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Polyhydroxyalkanoates (PHAs) – Production, Properties, and Biodegradation

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6.1 Introduction

Plastics are omnipresent in our daily lives; they are inexpensive, easy to process, and offer a multitude of functionalities. In the past 100 years, the industrial synthesis of fossil carbon-based chemosynthetic plastics and generally non-biodegradable plastics increased exponentially. Currently, 400 Megatons (Mt) of plastics is estimated to be produced and consumed annually. As a matter of fact, humankind uses and disposes fossil plastics with little afterthought on the implications; reduction, reuse, recycling, and litter elimination of spent plastics are urgently needed. The lack of biodegradability of the established plastics, which excludes their industrial composting, their lack of proper disposal methods, and therefore their leakage into diverse habitats, has resulted in rapid plastic accumulation in the environment. This has caused well-known detrimental effects such as the pollution of our oceans and ubiquitous generation of secondary microplastic particles in the environment and contaminating our food chain.

A seminal paper published in 2017 by Geyer et al. draws a comprehensive picture of the life cycle and destiny of all chemosynthetic plastics ever made. They calculated that until 2015, a total amount of about 10 Giga tons (Gt) of fossil plastics was synthesized globally; it is estimated that approximately 79% thereof has been deposited in landfills, or even simply released in the environment far more than the approximately 9% of plastics reported to have been recycled, or the roughly 12% of plastics which have been incinerated. The same study estimates that by 2050, 12 Gt of disposed plastic waste will be deposited in landfills or accumulated in the environment, which is double the quantity we have accumulated until 2015 [1]. In 2016, the Ellen MacArthur Foundation published a comprehensive report "The New Plastics Economy: Rethinking the future of plastics" highlighting the urgent need for applying the principles of circular economy to the production of plastics, especially those

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used for packaging. This report was based on contributions by experts from diverse industrial branches and various international organizations and presents a vision of an economy in which spent plastics should never become waste streams. The report concluded that in 2015, roughly 100 million tons of plastics was manufactured only for packaging purposes, making up the largest single source of plastic contamination. Moreover, they estimated that plastic manufacturing currently consumes about 6% of the total fossil mineral oil produced and causes approximately 1% of the total carbon emission that includes their incineration after their life span. At a first glance, these values do not seem to be significant. However, without serious efforts to change these trends, the study estimated that by 2050, plastic production would consume about 20% of the global fossil oil production and contribute to 15% of the atmospheric carbon emissions if the energy sector achieves its stated goal of reducing greenhouse gases by 80% by 2050 [2]. Although this report also mentions biodegradable/compostable materials as urgently needed, a comprehensive and authoritative study on how to incorporate such materials in our society is still lacking.

After the discovery of fossil fuels, such as coal, petroleum, and natural gas, which enabled the industrial revolution, carbon that was sequestered for hundreds of millions of years was suddenly released into the atmosphere. This resulted in the increase in atmospheric concentration of greenhouse gases (GHGs), especially CO_2, CH_4, and NO_x, which are the drivers of the climate change we are facing now. The increased temperatures due to increased GHGs have reduced CO_2 solubility in oceans, thus fueling further CO_2 evolution and further increasing the temperature. Plastics, typical products of crude oil and natural gas, have contributed to this development. At the very least, by accumulating plastics that do not decompose into CO_2, CH_4, or water in a reasonably short time frame, these fossil-based materials have other, even more destructive effects on our planet, including its oceans and all marine life forms, and eventually our primary sources of food [3]. Hence, according to the solutions by "Prevention is better than Cure" [4], plastic accumulation and contamination are the order of the day; however, this requires fundamentally new technological solutions applicable on a large scale and groundbreaking changes in the way the industry currently operates [5]. Although several approaches have been developed over decades and are currently being used, e.g. plastic collection followed by recycling or incineration, these procedures do not address the entire range of problems caused by plastic pollution as described above. This fact can be paraphrased by the famous quotation "less bad is not good enough" once coined by Schnitzer and Ulgiati when it comes to making industrial processes sustainable from a holistic point of view; therefore, we need to expend efforts to improve individual process steps as well as our fundamental approach to the types of industrial processes, feedstocks, and materials we should be using [6]. Although the current approaches for the treatment of spent plastics can be further enhanced and extended to capture significantly larger amounts of used plastics, there will always remain some sources of plastic pollution, which cannot be covered because of their de facto universal occurrence [1].

Recycling of plastic is essential, and the corresponding technologies need to be enhanced (cf. Chapter 6.5 in this book). As a downside, recycling, which includes chemical recycling in the future, will also come with significant expenses, including

massive energy requirements (in turn causing further GHG emissions), problems associated with separation of different types of plastics, and quality of degradation products with every recycling step. Therefore, recycling alone is an insufficient tool to address the entire plastic waste predicament. Moreover, plastic recycling just postpones the problem of plastic pollution but does not prevent or cure it. Most of all, recycling can in no way lead to a solution to the microplastic problem; secondary microplastic particles with a size range from 0.1 μm (the size of a SARS-CoV-2 virus particle) to 5 mm (approximately the length of an ant) are created by disintegration of larger plastic materials visible to the naked eye; they are detached from textiles as microplastic fibers (as reported by Napper and Thompson, washing 6 kg cloths made of synthetic fibers in a washing machine releases, depending on the nature of the synthetic material [polyester/cotton blend, polyester, or acrylic], roughly 138 000, 496 000, and 728 000, 700 000 plastic fibers, respectively, per wash into the sewage! [7]), arise as abrasion from plastic shoe soles, and, as a major source of pollution, as abrasion from car tires. In addition, primary microplastics, which are by default added to diverse industrial products such as cosmetics, dental care products, abrasives, detergents, paints, polymeric citrus fruit coatings, adhesives, and others, are also introduced into the environment. Despite the current commitments of the industry to at least reduce the use of primary microplastics, it cannot be expected that they will completely be abolished in the foreseeable future. Moreover, the ratio of primary to secondary microplastics is negligible, the latter being the dominant source over time [1]. Animal studies have already confirmed the detrimental effects of microplastics on their intestinal systems [8]; microplastics can wander through various stages of metamorphosis in insects, which are then consumed by birds [9], and the residual microplastics then disrupt fine-tuned zoological systems [10]. Moreover, microplastic particles traverse the food chain starting from zooplankton or mollusks via crustacea and fish to ultimately enter the digestive systems of marine mammals or reach our dining tables. Recent studies report the occurrence of microplastics in most diverse types of food items such as drinking water [11], table salt [12], honey [13], beer [14], or seafood [15].

As a consequence of these issues, 127 nations have already prohibited plastics for single-use applications such as shopping bags made of fossil plastics [16]. Yet, outlawing plastic use or trusting the existing technologies solely to treat spent fossil plastics are no real solutions for eliminating the adverse ecological effects of plastic waste and GHG emissions, now and tomorrow. The dimension of the problem requires further, more radical, and innovative technologies and specifically solutions that are rooted in what nature teaches us through its natural cycles of up-cycling and biodegrading of all natural materials and best of all its well-established cycling of carbon [3]. This is exactly when such biological materials from renewable resources, which have properties in use matching those of fossil plastics, while, at the same time, remain embedded in circular and natural cycles, are brought into play.

6.1.1 General Aspects of Biodegradation of Polymers

Since the emergence of the very first life forms, biological materials have demonstrated their circular nature; this has kept GHG generation and consumption in a

closed cycle. Following these successful principles of nature is the best approach when it comes to production of polymeric materials that act like plastics and prevent plastic pollution. This timeless strategy ensures from the very early stages of process development that accumulation of such xenobiotics, which have a fatal impact on the biosphere, can be obviated. This includes producing compounds from renewable sources such as GHGs like CO_2 and/or CH_4, such as biogas plus H_2O, and, after their life span, returning them to nature. Nature, in turn, transforms these compounds back into CO_2, CH_4, H_2O, and biomass during the biodegradation process, and the cycle gets closed. Biodegradability, and therefore industrial and home composting, needs and must be considered to be an important end-of-life option for single-use consumer plastics. Natural polymeric materials, including cellulose and polyhydroxyalkanoates (PHAs), typically biodegrade in the ecosphere under various conditions; they turn into CO_2, water, and biomass in the presence of O_2 and into CO_2, CH_4, water, and biomass during anaerobic digestion. Importantly, the process of biodegradation of natural materials is circular: photochemotroph-like plants use CO_2, water, and nutrients to grow, and, at their end of life, they biodegrade into the very products they consumed to thrive [3].

Technologically, biodegradation is already performed industrially by increasing the temperature and/or by adding specific microorganisms that consume the organic matter and turn them into CO_2, CH_4, water, and biomass or compost; these processes are called "Industrial Composting." In industrial composting facilities, compost temperatures exceed 50 °C because of the metabolic heat generated by the microbes thriving in it. This high temperature is a legislative requirement in many countries as it ensures sufficient pasteurization of the final product (compost). The same process of biodegrading natural materials can be carried out at lower temperatures; this process is known as "Home Composting." Here, temperatures as high as in industrial composters are typically not reached or reached only in the core, as the compost is agitated/turned less often and has a higher surface-to-volume ratio beneficial for cooling under ambient temperature. Because of a lower aeration in home composting, microbial growth is less intense, and less heat is generated. Timeline is also important in biodegradation and/or composting performance; higher temperature in Industrial Composting speeds up biodegradation, whereas the process of Home Composting takes longer; however, in both processes, biodegradation occurs [17].

6.1.2 General Aspects of Microbial Synthesis of PHAs

Indeed, there are myriads of materials being produced using CO_2 or CH_4 as renewable carbon sources, but only few of them have plastic-like properties and are also converted into CO_2, CH_4, H_2O, and biomass at their end of life by natural processes. PHAs, a group of microbial polyesters found in nature, are one such material family; remarkably, they also have many of the desired properties of the currently market-dominating types of fossil plastics, such as poly(ethylene terephthalate) (PET), polyethylene (PE), polypropylene (PP), polystyrene (PS), poly(vinyl chloride), or polyamides (e.g. nylon), and can be converted/processed

using the existing equipment with only minimal modifications in processing conditions. This makes PHAs one of the most auspicious groups of compounds to substitute many of the commercial fossil plastics today, particularly those produced for single-use applications and packaging, which are difficult to collect or recycle [18]. However, the market presence of PHAs is still negligible; although the total global plastic production is estimated with almost 400 Mt/yr, PHA production does not even encompass 0.01% thereof [19]. The large variety of chemical, physical, and mechanical properties of PHAs arises from the numerous types of monomers (150 of them have been identified to date [20]) available to build them. Then, there is the question of polymer design using these 150 different monomers along with our vast knowledge of biology and polymer chemistry – random or block copolymers having various molecular weights and distributions – hence, literally an infinite number of different PHA molecules can be designed to mimic fossil plastics in their functionality and yet be produced from renewable carbon and be biodegradable as an end-of-life option. The discovery of over 150 different monomers that allows for innumerable types of PHA polymers resulted in the introduction of the terminus "PHAome" in the recent scientific literature by a leading research group in this field, analogous to the genome, proteome, transcriptome, lipidome, metallome, or metabolome of organisms. The term "PHAome" reflects the diversity of PHAs already found in nature and to those that still need to be discovered in biological samples [21]. Critically, different types of PHAs may also exhibit different biodegradability behaviors, adding to our ability to tune end-of-life options for PHA polymers [22].

Microbiologically, PHAs are a group of polyoxoesters that are naturally produced and degraded. They accumulate as inert intracellular inclusion bodies in the cytoplasm of around 40% of the world's microbial species, predominantly from prokaryotic genera belonging to the two domains Bacteria and Archaea [23]. Some extraordinary reports on eukaryotic wild-type yeasts as PHA producers occurred in the scientific literature only during the past few years. Their chemical structure resembles a linear polyoxoester of helical structure of the individual PHA chains, which are intracellularly organized in granules, also referred to as "carbonosomes," in which the polyester chains form a hydrophobic core part, surrounded by a hydrophilic layer mainly consisting of enzymes responsible for the cyclic processes for PHA biosynthesis and degradation plus structural proteins (phasins) responsible for the formation, size, shape, and location stabilization of PHA granules [24].

Based on the consumption of heterotrophic (mainly carbohydrates, alcohols, lipids, organic acids, and methane) or autotrophic (fixation of CO_2 and utilization of CO by conversion to CO_2) renewable substrates, microorganisms typically start to accumulate increased quantities of PHA as products of their secondary metabolism when they are exposed to certain nutritional insufficiencies or stressors, which impede the increase of cell density in the culture. Such strict separation of cell growth and PHA accumulation ("non-growth-associated PHA biosynthesis") is reported, e.g. for *Paraburkholderia fungorum* DSM 1749 or *Azotobacter vinelandii* when cultivated on carbohydrates; here, the switch from balanced growth to predominant PHA formation can conveniently be accomplished by stopping the supply

of, e.g. the nitrogen source. In many cases, e.g. for the well-described PHA production strains *Cupriavidus necator* DSM 545 (an eubacterium) or *Haloferax mediterranei* DSM 1411 (a haloarchaeon), only low amounts of PHAs are formed during the phase of exponential growth, with PHA productivity suddenly increasing after the onset of the (nutritional) stress ("partially growth-associated PHA biosynthesis"). Some exceptions, however, are reported to synthesize significant fractions of PHAs in cell mass even under environmentally balanced conditions ("growth-associated PHA biosynthesis"), such as *Azohydromonas lata*, a species already used decades ago for (semi)industrial PHA production by Chemie Linz (Austria) or Imperial Chemical Industries (ICI, UK). When these microorganisms experience shortage of exogenous substrates/food, they remobilize and consume the accumulated PHAs as a source of energy and carbon, which gives them an advantage over polymer-free microbes when it comes to survival during periods of starvation (reviewed by [25]). In addition to their role as reserve materials, some intriguing novel functions of PHA were more recently uncovered, predominately substantiating that PHA biosynthesis and formation of their degradation products are the consequences of the "SOS-response" of microorganisms to diverse stressors [26–28] such as osmotic challenge caused by exposure to hypotonic or hypotonic media [29, 30], oxidative stress [31, 32], freezing [33, 34], thermal load [31], or UV irradiation [35].

6.1.3 Types and Properties of PHAs

The type and structure of PHAs that the microorganisms produce depend on their raw material source and the production organism. By 1995, 95 different types of PHA polymers and their corresponding unique building blocks (hydroxyalkanoic and some hydroxyalkenoic acids) were discovered, and to date, about 150 different types of PHA polymers and their corresponding building blocks have been described. Notably, many of these 150, often xenobiotic compounds were only polymerized in test tubes to PHAs by isolated PHA synthase enzymes and were never found in PHAs produced by living cells [36]. Nonetheless, all of them are produced using renewable carbon, and many of them are predicted to have the desired functionality for commercial use and most of them biodegrade at the end of life, the three essential criteria for circularity in use. Figure 6.1 shows the general chemical structure of PHAs and PHA granules ("carbonosomes") included in microbial cells.

