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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 earnt 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 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 analysis highlight 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)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

In another study, an improved artificial pathway of *C. necator* for the biosynthesis of P(3HB-co-3HHx) with high 3HHx compositionhas been established (Insomphun et al., 2015). An effective deletion of *phaB1* gene, which causes the depression of (R)-specific reduction of acetoacetyl-CoA, led to the formation of the C6–monomer unit from fructose driven by crotonyl-CoA carboxylase/reductase (Ccr). Furthermore, the co-overexpression of *phaJ4a* gene, which encodes medium-chain-length (R)-enoyl-CoA hydratase, with ccr promoted the incorporation of both 3HB and 3HHx units into the polymer (Insomphun et al., 2015). Further introduction of *emdMm* synthetic gene, encoding for an ethylmalonyl-CoA decarboxylase derived from mouse, shown to be remarkably effective for P(3HB-co-3HHx) biosynthesis, probably by converting ethylmalonyl-CoA into butyryl-CoA. Finally, a remarkable cellular content of P(3HB-co3HHx) composed of 22 mol% 3HHx has been synthesized starting from fructose using this engineered strain of *C. necator* with ΔphaB1 genotype expressing ccr, phaJ4a, and emd (Insomphun et al., 2015).

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

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

2.4. Biological PHA production

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

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

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

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

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

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

Engineering strategies for PHAs accumulation more often may involve genes related to the oxygen uptake, since oxygen limitation may occur after obtaining high cell densities when PHB production initiate, but also in genes related to PHAs biosynthetic pathways: these and other modifications may enhance PHAs production (Wang et al., 2014). For instance, as reported in a relevant and recent study, PHB pathway optimization has been also investigated in *E. coli* by modifying the expression levels of the three genes *phbC*, *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 *Rasltonia 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 CO_2 fixation via Calvin–Benson–Bassham (CBB) cycle and NiFe hydrogenases to oxidize
259 H_2 (Schwartz et al., 2003). *C. necator* has the ability to grow also in anoxic conditions, during which

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

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

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

C. necator H16 genome has been recently sequenced and published (Pohlmann et al., 2006), allowing the increase of genetic and metabolic studies, allowing several genetic modifications and encouraging different industrial applications of this very versatile microorganism. *C. necator* genome comprises 7,416,678 bp, of which 6,626 coding sequences (CDS) were identified. Almost all essential genes for the metabolism and for other essential cell functions are on Chromosome 1 (4,052,032 bp, 3,651 CDS), while a megaplasmid and chromosome 2 (2,912,490 bp, 2,555 CDS) encode a repertoire of genes related to the metabolism of a wide range of substrates and terminal electron acceptors, involved in CO_2 fixation, H_2 oxidation, denitrification, etc., and contain only very few essential genes (Pohlmann et al., 2006). The presence of these genes, encoding for enzymes involved in organic acid metabolism, allow the heterotrophic growth of *C. necator*; these all genes are located on chromosome 1 (Pohlmann et al., 2006).

3.2 *C. necator* industrial applications

Although *C. necator* has the great potential to be easily engineered to produce valuable compounds directly from CO_2, a lot of effort still must be done to develop genetic libraries to enable such attempts in an optimal way. A variety of transcriptome, proteome and metabolome studies occurred after the complete genome sequence of *C. necator* H16, making available valuable data, leading to elucidations about its

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

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

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

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

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

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

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

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

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

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

Among these different waste substrates, it is possible to find a wide repertoire of industrial by-products, which are the most promising renewable substrates, and residual compounds like glycerol, raw sugar-rich materials like molasses, starch and lignocellulose, but also other high carbon content substrates like soybean, palm and corn oils. During nutrient shortage growth, *C. necator* directs most of its carbon flux to the synthesis of PHB, while under autotrophic growth conditions with H_2/CO_2 as nutrient sources, *C. necator* can synthesize 61 g/L of PHB (representing ~70% of total cell weight) in 40 h (Jiang et al., 2016).

