



Review

Copolymers as a turning point for large scale polyhydroxyalkanoates applications

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ABSTRACT

Traditional plastics reshaped the society thanks to their brilliant properties and cut-price manufacturing costs. However, their protracted durability and limited recycling threaten the environment. Worthy alternatives seem to be polyhydroxyalkanoates, compostable biopolymers produced by several microbes. The most common 3-hydroxybutyrate homopolymer has limited applications calling for copolymers biosynthesis to enhance material properties. As a growing number of researches assess the discovery of novel comonomers, great endeavors are dedicated as well to copolymers production scale-up, where the choice of the microbial carbon source significantly affects the overall economic feasibility. Diving into novel metabolic pathways, engineered strains, and cutting-edge bioprocess strategies, this review aims to survey up-to-date publications about copolymers production, focusing primarily on precursors origins. Specifically, in the core of the review, copolymers precursors have been divided into three categories based on their economic value: the costliest structurally related ones, the structurally unrelated ones, and finally various low-cost waste streams. The combination of cheap biomasses, efficient pretreatment strategies, and robust microorganisms paths the way towards the development of versatile and circular polymers. Conceived to researchers and industries interested in tackling polyhydroxyalkanoates production, this review explores an angle often underestimated yet of prime importance: if PHAs copolymers offer advanced properties and sustainable end-of-life, the feedstock choice for their upstream becomes a major factor in the development of plastic substitutes.

1. Introduction

1.1. Traditional plastics: excellent properties and scalability but unsustainable end-life

Since its breakout in the market in the fifties, global plastic production is increasing steadily, up to >450 Mt. per year in 2023 [1,2]. The reasons for such an enormous consumption lie on the excellent physical properties and the cost-effectiveness of these materials. Between the industrial use sectors where plastic materials are employed, packaging represents by far the largest (35.9 %), followed by building and construction (16.0 %), textiles (14.5 %), consumer and institutional products (10.3 %), transportation (6.6 %), electrical/electronics (4.4 %), industrial machinery (0.7 %), and others (11.5 %) [1]. Plastic polymers

are indeed light, stiff and bio-inert but non-toxic, have extreme ductility, strength-to-weight ratio, corrosion resistance, durability and a great high thermal/electrical insulation properties [3]. Moreover, the fossil-based industry can indeed guarantee high scalability at low production expenditures for plastic manufacturing. As a result, in 2019 global plastic use per capita recorded 60.1 kg, peaking to 155.8 kg in Organization for Economic Co-operation and Development (OECD) countries.

Unfortunately, the durability of conventional plastics, previously enumerated as a major strength, takes with it a huge negative externality: the impact on the environment. >22 Mt. of plastic were leaked in 2019 in terrestrial and aquatic environments where they will not degrade before centuries. Furtherly, this quantity is projected to double by 2060 [2]. As a matter of fact, microplastics (fragments of plastics <5 mm) have been indeed found in very remote environments such as deep

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sea and Antarctica [4,5]. Besides, their impact threatens human health as well since they can be ingested or inhaled. Indeed, plastics have been proved to enter the food chain and have also been detected in people apartments, dispersed in air [6,7]. Biota is then seriously jeopardized: several plastic residues like fishing wires and nets or even every-day use shopping bags and traditional cans clips may result as deadly traps when animal species, especially in the marine environment, end up entangled or wrapped in these dispersed wastes [8]. Finally, in 2019 plastic production from fossil fuels and their conversion was responsible for 1.8 Gt of greenhouse gas (GHG) emissions, which are predicted to reach 4.3 Gt by 2060 under a business-as-usual scenario [2]. The gigantic accumulation of plastic debris in the environment is not surprising, since only 9 % of plastic is nowadays recycled (Fig. 1) while 20 % ends up in incinerators and 50 % in landfills, leaving the remaining fraction outside the management system, spread or burnt irregularly [2]. The reason why traditional plastic is seldom recycled lays mostly on a market disconnection: the quality and the volume of plastic feedstocks entering the waste management and recycling facilities mismatch the demand for recycled plastic [9]. In fact, the huge volume of plastic-based objects in use is highly heterogeneous: commodities are usually blends of different types of plastics which become too complex to be recycled [10]. If the improvement of the existing plastic waste management is thereby essential, the implementation of alternative biodegradable polymers becomes equally important.

1.2. Polyhydroxyalkanoates: bio-based and compostable polymers although still seldom adopted

Differently from the conventional ones, some plastics are capable of biodegrading in few months (Fig. 2A). More specifically, microorganisms present in the environment breakdown the material into CO₂, water and biomass [11]. Additionally, another parameter that is considered to distinguish different plastic materials is their carbon source origin, whether this is not renewable as for fossil-based plastic or renewable as for bio-based plastic [12]. For this reason, the exact same final polymer can either be bio-based or not, depending on the carbon source origin, as in the case of PE. The advantage of bio-based plastics is that they derive from biomass thus limiting the dependency from fossil fuels and allowing to save CO₂ emissions [13]. As a result, bioplastics are defined as polymers that are either biodegradable or bio-based or both. Overall instead, plastics can be divided into four separate categories as displayed

in Fig. 2A. Bioplastics thereby are all the polymers which are not part of the non-biodegradable, fossil-based fraction. Nevertheless, bioplastics production in 2022 accounted only for 2.23 Mt., <1 % of global plastic production, and its share is predicted to grow limitedly, up to 6.3 Mt. by 2027 [14]. Bio-based and biodegradable plastics gained profound attention in the last decades because of the embedded circularity of these biopolymers. PLA (polylactic acid) is the leader in terms of market share (20.7 %) followed by starch blends (17.9 %). However, the trend is forecast to turn in favor of PHAs (polyhydroxyalkanoates) which from 1.8 % share of 2021, will represent 8.9 % of bioplastics market by 2027, overtaking starch fraction (6.3 %) (Fig. 2B) [14]. Moreover, differently from polylactic acid (PLA), polybutylene succinate (PBS), and polycaprolactone (PCL), only PHA shows excellent biodegradability in seawater [15].

PHAs are a class of compostable polyesters first described by Maurice Lemoigne in 1926. These biopolymers are naturally occurring, accumulated by several organisms, mostly by many prokaryotes under excess of C source, in forms of intracellular granules as storage materials, often in contrast to stressing conditions such as extreme temperatures or environments where salinity or oxygenation are for instance altered [16]. As a matter of fact, PHAs-producing microorganisms can accumulate PHAs up to 90 % of their dry weight [17]. Their industrial production, therefore, occurs mainly, but not exclusively, via biotechnological processes [18,19]. Fermentation takes place in batch, fed-batch, or continuous mode, where a large range of microorganisms is exploited, including gram-negative *Cupriavidus necator* [20], engineered *Escherichia coli* [21], *Pseudomonas* strains [22], the halophilic bacterium *Halomonas bluephagenesis* [23], and cyanobacteria like *Nostoc muscorum* [24]. However, today the PHAs market size is still negligible because of their high production costs. Specifically, feedstocks prices are too elevated, calling for the transition towards second generation biorefineries where wastes find application as new low-cost substrates [25–27]. Additionally, strains performances must be enhanced, also throughout genetic engineering [25,28].

PHAs are linear polyesters formed by monomers of hydroxyl acids (HAs) linked together by ester bonds [29]. The HAs are fatty acids, whose lengths are comprised between 3 to >14 carbon atoms [30]. Short-chain-length (SCL) monomers have from 3 to 5 carbon atoms, whereas medium-chain-length (MCL) monomers have between 6 and 14 (Fig. 3A). Recently, the class of long-chain-length (LCL) monomers has been described for units longer than 14 carbon atoms [31]. Specifically,

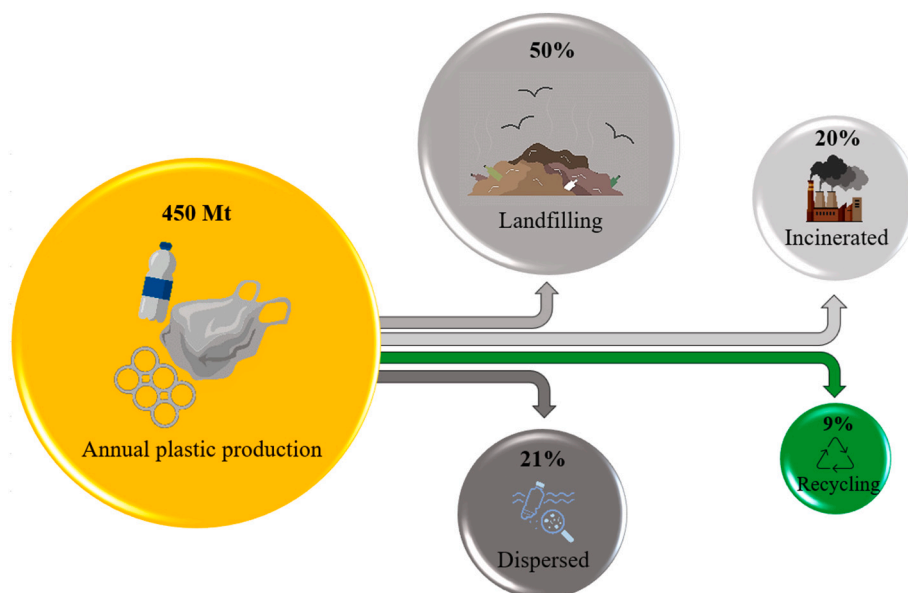


Fig. 1. Different plastic waste fates and relative proportions in 2019 [2].

