Review

From Organic Wastes and Hydrocarbons Pollutants to Polyhydroxyalkanoates: Bioconversion by Terrestrial and Marine Bacteria

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Abstract: The use of fossil-based plastics has become unsustainable because of the polluting production processes, difficulties for waste management sectors, and high environmental impact. Polyhydroxyalkanoates (PHA) are bio-based biodegradable polymers derived from renewable resources and synthesized by bacteria as intracellular energy and carbon storage materials under nutrients or oxygen limitation and through the optimization of cultivation conditions with both pure and mixed culture systems. The PHA properties are affected by the same principles of oil-derived polyolefins, with a broad range of compositions, due to the incorporation of different monomers into the polymer matrix. As a consequence, the properties of such materials are represented by a broad range depending on tunable PHA composition. Producing waste-derived PHA is technically feasible with mixed microbial cultures (MMC), since no sterilization is required; this technology may represent a solution for waste treatment and valorization, and it has recently been developed at the pilot scale level with different process configurations where aerobic microorganisms are usually subjected to a dynamic feeding regime for their selection and to a high organic load for the intracellular accumulation of PHA. In this review, we report on studies on terrestrial and marine bacteria PHA-producers. The available knowledge on PHA production from the use of different kinds of organic wastes, and otherwise, petroleum-polluted natural matrices coupling bioremediation treatment has been explored. The advancements in these areas have been significant; they generally concern the terrestrial environment, where pilot and industrial processes are already established. Recently, marine bacteria have also offered interesting perspectives due to their advantageous effects on production practices, which they can relieve several constraints. Studies on the use of hydrocarbons as carbon sources offer evidence for the feasibility of the bioconversion of fossil-derived plastics into bioplastics.

Keywords: polyhydroxyalkanoate; hydrocarbons; marine and terrestrial bacteria; waste carbon source; bioplastic

1. Introduction

Fossil plastics completely revolutionized the world of manufacturing in the 1900s [1]; today, they represent one of the most serious concerns, especially for the marine environment [2,3]. According to Jambeck and co-authors [4], the amount of plastic waste produced each year is equivalent to that of newly produced plastic (270 metric tons), and is mostly...
single-use. This makes fossil-based plastic production unsustainable. Of the coastal plastic waste (about 99 million tons), 31.9 million tons is poorly managed, and on average, 8 million tons reaches the sea. Plastic enters the marine environment through rivers, ship and air transport, atmospheric events, and through the air [5]. In addition, the huge amount of plastic single-use masks that we are using all over the world, and their incorrect disposal, is exacerbating plastic pollution in the marine environment [6]. Mechanical stresses, UV radiation, biodegradation, weathering and abrasions contribute to breaking up the plastic waste and producing pieces smaller than 5 mm, which increases the amount of microplastics in the sea (currently at 46,000 particles per km$^2$) that are not being removed [7]. It has been widely demonstrated that plastic in the sea has a negative impact on marine biota, on human and animal health, on the climate, and on the photosynthetic capacity of phytoplankton; moreover, plastic material can be a vector of pathogens, pollutants and invasive species [2,8,9]. In addition, the industrial production of plastic from fossil fuels causes further pollution, due to the release of toxic substances into the environment [10]. The replacement of plastic with eco-compatible and eco-sustainable materials is required urgently. An interesting option is represented by the use of the biopolymers polyhydroxyalkanoates (PHA), which are produced solely by microorganisms [11]. The most interesting features of PHA are their biodegradability and biocompatibility, as they are thermoplastics consisting of a repeating chain of various hydroxyalkanoate monomers [12,13]. As a function of the number of carbon atoms in the chain, the monomers are classified as short-chain length (scl-PHA; 3–5 carbon atoms), medium-chain length (mcl-PHA; 6–14) or long-chain length monomers (lcl-PHA; >14) [13]. Due to this variable structure, the PHA family covers a wide range of properties. The homopolymer poly(3-hydroxybutyrate) (P(3HB)) is the most extensively present in the market and it is quite similar to polypropylene; however, P(3HB) has a low elongation at break, which makes P(3HB) much more brittle than polypropylene. Copolymers such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV), poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (P(3HB-co-3HH)) and poly(3-hydroxybutyrate-co-3-hydroxyoctanoate) (P(3HB-co-3HO)) have improved properties compared to P(3HB). In fact, an increase in the content of 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HH) monomers decreases the degree of crystallinity, melting temperature and tensile strength, and increases the elongation at break [12]. In addition, scl-PHA and mcl-PHA copolymers such as P(3HB-co-3HH) and P(3HB-co-3HO) represent a good option for the plastic industry since they have rubber-like elastomeric properties and can be included in different market sectors than P(3HB) and P(3HB-co-3HV).

Now, PHAs are applied in several sectors in biomedicines, food packaging and electronics, and they are accepted in public opinion due to their low environmental impact [14,15]. However, the PHA market scenario is strongly limited by the high cost, which in turn is affected by the use of sterile pure-culture cultivation.

That said, PHA production processes have high costs, mostly resulting from the expensiveness of carbon sources (40–50% of total production cost) [16,17]. A more interesting and sustainable perspective is related to the bioconversion of waste products, such as food waste, bio-waste, and dairy wastes, into PHA with mixed microbial culture (MMC) [18–21]. In the terrestrial environment, optimized processes for PHA production have been extensively reported, and a pipeline for the market has almost been defined [22–25]. However, despite the increase in the technology readiness level (TRL) up to few demonstration-scale plants [21], the available amount of produced PHA is still too low to define potential applications. Thermoplastic and packaging applications cover a huge market sector and the entry of PHA could be feasible, even though the stability of thermal and mechanical PHA properties under routine operation must be still demonstrated. A more accessible market scenario is represented by the equally innovative technology of groundwater remediation, in which the PHA is utilized as an electron donor. Above all, the use of PHA produced from MMC and waste cannot be separated from a deep evaluation of the presence of impurities and pollutants, which need to comply with guidelines and regulations.
Recently, marine bacteria have attracted more attention due to several benefits they offer to the sustainable PHA production process [26]. An environmentally friendly option is the use of organic contaminants in the marine environment, such as oil or oil-derived plastics, as substrates for PHA production [15,27–29]. Blue bioeconomy, based on the marine and maritime economy, encourages the exploitation of marine resources and their use in a sustainable manner. In particular, marine bacteria are known to be greatly biodiverse and have enormous biotechnological potential; they can be used for the production of precursors of bioproducts and biomaterials. Moreover, marine bacteria can carry out processes for the degradation of toxic substances (bioremediation) such as hydrocarbons [30–33].

This review aims to explore the potential uses of terrestrial and marine microbial resources in the bioconversion of both urban wastes and hydrocarbons/fossil plastics into PHA. It is well known that PHAs are some of the carbon transformation products occurring in the treatment of wastewater by activated sludge. Different types of dynamic conditions, such as those caused by discontinuous feeding strategy and/or varying redox conditions (e.g., anaerobic/anoxic/aerobic), are used to boost the enrichment of PHA-storing microorganisms in a cultivated culture by applying a variety of process configurations. The methods of enrichment of such biomass with high PHA-storage capacity strongly affects the outcomes of PHA-related performances, which basically are summarized as the maximum content of the polymer in the cells and the PHA productivity and/or the global PHA yields. The last two parameters are not always present in the literature, since they are related to the real industrial scenario of the technology more than laboratory scale evaluation.

We first describe the bioconversion of organic wastes into PHA in terrestrial and marine environments, and then the bioconversion of hydrocarbons into PHA by terrestrial and marine bacteria will be reported.

2. Bioconversion of Organic Wastes to PHA

2.1. Bioprocess for PHA Production from Terrestrial Bacterial Strains

In the fields of bioprocess engineering, biotechnology and applied microbiology, the use of MMC is attracting increasing attention for the development of cost-effective processes, offering innovations in terms of waste recovery and valorization. As an example, the MMC technology has been largely applied in combined aerobic–anaerobic processes to produce PHA from low- or no-cost carbon sources. The large family of PHA consists of thermoplastic polyesters synthetized as intracellular carbon and energy reserves by numerous species of Gram-positive and Gram-negative microorganisms (predominantly aerobic, but also anaerobic, photosynthetic bacteria and certain archaea) [34]. The most important aspect causing the increasing interest in PHA production is linked to the possibility of adapting the bioprocess to the treatment of waste. Under a large variety of process operating conditions, such waste can be considered as a renewable bioresource or precursor for the microbial synthesis of PHA, which in turn are the precursors of bio-based plastics.

PHA can be aerobically converted into carbon dioxide and water, or into methane in the anaerobic digestion process [35]. For these reasons, they represent a reliable biodegradation approach compared to the more commonly used fossil fuel-derived polymers, such as polypropylene (PP) and polyethylene (PE). On the other hand, no structured market of commercialized MMC–PHA exists yet, since the technology remains at the low TRL of 5–6, indicating pilot-scale plant implementation.