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

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

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

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

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

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

4.1.1 General sugars utilization features in C. necator

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

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

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

4.1.2 Fructose uptake and utilization

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

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

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

4.1.3 Gluconate uptake and utilization

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

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

4.1.4 Glucose uptake and utilization

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

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

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

4.1.5 Mannose

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

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

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

4.1.6 Xylose and arabinose

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

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

Furthermore, a *C. necator* mutant strain, harbouring genes from *E. coli* for a xylose transporter (xylE), 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 thy 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

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

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

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

4.1.9 A focus on acetic acid

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

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

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

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

Despite these mechanisms occurring at high concentrations, so far, acetic acid has earnt a quite important success as an interesting alternative for its indirect sink of CO_2 and its conversion of into PHA in terms of CO_2 fixation, H_2 consumption, safety and process performance, and substrate cost (Marudkla et al., 2018). This approach has been recently tested for the synthesis of both the homopolymer PHB and the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), during which fed-batch cultivations strategies have been used and a pH-stat fed-batch feeding strategy in combination and an additional Dissolved Oxygen (DO)-dependent feed have been performed (Garcia-Gonzalez and De Wever, 2018). This approach has been selected because biomass growth on carboxylates, in this case on acetic acid, leads to a 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 CO_2 emissions into valuable
570 chemical products, including the biopolymers. Considering the biotechnologically relevant C1-substrates
571 such as methanol, formate, CO and CO_2, 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, CO_2 and formate follow a similar metabolization pathway via the CBB cycle.

574 When CO_2 is used as the sole carbon sink and H_2 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 H_2 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 CO_2, 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 CO_2 and water. In fact, recombinant strains of *C. necator* have
586 been used for electro-microbial conversion of CO_2/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 α1–4
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 μm) 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 H_2O_2 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 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 $g \cdot L^{-1} \cdot h^{-1}$.

654 In another study, lipid-rich surplus streams from slaughterhouses and the rendering industry have
655 been studied fot 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 a 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

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

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

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

692 From both economic and ecological point of view this could be a huge step forward, because the
693 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

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

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

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

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

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

747 cell growth has been obtained from biodiesel-wastewater due to high Na^+ in this medium. Apparently, crude
748 glycerol, $(NH_4)_2SO_4$, NaCl and K_2SO_4, and trace element concentration revealed significant effects ($P<0.1$)
749 influencing PHB accumulation (Mothes et al., 2007; Sangkharak and Prasertsan, 2011). From this analysis, the
750 highest PHB concentration of 24.98±1.87 g/L and of 54.01% of CDW have been obtained by modulating these
751 condition parameters, hence using 60 g/L of crude glycerol, 1.32 g/L $(NH_4)_2SO_4$ and 2.0 g/L trace element,
752 which allow the strain TISTR 1095 to reach the highest biomass (46.25±2.10 g/L).

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

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

763 *5.1.4 Waste potato starch*

764 Low cost carbon substrates have sparked wide interest for their application for industrial purposes,
765 including PHB production. Before its utilization, waste potato starch has to undergo saccharification process,
766 in order to make derived sugars (i.e. glucose and maltose) easily fermentable by microorganisms. This
767 strategy has been applied with *C. necator* NCIMB 11599, which can reach 1.47g/(L*h) PHB productivity under
768 phosphate limitation conditions (179g/L biomass, 94g/L PHB, Ybiomass/starch = 0.46g/g, YPHB/starch =
769 0.22g/g) (Haas et al., 2008). Interestingly, the performance achieved by using saccharified starch was virtually
770 identical to the one obtained by using glucose, which is the main sugar present in the substrate medium. In
771 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 alterative 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 $L^{-1}h^{-1}$, 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 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 pearlings 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 achieavable, even using cheap and available starting material.
855 A good PHA producing strain used so far for wastewater recovery is *Plasticicumulans 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

