucose (Volodina et al., 2016). *C. necator* also
468 possesses its own phosphomannose isomerase, which converts mannose-6-phosphate, previously
469 phosphorylated by *mak*, to fructose-6-phosphate.

470 Spontaneous mannose-utilizing mutants of *C. necator* have not been identified, yet. In order to
471 metabolically favour the utilization of mannose by *C. necator* H16, an artificial pathway has been recently
472 studied by expressing *mak* and *pmi* genes found to be fully functional in *E. coli* (Sichwart et al., 2011) and to
473 lead to a moderate growth of the recombinant strains of *C. necator*, but the bacterium moderately grew only
474 after prior cultivation on fructose (Sichwart et al., 2011). The PHB content obtained from the recombinant
475 gluconate and mannose grown strains were comparable (Sichwart et al., 2011; Volodina et al., 2016).

476 However, it is not convenient to use mannose as sole and pure carbon substrate, both because
477 genetically modified strains should be made, and this has a cost, but also because of the higher cost of the
478 mannose pure powder compared to glucose and fructose. Mutant strains growing on mannose may be useful
479 whether carbon sink would be used as waste material.

480 4.1.6 Xylose and arabinose

481 Xylose and arabinose are hemicelluloses deriving sugars that cannot be metabolized by *Cupriavidus*
482 *necator* H16, most probably because the lack of enzymes involved in both the uptake and the catabolism of
483 these. In fact, xylose specific transporters have not been found in genome analysis of *C. necator*, and not
484 even genes involved in xylose catabolism (Pohlmann et al., 2006).

485 However, *C. necator* grows on xylose-containing feedstocks using mixed culture combination with
486 *Lactococcus lactis* IO-1, as observed in a two-stage fermentation method (Tsuge et al., 2001). In a first phase
487 of the fermentation, *L. lactis* ferments xylose to lactate and acetate, and in the second stage these
488 intermediates are used as substrates to synthesize PHA by *C. necator*.

489 Furthermore, a *C. necator* mutant strain, harbouring genes from *E. coli* for a xylose transporter (*xylE*),
490 a xylose isomerase (*xylA*), which converts xylose to xylulose, and a xylulokinase (*xylB*) to form xylulose-5-

phosphate, resulted to be able of growing on these carbon substrates (Liu et al., 2014). However, this mutant strain could not utilize arabinose via heterologous expression of another set of genes from *E. coli* W3110, encoding for L-arabinose uptake and metabolism (Volodina et al., 2016).

For xylose and arabinose, the main problem remains the cost-production analysis, which is not favourable compared with other simple sugars.

4.1.7 Lactose and galactose

Lactose and galactose cannot be metabolized by *C. necator* H16. However, thanks to genetic engineering of glucose-utilizing *C. necator* G⁺1, cleavage of lactose has been obtained; *lacZ* (β -galactosidase), the inducer *lacI* and the operator encoded by *lacO* from *E. coli* have been heterologous expressed in *C. necator* G⁺1 strain, which resulted to be able to release galactose into the medium (Pries et al., 1990). The utilization of both glucose and galactose was possible only if *gal* genes and *lac* genes were heterologously co-expressed; still the galactose metabolism of *C. necator* remains to clarify.

Another mutant strain tested for growth on these disaccharides and on whey is *C. necator* DSM 545 (H1G⁺3) modified to harbor the *E. coli lac* genes (Povolo et al., 2010). In this study, the *lac* genes were integrated in the *phaZ* gene, then deleted, encoding for a PHA depolymerase, in order to minimize the mobilization of PHB and to maximize the growth and accumulation of PHB.

Still, lactose and galactose have a competitive cost with respect to xylose, arabinose and mannose, but not enough compared to glucose.

4.1.8 Fatty acids

Organic acids are fermentation products and common intermediates (in CoA-thioester form) of the central metabolism in *C. necator*. Fatty acids (FAs) are promising substrates for PHAs conversion, since they have a very high yield and the potential to lessen production costs. Moreover, they are easily uptaken up by *C. necator* H16 without the need for substrate range expansion or strain optimization, like may often occur for carbohydrates uptake and metabolism (Volodina et al., 2016). Furthermore, they are usually used as a

515 mixture of FAs, rather than one by one (except for acetic acid), which eliminates the purification of a single
516 FA problem.

517 However, some of the most used short chain length fatty acids (SCL-FAs), such acetic, propionic, butyric
518 and valeric acid, often referred to as volatile fatty acids (VFAs), are acidifying agents: they can freely diffuse
519 through the bacterium membrane, leading to the acidification of the cytoplasmic environment, which
520 provokes a toxic effect that inevitably slows down metabolites production (Yu and Wang, 2001).
521 Furthermore, dissociated FAs can lead to a final inefficient energy metabolism, by the alteration of the proton
522 gradient through the membrane and consequent increase in osmotic pressure (Wang et al., 2010).

523 Fortunately, *C. necator* possesses a detoxification mechanism which permits to overcome the toxic
524 effect of FAs (Lee et al., 2009, 2006). Besides the toxicity issue, there are other bottlenecks for the use of
525 pure-free FAs: they provide 3HB and 3HV-monomers for PHAs synthesis in *C. necator* H16 and they still remain
526 relatively expensive if they are not waste sources.

527 4.1.9 A focus on acetic acid

528 When a mixture of organic acids is supplied to a *C. necator* grow culture, propionic, lactic or butyric
529 acid are consumed first, and only when they are exhausted cells start to metabolize acetic acid (Yan et al.,
530 2003). The differences in utilization rates have to be considered to understand and estimate the PHB
531 production rate; two acetic acid molecules are needed to produce one molecule of PHB. Instead, butyric acid
532 is consumed to a higher rate and less toxic to the cell, hence if it is used as sole carbon source it provides one
533 molecule of PHB or two molecules acetyl-CoA, which enter then the Krebs's cycle (TCA), resulting in better
534 cell growth and PHB yield (Yan et al., 2003; Yang et al., 2010). Moreover, it has been recently shown that
535 butyric acid is also a precursor for C6-units PHAs (P(HB-co-HHx) copolymer) using engineered strains of *C.*
536 *necator* containing two modifications: deletions of the acetoacetyl-CoA reductase (phaB) genes and the
537 replacement of the native PHA synthase with phaC2 from *Rhodococcus aetherivorans* I24 (Jeon et al., 2014).
538 Notwithstanding all these interesting and potential applications for a good PHB production, the optimization
539 of the conversion of FAs to products has still to be achieved.

540 Among all acids, acetic acid is known to cause severe toxic effects in *C. necator* when it is used at high
541 concentrations (the inhibitory acetate concentration has been found to be about 3 gL⁻¹ for *C. necator* (Garcia-
542 Gonzalez and De Wever, 2018)), problem which can be easily understood also looking at its very high
543 dissociation constant for its metabolism. Therefore, acetic acid detoxification mechanism relies on its rapid
544 metabolization and on the upregulation of detoxifying enzymes (i.e. catalases) (Lee et al., 2009). It is also
545 known that the higher is the cell mass concentration of *C. necator* the higher the tolerance of the strain,
546 because of the increase in utilization rate of acetic acid (Yu & Wang, 2001).

547 Analyzing *C. necator* carbon flux came out that the majority of the consumed acetic acid is mainly split
548 between TCA and PHB synthesis and only a small part enters other pathways (Yu and Si, 2004). Therefore,
549 acetate is not the best suitable substrate for *C. necator* growth and fermentation, because of the low growth
550 yield due to its weak electron power (it has a pka of 4.75) and its toxic effects.

551 Once acetic acid enters the cytoplasm through membrane diffusion, H⁺ ion dissociates causing pH
552 lowering. The cascade of events is the following: cytoplasmic pH decreases, membrane potential changes
553 and, in turn, inner osmotic pressure rises leading to anions, hence negative charges, accumulation to balance
554 the positive charges due to K⁺ entry and forced H⁺ extrusion from the cell. This extrusion will not make
555 possible the use of these protons to generate ATP through the ATPase pump, hence decreasing energy
556 availability to the cell which will not grow further (Wang and Yu, 2000).

557 Despite these mechanisms occurring at high concentrations, so far, acetic acid has earned a quite
558 important success as an interesting alternative for its indirect sink of CO₂ and its conversion of into PHA in
559 terms of CO₂ fixation, H₂ consumption, safety and process performance, and substrate cost (Marudkla et al.,
560 2018). This approach has been recently tested for the synthesis of both the homopolymer PHB and the
561 copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), during which fed-batch cultivations
562 strategies have been used and a pH-stat fed-batch feeding strategy in combination and an additional
563 Dissolved Oxygen (DO)-dependent feed have been performed (Garcia-Gonzalez and De Wever, 2018). This
564 approach has been selected because biomass growth on carboxylates, in this case on acetic acid, leads to a
565 rise in media pH; therefore, coupling of the pH control with a carboxylate feed is a useful strategy to maintain

566 the pH at the desired level, which in the case of Garcia-Gonzalez and Wever (2018) has been set at 6.8-6.9,
567 while providing the carbon source, seems to be the best one for *C. necator* PHB production.

568 4.1.10 C-1 compounds

569 White biotechnology comprises promising approaches aimed to transform CO₂ emissions into valuable
570 chemical products, including the biopolymers. Considering the biotechnologically relevant C1-substrates
571 such as methanol, formate, CO and CO₂, only the first and the last are used by *C. necator* as the sole carbon
572 source (Friedrich et al., 1979). The others C-1 compounds can be supplied together with other carbon
573 sources; interestingly, CO₂ and formate follow a similar metabolization pathway via the CBB cycle.

574 When CO₂ is used as the sole carbon sink and H₂ as energy source, *C. necator* is able to assimilate
575 carbon dioxide thanks to the activity of the enzyme RuBisCO (Ribulose-1,5-bisphosphate carboxylase-
576 oxygenase), and at the same time three distinct oxygen-tolerant [NiFe]-hydrogenases carry on H₂ oxidation
577 for energy delivering (Lenz et al., 2010). Interestingly, when formate is supplied as substrate, hydrogenases
578 and enzymes of CBB cycle are still active (Friedrich et al., 1979), allowing oxidation of formate to CO₂, which
579 is in turn fixed in CBB cycle. In this case, the bottleneck is represented by the very toxic effect of formic acid,
580 which may cause inhibition of central metabolism enzymes. Therefore, formic acid could not be used at high
581 concentrations (Volodina et al., 2016).

582 In conclusion, for what concern PHAs bioproduction, due to formate toxicity, the low energy potential
583 and the low PHA/biomass yield, this substrate is not suitable as sole carbon source, unless future studies will
584 prove it differently. However, formic acid has a great potential for microbial conversion to diverse products:
585 it can be electrochemically generated from CO₂ and water. In fact, recombinant strains of *C. necator* have
586 been used for electro-microbial conversion of CO₂/formate to higher alcohols (Jeon et al., 2013; Li et al.,
587 2012).

588 5.1 Waste valorization: a circular economy strategy

589 The aim of *C. necator* fermentations, and most generally of each kind of industrial fermentation, is to
590 reduce as much as possible the costs of production of the desired product, in order to make the whole
591 process merchantable and valuable for large scale applications.

592 Beside the “blessing” of the wide repertoire of substrates used so far for *C. necator* PHB biosynthesis
593 fermentation, this biosynthetic process still requires to optimize and to balance cost production and
594 production yield.

595 Using by-products or waste products, from different biorefineries, as carbon sinks for *C. necator*
596 fermentative biopolymer production, this synthesis could be aligned to the circular economy approaches, in
597 which everything assumes a new commercial value for different applications and nothing is wasted, helping
598 in the reduction of production costs and increasing the ecological impact of the biopolymer (Karan et al.,
599 2019; Leipold and Petit-Boix, 2018). Different potential or applied substrates from biorefineries wastes for
600 fermentation and PHB biosynthesis of some of the most used *C. necator* strains are reported in the following
601 paragraphs.

602 5.1.1 Starch and lignin derivatives

603 Starch is one of the most abundant polysaccharides, consisting in glucose moieties linked by α 1–4
604 glycosidic bonds. It is present in several plants and food crops, such as potatoes, wheat, corn, rice, etc., and
605 consequently it is also quite abundant in food wastes and starchy wastewaters. Also the so called “third
606 generation” of starch produced by microalgae is an interesting solution considered as new feedstocks for
607 starch-based bioplastic production (Brányiková et al., 2011). Microalgae produce small starch granules
608 (narrow size distribution of 0.5–2.1 μ m) with the advantage of no lignin present (Mathiot et al., 2019). Starch
609 can be hydrolysed into glucose, maltose and maltotriose by amylases or glucoamylases enzymes; however,
610 *C. necator* lacks genes encoding for these enzymes. Therefore, starch can be used as feedstock for *C. necator*
611 only after a previous hydrolysis, which inevitably increases the growth and production costs. In addition,
612 since glucose is the end product of this hydrolytic step, a glucose-growing strain of *C. necator* must be used
613 instead of wild type strain.

614 It is known from literature that at least two different approaches have been tested to use starch for
615 PHAs production, so far. One approach implies the application of a two-step fermentation procedure (Yu and
616 Wang, 2001). In this case, acidogenic bacteria are used to convert the starchy wastes into volatile FAs (VFAs)
617 such as acetic, propionic, formic, and butyric acids. Then, *C. necator* is able to convert these VFAs to PHAs
618 during a second fermentation step. The second method is based on enzymatic hydrolysis of starch into
619 fermentable sugars, such as glucose (Haas et al., 2008).

620 However, starch still remains an inconvenient and not competitive feedstock for PHAs production,
621 because of the energy consumption during two-step fermentation, the down-stream processing and
622 saccharification costs. A possible solution to overcome these problems could be the heterologous expression
623 of genes for amylases in PHAs producing bacteria.

624 Similar problems occur with lignin derivatives: lignin has to undergo chemical, physical or biological
625 pre-treatment to be fermented by *C. necator*. From these treatments, aromatic derivatives as p-coumaric,
626 caffeic, ferulic and sinapinic acid are released (Tomizawa et al., 2014), which can be further metabolized to
627 oxaloacetate and pyruvate by some bacteria, such as *Pseudomonas putida* and *Sphingomonas paucimobilis*.
628 These second step produces intermediates, and some of them can be used by *Cupriavidus necator* H16 to
629 produce and store important amounts of PHB; among these intermediates there are 4-hydroxybenzoic (4-
630 HBA), 2,5-dihydroxybenzoic/gentisic (2,5-DHBA), 3,4-dihydroxybenzoic/protocatechuic (3,4-DHBA)
631 (Tomizawa et al., 2014).