To date, only around 15 different naturally occurring building blocks and their corresponding PHA polymers have been thoroughly studied. A lot of work has gone into elucidating their properties and applicability in industrial and consumer applications such as films, fibers, thermoformed and molded parts for use in packaging, food service, agriculture, medical devices, electronics, leisure industry, fabrics, paints and coatings, adhesives, etc. [37].

Like many types of natural polymers, PHAs can either consist of only one type of monomer, or two or more different building blocks. In the first case, the so-called homopolymers, poly(3-hydroxybutyrate) (PHB or P3HB) is the only important naturally occurring example discovered to date and the by far most frequently occurring and best-studied PHA from biological samples. However, its application is restricted

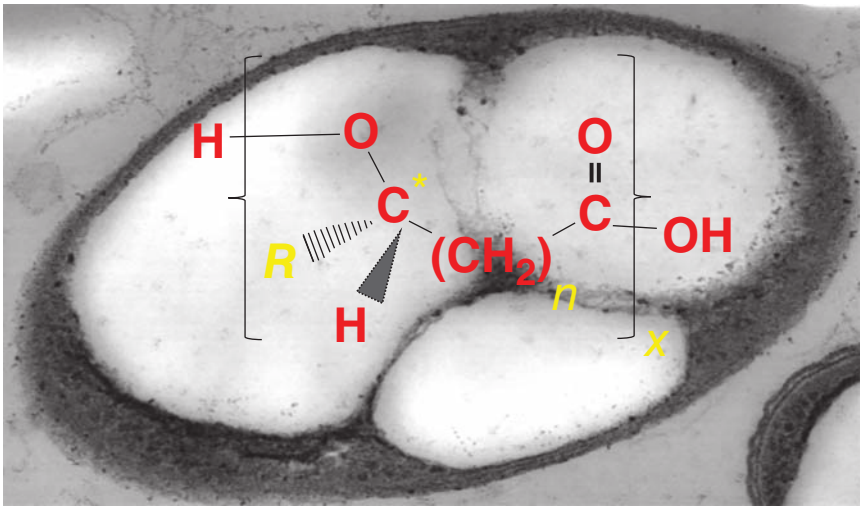

Figure 6.1 General formula for natural PHA biopolyesters; the asterisk (*) indicates the chiral center of most PHA constituents (important exception: achiral 4-hydroxybutyrate – 4HB). *n*: number of methylene groups in monomer's backbone; *x*: degree of polymerization (number of monomers building up the PHA polymer molecule); *R*: side chain of the monomer. Example: $R = CH_3$, $n = 1$ correspond to 3-hydroxybutyrate – 3HB. Gray background photograph: scanning transmission electron microscope (STEM) picture of a PHA-rich *Cupriavidus necator* cell cultivated on glucose (picture provided with courtesy by E. Inglolić, FELMI/ZFE, Graz, Austria); the white inclusion bodies, amounting to about 80% of the cell dry mass, are PHA granules. Source: Courtesy of E. Inglolić.

because of its high crystallinity, melting temperature close to its degradation temperature, and brittleness.

Better material properties and enhanced processability are achieved when interrupting this crystalline PHB matrix by inserting comonomers in the polymer backbone. The generated heteropolyesters are termed copolymers when two different building blocks are present, and terpolymers when three different monomers are present and infrequently quaterpolymers when four different monomers build up the PHAs. PHAs containing more than one comonomer generally have lower crystallinity, lower melting temperature, and enhanced processability in comparison to PHB (reviewed by [20]).

Depending on the chain length of monomers, PHAs can be divided into three major groups; PHAs consisting of monomers with three to five carbon atoms (3-hydroxypropionate [3HP; achiral], 3-hydroxybutyrate [3HB; chiral], 4-hydroxybutyrate [4HB; achiral], 3-hydroxyvalerate [3HV; chiral], 4-hydroxyvalerate [4HV; chiral], 5-hydroxyvalerate [5HV; achiral]) are termed short chain length PHAs (*scl*-PHA). Such *scl*-PHAs, with the exception of the highly flexible poly(4-hydroxybutyrate) (P4HB) and copolyesters of 3HB and 4HB with high 4HB fractions [38], are rather crystalline, have high melting points and glass transition temperature, and resemble typical thermoplastic materials

from fossil carbon, such as polypropylene (PP). The most important *scl*-PHA production microorganisms are *C. necator*, *Burkholderia sacchari*, *Alcaligenes latus*, (today: *Azahydromonas lata* and *Azahydromonas australica*) or *Haloferax mediterranei*. The best-studied representatives of *scl*-PHA are the homopolyesters PHB and P4HB, the copolyesters poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBHV) and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P3HB4HB), and the terpolyester poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) (P3HB3HV4HB) (reviewed by [20]).

Medium chain length PHAs (*mcl*-PHA), in contrast, are typically amorphous, rubber-like resins with low glass transition temperature and a low melting point or melting temperature range. They harbor monomers with 6–14 carbon atoms (3-hydroxyhexanoate, 3HHx, to 3-hydroxytetradecanoate, 3HTD) and are most frequently produced by members of the *Pseudomonas* genus. In addition, they typically have lower molecular mass than *scl*-PHA. Such *mcl*-PHAs are only experimentally produced in small quantities but are likely to enter the market as smart polymers such as "bio-latexes," thermo- or pressure-sensitive adhesives, or carriers for bioactive compounds [39, 40]. Using *mcl*-PHAs containing monomers with unsaturated side chains, readily biodegradable rubber-like polymers of superior product properties including those that are cross-linked can be produced by post-synthetic modification such as cross-linking [41].

The third group, long chain length PHAs (*lcl*-PHA) are scarcely described; these materials have monomers with more than 14 carbon atoms (3-hydroxypentadecanoate, 3-hydroxyhexadecanoate, 3-hydroxyheptadecanoate, 3-hydroxyoctadecanoate, unsaturated 3-hydroxyoctadecenoate, and others), with properties similar to those of *mcl*-PHAs with long side chain in monomers (reviewed by [20]).

6.2 Biosynthesis – Substrates and Strains

6.2.1 Principle Stoichiometry of PHA Biosynthesis

The following equations provide an overview about the principle stoichiometry of PHB biosynthesis under different conditions and theoretical substrate-to-PHB conversion yields:

(a) Chemoheterotrophic, aerobic conversion of glucose [42, 43]:

$$C_6H_{12}O_6\ (\text{glucose}) + (3p+1)\text{ADP} + (3p+1)P_i + (3/2)O_2$$
$$\rightarrow (1/n)(C_4H_6O_2)_n\ (\text{PHB}) + 2CO_2 + 3H_2O + (3p+1)\text{ATP}$$

p: P/O ratio; Pi: inorganic phosphate

Theoretical yield for aerobic PHB biosynthesis from glucose when converted via the Entner–Doudoroff (2-keto-3-desoxy-6-phosphogluconate [KDPG]) pathway: Based on the equation $Y = \frac{43\,x}{2(12x+y+16z)}$ (for glucose: $C_6H_{12}O_6$; $x = 6, y = 12, z = 6$) [41, 42]:

$$Y_{\text{PHB/glu (theor.)}} = 86/180\ \text{g/g} = 0.48\ \text{g/g}$$

Aerobic PHB formation from glucose is energetically favored; for the reaction of glucose to 3-hydroxybutyryl-CoA, the direct precursor of PHB, Pan et al. [44] report an energy gain of 193.72 kJ/mol, which is however lower than for the complete respiration of glucose to CO_2:

$$C_6H_{12}O_6 \text{ (glucose)} + \text{free CoA} + 3NAD^+ \rightarrow 3\text{HB-CoA} + 2CO_2 + 3NADH + 3H^+; \Delta G^0 = -193.72 \text{ kJ/mol}$$

$$C_6H_{12}O_6 \text{ (glucose)} + 6O_2 \rightarrow 6CO_2 + 6H_2O; \Delta G^0 = -2821.98 \text{ kJ/mol}$$

ΔG^0: Gibbs free energy change at standard conditions

(b) Chemoheterotrophic, aerobic conversion of fatty acids via β-oxidation (example: linoleic acid) [45]:

$$C_{18}H_{32}O_2 + 4.75O_2 \rightarrow 4.5\text{PHB} + 2.5H_2O$$

$$Y_{\text{PHB/acid (theor.)}} = 1.5 \text{ g/g}$$

(c) Chemoheterotrophic, aerobic conversion of glycerol [45]:

$$2C_3H_8O_3 + 2.5O_2 \rightarrow \text{PHB} + 2CO_2 + 5H_2O$$

$$Y_{\text{PHB/acid (theor.)}} = 0.46 \text{ g/g}$$

Energy generated by the formation of 1 mol 3HB-CoA from 2 mol glycerol: $\Delta G^0 = -996.15$ kJ

(d) Chemoheterotrophic, aerobic conversion of methane by type-II methanotrophs via the serine pathway [43]:

$CH_4 + O_2 + NADH + H^+ \rightarrow CH_3OH + NAD^+$

$CH_3OH + {}^1/_2O_2 + ADP + P_i \rightarrow HCOH + ATP$

(i) $HCOH + NAD^+ \rightarrow HCOOH + NADH$; $HCOOH + NAD^+ \rightarrow CO_2 + NADH$

(ii) $HCOH$ + glycine → serine →→→ malyl-CoA → acetyl-CoA

(iii) 2acetyl-CoA + $NADPH$ →→→ $\text{PHB} + NADP^+$

$$\text{Sum}: 5CH_4 + 4O_2 + (5q-3)\text{ADP} + (5q-3)P_i \rightarrow \text{PHB} + CO_2 + 4H_2O + (5q-3)\text{ATP}$$

q: mol adenosine triphosphate (ATP) to be produced from the conversion of methanol to methanal; some ATP is utilized in the serine cycle to generate malolyl-CoA from malate

$$Y_{\text{PHB/CH4 (theor.)}} = 0.54 \text{ g/g}$$

Energy generated by the formation of 1 mol 3HB-CoA from 5 mol CH_4: $\Delta G^0 = -1148$ kJ

(e) Photoautotrophic conversion of CO_2 by cyanobacteria via the Calvin cycle [46]:

(i) Calvin cycle: $3CO_2 + 6NADPH + 9ATP + 5H_2O \rightarrow GAP + 6NADP^+ + 9ADP + 8P_i$

(ii) $GAP + NAD^+ + ADP + P_i \rightarrow PEP + NADH + ATP$

(iii) $PEP + ADP \rightarrow$ pyruvate $+ ATP \rightarrow$ acetyl-CoA $+ CO_2$

(iv) $2\text{Acetyl-CoA} + \text{NADPH} \rightarrow\rightarrow\rightarrow \text{PHB} + \text{NADP}^+$

$$\text{Sum}: 4CO_2 + 14\text{ATP} + 10H_2O + 13\text{NADPH} + 2\text{NAD}^+ \rightarrow \text{PHB} + 14\text{ADP} + 14P_i + 13\text{NADP}^+ + 2\text{NADH}$$

GAP: glycerolaldehyde-3-phosphate; PEP: phosphoenolpyruvate

$$Y_{\text{PHB/CO2 (theor.)}} = 1.95\,\text{g/g}$$

(*Nota bene*: many side reactions take place for the formation of pigments, amino acids, lipids, glycogen, acetaldehyde, etc.!)
Energy generated by the formation of 1 mol 3HB-CoA from 4 mol CO_2: $\Delta G^0 = -4832.58\,\text{kJ}$

6.2.2 Biosynthesis of *scl*- and *mcl*-PHAs

The mechanism of microbial PHA biosynthesis has been extensively studied. In principle, carbon sources are converted to oxoacyl-CoA thioesters, often to acetoacetyl-CoA generated by the condensation of the central metabolite acetyl-CoA; this reaction is catalyzed by the enzyme 3-ketothiolase (PhaA; a.k.a. acetyl-CoA – acetyltransferase, formerly known as β-ketothiolase; EC 2.3.1.9). After being catalyzed by the NADPH-dependent oxoacyl-CoA reductase (PhaB), the formed oxoacyl-CoA thioesters are reduced to the corresponding (*R*)-hydroxyacyl-CoA thioesters. Finally, polyester synthases (PhaC; former name: PHA polymerases) catalyze the linking of (*R*)-hydroxyacyl-CoA thioesters to form growing polyester chains; this polycondensation releases one molecule of free CoA per docked monomer (reviewed by [20]).

In microorganisms, four different classes (I–IV) of PHA synthases have been identified based on their subunits, amino acid sequences, and substrate specificity [47]. In general, high intracellular concentrations of ATP, NAD(P)H, and acetyl-CoA, indicators for a high energy charge of the cell, and low concentrations of free CoA favor the activity of enzymes involved in PHA biosynthesis; such conditions are found, e.g. when the tricarboxylic acid cycle (TCC) or other central metabolic pathways are interrupted due to the deprivation of a growth-essential nutrient, e.g. at the limitation of nitrogen or phosphate sources, in parallel to sufficient availability of exogenous carbon source. Mechanistically, the PHA biosynthesis provides the cells with a tool to regenerate NADP^+ by the above-described redox reaction between NADPH and acetoacetyl-CoA; this "pseudofermentation" generates (*R*)-hydroxybutyryl-CoA and NADP^+, the latter is used by the microbial cell for substrate oxidation, thus enabling nutrient consumption for energy generation even during conditions blocking the activity of TCC [20]. Figure 6.2 illustrates the cycle of PHA biosynthesis and biodegradation.

Class I PHA synthases, e.g. the enzyme found in the best know PHA producer *C. necator*, polymerize (*R*)-hydroxyacyl-CoA thioesters with the acyl group consisting of three to five carbon atoms. Moreover, class III PHA synthases, such as the one of

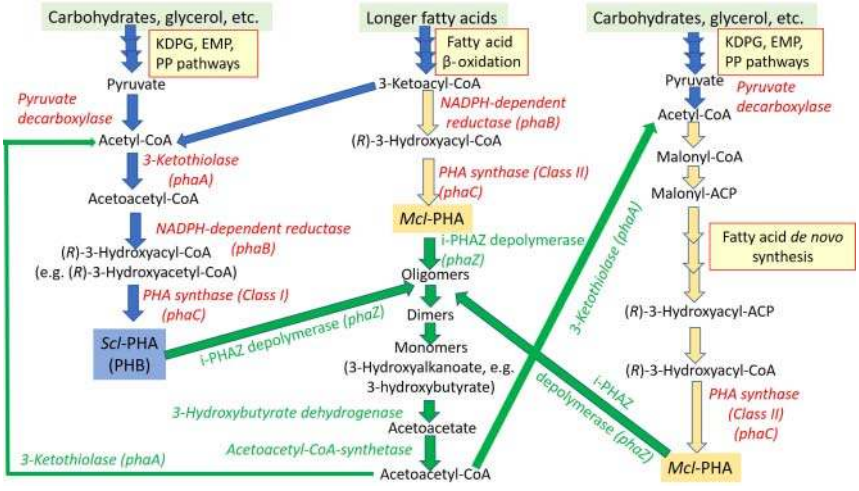

Figure 6.2 Cycle of intracellular PHA biosynthesis and degradation. Blue arrows lead to biosynthesis of short-chain-length PHAs (*scl*-PHA; e.g. PHB), beige arrows to biosynthesis of medium-chain-length PHA (*mcl*-PHA), and green arrows to remobilization of PHA reserves. Red font: PHA synthesis enzymes; green font: enzymes catalyzing PHA catabolism. EMP, Embden–Meyerhof–Parnas pathway.