872 bacteria isolated from mangrove environments, *Bacillus* sp. strain 27 and *Rhodococcus* sp. strain 36, resulted
873 to be able to grow in aqueous synthetic media containing PP microplastics and caused a weight loss of 4.0–
874 6.4% after 40 days (Auta et al., 2018). Also PUR could be used as sole nutrient source for the growth of
875 microorganisms like *Aspergillus niger* and *Cladosporium herbarum* (Filip and Giessen, 1979), together with
876 some *Pseudomonas* species, capable of degrading emulsified polyester PUR (Schmidt et al., 2017). For PET
877 degradation, in particular low-crystallinity PET (lcPET, 9%), the actinomycete *Thermobifida fusca* can
878 effectively degrade up to 50% of the initial weight of this polymer at 55°C for 3 weeks (Mueller, 2006). Last
879 but not least, also *C.necator* shows the peculiarity to use synthehic plastics for its growth. In particular, it
880 exhibited PHA accumulation when supplied with non-oxygenated PE pyrolytic hydrocarbons as a carbon
881 source in a nitrogen-containing medium (Johnston et al., 2017). In two recent papers, the production of
882 P(3HB-co-3HV-co-3HHx) by *C. necator* has been tested using oxidized PE wax and PP wax, after a pyrolysis
883 pretreatment under aerobic condition: 1,24 g/L (using PE wax) and 1,36 g/L (using PP wax) of copolymer have
884 been obtained (Johnston et al., 2019; Radecka et al., 2016). Moreover, PE gasification, instead of pyrolysis,
885 seems to enhance PHA accumulation in *C. necator* H16 (Qin et al., 2021).

886 **6.1 *C. necator* fermentation strategies**

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

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

For what concern *C. necator*, two cultivation methods can be applied to directly use CO_2 as a carbon sink for PHB production, which can be either pure synthetic CO_2 (Islam Mozumder et al., 2015) or industrial off-gases (Garcia-Gonzalez and De Wever, 2017). As already mentioned, this bacterium is able to shift between autotrophic growth and heterotrophic growth. The latter one is the most common and widely used, but efforts for PHB production using CO_2 in autotrophy can occurr.

Proceeding with the so called *"autotrophic-autotrophic"* PHB production process, a gas mixture containing CO_2, H_2 and O_2 is furnished to the bacteria growing into the bioreactor to induce both cell mass growth and PHB accumulation. Occurring reactions are reported below (1 and 2) (Garcia-Gonzalez and De Wever, 2018).

$$21.36\ H_2 + 6.21\ O_2 + 4.09\ CO_2 + 0.76\ NH_3 \rightarrow C_{4.09}H_{7.13}O_{1.89}N_{0.76} + 18.7\ H_2O \quad (1)$$

$$33\ H_2 + 12\ O_2 + 4\ CO_2 \rightarrow C_4H_6O_2 + 30\ H_2O \quad (2)$$

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

$$C_6H_{12}O_6 + 1.97\ O_2 + 0.72\ NH_4+ \rightarrow 3.79\ CH_{1.74}O_{0.46}N_{0.19} + 2.21\ CO_2 + 0.72\ H^+ + 3.78\ H_2O \quad (3)$$

There are several studies in which researchers have investigated mixotrophic PHAs copolymer production; in this third case, CO_2 is supplied in combination with an organic co-substrate at the same moment, during both growth and PHB production steps (Ghysels et al., 2018).

During gas fermentation, precaution must be taken regarding gas detonation when O_2 is supplied combined with H_2. This requirement can be achieved by checking over the O_2 concentration in the gas phase and by keeping it below the lower level of explosion. However, in this case, notwithstanding higher cell mass concentration can be reached, the lower gas concentration in the fully autotrophic cultivation process entails 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 CO_2 to PHA (Islam Mozumder et al., 2015), but also in terms of production costs, since less of the costly renewable H_2 is needed in this process. Shifting from autotrophic process to a heterotrophic one, characterized by a glucose-grown biomass, the H_2 consumption is theoretically reduced by 20% (Garcia-Gonzalez and De Wever, 2017). However, still a low amount of CO_2 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 CO_2. 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 H_2 and CO_2 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).