632 In a recent study, kenaf biomass (KB) has been employed (Saratale et al., 2019) as feedstock for PHB
633 synthesis using *Cupriavidus necator* and testing several pre-treatments for the ligneous-cellulosic raw
634 material, followed by enzymatic saccharification. PHAs accumulation, PHB yield, crystalline structure, and
635 thermal properties and *C. necator* growth reached using KB hydrolysates were similar to those obtained using
636 synthetic sugar mixture. In fact, during a 36h fermentation, sodium carbonate and sodium sulfite pretreated
637 KB hydrolysates (30 g/L), exhibited maximum 70.0% PHA accumulation, showing a PHB titers of 10.10 g/L and
638 PHB yields of about 0.488g/g of reducing sugar (Saratale et al., 2019). For what concern saccharification
639 process, a combined NaOH and H₂O₂ pre-treatment of hydrolysates resulted in significant sugar consumption
640 (80%) and better biomass production of 3.22g/L, with PHA accumulation at 65.9%, a PHB titer of 7.65g/L and

641 PHB yield of 0.478g/g of reducing sugar. Consequently, a green liquor pretreated KB hydrolysates are
642 promising and economically practicable substrate for PHB production (Saratale et al., 2019).

643 5.1.2 Animal fats and plant oils

644 There is a huge dilemma for the use of waste animal fats from slaughtering and food processing
645 industries, avoiding to consider the ethical issues related to these processes (“plate-vs-plastic” controversy
646 (Koller and Braunegg, 2015)), which is far beyond our topic: on one side they have a great potential as carbon
647 source for PHAs production, on the other side they result to be problematic during fermentation processes
648 due to their high melting temperature (Jiang et al., 2016). The amount of PHB per CDW reached so far is 79–
649 82% (w/w) when the wild type *C. necator* strain H16 is cultivated on various fats (Riedel et al., 2015).

650 Recently, it has been reported that PHAs synthesis has been tested in *C. necator* using tallow as
651 substrate and gum arabic as emulsifying agent in a 5-L fermenter and fed-batch mode. The strain reached
652 a cell dry weight (CDW) and a PHAs content of 45 and 26 g/L, respectively (Riedel et al., 2014). The yield was
653 of 0.40 g PHAs/g fat and a productivity of $0.36 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$.

654 In another study, lipid-rich surplus streams from slaughterhouses and the rendering industry have
655 been studied for PHA production. These raw materials can undergo chemical transformation to crude glycerol
656 phase (CGP) and biodiesel (Koller and Braunegg, 2015). This has been possible thanks to the project
657 ANIMPOL, funded by EU, during which a process for the production of PHA using diverse waste streams of
658 the animal processing industry has been elaborated (Koller and Braunegg, 2015; Shahzad et al., 2017). This
659 is very interesting from both environmental and economic point of view, since these organic wastes (e.g.
660 offal) have no market price (Shahzad et al., 2017). It has been estimated that 500 000 tons/year of lipid-rich
661 streams of bovine, porcine, avian etc. are produced in Europe (Titz et al., 2012). On the other hand, the
662 saturated share of biodiesel (SFAE) counteracts its’ applicability as biofuel, but it is possible to
663 biotechnologically convert it into 35.000 annual tons of PHAs, theoretically (Koller and Braunegg, 2015). Since
664 the conversion of the lipid fraction of slaughtering waste to CGP and biodiesel is well-established technique,
665 the optimization of each step could lead to an animal-derived PHA production at a price in the range of 2 €/kg
666 PHA (Koller and Braunegg, 2015). In fact, using SFAE as the main carbon source in a fed-batch cultivation

mode using *C. necator*, a maximum of 28.0 g/dm³ PHA were obtained, corresponding to a PHA mass fraction in CDM of 0.80 g/g, a specific growth rate μ_{\max} amounted to 0.17 1/h, and a very high yield for biomass production from SFAE of 0.6 g CDM per g SFAE (Shahzad et al., 2017). In a technical analysis considering both meat and bone meal (MBM) and biodiesel prices fluctuations, it has been estimated that the final PHA production cost varies from 1.41 €/kg to 1.64 €/kg using offal as waste or considering its market price, for which the fixed price of biodiesel is 0.97 €/L and MBM fixed price of 350 €/t (Shahzad et al., 2017).

Vegetable oils have been found to be possible substrates in the production of PHAs and their liquid form make them relatively easy to be fermented (Kahar et al., 2004; Verlinden et al., 2011). Most important, from economic and ecological point of view, is the application of wasted oils from mills or consumed oils, which are even cheaper than purified oils (Mumtaz et al., 2010; Verlinden et al., 2011). In fact, *C. necator* H16 can produce PHB homopolymer up to about 80% (w/w) of the CDW during its stationary growth phase starting from different plant oils (Fukui and Doi, 1998). The same w/w ratio has been obtained by a recombinant strain of *C. necator* (DSM 541, a PHA-negative mutant) harbouring a PHA synthase gene from *Aeromonas caviae* (Fukui and Doi, 1998), in which a copolymer P(3HB-co-3HHx) with 4%–5% of (R)-3-hydroxyhexanoate (3-HHx) was produced. This recombinant strain is now in use commercially by Procter & Gamble Co., Ltd. (Cincinnati, OH, USA), to produce PHB-co-3HHx: the strain is also capable to obtain similar PHAs production results using soybean oil as the sole carbon source (20 g · L⁻¹) (Kahar et al., 2004).

C. necator can also accumulate PHB from the rapeseed oils fermentation, achieving 1.2 g/l of PHB concentration from this waste frying oil, which is similar to a concentration that can be obtained from glucose (Verlinden et al., 2011). In this case, the PHB harvest content varies depending on the use of pure oil (0.62 g/l) and heated oil (0.9 g/l), suggesting that a feeding strategy using waste frying oil could even achieve higher PHB content than pure vegetable oil. The results obtained in that study were not interesting in comparison to fermentations with *C. necator* using several oils performed by del Rocio et al. (2007), in which PHAs concentrations ranged between 3 to 6 g/l in 25 hours (Lopez-Cuellar, Ma del Rocio, Gracida-Rodriguez and Fermín, 2007).

From both economic and ecological point of view this could be a huge step forward, because the collection of waste frying oil could be easily achieved, helping to save waste oils and reducing the starting

694 material costs. Waste oil is a good alternative with respect to purified oil or other purified sugars for PHB
695 production yield. Moreover, if the waste frying oil has to be filtered first for the production of soap or
696 biodiesel, this step is not needed nor required for PHAs production and this further decreases production
697 costs (Cibis et al., 2007).

698 From chemical and molecular point of view there are not differences: the molecular weights of
699 biopolymers produced from waste frying oil are similar to those obtained from other oils and glucose,
700 meanings that producing PHB from waste frying oil does not affect the molecular properties of the final
701 product. The application of these oils would not affect the global food chain and they do not allow food-feed
702 competition, having a great potential as renewable source needed for large scale industrial applications and
703 production of different types of biopolymers (Obruca et al., 2010).

704 5.1.3 Glycerol

705 Currently, one of the major contributions of glycerol as bioproduct comes from the transesterification
706 of oils and methanol; free FAs are bioproducts of this process too. About 10 tons of glycerol is accumulated
707 as waste product from transesterification of vegetable oils or animal fats to obtain 100 tons of biodiesel, so
708 far (Andreeßen et al., 2010). As one of the main by-products, glycerol is an attractive and cheap substrate for
709 several applications, especially considering its competitiveness for PHAs and other biobased production
710 (Posada et al., 2011).

711 One interesting fermentative possibility is the use of crude glycerol, but in this case it is important to
712 consider all the constituents and the impurities that could be found in it, including mono-/di-/triglycerides,
713 soap, methanol and salts, which may interfere with fermentation processes; despite the low cost of this raw
714 substrate, the impurity of the blend is the first bottleneck for glycerol application (Yang et al., 2012).

715 In *C. necator*, enzymes acting on glycerol metabolisms (two glycerol kinases GlyK and two glycerol-3-
716 phosphate dehydrogenases Gly3-DH) have been found on both chromosomes (Pohlmann et al., 2006).
717 Glycerol can enter the cells through passive diffusion and is then phosphorylated by a kinase, producing
718 glycerol-3-phosphate, which is in turn dehydrogenated to dihydroxyacetone-phosphate. This intermediate is
719 then metabolized in the sugar-degrading pathway. However, the growth rate of *C. necator* on glycerol is very

low and this is due to the presence of the overproduced reactive oxygen species (ROS), such as H_2O_2 , produced during the oxidative stress substrate-induced (Volodina et al., 2016).

So far, several attempts have been undertaken to optimize glycerol utilization by *C. necator* strains DSM 545 and IPT 026 for PHB production (Cavalheiro et al., 2009). This cheap by-product resulted to inhibit the growth at concentration higher than $40 \text{ g} \cdot \text{L}^{-1}$, probably due to impurities in the feedstock (Cavalheiro et al., 2009).

It has been observed that copolymers PHBV and poly(3-hydroxyvalerate-co-3hydroxyvalerate-co-4-hydroxybutyrate) can be produced using glycerol as substrate (Cavalheiro et al., 2012). In this recent work, a maximum CDW of $82.5 \text{ g} \cdot \text{L}^{-1}$ has been achieved, while the productivity was approximately $0.6\text{--}1.5 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$, with a P3HB content was 62% for pure glycerol. Instead, when crude glycerol from biodiesel production is used, lower values has been obtained (CDW was $68.8 \text{ g} \cdot \text{L}^{-1}$, productivity of $0.84 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ and a PHB content of 38%). From the metabolic analysis of *C. necator* DSM 545 glycerol fermentation turned out the facilitation of crude glycerol uptake and metabolization, which is carried out by GlyK and Gly3-DH enzymatic activity and their linkage with the anabolic EMP-pathway, could compensate the slow cell growth, which in turn can improve the saleability of the biopolymers (Lopar et al., 2014).

Higher PHB productivity has been gained in another study aimed to compensate the slow growth rate through genetic engineering modifications (Fukui et al., 2014). It has been demonstrated that even low glucose concentrations in the medium reduces the glycerol consumption in *C. necator* DSM 545, probably because of the negative feed-back controller of glycerol consumption due to the high intracellular pool of glycerol-3-phosphate and dihydroxyacetone-phosphate (Vrana Špoljarić et al., 2013). Therefore, the assumption about inhibition of initial glycerol activation reactions by the products of the EMP-pathway could be valid (Lopar et al., 2014). Interestingly, it has been found that an unspecific incorporation of glycerol by PhaC (one of the three enzymes acting on PHB biosynthesis) can occur during prolonged glycerol cultivation, leading to the polymer chain termination (the so called “*endcapping effect*”) (Tanadchangsang and Yu, 2012).

Wastewater from biodiesel refineries has been tested too, together with crude glycerol, to produce PHAs through fermentation of *C. necator* TISTR 1095 (Sangkharak and Prasertsan, 2011). Unfortunately, no

cell growth has been obtained from biodiesel-wastewater due to high Na^+ in this medium. Apparently, crude glycerol, $(\text{NH}_4)_2\text{SO}_4$, NaCl and K_2SO_4 , and trace element concentration revealed significant effects ($P < 0.1$) influencing PHB accumulation (Mothes et al., 2007; Sangkharak and Prasertsan, 2011). From this analysis, the highest PHB concentration of 24.98 ± 1.87 g/L and of 54.01% of CDW have been obtained by modulating these condition parameters, hence using 60 g/L of crude glycerol, 1.32 g/L $(\text{NH}_4)_2\text{SO}_4$ and 2.0 g/L trace element, which allow the strain TISTR 1095 to reach the highest biomass (46.25 ± 2.10 g/L).

In another study (Moita et al., 2014), a co-culture strategy has been performed: two strains, having the ability to consume both glycerol and methanol present in the crude substrate, have been selected; at the end, the two biopolymers accumulated (PHB and glucose biopolymer (GB)) resulted to be produced using glycerol as the only carbon. The aerobic mixed cultures and a real waste substrate with non-volatile fatty acids (VFA) organic matter reached a maximum PHB content of 47% and a productivity of 0.27 g X/L.d, where X is the biomass concentration.

In conclusion, the absence of the need for a pre-fermentation to use crude glycerol for PHB conversion makes overall production process more economically achievable and more sustainable with respect to other pre-treated feedstocks; several valid fermentation strategies have been performed and many others have to be defined, yet.

5.1.4 Waste potato starch

Low cost carbon substrates have sparked wide interest for their application for industrial purposes, including PHB production. Before its utilization, waste potato starch has to undergo saccharification process, in order to make derived sugars (i.e. glucose and maltose) easily fermentable by microorganisms. This strategy has been applied with *C. necator* NCIMB 11599, which can reach $1.47 \text{ g}/(\text{L} \cdot \text{h})$ PHB productivity under phosphate limitation conditions (179g/L biomass, 94g/L PHB, $\text{Y}_{\text{biomass/starch}} = 0.46 \text{ g/g}$, $\text{Y}_{\text{PHB/starch}} = 0.22 \text{ g/g}$) (Haas et al., 2008). Interestingly, the performance achieved by using saccharified starch was virtually identical to the one obtained by using glucose, which is the main sugar present in the substrate medium. In that work, Haas et al. (2008) used an unsophisticated liquefaction and saccharification method using

772 commercial enzymes, from which a part of the starch (about 9%) was lost to maltose, which could not be
773 used by *C. necator* NCIMB 11599, but neither inhibits the growth and PHB production of the strain. These
774 evidences suggest that saccharified waste starch potato is a viable and cheap alternative substrate for PHB
775 biosynthesis.

776 5.1.5 Waste office paper

777 Waste paper and paper-derived materials have been recently studied to delve into the utilization of
778 materials for by-product production for industrial purposes, such as for biofuel production (Dubey et al.,
779 2012).

780 Surprisingly, paper waste, with its high cellulose content and its abundance, has its potential to be used
781 as renewable feedstock for fuels, chemicals and materials biorefineries, helping to reduce production costs,
782 thanks to its abundance in municipal solid wastes (it accounts for more than 35% of total lignocellulosic
783 wastes) (Dubey et al., 2012).