Hence, the current price of PHA is related to pure-culture industrial-scale production, based on sterile single-microorganism cultivation, and it ranges from 4.0 to 8.0 EUR/kg [36,37]. Notwithstanding the environmental impacts of plastic waste and the burden of costs, the current PHA price cannot be considered commercially competitive when compared to that of the fossil fuel-derived polymers, which is typically less than 1.0 EUR/kg [34]. For this reason, although PHA with equivalent properties and functionality to conventional plastics can be produced by bacterial fermentation at the industrial scale, they still represent only a small fraction of global plastic production, including bio-based and non-bio-based plastics [38].
There is no doubt that many advantages arise from the employment of open MMC; however, the actual scenario of PHA production says otherwise, as it is based on the use of pure cultures, with wildtype or genetically modified microorganisms. The MMC technology represents an open system that does not require sterilization, and can be adapted to the treatment of waste, otherwise defined as low-cost feedstock (all the organic residues produced in urban and industrial scenarios) [22,39]. In turn, the wide range of substrates suitable for MMC-PHA production manifests in the wide range of thermoplastic and elastomeric properties of the synthetized PHA, including poly(3-hydroxybutyrate) (P(3HB)), poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)), copolymers with variable 3-hydroxyvalerate (3HV) content, poly(4-hydroxybutyrate) (P(4HB)) and related copolymers, and poly(3-hydroxyhexanoate) (P(3HH)) and related copolymers [40–42]. PHA’s properties mainly depend on the length and composition of the side chains—the literature states that the addition of monomers such as 3HV, and less frequently 3HH, to a P(3HB) backbone causes a reduction in the crystallinity and the melting temperature, as well as increasing the flexibility [43]. Indeed, P(3HB) is a very highly crystalline and stiff material, but at the same time, it is also brittle; its melting temperature is generally higher than those of copolymers (around 170–180 °C) and close to the degradation temperature (165–170 °C) [44]. Therefore, P(3HB-co-3HV), and copolymers in general, are more attractive than homopolymers such as P(3HB); this enlarges the already broad range of PHA applications.

In the context of waste utilization, MMC–PHA production can be easily integrated into existing infrastructures for the biological treatment of organic waste residues and wastewaters [45]. In the last decade, many research groups have contributed to the scaling-up of the process from laboratory-scale experiments to new and integrated pilot-scale facilities [46–54]. Regardless of the operating conditions and the adopted scale of the application, the inoculum typically used for MMC systems is the activated sludge from municipal wastewater treatment plants [25]. The microbial composition of the sludge needs to be selectively enriched in microorganisms with a high ability to synthesize PHA; such potential, referred to as storage response [55,56], is reached by applying a dynamic feeding regime to microorganisms in bioreactors, where the conditions (substrate concentration, oxygen, redox potential) change according to the well-known feast–famine (FF) regime [34].

2.1.1. Role of the “Feast and Famine Regime” in MMC-PHA Production from Waste Feedstock

The synthesis of PHA by MMC involves a multi-stage process, with a sequence of elements consisting of aerobic and anaerobic units. Generally, the first step involves a dark acidogenic fermentation of the waste feedstock to obtain a stream rich in volatile fatty acids (VFA). This step is particularly important for wastes rich in soluble chemical oxygen demand (COD), which must be acidified into VFA to maximize the storage response [34,57]. The produced VFA-rich stream represents the C source for the following aerobic stages: a first sequencing batch reactor (SBR) for MMC selection and the enrichment of PHA-accumulating microorganisms, and a second fed-batch reactor for the accumulation of PHA within cellular walls. MMC have shown a broad PHA-accumulation ability, frequently quantified as the achievable PHA content in the biomass (Figure 1). This value ranges between 0.3 and 0.8 g PHA/g VSS (volatile suspended solids) depending on the process operating conditions and the type of waste substrate [22].

The optimal operating conditions of each single stage have been largely investigated in the literature (at the laboratory and pilot scale)—the overall process performance is basically calculated as the overall yield of synthetized PHA per kg of VS (volatile solids) in the waste feedstock [49,50,58]. An essential requirement for efficient microbial selection is the application of dynamic feeding conditions, which are generally characterized by transient C source availability, whereby microorganisms are faced with an alternation between high (feast) and low or zero (famine), concentrations of external organic substrate. The “feast and famine” (FF) regime is usually applied with a short hydraulic retention time (HRT; 1–2 days).
Both of these conditions force a physiological adaptation in those microorganisms that are fast enough and able to thrive under such unbalanced growth conditions—accumulating the external C source as PHA in the feast phase and using it as C-reserve to grow in the famine phase [22].

Figure 1. Main process outline utilized for PHA production from waste feedstock: (A) simplified version with low-solids waste or wastewaters; (B) integrated version with overflow recovery with high-solids waste.

SBR is typically operated through a succession of cycles, whose duration can be adapted according to the characteristics of the fermented feedstock (e.g., VFA concentration) [59,60]. Such cycles guarantee the occurrence of the feast period (C source availability) during the feed phase and part of the reaction phase (feast), whereas the culture faces starvation in the rest of the cycle (famine) [61]. The ratio between the length of feast and the overall cycle length has been identified as the crucial parameter in establishing selective pressure for the dominance of PHA-accumulating microorganisms in the MMC [34]. Generally, the threshold value is recognized as 0.20 h/h; above this value, the famine phase is not long enough, and is less effective in the stimulation of physiological adaptation to the FF regime. In addition, it is often reported that the “feast to famine” ratio (F/F) is a key parameter; accordingly, a microbial storage response is evident at low F/F ratios (up to 0.25), while a growth response becomes predominant at high F/F ratios (equal to 0.90 or higher) [62].
Operatively, in fully aerobic FF conditions (also called aerobic dynamic feeding, ADF), the profile of dissolved oxygen concentration in the SBR is an indication of the establishment of selective pressure, and it is used as a simple tool to monitor the length of both the feast and the famine periods [63]. Such an aerobic FF regime has been applied to treat organic waste residuals and wastewaters (e.g., olive oil mill wastewaters, primary and secondary sludges, paper mill effluents, molasses, food and fruit waste, etc.) [18,42,48,64–67]. This type of configuration generally does not require nutrient removal. In fact, the integrated approach recently used for waste treatment includes a section for anaerobic overflow recovery, wherein biogas is produced and nutrients are recovered in the composting section of the digestates [49].

The selection of PHA-accumulating microorganisms can also be integrated with the side stream treatment of nitrogen using nitrite from sewage sludge reject water. In this case, microbial selection is coupled with nitrogen removal, and is favored by the establishment of aerobic (feast) and anoxic (famine) conditions in the SBR [51].

Nitrogen plays a crucial role in MMC selection. Besides seeking strategies for the simultaneous removal of carbon and nitrogen (C-N), recent research has also been focused on investigating the optimal nitrogen concentration in the SBR. Some waste streams (e.g., paper mill and olive oil mill wastewaters, fruit waste, cheese whey permeate) are often nitrogen-deficient, and their supplementation in the SBR is required for efficient microbial growth. Recent studies have examined different approaches to nitrogen supply, referred to as uncoupled and coupled feeding with a carbon source [42,52,67–69]. The most commonly applied coupled C-N feeding strategy consists of simultaneous carbon and nitrogen feeding; as a consequence, nitrogen is only available in the feast period (but also maybe in the famine period—either the whole famine or part of it). On the contrary, in the uncoupled C-N feeding strategy, nitrogen addition is delayed at the end of the feast, when the carbon is completely consumed. The lack of nitrogen in the feast period strongly favors the PHA storage response, and its presence in the famine period drives the microbial growth of PHA-storing microorganisms. The adaptation of such processes to the substrate’s characteristics substantially increases the biomass storage response, as quantified by the storage yield in the feast period ($Y_{PS_{feast}}$), which almost doubles compared to the storage yield in coupled C-N feeding. Such a modification may imply a change in the PHA composition (high 3HV content [68,69]), or a change in the MMC composition [57,67].

2.1.2. Terrestrial PHA-Accumulating Bacteria in Open Mixed Culture

All the above-mentioned “feast and famine” conditions have been rigorously investigated, and proven useful for the operation of SBR (both at the laboratory and the pilot scale) and the establishment of the dynamic feeding required to enrich an inoculum (activated sludge) into PHA-accumulating microorganisms. Table 1 shows a variety of PHA-accumulating bacteria identified in different processes to produce PHA, and reports on the different MMC characterizations and the compositions of stored polymers outlined in the most recent literature. These results are divided according to different aspects of the biological process and the properties of the selected biomass, as follows: type of substrate (feed); operating conditions such as HRT and sludge retention time (SRT); organic loading rate (OLR) of the reactors (mainly for biomass accumulation), and polymer storage properties.