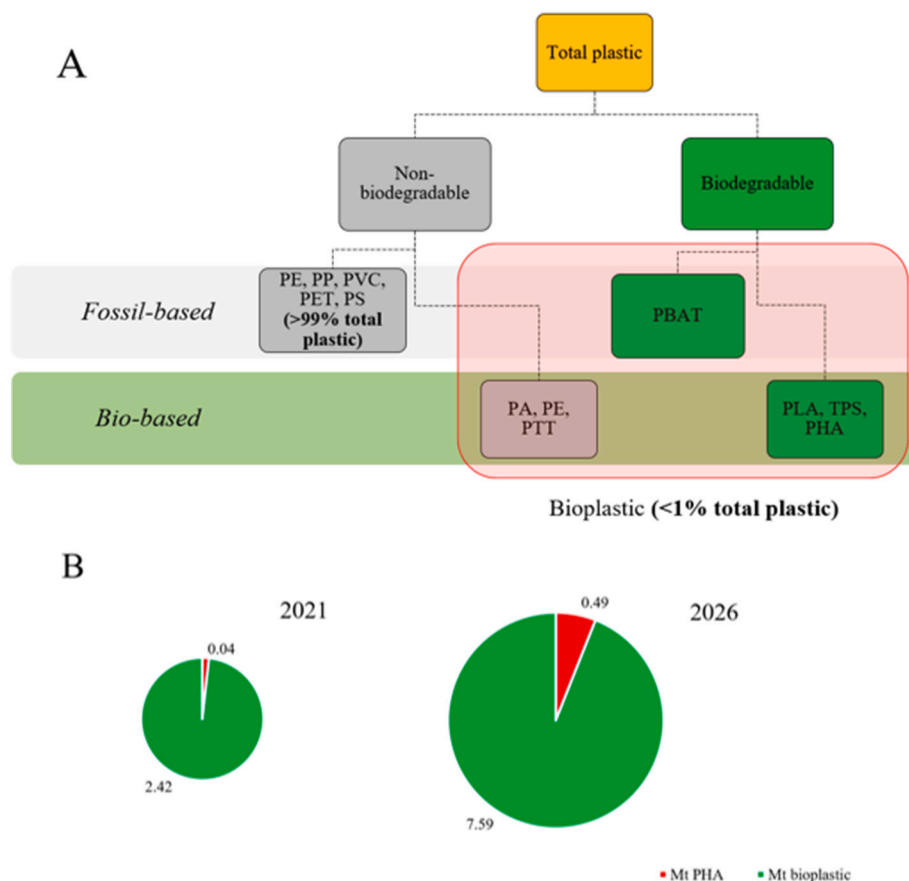


Fig. 2. Plastics are classified for the degradability rate and the nature of the feedstock. Bioplastics are highlighted in red (A). Global production (Mt) of bioplastics (green) and PHA (red) in 2021 and forecast for 2026 (B) [14]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

as indicated in Fig. 3, the backbone accounts for 3 to 6 carbon atoms, depending on the number of methyl units (indicated by the letter “x” in the formula). While the length of the side chain, or residue “R” in the formula, can vary significantly (e.g., 3-hydroxyhexadecanoate) (Fig. 3B) [32]. As a consequence, theoretically a great number of combinations can be generated, and, as a matter of fact, already >160 different monomers have been experimentally measured so far [33]. Additionally, when a single monomer unit is repeated it forms a homopolymer, while if two or more different units are bound together the PHAs are classified as copolymers [34]. The properties of PHAs are tightly related to their molecular composition, in terms of percentages of different monomeric units. Therefore, the wide spectrum of combinations offered by the building blocks, entails a broad range of applications for the PHAs [35]. Poly (3-hydroxybutyrate) P(3HB) homopolymer represents the PHA *par excellence* since it was the first discovered, the easiest to produce from innumerable carbon sources, and thereby the most studied. Accordingly, nowadays P(3HB) is commercialized by Biomer, PHB Industrial S.A., COFCO Cooperation Ltd., Mango Materials, Newlight Technologies, and Nafigate Corporation [36]. P(3HB) structure is extremely regular, making this material very hard and suitable for applications such as 3D printing and packaging [37,38]. However, the high crystallinity makes P(3HB) brittle and with a low elongation at break, heavily limiting its applications [39]. The incorporation of comonomers becomes therefore necessary to increase PHB performance and make it competitive with traditional plastics. This review offers an extremely vast and updated view on PHAs copolymers. Despite several review papers diving into PHAs copolymer production [40], this work highlights with unprecedented details the relationship between feedstock of use and final product obtained.

2. Copolymers as the keystone to enlarge industrial applications of polyhydroxyalkanoates: upstream from diverse precursors

Typically, the most widespread comonomers are 3-hydroxyvalerate (3 HV), 4-hydroxybutyrate (4HB), and 3-hydroxyhexanoate (3HHx). When 3 HV is included, the melting point of the polymer shrinks together with its crystallinity, while its flexibility and toughness increase [41]. Indeed, some applications of P(3HB-co-3 HV) are semi-structural adhesives, bottles, and films [36]. Currently, Chinese Tianan Biologic Materials Co. is the largest P(3HB-co-3 HV) player worldwide, recording 2 kt yearly production, while Brazilian PHB Industrial S.A. manufactures two different P(3HB-co-3 HV) materials with a 3 HV content of 9 and 17 % respectively, using propionic and valeric acids as 3 HV precursors [36,42]. When 3 HV was incorporated in nanocomposites materials, it showed significant antimicrobial properties, suitable for food packaging [43]. On the other hand, 4HB brings to the material improved elastomeric properties, and increased melting and crystallization temperatures, rendering the polymer extremely attractive for packaging and medical applications [44,45]. Recently Korean CJ White Bio started a 5 kt per year production of P(3HB-co-4HB) aiming to reach 65 kt by 2025, while Chinese PhaBuilder implemented the fermentation of glucose, corn steep liquor and γ -butyrolactone by *H. bluephagenesis*, obtaining 13.5 % 4HB copolymer [23,46]. The larger the 4HB fraction within the polymer the higher the tensile strength of the material. Indeed, when the homopolymer P(4HB) is obtained, the tensile strength becomes higher than for PP (polypropylene) and the material is well suited for biomedical applications, as in the case of TephafLEX™ that was accepted by the Food and Drug Administration (FDA) [47,48]. Both P(3HB-co-4HB) and P(4HB) are excellent biomaterials. In fact, P(3HB-

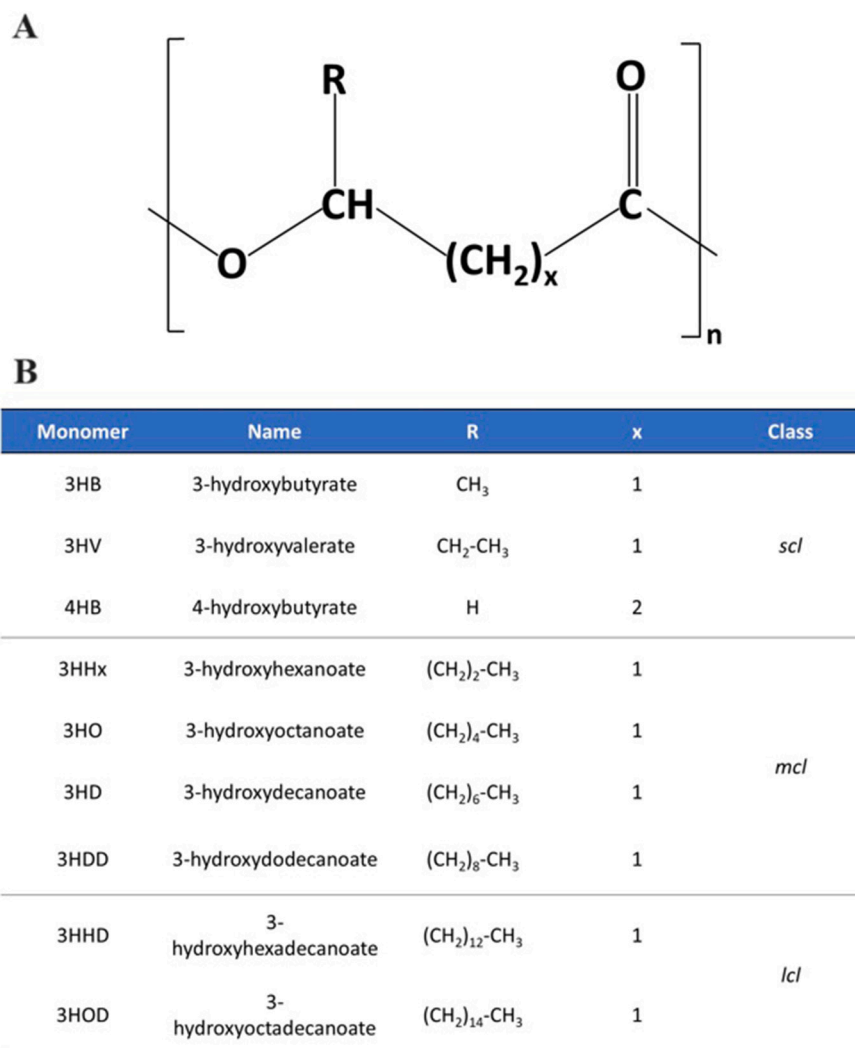


Fig. 3. PHAs monomers structural formula: R-group represents the side chain. The number of central methyl units (X) is one for 3HAs, while it increases for monomers like 4HB and 5 HV (A). The class of the PHA monomer is determined by the overall length of the carbon backbone. The number of specific monomers units within the polymer is summarized by n (B).

co-4HB) and P(4HB) are characterized by a slow degradation rate, and its degradation products are less toxic for the body when compared to other biomaterials used for medical implants, like polyglycolide (PGA) [47]. These features are desirable since the polymer durability must match with healing time and the release of toxic by-products must be avoided. As a result, the spectrum of applications of P(3HB-co-4HB) and P(4HB) comprises threads of suture, scaffold, and other medical implants [47]. Regarding 3HHx, the longer side chain of this monomer strongly disturbs the crystallinity of the polymer. As a result, 3HHx supplementation renders the material particularly flexible, softer and easier to process, hence ideal for single-use commodities [39]. For instance, P(3HB-co-HHx) is used for bottles, straws, cutlery, shopping bags, makeup containers [39]. Nevertheless, P(3HB-co-HHx) finds application also in scaffolds and for other biomedical purposes, thanks to its biocompatibility [39]. American Danimer Scientific manufactures around 10 kt per year of P(3HB-co-HHx) using canola, palm and soy oils as feedstocks, similarly to Japanese Kaneka which in 2020 reported a 5 kt per year capacity of P(3HB-co-HHx) produced from palm oil [49,50].