784 Waste office paper (WOP) can be surely considered an “unthinkable substrate” for PHB production.
785 Clearly, this waste can not be utilized for PHB production as it is, but it has to undergo two pre-treatments:
786 enzymatical saccharification and hydrolyzation, as reported in a recent paper (Neelamegam et al., 2018).

787 Glucose (22.70 g/L) and xylose (1.78 g/L) are the main sugars composing the hydrolysate, accounting
788 for about 816 mg/g of the sugar yield. In Annamalai et al. (2018) a batch fermentation of *C. necator* using the
789 pretreated WOP hydrolysate has been performed, achieving 7.74 g/L of cell biomass, 4.4 g/L PHB production
790 and 57.52% PHB content, and a volumetric productivity and yield of 0.061 g/L/h and 0.210 g/g sugar,
791 respectively. These definitely improvable results evidenced that WOP is a potential alternative feedstock for
792 PHB biorefinery production, but still further enhancements should be done to enhance PHB content,
793 volumetric productivity and yield, in order to become a commercially competitive strategy.

794 5.1.6 Wine lees

795 Wine lees (WL) (EC No. 337/ 79) are defined as “the residue that forms at the bottom of recipients
796 containing wine, after fermentation, during storage or after authorized treatments, as well as the residue
797 obtained following the filtration or centrifugation of this product”.

798 During wine production, about 2–6% of WL of the total volume of wine produced is obtained. The
799 development of a novel wine lees (WL) based integrated biorefinery can be applied for the production of
800 several added-value products, including PHB (Bai et al., 2008; Dimou et al., 2015).

801 In a recent study, WL has been previously fractionated for the production of antioxidants, tartrate and
802 ethanol, from which a remaining stream is obtained too. This was converted into a fermentation nutrient
803 supplement for PHB biosynthesis by *C. necator* DSM 7237 strain (Dimou et al., 2015). Furthermore, the
804 pretreated WL has been hydrolyzed using crude enzyme repertoire produced via solid state fermentation of
805 *Aspergillus oryzae*. During batch and fed-batch fermentations for the production of PHB, WL hydrolysates
806 have been used together with crude glycerol as carbon sinks (Dimou et al., 2015). The free amino nitrogen
807 (FANs) content, present in the WL derived hydrolysates, significantly affects both bacterial growth and PHB
808 production. This fed-batch fermentation strategy, with an initial FANs concentration of 700 mg L⁻¹ and
809 supplemented with trace elements, achieved up to 30.1 g L⁻¹ of PHB and an intracellular content of 71.3%
810 (w/w), with a productivity of 0.56 g L⁻¹h⁻¹, suggesting that WL could be used for the production of nutrient-
811 rich fermentation supplements for highly efficient PHB production after the extraction of value-added
812 components with diversified market outlets (Dimou et al., 2015). Synergistic utilization of renewable
813 resources, as presented in this study, with the utilization of crude glycerol and wine lees derived hydrolysates,
814 could lead to highly efficient bioprocesses.

815 5.1.7 Wheat based

816 Wheat-based fermentation strategy has been evaluated for PHB biorefineries production by *C.*
817 *necator*. The wheat kernel consists in layers which should be initially removed through pearling technologies,
818 from which valuable co-products can be extracted too, improving the economic competitiveness of the
819 overall process. (Dexter and Wood, 1996; Koutinas et al., 2007). A further step consists in a fungal
820 fermentation aimed to the production of enzymes needed to hydrolyse pearled wheat flour suspensions into

821 a rich in glucose medium (wheat hydrolysate - WH). From this step, another merchantable by-product could
822 be extracted too: gluten (Hernández-Muñoz et al., 2003). This process produces also solids wastes with high
823 potential application, that contain fungal mycelia and undigested wheat components, which can be further
824 utilized to produce nutrient-rich supplements, particularly rich in nitrogen, for bacterial bioconversions after
825 fungal autolysis (Koutinas et al., 2005). This fungal extract (FE) has similar composition to yeast extract, whose
826 cost hinders its industrial utilization.

827 Both WH and FE can be used and mixed in appropriate proportions, with varying glucose (i.e. 5–26 g l⁻¹
828 ¹) and FAN (0.1–1.2 g L⁻¹) concentrations for batch shake flask fermentations, to provide a medium allowing
829 bacterial growth and PHB accumulation (Koutinas et al., 2007). Higher microbial growth but less PHB
830 accumulation, though, have been obtained by rising FAN concentration. High growth yields (up to 1.07 g cells
831 (g glucose)⁻¹) have been achieved by the consumption of various carbon sources (carbohydrates, mainly
832 glucose, amino acids, peptides), with a growth rate which can reach up to 0.16 h⁻¹.

833 Three WH (WH1, WH2 and WH3) with similar glucose concentration (200–220 g L⁻¹) and different FAN
834 (0.3–1.48 g L⁻¹) concentrations have been tested for *C. necator* growth and PHB accumulation in fed-batch
835 shake flask fermentation (Koutinas et al., 2007). The highest microbial biomass concentration (29.9 g L⁻¹),
836 growth yield (0.28 g residual microbial biomass (g glucose)⁻¹) and PHB yield (0.43 g (g glucose)⁻¹) have been
837 recorded using the WH3 medium, which was the one with the highest nitrogen concentration. In comparison,
838 WH2 gave the highest PHB concentration (51.1 g L⁻¹) and content (0.7 g g⁻¹).

839 Therefore, applying these processing steps, production costs for PHB biosynthesis is importantly
840 reduced by three factors: the production of merchantable value-added co-products, such as pearlins and
841 gluten, the reduction costs for feedstock thanks to the integration process, and last but not least, the
842 increasing conversion yield of glucose into PHB during fed-batch fermentation.

843 5.1.8 Wastewater

844 Thanks to the variability of the possible applicable processes, PHAs production can be managed in the
845 circular economy strategy for the use of waste and wastewater, mainly consisting of industrial process
846 effluent waters and wastewater, previously biological pre-treated.

847 This PHAs production approach has been pointed towards the establishment of technical possibility to
848 use mixed microbial cultures (MMC) using wastes and residuals as feedstock. During the last years, the
849 laboratory scale research over this topic has been conducted following three main steps: an acidogenic
850 fermentation of the raw substrate to obtain a volatile fatty acid (VFA)-rich stream, followed by MMCs
851 biomass growth to improve PHAs production rate, and the final PHAs accumulation step (Valentino et al.,
852 2017). This strategy has to overcome the problem of being merchantable for high quality of production and
853 recovery of the polymers over an extended period of time; another issue to solve is the commercially scaling
854 up, which is not easily achievable, even using cheap and available starting material.

855 A good PHA producing strain used so far for wastewater recovery is *Plasticumulans acidovorans*
856 (Jiang et al., 2012). This approach has been here reported because *C. necator* could be included among MMC
857 for wastewaters recovery, thanks to its ability to ferment VFA for PHAs production and its high versatility. In
858 conclusion, any significant improvement in the downstream processes (i.e. treatments to upgrade PHAs-in-
859 biomass properties before PHAs extraction from the biomass and following steps) will results in higher added
860 value to the process chain and lower overall costs. The supplied sources have to be enlarged to the potential
861 and to widen the PHAs producing MMC, in order to boost circular economy approaches.

862 5.1.9 Conventional plastics

863 Since the increasing accumulation of plastic wastes, a very interesting and smart innovative approach has
864 been found recently, basing on several reports on biodegradation of synthetic plastics by microorganisms or
865 enzymes, offering the possibility to develop biological treatment technology for petroleum plastic wastes (Ru
866 et al., 2020). Several microorganisms are able to degrade a wide repertoire of synthetic plastics, such as
867 polypropylene (PP), polyethylene (PE), polystyrene (PS), polyurethane (PUR), polyvinyl chloride (PVC) and
868 polyethylene terephthalate (PET) (Ru et al., 2020). Some of these microorganisms are reported here for each
869 synthetic polymer: two cyanobacteria, *Phormidium lucidum* and *Oscillatoria subbrevis*, are able of degrading
870 30% of the initial weight of tested PE over a 42-day period (Ru et al., 2020; Sarmah and Rout, 2018), while
871 *Xanthomonas* sp., *Sphingobacterium* sp., and *Bacillus* sp. could degrade PS. Furthermore, two marine

bacteria isolated from mangrove environments, *Bacillus* sp. strain 27 and *Rhodococcus* sp. strain 36, resulted to be able to grow in aqueous synthetic media containing PP microplastics and caused a weight loss of 4.0–6.4% after 40 days (Auta et al., 2018). Also PUR could be used as sole nutrient source for the growth of microorganisms like *Aspergillus niger* and *Cladosporium herbarum* (Filip and Giessen, 1979), together with some *Pseudomonas* species, capable of degrading emulsified polyester PUR (Schmidt et al., 2017). For PET degradation, in particular low-crystallinity PET (IcPET, 9%), the actinomycete *Thermobifida fusca* can effectively degrade up to 50% of the initial weight of this polymer at 55°C for 3 weeks (Mueller, 2006). Last but not least, also *C. necator* shows the peculiarity to use synthetic plastics for its growth. In particular, it exhibited PHA accumulation when supplied with non-oxygenated PE pyrolytic hydrocarbons as a carbon source in a nitrogen-containing medium (Johnston et al., 2017). In two recent papers, the production of P(3HB-co-3HV-co-3HHx) by *C. necator* has been tested using oxidized PE wax and PP wax, after a pyrolysis pretreatment under aerobic condition: 1,24 g/L (using PE wax) and 1,36 g/L (using PP wax) of copolymer have been obtained (Johnston et al., 2019; Radecka et al., 2016). Moreover, PE gasification, instead of pyrolysis, seems to enhance PHA accumulation in *C. necator* H16 (Qin et al., 2021).

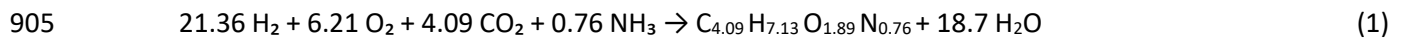
6.1 *C. necator* fermentation strategies

Several substrate utilizations have been mentioned in this review, but fermentation is not only performed concerning only the substrates used. Fermentation strategies have to be studied and set in order to get the highest yield production, indeed. The same substrate supplied to fermentative bacteria can result in different product yield and rate of production by changing fermentation method. Here we briefly discuss some main considerations to deal with for PHAs fermentative production.

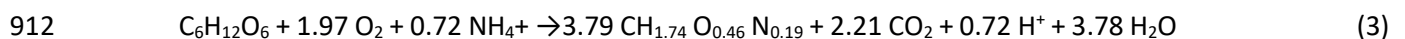
PHAs production is mostly carried out by applying a cultivation method consisting, generally, in two steps: i) firstly, a cell growth under favourable growth conditions is performed using a rich, containing all macro and micronutrients needed to reach high cell density; ii) secondly, PHAs synthesis and accumulation occurs under imbalanced growth conditions, mostly characterized by phosphorous and nitrogen shortage.

896 For what concern *C. necator*, two cultivation methods can be applied to directly use CO₂ as a carbon
 897 sink for PHB production, which can be either pure synthetic CO₂ (Islam Mozumder et al., 2015) or industrial
 898 off-gases (Garcia-Gonzalez and De Wever, 2017). As already mentioned, this bacterium is able to shift
 899 between autotrophic growth and heterotrophic growth. The latter one is the most common and widely used,
 900 but efforts for PHB production using CO₂ in autotrophy can occur.

901 Proceeding with the so called “*autotrophic-autotrophic*” PHB production process, a gas mixture
 902 containing CO₂, H₂ and O₂ is furnished to the bacteria growing into the bioreactor to induce both cell mass
 903 growth and PHB accumulation. Occurring reactions are reported below (1 and 2) (Garcia-Gonzalez and De
 904 Wever, 2018).



907 Whereas, the “*heterotrophic-autotrophic*” PHB production process, relies on a feeding approach based
 908 on an organic substrate as a source of carbon (i.e. glucose, fructose, glycerol etc) to support the heterotrophic
 909 growth, which is then followed by a shift to autotrophic conditions allowing PHB production, as reported in
 910 the following schematic reaction, in which glucose is used to represent the carbon sink in the reaction (3)
 911 (Garcia-Gonzalez and De Wever, 2018, 2017; Ishizaki et al., 2001).



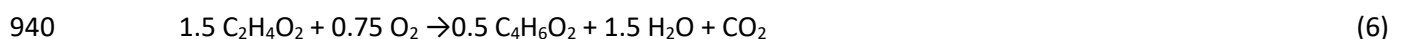
913 There are several studies in which researchers have investigated mixotrophic PHAs copolymer
 914 production; in this third case, CO₂ is supplied in combination with an organic co-substrate at the same
 915 moment, during both growth and PHB production steps (Ghysels et al., 2018).

916 During gas fermentation, precaution must be taken regarding gas detonation when O₂ is supplied
 917 combined with H₂. This requirement can be achieved by checking over the O₂ concentration in the gas phase
 918 and by keeping it below the lower level of explosion. However, in this case, notwithstanding higher cell mass
 919 concentration can be reached, the lower gas concentration in the fully autotrophic cultivation process entails
 920 an increased risk of mass transfer limitation, which in turn, may induce premature shifting to the PHAs

production phase, or the incomplete PHB accumulation in the case of heterotrophic–autotrophic process. In terms of cell mass concentration and growth rate, this latter approach has been considered more promising for the direct conversion of CO₂ to PHA (Islam Mozumder et al., 2015), but also in terms of production costs, since less of the costly renewable H₂ is needed in this process. Shifting from autotrophic process to a heterotrophic one, characterized by a glucose-grown biomass, the H₂ consumption is theoretically reduced by 20% (Garcia-Gonzalez and De Wever, 2017). However, still a low amount of CO₂ of the total in the gas mixture can be converted into biopolymers.

Beside sugars supplying, another interesting carbon source which may be used during the autotrophic-heterotrophic-heterotrophic PHA production process is acetic acid, allowing a production approach in which PHB can be indirectly produced from CO₂. This can be achieved mainly using two types of bacteria, preferably in a two-stage fermentation process, in which the first group of bacteria (i.e. acetogenic bacteria) has the ability to produce acetic acid by reducing H₂ and CO₂ as sole energy and carbon sources (Equation 4) (Ragsdale, 2008). The resulting acetic acid can be used subsequently by another organism for both biomass growth, by also supplying a nitrogen source, and PHA production (Equation 5 and 6).