Most of the PHA-accumulating bacteria belong to the classes $\alpha$-Proteobacteria and $\beta$-Proteobacteria. The differences become more visible when microbiological analyses are performed at higher taxonomic resolution. Several studies on the storage and production of PHA have used different operating conditions and substrates. In general, the processes are elicited through feast and famine methods, using SBR under aerobic conditions. Anaerobic conditions are applied in both SBR and accumulation stages.
Table 1. Terrestrial bacteria producing PHA.

| Main Microorganisms          | Feedstock                                                                 | Operating Parameters            | Biomass PHA Content (wt%) and PHA Composition (%) | Reference |
|------------------------------|---------------------------------------------------------------------------|--------------------------------|--------------------------------------------------|-----------|
| **Thauera 48.9%, Hypomonas 3.9%, Aquimonas 1.8%** | HAc rich wastewater (synthetic)                                           | HRT = 2 d, SRT = 10 d, OLR = 1.5 gCOD/(L d) | 3HB/3HV: 100 wt%                                    | [70]      |
| **Bdellovibrio bacteriovorus** (3.5%) | fermented sewage sludge/wet air oxidation of sewage sludge: HAc 2734 mg/L, HPr 460 mg/L | HRT = 1 d, SRT = 4 d            | 41% gPHA/gVSS; 3HB/3HV: 77/23 wt%                  | [71]      |
| **Rhodobacter and Rosobacter 20–50%, Amaranococcus 20–50%, Paracoccus 5–20%, Zoogloea 20–50%, Plasticimulans 5–20%** | sewage sludge and fermented fruit waste: HLaC 0.65 g/gCOD<sub>equil</sub>, HAc 0.95, HPr 0.07, EtOH 0.48, HBut 2.25, HVal 0.17, HCap 9.97. | HRT = 2 d, SRT = 4 d, OLR = 3 gCOD/(L d) | 55–75% gPHA/gVSS; 3HB/3HV: 84–87/13–16 wt% | [52] |
| **Paracoccus up to 87.2%, Larnopredia up to 33.0%, Rhodobacteraceae up to 21.7%, Rhizobiales Incertae Sedis up to 18.5%, Amurcococcus up to 9.6%, Thiobirchup to 14.9%, Shinella up to 10%, Leucobacter up to 10.5%, Paracoccus up to 9.6%, Gemmobacter up to 10.7%** | fermented fruit waste: HAc 2%, HAc 31%, HPr 13%, EtOH 9%, HBut 68%, HVal 12%. | HRT = 1 d, SRT = 4 d, OLR = 2–14 gCOD/(L d) | - | [73] |
| **Paracoccus and Rhodobacter (up to 43%)** | hardwood sulfite spent liquor                                           | HRT = 1 d, SRT = 5 d, OLR = 17 gCOD/(L d) | 10% PHA/VSS (molar)                                | [72]      |
| **Acidovorax 16.9%, Alcaligenes 13.0%, Paracoccus, Rhodobacter, Rhizobium 13.6%, Comamonas (up to 43.3%)** | fermented hardwood spent sulfite liquor: HAc/HPr/HBut/HLaC/HVal = 62:18:13:10:1 | HRT = 1–2 d, SRT = 5 d, OLR = 2–7 gCOD/(L d) | - | [73] |
| **Hydrogenophaga, Thauera, Pseudoxanthomonas, Flavobacterium, Paracoccus, Leifsonia, Exiguobacterium, Rhodobacter** | fermented fruit waste and sewage sludge                                    | HRT = 1 d, SRT = 1 d, OLR = 3–6 gCOD/(L d) | 45–55% gPHA/gVSS; 3HB/3HV: 90/10 wt% | [49] |
| **Acidovorax and Hydrogenophaga (52–79%), Thauera and Azorlans (12%)** | fermented food waste: HAc 21.5%, HBut 38.0%, HPr 12.7%, HVal 11.6%, HCap 10.0% | HRT = 1 d, SRT = 1 d, OLR = 2.5 gCOD/(L d) | 40–45% gPHA/gVSS; 3HB/3HV: 88/12 wt% | [46] |
| **Allorhizobium, Neorhizobium, Pararhizobium, Rhizobium (up to 38.3%); Acidivorax, Aquimonas, Comamonas, Hydrogenophaga, Ramlibacter, Zoogloea (up to 35.3%)** | fermented food waste: HAc 23%, HPr 19%, HBut 46% (COD basin) | HRT = 1 d, SRT = 1 d, OLR = 3.5 gCOD/(L d) | 40–45% gPHA/gVSS; 3HB/3HV: 90/10 wt% | [74] |
| **Clostridium (29%), Pseudomonas (8%), Rhodopseudomonas (5%)** | synthetic VFA: 3 g/L HAc and 0.5 g/L HBut | OLR = 2 gCOD/(L d) | - | [75] |
Table 1. Cont.

| Main Microorganisms | Feedstock | Operating Parameters | Biomass PHA Content (wt%) and PHA Composition (%) | Reference |
|---------------------|-----------|----------------------|--------------------------------------------------|-----------|
| Enterobacter and Pseudomonas (66.6%) | synthetic wastewater and glucose | HRT = 1 d SRT = 1 d OLR = 3.7 gCOD/(L d) | - | 3Hb/3HV: 76–88/8–21 wt% [76] |
| Amaricoccus and Thauera from 56.3% to 72.4% | crude glycerol fermentation (90 Cmmol/L): HAc 2.38 Cmmol/L, HPr 12.10 Cmmol/L, HBut 30.52 Cmmol/L. | HRT = 1 d SRT = 1 d OLR = 3.7 gCOD/(L d) | - | 3Hb/3HV: 75/25 wt% [77] |
| Zoogloea (10.1%) Zoogloea resinihila, Dechloromonas (4.45%), Azospira (2.82%) | sodium acetate | HRT = 2 d | - | 68% gPHA/gVSS [78] |
| Uncultured Rhodocyclusae | fermented food waste | HRT = 0.7 d SRT = 1 d OLR = 8 gCOD/(L d) | - | 3Hb/3HV: 50/50 wt% [79] |
| Plasticicumulans Acidivorans | fermented paper mill wastewater: VFA/COD<sub>сол</sub> 0.72; HAc 37%, HPr 21%, HBut 29%, HVal 16% | HRT = 1 d SRT = 1 d OLR = 3 gCOD/(L d) | - | 3Hb/3HV: 75/25 wt% [47] |
| Corynebacterium, Xantomonadaceae, Borea, Amaricoccus, Paracoccus | fermented cheese whey: EtOH 41 mg/L, HAc 52 mg/L, HBu 14.8 mg/L, TOA 294 mg/L | HRT = 1 d SRT = 4–5 d OLR = 2 gCOD/(L d) | - | 3Hb/3HV: 87/13% (molar) [67] |
| Proteobacteria (77.6%), Bacteroidetes (77.6%), Nitrospira (1.75%), Arnimalmonadetes (1.3%) | fermented paperboard mill wastewater: COD<sub>сол</sub> 0.92 g/L; VFA 0.34 g/L | HRT = 1 d SRT = 10 d OLR = 3 gCOD/(L d) | - | 3Hb/3HV: 84–92/8–16 wt% [80] |
| On HAc: | | | | |
| Moraxellaceae (12%), Rhodobacteraceae (11.7%), Bacillaceae (11.6%), Flavobacteriaceae (7%), Comamonadaceae (6.7%); | | | | |
| On HCap: | | | | |
| Moraxellaceae (18%), Rhodobacteraceae (15.4%), Flavobacteriaceae (8.5%), Comamonadaceae (5.6%) | fermented food waste (30 v/v%) and sewage sludge (70 v/v%); VFA up to 29.5 g/L | HRT = 1 d | - | 3Hb/3HV: 94–97/3–6 wt% [81] |
| (a) Paracoccus 26%, Lactococcus 28%, Enterococcus 15%; (b) Azospirillum 90% | synthetic hemicellulose hydrolysates: (a) xylose 79.7%, Hac 8.9%; (b) xylose 42%, HAc 50% | HRT = 1 d SRT = 1 d | (a) 4% gPHA/gVSS (b) 18% gPHA/gVSS | - | [82] |
| Paracoccus, Comamonas, Azoarcus, Thauera | acidified cooked mussel wastewater (62% gVFA/gCOD<sub>сол</sub>) | HRT = 1 d SRT = 1 d OLR = 1–2 gCOD/(L d) | - | 3Hb/3HV: 70–83/17–30 wt% [83] |
| β-Proteobacteria up to 54% | | | | |
| synthetic VFA: 4.8 g COD/L; HAc/Hpr/Hbu = 16/1.5/8 (COD based) | HRT = 2 d SRT = 10 d OLR = 1.2 gCOD/(L d) | 71.4% gPHA/gVSS | - | [84] |
As shown in Table 1, when acetate is used as the sole substrate or main carbon source, the dominance of *Azoarcus* and *Thauera* spp. (Gram-negative bacteria of the Zoogloeaaceae family, of the order *β*-Proteobacteria) is evident. *Thauera* was found to represent approximately 50% of the MMC, with acetic acid as the sole substrate and producing 3HB exclusively [70]. Wijeyekoon et al. [71] selected a MMC with 84% *Thauera*, using a substrate of 83% COD/COD acetic acid and 17% COD/COD propionic acid; accordingly, the PHA composition was different from that obtained in the work of Sruamsiri et al. [70], reaching 77% \(\text{w/w} 3\text{HB and 13% w/w 3HV} \) [86].