Literature on PHAs copolymers is growing and flourishing. Several strategies are considered to maximize copolymers' upstream processes, and above all the identification of new PHAs-producing strains, the genetic engineering of natural ones, the selection of novel feedstocks, and process optimization. Review papers mostly describe the range of

different wastes or microbial strains adopted for PHAs production [51], and in some circumstances are dedicated to specific feedstocks or microbes [52,53]. Alternatively, when the focus of the authors is on copolymers, reviews either touch upon the physical properties and biosynthetic routes of a specific copolymer [39,44,54] or broadly describe copolymers from some substrates of interest such as specific wastes [31]. On top of that, some other papers address brilliantly the vast plethora of PHAs copolymers and the different fermentation strategies that can be adopted, putting however the accent on the final product more than on the feedstock of origin, which is conceptually the real focus of this review [55]. Finally, other reviews, as in the case of Rodríguez-Perez and colleagues' article, offer in-depth analysis of copolymers production from various wastes, yet excluding the conversion of non-waste substrates and ultimately updated to contributions from up to 2018 [56]. This review, instead, presents an overview of the most updated works about manifold copolymers productions, classified for the choice of the substrate, a major bottleneck for the development of PHAs [57]. Specifically, carbon sources have been conceived in three separate sets (Fig. 4): (i) most expensive structurally related precursors, (ii) structurally unrelated precursors and (iii), more extensively, cheap industrial and/or agricultural waste streams. Structurally related copolymer precursors such as valerate, butyrate or octanoate are compounds with similar chemical structure to PHAs monomers and undergo

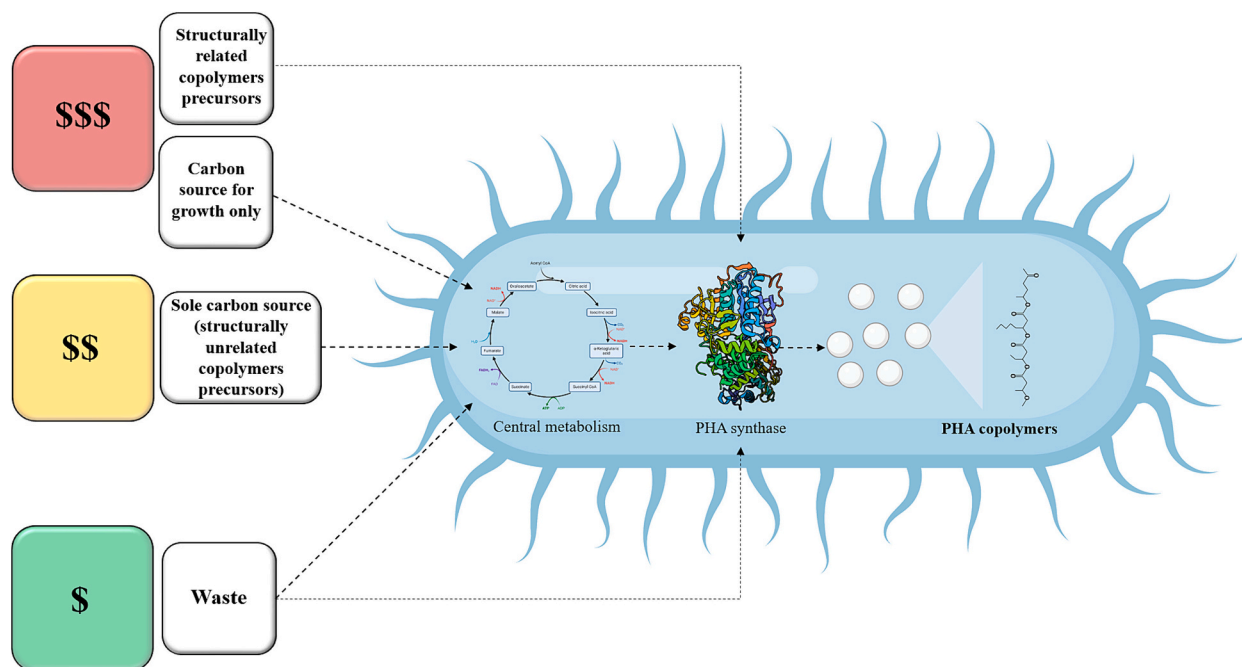


Fig. 4. Copolymers production using different feeding strategies, reported from the most expensive (top) to the cheapest one (bottom). High-cost strategy (red): optimized little amount of expensive structurally related copolymers precursors is directly converted by PHA synthase, although biomass production is supported by a separate carbon source provided in larger proportion. Intermediate-cost strategy (yellow): structurally unrelated copolymers precursors support biomass production and can be converted by peculiar central metabolisms into PHA synthase substrates. Low-cost strategy (green): wastes can hold both structurally related and unrelated copolymers precursors, besides other carbon sources for further biomass production. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

a single direct transformation mediated by the PHA synthase [58]. On the other hand, structurally unrelated copolymer precursors are molecules whose chemical structure is highly different from the one of the final monomers and thus require several enzymatic transformations, throughout the central metabolism, before being metabolized by the PHA synthase [58]. Finally, wastes are more complex feedstocks which can have either an elevated sugar content (usually in form of complex polysaccharides that require to be firstly hydrolyzed) or are rich of lipids to support both biomass production and PHAs accumulation. Wastes ultimately contain impurities and are more heterogenous, yet they represent a burden for the environment if not processed in a sustainable manner and their adoption for PHAs copolymers production is the most circular and cheapest strategy compared to using structurally related on unrelated precursors.

2.1. Copolymers production from structurally related precursors

Structurally related precursors for copolymer synthesis are typically expensive and sometimes toxic, after certain titers, for the bacterial strains. However, their conversion yields are significantly high (Table 1).

1,4-butanediol represents one of the most classical precursors of 4HB. The metabolic route involves an alcohol dehydrogenase and a reductase enzyme, and it is known for the inverse pathway, where 1,4-butanediol is the product of interest. Nonetheless, 1,4-butanediol together with glycerol has been converted into P(3HB-co-66 %-4HB) by *Aneurinibacillus* sp. H1 to form a film with great elongation at break and reduced hydrophobicity, making it suitable for medical applications [59]. Other precursors of 4HB are γ -butyrolactone and sodium-4HB, which were efficiently converted up to 51 % of cell dried matter by a modified strain of *C. necator* whose PHA synthase was substituted with the one of *Burkholderia contaminans* Kad1, the $pha_{C_{Bcon}}$ that has been demonstrated after BLASTp and neighbor-joining phylogenetic tree to be a class I PHA synthase [60]. However, the highest yields so far ever

reported were 46 g/L of P(3HB-co-99%4HB) using *Cupriavidus malaysiensis* USMAA1020 and 73 g/L P(3HB-co-21 %-4HB) throughout the engineered *H. bluephagenesis* TDB141 Δ AC strain [61,62]. Using an analog approach, γ -butyrolactone and valerate triggered in *C. necator* [63] the accumulation of both P(3HB-co-10 %-4HB) and P(3HB-co-20 %-3 HV), respectively. While Le Meur and colleagues produced the homopolymer P(4HB) with a recombinant *E. coli*, holding the pKSSE5.3 plasmid, using glycerol for biomass production and Na-4HB as 4HB precursor. Besides, they optimized the feeding strategy by limiting amino acids availability, adding weak organic acids, to produce 15 g/L of P(4HB). Indeed, they speculated how acetate may influence the amino acids availability for *E. coli*, while increasing the acetyl-CoA biosynthesis, which ultimately donates the CoA group for 4HB accumulation [64].

For what concerns 3HB-co-3 HV biosynthesis, propionate represents one of the main structurally related precursors of the 3 HV unit. In this metabolic pathway, a CoA group is added first to propionate. Then, throughout the action of a β -ketothiolase propionyl-CoA undergoes a condensation step with acetyl-CoA to form 3-ketovaleryl-CoA. Finally, acetoacetyl-CoA reductase catalyzes the formation of 3 HV-CoA, direct substrate of the PHA synthase [65]. Exploiting this route, *E. coli*, genetically equipped with PHA synthase genes, was found to accumulate up to 10.9 % of 3 HV molar fraction when incubated with 1.5 g/L propionate [66]. Additionally, to further boost 3 HV accumulation, the succinyl-CoA synthase gene (*sucD*) has been overexpressed in *E. coli* YH090 and incubated with glycerol, succinate, and propionate. As a result, the 3 HV fraction ramped up to 72 % of total PHA content. [67]. Ter-polymers comprising both 3 HV and 4HB have also been described when 4.1 g/L of P(3HB-co-17.9 %-3 HV-co-16.5 %-4HB) was synthesized from 1,4-butanediol and valeric acid [68]. As mentioned previously, 3HHx is one of the most attractive comonomers, and can be obtained from hexanoate. The typical route for the synthesis of 3HHx from fatty acids starts with their conversion into acyl-CoA, which afterwards undergoes two rounds of oxidation followed by one hydration. Ultimately,

Table 1
Copolymers production from structurally related copolymers precursor.