C. necator is a perfect candidate to carry on PHB production starting from acetic acid, if its concentration is kept below the inhibitory concentrations for PHAs synthesis (Garcia-Gonzalez and De Wever, 2018).



With respect to the autotrophic–autotrophic PHB production process, this cultivation method, implying indirect production of PHB from CO₂, could theoretically reduce the amount of CO₂ needed, up to 2.84ton CO₂/ton PHB, but most importantly the H₂ consumption could be reduced by 50%. According to the mass balances, this method results far better even compared to the glucose based PHB production, during

945 which CO₂ is emitted rather than consumed (at least 2.81ton CO₂/ton PHB) (Garcia-Gonzalez and De Wever,
946 2018, 2017).

947 Because of the inhibitory effect that acetic acid could have at certain concentration on different strains,
948 the use of this intermediate for PHB production is preferably applied on mixed cultures or two separated
949 cultures rather than a pure culture. Acetic acid can also be produced, together with propionic and butyric
950 acid, by anaerobic conversion of organic wastes, from which both pure and mixed cultures can produce these
951 short-chain carboxylates to sustain the carbon flux toward PHAs biosynthesis (Valentino et al., 2017).
952 However, the PHAs monomeric structure, which in turn strongly influence the polymer physical/mechanical
953 properties, is affected by this variation in the carboxylate composition (Garcia-Gonzalez and De Wever, 2018;
954 Valentino et al., 2017; Wang and Yu, 2000); therefore, such undefined carboxylate substrates are
955 undesirable. Furthermore, the consumption rate of these acids for PHAs production is preferential, this
956 means that one carboxylate is usually preferred with respect the others, and these less preferred
957 carboxylates could increase and accumulate in the fermentation medium, resulting in a toxic and/or
958 inhibiting effect on the producing host (Huschner et al., 2015).

959 6.1.2 Nutrient availability: C/N ratio

960 It is now clear that PHAs are mainly produced and accumulated under unbalanced nutrient conditions,
961 for one or multiple nutrients, especially in the case of PHAs accumulation using non-growth-associated
962 bacterium like *C. necator*, which gives its best when feedstock lacks an essential nutrient (or more than one)
963 for growing such as nitrogen, phosphorous, oxygen etc (Repaske and Repaske, 1976; Valentino et al., 2015).

964 These parameters, together with carbon source, environmental conditions, such as pH and the
965 nitrogen/carbon ratio, affect the growth rate, the cell density and the PHB molecular size (Wang and Yu,
966 2000). In particular, the PHB productivity seems to be higher during nutrient limitation rather than deficiency
967 (Johnson et al., 2010; Valentino et al., 2015). In fact, the absence of an essential nutrient for the growth
968 inevitably induce to cellular PHAs saturation, while nutrient limitation still allows cells to slowly duplicate,
969 still keeping on PHAs accumulation period without empowering excessive growth response (Kourmentza et
970 al., 2017b).

971 The nitrogen content should be also monitored to manipulate the fine balance between biomass
972 growth and PHAs biosynthesis; a high nitrogen content provides biomass growth instead of PHAs
973 accumulation (Yan et al., 2003). This can be explained as follows: when the medium contains high amount of
974 nitrogen, all these elements are directed toward macromolecules biosynthesis, like DNA, RNA and proteins
975 and energy molecules, allowing cell division; instead, with little or no nitrogen concentration in the medium,
976 this cannot happen and all apparent increase in biomass concentration which can be recorded
977 spectrophotometrically is actually due to accumulation of PHB in the cells (Marudkla et al., 2018).

978 In one study, it has been reported that nitrogen shortage did not enhance the PHAs accumulation in
979 mixed cultures fermentations. (Dionisi et al., 2006). As reported in another paper, the main reason for limiting
980 the nitrogen concentration would be to hamper bacterial growth of non PHA-accumulating bacteria (Marang
981 et al., 2014). These different observations and results coming from the analysis of different cultures may lead
982 to conclude that several contradictions should be clarified; however, both researcher groups underline that
983 the requirement for nitrogen limitation for a high PHAs content is mainly based on the general composition
984 of the medium and on the supplied substrate. (Kourmentza et al., 2017b).

985 Basing on the C/N ratio as a measure of nutrient availability variation, fermentation strategies can be
986 classified into two-stage and three stage fermentation. The first one consists of two phases in which C/N ratio
987 changes to favour firstly cell growth, for instance using a pH-stat feeding with a C/N = 10 like occurred in
988 Garcia-Gonzalez (2018), and then biopolymer accumulation, thanks to the limitation or deficiency of nitrogen
989 content in the medium ($C/N = \infty$). In this case, only one strain has been used to carry on the PHA biosynthesis,
990 but this strategy can be applied also using two different strains.

991 Instead, the three-phase fed-batch culture strategy consists in an intermediate phase, between C/N =
992 10 and $C/N = \infty$, to allow a low residual biomass growth rate, in order to maintain an active metabolism and
993 to increase PHB productivity. For Garcia-Gonzalez et al. (2018), this intermediate phase, considered as an
994 enrichment step before the PHB accumulation phase, has been set by using a solution with a high C/N ratio
995 ($C/N = 90$) instead of the initial pH-stat solution (Garcia-Gonzalez and De Wever, 2018; Huschner et al., 2015).
996 This three-step strategy, tailored by Huschner (2015) and Garcia-Gonzalez (2018), has been proven to be
997 efficient for reaching high PHB content on CDW. However, this separate enrichment step created some

998 criticism; the main critic is that during this intermediate phase, PHB is produced, but not accumulated, since
999 it is possibly consumed to drive the selection; moreover it seems having a lowering effect on the overall
1000 PHA/Substrate yields of the system. Further analysis and studies have to be performed, yet, in order to
1001 improve this system.

1002 **6.2 Life cycle assessment for PHA production in *C. necator***

1003 What it has been reported so far about waste substrates it is only a relatively small part of an entire
1004 research field based on Life Cycle Assessment (LCA) and economic strategies aimed to reduce the whole
1005 production impact to reach a final equation equal to zero, in which everything enters the production process
1006 results in zero impact, or even less than zero, in which the waste becomes productive from several point of
1007 view (environmental and economical, mainly).

1008 From the economic perspective, it is possible to distinguish two kind of economic strategies. One is the
1009 so called “linear economy”, based on the assumption that planet resources are abundant, available, easy to
1010 achieve, and the wastes coming from them are easy to dispose. The other is the circular bioeconomy, which
1011 concerns the “*sustainable resource-efficient valorization of biomass in integrated, multi-output production*
1012 *chains (e.g. biorefineries) while also making use of residues and wastes and optimizing the value of biomass*
1013 *over time via cascading*” (Stegmann et al., 2020).

1014 Economic, environmental and social aspects are all linked and all the necessary for sustainability
1015 contribution. Circular bioeconomy principles are considered for economic policies of bioplastics production
1016 using waste feedstocks and with all the sustainability assessments based on environmental and economic
1017 paths. Key points that circular economy has to challenge to spread are policies and political agreements, the
1018 costs and the current small size of bio-based markets (Stegmann et al., 2020). The most promising product
1019 sectors for the bioeconomy are plastics and construction & building materials, since they have most recycling
1020 and cascading potential. These strategies have been extensively applied for bioplastics production, such as
1021 PHA, since this process can be highly beneficial, but still the “Zero Emission” path in the PHA production
1022 needs to get a step closer towards sustainability.

1023 The technical challenges of bioplastics production struggle to enter mainstream markets and they are
1024 still confined in their niche markets. The main limitation is the very high production cost; in fact, the
1025 conventional petroleum-based polymers have a cost of approximately US \$ 1000–1500/MT, compared to US
1026 \$ 4000/MT up to US \$ 15000/MT for biopolymers such as PHB (Kosseva and Rusbandi, 2018).

1027 In particular, for what concern PHAs, the high production costs are due to waste disposal and pure
1028 substrates used. From this, the need to improve and upgrade the waste, particularly the industrial wastes,
1029 into feedstocks for production of PHAs, replacing expensive carbon sources. Moreover, in order to maintain
1030 the circularity of the entire process, efficient recycling of waste streams and both good upstream and
1031 downstream processes are required.

1032 Recycling from and for PHA production is important both for cost reduction and for minimization of
1033 the environmental load. An interesting approach in recycling step aimed to increase the PHA production is
1034 the feed forward method. This strategy is carried out by furnishing the same biomass obtained in the previous
1035 reaction cycle and from which the PHAs is extracted to the culture which will be used during the next reaction
1036 step for further carbon conversion into PHA from the PHA-reduced biomass. This approach has the advantage
1037 to avoid the need for new biomass, thus reducing carbon requirement and costs (Kosseva and Rusbandi,
1038 2018; Yadav et al., 2020). The same strategy can be applied for reutilization of waste streams for industrial
1039 PHAs production (Dias et al., 2006; Valentino et al., 2017).

1040 Another possibility in the recycling approach is the use of derivatives sources from PHA production and
1041 extraction for other purposes. This might be applied for components released after cell-disruption, such as
1042 proteins released, which account about 50% of the dry weight of bacterial cells; the waste stream containing
1043 the protein could be used as nitrogen source for the next batch of fermentation as well as proteins can be
1044 easily recovered by precipitation and then used as animal feed (Gherghel et al., 2019).

1045 Life cycle assessment (LCA) tools are needed to determine if a process can be defined as sustainable
1046 or not in terms of environmental impacts associated with any manufacturing steps involved in it, from raw
1047 material acquisition to disposal or recycling. It is aimed to analyze the whole process, from the cradle to the
1048 grave, in order to optimize the eco-design or environmental performance of the product or manufacturing
1049 process. The LCA analysis underlines the major process steps that have the greatest environmental impact,

1050 helping for researchers, decision-makers, policy-makers. However, LCA tools aimed to find and quantify the
1051 environmental impact of PHA production have been focussed so far on isolated aspects of production,
1052 without analyzing the whole production performances, such as energy requirements or only CO₂ emissions,
1053 which may sometimes be in contrast with each other (Atlić et al., 2011; Koller et al., 2018, 2017). The
1054 identification of the ecological hot spots it is important for the analysis and application of the whole process.

1055 Another tool which can be used to reach this issue is Sustainable process index (SPI). The SPI underlines
1056 the main and important parameters meaningful for the ecological pressure of PHA production, such as
1057 process yield, energy consumption and release of CO₂ (Koller et al., 2011).

1058 The Cleaner Production (CP) is another tool for environmental assessment, aiming to the minimization
1059 of the waste and emissions, still maximizing the output flow. The main point is helping optimization of the
1060 future PHA production processes, saving energy and minimizing waste. This is important for Industries which
1061 may analyse the materials and energy flow in order to identify key points of improvements (i.e. use of
1062 materials, less or absence of formation of wastewater, no waste streams, neither surplus heat, global
1063 warming gaseous emissions and no solid waste)(Koller et al., 2011; Yadav et al., 2020).

1064 Other sustainability assessment tools need to be mentioned, such as carbon footprint, carbon
1065 efficiency, health and safety score cards and biomass utilisation efficiency. All these strategies should be
1066 discussed and connected for a global sustainable development.

1067 **7.1 Conclusions**

1068 This review provides an overview on the main substrates used to produce PHAs, especially PHB, in *C.*
1069 *necator* strains, comparing the different yields and rate of biosynthesis in order to make an easier selection
1070 for both substrates and the most proper feeding strategy. The focus chosen is the comparison between pure
1071 and waste substrates, with the aim to encourage the application of circular economy strategies towards the
1072 selection and the application of substrates which might lead to a sustainable produced product.

1073 As we explained, the selected substrate will not define by itself the rate and yield of PHAs production
1074 for *C. necator*, since cultivation methods and downstream processes for biopolymer purification have their
1075 impact on the final product too. Moreover, the choice of a waste substrate does not define the entire system

1076 of production as sustainable, but different tools (e.g. LCA, SPI, CP) should be used to asses the sustainability
1077 of the whole process, from cradle to grave.

1078 As reported here, *C. necator* is a promising and versatile bacterium for PHAs bioaccumulation and
1079 further analysis about its metabolism may lead to the possibility to enhance the wide range of substrates
1080 which may be used to produce bioplastics using this chemolithoautotrophic bacterium. *C. necator* could be
1081 used for several industrial applications, not only strictly related to biopolymers synthesis, for which it became
1082 popular.

1083 Notwithstanding its wide potential, a lot have to be done in order to make industrial scale up easier to
1084 be achieved in a way to reuse waste material and reduce the cost impact, but mainly in order to make the
1085 overall process environmentally sustainable, dealing with recently increasing concern about the so called
1086 “green agreement”, focussed and interested in upcoming green chemistry and innovative applications.