The presence of *Amaricoccus* was identified when acetic, propionic, butyric acid, crude glycerol or ethanol were present. In particular, it represented up to 50% of the MMC in Silva et al. [42], wherein fermented sewage sludge and fruit waste were used as the substrate (the presence of ethanol was close to 50% COD/COD). Matos et al. [52] found a lower level (close to 10%) of *Amaricoccus* when using fermented fruit waste as the sole substrate, with ethanol at 9% COD/COD. Similar results have been reported by Oliveira et al. [67], using fermented cheese whey and ethanol as the substrate. Several studies have shown high contents of *Paracoccus*, a coccoid bacterium known for its nitrate-reducing properties. It was found in two trials using spent hardwood sulfite liquor [72] and fermented hardwood spent sulfite liquor [73], with a relative abundance of up to 43%.

Matos et al. [52] found a relative abundance of *Paracoccus* of over 87%, using fermented fruit waste as the substrate. *Paracoccus* was also found in another study by Silva et al. [42], wherein fermented fruit waste was used as the substrate for the SBR. The obtained MMC was characterized by high storage capacity (up to 71.3% \(\text{w/w} \) of PHA content in the biomass), by using sewage sludge and fermented fruit waste mixture as feedstock. The tetra-polymers 3HB:3HV:3HH, at 33:1:66%, were enriched in a pilot-scale SBR operated at 20–25 °C (having a conversion yield of around 1.0 COD\(_{\text{PHA}}\)/COD and a PHA content above 70% PHA/g TS). These are the highest values of medium-chain length PHA production so far obtained using MMC and real feedstock. The culture was employed in an SBR operating...
at a 3.0 gCOD/(L d) OLR, with fermented fruit waste as the substrate with the following composition (gCOD/L): lactate (0.05 ± 0.06), acetate (1.0 ± 0.2), propionate (0.07 ± 0.06), butyrate (2.3 ± 0.4), valerate (0.2 ± 0.2), caproate (10 ± 2) and ethanol (0.5 ± 0.1). The SRT was equal to 4.0 days, and the HRT was 1.0 day. The cycle length in the SBR was 12 h, divided into 11 h of aeration and 1 h of settling (with no aeration and mixing). Rhodobacter, Roseobacter, Amaricoccus, and Zoogloeoa showed peak relative abundances of 50% in the microbial community, and dominated the SBR. Plasticicumulans and Paracoccus showed peak relative abundances of 20%; Lampropedia, Azorarcus, and Thauera reached up to 5%.

Three different studies, with similar OLR values applied using food waste alone and/or in a mixture with sewage sludge for the selection of PHA-accumulating microorganisms, showed a high content of Hydrogenophaga bacteria [48,49,74], which has been described as a microorganism with high storage capabilities, producing PHA with high content of 3HB monomers compared to 3HV monomers (approximately 90:10% of 3HB:3HV w/w).

Guerra-Blanco et al. [75] and Amulya et al. [76] found Pseudomonas (relative abundance up to 66.6%) using a synthetic VFA mixture to simulate real fermented wastewater. In both studies the MMC approach was conceived as an integrated technology to be coupled with wastewater treatment plant; hence, particularly focused on the water-line treatment services more than waste management practices.

Another study showed the high storage capacity (76% PHA w/w) of a selected consortium [77] using fermented crude glycerol as the substrate. This study showed a high selectivity in synthesizing PHA from VFA only, leaving 1,3-propanediol (1,3-PDO) in the supernatant. The polymer analysis showed a final composition of 75% w/w 3HB and 25% w/w 3HV. The SBR was dominated by Amaricoccus and Thauera by up to 72.4%.

Inoue et al. [78] used sodium acetate as the substrate, and the presence of 10% Zoogloeoa has been reported. A study previously discussed [74] also reported the presence of Zoogloeoa together with Acidivorax, Aquimonas, Comannonas, Hydrogenophaga, and Ramlibacter up to 35%; in this case, fermented food waste was used as the only substrate, with relative VFA contents of 23% COD/COD acetic, 19% propionic and 46% butyric acid, respectively.

The presence of acetic and butyric acid in the feedstock contributes to a final product with a high content of P(3HB). The literature suggests that the production of 3HB is mainly related to the presence of Thauera, Amaricoccus and Azoarcus in the selected MMC.

The study of Mulders et al. [79] is of particular interest, since here, a polymer with 50% w/w 3HV was produced by the selected MMC; in this case, uncultured Rhodocyclaceae was grown on fermented organic waste at a high OLR (up to 8.0 gCOD/L d). Additionally, Pereira et al. [73] showed the presence of Rhodobacter and Rhizobium (16.3%) at a high OLR, producing PHA at a high 3HV level (38% w/w).

Particularly representative is the case of the open PHA-producing culture that was strongly dominated by Plasticicumulans acidivorans; this culture was enriched in a pilot-scale plant fed with industrial fermented wastewater containing 64% VFA (as a fraction of the total soluble COD) and 22% ethanol (as a fraction of the total soluble COD) [47]. The culture was rendered almost pure in a boosted selection process, carried out at a low OLR and at 30 °C. Such culture was able to accumulate PHA in the range 0.70–0.90 g PHA/g VSS. The selection process limited the growth of a non-storing population, as well as the accumulation of non-PHA storage compounds, which was apparently related to the uptake of ethanol.

Going further with Table 1, additional examples from literature are reported [67,80–88] with PHA-producing bacteria already presented and discussed in the previous examples.

In the context of MMC-PHA production technology, successful microbial selection is mandatory, since the types of selected microorganisms have a strong impact on both PHA accumulation capacity and PHA composition, which in turn affect the properties of the purified PHA and its potential applications.
2.2. Marine Strains PHA-Producers

The marine environment represents one of the most intriguing challenges for bacteria, as they are faced with a great variety of conditions in terms of temperature, pH, salinity, availability of nutrients, hydrodynamics and pollution [89]. PHAs are produced by bacteria as a response to stress conditions, such as UV, drying, imbalances in the nutrients C/N/P, and salinity [90]. Salinity has been widely investigated as a key factor in PHA production [91]. Moreover, the ecological importance of PHA was highlighted as a source of carbon and energy in low-nutrient marine habitats [92,93]. Halophiles, from moderate to extremophile microorganisms (Archaea), exhibit special properties related to PHA production [26].

Table 2 shows some examples of marine PHA-producing bacteria. They present a great variability, in terms of both taxonomy and source environment. Marine PHA-producers have been found living in free form or associated as symbionts with higher organisms; in coastal waters and sediments, in deep waters and sediments, as well as in sea ice and hydrothermal environments [26,94,95]. They mainly belong to the Proteobacteria phylum, but there are also representative strains of Firmicutes and Actinobacteria. Among the Proteobacteria, signatures related to α, β and γ classes have been reported.

Table 2. Marine bacteria producing PHA *. (See Section 2.2 for the operating conditions).

| Strain | Phylum | Isolation Source | PHA Produced | Carbon Source Used |
|--------|--------|-----------------|--------------|--------------------|
| Afifella marina | α-Proteobacteria | seawater | P(3HB) | nutrient rich medium |
| Alcanivorax borkumensis | γ-Proteobacteria | seawater sediments | PHA | sodium acetate |
| Alteromonas lipolytica | γ-Proteobacteria | seawater | P(3HB) | marine broth |
| Bacillus cereus MCCB 281 | Firmicutes | marine sponge callyspongia diffusa | P(3HB-co-3HV) | palm jaggery |
| Bacillus licheniformis MSBN12 | Marine sponge | seawater | P(3HB-co-3HV) | glycerol |
| Bacillus megaterium | Firmicutes | sediment | P(3HB) | glucose |
| Bacillus sp. NQ-11/A2, | Firmicutes | sediment | P(3HB) | glucose |
| Brevibacterium casei MS104 | Actinobacteria | marine sponge dendrilla nigra | P(3HB) | starch |
| Burkholderia sp. AIU M5M02 | β-Proteobacteria | shallow sea mud | P(3HB-co-3HV) | nutrient-limiting mineral salt medium mannitol as a carbon source |
| Colwellia sp. JAMM-0421 | γ-Proteobacteria | deep sea | P(3HB), P(3HB-co-3HV) | glucose, fructose, sodium gluconate or soybean oil |
| Desulfobacterium autotrophicum | δ-Proteobacteria | sediment | P(3HB), P(3HB-co-3HV) | caproate |
| Desulfobotulus saporovans | δ-Proteobacteria | sediment | P(3HB), P(3HB-co-3HV) | caproate |
| Desulfococcus multivorans | δ-Proteobacteria | sediment | P(3HB), P(3HB-co-3HV) | benzoate |
| Desulfonema magnsum | δ-Proteobacteria | sediment | P(3HB), P(3HB-co-3HV) | benzoate |
| Desulfosarcina variabolis | δ-Proteobacteria | sediment | P(3HB-co-3HV) | benzoate |
| Dinoreoseobacter shihae DFL 12T | α-Proteobacteria | prorocentrum lima | PHA | sodium acetate |
| Erythrobacter longus DSMZ 6997 | α-Proteobacteria | enteromorpha linza | PHA | glucose |
| Halomonas boliviensis | γ-Proteobacteria | seawater | P(3HB) | different combinations of carbohydrates and hydrolysed polysaccharides |
| Halomonas campisalis | γ-Proteobacteria | seawater | P(3HB-co-3HV), 3.6 mol% 3HV | maltose and yeast extract |
Table 2. Cont.