Allochromatium vinosum, and class IV PHA synthases, e.g. in *Bacillus megaterium*, also generate *scl*-PHAs [20].

Class II PHA synthases preferentially polymerize longer (*R*)-hydroxyacyl-CoA thioesters with the acyl group consisting of 6–14 carbon atoms as substrates, thus forming *mcl*-PHAs [48]. Such compounds are often generated by β-oxidation of the respective fatty acids. This β-oxidation first generates (*S*)-3-hydroxyacyl-CoA, which cannot be directly incorporated into *mcl*-PHAs due to stereospecificity of the synthase's subunit. Therefore, specific isomerases that convert the (*S*)- into the (*R*)-isomers are active in such organisms. In addition, *mcl*-PHAs can also be produced from unrelated carbon sources such as sugars or glycerol via the fatty acid *de novo* synthesis. Different from β-oxidation, fatty acid *de novo* synthesis generates the (*R*)-isomers of acyl-CoAs, which are directly used as substrates for polymerization by class II synthases [39, 40]. Natural *mcl*-PHAs produced by wild-type organisms exclusively present heteropolyesters consisting of different building blocks. Homopolyesters, e.g. P(3HHx), poly(3-hydroxyoctanoate) (P3HO), or poly(3-hydroxydecanoate) (P3HD), can only be obtained by genetically engineered organism; in this case, the β-oxidation pathway needs to be weakened to avoid generation of diverse substrates for the PHA polymerase [47]. Similar to *mcl*-PHAs, the polymerization of *lcl*-PHA is also catalyzed by class II synthases (reviewed by [47]).

6.2.3 Heterotrophic Feedstocks

Typical substrates for PHA biosynthesis by heterotrophic microorganisms (dependent on organic substrates as carbon sources) are either hydrophilic compounds

such as carbohydrates (mono-, di-, oligo-, and polysaccharides), alcohols (glycerol, methanol, etc.), or water-soluble organic acids. In such cases, homogeneous substrate distribution in the bioreactor system can easily be accomplished by established agitation systems (stirrer). Also, various hydrophobic compounds are used as starting materials for PHA production. For example, triacylglycerides or methylesters of fatty acids have been described. In the case of substrates that have poor water solubility, a sufficient distribution in the aqueous cultivation phase needs to be warranted in order to allow cell convenience and ready access to the nutrient source; this can be accomplished by the addition of adequate non-toxic emulsifiers and appropriate agitation of the cultivation broth to generate substrate (such as lipid) droplets with a maximum surface-to-volume ratio (reviewed by [25]). In some cases, the PHA production strain generates its own emulsifier, as shown for the *mcl*-PHA producer *Pseudomonas putida* [49].

The discovery of such novel heterotrophic substrates for bioplastic production has created a paradigm shift during the past two decades in the production of PHAs. Before, PHA production was predominately studied and carried out using "first-generation" substrates; hence, bioplastic manufacturing competes with food sources, such as glucose, sucrose, or edible oils. The use of "second-generation" feedstocks such as carbon-rich waste and surplus materials from diverse agricultural and industrial sources, has allowed biopolymer industry to overcome the "Food vs. Bioplastic" debate. The prime examples of such second-generation carbon sources for PHA production include hydrophilic, often complex, substrate cocktails such as ultra-filtrated surplus whey [50], molasses [51], crude glycerol from the biodiesel industry [52], hydrolyzed lignocellulose materials of diverse origins [53–55], sugar-rich waste from fruits and wine production [56, 57], yeast industrial wastewater [58], or hydrolyzed abundant chitin waste from crustacea aquaculture [59]. The lipophilic surplus feedstocks for PHA production are waste cooking oil [60], waste water from olive oil production [61], non-edible plant oils [62], or low-quality biodiesel fractions produced from lipophilic slaughtering waste [63]. Currently, numerous efforts are carried out globally to enhance PHA bioproduction from these diverse waste streams via genetically engineering of the production organisms [64].

An actual trend on the conversion of liquid waste streams with high biological oxygen demand (BOD) is its acidogenic conversion to a mixture of diverse organic acids, which is followed by conversion of these organic acids to PHA by mixed microbial cultures enriched in powerful PHA-producing microorganisms. Such enrichment is typically achieved by cyclic feast-and-famine feeding of mixed cultures; during the famine phases, PHA-rich cells have a survival advantage by their biopolymer storage and can refill this storage in the next feast phase; in contrast, the share of organisms not able to accumulate PHA will progressively decrease with time. Here, process conditions are highly decisive to achieve high productivity; the composition of the organic acid mix achieved during acidogenesis significantly impacts the composition of the PHA accumulated by the microbial community in the next step [65–67]. Among such liquid waste streams, the use of municipal solid waste [68], dairy waste [69], food waste [70], or crude glycerol [71] is reported in the literature.

6.2.4 Autotrophic Feedstocks

Beside heterotrophic PHA biosynthesis, several microbes are able to convert CO_2 as a carbon source in photoautotrophic (light as the energy source and inorganic compounds as the carbon source) or mixotrophic (supply of inorganic carbon source – CO_2 – plus organic compounds) cultivations. Especially, many cyanobacteria are highly versatile cell factories for solar-driven generation of biomass, biopolyesters, and other valuable bioproducts such as pigments, especially phycobiliproteins, in photoautotrophic setups. Such microorganisms fit well into the currently topical biorefinery concepts, which do not only utilize the valuable intracellular bioproducts but also upgrade the residual biomass for use as fertilizers or substrates for anaerobic biogas generation [72]. A range of diverse cyanobacteria from different genera have already been tested in lab scale for their potential as PHA producers in order to evaluate their productivity and dependence on light regime, cultivation vessels, macro- and micronutrient supply, feeding regime (autotrophic and mixotrophic), and type of CO_2 source. Among all these strains, representatives of the genera *Synechocystis*, *Synechococcus*, *Arthrospira* (*Spirulina*), *Oscillatoria*, *Calothrix*, and *Nostoc* currently appear most promising for large-scale processes, while among them, *Synechocystis* sp. was heavily subjected toward genetic engineering studies to improve PHA productivity and to close diverse metabolic bottlenecks [73]. High concentration of CO_2 can be found, e.g. in combustion flue gas from energy generation plants or "off gases" from different industrial-scale chemical processes such as cement production or fermentation industries (e.g. breweries). Mostly, such CO_2 sources need to be pretreated by different processes to remove chemical contaminants such as SO_x and NO_x before they can be supplied to the cyanobacterial culture [74]. However, this approach of using waste gas as the substrate combines mitigation of CO_2 from gaseous industrial effluents with bioproduction of marketable products such as PHA, as it was already successfully shown by Austrian researchers on a pilot plant-scale process operated for three years [75]. Unfortunately, such setups did not exceed yet pilot-scale processes; additional investigations need to be carried out to achieve adequate volumetric productivity by developing advanced photobioreactor setups, selection of cyanobacteria with excellent PHA accumulation capacity, and application of tools of genetic engineering to close metabolic bottlenecks [73]. If successfully optimized, such processes for production of PHAs, pigments, etc., based exclusively on the conversion of waste streams (flue gas for CO_2 supply plus agro-industrial effluents and wastewater and anaerobic digestates as nutrient sources), would constitute prime examples of "The Circular Bioeconomy" [76]. Research has successfully demonstrated that after extracting the PHA from *Spirulina* sp. LEB 18, the residual cyanobacterial biomass can be successfully recycled as the nutrient source to subsequent cultivations of the same organism [77]. To date, such cyanobacteria have been demonstrated to exclusively produce PHB homopolyesters from CO_2 as a sole carbon source, while copolyesters containing 3HV as comonomers were only obtained when cofeeding heterotrophic, structurally related cosubstrates such as valerate. This copolyester biosynthesis was demonstrated for the microorganism

Nostoc muscorum Argadh [78]. Recently, Tarawat et al. have shown that *Nostoc* sp. TISTR 9131 and TISTR 8164 and some *Anabaena* sp. were able to synthetize PHBHV from mixotrophic CO_2 and acetate supply, while *Anabaena spiroides* TISTR 8075 generated PHBHV even from photoautotrophic cultivation on CO_2 as a sole carbon source [79].

6.2.5 Syngas

Syngas, containing the carbon compounds CO and CO_2, can be used as a substrate for PHA bioproduction by *Rhodospirillum rubrum*, a Gram-negative, facultatively phototrophic purple non-sulfur bacterium. This organism is able to fix CO_2 via the well-known enzyme Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo; EC 4.1.1.39) in the Calvin cycle and oxidize CO via a carbon monoxide dehydrogenase (EC 1.2.7.4) in the so-called "water-gas shift reaction" to CO_2 and H_2 (reviewed by [80]). Both artificial syngas (mixtures of CO, CO_2, H_2, and N_2) [81] and the pyrolysis products of organic waste materials were successfully applied for this purpose. Predominately, *scl*-PHAs were to this day obtained by syngas-based cultivations; only after genetic engineering of *R. rubrum*, it was possible to produce a rubber-like *mcl*-PHA bio-latex from syngas [82]. Organic waste converted to syngas for PHA production encompasses household waste [83], hydrocarbons [84], and food waste [85].

Recently, syngas was used as a substrate for anaerobic production of PHAs by obligate anaerobic acetogenic *Clostridium* strains, which are already routinely cultivated on the industrial scale for fodder production. Here, recombinant strains of *Clostridium coskatii* and *Clostridium ljungdahlii* expressing a new artificial PHA biosynthesis pathway were successfully tested for biopolymer production in flask-scale setups. The fermentation technology of gaseous substrates by wild-type Clostridia has considerably advanced during the past decade and has already reached multi-tons industrial scale cultivation setups in PR China. The authors of the study consider this process as an economically feasible process toward PHA production from waste and greenhouse gases [86].

6.2.6 Methane

Beside CO_2 and syngas, the greenhouse gas methane is widely available in natural gas at a volume share of greater than 90%, in emissions from landfills, and constitutes the flammable component in biogas originating from anaerobic digestion of organic matter (including spent PHAs or non-PHA residual biomass after biopolymer recovery!). Methanotrophic bacteria, a group of Gram-negative organisms of the methylotroph subgroup, are able to convert methane as a source of carbon and energy via the ribulose monophosphate pathway (RMP) or via the serine pathway; several methanotrophs are described to accumulate PHB. The *Methylocystis* genus encompasses various type II methanotrophic species, which resort to the serine pathway for PHB biosynthesis. Such organisms, such as *Methylocystis hirsuta*, can be found in various environments. For water-insoluble gases such as methane, mass

transfer in bioreactors plays a crucial role for the cultivation process. In this context, Moradi et al. modeled the optimum conditions for PHB production from natural gas in a bubble column bioreactor using the COMSOL software, considering factors such as methane-to-air ratio, inlet gas rate, and gas hold up [87]. Rahnama et al. described PHB production by *M. hirsuta* from mixtures of natural gas and air in a simple bubble column and a forced liquid vertical tubular loop bioreactor, achieving an average PHB content in biomass of about 52 wt% [88]. This process was improved by Ghoddosi et al. who achieved increased methane uptake by the strain and high density by adding organic cosubstrates such as acetate, glucose, and methanol. In a bubble column bioreactor operated at optimum conditions, 8 g/l of biomass with a PHB content of 73.4 wt% was obtained [89].

From the technoeconomic point of view, methane-based PHA production is feasible and has been demonstrated to have the potential to be economically competitive. A study by Levett et al. reports a production price of PHA in a similar range like PHA biosynthesis from purified sugars; these authors claim high cooling costs as the decisive cost factor in this process, which compensates the gain from lower raw material costs [90]. However, they failed to consider more modern and advanced gas fermentation reactor designs that are already commercially available and are claimed to produce single-cell proteins (SCPs) economically using methane fermentation (commercialized by the companies Calysta [91] and Unibio [92]). In addition, the companies Newlight Technologies [93] and Mango Materials [94] have also successfully demonstrated PHB production using methane fermentation. The breakthrough in this regard is imminent because methane represents one of the most suitable and pure forms of carbon for industrial production of materials, and polyolefins from natural gas in North America represent one of the most inexpensive fossil plastics available today, primarily because of the currently low price of natural gas. Methane emissions from landfills and anaerobic digesters constitute a viable alternative to methane from natural gas; when used for PHA synthesis, this could theoretically replace about 20–30% of the total plastics produced annually today, but having the biodegradability characteristics that are so desired. Therefore, methane-based PHA production not only opens new avenues for biopolyester production but also mitigates the issues related to GHG emissions. As recently reviewed by Liu et al., factors such as the typically low efficiency of methanotrophs and often insufficient gas–liquid mass transfer in the bioreactors used need to be overcome. Moreover, most reports on PHA production to date are restricted to PHB homopolyester, which results in modest material properties. These authors therefore suggest the application of feast-famine feeding regimes, genetic engineering of microorganisms to increase specific PHA productivity, development of novel gas-permeable membrane bioreactors and two-phase partitioning bioreactors to overcome low solubility of methane and oxygen, and development of protocols for the production of PHA copolyesters with tailored molecular mass (by optimizing the feeding regime of 3HV-related precursors and selection of the appropriate growth-limiting factor) [95]. New methanotrophic strains as potential PHA producers are steadily isolated and described, as shown by studies of Khosravi-Darani et al. for the new *Microbacterium* sp. PBCC6 [96] or for Orita et al.'s *Methylobacterium*

extorquens AM1, a strain that interestingly accumulates the PHB homopolyester during methylotrophic cultivation, but PHBHV copolyester under cobalt-limited conditions on methanol as the sole carbon source [97].

6.2.7 Production Strains

PHA biosynthesis is described for diverse groups of prokaryotes, such as Gram-negative and Gram-positive eubacteria, chemoheterotrophs, photoautotrophs, mixotrophs, and a plethora of typically extremophilic archaea. Currently, the quest for new powerful PHA production strains is steadily ongoing. Table 6.1 provides an updated overview of genera encompassing PHA-accumulating wild-type species.