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1090 **Conflicts of Interest**

1091 The authors declare no conflict of interest.

Figures and Tables

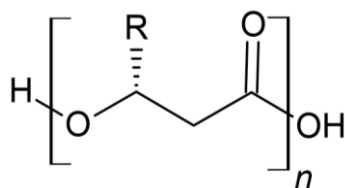


Figure 1. Polyhydroxyalkanoates (PHAs) general structure (ChemSketch). The *R* group which is the substituent group different for each PHAs. The *n* stands for the number of repeated unit for polymer. Both the side chain *R* and the monomer backbone (number of methylene -CH₂ groups) determine the type of biopolymer, as reported in figure 2.

| Biopolymer | Carbon | <i>R</i> group |
|--------------------------------|-----------------|----------------|
| Poly(3-hydroxybutyrate) PHB | C ₄ | Methyl |
| Poly(4-dihydroxybutyrate) P4HB | C ₄ | - |
| Poly(3-hydroxyvalerate) PHV | C ₅ | Ethyl |
| Poly(3-hydroxyhexanoate) | C ₆ | Propyl |
| Poly(3-hydroxyheptanoate) | C ₇ | Butyl |
| Poly(3-hydroxyoctanoate) | C ₈ | Pentyl |
| Poly(3-hydroxynonanoate) | C ₉ | Hexyl |
| Poly(3-hydroxydecanoate) | C ₁₀ | Heptyl |
| Poly(3-hydroxyundecanoate) | C ₁₁ | Octyl |
| Poly(3-hydroxydodecanoate) | C ₁₂ | Nonyl |
| Poly(3-hydroxytridecanoate) | C ₁₃ | Decyl |
| Poly(3-hydroxytetradecanoate) | C ₁₄ | Undecyl |
| Poly(3-hydroxypentadecanoate) | C ₁₅ | Dodecyl |
| Poly(3-hydroxyhexadecanoate) | C ₁₆ | Tridecyl |

Figure 2. PHAs general structures and substituent groups (modified from Mutiara et al., 2014). The number of carbon atoms of the substituent group and *R* group name are reported for each PHA.

| Parameters | Conventional plastic | Biopolymers | | | |
|-----------------------------------|----------------------|-------------|------------|--------------|--------------|
| | <i>PP</i> | <i>PHB</i> | <i>PHV</i> | <i>PHB4B</i> | <i>PHBHx</i> |
| Melting temperature (°C) | 170 | 168-182 | 145 | 150 | 127 |
| Cristallinity (%) | 50-70 | 60 | 56 | 45 | 34 |
| Glass transition temperature (C°) | -10 | 2-4 | -1 | -7 | -1 |
| Extension to break (%) | 400 | 5 | 50 | 444 | 400 |
| Tensile strength (Mpa) | 38 | 43 | 20 | 26 | 21 |

Figure 3. Chemical and physical properties of PHAs and polypropylene (PP) (modified from (Georgios et al., 2016; Verlinden et al., 2007)). “PHB” and “PHV” stand for poly(3-hydroxybutyrate) and poly(3-hydroxyvalerate) respectively, while “PHB4B” stands for poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (scl-copolymers) and “PHBHx” stands for copolymers that contain 3-hydroxyhexanoate (mcl-copolymers). Biopolymers properties are compared to those of conventional plastics to highlight differences and similar properties.

| Name of the company | Biocatalyst | Substrate | Product (trademark) | Production capacity |
|--|--|--|---|--------------------------------|
| Biomatera, Canada | Non-pathogenic, non-transgenic bacteria isolated from soil | Renewable raw materials | PHA resins (Biomatera) | - |
| Bio-ON srl, Italy | <i>C. necator</i> | Sugar beets | PHB, PHBV spheres (minerv®-PHA) | 10,000 t/y |
| Biomer, Germany | - | Sugar (sucrose) | PHB pellets (Biomer®) | - |
| BluePHA, China | Development of microbial strains via synthetic biology | - | Customized PHBVHHx, PHV, P3HP3HB, P3HP4HB, P3HP, P4HB synthesis | - |
| Danimer Scientific, USA | - | Cold pressed canola oil | mcl-PHA (Nodax® PHA) | - |
| PHB Industrial S.A., Brazil | Alcaligenes sp. | Saccharose | PHB, PHBV (BIOCYCLE®) | 3000 t/y |
| PolyFerm, Canada | Naturally selected microorganisms | Sugars, vegetable oils | mcl-PHA (VersaMer™ PHA) | - |
| Biotec, Italy | - | Potato starch | Bioplast® | - |
| TianAn Biologic Materials Co. Ltd. China | <i>C. necator</i> | Dextrose deriving from local corn of cassava | PHB, PHBV (ENMAT™) | 10,000 t/y, 50,000 t/y by 2020 |
| Metabolix (Woburn, MA, USA) | - | Corn | Mirel™-PHA | 50,000 t/y |
| Biomer (Krailling, Germany) | - | Sugar | PHB | - |
| Tianjin GreenBio Material Co. China | - | Sugar | P (3, 4HB) films, pellets/foam pellets (Sogreen®) | 10,000 t/y |
| Kaneka, Japan | <i>C. necator</i> KNK005 | Plant oil | PHBH™ (P(3HB-co-3HHx)) | 5000 t/y |
| Novamont, Italy | - | Starch, cellulose, vegetable oils | Mater-Bi® | - |

Figure 4. Pilot and industrial scale PHAs of some manufacturers active worldwide (modified from (Jiang et al., 2016; Kourmentza et al., 2017b; Miyahara et al., 2021)). Biocatalyst, substrate, product (trademark) and production capacity are listed, if known, for each manufacturer.

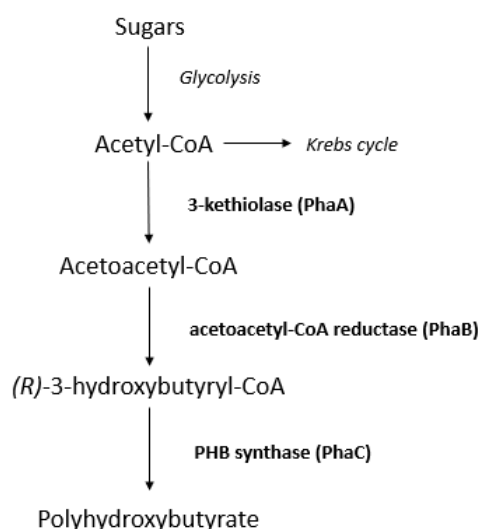


Figure 5. Schematic representation of poly (3-hydroxybutyrate) (PHB) biosynthetic pathway. The pathway is simplified starting from sugars.

| Waste substrates | Strain | Cell dry weight (g/L) | PHA content (% or g/L) | Productivity (g/ L · h) | Yield (gPHA/gsubstrate) | Source |
|----------------------------|-------------------------------|-----------------------|------------------------|-------------------------|-------------------------|---------------------------|
| Kenaf biomass | <i>C. necator</i> H16 | 12.4 | 70.10% | / | 0.494 | (Saratale et al., 2019) |
| Saccharified potato starch | <i>C. necator</i> NCIMB 11599 | 179 | about 55% | 1.47 | 0.22-0.46 | (Haas et al., 2008) |
| Lignin derivatives | <i>C. necator</i> H16 | 0.69-1.6 | 63-65% | / | / | (Tomizawa et al., 2014) |
| Animal fats | <i>C. necator</i> H16 | 45 | 26 g/L | 0.36 | 0.4 | (Riedel et al., 2015) |
| Waste frying oil | <i>C. necator</i> H16 | 4.2 | 79% | / | / | (Verlinden et al., 2011) |
| Corn oil | <i>C. necator</i> H16 | 3.5-3.6 | 81% | / | / | (Fukui and Doi, 1998) |
| Waste glycerol | <i>C. necator</i> DSM 545 | 48.6 | 50% | 1.1 | / | (Cavalheiro et al., 2009) |
| Waste office paper | <i>C. necator</i> H16 | 7.74 | 0.58 | 0.061 | 0.21 | (Neelamegam et al., 2018) |
| Wine lees | <i>C. necator</i> DSM 7237 | / | 71.30% | 0.56 | / | (Dimou et al., 2015) |
| Wheat hydrolysate | <i>C. necator</i> H16 | 29.9 | 51.1 g/L | 0.29 | 0.7 | (Koutinas et al., 2007) |

Figure 6. Waste substrates used to produce PHB through different strains of *C. necator* fermentation analysed in this review. This table resumes the waste substrates highlighted in this review for *C. necator* biopolymer accumulation, comparing the cell dry weight (CDW), PHA content (% or g/L), productivity (g/ L · h) and yield

(g PHA/g substrate), if known, for each substrate used with different *C. necator* strains. The references from this paper are reported.

References

- 1092 Andreesen, J.R., Bahl, H., Gottschalk, G., 1989. Introduction to the Physiology and Biochemistry of the Genus
1093 *Clostridium*. *Clostridia* 27–62. https://doi.org/10.1007/978-1-4757-9718-3_2
- 1094 Andreeßen, B., Lange, A.B., Robenek, H., Steinbüchel, A., 2010. Conversion of glycerol to poly(3-
1095 hydroxypropionate) in recombinant *Escherichia coli*. *Appl. Environ. Microbiol.* 76, 622–626.
1096 <https://doi.org/10.1128/AEM.02097-09>
- 1097 Atlić, A., Koller, M., Scherzer, D., Kutschera, C., Grillo-Fernandes, E., Horvat, P., Chiellini, E., Braunegg, G.,
1098 2011. Continuous production of poly([R]-3-hydroxybutyrate) by *Cupriavidus necator* in a multistage
1099 bioreactor cascade. *Appl. Microbiol. Biotechnol.* 91, 295–304. [https://doi.org/10.1007/s00253-011-](https://doi.org/10.1007/s00253-011-3260-0)
1100 3260-0
- 1101 Auta, H.S., Emenike, C.U., Jayanthi, B., Fauziah, S.H., 2018. Growth kinetics and biodeterioration of
1102 polypropylene microplastics by *Bacillus* sp. and *Rhodococcus* sp. isolated from mangrove sediment.
1103 *Mar. Pollut. Bull.* 127, 15–21. <https://doi.org/10.1016/j.marpolbul.2017.11.036>
- 1104 Bai, Z., Jin, B., Li, Y., Chen, J., Li, Z., 2008. Utilization of winery wastes for *Trichoderma viride* biocontrol agent
1105 production by solid state fermentation. *J. Environ. Sci.* 20, 353–358. [https://doi.org/10.1016/S1001-](https://doi.org/10.1016/S1001-0742(08)60055-8)
1106 0742(08)60055-8
- 1107 Barabote, R.D., Saier, M.H., 2005. Comparative Genomic Analyses of the Bacterial Phosphotransferase
1108 System. *Microbiol. Mol. Biol. Rev.* 69, 608–634. <https://doi.org/10.1128/mmbr.69.4.608-634.2005>
- 1109 Basso, L.C., De Amorim, H. V., De Oliveira, A.J., Lopes, M.L., 2008. Yeast selection for fuel ethanol production
1110 in Brazil. *FEMS Yeast Res.* 8, 1155–1163. <https://doi.org/10.1111/j.1567-1364.2008.00428.x>
- 1111 Bi, C., Su, P., Müller, J., Yeh, Y.C., Chhabra, S.R., Beller, H.R., Singer, S.W., Hillson, N.J., 2013. Development of
1112 a broad-host synthetic biology toolbox for *Ralstonia eutropha* and its application to engineering
1113 hydrocarbon biofuel production. *Microb. Cell Fact.* 12, 1–10. [https://doi.org/10.1186/1475-2859-12-](https://doi.org/10.1186/1475-2859-12-107)
1114 107
- 1115 Bowien and Schlegel, H., 1981. Physiology and biochemistry of aerobic hydrogen-oxidizing bacteria. *Annu*
1116 *Rev Microbiol* 35. <https://doi.org/10.1146/annurev.mi.35.100181.002201>
- 1117 Brányiková, I., Maršáľková, B., Doucha, J., Brányik, T., Bišová, K., Zachleder, V., Vítová, M., 2011. Microalgae-
1118 novel highly efficient starch producers. *Biotechnol. Bioeng.* 108, 766–776.
1119 <https://doi.org/10.1002/bit.23016>
- 1120 Bugnicourt, E., Cinelli, P., Lazzeri, A., Alvarez, V., 2014. Polyhydroxyalkanoate (PHA): Review of synthesis,
1121 characteristics, processing and potential applications in packaging. *Express Polym. Lett.* 8, 791–808.
1122 <https://doi.org/10.3144/expresspolymlett.2014.82>
- 1123 Cavaleiro, J.M.B.T., de Almeida, M.C.M.D., Grandfils, C., da Fonseca, M.M.R., 2009. Poly(3-hydroxybutyrate)
1124 production by *Cupriavidus necator* using waste glycerol. *Process Biochem.* 44, 509–515.
1125 <https://doi.org/10.1016/j.procbio.2009.01.008>
- 1126 Cavaleiro, J.M.B.T., Raposo, R.S., de Almeida, M.C.M.D., Teresa Cesário, M., Sevrin, C., Grandfils, C., da
1127 Fonseca, M.M.R., 2012. Effect of cultivation parameters on the production of poly(3-hydroxybutyrate-
1128 co-4-hydroxybutyrate) and poly(3-hydroxybutyrate-4-hydroxybutyrate-3-hydroxyvalerate) by
1129 *Cupriavidus necator* using waste glycerol. *Bioresour. Technol.* 111, 391–397.
1130 <https://doi.org/10.1016/j.biortech.2012.01.176>
- 1131 Chakravarty, J., Brigham, C.J., 2018. Solvent production by engineered *Ralstonia eutropha*: channeling carbon
1132 to biofuel. *Appl. Microbiol. Biotechnol.* <https://doi.org/10.1007/s00253-018-9026-1>
- 1133 Chen, G.Q., 2012. New challenges and opportunities for industrial biotechnology. *Microb. Cell Fact.*
1134 <https://doi.org/10.1186/1475-2859-11-111>
- 1135 Chia, K.H., Ooi, T.F., Saika, A., Tsuge, T., Sudesh, K., 2010. Biosynthesis and characterization of novel
1136 polyhydroxyalkanoate polymers with high elastic property by *Cupriavidus necator* PHB-4 transformant.
1137 *Polym. Degrad. Stab.* 95, 2226–2232. <https://doi.org/10.1016/j.polymdegradstab.2010.09.011>
- 1138 Cibis, E., Ryznar-Luty, A., Krzywonos, M., Miskiewicz, T., 2007. Aerobic biodegradation of vinasse: Effect of
1139 temperature, initial pH and pH control. *J. Biotechnol.* 131, S155–S156.
1140 <https://doi.org/10.1016/j.jbiotec.2007.07.875>
- 1141 Dexter, J.E., Wood, P.J., 1996. Recent applications of debranning of wheat before milling. *Trends Food Sci.*

Technol. [https://doi.org/10.1016/0924-2244\(96\)81326-4](https://doi.org/10.1016/0924-2244(96)81326-4)

Dias, J.M.L., Lemos, P.C., Serafim, L.S., Oliveira, C., Eiroa, M., Albuquerque, M.G.E., Ramos, A.M., Oliveira, R., Reis, M.A.M., 2006. Recent advances in polyhydroxyalkanoate production by mixed aerobic cultures: From the substrate to the final product. *Macromol. Biosci.* <https://doi.org/10.1002/mabi.200600112>

Dimou, C., Kopsahelis, N., Papadaki, A., Papanikolaou, S., Kookos, I.K., Mandala, I., Koutinas, A.A., 2015. Wine lees valorization: Biorefinery development including production of a generic fermentation feedstock employed for poly(3-hydroxybutyrate) synthesis. *Food Res. Int.* 73, 81–87. <https://doi.org/10.1016/j.foodres.2015.02.020>

Dionisi, D., Majone, M., Vallini, G., Di Gregorio, S., Beccari, M., 2006. Effect of the applied organic load rate on biodegradable polymer production by mixed microbial cultures in a sequencing batch reactor. *Biotechnol. Bioeng.* 93, 76–88. <https://doi.org/10.1002/bit.20683>

Doi, Y., Kitamura, S., Abe, H., 1995. Microbial Synthesis and Characterization of Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). *Macromolecules* 28, 4822–4828. <https://doi.org/10.1021/ma00118a007>

Dubey, A.K., Gupta, P.K., Garg, N., Naithani, S., 2012. Bioethanol production from waste paper acid pretreated hydrolyzate with xylose fermenting *Pichia stipitis*. *Carbohydr. Polym.* 88, 825–829. <https://doi.org/10.1016/j.carbpol.2012.01.004>

Fernández Dacosta, C., 2018. Alternative sources to fossil carbon: Ex-ante assessment of novel technologies using waste as a resource.