| Strain                  | Phylum            | Isolation Source          | PHA Produced               | Carbon Source Used                                      |
|-------------------------|-------------------|---------------------------|----------------------------|--------------------------------------------------------|
| Halomonas halophila     | γ-Proteobacteria  | seawater                  | P(3HB)                     | hydrolysates of cheese whey, spent coffee grounds, sawdust and corn stover, lignocellulose |
| Halomonas hydrothermalis| γ-Proteobacteria  | seawater                  | P(3HB)                     | waste frying oil                                       |
| Halomonas marina        | γ-Proteobacteria  | seawater                  | P(3HB-co-3HV), 12.8 mol% 3HV | glucose yeast extract alkanolic acids (C₃–C₆)            |
| Halomonas profundus     | γ-Proteobacteria  | deep sea hydrothermal vent shrimp | P(3HB), P(3HB-co-3HV)      | acetate, pyruvate, propionate, valerate, octanoate, glucose and glycerol |
| Labrenzia alexandrii DFL 11T | α-Proteobacteria | alexandrium lusitanicum | PHA                        | ASW with 1 g/L peptone and 1 g/L yeast extract          |
| Marinobacter guineae    | γ-Proteobacteria  | seawater                  | PHA                        | nutrient rich medium                                   |
| Massilia sp. UMI-21     | β-Proteobacteria  | seaweed                   | PHA                        | starch, maltoltriose, or maltose as a sole carbon source |
| Methylarcula marina     | α-Proteobacteria  | coastal seawater          | P(3HB)                     | starch hydrolysate                                      |
| Methylarcula terricola  | α-Proteobacteria  | coastal sediment          | P(3HB)                     | starch hydrolysate                                      |
| Methylbacterium sp.     | α-Proteobacteria  | sediment                  | P(3HB)                     | valeric acid and methanol                               |
| Moritella sp. JCM21335  | γ-Proteobacteria  | deep sea                  | P(3HB-co-3HV)              | glucose, fructose, gluconate and plant oils             |
| Neptunomonas antarctica | γ-Proteobacteria  | sediment                  | P(3HB)                     | bacto tryptone, yeast extract and fructose             |
| Oceanicola granulosus   | α-Proteobacteria  | seawater                  | P(3HB)                     | pentoses, hexoses, oligosaccharides, sugar alcohols, organic acids and amino acids. |
| Oceanimonas doudoroffii | γ-Proteobacteria  | seawater                  | P(3HB)                     | lignin or several lignin derivatives                    |
| Paracoccus sp. LL1      | α-Proteobacteria  | seawater                  | P(3HB)                     | waste cooking oil                                       |
| Paracoccus serinphilus  | α-Proteobacteria  | marine bryozoan           | PHA                        | peptone–yeast marine medium                             |
| Photobacterium leiognathi 208 | γ-Proteobacteria | seawater                  | P(3HB)                     | peptone, glycerol and valeric acid                      |
| Photobacterium leiognathi 683 | γ-Proteobacteria | fish                      | P(3HB-co-3HV)              | water fish extract followed by peptone, glycerol and valeric acid glucose, decanoic acid, or olive oil |
| Pseudoalteromonas sp. SM9913 | γ-Proteobacteria | deep sea sediment          | P(3HD-co-3HDD)             | glucose, decanoic acid, or olive oil                    |
| Pseudomonas guezennei   | γ-Proteobacteria  | marine microbial mat      | PHA                        | glucose                                                |
| Rhodovulum eurhalinum   | α-Proteobacteria  | seawater                  | PHA                        | malate, pyruvate and acetate                            |
| Roseobacter denitrificans OCh 114 | α-Proteobacteria | enteromorpha linza        | PHA                        | sodium acetate followed by glucose                      |
| Roseospira goensis      | α-Proteobacteria  | sediment                  | P(3HB-co-3HV)              | sodium acetate                                          |
| Saccharophagus degradans ATCC 43961 | γ-Proteobacteria | salt marsh grass          | P(3HB)                     | glucose                                                |
| Shewanella basaltis     | γ-Proteobacteria  | seawater                  | PHA                        | nutrient rich medium                                    |
| Shewanella surangensis JMM-0036 | γ-Proteobacteria | deep sea                  | Oligohydroxyalkanoate      | glucose, fructose, sodium gluconate, or soybean oil    |
| Sphingopyxis alaskensis | α-Proteobacteria  | seawater                  | P(3HB)                     | waste vegetable oil                                     |
Table 2. Cont.

| Strain                        | Phylum       | Isolation Source                  | PHA Produced                  | Carbon Source Used       |
|-------------------------------|--------------|-----------------------------------|------------------------------|--------------------------|
| Thiohalocapsa marina         | γ-Proteobacteria | seawater                          | P(3HB)                       | sodium acetate           |
| Vibrio azureus BTKB33        | γ-Proteobacteria | sediment                          | P(3HB)                       | glucose                  |
| Vibrio harvei MCCB 284       | γ-Proteobacteria | tunicate phallusia nigra          | P(3HB)                       | fructose, yeast extract   |
| Vibrio proteolyticus         | γ-Proteobacteria | seashore                           | P(3HB), P(3HB-co-3HV)        | fructose, glucose, gluconate (sodium gluconate), or soybean oil |
| Vibrio sp. KN01              | γ-Proteobacteria | seawater                           | P(3HB), P(3HB-co-5HV-co-3HP) *** | glucose, fructose, gluconate (sodium gluconate), or soybean oil |
| Yangia sp. ND199             | α-Proteobacteria | mangrove samples                  | P(3HB-co-3HV)                | glucose                  |

* [26,94]; ** poly-3-hydroxyoctanoate (P(3HO)); *** poly-3-hydroxypropionate (P(3HP)).

As it is showed in the Table 2, α-Proteobacteria PHA producers include aerobic anoxygenic photosynthetic bacteria that carry out phototrophy without producing oxygen. PHA formation may play an important role in regulating these bacteria’s trophic metabolism, according to the availability of organic carbon in the marine environment. Most of these have been isolated from marine microalgae, and are able to produce PHA in the presence of sugars or organic acids [96]. Dinoseobacter shibae, Roseobacter denitrificans, and Erythrobacter longus performed PHA production when irradiated in the presence of sodium acetate and glucose [97]. Labrenzia alexandrii, which was isolated from the toxic dinoflagellate Alexandrium lusitanicum, produced PHA in seawater enriched with peptone and yeast extract [98].

The α-Proteobacteria also include Methylarcula marina and Methylobacterium sp. isolated from coastal seawater, which had accumulated β-hydroxybutyrate and compatible solutes under conditions of high salinity, indicating that β-hydroxybutyrate could serve as an osmolyte that helps cells to maintain their turgor pressure [99]. Moreover, the same genera were found to produce P(3HB) in the presence of starch hydrolysate, valeric acid and methanol. Methylotrophus bacteria have also been found to be associated with algae, using methanol derived from pectin metabolism and converting it into value-added products, such as P(3HB) [100]. The ability of strains belonging to the Paracoccus genus to produce PHA from wastes is noticeable [101]. In particular, marine Paracoccus species have been described as consumers of glycerol, methanol or n-pentanol, as well as mixtures, such as lignocellulosic biomass hydrolysates or waste cooking oils. An additional benefit was also reported in the form of the co-accumulation of PHA and carotenoid [102].

Among γ-Proteobacteria, strains belonging to the Halomonas genus have been widely reported as PHA producers (Table 2) [26,94].

Several species belonging to the genus Halomonas are listed in Table 2; in particular, Halomonas boliviensis is moderately halophilic, and produces P(3HB) using glucose, sucrose, maltose, xylose and wheat bran. The Halomonas sp. KM-1 strain produces P(3HB) using waste glycerol as the sole carbon source. Halomonas bluephagenesis TD01 produces P(3HB-co-3HV) using glucose and propionic acid or valeric acid [103]. Similarly, Halomonas marina, Halomonas profundus and Halomonas venusta produce P(3HB-co-3HV) in a medium supplemented with glucose or valeric acid [104,105]. Halomonas campisalis achieves P(3HB-co-3HV) accumulation when grown in the presence of maltose. Halomonas dagingensis was shown to accumulate PHA when grown in an algal biodiesel waste residue rich in crude glycerol [106]. Halomonas salina has been reported to accumulate the P(3HB) homopolymer using a variety of substrates, such as starch hydrolysate, glucose, and glycerol. An ability to convert low-cost substrate into valuable PHA was frequently described in Halomonas species. As an example, Halomonas hydrothermalis is able to produce poly(hydroxybutyrate) (P(3HB)) when cultivated in the residual glycerol from biodiesel made from Jatropha in a seawater medium [107]. Halomonas campaniensis LS21 can be grown in artificial seawater using food waste consisting of cellulose, proteins, fats, fatty acids and starch. A recombinant
Halomonas elongata A1 can produce 90.76% P(3HB) in a mineral medium enriched only with glucose [26].