In addition to prokaryotes, a very limited number of reports are available for PHA accumulation by eukaryotic wild-type strains. Oligomers of 3HB with low molecular mass, however, are found in almost all life forms, also in the human metabolism. Moreover, PHA biosynthesis genes were successfully transferred to different eukaryotes, which then displayed PHA accumulation in their biomass. Here, the pioneering work of Yves Poirier's research group from the past 30 years on photoautotrophic PHA accumulation by green plants, especially by the heavily studied genetic and plant-metabolic model organism *Arabidopsis thaliana*, needs to be emphasized. Starting from CO_2 fixation, these recombinant plants provide real phyto-factories, producing in parallel PHAs, carbohydrates, lipids, pigments, and diverse phytochemicals with the potential market value. Although first generation of transgenic plants for PHA production produced the biopolyester in the plant cells' cytoplasm, nucleus, and vacuole at rather low productivity [101], the second generation showed increased PHA yields by storing the material in the plastids [102]. For relevant reviews on *in planta* PHA biosynthesis, please refer to Refs. [103, 104].

Mcl-PHA biosynthesis was achieved in the peroxisomes of the yeast *Saccharomyces cerevisiae* when equipping the organism with *P. putida* PHA synthesis genes, which express the class II PHA synthase able to metabolize the intermediate of β-oxidation for *mcl*-PHA synthesis [105]. Similar experiments were also carried out with the yeast *Pichia pastoris*, which was equipped with *Pseudomonas aeruginosa* PHA synthase genes [106]. More recently, the oleaginous yeast *Yarrowia lipolytica* was genetically modified. It was shown that by variation of the yeast's β-oxidation multifunctional enzyme, it was possible to trigger the monomeric composition of the produced PHA; both homo- and heteropolyesters were obtained by this strategy [107]. When expressing the *P. aeruginosa* PAO1 *PhaC1* gene together with a PTS1 peroxisomal signal in *Y. lipolytica*, cultivation on oleic acid resulted in the accumulation of *mcl*-PHA heteropolyesters with monomers from C6 (3HHx) to C14 (3HTD) [108]. However, PHA production in eukaryotic cells such as yeasts is more complex compared to prokaryotes because of the compartmentation of the metabolic pathways in such higher developed organisms; hence, it would be beneficial to have wild-type yeasts available for this purpose. Indeed, it was reported that the wild-type isolate *Rhodotorula minuta* Y4 accumulated 2 wt% of PHBHV copolyester when cultivated on glucose and oleic acid [109].

Table 6.1 Compilation of PHA-accumulating microbial genera. Update from [98]

Eubacteria		Archaea
Gram-negative genera	**Gram-positive genera**	**Three-letter code for haloarchaea according to Litchfield [99]**
(A) *Accumulibacter*, *Acidovorax*, *Acinetobacter*, *Actinobacillus*, *Aeromonas*, *Alcaligenes*, *Alcanivorax*, *Alkalilimuicola*, *Allochromatium*, *Amphritea*, *Anabaena*[a], *Anacystis*[a], *Aphanocapsa*[a], *Aphanothece*[a], *Aquaspirillum*, *Aquitalea*, *Aromatoleum*, *Arthrospira*[a], *Asticcaulus*, *Aulosira*[a], *Aureobasidium*, *Axobacter*, *Azohydromonas*, *Azomonas*, *Azospirillum*, and *Azotobacter*	***(A)*** *Actinomycetes* and *Aneurinibacillus*	***(C)*** *Cenarchaenum*
(B) *Beggiatoa*, *Beijerinckia*, *Beneckea*, *Brachymonas*, *Bradyrhizobium*, *Brevundimonas*, and *Burkholderia*	***(B)*** *Bacillus* and *Brochothrix*	***(H)*** *Halalkalicoccus* (*Hac.*) *Halobacterium* (*Hbt.*) *Haloarcula* (*Har.*) *Halobiforma* (*Hbf.*) *Halococcus* (*Hcc.*) *Haloferax* (*Hfx.*) *Halogeometricum* (*Hgm.*) *Halogranum* (*Hgn.*) *Halomicrobium* (*Hmc.*) *Halopiger* (*Hpg.*) *Haloquadratum* (*Hqr.*) *Haloterrigena* (*Htg.*) *Halorhabdus* (*Hrd.*) *Halorubrum* (*Hrr.*) *Halostagnicola* (*Hst.*)
(C) *Caenibacterium*, *Caldimonas*, *Calothrix*[a], *Caryophanon*, *Caulobacter*, *Chelatococcus*, *Chloroflexus*[a], *Chlorogleopsis*[a], *Chroococcus*[a], *Chromatium*, *Chromobacterium*, *Chromohalobacter*, *Cobetia*, *Comamonas Competibacter*, *Cupriavidus* (formerly: *Alcaligenes*, *Ralstonia*, and *Wautersia*), *Cyanobacterium*[a], and *Cyanothece*[a]	***(C)*** *Clostridium* and *Corynebacterium*	
(D) *Dechloromonas*, *Defluviicoccus*, *Delftia*, *Desulfococcus*, and *Desulfobacterium*	***(D)*** *Derxia*	
(E) *Ectothiorhodospira*, *Ensifer* (formerly: *Sinorhizobium*), and *Erwinia*		
(F) *Ferrobacillus*, *Fischerella*[a], and *Fundibacter*	***(I)*** *Isoptericola*	
(G) *Gamphosphaeria*, *Gloeocapsa*[a], and *Gloeothece*[a]	***(L)*** *Lysinibacillus*	
(H) *Haemophilus*, *Haererehalobacter*, *Hahella*, *Halomonas*, *Halorhodospira*, *Hapalosiphon*[a], *Hydrogenophaga*, *Herbaspirillum*, and *Hyphomicrobium*	***(M)*** *Microbacterium*, *Micrococcus*, and *Microlunatus*	***(N)*** *Natrialba* (*Nab.*) *Natrinema* (*Nnm.*) *Natronobacterium* (*Nbt.*) *Natronococcus* (*Ncc.*) *Natronomonas* (*Nmn.*) *Natronorubrum* (*Nrr.*) *Nitrosopumilus*
	(N) *Nocardia*	
	(P) *Paucispirillum* and *Piscicoccus*	
	(R) *Rhodococcus*	
	(S) *Staphylococcus*, *Streptomyces*, and *Syntrophomonas*	
	Eukaryotes	
	(R) *Rhodotorula*[e]	
	(S) *Scenedesmus*[f]	

Table 6.1 (Continued)

Eubacteria		Archaea
Gram-negative genera	**Gram-positive genera**	**Three-letter code for haloarchaea according to Litchfield [99]**
(I–K) *Ideonella*, *Iodobacter*, *Janthinobacterium*, *Jeongeupia*, *Klebsiella*, and *Kushneria*		
(L) *Labrenzia*, *Lampropedia*, *Leptothrix*, and *Legionella*		
(M) *Magnetospirillum*, *Marinobacter*, *Marinospirillum*, *Massilia*, *Mesorhizobium*, *Methanomonas*, *Methylarcula*, *Methylibium*, *Methylobacterium*, *Methylocystis*, *Methylosinus*, *Methylomonas*, *Methylovibrio*, *Microcoleus*[a], *Microcystis*[a], *Moraxella*, and *Mycoplana*		
(N) *Naxibacter* (today: *Massilia*), *Neptunomonas*, *Nitrobacter*, *Nitrococcus*, *Nodularia*[a], *Novosphingobium*, and *Nostoc*[a]		
(O) *Ocenicella*, *Oceanospirillum*, *Oligotropha*, and *Oscillatoria*[a]		
(P) *Pandoraea*, *Pannonibacter*, *Paracoccus*, *Paraburkholderia*, *Parafunghorum (*formerly: *Pseudomonas*), *Pedomicrobium*, *Pelomonas*, *Phormidium*[a], *Photobacterium*, *Plasticicumulans*, *Pleurocapsa*[a], *Polaromonas*, *Pleomorphobacterium* (=*Oceanicella*), *Protomonas*, *Pseudoanabaena*[a], *Pseudogulbenkiania*, and *Pseudomonas*		
(R) *Rhizobium*, *Rhodobacter*[b], *Rhodoferax*[b], *Rhodopseudomonas*, *Rhodospirillum*, *Rivularia*[a], *Rubrivivax*[b], *Roseateles*[c], and *Ruegeria*		
(S) *Saccharophagus*, *Salinivibrio*, *Schlegelella*, *Scytonema*[a], *Sinorhizobium*, *Sphaerotilus*, *Sphingomonas*, *Sphingopyxis*, *Spirillum*, *Spirulina*[a], *Stappia*, *Stella*, *Stenotrophomonas*, *Synechococcus*[a], and *Synechocystis*[a]		

Table 6.1 (Continued)

Eubacteria		Archaea
Gram-negative genera	**Gram-positive genera**	**Three-letter code for haloarchaea according to Litchfield [99]**
(T) *Thauera*, *Thermus*, *Thiobacillus*, *Thiocapsa*[d], *Thiococcus*[d], *Thiocystis*[d], *Thiodictyon*[d], *Thiopedia*[d], *Thiosphaera*[d], and *Tolypothrix*[a]		
(V) *Variovorax*, *Vibrio*		
(W) *Wautersia* (today: *Cupriavidus*), *Westielopsis*[a]		
(X) *Xanthobacter*		
(Y) *Yangia*, *Yokenella*		
(Z) *Zobellella*, *Zoogloe*		

a) Cyanobacteria.
b) Purple non-sulfur photosynthetic bacteria.
c) Aerobic photosynthetic bacteria with bacteriochlorophyll a.
d) Anoxygenic purple sulfur bacteria.
e) Yeasts.
f) Eukaryotic microalgae.
Source: Adaptation and extension from [98, 100].

Even animals were modified to produce PHA; in this context, Williams et al. achieved simultaneous expression of the dehydrase-domain mutant rat fatty acid synthase cDNA (provides *de novo* synthesis of the PHB precursor *R*-(−)-3-hydroxybutyryl-coenzyme A) and the *phbC* gene encoding class I PHA synthase from *C. nector* genes into insect cells of *Spodoptera frugiperda* (the fall armyworm, a butterfly); here, gene transfer was carried out by baculovirus vectors [110].

6.3 Bioengineering: Bioreactor Design and Feeding Regime

6.3.1 Feeding Regime

PHA production on a larger scale is performed under controlled conditions carried out in bioreactors, which can constitute batch (substrate feeding occurs only at the start, emptying the bioreactor for harvesting PHA-rich biomass only after stopping the process), fed-batch (substrate addition by pulse-feeding when needed according to consumption by the cells, harvest after stopping the process), and continuous (both substrate feed and product harvest occur during the entire process) processes. The feeding strategy and the bioreactor operation mode (batch, fed-batch, or continuous) need to be well adapted to the applied microbial strain, the substrate used, and

the biomass and product formation kinetics [111, 112]. Processes for PHA production carried out in batch [113], repeated batch ("drain-and-fill" and "fill-and-empty" processes) [114], fed-batch [115], or cyclic fed-batch mode [116] display the typical disadvantage of insufficient controllability of PHA composition on the level of monomers and monomer distribution and are often of low volumetric productivity [117].

Figure 6.3 provides an overview of different cultivation regimes used to produce PHAs.

6.3.2 Continuously Operated Bioreactors for Liquid Feed

Detailed knowledge about the growth and PHA biosynthesis kinetics is required to develop efficient continuous production processes. To optimize such continuous processes, the operation mode (dilution rate) needs to be in accordance with the kinetics of biomass growth and PHA biosynthesis. Such continuous processes often enable high volumetric productivity, better control of the product composition and properties, and use of substrates, which may otherwise inhibit the organisms at low concentrations. The latter is typical for compounds such as propionic acid, valeric acid, or γ-butyrolactone (GBL), acting as precursors of desired PHA monomers such as 3HV or 4HB, respectively.

Single-, two-, and multi-stage bioreactor setups are reported for continuous processes for PHA biosynthesis. As mentioned before, PHAs are typical products of the secondary metabolism, synthesized by multi-stage metabolic processes; therefore, it is reasonable to switch to two-stage continuous processes to produce such biopolyesters, where catalytically active, almost PHA-free biomass is generated at high cell density in a first continuously operated bioreactor; this biomass is continuously transferred into the second continuously operated bioreactor, where nutritional conditions stop biomass growth but favor PHA biosynthesis (e.g. limitation in nitrogen source); in this second stage, the cells get filled with PHA granules at typically high productivity. In such two-stage continuous processes, both the growth-limiting component (e.g. nitrogen source) and the carbon source can be supplied to the system at feeding rates according to the consumption rate, which results in an almost complete consumption of substrates by the cells and allow the spent fermentation broth to be free of substrates. This, in turn, saves the substrate cost, while resulting in lower fraction of PHA in biomass compared to a surplus of carbon source in the second stage, which, however, needs to be disposed of with the spent fermentation broth.

In multistage continuous processes, the so-called "bioreactor cascades," a range of individual stirred tank bioreactors are serially connected; with increasing number of bioreactors, such setups mimic the tubular plug flow reactor characteristics [118]. It was demonstrated that in bioreactor cascades, volumetric productivity can be further increased in comparison to two-stage setups, as shown by Atlić et al., who reported the highest volumetric PHA productivity (1.85 g/(l·h) for the entire cascade, 3 g/(l·h) for vessel 4) in continuous processes ever for a continuously operated bioreactor cascade consisting of five bioreactor vessels. In this setup, the first bioreactor served to form PHA-poor biomass under nutritionally balanced conditions, while the second

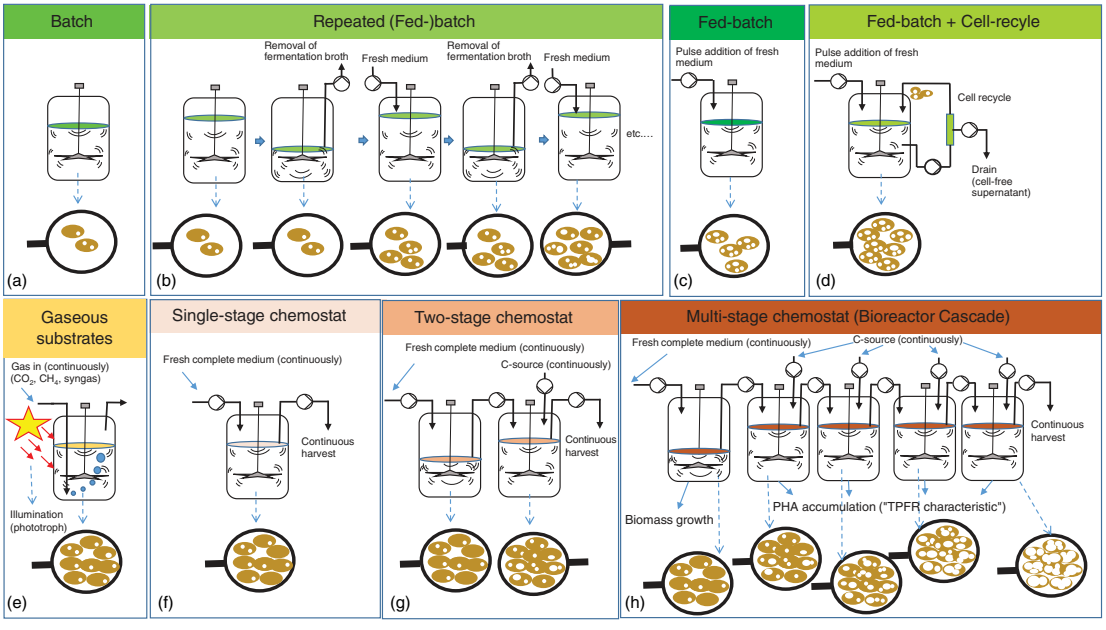

Figure 6.3 Different operation strategies to run PHA production processes in bioreactors. (a) batch, (b) repeated (fed-) batch, (c): fed-batch, (d) fed-batch with cell-recycle, (e) gaseous substrates, (f) single-stage chemostat, (g) two-stage chemostat, (h) multi-stage chemostat (bioreactor cascade). Source: [111] Reproduced with permission of MDPI publishers, Basel.

bioreactor (transient stage) served for depletion of residual nitrogen sources not utilized by the cells in bioreactor 1. In the vessels 3 to 5, increasing quantities of PHA were accumulated by the cells run under nitrogen-limited conditions with ample supply with the carbon source. In addition, such cascade setups enable the exact adaptation of the cultivation conditions in each individual bioreactor by triggering the substrate (main carbon source or precursor substrates) supply to each bioreactor; this allows designing blocky structured PHA with predefined compositions and properties [119]. Data from this cascade process were later used to develop mathematical models of the bioprocess; these models encompass formal kinetic [120] and high structured metabolic models [121], as well as footprint area analysis of imaged PHA-containing cells from individual reactors [122]; all these models provided new insights into the various screws that you have to turn to optimize cascade processes for PHA production, especially regarding nutrient supply to individual bioreactors and optimum number of cascade vessels [123].