Filip, Z., Giessen, U., 1979. Polyurethane as the sole nutrient source for *Aspergillus niger*, and *Cladosporium herbarum*. *Appl. Microbiol. Biotechnol.* 7, 277–280. <https://doi.org/doi:10.1007/bf00498022>

Franz, A., Rehner, R., Kienle, A., Grammel, H., 2012. Rapid selection of glucose-utilizing variants of the polyhydroxyalkanoate producer *Ralstonia eutropha* H16 by incubation with high substrate levels. *Lett. Appl. Microbiol.* 54, 45–51. <https://doi.org/10.1111/j.1472-765X.2011.03171.x>

Friedrich, C.G., Bowien, B., Friedrich, B., 1979. Formate and oxalate metabolism in *Alcaligenes eutrophus*. *J. Gen. Microbiol.* 115, 185–192. <https://doi.org/10.1099/00221287-115-1-185>

Fukui, T., Doi, Y., 1998. Efficient production of polyhydroxyalkanoates from plant oils by *Alcaligenes eutrophus* and its recombinant strain. *Appl. Microbiol. Biotechnol.* 49, 333–336. <https://doi.org/10.1007/s002530051178>

Fukui, T., Mukoyama, M., Orita, I., Nakamura, S., 2014. Enhancement of glycerol utilization ability of *Ralstonia eutropha* H16 for production of polyhydroxyalkanoates. *Appl. Microbiol. Biotechnol.* 98, 7559–7568. <https://doi.org/10.1007/s00253-014-5831-3>

Garcia-Gonzalez, L., De Wever, H., 2018. Acetic acid as an indirect sink of CO₂ for the synthesis of polyhydroxyalkanoates (PHA): Comparison with PHA production processes directly using CO₂ as feedstock. *Appl. Sci.* 8. <https://doi.org/10.3390/app8091416>

Garcia-Gonzalez, L., De Wever, H., 2017. Valorisation of CO₂-rich off-gases to biopolymers through biotechnological process. *FEMS Microbiol. Lett.* 364, 1–8. <https://doi.org/10.1093/femsle/fnx196>

Georgios, K., Silva, A., Furtado, S., 2016. Applications of Green Composite Materials, in: *Biodegradable Green Composites*. pp. 312–337. <https://doi.org/10.1002/9781118911068.ch10>

Gherghel, A., Teodosiu, C., De Gisi, S., 2019. A review on wastewater sludge valorisation and its challenges in the context of circular economy. *J. Clean. Prod.* <https://doi.org/10.1016/j.jclepro.2019.04.240>

Ghysels, S., Islam, S., Wever, H. De, Volcke, E.I.P., 2018. Bioresource Technology Targeted poly (3-hydroxybutyrate-co-3-hydroxyvalerate) bioplastic production from carbon dioxide. *Bioresour. Technol.* 249, 858–868. <https://doi.org/10.1016/j.biortech.2017.10.081>

Grousseau, E., Lu, J., Gorret, N., Guillouet, S.E., Sinskey, A.J., 2014. Isopropanol production with engineered *Cupriavidus necator* as bioproduction platform. *Appl. Microbiol. Biotechnol.* 98, 4277–4290. <https://doi.org/10.1007/s00253-014-5591-0>

Haas, R., Jin, B., Zepf, F.T., 2008. Production of poly(3-hydroxybutyrate) from waste potato starch. *Biosci. Biotechnol. Biochem.* 72, 253–256. <https://doi.org/10.1271/bbb.70503>

Hanisch, J., 1, 3 Marc Waßltermann, 1, Robenek, 2, 3 Horst, Chel, and A.S., 2006. The *Ralstonia eutropha* H16 phasin PhaP1 is targeted to intracellular triacylglycerol inclusions in *Rhodococcus opacus* PD630 and *Mycobacterium smegmatis* mc 2 155 , and provides an anchor to target other proteins Jan Ha Printed in Great Britain. *Microbiol.* 152, 3271–3280 3271–3280. <https://doi.org/10.1099/mic.0.28969-0>

Hernández-Muñoz, P., Kanavouras, A., Ng, P.K.W., Gavara, R., 2003. Development and Characterization of

Biodegradable Films Made from Wheat Gluten Protein Fractions. *J. Agric. Food Chem.* 51, 7647–7654.
<https://doi.org/10.1021/jf034646x>

Huschner, F., Grousseau, E., Brigham, C.J., Plassmeier, J., Popovic, M., Rha, C., Sinskey, A.J., 2015. Development of a feeding strategy for high cell and PHA density fed-batch fermentation of *Ralstonia eutropha* H16 from organic acids and their salts. *Process Biochem.* 50, 165–172.
<https://doi.org/10.1016/j.procbio.2014.12.004>

Insomphun, C., Xie, H., Mifune, J., Kawashima, Y., Orita, I., Nakamura, S., Fukui, T., 2015. Improved artificial pathway for biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) with high C6-monomer composition from fructose in *Ralstonia eutropha*. *Metab. Eng.* 27, 38–45.
<https://doi.org/10.1016/j.ymben.2014.10.006>

Ishizaki, A., Tanaka, K., Taga, N., 2001. Microbial production of poly-D-3-hydroxybutyrate from CO₂. *Appl. Microbiol. Biotechnol.* 57, 6–12. <https://doi.org/10.1007/s002530100775>

Islam Mozumder, M.S., Garcia-Gonzalez, L., Wever, H. De, Volcke, E.I.P., 2015. Poly(3-hydroxybutyrate) (PHB) production from CO₂: Model development and process optimization. *Biochem. Eng. J.* 98, 107–116.
<https://doi.org/10.1016/j.bej.2015.02.031>

Jendrossek, D., 2009. Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes). *J. Bacteriol.* 191, 3195–3202. <https://doi.org/10.1128/JB.01723-08>

Jeon, B.Y., Yi, J.Y., Jung, I.L., Park, D.H., 2013. Activation of Ethanol Production by Combination of Recombinant *Ralstonia eutropha* and Electrochemical Reducing Power. *Adv. Microbiol.* 03, 42–45.
<https://doi.org/10.4236/aim.2013.31006>

Jeon, J.M., Brigham, C.J., Kim, Y.H., Kim, H.J., Yi, D.H., Kim, H., Rha, C.K., Sinskey, A.J., Yang, Y.H., 2014. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (P(HB-co-HHx)) from butyrate using engineered *Ralstonia eutropha*. *Appl. Microbiol. Biotechnol.* 98, 5461–5469.
<https://doi.org/10.1007/s00253-014-5617-7>

Jiang, G., Hill, D.J., Kowalczyk, M., Johnston, B., Adamus, G., Irorere, V., Radecka, I., 2016. Carbon sources for polyhydroxyalkanoates and an integrated biorefinery. *Int. J. Mol. Sci.*
<https://doi.org/10.3390/ijms17071157>

Jiang, Y., Marang, L., Tamis, J., Loosdrecht, M.C.M. Van, Dijkman, H., Kleerebezem, R., 2012. Waste to resource: Converting paper mill wastewater to bioplastic. *Water Res.* 46, 5517–5530.
<https://doi.org/10.1016/j.watres.2012.07.028>

Johnson, B.F., Stanier, R.Y., 1971. Dissimilation of aromatic compounds by *Alcaligenes eutrophus*. *J. Bacteriol.* 107, 468–475. <https://doi.org/10.1128/jb.107.2.468-475.1971>

Johnson, K., Kleerebezem, R., van Loosdrecht, M.C.M., 2010. Influence of ammonium on the accumulation of polyhydroxybutyrate (PHB) in aerobic open mixed cultures. *J. Biotechnol.* 147, 73–79.
<https://doi.org/10.1016/j.jbiotec.2010.02.003>

Johnston, B., Jiang, G., Hill, D., Adamus, G., Kwiecień, I., Zięba, M., Sikorska, W., Green, M., Kowalczyk, M., Radecka, I., 2017. The molecular level characterization of biodegradable polymers originated from polyethylene using non-oxygenated polyethylene wax as a carbon source for polyhydroxyalkanoate production. *Bioengineering* 4. <https://doi.org/10.3390/bioengineering4030073>

Johnston, B., Radecka, I., Chiellini, E., Barsi, D., Ilieva, V.I., Sikorska, W., Musiol, M., Zięba, M., Chaber, P., Marek, A.A., Mendrek, B., Ekere, A.I., Adamus, G., Kowalczyk, M., 2019. Mass spectrometry reveals molecular structure of polyhydroxyalkanoates attained by bioconversion of oxidized polypropylene waste fragments. *Polymers (Basel)*. 11. <https://doi.org/10.3390/polym11101580>

Jurgens, G., Survase, S., Berezina, O., Sklavounos, E., Linnekoski, J., Kurkijärvi, A., Väkevä, M., van Heiningen, A., Granström, T., 2012. Butanol production from lignocellulosics. *Biotechnol. Lett.* 34, 1415–1434.
<https://doi.org/10.1007/s10529-012-0926-3>

Kaddor, C., Steinbüchel, A., 2011. Effects of homologous phosphoenolpyruvate-carbohydrate phosphotransferase system proteins on carbohydrate uptake and poly(3-Hydroxybutyrate) accumulation in *Ralstonia eutropha* H16. *Appl. Environ. Microbiol.* 77, 3582–3590.
<https://doi.org/10.1128/AEM.00218-11>

Kahar, P., Tsuge, T., Taguchi, K., Doi, Y., 2004. High yield production of polyhydroxyalkanoates from soybean oil by *Ralstonia eutropha* and its recombinant strain. *Polym. Degrad. Stab.* 83, 79–86.
[https://doi.org/10.1016/S0141-3910\(03\)00227-1](https://doi.org/10.1016/S0141-3910(03)00227-1)

1248 Karan, H., Funk, C., Grabert, M., Oey, M., Hankamer, B., 2019. Green Bioplastics as Part of a Circular
1249 Bioeconomy. *Trends Plant Sci.* <https://doi.org/10.1016/j.tplants.2018.11.010>

1250 Khanna, S., Srivastava, A.K., 2005. Recent advances in microbial polyhydroxyalkanoates. *Process Biochem.*
1251 40, 607–619. <https://doi.org/10.1016/j.procbio.2004.01.053>

1252 Kharkwal, S., Karimi, I., Chang, M., Lee, D.-Y., 2009. Strain Improvement and Process Development for
1253 Biobutanol Production. *Recent Pat. Biotechnol.* 3, 202–210.
1254 <https://doi.org/10.2174/187220809789389117>

1255 Khosravi-Darani, K., Mokhtari, Z.B., Amai, T., Tanaka, K., 2013. Microbial production of poly(hydroxybutyrate)
1256 from C1 carbon sources. *Appl. Microbiol. Biotechnol.* <https://doi.org/10.1007/s00253-012-4649-0>

1257 Kim, H.S., Oh, Y.H., Jang, Y.A., Kang, K.H., David, Y., Yu, J.H., Song, B.K., Choi, J. il, Chang, Y.K., Joo, J.C., Park,
1258 S.J., 2016. Recombinant *Ralstonia eutropha* engineered to utilize xylose and its use for the production
1259 of poly(3-hydroxybutyrate) from sunflower stalk hydrolysate solution. *Microb. Cell Fact.* 15, 1–13.
1260 <https://doi.org/10.1186/s12934-016-0495-6>

1261 Koller, M., Braunegg, G., 2015. Biomediated production of structurally diverse poly(hydroxyalkanoates) from
1262 surplus streams of the animal processing industry. *Polimery/Polymers* 60, 298–308.
1263 <https://doi.org/10.14314/polimery.2015.298>

1264 Koller, M., Gasser, I., Schmid, F., Berg, G., 2011. Linking ecology with economy: Insights into
1265 polyhydroxyalkanoate-producing microorganisms. *Eng. Life Sci.*
1266 <https://doi.org/10.1002/elsc.201000190>

1267 Koller, M., Maršálek, L., de Sousa Dias, M.M., Braunegg, G., 2017. Producing microbial polyhydroxyalkanoate
1268 (PHA) biopolyesters in a sustainable manner. *N. Biotechnol.* <https://doi.org/10.1016/j.nbt.2016.05.001>

1269 Koller, M., Shahzad, K., Braunegg, G., 2018. Waste streams of the animal-processing industry as feedstocks
1270 to produce polyhydroxyalkanoate biopolyesters. *Appl. Food Biotechnol.*
1271 <https://doi.org/10.22037/afb.v%vi%i.18557>

1272 Kookos, I.K., Koutinas, A., Vlysidis, A., 2019. Life cycle assessment of bioprocessing schemes for poly(3-
1273 hydroxybutyrate) production using soybean oil and sucrose as carbon sources. *Resour. Conserv. Recycl.*
1274 141, 317–328. <https://doi.org/10.1016/j.resconrec.2018.10.025>

1275 Kosseva, M.R., Rusbandi, E., 2018. Trends in the biomanufacture of polyhydroxyalkanoates with focus on
1276 downstream processing. *Int. J. Biol. Macromol.* <https://doi.org/10.1016/j.ijbiomac.2017.09.054>

1277 Kotrba, P., Inui, M., Yukawa, H., 2001. Bacterial phosphotransferase system (PTS) in carbohydrate uptake and
1278 control of carbon metabolism. *J. Biosci. Bioeng.* [https://doi.org/10.1016/S1389-1723\(01\)80308-X](https://doi.org/10.1016/S1389-1723(01)80308-X)