PHA producers have also been isolated from deep sea Colwellia sp. JAMM-0421 and Moritella sp. JCM21335 (Table 2); these metabolize sugars and fats to accumulate PHA. Furthermore, Pseudoalteromonas sp. SM9913 isolated from deep sediments produces copolymers of 3-hydroxydecanoate (3HD) and 3-hydroxydodecanoate (3HDD) from glucose, decanoic acid or olive oil. Halomonas profundus AT1214, isolated from hydrothermal shrimp from the deep sea, can provide P(3HB) starting from sugars and organic acids [94].

Waste products from algae have also been reported as useful carbon sources for the production of PHA by β-Proteobacteria, such as the Burkholderia sp. AIU M5M02 isolated from marine mats, which is able to convert algal mannitol into P(3HB). Additionally, strain UMI-21, belonging to the Massilia genus isolated from algae, showed an ability to synthesize PHA from starch, maltotriose, or maltose (Table 2) [108].

In coastal marine areas or in shallow waters, bacteria can find a wide variety of organic compounds derived from algae, their by-products, or products of their degradation—the opportunistic pathogen Vibrio sp. KN01 produces P(3HB) using sugars, as well as gluconic acid and soybean oil.

The interest in PHA-producing halophiles has grown in recent years thanks to the efficiency of the process, as well as the cost reductions due to the halophilic nature of marine bacteria. Their use has considerable advantages: (1) they are easily cultivated; (2) the costs of cultivation medium are low, and in some cases it is actually possible to use sea water; (3) they have limited economic and nutritional requirements; (4) the fermentation process can be conducted in non-sterile conditions (long-lasting, open, continuous, energy-saving bioprocessing); (5) cell lysis can be performed with tap water; (6) they are cheap and their cultivation are characterized by low environmental impact. Given that the marine bacteria require saline environment, the amount of NaCl should be optimized. As an example, Dubey and Mishra have demonstrated that the strain Halomonas daqingensis showed the best performance when grown in media supplemented with NaCl up to 5% [106]. Moreover, since the high salinity may damage the steel tanks, the bioreactor can be provided with plastic tanks instead [109]. Ref. [26] outlines the various strategies developed to improve PHA yield; for example, Halomonas venusta KT832796 was tested in a 2 L bioreactor by adding glucose (20 g/L) and ammonium citrate (2 g/L). Under these conditions, the strain produces 3.86 g/L cell dry weight (CDW), containing 70.56% (by weight) of P(3HB) produced at 0.160 g/L/h. The volumetric productivity was improved with a high concentration of glucose (100 g/L) in a single solution, which was maintained at 1–2 g/L. This helped maintain acceptable pH values, with an 88.12% net content of P(3HB). Furthermore, a volumetric productivity value for P(3HB) of 0.248 g/L/h was achieved. In another study by Ortiz-Veizán and co-authors [110], limited oxygen conditions were applied to overcome the low productivity of Halomonas boliviensis, high quantities of monosodium glutamate were added, and the nitrogen and phosphorus intakes were regulated, thus achieving a satisfactory yield. The process was particularly advantageous for the co-production of ectoine, operating in a fed-batch system and in different phases. The open and continuous fermentation was performed as a fed-batch system with H. bluephagenesis TD01, using two fermenters in succession and limiting the nitrogen supply during the second process. The biomass from the first fermentation was transferred to a second fermenter to promote PHA accumulation, while limiting the nitrogen supply in between. The first fermentation achieved a yield of 40 g/L CDW, equal to 60% (wt%) P(3HB); in the second, the yield was 20 g/L CDW, containing 65% (wt%) P(3HB). Halomonas campaniensis LS2 gave interesting results when grown in seawater enriched with kitchen waste, in an open, continuous and non-sterile system. They obtained 73 g/L of CDW, containing up to 70% (wt%) P(3HB), and also avoided contamination. Vibrio proteolyticus provided the highest amount of PHA (47.68%) and biomass (3.62 g/L) when grown in an M9 minimal medium supplemented with 5% NaCl and 2% fructose as the sole carbon source. Neptunomonas antarctica was cultivated in flasks containing two different basal media—one with natural...
seawater and the second with artificial seawater, obtaining similar yields of 0.18 g P(3HB)/g fructose (2.13 g/L of (P3HB)).

2.3. Known Metabolic Pathways for PHA Production from Organic Substrates

Figure 2 shows three metabolic pathways for the production of PHA. The pathway of PHA synthesis in bacteria is characterized by several reactions, starting with acetyl coenzyme A catalyzed by a substrate-specific PHA synthase, which performs its function in the cytosol where the polymerization of hydroxyacyl thioesters takes place [111]. The genes involved in PHA biosynthesis encoding for the assembling of granules (phaP), catalysis (phaC, phaM), precursor production (phaA, phbB, phaG, phaJ), and PHA degradation (phaZ) [112]. As an example, three biosynthetic pathways have been described (Figure 2).

![Figure 2. Overview of the principal metabolic pathways of PHA production in bacteria.](image)

Two acetyl-CoA molecules are initially combined by β-ketoacyl-CoA thiolase (phbA) to form acetoacetyl-CoA. Acetoacetyl-CoA is then reduced to (R)-3-hydroxybutyryl-CoA by acetoacetyl-CoA dehydrogenase/reductase (phbB). The (R)-3-hydroxybutyryl-CoA is the monomer for the following P(3HB) polymerization (phbC), resulting in the production of P(3HB). The (R)-3-hydroxybutyryl-CoA monomers, precursors of P(3HB), can be produced by sugar or fatty acid metabolism (de novo fatty acid biosynthesis or β-oxidation). mcl-PHA (mcl-(R)-3-hydroxyfatty acids) production begins with the conversion of compounds derived from fatty acid metabolism to (R)-3-hydroxyacyl-CoA (Figure 2). When the substrate is oxidized to acetyl-CoA via fatty-acid de novo biosynthesis, a precursor of PHA is produced by transacylase (phatA). If the fatty-acid β-oxidation pathway is used, then the hydratase (PhaJ) catalyzes (R)-3-hydroxyacyl-CoA formation [95,112–114].

As regards hydroxyl fatty-acid chains, C3 to C5 include the short-chain scl-PHA (e.g., P(3HB)), while the medium-length C6 to C14 include mcl-PHA (e.g., P(3HO)) [115].

Based on substrate specificity, PHA synthases are divided into four classes (Figure 3). Class I utilizes CoA thioesters of 3-HAs, 4-HAs, and 5-HAs, comprising three to five carbon atoms. For example, Halomonas sp. SF2003 harbors two distinct PHA synthases, PhaC1 and PhaC2, both belonging to class I [113]. Additionally, in the Vibrio azureus strain BTKB33, isolated from marine sediments, the presence of class I PHA synthases was detected, particularly polyhydroxybutyrate polymerase [116].
The occurrence of class III PHA synthases in marine PHA producers has been frequently reported. In this respect, the possibility of optimizing processes for hydrocarbon bioconversion of industrial waste streams but the bioconversion of pollutants is still far too high. Substrates for the biosynthesis of PHAs can markedly reduce the production costs.

| Class | Subunits | Species            |
|-------|----------|--------------------|
| I     | PhaC     | Halomonas elongata |
| II    | PhaC     | Pseudomonas oleovorans |
| III   | PhaC, PhaB | Neptunomonas concharum |
| IV    | PhaC, PhaR | Bacillus cereus |

Figure 3. PHA synthase class in bacteria isolated from marine matrices both in marine and terrestrial strains.

On the other hand, class II polymerases are specific to 3-HAs with 6 to 14 carbon atoms, as well as 4-HAs and 5-HAs. *Pseudomonas* spp. contain PHA synthases belonging to class II, and the PHA synthesis operon of most *Pseudomonas* spp. has two PHA synthases: PhaC1 and PhaC2.

Synthases of both classes I and II are encoded by the *phaC* gene. Class III synthases are encoded by two genes (*phaC* and *phaE*) with substrate specificities similar to class I. The occurrence of class III PHA synthases in marine PHA producers has been frequently detected in sulfate-reducing bacteria isolated from marine anaerobic sediments.

The homologous PhaC and PhaE proteins have been described in *Desulfovoccus multivorans* [117], as well as in anoxicogenic purple sulfurbacteria *Allochromatium vinosum* [118] and in cyanobacteria [119]. Two class I PHA synthases, one class III PHA synthase, and other PHA-related enzymes have been predicted on the genome of the marine bacterium *Neptunomonas concharum* JCM17730.

The presence of multiple PHA synthases with different properties probably grounds the strain’s ability to accumulate P(3HB) under diverse growth conditions in changeable marine environments [120]. Class IV synthases consist of two genes (PhaC and PhaR) and utilize monomers of three to five carbon atoms. *Bacillus cereus* isolated from seawater samples produces PHA using PHA synthase class IV, with high efficiency [121].

Within class I and class II of the PHA synthases, only one type of subunit (PhaC) has been detected. Class III PHA synthases (e.g., in the halotolerant *Neptunomonas* sp.) consist of two different types of subunits: PhaC and PhaE. Class IV PHA synthases (e.g., in *Bacillus cereus*) consist of PhaC and PhaR subunits.