6.3.3 Bioreactors for Gas Feed

Methane can be converted to PHA by methanotrophic production strains such as *Methylocystis* sp.; for such cases, high PHA yields of more than 0.5 g PHA per gram CH_4 are reported, which surmounts the theoretical yield of sugar-based heterotrophic PHA production of 0.48 g/g (reviewed by [124]).

For large-scale PHA production from methane, bioreactor systems with optimized capability to transfer the gas (air enriched with CH_4) to the microorganism is needed; various reactor types, including bubble columns and airlift bioreactors, come into play. A recent review reports the application of loop bioreactors for PHA production based on methane-rich natural gas; computational fluid dynamics and mathematical modeling were implemented to optimize the process. It was shown that gas holdup, shear stress, gas transfer coefficients, and liquid velocity vectors of the systems need to be investigated to conceive the interrelations between process parameters and PHA biosynthesis [96].

Syngas, in turn, is generated by gasification of organic waste materials; it can be metabolized into PHAs by, e.g. *R. rubrum*, a facultative anaerobic photosynthetic bacterium. However, using this substrate presents some safety risks regarding toxicity and risk of explosion. This was addressed by Karmann et al. who developed a platform of syngas fermentation, equipped with sophisticated technical safety precautions, tools to measure and control gas supply and consumption by cells, and modern process analytical technology (PAT), including an in-line flow cytometer to determine the cell density and PHA content in *R. rubrum* in fed-batch and continuous processes [125].

6.3.4 Photo-reactors for CO_2 Feed

Gaseous substrates such as CO_2, syngas, and methane are increasing being investigated for PHA production, given the availability of these carbon-rich gases and the importance in GHG reduction. The primary issue in gas-based substrate use is their

lack of solubility in water, where the microorganisms and other nutrients reside during fermentation.

CO_2-based PHA biosynthesis occurs with photoautotrophic cyanobacteria, a group of prokaryotes formerly designated as "blue-green algae". Both discontinuously and continuously operated illuminated photobioreactors are reported in the literature, similar to photobioreactors used to cultivate eukaryotic microalgal species. The illumination regime (light intensity, wave length, dark–light cycles, etc.), geometry, mixing characteristics, and gassing/degassing behavior of such photobioreactors need to be optimized for efficient cultivation of cyanobacteria [126–129]. A recent review presents different photobioreactor types up to now tested to farm cyanobacteria, demonstrating that the most suitable photobioreactor setups predominantly depend on the specific light and nutrient requirements of the studied cyanobacterium [76]. Based on the results from pilot-scale runs with the cyanobacterium *Synechocystis* sp., this and other studies concluded that tubular photobioreactors might be the optimum solution for PHA production by such phototrophic production strains [75].

6.4 Downstream Processing for PHA Recovery

Downstream processing for the recovery of microbial PHA biopolyesters from biomass constitutes an integral part of the entire PHA production chain. This process is currently considered the major cost factor for PHA production besides feedstock cost. Moreover, the downstream processing is also contributing significantly to the ecological footprint of the product, e.g. by the use of solvent, chemicals, and energy. As comprehensively summarized in recent review articles, there are various current trends and ongoing efforts to optimize PHA recovery, aimed at reducing or even eliminating solvents and time expenditure, minimizing the use of chemicals in general and energy input; maximizing recovery yields and product purity, and maintaining the properties of native PHAs [130]. To an increasing extent, biological methods for PHA recovery replace chemical approaches, at least on a laboratory scale [131]. Moreover, the applied PHA recovery method is decisive for the molecular mass and purity (important for polymer processing and use of all kinds, including medical use) of the obtained product and the achievable recovery yield. Finally, the PHA recovery technique affects the PHA content recovered from biomass. For example, not all recovery techniques are applicable for all types of PHAs – crystalline *scl*-PHA requires different recovery types compared to amorphous *mcl*-PHA – or to all types of PHA-producing microorganisms – robust strains need other recovery techniques than organisms with fragile cell structures. In principle, solvent-based extraction methods, the use of ionic liquids (ILs) and supercritical solvents, chemical, mechanical, and biological methods for disintegration of residual biomass are described for PHA recovery. The applied recovery method not only determined product purity and recovery yield but also the fate of remaining non-PHA biomass [132].

Figure 6.4 provides a schematic illustration of diverse established and emerging methods for PHA recovery from microbial biomass.

6.4.1 Classical Solvents

The state-of-the-art in PHA recovery involves the use of halogenated solvents, especially chloroform, because it gives high extraction yields, excellent PHA polymer purity, and minimal loss of PHA polymer properties during melt processing. The downsides of this method are of course the use of high volumes of a toxic solvent that, despite the presence of state-of-the-art solvent recovery and recycling processes, can be hazardous and pose human and environmental risks. Besides, many of these outstanding PHA solvents such as chloroform have petrochemical origin, which clearly contradicts the paradigm of PHAs being renewable and benign green materials produced by sustainable process steps. Although resulting in high product recovery yields and purity, solvent-based PHA extraction typically destroys the natural (native) characteristics of PHA granules by reducing molecular mass via random and chain end scission, particularly at higher temperature and prolonged extraction time; this can obstruct their further processing [130].

After dissolving in halogenated solvents, the PHA requires a second precipitating solvent in order to obtain high-purity PHAs because simply evaporating the solvent would leave impurities in the PHA polymer. These "PHA anti-solvents" (ethanol, acetone, methanol, hexane, etc.) are typically added in excess and in their presence, together with lowering the temperature, dramatically reduces the PHA solubility in chloroform, thus precipitating the polymer. After precipitating the PHA polymer, the mixture of PHA solvent and "PHA anti-solvent" is removed and separated into their individual components via distillation, expending substantial energy. Typically, about 3 wt% PHA is dissolved after extraction in classical PHA solvents such as chloroform and need about 10-fold volume of anti-solvent for precipitation [132].

As a way out, a convenient, lower energy consuming separation process for the chloroform–ethanol mixture was developed by Rodolfo Bona in the research labs of Gerhart Braunegg, one of the pioneers of PHAs. This method is based on simply adding appropriate amounts of water. At the appropriate chloroform–water–ethanol ratio, a ternary three-component, two-phase system is created, which allowed recovery of the high-density chloroform at around 95% purity with the rest being ethanol. They showed that this chloroform containing some ethanol residue (ethanol is present as a stabilizer in commercial chloroform anyway) can be reused in subsequent PHA extraction cycles. Removal of minor water residues from this phase is possible by adding drying agents. The second phase, a low-density ethanol/water mixture, contains only minor quantities of chloroform (1%) and about 18% ethanol. The phase diagram of this three-component, two-phase system is presented in Figure 6.5. Despite the convenience and ease of using this separation technique, the authors of this chapter are not aware of its industrial implementation [132].

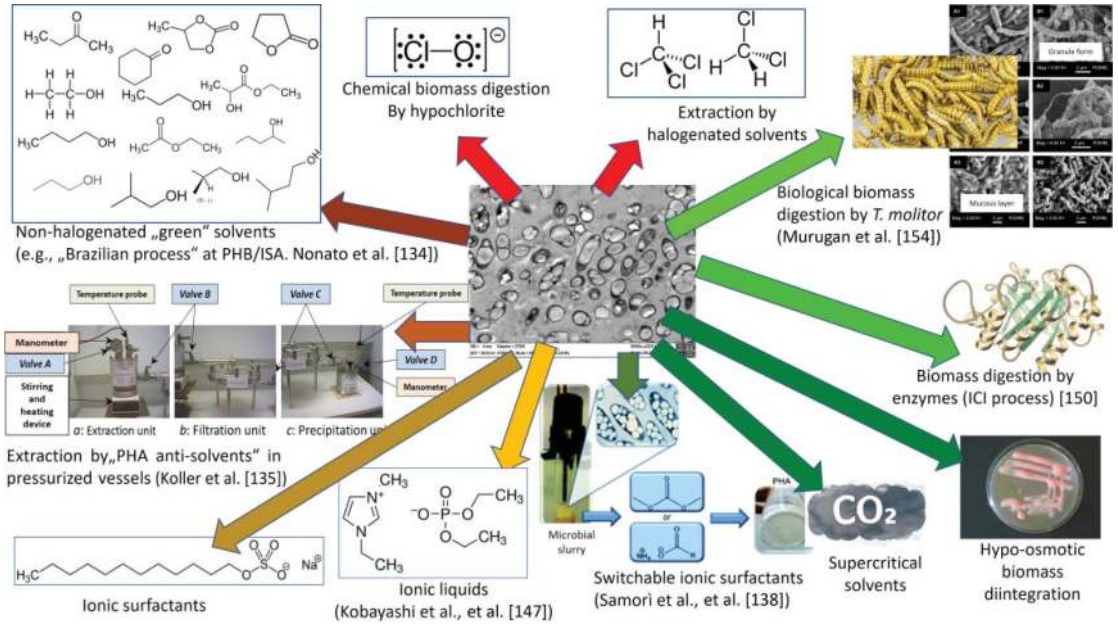

Figure 6.4 Compilation of established and emerging PHA recovery methods by chemical and biological methods; coloration of arrows provides a rough, maybe preliminary, estimation of the overall sustainability of individual methods, with red being not or hardly sustainable and green indicating high sustainability. Source: Koller [130]/with permission from Sciendo.

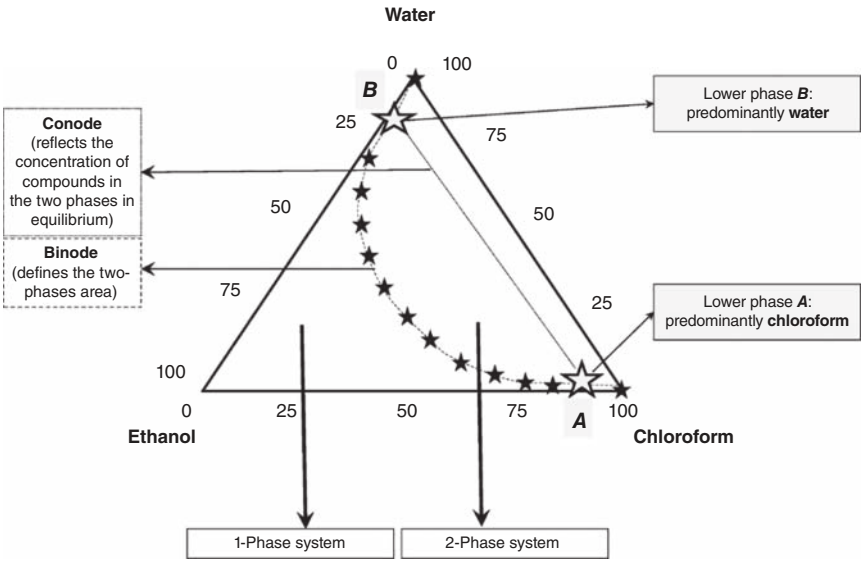

Figure 6.5 One- and two-phase areas in the ternary system chloroform–ethanol–water. Source: Koller et al. [132]/with permission from Sciendo.

6.4.2 Halogen-Free Solvents

A Brazilian PHA company, PHB Industrial S.A. (PHB/ISA), demonstrated the use of distillation by-products from ethanol production (fusel oils containing 1-propanol, iso-butanol, (*S*)-2-methyl-1-butanol, and isopentanol) as a solvent for PHA extraction. This mixture is produced in house during sugarcane and ethanol production from sugarcane. This allows the company to have little or no additional cost for solvents needed for the separation and purification of PHAs. Cultures of *Cupriavidus necator* or *Burkholderia* sp. are used for the semi-industrial PHA production process; sucrose and molasses serve as a carbon source. After the fermentation, cells are thermally inactivated, flocculated, and concentrated to a cell slurry of up to 300 g/l density. This slurry undergoes a multi-step extraction process with the fusel oils in continuously stirred tanks. From the generated extract, cell debris is removed, and, by cooling down, the PHA solution turns into a gel. By pressing and evaporation, the major part of the solvent is removed, and the residual fusel oil is withdrawn from the PHA by dispersing the PHA in water (subsequent additional distillation-based recovery of solvent) and vacuum drying the polymer into a PHA granulate. The purity of the obtained polymer is reported to be in a similar range (about 98%) to the purity obtained by established extraction methods based on chloroform [133].