1279 Kourmentza, C., Pl, J., Venetsaneas, N., Burniol-figols, A., Varrone, C., Gavala, H.N., Reis, M.A.M., 2017a.
1280 Recent Advances and Challenges towards Sustainable Polyhydroxyalkanoate (PHA) Production.
1281 *Bioingenireeing* 1–43. <https://doi.org/10.3390/bioengineering4020055>

1282 Kourmentza, C., Plácido, J., Venetsaneas, N., Burniol-Figols, A., Varrone, C., Gavala, H.N., Reis, M.A.M., 2017b.
1283 Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production.
1284 *Bioengineering.* <https://doi.org/10.3390/bioengineering4020055>

1285 Koutinas, A.A., Wang, R.H., Webb, C., 2005. Development of a process for the production of nutrient
1286 supplements for fermentations based on fungal autolysis. *Enzyme Microb. Technol.* 36, 629–638.
1287 <https://doi.org/10.1016/j.enzmictec.2004.01.015>

1288 Koutinas, A.A., Xu, Y., Wang, R., Webb, C., 2007. Polyhydroxybutyrate production from a novel feedstock
1289 derived from a wheat-based biorefinery. *Enzyme Microb. Technol.* 40, 1035–1044.
1290 <https://doi.org/10.1016/j.enzmictec.2006.08.002>

1291 Krauß, D., Hunold, K., Kusian, B., Lenz, O., Stülke, J., Bowien, B., Deutscher, J., 2009. Essential role of the
1292 hprK gene in *ralstonia eutropha* H16. *J. Mol. Microbiol. Biotechnol.* 17, 146–152.
1293 <https://doi.org/10.1159/000233505>

1294 Lee, H.M., Jeon, B.Y., Oh, M.K., 2016. Microbial production of ethanol from acetate by engineered *Ralstonia*
1295 *eutropha*. *Biotechnol. Bioprocess Eng.* 21, 402–407. <https://doi.org/10.1007/s12257-016-0197-2>

1296 Lee, S.E., Li, Q.X., Yu, J., 2009. Diverse protein regulations on PHA formation in *Ralstonia eutropha* on short
1297 chain organic acids. *Int. J. Biol. Sci.* 5, 215–225. <https://doi.org/10.7150/ijbs.5.215>

1298 Lee, S.E., Li, Q.X., Yu, J., 2006. Proteomic examination of *Ralstonia eutropha* in cellular responses to formic
1299 acid. *Proteomics* 6, 4259–4268. <https://doi.org/10.1002/pmic.200500824>

1300 Leipold, S., Petit-Boix, A., 2018. The circular economy and the bio-based sector - Perspectives of European

1301 and German stakeholders. J. Clean. Prod. 201, 1125–1137.
 1302 <https://doi.org/10.1016/j.jclepro.2018.08.019>
 1303 Lenz, O., Ludwig, M., Schubert, T., Bürstel, I., Ganskow, S., Goris, T., Schwarze, A., Friedrich, B., 2010. H₂
 1304 conversion in the presence of O₂ as performed by the membrane-bound [NiFe]-Hydrogenase of
 1305 *Ralstonia eutropha*. ChemPhysChem. <https://doi.org/10.1002/cphc.200901002>
 1306 Li, H., Opgenorth, P.H., Wernick, D.G., Rogers, S., Wu, T.Y., Higashide, W., Malati, P., Huo, Y.X., Cho, K.M.,
 1307 Liao, J.C., 2012. Integrated electromicrobial conversion of CO₂ to higher alcohols. Science (80-.).
 1308 <https://doi.org/10.1126/science.1217643>
 1309 Li, T., Ye, J., Shen, R., Zong, Y., Zhao, X., Lou, C., Chen, G.Q., 2016. Semirational Approach for Ultrahigh Poly(3-
 1310 hydroxybutyrate) Accumulation in *Escherichia coli* by Combining One-Step Library Construction and
 1311 High-Throughput Screening. ACS Synth. Biol. 5, 1308–1317.
 1312 <https://doi.org/10.1021/acssynbio.6b00083>
 1313 Lopar, M., Špoljarić, I.V., Cepanec, N., Koller, M., Braunegg, G., Horvat, P., 2014. Study of metabolic network
 1314 of *Cupriavidus necator* DSM 545 growing on glycerol by applying elementary flux modes and yield space
 1315 analysis. J. Ind. Microbiol. Biotechnol. 41, 913–930. <https://doi.org/10.1007/s10295-014-1439-y>
 1316 Lopez-Cuellar, Ma del Rocio, Gracida-Rodriguez, J.N., Fermín, P.-G., 2007. Production of
 1317 polyhydroxyalkanoates by *Wautersia eutropha* using vegetal oils as carbon source. J. Biotechnol. 131,
 1318 S156. <https://doi.org/doi:10.1016/j.jbiotec.2007.07.876>
 1319 Lu, J., Brigham, C.J., Gai, C.S., Sinskey, A.J., 2012. Studies on the production of branched-chain alcohols in
 1320 engineered *Ralstonia eutropha*. Appl. Microbiol. Biotechnol. 96, 283–297.
 1321 <https://doi.org/10.1007/s00253-012-4320-9>
 1322 Lu, J., Tappel, R.C., Nomura, C.T., 2009. Mini-review: Biosynthesis of poly(hydroxyalkanoates). Polym. Rev.
 1323 <https://doi.org/10.1080/15583720903048243>
 1324 Marang, L., Jiang, Y., van Loosdrecht, M.C.M., Kleerebezem, R., 2014. Impact of non-storing biomass on PHA
 1325 production: An enrichment culture on acetate and methanol. Int. J. Biol. Macromol. 71, 74–80.
 1326 <https://doi.org/10.1016/j.ijbiomac.2014.04.051>
 1327 Marudkla, J., Lee, W.C., Wannawilai, S., Chisti, Y., Sirisansaneeyakul, S., 2018. Model of acetic acid-affected
 1328 growth and poly(3-hydroxybutyrate) production by *Cupriavidus necator* DSM 545. J. Biotechnol. 268,
 1329 12–20. <https://doi.org/10.1016/j.jbiotec.2018.01.004>
 1330 Mathiot, C., Ponge, P., Gallard, B., Sassi, J.F., Delrue, F., Le Moigne, N., 2019. Microalgae starch-based
 1331 bioplastics: Screening of ten strains and plasticization of unfractionated microalgae by extrusion.
 1332 Carbohydr. Polym. 208, 142–151. <https://doi.org/10.1016/j.carbpol.2018.12.057>
 1333 Miyahara, Y., Hiroe, A., Sato, S., Tsuge, T., Taguchi, S., 2021. Microbial Polyhydroxyalkanoates (PHAs): From
 1334 Synthetic Biology to Industrialization . Biopolym. Biomed. Biotechnol. Appl. 231–264.
 1335 <https://doi.org/10.1002/9783527818310.ch8>
 1336 Moita, R., Freches, A., Lemos, P.C., 2014. Crude glycerol as feedstock for polyhydroxyalkanoates production
 1337 by mixed microbial cultures. Water Res. 58, 9–20. <https://doi.org/10.1016/j.watres.2014.03.066>
 1338 Mothes, G., Schnorpfeil, C., Ackermann, J.U., 2007. Production of PHB from crude glycerol. Eng. Life Sci. 7,
 1339 475–479. <https://doi.org/10.1002/elsc.200620210>
 1340 Mueller, R.J., 2006. Biological degradation of synthetic polyesters-Enzymes as potential catalysts for
 1341 polyester recycling. Process Biochem. 41, 2124–2128. <https://doi.org/10.1016/j.procbio.2006.05.018>
 1342 Mumtaz, T., Yahaya, N.A., Abd-Aziz, S., Abdul Rahman, N., Yee, P.L., Shirai, Y., Hassan, M.A., 2010. Turning
 1343 waste to wealth-biodegradable plastics polyhydroxyalkanoates from palm oil mill effluent-a Malaysian
 1344 perspective. J. Clean. Prod. 18, 1393–1402. <https://doi.org/10.1016/j.jclepro.2010.05.016>
 1345 Murugan, P., Gan, C.Y., Sudesh, K., 2017. Biosynthesis of P(3HB-co-3HHx) with improved molecular weights
 1346 from a mixture of palm olein and fructose by *Cupriavidus necator* Re2058/pCB113. Int. J. Biol.
 1347 Macromol. 102, 1112–1119. <https://doi.org/10.1016/j.ijbiomac.2017.05.006>
 1348 Neelamegam, A., Al-Battashi, H., Al-Bahry, S., Nallusamy, S., 2018. Biorefinery production of poly-3-
 1349 hydroxybutyrate using waste office paper hydrolysate as feedstock for microbial fermentation. J.
 1350 Biotechnol. 265, 25–30. <https://doi.org/10.1016/j.jbiotec.2017.11.002>
 1351 Ng, Z.R., Takahashi, K., Liu, Z., 2013. Isolation, characterization and evaluation of hyper 2-propanol producing
 1352 bacteria from Singapore environment. World J. Microbiol. Biotechnol. 29, 1059–1065.
 1353 <https://doi.org/10.1007/s11274-013-1269-5>

1354 Obruca, S., Marova, I., Snajdar, O., Mravcova, L., Svoboda, Z., 2010. Production of poly(3-hydroxybutyrate-
 1355 co-3-hydroxyvalerate) by *Cupriavidus necator* from waste rapeseed oil using propanol as a precursor of
 1356 3-hydroxyvalerate. *Biotechnol. Lett.* 32, 1925–1932. <https://doi.org/10.1007/s10529-010-0376-8>
 1357 Orita, I., Iwazawa, R., Nakamura, S., Fukui, T., 2012. Identification of mutation points in *Cupriavidus necator*
 1358 NCIMB 11599 and genetic reconstitution of glucose-utilization ability in wild strain H16 for
 1359 polyhydroxyalkanoate production. *J. Biosci. Bioeng.* 113, 63–69.
 1360 <https://doi.org/10.1016/j.jbiosc.2011.09.014>
 1361 Osburn, O.L., Brown, R.W., Werkman, C.H., 1937. The Butyl Alcohol-Isopropyl Alcohol Fermentation. *J. Biol.*
 1362 *Chem.* 121, 685–695. [https://doi.org/10.1016/s0021-9258\(18\)74298-1](https://doi.org/10.1016/s0021-9258(18)74298-1)
 1363 Patakova, P., Linhova, M., Rychtera, M., Paulova, L., Melzoch, K., 2013. Novel and neglected issues of acetone-
 1364 butanol-ethanol (ABE) fermentation by clostridia: *Clostridium* metabolic diversity, tools for process
 1365 mapping and continuous fermentation systems. *Biotechnol. Adv.* 31, 58–67.
 1366 <https://doi.org/10.1016/j.biotechadv.2012.01.010>
 1367 Pohlmann, A., Fricke, W.F., Reinecke, F., Kusian, B., Liesegang, H., Cramm, R., Eitingier, T., Ewering, C., Pötter,
 1368 M., Schwartz, E., Strittmatter, A., Voß, I., Gottschalk, G., Steinbüchel, A., Friedrich, B., Bowien, B., 2006.
 1369 Genome sequence of the bioplastic-producing “Knallgas” bacterium *Ralstonia eutropha* H16. *Nat.*
 1370 *Biotechnol.* 24, 1257–1262. <https://doi.org/10.1038/nbt1244>
 1371 Poirier, Y., Somerville, C., Schechtman, L.A., Satkowski, M.M., Noda, I., 1995. Synthesis of high-molecular-
 1372 weight poly([r]-(-)-3-hydroxybutyrate) in transgenic *Arabidopsis thaliana* plant cells. *Int. J. Biol.*
 1373 *Macromol.* 17, 7–12. [https://doi.org/10.1016/0141-8130\(95\)93511-U](https://doi.org/10.1016/0141-8130(95)93511-U)
 1374 Posada, J.A., Naranjo, J.M., López, J.A., Higuera, J.C., Cardona, C.A., 2011. Design and analysis of poly-3-
 1375 hydroxybutyrate production processes from crude glycerol. *Process Biochem.* 46, 310–317.
 1376 <https://doi.org/10.1016/j.procbio.2010.09.003>
 1377 Povolo, S., Toffano, P., Basaglia, M., Casella, S., 2010. Bioresource Technology Polyhydroxyalkanoates
 1378 production by engineered *Cupriavidus necator* from waste material containing lactose. *Bioresour.*
 1379 *Technol.* 101, 7902–7907. <https://doi.org/10.1016/j.biortech.2010.05.029>
 1380 Priefert, H., Hein, S., Kruger, N., Zeh, K., Schmidt, B., Steinbüchel, A., 1991. Identification and molecular
 1381 characterization of the *Alcaligenes eutrophus* H16 *aco* operon genes involved in acetoin catabolism. *J.*
 1382 *Bacteriol.* 173, 4056–4071. <https://doi.org/10.1128/jb.173.13.4056-4071.1991>
 1383 Qin, Z.H., Mou, J.H., Chao, C.Y.H., Chopra, S.S., Daoud, W., Leu, S. yuan, Ning, Z., Tso, C.Y., Chan, C.K., Tang,
 1384 S., Hathi, Z.J., Haque, M.A., Wang, X., Lin, C.S.K., 2021. Biotechnology of Plastic Waste Degradation,
 1385 Recycling, and Valorization: Current Advances and Future Perspectives. *ChemSusChem.*
 1386 <https://doi.org/10.1002/cssc.202100752>
 1387 Raberg, M., Peplinski, K., Heiss, S., Ehrenreich, A., Voigt, B., Döring, C., Bömeke, M., Hecker, M., Steinbüchel,
 1388 A., 2011. Proteomic and transcriptomic elucidation of the mutant *Ralstonia eutropha* G+1 with regard
 1389 to glucose utilization. *Appl. Environ. Microbiol.* 77, 2058–2070. <https://doi.org/10.1128/AEM.02015-10>
 1390 Radecka, I., Irorere, V., Jiang, G., Hill, D., Williams, C., Adamus, G., Kwiecień, M., Marek, A.A., Zawadiak, J.,
 1391 Johnston, B., Kowalczyk, M., 2016. Oxidized polyethylene wax as a potential carbon source for PHA
 1392 production. *Materials (Basel)*. 9, 1–16. <https://doi.org/10.3390/ma9050367>
 1393 Ragsdale, S.W., 2008. Enzymology of the Wood-Ljungdahl pathway of acetogenesis, in: *Annals of the New*
 1394 *York Academy of Sciences*. pp. 129–136. <https://doi.org/10.1196/annals.1419.015>
 1395 Repaske, R., Repaske, A.C., 1976. Quantitative requirements for exponential growth of *Alcaligenes*
 1396 *eutrophus*. *Appl. Environ. Microbiol.* 32, 585–591. <https://doi.org/10.1128/aem.32.4.585-591.1976>
 1397 Riedel, S.L., Jahns, S., Koenig, S., Bock, M.C.E., Brigham, C.J., Bader, J., Stahl, U., 2015. Polyhydroxyalkanoates
 1398 production with *Ralstonia eutropha* from low quality waste animal fats. *J. Biotechnol.* 214, 119–127.
 1399 <https://doi.org/10.1016/j.jbiotec.2015.09.002>
 1400 Riedel, S.L., Lu, J., Stahl, U., Brigham, C.J., 2014. Lipid and fatty acid metabolism in *Ralstonia eutropha*:
 1401 Relevance for the biotechnological production of value-added products. *Appl. Microbiol. Biotechnol.*
 1402 <https://doi.org/10.1007/s00253-013-5430-8>
 1403 Ru, J., Huo, Y., Yang, Y., 2020. Microbial Degradation and Valorization of Plastic Wastes. *Front. Microbiol.* 11.
 1404 <https://doi.org/10.3389/fmicb.2020.00442>
 1405 Ryberg, M.W., Hauschild, M.Z., Wang, F., Averous-monneray, S., Laurent, A., 2019. Resources , Conservation
 1406 & Recycling Global environmental losses of plastics across their value chains. *Resour. Conserv. Recycl.*