3. Bioconversion of Hydrocarbons to PHA by Terrestrial and Marine Bacteria

Despite the scientific research and the increasing demand for novel materials, especially bioplastics, the pipeline for the commercialization of PHAs is still in its infancy [122]. One of the major constraints is the production cost, which is still far too high. Substrates for the biosynthesis of PHAs can markedly reduce the production costs [123,124].

In response to this issue, many studies have investigated industrial waste streams as sustainable methods to produce PHAs. Among the explored substrates, interesting results have been obtained by the use of waste plant oils, molasses from the sugar industry, lignocellulosic materials, oil palm shell, pressed fruit fibers, biodiesel waste, and waste animal oil [125]. These are biogenic waste streams but the bioconversion of pollutants into valuable products can represent a fascinating challenge of processes related to circular economy. In this respect, the possibility of optimizing processes for hydrocarbon...
biodegradation and PHA accumulation has been explored in both terrestrial and marine hydrocarbon-degrading bacteria.

The production of PHA in bacteria has frequently been described as a survival strategy under stress conditions, in particular PHAs are usually produced when the microbes experience environmental conditions such as nutrient-limiting concentrations of nitrogen, phosphorus, sulfur, or oxygen and excess carbon source [126]. Oil-polluted environments induce bacterial stress [127–129]. As an example, hydrocarbon-polluted environments are characterized by an imbalanced C:N ratio, which is a known condition under which bacteria produce PHAs. The hope is that such bacteria can be exploited in such a way that combines bioremediation for environmental clean-up with the production of a high-value-added material [130].

The ability of bacteria to degrade hydrocarbons has been known for several decades. As an example, the same microorganisms can grow on octane and accumulate mcl-PHAs, while various aromatic hydrocarbons can be used as substrates for PHA production by a number of *Pseudomonas* strains [33,131–133].

Hydrocarbon-degrading bacteria play a primary role in the biodegradation of oil hydrocarbons, and consequently affect the evolution of oil in the environment, both in terms of composition and toxicity [32]. The hydrocarbon degraders identified up to now belong to the major bacterial classes (α, β, and γ-Proteobacteria, Actinomycetia, Bacteroidia), and cover more than 175 genera [134]. Some of these are defined generalist degraders, since they can utilize hydrocarbons as well as non-hydrocarbon substrates [135], while a subset of hydrocarbon-degrading bacteria are specialist members, as they use aliphatic hydrocarbons (*Alcanivorax*, *Oleiphilus*, *Oleispira*, and *Thalassolitus* genera), or the aromatic fraction (*Cyclomicrobium* and *Neptunomonas* genera), as the sole source of carbon and energy [32,136,137].

As listed in Table 3, various species of bacteria that are able to convert hydrocarbons into PHA belong to the Proteobacteria phylum with representative signature of classes γ, α and β. They are taxonomically diverse, mainly belonging to the genera *Pseudomonas*, *Alcanivorax*, *Ralstonia*, *Ochrobactrum*, *Novosphingobium*, *Methylosinus*, and *Cupriavidus*.

As early as 1983, de Smet et al. [138] demonstrated that *Pseudomonas oleovorans* accumulates P(3HO) granules when grown on n-octane. Further studies have highlighted that PHAs from hydrocarbons exhibit a variety of structures with different alkyl groups, dependent on the substrates used [139].

Nikodinovic and co-authors [140] studied several *Pseudomonas* strains cultivated in single or mixed cultures. The results show that the maximum yield was reached when using a mixed bacterial consortium, which successfully degraded BTEX and simultaneously produced PHA at up to 24% of the cell dry weight.

The production of medium-chain length poly(3-hydroxyalkanoates) was also reported by Ni and co-authors [141]. *Pseudomonas fulva* TY16, grown in a medium supplemented with BTEX as the sole source of carbon, biosynthesized MCL-PHAs.

*Rhodococcus aetherivorans* is able to use toluene as its sole carbon source, and accumulates poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [142]. *Alcaligenes denitrificans* A41 was cultivated in the presence of biphenyl as the sole substrate, in two steps: the first aimed at obtaining biomass in the stationary growth phase; during the second phase, the medium was depleted of nitrogen and supplemented with biphenyl. The PHA content reached was up to 47.6% of the CDW [143].

In the context of the marine environment, the *Alcanivorax* genus, an obligate hydrocarbonoclastic bacterium, is able to produce mcl-PHA from octadecane [144]. Among the α-Proteobacteria, *Ochrobactrum intermedium* was shown to produce P(3HB) from oil bilge water [145], while the methanotrophic bacterium *Methylosinus trichosporium OB3b* accumulates polyhydroxybutyrate when grown in trichloroethylene or methane under oxygen-limiting conditions [146,147].

Notably, most available studies are based on hydrocarbons derived from either petroleum or from petro-plastics. For instance, it was previously reported that *Pseudomonas putida* CA-3 is able to convert styrene or polystyrene into biodegradable plastic polyhydrox-
alkanoate (PHA) [148]. Moreover, Kenny and co-authors [149] have used a combination of heat treatment (pyrolysis) and bacterial fermentation in nitrogen-limiting conditions to convert polyethylene terephthalate into PHA. Guzik et al. [27] developed and implemented a two-step chemo-biological method for the conversion of polyethylene into PHA. The first step was polyethylene pyrolysis, with the production of a mixture of hydrocarbons; the second step was the direct supplementation of pyrolysate wax to the microorganisms to encourage growth and PHA accumulation. The PHA production was enhanced by supplementing with inorganic nitrogen and biosurfactants. *Cupriavidus necator* (also known as *Ralstonia eutropha*) was used as a model for PHA production [150], and produced P(3HB) and P(3HB-co-3HV) when a mixed substrate was supplemented, composed of plant oil and 3-hydroxyvalerate [151]. *Cupriavidus necator* is also able to degrade a wide range of aromatic and chloroaromatic compounds [152]. A recent study [153] demonstrated that *Cupriavidus necator* is able to convert hydrocarbons from pre-treated low-density polyethylene (widely represented among plastic products) into monomeric units of 3HB, 3HV and 3HH.

### Table 3. Hydrocarbon-degrading strains able to produce PHA.

| Microorganism                          | PHA Produced | Carbon Source Used                              | Isolation Source                     | Reference          |
|----------------------------------------|--------------|-------------------------------------------------|--------------------------------------|--------------------|
| *Alcanivorax borkumensis*              | PHA          | Octadecane                                      | Seawater                             | [144,154]          |
| Pseudomonas fulva TY16                 | mcl-PHAs *   | Benzene, toluene, and ethylbenzene              | Soil                                 | [141]              |
| Pseudomonas putida, Pseudomonas sp. and *Ralstonia eutropha* | mcl-PHA | Phenanthrene, pyrene and fluoranthene | PAH-contaminated site in Ao Tap Lamu, Phang-nga (Thailand) | [155] |
| *P. putida CA-3*                       | mcl-PHA      | Styrene and phenylacetic acid                   | Industrial bioreactor                | [156]              |
| *Ochrobactrum intermedium*             | P(3HB)       | Oily bilge water                                | Oily bilge waste contaminated seawater | [145]              |
| Methylosinus trichosporium OB3b        | P(3HB)       | Trichloroethylene, methane                      | Terrestrial and aquatic environment  | [146,147]         |
| *P. oleovorans ATCC 29347*             | PHA          | C8-C12 alkanes                                  | Terrestrial and aquatic environment  | [156]              |
| *Ralstonia eutropha H16*               | a blend of P(3HB) and P(3HB-co-3HV) | Plant oils and 3-hydroxyvalerate | Sludge | [151] |
| *Pseudomonas aeruginosa 47T2*          | PHA          | Waste frying oil                                | Waste frying oil                     | [157]              |
| *P. putida F1*                         | mcl-PHA      | Toluene, benzene, or ethylbenzene               | Terrestrial and aquatic environment  | [140]              |
| *P. putida mt-2*                       | mcl-PHA      | Toluene or p-xylene                             | Terrestrial and aquatic environment  | [140]              |
| Mixed culture of *P. putida F1, mt-2, and CA-3* | mcl-PHA | Benzene, toluene, ethylbenzene, p-xylene, and styrene | Terrestrial and aquatic environment | [140] |
| *P. saccharophila NRRL B-628*          | mcl-PHA      | Coconut oil, tallow                             | Terrestrial and aquatic environment  | [158]              |

* medium chain length polyhydroxyalkanoate (mcl-PHA).
Known Pathways for PHA Production from Hydrocarbons

The β-oxidation pathway is involved in the biosynthesis of PHAs from hydrocarbons; in fact, metabolic precursors of PHA are produced by the degradation of hydrocarbons [159]. The degradation of hydrocarbons via terminal oxidation produces free fatty acids, which are subjected to β-oxidation after their activation by an acyl-CoA synthase. This process produces (S)-3-OH-acyl-CoAs, which are isomerized into (R)-3-OH-acyl-CoAs by the action of an isomerase and then converted to PHA through the action of the PhaC synthase.