$$2\ CO_2 + 4\ H_2 \rightarrow C_2H_4O_2 + 2\ H_2O \quad (4)$$

$$C_2H_4O_2 + 0.79\ O_2 + 0.22\ NH_4 + \rightarrow 1.14\ CH_{1.74}O_{0.46}N_{0.19} + 1.33\ H_2O + 0.86\ CO_2 + 0.22\ H^+ \quad (5)$$

$$1.5\ C_2H_4O_2 + 0.75\ O_2 \rightarrow 0.5\ C_4H_6O_2 + 1.5\ H_2O + CO_2 \quad (6)$$

With respect to the autotrophic–autotrophic PHB production process, this cultivation method, implying indirect production of PHB from CO_2, could theoretically reduce the amount of CO_2 needed, up to 2.84ton CO2/ton PHB, but most importantly the H_2 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 CO_2 is emitted rather than consumed (at least 2.81ton CO_2/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 = ∞). 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 = ∞, 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

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

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

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

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

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

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

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

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

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

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

helping for researchers, decision-makers, policy-makers. However, LCA tools aimed to find and quantify the environmental impact of PHA production have been focussed so far on isolated aspects of production, without analyzing the whole production performances, such as energy requirements or only CO_2 emissions, which may sometimes be in contrast with each other (Atlić et al., 2011; Koller et al., 2018, 2017). The identification of the ecological hot spots it is important for the analysis and application of the whole process.

Another tool which can be used to reach this issue is Sustainable process index (SPI). The SPI underlines the main and important parameters meaningful for the ecological pressure of PHA production, such as process yield, energy consumption and release of CO_2 (Koller et al., 2011).

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

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

7.1 Conclusions

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

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

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

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

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

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Conflicts of Interest

The authors declare no conflict of interest.

Figures and Tables

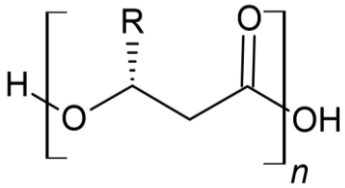

Figure 1. Polyhydroxyalkanoates (PHAs) general structure (ChemSketch). The *R* group which is the substituent group different for each PHAs. The *n* stands for the number of repeated unit for polymer. Both the side chain *R* and the monomer backcone (number of methylene -CH_2 groups) determine the type of biopolymer, as reported in figure 2.

Biopolymer	**Carbon**	***R* group**
Poly(3-hydroxybutyrate) PHB	C_4	Methyl
Poly(4-dyhroxybutyrate) 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			
	PP	*PHB*	*PHV*	*PHB4B*	*PHBHx*
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	*C. necator*	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 (VersaMerTM PHA)	-
Biotec, Italy	-	Potato starch	Bioplast®	-
TianAn Biologic Materials Co. Ltd. China	*C. necator*	Dextrose deriving from local corn of cassava	PHB, PHBV (ENMATTM)	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	*C. necator* 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.

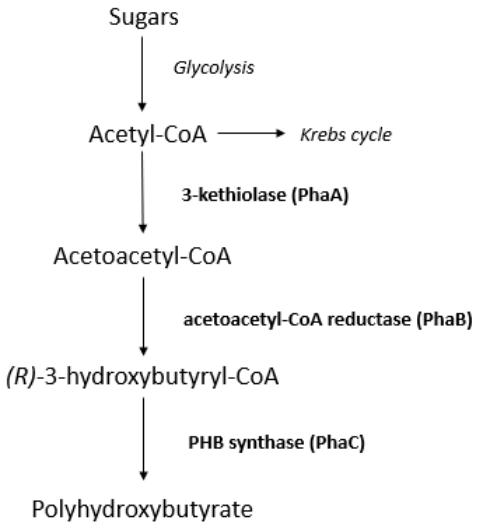

Figure 5. Schematic representation of poly (3-hydroxybutyrate) (PHB) biosynthetic pathway. The pathway is simplified starting from sugars.

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

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