Structurally related copolymers precursor	Carbon source for microbial growth	Type of PHAs	Operation mode	Strain	PHAs concentration (g/L)	PHAs content (% CDM)	Process improvement	Reference
1,4-Butanediol	Glycerol	P(3HB-co-66.0 %-4HB)	Flask	<i>Aneurinibacillus</i> sp. H1	0.6	32.1	New thermophilic strain isolated from compost	[59]
Na-4HB	Nutrient rich	P(3HB-co-34.0 %-4HB)	Flask (two-stage)	<i>C. necator</i> PHB ⁻ 4 Cn-Kad1	0.5	20.0	Engineered <i>C. necator</i> expressing the PHA synthase of <i>B. contaminans</i> Kad1	[60]
1,6-Hexanediol	1,4-Butanediol	P(3HB-co-99.0 %-4HB)	Fed-batch (twice pulse feed)	<i>Cupriavidus malaysiensis</i> USMAA1020	46.0	92.0	Extra copy of the PHA synthase gene; pulse, constant and mixed feeding	[61]
1,4-Butanediol	Glucose	P(3HB-co-21.0 %-4HB)	Fed-batch	<i>H. bluephagenesis</i> TDB141 Δ AC	73	79.6	Deletion of 4HB degradation pathway	[62]
γ -Butyrolactone	Glucose	P(3HB-co-10.0 %-4HB)	Flask (two-stage)	<i>C. necator</i> DSM 545	7.7	64.0	Polymer was not affected by new, green, extraction methodology	[63]
Valeric acid	Glucose	P(3HB-co-20.0 %-3 HV)	Flask (two-stage)	<i>C. necator</i> DSM 545	6.5	54.0	Polymer was not affected by new, green, extraction methodology	[63]
Na-4HB	Glycerol	P(4HB)	Fed-batch (exponential feeding)	Recombinant <i>E. coli</i>	15	33.0	pKSSE5.3 plasmid expression; limiting amino acids availability	[64]
Propionate	Glucose	P(3HB-co-10.9 %-3 HV)	Flask	Recombinant <i>E. coli</i>	0.85	24.7	<i>E. coli</i> genetically equipped with PHA synthase genes	[66]
Propionate	Glycerol and succinate	P(3HB-co-72 %-3 HV)	Flask	<i>E. coli</i> YH090	0.36	80.0	Succinyl-CoA synthase genes (<i>sucD</i>) has been overexpressed	[67]
1,4-Butanediol and valeric acid	Glycerol	P(3HB-co-17.9 %-3 HV-co-16.5 %-4HB)	Flask (two-stage)	<i>C. malaysiensis</i> USMAA2-4	4.1	69.6	New strain; several new cultivation strategies	[68]
Hexanoate	Xylose	P(3HB-co-34.4 %-3HHx)	Fed-batch	<i>B. sacchari</i> LFM11	22.6	50.3	Expression of <i>B. sacchari</i> xylose consumption genes and <i>Aeromonas</i> sp. <i>phaC</i> (PHA synthase)	[69]
Propionate, butyrate	Fructose	P(54 % 3HB-co-36 % 3 HV-co-10 % 3HHx)	Flask	<i>Ralstonia eutropha</i> strain (Re2133/pCB81)	1.6	42.0	<i>phaB1</i> , <i>phaB2</i> , and <i>phaB3</i> genes were deleted, while synthetic PHA operon formed by <i>phaC2</i> , <i>phaA</i> , <i>phaJ</i> was heterologously expressed	[70]
Valerolactone	Glycerol	P(3HB-co-2.1 %-3 HV-co-47.4 %-4 HV)	Flask	<i>Aneurinibacillus</i> AH30	1.21	66.1	Direct conversions of lactones into 4 HV,	[71]
γ -Hexalactone	Glycerol	P(3HB-co-5.4 %-3 HV-co-7.0 %-4HHx)	Flask	<i>Aneurinibacillus</i> AFN2	0.44	43.9	Direct conversions of lactones into 4HHx	[71]
δ -Valerolactone	Glycerol	P(3HB-co-41.5 %-5 HV)	Flask	<i>Aneurinibacillus</i> AFN2	0.7	59.6	Direct conversions of lactones into 5 HV	[71]
Tiglic acid and crotonic acid	Glucose	P(3HB-co-24.0 %-3H2MB)	Flask	Recombinant <i>E. coli</i>	0.41	29.3	Production of a novel monomer	[72]
Diols	Glucose	P(63.5 %-3HB-co-8.4 %-3HP-co-16.5 %-4HB-co-11.6 %-5 HV)	Fed-batch (three stage)	<i>H. bluephagenesis</i> TD-141	22.4	54.8	Expression of specific alcohol/aldehyde dehydrogenases to convert diols into 5 HV	[73]
3,3'-Dithiodipropionic acid	Na-gluconate	P(3HB-co-22.0 %-3MP)	Flask	<i>C. necator</i> H16	n.r.	n.r.	Production of a novel monomer	[75]
Na-dodecanoate	Glucose	P(3HDD)	Flask	<i>P. putida</i> KT2440	0.35	25.2	Deletion of genes involved in fatty acid degradation	[77]
Decanoic acid and fatty acids	Citrate	P(3HD-co-20.0 %-3HPhV)	Multistage chemostats	<i>P. putida</i> KTQQ20	0.1	12.0	Deletion of <i>fadA</i> encoding 3-ketothiolase	[78]
Decanoic acid, dodecanoic acid or 9-decenol	Glucose	P(3HB-co-mcl-3HA)	Flask	Engineered <i>Pseudomonas entomophila</i>	3.5	60.0	Deletion <i>fadB</i> encoding S-3-hydroxyacyl-CoA dehydrogenase	[79]
Na-3HB and Na-2HB	Glucose	P(88.0 %-2HB-b-3HB)	Flask	Recombinant <i>E. coli</i>	0.2	11.5	Production of block copolymers	[80]
Na-3HB, Na-2HB and Na-3HP	Glucose	P(3HB-co-LMC HA)-b-P(2HB)	Flask	Recombinant <i>E. coli</i>	0.5	14.3	Production of block copolymers	[81]

the 3-ketoacyl-CoA thiolase (FadA) catalyzes the production of acetyl-CoA, which can eventually be transformed into PHAs [39]. Oliveira-Filho and colleagues, reported 22.6 g/L of genetically modified *Burkholderia sacchari* cell dried matter (CDM) production from hexanoic acid, with 50.3 % of P(3HB-co-34.4 %-3HHx) copolymer [69]. Finally, ter-polymers formed by units of 3HB, 3 HV, and 3HHx have also been biosynthesized by Jung and colleagues, who adopted a fermentation with propionate and butyrate, together with the recombinant *Ralstonia eutropha* strain (Re2133/pCB81), whose phaB1, phaB2, and phaB3 genes were deleted, while synthetic PHA operon formed by phaC2, phaA, phaJ was heterologously expressed [70].

According to the possible wide combinations of monomers, several papers focused on the research of innovative copolymers such as 3-hydroxy-2-methylbutyrate, 3-mercaptopropionate or block copolymers. As a first instance, novel thermophilic strains of the genus *Aneurinibacillus* have been firstly described for their surprising metabolism, able to convert lactones into directly related 4 HV, 4HHx and 5 HV. Although cell growth was limited, key genes identified might be transferred to more robust PHA-producers. Specifically the strains express both lactonase enzymes, able to open the lactone ring, and aspecific PHA synthases that can incorporate a wide range of substrates, including the very products originating from the lactonases activity [71]. Furutate and colleagues aimed instead to produce 3-hydroxy-2-methylbutyrate (3H2MB) as a novel monomer to combine with 3HHx and 3HB, employing tiglic acid as precursor. It was specifically observed that 3H2MB monomers have a faster crystallization behavior compared to 3HB [72]. In another work, since 5 HV monomer confers flexibility to

the material, an engineered *H. bluephagenesis* TD-141 was developed to express specific alcohol/aldehyde dehydrogenases to successfully transform several diols into copolymers comprising 5 HV units, leveraging the wide sensibility of the OrfZ PHA synthase enzyme for various substrate to ligate. As a result, highly transparent P(3HB-co-8.4 %-3HP-co-16.5 %-4HB-co-11.6 %-5 HV) was accumulated [73]. On the other hand, Huang et al. investigated particular copolymers characterized by thio/oxo ester linkages, specifically between 3HB and the novel discovered comonomer 3-mercaptopropionate (3MP) derived from 3,3'-dithiodipropionic acid, exploiting an interesting mercaptoalkanoates producer such as the *C. necator* H16 [74]. It was found that the resulting polymer had higher thermal stability [75]. A further homopolymer investigated is poly(3-hydroxydodecanoate) P(3HDD), which is highly transparent, glossy and flexible [76]. Hiroe and colleagues obtained a library of *E. coli* harboring mutant PHA synthase forms of *P. putida* KT2440 to optimize the conversion of sodium dodecanoate into P(3HDD) [77]. Differently, a strategy to address *mcl*-copolymers is to weaken the β -oxidation pathway, deviating fatty acids route towards PHAs biosynthesis, by deleting β -oxidation enzymes such as *fadA* encoding 3-ketothiolase, and *fadB* encoding S-3-hydroxyacyl-CoA dehydrogenase, and exploiting strategies like serial bioreactors [78,79]. Finally, copolymers can be distinguished for their nanostructure: while random copolymers present comonomers distributed in a not-ordinate manner within the polymeric chain, block copolymers comprise immiscible segments conferring captivating physical properties. Specific PHA synthases like PhaCAR, a chimeric class I PHA synthase formed by PhaCs from *Aeromonas caviae* and *C. necator*, allow the production of

Table 2
Copolymers production from structurally unrelated precursors as sole carbon source.