Concerning one of the most widely studied oil-degrading marine bacteria, *Alcanivorax borkumensis*, Sabirova et al. [144] experimentally demonstrated that when hydroxyacyl-CoA-specific thioesterase, acting exclusively on hydroxylated acyl-CoAs, was blocked in an *A. borkumensis* mutant strain, a notable increase in PHA formation was recorded. This is due to the rechanneling of CoA-activated hydroxylated fatty acids, alkane degradation cellular intermediates, towards PHA. When a tesB-like mutant was grown on alkane, the formation of PHA was 20 times higher than in a wild-type strain grown under the same conditions. Contrary to other bacteria, the PHAs in this mutant are excreted and accumulate extracellularly, improving their biotechnological value.

Yoon and other authors [160] reported the degradation of pyrolytic hydrocarbons of polyethylene via a terminal oxidation process comparable to the microbial degradation pathway of n-alkanes [160,161]. In this process, an alkane hydroxylase determines the oxidation of a terminal methyl group generating a primary alcohol, which is further oxidized to the corresponding aldehyde by an alcohol dehydrogenase, and then converted into fatty acids by an aldehyde dehydrogenase [162].

Ward et al. [162] demonstrated that *P. putida* CA-3 is able to convert styrene into PHA if grown under nutrient-limited conditions. This finding establishes the metabolic link between styrene degradation and PHA [163,164].

PHA production from aromatic hydrocarbon styrenes follows one of two available pathways:

(i) The hydroxylation of the aromatic ring of styrene to styrene cis-glycol by styrene dioxygenase, which is then oxidized to form 3-vinylcatechol by a cis glycol dehydrogenase. The 3-vinylcatechol is depredated into pyruvate, which is converted by the pyruvate dehydrogenase complex into acetyl-CoA. Acetyl-CoA will enter the TCA cycle to generate succinic acid, which could form β-D-Hydroxybutyryl-CoA or could be converted into PHA, by an acetoacetyl-CoA reductase (PhaB) or a PHA synthase (PhaC), respectively [165];

(ii) styrene is converted into phenylacetic acid, which, after hydroxylation, goes through the β-oxidation process to yield acetyl-CoA, which will then enter the TCA cycle or be converted into PHA [166,167].

4. Biodegradation of PHA by Marine Bacteria

The recent issue of marine microplastic contamination has led to an urgent need to substitute fossil-derived plastics with ecofriendly materials that are biobased, recyclable and biodegradable, specifically in marine environments.

The alternative use of bioplastics (especially PHA) instead of fossil plastics is a promising option, encouraged by recent studies on the biodegradability of bio-based plastics in marine environments.

Marine microorganisms able to metabolize PHA are widely distributed [94]. Chemically synthesized biodegradable plastics can be degraded by microorganisms via co-metabolism, since they lack a dedicated pathway. On the contrary, biologically synthesized plastics, such as PHA, are degraded by means of specific P(3HB) depolymerases [168].

Kroeker [169] has demonstrated that all marine P(3HB) depolymerases are adapted to catalyze PHA depolymerization at values of pH, temperature and salinity similar to those of seawater.

Among marine PHA-degrading bacteria, many hydrocarbon-degrading bacteria have been recognized, such as *Pseudomonas*, *Alcanivorax* and *Marinobacter* spp.

For instance, Zadjelovic and co-authors [94] demonstrated that several species belonging to the *Alcanivorax* genus harbor genes encoding PHA depolymerase ALC24_4107. Such
an enzyme is able to hydrolyze aliphatic polyesters of both natural and synthetic origin, such as P(3HB), P(3HB-co-3HV), PES, PBS and PCL.

Moreover, the *P. stutzeri* YM1006 and *Marinobacter* sp. NK-1 strains produce an extracellular P(3HB) depolymerase, which is active against P(3HB) and (R)-3HB [170,171].

5. Market Projections for Bio-Plastics

The current stage of development of the MMC-PHA technology and its TRL is not sufficient to support the broad characterization of PHA and the following bioplastic formulations. Large amounts of material, and therefore high carbon source availability, are required to perform the MMC–PHA production process routinely. This condition is very important, and extremely helpful for defining the possible product applications. The pilot-scale examples have been characterized by PHA production in kilograms, which is still too low an amount for compounders and/or product market developers. Some options are available as regards the high TRL that will be achievable in the coming years.

For commodity applications, there are some mechanical properties that must be prioritized: elongation at break, Young’s modulus and tensile strength [172]. It is widely known that the manipulation of these properties is necessary to reduce the polymer’s brittleness, which is a severe obstacle limiting PHA applicability. Based on the most common compositional features of PHA synthetized by MMC (3HV content of roughly 10–20 wt%), the material would have good stiffness, good flexibility, and improved toughness (and/or brittleness) compared to P(3HB) homopolymers. However, the PHA’s mechanical properties may not be suitable when its molecular weight ($M_W$) is lower than 400 kDa. Some of the studies reported above found a $M_W$ higher than or equal to 600 kDa, which ensures such PHA are suitable for thermoplastic applications.

One of the most probable market scenarios requiring PHA with low $M_W$ (and eventually PHA with a purity level far above 100 wt%) is the remediation of groundwater polluted by chlorinated hydrocarbons, wherein PHA (or PHA-rich biomass) can be utilized as an electron donor for biologically reductive dechlorination [173]. This technology involves the use of permeable reactive barriers (PRBs) with zero-valent iron (ZVI), which is the reactive media used for treating insoluble contamination [173]. The PRB–ZVI technology requires a slow-release carbon source (e.g., PHA or PHA-rich biomass) that is active for a long time in order to enhance the activity of the ZVI barriers, stimulating their activity and enabling reductive dechlorination.

Other studies investigated the utilization of PHA-based biodegradable films (at different PHA contents), with a wide range of mechanical properties and acceptable permeability properties, devised through melt compounding and blown extrusion, for agricultural purposes [36]. In addition, the packaging applications of fiber-based continuous films obtained from electrospun PHA have been explored [174]. In this study, a unique material with balanced mechanical, thermal and barrier properties was obtained via the combination of electrospinning and a mild annealing post-process, carried out below the melting temperature of the PHA.

Apart from the mechanical and thermal properties, one of the main concerns related to the use of organic waste as a source for PHA is the nature of the impurities and, in particular, the pollutants. These substances can migrate through the technology chain into the final product. Different PHA samples produced using the pilot-scale platform described in Moretto et al. [49] were analyzed to quantify relevant contaminants, such as polycyclic aromatic hydrocarbons [175], polychlorinated biphenyls (PCB) [176] and heavy metals [177]. In these PHA samples derived from urban organic waste, the total contents of the three categories of substances complied with the present guidelines and regulations. Hence, the use of such waste as a C source for the PHA production process appears to be safe for human health and the environment. Further research is necessary in this area, since this is the only report in the literature concerning contaminant quantification in waste-derived PHA.
The future developments of MMC–PHA production technology need to be oriented towards downstream processing and material formulation, which in turn require large-scale production, in the order of tons of PHA produced per year (demonstration plant).

As revealed by a recent review, the current trends for PHA applications are related to the single strain production, which can give viable routes for the PHA recovered from waste by MMC [178]. Given their biodegradability and non-toxicity, various PHA-based therapeutics have been developed: guided tissue repair/regeneration devices, sutures, cardiovascular patches, 3D custom-made bone marrow scaffolds, etc. [178]. Apart from medical devices, PHA can be utilized as coating agent since they are water resistant (food-packaging items with barrier coatings for moisture maintenance and/or oxygen lock); the paper industry also uses biopolymers as coatings to replace the synthetic ones due to the high hydrophobicity of PHA and their resistance to hydrolytic degradation, oxygen, and UV rays. In agriculture, PHA can be used as mulch, compostable and soil-friendly films for optimal soil integrity, moisture retention and growing weed control (biodegradable PHA-based mulch has been designed by Nodax™ and it is patented by Danimer Scientific [178]. In the frame of nanotechnology, bioactive compounds can be encapsulated in polymeric nanoparticles as drug delivery system (the drugs form a bond with the surface of the nanoparticles and perform a controlled drug release). Since P(3HB) is used to activate the formation of tissues or organs and given that human body does not make any immune response to P(3HB)-based implant materials, it is an ideal polymer for nano-entrapment and the delivery of antimicrobial compounds to a target site [178].

As regards marine bacteria, several enterprises have already explored *Halomonas* sp. PHA, demonstrating that marine bacteria promise competitive industrial-scale production. Nevertheless, it is necessary to widen our knowledge on the relevant physiology, taxonomy, biochemistry and biomolecular features, including the regulatory mechanisms controlling polymer synthesis, in order to improve PHA production.

Until now, despite of the numerous data available in the literature regarding the PHA production from waste and the advancements related to the identification of the potential application of waste-derived PHA, legislative barriers such as its End of Waste (EoW) status has been only preliminarily evaluated [50]. The new waste Directive 2018/851 [179] suggests the need to define the EoW status for each type of waste (case by case). The specific decree will be formulated by the competent Ministry or Agency for a specific bio-based product. At a national level, such a decree has to be based on: (a) definition of waste type (by using the European Catalogue Code) and its acceptability definitions/standards; (b) technical characteristics and definition of limits of the new recovered material and/or product; and (c) definition of the specific use and market for the recovered material. The finalized dossier will be collected in the specific databases of the Directorate-General Growth at European level.

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