In the frame of an industrial project with Partner BDI – BioEnergy International AG, Austria, one of the authors of this chapter developed a novel extraction strategy for PHA recovery from biomass by using the acetone that operates as a solvent at an elevated temperature and pressure but becomes anti-solvent upon cooling to room temperature. An aluminum device consisting of an extraction-, filtration-, and precipitation unit was designed and built to recover the PHA from freeze-dried

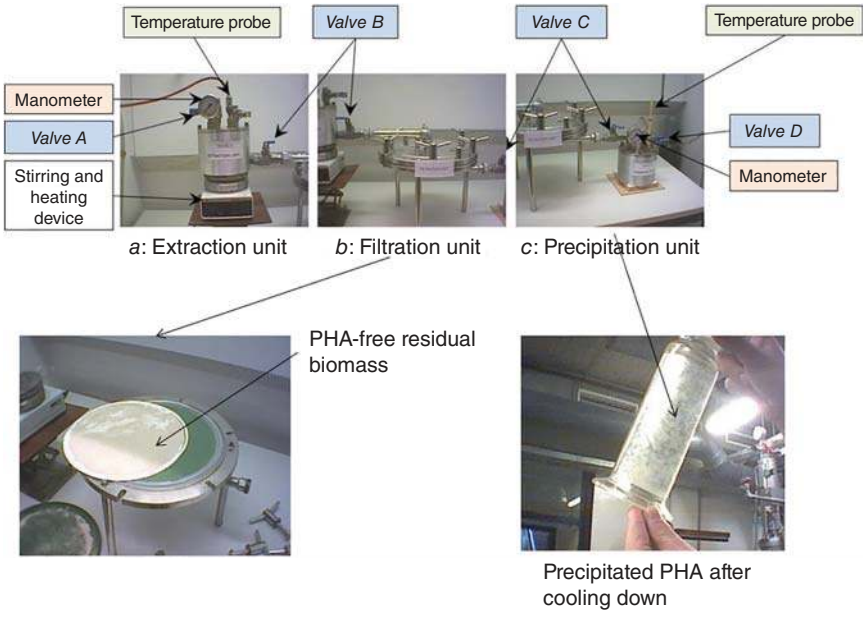

Figure 6.6 Setup for extraction of PHAs from microbial biomass with acetone under high p/T conditions. Modified from [72].

biomass of the haloarchaeon *Hfx. mediterranei* under high temperature (121 °C) and pressure (7 bar). It must be noted that acetone does not dissolve crystalline *scl*-PHAs as PHB or PHBHV under ambient temperature and pressure. The rest of the cell debris was then filtered out, and upon lowering the temperature and pressure, the PHA precipitated, leaving the lipids in acetone, which was then recycled after evaporation of the solvent. Prior to pressuring and raising the temperature of the acetone-PHA-cell debris mixture, the entire system was purged with nitrogen to remove oxygen and thus eliminating the risk of explosion. Recovery yields and product purity were competitive with results obtained for parallel control experimental setups that used chloroform [134]. Figure 6.6 illustrates this process.

In contrast to *scl*-PHAs, *mcl*-PHAs of low crystallinity can be extracted by acetone from biomass even at room temperature and ambient pressure; this was shown by Cerrone et al. for extraction of amorphous PHA copolyester consisting of 3-hydroxydodecanoate (3HDD), 3-hydroxydecanoate (3HD), 3-hydroxyoctanoate (3HO), and 3HHx (poly(3HDD-*co*-3HD-*co*-3HO-*co*-3HHx)) from lyophilized *Pseudomonas chlororaphis* biomass [135].

More recently, Jiang et al. studied the potential of cyclohexanone and GBL as extraction solvents for the *scl*-PHA PHB from *C. necator* biomass; extraction kinetics and its dependence on temperature were studied. Using cyclohexanone at 120 °C, 95% of present PHA was extracted from biomass within three minutes, while reduced temperature significantly decreased the extraction yield; purity was similar to control setups using chloroform. Using GBL at 120 °C, considerably

lower recovery yields (only 50% recovered after three minutes) were obtained in comparison to cyclohexanone. For both solvents, the molar mass and the molecular mass distribution (polydispersity, *Đ*) of PHAs were similar to process setups that used chloroform as the extraction solvent. According to the authors, cyclohexanone constitutes an auspicious candidate for sustainable, halogen-free PHA recovery, *inter alia* because of the expedient recovery yields achieved. As a drawback, cyclohexanone is a petrochemical product [136].

Samorì et al. used dimethyl carbonate (DMC), a fully biodegradable green solvent with minor toxicity in humans and to the environment, for the extraction of different types of *scl*-PHAs either from dried biomass or directly from concentrated wet bacterial slurry. Polymer recovery yields above 85%, and product purity exceeding 95% was reported. Both for dry and wet biomass, no molecular mass reduction was observed in the recovered PHA. For these experiments, the PHB-rich *C. necator* biomass was treated for four hours at DMC's boiling point (90 °C); PHA recovery from solution was either accomplished via solvent evaporation or precipitation by addition of cooled ethanol [137].

Examples for other promising non-halogenated solvents, which were used for PHA extraction at least on a laboratory scale, encompass the non-cyclic ketones methyl ethyl ketone (MEK) and methyl isobutyl ketone (MIBK), which were shown to extract P(3HB-*co*-3HHx) copolyesters, both from wet and dry biomass, while ethyl acetate was suitable to extract PHAs from dry biomass. Extractions were carried out under temperatures of 100 °C in sealed screw top test tubes. Among them, especially MEK was suggested as a promising alternative to halogenated solvents because of its relatively low price and its low density, which facilitates separation of cell debris from the polymer solution. In addition, its higher boiling point makes it less dangerous than acetone [138].

6.4.3 Supercritical Solvents

Supercritical fluids used as agents for PHA extraction offer an excellent combination of solvation power similar to liquids plus expedient diffusion power (lower density and viscosity than liquids) similar to gases. Supercritical CO_2 (sCO_2), the best-studied supercritical fluid, exists above the critical point of CO_2 at 304 K and 74 bar. After the extraction, it is depressurized to evaporate as gaseous CO_2 and could be recompressed and recycled. No solvent residues remain in contrast to solvent-based PHA extraction processes [139]. For different intracellular bioproducts that could be extracted by sCO_2, Williams et al. offered various organic solvents such as alcohols that could act as modifiers for overcoming the polarity difference between CO_2 and the intracellular product to be extracted, thereby enhancing the solubility of sCO_2 in the highly polar intracellular space [140]. Hampson and Ashby reported that small amounts of chloroform indeed facilitated sCO_2-mediated recovery of *mcl*-PHAs from *Pseudomonas* sp. biomass [141]. This was later confirmed by Hejazi et al., who achieved excellent *scl*-PHA recovery yields of almost 90% when applying sCO_2 together with methanol as the modifier solvent. These authors reported that 0.2 ml of methanol was added to 4 ml of CO_2 along with

an exposure time of 100 minutes at 40 °C and 200 atm as the optimum conditions for cell disintegration and PHB recovery [142]. Another study by the same team of researchers reported that an alkaline pretreatment of the lyophilized biomass of highly crystalline *scl*-PHAs can be recovered by sCO_2, at an even higher yield than what they showed possible by adding modifier solvents. It is noteworthy to also mention that sCO_2 can also be used to recover PHAs from wet PHA biomass, thus saving on PHA drying cost without sacrificing the quality of the recovered PHAs as shown by their retention of both molecular weight and distribution before and after the recovery process as compared to the recovery process that used lyophilized biomass. There was, however, slightly lower product purity noticed while using wet PHA biomass as the starting point as compared to dried lyophilized PHA biomass as the starting point [143].

6.4.4 Recovery by Chemical and Mechanical Disintegration of Biomass

Surfactants disrupt cell membranes, and intact PHA granules are set free. Samorì et al. used salts of longer fatty acids as surfactants; this process turned out to be applicable also to wet biomass, which in turn saves drying expenses. Polymer recovery yields above 99%, and a purity of more than 90% have been reported. In this investigation, ammonium laurate (dodecanoate) stood out as the so-called "switchable anionic surfactant"; it reversibly converts from a neutral (protonated) water-insoluble form at low pH-values to a deprotonated anionic water-soluble form at an elevated pH-value; the pH shift was done by adding and releasing CO_2. A suspension of *C. necator* biomass in an ammonium laurate solution was adjusted to pH-value 10, corresponding to the surfactant's anionic form. This dissolved the non-PHA bacterial matter and precipitated the PHA granules, which were then separated by centrifugation. The remaining supernatant liquid consisting of the ammonium laurate and disintegrated cells was neutralized by bubbling in CO_2, converting the laurate into protonated, water-insoluble lauric acid, which was then decanted from the resulting aqueous bicarbonate solution and recovered by centrifugation and reused in the next PHA recovery step. The remaining solution of dissolved cell constituents and NH_4HCO_3 can also be utilized as a nitrogen source in subsequent cultivations of the PHA production strain, thereby allowing for virtually no losses and complete circularity of the process [137].

Other well-established chemical methods based on the disintegration of the non-PHA cell material, leaving PHA granules intact, resort to the use of sodium hypochlorite (NaOCl) solution with sodium dodecyl sulfate (SDS). For example, Marudkla et al. reported a maximum PHA recovery of 78.7% when using 0.5% w/v SDS combined with 6% v/v NaOCl. However, SDS is an irritant, and NaOCl is a halogenated compound, contradicting the aim of abolishing chlorine from PHA manufacturing [144]. Researchers also observed that the presence of NaOCl reduced PHA molecular mass and generated different halogenated compounds (reviewed by [130]).

Several researchers have investigated using ILs; ILs are regarded as "molten salts" with melting points lower than 100 °C and often even below room temperature. Such

ILs have special properties, such as high thermal stability, insignificant vapor pressure, low flammability, and expedient ionic conductivity, and are considered superior to those reported for classical organic solvents. Fujita et al. have reported the direct dissolution of wet (95% water content!) biomass of the cyanobacterium *Synechocystis* sp. using the hydrophilic IL 1-ethyl-3-methylimidazolium methylphosphonate ([C2mim][MeO(H)PO2]). In this case, no heating or pretreatment was needed, and the authors successfully recovered "intracellular products such as PHA" [145]. This was verified by Kobayashi et al., who studied [C2mim][MeO(H)PO2] and other ILs for their potential to remove the non-PHA biomass of the cyanobacterium *Synechocystis* sp. PCC 6803, explicitly with the aim of releasing intact PHB granules. Again, [C2mim][MeO(H)PO2] stood out as the best IL to dissolve non-PHA matter while leaving PHB granules intact among the ILs tested. After cell disintegration, PHB was separated by filtration, with a recovery yield of more than 98%. ILs are generally recyclable, and this one in particular was readily recyclable by adding a small amount of solvent to precipitate the cyanobacterial debris, which were removed by filtration, and the mixture of IL and solvent was then evaporated at 40 °C for six hours for solvent removal. [146].

Haloarchaea, the extremely halophilic branch of the archaea domain, thrive at optimum salinities in the range between 2 and 5 M NaCl. This causes the generation of extraordinarily high inner-osmotic pressure to balance the high medium osmolarity outside the cells and opens the door for a convenient approach to release intracellular products from such organisms. Described for the first time in 1990 by Rodriguez-Valera and Lillo for PHA-rich *Hfx. mediterranei* biomass, the exposure of such extremely halophiles to hypotonic media (i.e. distilled water) rapidly and immediately disrupted cells and released the PHA granules without the addition of any organic extraction solvents. Due to the lower density of PHA granules in comparison to the non-PHA cell material, the thus treated broth formed a two-phase system, consisting of an upper layer of lower density PHA granule containing phase and a higher density cell debris phase at the bottom. The two phases are then separated by sedimentation or centrifugation. The top layer, "PHA cream," is then skimmed off, and the biopolymer further purified if required (reviewed by [147]).

Wild-type strains naturally accumulating high quantities of PHAs are equipped by nature to keep cell integrity at very high loads of intracellular storage materials. When expressing PHA synthesis genes in strains such as *Escherichia coli*, which are not natural PHA producers, the cell wall cannot stay intact at excessive loads of inclusion bodies such as PHAs. After cell harvest, high intracellular PHA fractions make cells burst even under mild biomass treatment. As an example, Choi and Lee transformed *E. coli* cells with *C. necator* PHA synthesis genes. These recombinant *E. coli* cells accumulated more than 70 wt% PHA in biomass. By simply stirring the biomass with 0.2 N NaOH at 30 °C for one hour, highly pure PHA (purity more than 95%) was recovered [148] primarily because of the poor cell integrity of the recombinant *E. coli* strains.

Advanced mechanical methods for cell disintegration for PHA recovery were also reported in the literature. Researchers have reported using high-pressure homogenization (HPH) where the disruption performance strongly depends on the biomass

concentration and operates rather modestly at low biomass concentrations. Ghatnekar et al. described using HPH in the presence of SDS for recovery of PHB granules from *Methylobacterium* sp. V49. They obtained a maximum recovery yield of 98% and a product purity of 95% using 5% SDS solution and a pressure of 400 kg/cm^2 for HPH [149]. Other researchers studied the disruption performance of HPH for PHA-rich culture broth of *C. necator* with biomass concentrations in the range of 1–6 wt%. They investigated HPH both with and without pretreating the fermentation broth with NaOH and SDS solutions. Two cycles at 800 kg/cm^2 were carried out. The results indicated excellent performance of HPH in cell disintegration. Best results (>99.99%) were achieved by combined pretreatment with NaOH (strongly alkaline conditions of pH 12) and SDS (1%). The results obtained without any pretreatment were also very promising, and they achieved a degree of cell disruption of 91–99%. Separation of the released PHA granules was then carried out by dissolved air floatation (DAF) [132].

6.4.5 Biological PHA Recovery

Chemical methods described above to disintegrate the microorganisms are often expensive, toxic, and/or corrosive to machinery and humans. Frequently, these chemicals are not recyclable (such as sodium hypochlorite, strong bases or acids, or the irritating anionic surfactant SDS). The alternative, using green biocatalysts (enzymes), to disrupt the microorganisms is not necessarily cost-efficient because the enzymes are frequently lost after cell disrupture, the resulting PHA biomass has poor product purity, and the overall rates of reactions can be low, thus resulting high costs, as reported for industrial scale tests [133]. Initial large-scale enzymatic cell disintegration was developed, practiced, and reported decades ago by Imperial Chemical Industries (ICI), UK. They used enzyme mixtures containing proteases, phospholipases, lysozyme, and nucleases, which were typically applied, often in combination with surfactants and strong oxidants such as hydrogen peroxide for the subsequent chemical purification of the extracted PHA [150]. A novel enzymatic approach for digestion of non-PHA biomass has been presented by Kachrimanidiou et al. These authors prepared a crude enzyme cocktail via solid-state fermentation (SSF) of the fungus *Aspergillus oryzae*. This enzyme mixture was applied to lyse *C. necator* cells for PHA recovery. Temperature and pH-value were optimized for maximum *C. necator* lysis and reached 90% PHA purity and a recovery yield of 97–98% [151].

Typically, cell homogenization during PHA recovery generates high quantities of DNA, which increases the viscosity of the fermentation broth, thus complicating the recovery of the PHA granules. Another approach to improve PHA recovery involved expressing nuclease enzymes in PHA accumulating cells. Generally, viscosity reduction is achieved by heat treatment, addition of commercially available nucleases, or by adding sodium hypochlorite, all adding to the cost of PHA polymer recovery. Rodríguez Gamero et al. integrated *Staphylococcus aureus* nuclease genes into the PHA production strains *C. necator* and *Delftia acidovorans*. The nuclease enzyme has been readily expressed in both bacterial host strains without negatively affecting the PHA biosynthesis. They observed significant viscosity reduction in

the rec. *C. necator* homogenate. This process of expressing the nuclease in the PHA producing microorganism could greatly reduce the overall production cost of PHA polymer recovery process [152].