Sole carbon source	Type of PHAs	Operation mode	Strain	PHAs concentration (g/L)	PHAs content (% CDM)	Process improvement	Reference
Glucose	P(3HB-co-17.0 %-4HB)	Fed-batch	Recombinant <i>H. bluephagenesis</i> TD01	14.3	60.5	Succinyl-CoA deviated to succinate semialdehyde and 4HB	[82]
Glucose	P(3HB-co-25.0 %-3 HV)	Flask	Recombinant <i>H. bluephagenesis</i> TD01	4.1	65.0	Strengthening succinyl-CoA conversion into propionyl-CoA	[83]
Levulinic acid	P(3HB-co-20.6 %-3 HV)	Flask	Adapted <i>C. necator</i> H16	3.9	55.4	Evolutionary engineering	[85]
Levulinic acid	P(3HB-co-60.2 %-3 HV-co-39.1 % 4 HV)	Flask	Recombinant <i>P. putida</i> EM42	3.4	38.2	Levulinic acid catabolism pathway was inserted	[86]
Fructose	P(3HB-co-1.5 %-3HHx)	Flask	Recombinant <i>C. necator</i> DSM541	0.7	48.0	Insertion of crotonyl-CoA reductase from <i>S. cinnamonensis</i> and for PHA synthase and (R)-specific enoylCoA hydratase from <i>A. caviae</i>	[87]
Glucose	P(3HB-co-4.0 %-3HDD)	Unsterile fermenter	<i>H. cupida</i> J9	2.9	22.9	Expression of low-specific <i>PhaC₁₉</i> PHA synthase	[88]
Glucose	P(3HB-co-3HA _{MCL}) partially block	Flask	Recombinant <i>Pseudomonas</i> sp. LFM461	0.8	25.0–30.0	PHA synthase has been substituted with the one of <i>Aeromonas</i> sp. TSM81 throughout the transposon system mini-Tn7	[89]
Glucose	P(3HB-co-6.3 %-3HHx-co-9.5 %-3HD)	Flask	Recombinant <i>Pseudomonas</i> sp. LFM461	0.1	2.0	PHA synthase has been substituted with the one of <i>A. hydrophila</i> ATCC7966 phaC throughout the transposon system mini-Tn7	[90]
Glucose	P(3HB-co-7.4 %-mcl-3HAs)	Flask	Recombinant <i>E. coli</i>	1.1	20.3	(R)-3HA-CoA ligase from <i>P. aeruginosa</i> PAO, and PHA synthases from <i>C. necator</i> and <i>Pseudomonas</i> sp. 61–3	[91]
Glycerol	P(3HB-co-48.2-3HP)	Fed-batch	Recombinant <i>E. coli</i>	9.8	46.2	Two parallel synthetic pathways for glycerol were introduced, and controlled by two independent regulatory systems	[93]
Glycerol	P(3HB-co-2.1 %-3HP)	Fed-batch	<i>S. blattae</i> ATCC33430	7.1	30.7	No supplementation of vitamin B ₁₂ ; expression of PHA synthase	[94]
Glycerol	P(3HB-co-3HO-co-3HD-co-3HDD-co-HTD)	Bioreactor batch	<i>Pseudomonas</i> sp. PAMC 28620	4.1	52.2	Novel identification of extremophiles able to convert directly glycerol into copolymers	[96]
Glycerol	P(3HB-co-3HHx-co-3HO-co-3HD-co-3HDD)	Bioreactor batch	<i>Pseudomonas</i> sp. MPC6	1.2	47.3	Novel identification of extremophiles able to convert directly glycerol into copolymers	[97]
Glucose	P(3HB-co-17.0 %-2HAs)	Flask	Recombinant <i>E. coli</i>	1.1	42.0	(R)-2-hydroxy-4-methylvalerate (2H4MV) dehydrogenase (<i>LdhA</i>) and 2H4MV-CoA transferase (<i>HadA</i>) from <i>P. difficile</i>	[98]

n.r.: not reported.

block copolymers, such as poly(2-hydroxybutyrate-*b*-3-hydroxybutyrate) [P(2HB-*b*-3HB)] and segment-containing different shares of block copolymers P(3HB-*co*-LMC HA)-*b*-P(2HB)s [80,81].

2.2. Copolymers production from structurally unrelated precursors

To produce copolymers and avoid the use of the expensive, structurally related precursors listed above, a widespread alternative is represented by the adoption of structurally unrelated carbon sources (Table 2). These compounds, sugars or alcohols like glucose and glycerol, are advantageous since they foster a large growth of biomass although typically promote the production of sole P(3HB). Therefore, to directly convert these molecules into copolymers, the creation or discovery of new metabolic pathways becomes essential. Accordingly, the most common strategy is to genetically modify well-known microorganisms: either by adding genes for the conversion of such carbon sources into copolymer direct precursors in classic PHA-producers like *C. necator* and *P. putida*, or, vice versa, by inserting PHA synthase cassette genes in strains able to transform structurally unrelated precursors into copolymer substrates. Alternatively, novel strains able to metabolize structurally distant precursors in copolymers are identified and isolated from their habitats.

Succinyl-CoA is a key element of tricarboxylic acid cycle (TCA), thus deviating its metabolism towards comonomers precursors might affect cell growth. Nevertheless, *H. bluephagenesis* TD01 has also been modified to produce 4HB comonomers from glucose. In fact, succinyl-CoA metabolism has been deviated towards succinate semialdehyde, whose reconversion backwards has been stopped by deleting two succinate semialdehyde dehydrogenases. The latter modifications severely boosted 4HB synthesis, up to 17.0 % mol, considering 23.6 g/L CDM containing 60.5 % P(3HB-*co*-17 % 4HB) [82]. Similarly, Chen and colleagues managed to produce 6.3 g/L CDM, 65.0 % P(3HB-*co*-25 % 3HV) from glucose by adding several chromosomal modifications to *H. bluephagenesis* TD01, targeted to both weaken the consumption of succinyl-CoA within the Krebs cycle, and strengthening instead its conversion into propionyl-CoA [83]. Likewise, levulinic acid has been adopted as a 3 HV/4 HV precursor. Although levulinic acid is structurally related to 3 HV and toxic for cells, it represents an extremely low-cost substrate that can be produced from cellulosic biomass [84]. Therefore, from a process-economic point of view, its application attracts as pure glucose or glycerol do and therefore was mentioned in this session. Microorganisms have been modified either by evolutionary engineering approaches to produce P(3HB-*co*-3 HV) from levulinic acid [85] or by classic genetic engineering achieving the synthesis of 3 HV and 4 HV comonomers as well from levulinic acid [86]. Regarding 3HHx, its production is generally tackled using fatty acids, thereby lipidic wastes. However, fructose was successfully converted into P(3HB-*co*-1.5 % 3HHx) by a recombinant *C. necator* DSM541 harboring genes for crotonyl-CoA reductase from *Streptomyces cinnamomensis* (*ccrSc*) and for PHA synthase and (*R*)-specific enoylCoA hydratase from *Aeromonas caviae* (*phaC-JAc*) [87]. In the wake of the next-generation industrial biotechnology (NGIB), another *Halomonas* strain was studied to promote copolymers production from structurally unrelated precursors under high salinity and non-sterile conditions. *H. cupida* J9 was found to express *PhaC*_{J9} whose catalytic pocket was demonstrated to be low-specific and eventually eliciting P(3HB-*co*-3HDD) production from glucose and glycerol [88]. In two separate works, glucose was as well successfully converted into *mcl*-PHAs by using *Pseudomonas* LFM461 strain whose PHA synthase has been substituted with either the one of *Aeromonas* sp. TSM81 or the *A. hydrophila* ATCC7966 *phaC* throughout the transposon system mini-Tn7. In both cases, the non-specificity of the inserted PHA synthases elicited the production of P(3HB-*co*-3HA_{MCL}) comprising fractions of 3HB, 3HHx, 3HD, partially in form of block copolymers [89,90]. Finally, since it represents an ideal platform for genetic modifications, *E. coli* has also been exploited for glucose conversion into comonomers. Hokamura et al. leveraged the enzymatic

activity of a (*R*)-3HA-CoA ligase from *Pseudomonas aeruginosa* PAO, together with the co-expression of recombinant PHA synthases from *C. necator* and *Pseudomonas* sp. 61–3, to transform glucose into *mcl*-3HAs consisting of C₈, and C₁₀, accounting for 7.4 % mol of the copolymer [91].

Regarding the use of glycerol as precursor for copolymers biosynthesis, a specific metabolic pathway that encompasses glycerol dehydratase and a propionaldehyde dehydrogenase allows the conversion of glycerol into 3-hydroxypropionate (3HP) monomer that diminishes glass transition, crystallinity and melting point of the material [92]. Wang and colleagues introduced the required genes in *E. coli* to successfully produce 9.8 g/L P(3HB-*co*-48.2 % 3HP) from glycerol [93]. Besides, 3HP fraction was tuned by inducing the expression of the recombinant genes with increasing concentrations of L-arabinose. However, vitamin B₁₂ must be periodically supplemented as a key co-enzyme and thus is consumed at high rates. Vitamins B₁₂ producer *Shimwellia blattae* ATCC33430, which is not a PHAs native accumulator, becomes therefore attractive and, after being genetically modified to express a PHA synthase, was able to accumulate P(3HB-*co*-3HP) from glycerol without supplementing vitamin B₁₂ [94]. Nevertheless, a more successful alternative to exploit pure glycerol was given by the isolation of novel strains, especially from extremely cold environments, where PHAs granules function as a protective barrier [95]. Two papers report the identification of psychrophilic microorganisms from Antarctic and Arctic environments. Curiously, they both belong to the *Pseudomonas* sp. and turn glycerol into copolymers. Specifically, while arctic *Pseudomonas* sp. PAMC 28620 produced at 35 °C up to 7.95 g/L biomass on 3 % glycerol, accumulating 52.2 % of a copolymer comprising 3HO, 3HD, 3HDD and 3HTD units [96], the other strain, *Pseudomonas* sp. MPC6, recorded 2.6 g/L biomass with 47.3 % P(3HB-*co*-3HHx-*co*-3HO-*co*-3HDD-*co*-3HDD) content once grown at lower temperature (30 °C), thus avoiding expensive temperature control systems [97].

Finally, alternative sources of structurally unrelated precursors for comonomers are amino acids. *E. coli* was engineered to synthesize 2HAs by inserting (*R*)-2-hydroxy-4-methylvalerate (2H4MV) dehydrogenase (*LdhA*) and 2H4MV-CoA transferase (*HadA*) from *Peptoclostridium difficile*, which enable the conversion of endogenous leucine into 2H4MV precursors. Without the supplementation of any amino acid, the recombinant strain accumulated P(3HB-*co*-17 % 2HA) on glucose [98]. Similarly, *Rhodospirillum rubrum* S1 (ATCC11170) was genetically modified to co-express transhydrogenase *PntAB* from *E. coli* MG1655 and *phaB1* acetoacetyl-CoA reductase from *C. necator*, which led to threonine-derived propionyl-CoA accumulation, a precursor for 3 HV biosynthesis [99].