151, 104459. <https://doi.org/10.1016/j.resconrec.2019.104459>

Sangkharak, K., Prasertsan, P., 2011. Utilization of biodiesel waste as a feedstock for the production of polyhydroxybutyrate by *Cupriavidus necator*. *African J. Biotechnol.* 10, 17812–17824. <https://doi.org/10.5897/AJB11.2184>

Saratale, R.G., Saratale, G.D., Cho, S.K., Kim, D.S., Ghodake, G.S., Kadam, A., Kumar, G., Bharagava, R.N., Banu, R., Shin, H.S., 2019. Pretreatment of kenaf (*Hibiscus cannabinus* L.) biomass feedstock for polyhydroxybutyrate (PHB) production and characterization. *Bioresour. Technol.* 282, 75–80. <https://doi.org/10.1016/j.biortech.2019.02.083>

Sarmah, P., Rout, J., 2018. Efficient biodegradation of low-density polyethylene by cyanobacteria isolated from submerged polyethylene surface in domestic sewage water. *Environ. Sci. Pollut. Res.* 25, 33508–33520. <https://doi.org/10.1007/s11356-018-3079-7>

Schmidt, J., Wei, R., Oeser, T., Silva, L.A.D. e. S., Breite, D., Schulze, A., Zimmermann, W., 2017. Degradation of polyester polyurethane by bacterial polyester hydrolases. *Polymers (Basel)*. 9, 65. <https://doi.org/10.3390/polym9020065>

Schubert, P., Steinbüchel, A., Schlegel, H.G., 1988. Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. *J. Bacteriol.* 170, 5837–5847. <https://doi.org/10.1128/jb.170.12.5837-5847.1988>

Schwartz, E., Henne, A., Cramm, R., Eitinger, T., Friedrich, B., Gottschalk, G., 2003. Complete nucleotide sequence of pHG1: A *Ralstonia eutropha* H16 megaplasmid encoding key enzymes of H₂-based lithoautotrophy and anaerobiosis. *J. Mol. Biol.* 332, 369–383. [https://doi.org/10.1016/S0022-2836\(03\)00894-5](https://doi.org/10.1016/S0022-2836(03)00894-5)

Shahzad, K., Narodoslawsky, M., Sagir, M., Ali, N., Ali, S., Rashid, M.I., Ismail, I.M.I., Koller, M., 2017. Techno-economic feasibility of waste biorefinery: Using slaughtering waste streams as starting material for biopolyester production. *Waste Manag.* 67, 73–85. <https://doi.org/10.1016/j.wasman.2017.05.047>

Shang, L., Jiang, M., Chang, H.N., 2003. Poly(3-hydroxybutyrate) synthesis in fed-batch culture of *Ralstonia eutropha* with phosphate limitation under different glucose concentrations. *Biotechnol. Lett.* 25, 1415–1419. <https://doi.org/10.1023/A:1025047410699>

Sichwart, S., Hetzler, S., Bröker, D., Steinbüchel, A., 2011. Extension of the substrate utilization range of *Ralstonia eutropha* strain H16 by metabolic engineering to include mannose and glucose. *Appl. Environ. Microbiol.* 77, 1325–1334. <https://doi.org/10.1128/AEM.01977-10>

Simó-Cabrera, L., García-Chumillas, S., Hagagy, N., Saddiq, A., Tag, H., Selim, S., AbdElgawad, H., Arribas Agüero, A., Monzó Sánchez, F., Cánovas, V., Pire, C., Martínez-Espinosa, R.M., 2021. Haloarchaea as Cell Factories to Produce Bioplastics. *Mar. Drugs* 19. <https://doi.org/10.3390/md19030159>

Singh Saharan, B., Grewal, A., Kumar, P., 2014. Biotechnological Production of Polyhydroxyalkanoates: A Review on Trends and Latest Developments. *Chinese J. Biol.* 2014, 1–18. <https://doi.org/10.1155/2014/802984>

Stegmann, P., Londo, M., Junginger, M., 2020. The circular bioeconomy: Its elements and role in European bioeconomy clusters. *Resour. Conserv. Recycl.* X. <https://doi.org/10.1016/j.rcrx.2019.100029>

Sudesh, K., Abe, H., Doi, Y., 2000. Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters. *Prog. Polym. Sci.* 25, 1503–1555. [https://doi.org/10.1016/S0079-6700\(00\)00035-6](https://doi.org/10.1016/S0079-6700(00)00035-6)

Tan, G.Y.A., Chen, C.L., Li, L., Ge, L., Wang, L., Razaad, I.M.N., Li, Y., Zhao, L., Mo, Y., Wang, J.Y., 2014. Start a research on biopolymer polyhydroxyalkanoate (PHA): A review. *Polymers (Basel)*. <https://doi.org/10.3390/polym6030706>

Tanadchangsang, N., Yu, J., 2012. Microbial synthesis of polyhydroxybutyrate from glycerol: Gluconeogenesis, molecular weight and material properties of biopolyester. *Biotechnol. Bioeng.* 109, 2808–2818. <https://doi.org/10.1002/bit.24546>

Titz, M., Kettl, K.H., Shahzad, K., Koller, M., Schnitzer, H., Narodoslawsky, M., 2012. Process optimization for efficient biomediated PHA production from animal-based waste streams. *Clean Technol. Environ. Policy* 14, 495–503. <https://doi.org/10.1007/s10098-012-0464-7>

Tomizawa, S., Chuah, J.A., Matsumoto, K., Doi, Y., Numata, K., 2014. Understanding the limitations in the biosynthesis of polyhydroxyalkanoate (PHA) from lignin derivatives. *ACS Sustain. Chem. Eng.* 2, 1106–1113. <https://doi.org/10.1021/sc500066f>

Tsuge, T., Tanaka, K., Ishizaki, A., 2001. Development of a novel method for feeding a mixture of L-lactic acid

and acetic acid in fed-batch culture of *Ralstonia eutropha* for poly-D-3-hydroxybutyrate production. *J. Biosci. Bioeng.* 91, 545–550. [https://doi.org/10.1016/S1389-1723\(01\)80171-7](https://doi.org/10.1016/S1389-1723(01)80171-7)

Valentino, F., Karabegovic, L., Majone, M., Morgan-Sagastume, F., Werker, A., 2015. Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels. *Water Res.* 77, 49–63. <https://doi.org/10.1016/j.watres.2015.03.016>

Valentino, F., Morgan-Sagastume, F., Campanari, S., Villano, M., Werker, A., Majone, M., 2017. Carbon recovery from wastewater through bioconversion into biodegradable polymers. *N. Biotechnol.* <https://doi.org/10.1016/j.nbt.2016.05.007>

Vandamme, P., Coenye, T., 2004. Taxonomy of the genus *Cupriavidus*: A tale of lost and found. *Int. J. Syst. Evol. Microbiol.* 54, 2285–2289. <https://doi.org/10.1099/ijls.0.63247-0>

Verlinden, R.A.J., Hill, D.J., Kenward, M.A., Williams, C.D., Piotrowska-Seget, Z., Radecka, I.K., 2011. Production of polyhydroxyalkanoates from waste frying oil by *Cupriavidus necator*. *AMB Express* 1, 1–8. <https://doi.org/10.1186/2191-0855-1-11>

Verlinden, R.A.J., Hill, D.J., Kenward, M.A., Williams, C.D., Radecka, I., 2007. Bacterial synthesis of biodegradable polyhydroxyalkanoates. *J. Appl. Microbiol.* ISSN 1364-5072 102, 1437–1449. <https://doi.org/10.1111/j.1365-2672.2007.03335.x>

Volodina, E., Raberg, M., Steinbüchel, A., 2016. Engineering the heterotrophic carbon sources utilization range of *Ralstonia eutropha* H16 for applications in biotechnology. *Crit. Rev. Biotechnol.* <https://doi.org/10.3109/07388551.2015.1079698>

Vrana Špoljarić, I., Lopar, M., Koller, M., Muhr, A., Salerno, A., Reiterer, A., Malli, K., Angerer, H., Strohmeier, K., Schober, S., Mittelbach, M., Horvat, P., 2013. Mathematical modeling of poly[(R)-3-hydroxyalkanoate] synthesis by *Cupriavidus necator* DSM 545 on substrates stemming from biodiesel production. *Bioresour. Technol.* 133, 482–494. <https://doi.org/10.1016/j.biortech.2013.01.126>

Wang, J., Yu, J., 2000. Kinetic analysis on inhibited growth and poly(3-hydroxybutyrate) formation of *Alcaligenes eutrophus* on acetate under nutrient-rich conditions. *Process Biochem.* 36, 201–207. [https://doi.org/10.1016/S0032-9592\(00\)00169-2](https://doi.org/10.1016/S0032-9592(00)00169-2)

Wang, J., Yue, Z., Sheng, G., Yu, H., 2010. Kinetic analysis on the production of polyhydroxyalkanoates from volatile fatty acids by *Cupriavidus necator* with a consideration of substrate inhibition , cell growth , maintenance , and product formation. *Biochem. Eng. J.* 49, 422–428. <https://doi.org/10.1016/j.bej.2010.02.005>

Wang, Y., Chen, R., Cai, J.Y., Liu, Z., Zheng, Y., Wang, H., Li, Q., He, N., 2013. Biosynthesis and Thermal Properties of PHBV Produced from Levulinic Acid by *Ralstonia eutropha*. *PLoS One* 8, 4–11. <https://doi.org/10.1371/journal.pone.0060318>

Wang, Y., Yin, J., Chen, G.Q., 2014. Polyhydroxyalkanoates, challenges and opportunities. *Curr. Opin. Biotechnol.* <https://doi.org/10.1016/j.copbio.2014.06.001>

Yabuuchi*, E., Kosako, Y., Yano, I., Hotta, H., Nishiuchi, Y., 1995. Transfer of Two Burkholderia and An Species to *Ralstonia* Proposal of *Ralstonia pickettii* (Ralston , Palleroni and Doudoroff 1973) Comb . Nov ., *Ralstonia solanacearum*. *Microbial. Immunol.* 39, 897–904. <https://doi.org/doi: 10.1111/j.1348-0421.1995.tb03275.x>

Yadav, B., Pandey, A., Kumar, L.R., Tyagi, R.D., 2020. Bioresource Technology Bioconversion of waste (water)/ residues to bioplastics- A circular bioeconomy approach. *Bioresour. Technol.* 298, 122584. <https://doi.org/10.1016/j.biortech.2019.122584>

Yamane, T., 1993. Yield of poly-D(-)-3-hydroxybutyrate from various carbon sources: A theoretical study. *Biotechnol. Bioeng.* <https://doi.org/10.1002/bit.260410122>

Yan, Q., Du, G., Chen, J., 2003. Biosynthesis of polyhydroxyalkanoates (PHAs) with continuous feeding of mixed organic acids as carbon sources by *Ralstonia eutropha*. *Process Biochem.* 39, 387–391. [https://doi.org/10.1016/S0032-9592\(03\)00127-4](https://doi.org/10.1016/S0032-9592(03)00127-4)

Yang, F., Hanna, M.A., Sun, R., 2012. Value-added uses for crude glycerol - A byproduct of biodiesel production. *Biotechnol. Biofuels.* <https://doi.org/10.1186/1754-6834-5-1322413907>

Yang, Y.H., Brigham, C.J., Budde, C.F., Boccazzi, P., Willis, L.B., Hassan, M.A., Yusof, Z.A.M., Rha, C., Sinskey, A.J., 2010. Optimization of growth media components for polyhydroxyalkanoate (PHA) production from organic acids by *Ralstonia eutropha*. *Appl. Microbiol. Biotechnol.* 87, 2037–2045.

1513 <https://doi.org/10.1007/s00253-010-2699-8>
1514 Yeo, J.C.C., Muiruri, J.K., Thitsartarn, W., Li, Z., He, C., 2018. Recent advances in the development of
1515 biodegradable PHB-based toughening materials: Approaches, advantages and applications. *Mater. Sci.*
1516 *Eng. C.* <https://doi.org/10.1016/j.msec.2017.11.006>
1517 Yu, J., Si, Y., 2004. Metabolic carbon fluxes and biosynthesis of polyhydroxyalkanoates in *Ralstonia eutropha*
1518 on short chain fatty acids. *Biotechnol. Prog.* 20, 1015–1024. <https://doi.org/10.1021/bp034380e>
1519 Yu, J., Wang, J., 2001. Metabolic flux modeling of detoxification of acetic acid by *ralstonia eutropha* at slightly
1520 alkaline ph levels. *Biotechnol. Bioeng.* 73, 458–464. <https://doi.org/10.1002/bit.1080>
1521 Zhao, X.Q., Bai, F.W., 2009. Yeast flocculation: New story in fuel ethanol production. *Biotechnol. Adv.* 27,
1522 849–856. <https://doi.org/10.1016/j.biotechadv.2009.06.006>
1523