A novel approach to PHA recovery involved the use of insects. *Tenebrio molitor* – meal worms in different stages of their life cycle were fed with freeze-dried PHA-rich *C. necator* biomass at the research group of Kumar Sudesh in Malaysia [153]. Residual biomass (proteins, etc.) was readily digested by the animals as SCP source, while the PHA granules were excreted by these worms in highly pure form. Subsequent purification steps using water, detergents, and heat treatment generated almost 100% pure PHA granules. Apart from PHA recovery, the cultivated meal worms might also serve as potential source of food or animal feed as an additional source of protein for human nutrition [154].

6.5 End-of-Life Options: Recycling and Biodegradation of PHAs

Just like other biopolymers such as thermoplastic starch (TPS), other polysaccharides such as chitin or cellulose, and proteins (e.g. gelatin, processed whey proteins), oxidative (aerobic) breakdown of PHA by bacteria or fungi, e.g. during composting, produces CO_2 and water, while anaerobic (anoxic) PHA degradation by living organisms, e.g. in biogas plants, generates CH_4, a potential substrate for new PHA production using methanotrophs, in addition to water and CO_2 [3, 155]. Biodegradability of PHA-based items is dependent on various factors, such as shape and thickness of polymer specimens (packaging films will biodegrade faster than bulky objects), material properties such as crystallinity, molecular mass, and composition of the PHA, and diverse environmental factors such as surrounding microflora, humidity, pH-value, temperature, or UV radiation; the details are elaborated below. This variability in the biodegradation of PHA matches all other biopolymers including starch, cellulose, proteins, or chitin, and elucidation of involved factors requires further investigation, especially to understand biodegradation rate for various types of PHAs, a subject with contradictory findings in the literature [156]. While most studies have reported that high crystallinity reduces the PHA degradation rate (which typically results in higher degradability of *mcl*-PHA than *scl*-PHA) [22], others report no impact of crystallinity on biodegradation [157]. Noteworthy, the scarcely studied homopolyester PHO, which has been biosynthesized in the laboratory and not found in nature, was even reported as not biodegradable at all in one study that has not yet been duplicated [158]. It can be authoritatively acknowledged that all types of PHAs currently produced in processable amounts are biodegradable [22]; as shown for commercially available PHAs like those produced by Kaneka, RWDC Industries, etc., they are not only biodegradable but also certified as "industrial compostable" and "home compostable" [3].

6.5.1 Recycling

Besides biodegradability, PHAs can also be mechanically recycled, chemically pyrolyzed into high value and widely used chemicals, and biologically recycled

to generate methane (and compost); methane can then be upcycled into PHA again [159]. This post-use recycling of PHA is important to avoid the limitation of using PHA for low-value applications only. As is the case with all materials used and recycled today, PHAs would achieve that status of being recycled effectively once a significant quantity of spent PHA becomes available in the future, and it is selectively collected and not mixed with other polymers. Extrusion and injection molding were demonstrated as viable techniques for mechanical recycling of PHA, as shown by Zaverl et al. for the copolyester PHBHV, which did not show any significantly impaired material properties even after five recycling rounds [160], or by Zembouai et al. who recycled PHBHV/PLA blends six times without substantial quality loss [161]. A combination of melt extrusion and compression molding was used by Rivas et al. to process PHB homopolyester; however, significant quality loss was noticed already after two recycling cycles [162]. Nevertheless, we need to consider that mechanical recycling of the spent PHA is still in a very early stage of development, and further studies are needed to evaluate and understand the parameters determining PHA's recyclability, such as composition, crystallinity, and molecular mass. Vu et al. recently reviewed the current lack of knowledge about the interaction of type and composition of PHAs and accessibility of such differently composed types of PHA items to mechanical recycling strategies [159]. Understanding this correlation, however, is the key for the next steps in PHA post-use treatment; it will ultimately decide if spent bioplastic materials need to be separated at the source [163]. Moreover, we should consider that too many different types and grades of PHAs being on the market might complicate its mechanical recycling. For these reasons, PHA is the "Gold Standard" among classes of materials within the contemporary vision of a "Circular Economy," based on the principles of managing waste and pollution in a smart and sustainable way, keeping products and materials in use, and regenerating natural systems. This concept is currently pursued by numerous organizations worldwide [3].

As an alternative to mechanical recycling, spent PHAs can be upgraded chemically via pyrolysis. Known already since the early 1980s, this chemical recycling process generates, depending on the type of treated PHA, compounds such as crotonic acid and oligomers of 3HB (from PHB), and also 2-pentenoic acid (from pyrolyzing PHBHV) [164]. Pyrolysis of the *mcl*-PHA poly(3-hydroxydecanoate) in turn generates 2-decenoic acid [165]. Another advanced recycling approach is based on the microwave-supported reaction of PHA with solvents such as alkaline methanol; this way, the PHB homopolyester undergoes conversion to 3HB, 3-methoxybutyrate, and crotonic acid, as reported by Yang et al. [166]. In addition, poly(3-hydroxypropionate) (P3HP) yields 3HPA, the precursor to acrylic acid [167], while P4HB could yield GBL, 1,4-butane diol or even succinic acid: all important chemical intermediates [168]. This approach shows that several of the bulk chemicals produced today could eventually be produced from renewable sources via the production of PHA as the intermediate material, thereby injecting further circularity to our fossil fuel-dependent world. Moreover, spent PHA can also undergo hydrolysis to generate optically pure building blocks, which in turn can be used as chiral synthons for the synthesis of marketable compounds, such as pharmaceuticals, synthons for organic synthesis, or fragrances. Such chiral

monomers, which have higher market values than the polymers themselves, could considerably contribute to making the entire PHA production process more valuable [169].

6.5.2 Incineration

When neither mechanical or chemical recycling nor composting of PHA is feasible, they can also be incinerated, which is the fate of more than 80% of collected petroleum plastics today in the European Union. Here, we need to consider that the incineration of PHA, based on its chemical composition, creates much less energy than incinerating fossil plastics such as polyolefins; hence, in the PHA case, incineration rather serves waste reduction than energy generation.

Figure 6.7 illustrates the cycle of PHA production and potential end-of-life options for the spent PHA.

6.5.3 Mechanistic Considerations of PHA Degradation

In nature, polymer biodegradation is catalyzed by depolymerases, a specific group of enzymes, mainly hydrolase enzymes. Depolymerases disintegrate the complex polymer molecules into constituent monomers to be used by microbes to generate energy for themselves and/or build up other cellar building blocks [170]. Such depolymerase enzymes are synthesized by numerous microorganisms; some secrete them extracellularly [171], while others have intracellular polymer

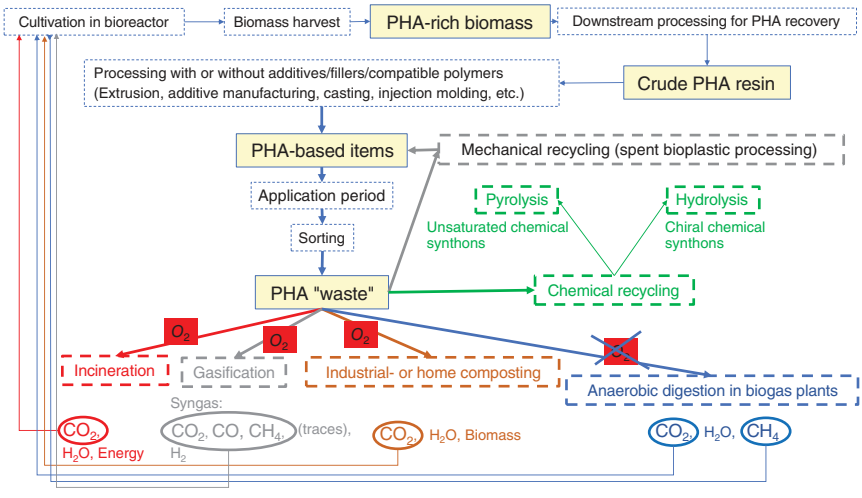

Figure 6.7 Cycle of PHA production, processing, sorting, and potential end-of-life options for spent PHA (PHA "waste"). CO_2 generated by incineration, composting, gasification, or anaerobic digestion can serve as a substrate for autotrophic PHA biosynthesis, e.g. by cyanobacteria. CO originating from gasification can be converted by *Rhodospirillum* sp. to CO_2 and converted to PHA, while CH_4 from biogas plants (anaerobic digestion) can be used for PHA biosynthesis by type II methanotrophs. Modified from [82].

degrading enzymes [172]. PHA depolymerases (PhaZs) specifically hydrolyze PHA polyoxoesters; these enzymes are encoded by *phaZ* genes. Two classes of PHA depolymerases are described, namely extracellular representatives (e-PhaZs), which are of low specificity and degrade almost all PHA polymers into microbially convertible oligomers and monomers, and their intracellular counterparts (i-PhaZs), which are specific to various types of PHA polymers. Typically, PHA are broken down *ex vivo* by extracellular depolymerases excreted by bacteria (e.g. streptomycetes) and fungi and by additional hydrolytic effects with a period of up to one year [173]. Further, e-PhaZs are typically characterized by low molecular mass with the pH optimum in the slightly alkaline range [170].

PHA biodegradation has been investigated over the past four decades and has been compared to various (semi)synthetic polymers. Many reviews summarize PHA biodegradation in soil, buffer solutions, freshwater, and seawater [169, 174]. The best studied PHA, PHB, showed a progressive non-biocatalytic molar mass reduction by almost 50% after 650 days of immersion at 37 °C even in pure water or phosphate-buffered saline [175].

Various factors impact the biodegradability of PHAs, such as [156, 176]

- composition (PHA homopolyesters such as PHB typically degrade slower than copolyesters)
- crystallinity (known to slow down PHA biodegradation compared to less-crystalline PHA polymers)
- molar mass (PHA polymers with lower molar mass typically biodegrade faster than those with higher molar mass)
- stereoregularity
- biodegradation conditions (temperature, humidity, pH-value, and the presence of nutrients for the degrading microorganisms).

In addition, extracellular depolymerases biodegrading *scl*-PHAs are different from those enzymes biodegrading *mcl*-PHA polymers. A range of *scl*-PHA depolymerases (EC 3.1.1.75) have been isolated and studied, while a limited number of *mcl*-PHA depolymerases (EC 3.1.1.76) have been characterized in detail. Specific *mcl*-PHA-degrading enzymes are rarer in microbes; they have been recently reviewed by Urbanek et al. and are known to exist mainly in some representatives of the genera *Pseudomonas* and *Streptomyces* and in some thermophiles [175]. Despite their diverse substrate specificity and amino acid sequence, all PHA depolymerases share a common α/β-hydrolase fold and a catalytic triad, which is also present in other α/β-hydrolases [177].

PHA polymers are also depolymerized by microorganisms intracellularly, catalyzed by i-PhaZs synthesized in the host cells; they mobilize the carbon and energy in the PHA reserves during periods when the microorganisms are lacking exogenous carbon source (see Figure 6.1). This *in vivo* degradation has its onset via i-PhaZs (EC 3.1.1.7x), generating (*R*)-3-hydroxyalkanoate monomers and the corresponding oligomers. The monomers are then reversibly oxidized to acetoacetate by the oxidoreductase (*R*)-3HB dehydrogenase (EC 1.1.1.30). Subsequently, the monomers are catalyzed by the transferase acetoacetyl-CoA synthetase (EC 2.3.1.194) into

acetoacetate, which then is converted to acetoacetyl-CoA and finally hydrolyzed to acetyl-CoA, the central metabolite in all microorganisms [178]. This final reaction is catalyzed by the reversible enzyme 3-ketothiolase (EC 2.3.1.9) described previously in this chapter.

Remarkably, i-PhaZs are not able to hydrolyze isolated, extracellular PHAs, whereas e-PhaZs, when expressed *in vivo*, do not hydrolyze intracellular granules. Two reasons have been identified as the causes: the substantial differences in the physical structure and the crystallinity of native granules *in vivo* and denaturation of extracellular PHA polymers after extraction from biomass [179, 180]. Intracellular PHA depolymerization is known to occur in parallel to PHA accumulation even under conditions favoring PHA biosynthesis (excess carbon source in parallel to limitation of other growth essential nutrients). This causes continuous and simultaneous PHA biosynthesis and PHA biodegradation in living microbes; thus, the polyester chains undergo a steady modification and reorganization, which is frequently described in the literature as the "cyclic nature of the PHA metabolism" [181]. However, under conditions beneficial for PHA biosynthesis, i-PhaZs have considerably lower activity than the PHA synthases active in the same cell. *E. coli* cells generally do not have the i-PhaZs gene; therefore, recombinant *E. coli* containing PHA synthesis genes (*phaCAB*) are able to produce PHA polymers with ultra-high molar mass and low polydispersity [182]. This can be emulated in other natural PHA polymers producing microorganisms by deactivating (or knocking out) the PHA depolymerases from there in the genome, demonstrated recently by Adaya et al. in *A. vinelandii* [183].

The significant impact of PHA crystallinity on its extracellular biodegradation was demonstrated by Choi et al. They produced PHBHV copolyesters with different 3HV fractions produced by the bacterium *Alcaligenes* sp. MT-16 using glucose plus the 3HV-precursor levulinic acid. Increasing 3HV fractions in PHBHV decreased the PHA polymer's crystallinity and resulted in faster extracellular degradation when immersed in solutions of fungal (strain *Emericellopsis minima* W2) e-PhaZ, demonstrating the effect of crystallinity, which in turn depended on the PHA polymer's composition. Biodegradation experiments carried out with the same types of PHA for 20 weeks in non-biotic alkaline medium without the addition of enzymes did not result in any PHA degradation at all, demonstrating the need for these PHA depolymerases in the biodegradation process [184].

All described i- and e-PhaZs in the literature have been demonstrated to be specific to oxoester bonds; e.g. they do not cleave thioester bonds as present in polythioesters (PTEs), a group of sulfur-containing PHA analogs. PTE copolyesters of 3-hydroxyalkanotes and 3-mercaptoalknaotes, such as poly(3HB-*co*-3MP) or poly(3HB-*co*-3MB), are obtained by feeding PHA producers such as *C. necator* precursor compounds such as 3-mercaptobutyrate (3MB) or 3-mercaptovalerate (3MV), in addition to carbon sources such as gluconate. These sulfur-containing substrates are not natural products and are chemically produced, and only a limited number of bacteria such as *Schlegellella thermodepolymerans* biodegraded these PTE polyesters [185]. Interestingly, this strain microorganism was recently identified to produce PHA polymers from inexpensive, xylose-rich carbon sources [186].