2.3. Copolymers production from waste streams

The concept of second generation biorefineries [100], where biodegradable commodities are manufactured from recyclable feedstocks, matches with a circular economic view [101]. Indeed, it allows to elongate the life cycle of wastes that are transformed into new commodities, therefore closing the loop. The use of by-products to produce PHAs has been explored for decades and yet it's constantly updated: novel substrates are discovered and bioprocess strategies are relentlessly tailored to become more and more efficient (Table 3). Besides, wastes are heterogeneous matrixes characterized by pools of highly variegated compounds. While some curb cell growth or PHAs accumulation, others, on the other hand, can function as copolymers precursors.

2.3.1. Wastes rich in simple sugars

The food industry represents a major source of sugar-rich waste streams, ideal for large cellular growths. Indeed, *C. necator* DSM 545 was screened both on several food residues and on different scales. The resulting most promising performance occurred with melon waste, accounting for a biomass production of 5.1 g/L, 74.9 % 3HB and 4.6 % 3 HV when the food residue was autoclaved and no medium salts were

Table 3
Copolymers production from wastes as sole carbon source.

Waste	Type of PHAs	Operation mode	Strain	PHAs concentration (g/L)	PHAs content (% CDM)	Process improvement	Reference
Melon waste	P(3HB-co-6.1 %-3 HV)	Flask	<i>C. necator</i> DSM 545	4.1	79.5	Low-cost, fast, and clean nutrients extraction	[26]
Date waste	P(3HB-co-18.0 %-3 HV)	Fed-batch	<i>Haloferax mediterranei</i> DSM1411	4.5	25.0	No need for trace elements supplementation	[102]
Liquid and gaseous streams from acetogenesis of melon waste	P(3HB-co-11.3 %-3 HV)	Batch, fermenter	<i>C. necator</i> DSM 545	2.7	44.3	Conversion of both liquid and gaseous streams from an acetogenic reactor	[103]
Liquid stream from acidogenesis of fruit waste	P(3HB-co-24.0 %-3 HV)	Fed-batch	Enriched MMC	1.7	80.5	Three-stage pilot scale experiment	[104]
Liquid stream from acidogenesis of fruit waste	P(3HB-co-1.0 %-3 HV-co-66.0 %-3HHx)	Fed-batch	Enriched MMC	0.8 gCOD/L	71.3	Three-stage pilot scale experiment	[105]
Apple waste	P(3HB-co-52.0 %-mcl-PHAs)	Bioreactor (batch)	<i>C. necator</i> DSM428 and <i>Pseudomonas citronellolis</i> NRRL B-2504	1.8	33.6	Sole apple waste without further supplementations	[106]
Liquid stream from acidogenesis of cheese whey	P(3HB-co-24.0 %-3 HV)	Fed-batch	Enriched MMC	n.r.	50	Two-step conversion	[108]
Cheese whey	P(3HB-co-18.4 %-4HB-co-2.2 %-3 HV)	Flask	<i>H. pseudoflava</i> DSM 1034	0.1	2.9	Direct conversion of cheese whey into comonomers	[109]
Spent coffee ground	P(3HB-co-3.7 %-3 HV)	Flask	<i>C. necator</i> DSM 545	10.0	88.9	Supplementation of DMSO	[111]
Sunflower oil	P(3HB-co-9.1 %-3 HV)	Flask	<i>C. necator</i> DSM 545	4.8	76.6	Direct conversion of sunflower oil	[111]
Rubber seed oil	P(3HB-co-3 HV)	Flask	<i>B. cereus</i> STV1180	2.6	64.1	Optimization via surface response methodology	[113]
Waste frying oil	mcl-PHAs	Bioreactor (batch)	Recombinant <i>P. putida</i>	1.9	38.3	Engineered to weaken the tripartite carboxylate transport system, in favor of the β -oxidation catabolism	[114]
Waste cooking oil	P(3HB-co-20.0 %-3HO-co-37.0 %-3HD-co-36.7 %-3HDD)	Pulse fed-batch	<i>P. chlororaphis</i> 555	13.9	26.2	No need of surfactants	[115]
Date seed oil	P(3HB-co-28.0 %-3HHx)	Flask	<i>C. necator</i> H16 Re2058/pCB113	6.1	80.0	PHA synthase from <i>R. aetherivorans</i> strain I24 (<i>phaC2Ra</i>) and (<i>R</i>)-specific enoyl coenzyme A hydratase (<i>phaJ</i>) gene from <i>P. aeruginosa</i>	[116]
Crude palm kernel oil	P(3HB-co-68.0 %-3HHx)	Flask	<i>C. necator</i> Re2160/pCB113	1.3	45.0	PHA synthase of <i>R. aetherivorans</i> I24 and an enoyl-CoA hydratase (<i>phaJ</i>) gene from <i>P. aeruginosa</i>	[117]
Tung oil	P(3HB-co-2 % 3 HV-co-45 % 3-HHx)	Fermenter, batch	<i>R. eutropha</i> Re2133/pCB81	5.4	20.4	Δ <i>phaB1</i> , Δ <i>phaB2</i> , Δ <i>phaB3</i> , Δ <i>phaC1</i> , and <i>phaC2Ra-phaA-phaJ1Pa</i>	[118]
Tuna condensate	P(3HB-co-20.0 %-3 HV)	Repeated batch in bioreactor	<i>C. necator</i> TISTR 1095	3.8	42.1	Novel waste stream	[119]
Fat/protein emulsions	P(3HB-co-20.0 %-3HHx)	Fed-batch	<i>C. necator</i> Re2058/pCB113	36	71.0	PHA synthase from <i>R. aetherivorans</i> strain I24 (<i>phaC2Ra</i>) and (<i>R</i>)-specific enoyl coenzyme A hydratase (<i>phaJ</i>) gene from <i>P. aeruginosa</i>	[120]
Udder and lard	P(3HB-co-7.0 %-4HB)	Flask (two step)	<i>D. acidovorans</i> DSM39	n.r.	43.0 (udder) 39.0 (lard)	<i>lipC</i> and <i>lipH</i> from <i>P. stutzeri</i> BT3 have been inserted to generate an efficient lipase	[121]
Rice straw waste	P(3HB-co-22.9 %-3 HV)	Flask (two stage)	<i>C. necator</i> ATCC 17697 ^T	0.8	39.0	Tuned extraction of 3 HV precursors with sulfuric acid and heat	[130]
Liquid stream from acidogenesis of broken rice	P(3HB-co-2.1 %-3 HV)	Flask	<i>C. necator</i> DSM 545	1.0	76.6	Two-step fermentation	[131]
Hydrolysate of a Japanese hemicellulosic cedar	P(3HB-co-19.9 %-2H4MV-co-9.6 %-2H3PhP)	Flask	Recombinant <i>E. coli</i>	0.6	25.0	Engineered to express (<i>R</i>)-2-hydroxy-4-methylvalerate (2H4MV) dehydrogenase (<i>LdhA</i>) and 2H4MV-CoA transferase (<i>HadA</i>) from <i>C. difficile</i>	[132]
Hydrolyzed bamboo	P(3HB-co-3 HV-co-3HDD)	Flask	<i>Halomonas alkalicola</i> M2	5.9 g/L	67.0	Alkaline pretreatment followed by enzymatic digestion. Halophilic strain: NaCl 70 g/L	[133]
Alkaline pretreated liquor	P(3HB-co-mcl-PHAs-co-lcl PHAs)	Fed-batch	<i>B. cepacia</i> B1-2	0.1	9.0	New isolated lignocellulosic strain	[134]

(continued on next page)

Table 3 (continued)

Waste	Type of PHAs	Operation mode	Strain	PHAs concentration (g/L)	PHAs content (% CDM)	Process improvement	Reference
Crude glycerol	P(3HB-co-10.0 %-3 HV)	Fed-batch	<i>H. mediterranei</i> DSM 1411	16.2	76.0	Metabolism favors propionyl-CoA synthesis and low-specific class III PHA synthase	[137]
Crude glycerol	<i>mcl</i> -PHAs	Fed-batch	<i>P. putida</i> KT2440 <i>ΔphaZ</i>	20.4	38.9	The polymer does not undergo the depolymerizing action of the <i>phaZ</i> enzyme	[138]
Crude glycerol	<i>mcl</i> -PHAs	Membrane reactor	<i>P. chlororaphis</i> subsp. <i>aurantiaca</i> (DSM 19603)	19.1	32.2	Direct conversion of CG into <i>mcl</i> -PHAs	[139]
Pyrolyzed PE	<i>mcl</i> -PHAs	Fed-batch	<i>P. putida</i> KT2440	54.0	65.0	After pyrolysis and oxidation, PE resulted into a mixture of fatty acids	[140]
Isobutyrate	P(3HB-co-37.0 %-PHiB-co-3.0 %-3 HV)	Fed-batch	Aerobic activated sludge	n.r.	41.1	An aerobic activated sludge of a wastewater treatment plant was enriched with iso-butyric acid	[142]
Liquid stream of syngas fermentation	P(3HB-co-23.0 %-3 HV)	Fed-batch	Enriched MMC	n.r.	41.5	Liquid effluents originating from syngas fermentations of <i>C. carboxidivorans</i> or <i>C. acetivum</i> , abundant of VFA and alcohols	[144]

n.r.: not reported.

supplemented, suggesting that melon provides a balanced amount of nutrients to the microbe to boost cell growth and PHAs accumulation [26]. Similarly, date waste demonstrated an enormous potential for copolymer application, due to its high sugar's concentration. The halophilic archaeon *Haloferax mediterranei* DSM1411 was adopted to produce 18 g/L of PHBV with 18 % 3 HV in non-sterile conditions. Again, supplementation of trace elements was found to negatively affect the biomass and PHAs production. It was speculated that the higher concentration of metals elicits EPS synthesis, as a barrier, instead of PHAs accumulation [102]. Alternatively, the effluents from an acetogenic reactor fed with melon waste, abundant of VFAs and alcohols, were converted in 2.7 g/L P(3HB-co-11.3 %-3 HV) by *C. necator* DSM 545 [103]. In the same work, C1 carbon sources derived from black fermentation, specifically CO₂, were converted in PHAs [103]. Further, in an optimized three-stage pilot scale experiment, fruit waste first fed an acidogenic reactor, whose liquid streams were abundant in butyrate and 3-hydroxyvalerate. After the Mixed Microbial Community (MMC) selection phase, PHAs production from the liquid effluent was maximized in an accumulation reactor where productivity peaked up to 8.1 g/L/d of P(3HB-co-24 %-3 HV) [104]. Following the same strategy, the ter-polymer including 3HHx was obtained by driving the acidogenic reactor towards caproic acid production. In this case, the reactor worked at microaerophilic conditions which favored the genera *Ruminiclostridium*, *Atopobium*, and *Olsenella*, able to convert lactate into caproic acid. This metabolite worked as a 3HHx precursor for a selected MMC whose PHAs production was optimized up to 3.29 gCOD/L h of P(3HB-co-1 %-3 HV-co-66 %-3HHx) [105]. Sole apple waste also found application for copolymers production: a co-culture of *C. necator* DSM428 and *Pseudomonas citronellois* NRRL B-2504 reached the production of 1.8 g/L of flexible P(3HB-co-52 %-*mcl*-PHAs) where the *mcl*-PHAs fraction was fully accumulated by *P. citronellois* [106].

Cheese whey, a by-product of the dairy industry, rich in lactose, is another abundant biomass, and annually almost 200 millions of tons (Mt) are produced in Europe [107]. MMCs can be exploited for cheese whey conversion into copolymers, as accomplished by Lagoa-Costa and colleagues who obtained a biomass accumulating PHAs up to 50 % of intracellular content, with 24 % mol fraction of 3 HV, from the liquid stream of an acidogenic reactor fed with cheese whey [108]. On the other hand, throughout pure cultivations of *Hydrogenophaga pseudoflava* DSM1034, a Gram-negative that showed significant suitability for a wide range of hexose and pentose sugars, after 72 h of 1-step fermentation, 0.1 g/L of terpolymer P(3HB-co-18.4 %-4HB-co-2.2 %-3 HV) was obtained from cheese whey [109].

2.3.2. Lipidic waste streams

Lipids represent the other main family of substrates for PHAs production. Lipid wastes and animal fats are mixtures of fatty acids, thereby ideal precursors for *mcl*-PHAs biosynthesis, as they require a reduced number of enzymatic steps [110]. Moreover, since these residues are not recalcitrant as lignocellulosic ones, costly pre-treatments are skipped, and the wastes are directly fermented into copolymers. Among the major residues, a wide range of vegetal spent oils and by-products from the related industry has been studied and already found applications on the market, manufactured and distributed mostly by Scientific Danimer and Kaneka. Accordingly, Ingram and Winterburn screened five different vegetable oils for the accumulation of P(3HB-co-3 HV) with *C. necator* DSM 545. On shaking flasks, the experiment showed the remarkable PHAs accumulation of 88.9 % (3.7 % mol 3 HV) of total CDM using spent coffee ground oil emulsified with dimethyl sulfoxide (DMSO) out of 11.3 g/L final biomass. The 3 HV fraction increased to 9.1 % out of 6.3 g/L biomass when *C. necator* was grown on sunflower oil [111]. Playing a key role is the fatty acids mixtures of these oils and, accordingly, *C. necator* preference for some of them. It was speculated that the specificity of the expressed lipase or the membrane transport system for fatty acids might be determinant in this sense [112]. Interestingly, using a response surface method, P(3HB-co-3 HV) production from rubber seed oil was firstly demonstrated and optimized, adopting *Bacillus cereus*, a microbe isolated from an oil contaminated field. The strain accumulated P(3HB-co-3 HV) up to 60.5 % of CDM, accounting for 2.6 g/L maximized production [113]. A downside of lipid-rich substrates is their hydrophobicity and the challenging emulsification with cells. To avoid the use of expensive amphiphilic surfactants, strains expressing natural extracellular lipases become an appealing option. *P. putida* falls under the category and was chosen to convert waste frying oil into *mcl*-PHAs such as 3HO and 3HD. Additionally, the strain KT2440 (DSM 6125) was engineered to weaken the tripartite carboxylate transport system, in favor of the β -oxidation catabolism [114] resulting in a doubled copolymer accumulation (1.9 g/L). Nevertheless, waste cooking oil composition may vary significantly, as explained by Ruiz and colleagues [115]. In their work, waste cooking oil comprised a large fraction of unsaturated fatty acids which influenced copolymer composition. Specifically, they set up a pulse fed-batch fermentation in a 5-L reactor with *Pseudomonas chlororaphis* 555. As a result, 13.9 g/L of light, amorphous copolymer (3HO, 3HD and 3HDD monomers) were obtained and productivity scored 0.3 g/L/h [115]. A further by-product of the food industry is date seed: their oil can be extracted and adopted for copolymers production, since it is ample of oleic acid. Using date seed oil, Purama et al. reported an accumulation of 80 % P(3HB-co-28

%-3HHx) from 7.6 g/L of recombinant *C. necator* H16 Re2058/pCB113 biomass [116]. Yet, a distinct set of vegetable oils including jatropha oil, crude palm oil, palm olein, soybean oil, corn oil, coconut oil and crude palm kernel oil was tested with *C. necator* Re2160/pCB113. Results were remarkable for all the substrates, recording high biomass levels and PHAs production with large fractions of 3HHx. However, crude palm kernel oil showed the most promising results: from 2.5 g/L of oil 1.3 g/L of P(3HB-co-68 %-3HHx) was produced. The polymer was characterized by great elongation to break, although little tensile strength and Young's modulus, which can be increased by diminishing to around 32 % the 3HHx fraction, resembling LDPE (low-density polyethylene) properties [117]. Additionally, the conversion of tung oil into PHAs is noteworthy. Tung oil is abundant of α -telostearic acid which fostered the production of P(3HB-co-2 % 3 HV-co-45 % 3-HHx) and whose residual traces provided an antioxidant effect to the material [118].

Another source of lipids is animal fat wastes, as in the case of tuna condensate, a by-product of the tuna processing operation, a key-sector in Thailand. The residue consists of 8.5 % proteins and 2 % lipids and finds application like animal feed, biodiesel production and has been evaluated also as a PHAs copolymer substrate. *C. necator* TISTR 1095 accumulated 3.8 g/L copolymer when grown on tuna condensate with 20 % mol of 3 HV [119]. Additionally, since heparin derivates from porcine intestinal mucosa, hundreds of tons of fat/protein emulsions are generated every year. Using a pneumatic feeding system, a fed-batch fermentation of fat/protein emulsions with *C. necator* Re2058/pCB113 led to the production of 51 g/L biomass, with 71 % P(3HB-co-20 %-3HHx) and an overall productivity of 0.6 g/L/h [120]. Finally, intriguing evidence was observed by using *Delfia acidovorans* DSM39, a strain that naturally accumulates 4HB monomers. Lacking lipolytic behaviors, the two genes *lipC* and *lipH* from the phylogenetically related *Pseudomonas stutzeri* BT3 have been inserted into the microbe to generate an efficient lipase: as a result, the PHAs accumulation from udder and lard recorded 43 and 39 % CDM, respectively, comprising 7 % mol 4HB [121].

2.3.3. Lignocellulosic biomass

Lignocellulose represents the most abundant biomass on earth [122]. Its structure is a complex and recalcitrant matrix of cellulose, hemicellulose, and lignin. Therefore, a pretreatment stage is needed to extract the monomeric sugars units, a process that goes under the name of saccharification. During lignocellulose saccharification though, organic acids and aromatic compounds are released as well from the biomass. At the end, whereas the sugar monomers will mostly support cellular growth, the organic acids and the aromatic compounds will prompt PHAs copolymers accumulation [123,124]. However, besides adding extra costs to the process, the required pre-treatment, as a side-effect, frees toxic compounds in the hydrolysate which affect microbial growth. These include for instance 5-Hydroxymethylfurfural, furfurals, vanillin, sorbic acid, and phenylacetaldehyde [125,126]. One solution is, in this sense, offered by detoxification treatments of the hydrolysates. Several chemical molecules, like reducing or sulfonation agents, have indeed the potential to block the inhibitory effect of these toxic compounds [127]. Detoxification from inhibitory compounds has also been applied in the context of PHAs production, exploiting a combination of overliming and low-temperature sterilization [128]. In the fermentation stage, the incorporation of these molecules cause a drop of the cytoplasmic pH that leads to an energetic depletion or even cell death [129]. Saccharification by acidic pre-treatments is one of the most common approaches, Ahn and colleagues tuned sulfuric acid concentrations together with a heating stage to extract larger amounts of reducing sugars and 3 HV precursor (levelunic acid) from rice straw waste [130]. As a result, higher volumes of sulfuric acid or heating incubation times were followed by larger concentrations of 3 HV comonomer (22.9 %) but an overall reduced accumulation of PHAs. Another by-product of rice is broken rice and its conversion into PHAs was successfully achieved by Brojanigo et al. even without any saccharification

phase. Accordingly, an acidogenic reactor was fed with broken rice and the liquid effluent, rich in VFAs, subsequently adopted for *C. necator* DSM 545 growth and production of 0.95 g/L P(3HB-co-3 HV) [131]. Furthermore, *E. coli*, engineered to express (R)-2-hydroxy-4-methylvalerate (2H4MV) dehydrogenase (*LdhA*) and 2H4MV-CoA transferase (*HadA*) from *Clostridium difficile*, was grown up to 2.2 g/L on the hydrolysate of Japanese hemicellulosic cedar, obtained by the activity of a mix of commercial cellulases. As a result, 2HAs copolymers 2H4MV (19.9 %) and 2H3PhP (9.6 %) were accumulated [132]. Additionally, bamboo, another attractive lignocellulosic biomass, was hydrolyzed through an alkaline pretreatment followed by an enzymatic digestion of the solid fraction by commercial cellulases, resulting in a mixture of sugars and copolymers precursors. In combination, the extremophilic bacterium *Halomonas alkalicola* M2 was isolated from a paper mill. Since the microbe tolerates extremely high pH levels (alkaliphile) and displays optimum growth under high concentration of NaCl (halophile) it was grown on the bamboo alkaline hydrolysate at pH 10 and NaCl 70 g/L, resulting in 5.9 g/L of PHAs, including 3 HV and 3HDD comonomers [133]. Eventually, the identification of novel lignocellulosic strains was the strategy of Xu et al. as well, who isolated bacteria such as *Burkholderia cepacia* B1-2 and *P. putida* B6-2 from soil to produce mixtures of scl/mcl and lcl-PHAs from the alkaline pretreated liquor originating from corn stover [134].

2.3.4. Other waste streams

For every 10 kg of biodiesel produced, 1 kg of crude glycerol (CG) is generated. Although this by-product is not clean as pure glycerol and calls for purification under some circumstances [135], it also finds applications in several industries [136]. Accordingly, CG constitutes a valuable option for PHAs copolymers biosynthesis as well. In a study published by Hermann-Krauss and colleagues, archaeon *H. mediterranei* DSM 1411 was grown either on pure glycerol or on CG and the different P(3HB-co-3 HV) production was assessed. Remarkably, both the PHAs productivity and the 3 HV fraction in the polymer were identical for pure glycerol and CG: 0.1 g/L/h and 10 %, respectively. Overall, the strain accumulated 16.2 g/L PHAs from CG, accounting for 0.2 g/g yield, demonstrating its potential for producing 3 HV comonomer from unrelated precursors, thanks to its metabolism aimed towards propionyl-CoA synthesis (precursor for 3 HV) and its PHA synthase, a class III PHA synthase formed by two subunits PhaC and PhaE that induce PHBV accumulation [137]. On the other hand, *P. putida* KT2440 Δ *phaZ* holding a mutated *phaZ* PHA depolymerase gene accumulates larger amounts of PHAs if grown on CG, as the polymer does not undergo the depolymerizing action of the *phaZ* enzyme: adopting a dissolved oxygen-stat feeding approach, 20.4 g/L of *mcl*-PHAs including a large range of monomers (76 % C₁₀) were obtained, and productivity recorded 0.3 g/L/h [138]. In another work, co-production of phenazines and *mcl*-PHAs was tackled by the CG fermentation with *Pseudomonas chlororaphis* subsp. *aurantiaca* (DSM 19603) using a cell retention culture approach in a membrane reactor which, throughout specific filters, allows to obtain higher cell density when compared to batch strategy: cell concentration resulted to be 28-folds higher, and consequently 19 g/L *mcl*-PHAs were produced, characterized by 3HD and 3-hydroxytetradecanoate as main monomers fractions [139].

Recently, researchers focused also on the identification of novel and alternative wastes for copolymers production. For instance, traditional PE was processed to be converted into *mcl*-PHAs as a non-standard route of recycling. After pyrolysis and oxidation, PE resulted in a mixture of fatty acids, thus ideal copolymers precursors. *P. putida* KT2440 grew up to 83 g/L CDM within 25 h on the resulting mix, accumulating 65 % of PHAs, formed by 3HO, 3-hydroxynonanoate, 3-hydroxyheptanoate and 3HD units [140]. An additional approach to discover novel copolymers consists in leveraging MMCs fermentation strategies [141], as brilliantly succeeded by Vermeer and colleagues. In their work, an aerobic activated sludge of a wastewater treatment plant was enriched with isobutyric acid, and poly(3-hydroxyisobutyrate) (PHiB) was hence

measured for the first time [142]. As a matter of fact, if the down side of MMCs is represented by the significant variability between different batches, on the other hand, complex substrates such as organic fraction of municipal solid waste (OFMSW) are successfully converted into variable amount and types of PHAs [143]. Eventually, syngas represents another intriguing waste, indeed autotrophic growth of PHA-accumulating strains is deeply studied in recent years. However, syngas carbon sources CO and CO₂ conversion, so far, has been limited to P(3HB). Portela-Grandío and colleagues proposed a two-step fermentation, where liquid effluents originating from syngas fermentations of *Clostridium carboxidivorans* or *Clostridium acetivum*, abundant of VFA and alcohols, were transformed into P(3HB-co-23 %3 HV) by a MMC accumulating up to 41.5 % CDM [144].

3. Future perspectives

Despite a growing market and clear-cut attractiveness [145], PHAs copolymers manufacturing is still scant. Looking at the overall PHAs market, hence including both PHAs copolymers and P(3HB), in 2023 around 0.1 Mt. have been produced [146]. Therefore, commercially available PHAs copolymers represent a tiny fraction of the total global plastic market (450 Mt./year). Indeed, the scale of magnitude PHAs copolymers producers deal with is in the order of kt/year more than Mt./year. Besides the already mentioned Kaneka, Danimer Scientific, CJ, PhaBuilder, and Tianan, other PHAs copolymers manufacturers currently, in 2024, are Bluepha P(3HB-co-3HH), Biotic P(3HB-co-3 HV), TerraVerdae Bioworks (former PolyFerm) *mcl*-PHAs, RWDC P(3HB-co-3HH), and Helian Polymers P(3HB-co-3 HV). However, none of the mentioned players' capacity reaches 100 kt/year. Such limited capacities do not concern PHAs copolymers producers only, but all PHAs manufacturers, including P(3HB) producers as well. As a matter of fact, it is an issue of scalability and, as already anticipated, manufacturing costs. Biotechnological production by fermentation entails maintaining a stable temperature, a sterile environment, adopting expensive substrates, and dealing with microorganisms' kinetics of growth, sometimes slow and characterized by phases when the production of PHAs is limited. For all these reasons, expenditure is still too high especially at such reduced scales to compete with petroleum-derived plastics, which instead rely on decades of process-optimization at much larger scales adopting an incredibly low-cost feedstock. Thereby, as an alternative solution, also plants derived PHAs have been conceived by the American Yield10 Bioscience, who relies on large arable lands to grow low-cost PHAs accumulating *Camelina sativa* plants [147].

On one hand, manifold research reports the production of novel copolymers at interesting lab-scale yields, characterized by appealing properties. Nevertheless, pilot-scale studies are yet limited and accounting mainly for MMCs-driven processes [148,149] calling thereby for broader endeavors on pure culture-based fermentations with larger volumes. Together with higher scale trials, life-cycle assessments (LCA) and techno-economic assessments (TEA) are demanded to evaluate the sustainability and the feasibility of the process. Moreover, fermentation costs and PHAs yields are the most critical factors concerning the economics of PHAs production [150]. Regarding the feedstock, if crops are cultivated exclusively to be converted into the biopolymer of interest, then land use would increase, turning into an environmental hazard [151]. Instead, the adoption of wastes may be a preferable strategy [151]. For instance, the EU-ANIMPOL project aimed to access the techno-economic feasibility to produce P(3HB-co-3 HV) from slaughtering residues: carried out in 2017, considering overall costs and revenues, the calculated investment payback time was spanning between 3.25 and 4.5 years [152]. Similarly, within the EU-WHEYPOL project, a LCA study based on results of whey transformation by *H. mediterranei* DSM 1411 into P(3HB-co-3 HV) pointed out the importance of electricity price used for fermentation and its impact in terms of ecological footprint [153].

On top of that, lignocellulosic residues' huge availability can't be

underestimated. Nevertheless, their recalcitrance implicates expensive pre-treatments which should be heavily optimized or utterly avoided if possible, in favor of cleaner and milder solutions such as hybrid catalytic systems or enzymatic pretreatments [154,155]. Additionally, autotrophic fermentation of gases such as syngas gave birth to the 3rd generation of bioplastics, including PHAs, representing a cutting-edge and circular strategy [103,156]. Importantly, both academia and industry are required to improve the existing collection of industrial copolymers-producer microbes in quantitative and qualitative terms [124,157]. As reported in this review, extreme environments and industrial waste streams are perfect niches to explore the enormous biodiversity available in nature. In combination, innovative genetic modification technologies targeted to unlock novel copolymers pathways and strengthen them are indeed requisite. In the end, the design process must always be considered: research should meet final product requirements and seek for demanded material properties, instead of adapting its results to potential applications. If the market calls for greener plastics maintaining high-standard physical properties, the starting point is identifying PHAs copolymers that can assure these performances.

4. Summary and conclusions

Close loop life cycle PHAs are technologically constrained by high production costs and limited material properties. However, the inclusion of diverse monomers renders PHAs polymer performances comparable with traditional plastics ones. In this review, the most updated works on PHA copolymers production have been collected and divided in groups according to the precursors of origin. In summary, three sets of PHAs copolymers precursors have been investigated: structurally related ones, structurally unrelated, and wastes. We conclude that precursors belonging to either one of the three categories are characterized by different economic values, nevertheless common approaches to optimize production yields and cut process costs have been identified. As such, to smooth out precursor's expenditures, three tracks are outlined. First, the quest for cheap substrates such as waste. Secondly, the selection of prime strains, whose metabolisms entail the heavy accumulation of copolymers. Finally, tailored technologies like efficient substrate pre-treatment. While fossil-based plastics pay the abundance of properties offered by polymers blends with the difficulties to operate extensive recycling, PHAs copolymers maintain great versatility together with clean end-life destinies.

CRedit authorship contribution statement

Paolo Costa: Writing – original draft, Investigation, Conceptualization. **Marina Basaglia:** Writing – review & editing. **Sergio Casella:** Writing – review & editing. **Lorenzo Favaro:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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