6.6 Biodegradation – Added Value for Selected Applications

6.6.1 Packaging

The diversity of PHA monomers identified to date and the thermoplastic nature of these polymers make them a "Platform Polymer" capable of substituting numerous petroleum polymers for durable as well as single-use applications. Among the diverse areas of application, production of PHA-based biodegradable packaging materials is the most obvious one, especially for food packaging, where compostable, transparent packaging materials with high O_2, CO_2, and moisture barrier are desired. After consuming the food, the PHA-based packaging material, which is frequently contaminated with food residues, can simply be disposed of as bio-waste that can be home or industrially composted, reducing plastic waste considerably [187]. PHA polymers exhibit high O_2-barrier properties, which have attracted enormous interest in these materials for food packaging helping to reduce food waste due to oxidation [188]. PHA compares favorably to HDPE in the preservation of quality of packaged food [189]. As far back as 1996, PHBHV received European Union approval for food contact applications in trays, containers, and films for packaging food [190]. The entire food service packaging area could benefit. Recently, the beverage company Bacardi® announced to reduce about 3000 annual tons (corresponding to 80 million bottles) of PET currently used to bottle their alcoholic drinks; the company plans to use biodegradable poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (P(3HB-*co*-3HHx)), a highly auspicious and patented PHA copolyester having the trade name Nodax™ and produced by Danimer Scientific on a commercial scale. Production of this hybrid *scl-mcl*-PHA starts from bioconversion of palm, canola, or soy seed oil. According to Barcardi®, this "100% biodegradable spirit bottles" will biodegrade within 18 months in composting facilities, soil, freshwater, and seawater [191]; the rate of abiotic hydrolysis of PHA-based items in marine environment, however, depends on the shape, thickness, and monomeric composition.

One area that has captured attention recently includes coated paper for food service applications. Most of the food service paper is coated with a petroleum plastic such as PE. Such materials are difficult to recycle, thereby ending up in landfills or being incinerated. The renewable cellulose fiber could be recycled up to seven times if the coating could be separated economically from the fiber. With PE coating, this separation is difficult and often results in high cellulose fiber losses generating PE microplastics that clogs up recycling screens in paper mills or contaminates wastewater processing plants, thus making cellulose fiber recycling uneconomical [192]. The separated PHA polymer particles being biodegradable would aid in the denitrification of wastewater/sludge at wastewater treatment plants; PHA is already used as a denitrification agent in wastewater treatment plants [193]. Coating of paper with PHA can occur via solvent casting [194] or via compression molding of PHA nanocomposite films [195]. At the moment, Danimer Scientific is announcing its partnership with the company Kemira Pulp & Paper to

develop repulpable and biodegradable paper and board coated with Nodax™ PHA instead of PE [196].

Moreover, PHA can also be used to produce mulching films for agricultural use; indeed, the major global PHA manufacturers such as Nafigate, Tianjin GreenBio Materials Co., Ltd. (GreenBio), or Shenzhen Ecomann Biotechnology Co., Ltd. currently produce PHA resins explicitly for manufacturing of mulch films and packaging purposes (reviewed by [197]). Another field of future application could be textile fibers obtained from electrospinning of PHA; this could also contribute to mitigating the microplastic problem when considering the high number of microplastic fibers typically released when washing, e.g. fleece pullovers [198].

6.6.2 Hygiene/Care/Cosmetics

Other applications for PHA polymers exist where recycling is not feasible, and used products generally end up in landfills or are incinerated, such as diapers, wet wipes, and other hygiene-related articles. Currently, many of these products use petroleum plastics that simply accumulate in landfills; however, when produced from PHA polymers, consumers or waste management companies would have multiple end-of-life options, especially in their ability to compost, recover, and recycle the carbon, making such products circular [3].

Moreover, the company NAFIGATE uses PHB produced from waste cooking oil to replace petrochemical primary microplastics for peeling by shower milks and substitutes polluting chemicals such as oxybenzone chemical UV filters in sunscreen creams; these products are already available on the market [199]. Similar products were also commercialized by the Italian company Bio-On under the trademark "Minerv-Bio Cosmetics" and contain microbeads for peeling emulsions such as body and face peelings, cleaning agents, or toothpastes. As micro-sized powder particles, only 5–20 μm in size, they give texture to creamy cosmetic products. Moreover, the lipophilic and hydrophobic nature of such PHA microparticles makes them especially interesting for skin care; they do not remove water from skin but provide greasy skin a natural matt appearance. In the sun protection sector, Bio-On produces sunscreens containing marine biodegradable PHA microparticles (brand name "MyKAI"); here, the high light-scattering effect of nano-sized PHA granules makes this biopolymer the material of choice for this application. In all these cases, PHA replaces petrochemical primary microplastic particles [200].

6.6.3 Medical – Drug Delivery

PHA's biodegradability under different environmental conditions especially *in vivo* makes this class of biopolymers useful candidates for drug carriers. As stated above, extracellular PHA depolymerases and other non-specific enzymes excreted by different microorganisms are thus able to disintegrate PHA polymers into soluble oligomers and monomers and eventually to CO_2 and water. The drug release rate of PHA-based drug delivery systems has been demonstrated to be tuned by the PHA polymer's composition and molecular mass. PHA polymers

have shown to substantially influence the bioavailability of bioactive compounds, improved drug encapsulation with reduced toxicity compared to other biodegradable polymers [201, 202]. As an illustrative example for use of PHA in drug delivery, P(3HB-*co*-3HHx) nanoparticles of a mean size of about 150 nm were loaded with rhodamine-B. These nanoparticles were coated with subcytotoxic poly(ethylene imine) concentrations to support attachment to different cell types and the uptake by these cells. The reaction of cells to these loaded nanoparticles was investigated *in vivo* and *ex vivo*; the nanoparticles were transported along endolysosomal cell compartments, the endoplasmic reticulum, and the Golgi apparatus, without causing any negative effects on cell morphology or respiration [203]. In addition, P(3HB-*co*-3HHx) with 12 mol% 3HHx was processed by electrospinning together with graphene-coated silver nanoparticles. The generated fiber material was used to test its antibacterial activity against the test organisms *S. aureus* and *E. coli*. The results showed that the fibers with embedded nanoparticles strongly reduced the number of living cells; therefore, these new nanocomposites may be efficient in the dressing of chronic wounds and in sanitizing applications [204]. Other "smart wound dressing" based on non-woven films consisting of poly(3HB-*co*-4HB) were successfully tested in reducing inflammation, improving the angiogenic properties of skin and enhancing the wound healing process in rat model experiments. The presence of fibroblast cells on the biopolymer films accelerated wound healing, vascularization, and regeneration; it was shown that cells excreted proteins that formed a layer on the membrane surface and supported the movement of epidermal cells from wound-surrounding tissues [205]. Moreover, the adhesive properties of a *mcl*-PHA consisting of at least five different monomers, produced by the strain *Pseudomonas chlororaphis* from crude glycerol phase from the biodiesel production, were recently demonstrated by Pereira et al.; this semicrystalline material revealed very low melting temperature T_m (44 °C) and glass transition temperature T_g (−48 °C). Films made of this *mcl*-PHA were translucid, highly elastic, water repellent, and relatively permeable to O_2 and CO_2 and demonstrated excellent adhesion properties toward skin, which make them potential candidates for a new class of natural adhesives for wound dressings or closure [206].

Using biodegradable PHA polymers together with other biodegradable nanoparticles such as nanosized cellulose [19] or lignocellulose nanofibers [207] is already yielding promising solutions as bionanocomposites with specific features, such as enhanced gas barrier properties, which in turn can replace multilayered petroleum plastic-based packaging materials that would preserve food from oxidative degradation [208]. In addition, at end of life, such bionanocomposites could be composted to recover the carbon in the form of biogas for subsequent use as a model for a circular economy.

The mechanical properties of PHAs, such as the elastic modulus, tensile strain, and tensile strength of PHAs such as PHB and their composite materials are similar to those measured for human bones; therefore, they hold significant promise for application as implant materials, e.g. to cure femoral fractures. In comparison to polymers used in surgery, such as PLA, poly(glycolate) (PGA), or poly(lactid-*co*-glycolid) (PLGA), PHA-based implants have the added benefit of

not reducing the local pH-value during *in vivo* degradation; this positive attribute makes PHAs compatible with human cells and the immune system. The low *in vivo* degradation rate of *scl*-PHA-based implants and its crystallinity need to be tuned to regulate the enzymatic degradation of the implants, as demonstrated for the remarkable recalcitrance of tiny bar-shaped PHB-based femoral implants against *in vivo* degradation in living rats [209]. Because of their versatility such as tunable mechanical properties, combined with excellent biocompatibility and *in vivo* degradability, PHA biopolyesters are among the most auspicious biomaterials for tissue engineering and are being used to replace and heal different types of hard or soft tissue; PHA-based tissue engineering is described for restoring cartilage, skin, cardiovascular tissues, bone marrow, and nerve conduits (recent reviews by [210, 211]). Moreover, PHAs were tested successfully for bone plates, bone plating systems, and bone marrow scaffolds (reviewed by [212]). Especially, we currently witness various established and new processing techniques in the biomedical field to design medical devices and products from PHAs, such as solvent casting, melt spinning, electrospinning, and additive manufacturing (reviewed by [213]).

6.6.4 Other Applications

For agricultural applications, blends consisting of PLA and PHA were successfully processed into mulches by non-woven textile technology. The authors of the study stated these new mulches to be useful as durable and compostable materials for long-term agricultural applications, such as row covers [214].

Currently, PHAs also start penetrating the toy sector. In 2018, the company Bio-On started commercializing their so-called "Minerv Supertoys," which are LEGO™-like toy bricks made of stained PHA [215].

The ban of plastic materials in various countries offered new chances for PHA to enter new markets; this was shown, e.g. by the US company Newlight, which produces PHAs from GHGs by marine microbes. The produced PHAs (commercialized as "AirCarbon" PHA) are processed to drinking straws and cutlery, which are sold as "Restore™" foodware products [216]. Moreover, the company uses this "AirCarbon" PHA as components of leather replacements in smartphone covers or fashion accessories and for production of eyewear frames [217].

A completely different application of PHAs is the conversion of PHB and *mcl*-PHA to methyl esters of the corresponding building blocks using acid methanolysis. The combustion heat of these products, a new class of biodiesel-like biofuels, was compared with established fuels and fuel additives such as gasoline, ethanol, or 1-butanol. The combustion heat of 3-hydroxyalkanoate methyl esters amounted to 20–30 kJ/g, similar to the value for ethanol (27 kJ/g) [218]. Later, it was shown that addition of 3HB-methyl ester to gasoline even outperforms the addition of ethanol in terms of oxygen content, flash point, boiling point, and dynamic viscosity; blending of gasoline with approximately 10% 3HB-methyl ester only insignificantly reduced the octane number [219]. Notably, PHAs used for the production of such novel biofuels do not need high molecular mass nor high purity; it can therefore be produced in simple, inexpensive, non-sterile setups by mixed microbial cultures using sewage water as a substrate [218].

6.7 Conclusions

The awareness of plastic pollution has thus far resulted in rigid regulations across the world to eliminate or strictly reduce the production and use of many articles from established petroleum plastics. These legislations have frequently banned the use of PHA polymers as well, without having conducted a system-based assessment of the circularity of renewable and biodegradable PHA polymers and the vast potential for benefits to society that these naturally occurring materials can have without the burden of end-of-life problems well known for petroleum plastics.

However, increasing awareness of the damage from fossil fuel use, emissions thereof, and the growing accumulation of plastics waste, especially in ecologically sensitive environment and their deleterious effects on our well-being are becoming increasingly clear to everybody, including consumers; brand owners are looking for solutions with limited success thus far. In this context, it should be emphasized that there is no generally valid recipe for an "ideal PHA" to be produced; as discussed in the text, different applications require different material properties, and PHAs offer a broad variety of such properties for most diverse final fields of application.

Several commercial efforts are underway worldwide to increase PHA polymer production and to translate them into applications to mitigate the negative effects of GHG emissions and plastics waste. Many of these PHAs have suitable attributes to replace fossil plastics. However, it must be recognized that the use of fossil plastics, their incineration and the resulting GHG emissions, and their accumulation in landfills and in our environment are issues that stand on their own. There needs to be a paradigm shift in terms of our use of fossil resource usage and disposal without which their consequences will take on catastrophic consequences. The use of PHA is one such solution. The primary drivers in the adaptation of any material are (i) their safety and suitability in use, (ii) their price, and (iii) their availability. Despite the pioneering work of many researchers since the late 1950s, industry has shown merely sporadic interest in their use. Therefore, PHA price and availability relative to fossil plastics have remained their greatest impediments. Current prices of PHA vary between US$ 4 and US$ 8 per kilogram, while fossil plastics they could replace are priced in the US$ 1 to US$ 2 per kilogram range. Their availability has also remained low; therefore, proliferation has been limited. Recently, Danimer Scientific has announced several joint development and commercialization agreements with Mars Wrigley, Pepsi, and Nestle. They have also announced significant volume ramp-up to several hundred thousand tons per year by 2025. The primary challenges in PHA production continue to be the cost and availability of carbon sources for the production of PHA polymers that do not compete with food production or do not destroy our fragile ecosystems any further. Other challenges in bringing down the overall cost of production are (i) innovating in highly productive microbial cell factories, including the use of mixed microbial cell cultures, (ii) new bioengineering concepts well adapted to cope with the requirements of these cell factories, (iii) challenging raw materials such as CO_2, CH_4, H_2, and other mixed carbon-rich waste streams, and (iv) the efficient and safe methods for PHA polymer recovery from microbial biomass.

The low fossil fuel prices due to the currently slowing global economy as a consequence of the COVID-19 pandemic, resulting in increased use of fossil plastics, in combination with their hygienic benefits, do not necessarily bode well for increased innovation and commercialization efforts in PHA polymers. However, rising public awareness of marine and terrestrial pollution by persistent petroleum plastics, the emerging ubiquitous occurrence of microplastic particles in the ecosphere, the commitment of many nations toward plastic pollution, and the fulfillment of climate targets nations have mutually agreed to, and the indisputable limitation of fossil raw materials will lead to a growing demand for the versatile, biodegradable PHA polymers. Finally, it should be emphasized that instable political situations in important oil-exporting countries can suddenly result in a rocketing of the crude oil price, as witnessed in late February 2022 after the Russian invasion in Ukraine. Such situations might significantly accelerate the switch towards renewables for polymer production.

References

1 Geyer, R., Jambeck, J.R., and Law, K.L. (2017). Production use and fate of all plastics ever made. *Science Advances* 3: e1700782. https://doi.org/10.1126/sciadv.1700782.

2 How to build a circular economy. https://www.ellenmacarthurfoundation.org/publications/the-new-plastics-economy-rethinking-the-future-ofplastics. Ellen MacArthur Foundation (accessed 11 November 2021).

3 Koller, M. and Mukherjee, A. (2020). Polyhydroxyalkanoates–linking properties, applications, and end-of-life options. *Chemical and Biochemical Engineering Quarterly* 34 (3): 115–129. https://doi.org/10.15255/CABEQ.2020.1819.

4 Anastas, P.T. and Warner, J.C. (1998). *Green Chemistry: Theory and Practice*. Oxford/New York: Oxford University Press ISBN 9780198502340.

5 Narodoslawsky, M. (2010). Structural prospects and challenges for bio commodity processes. *Food Technology and Biotechnology* 48 (3): 270–275. https://doaj.org/toc/1334-2606.

6 Schnitzer, H. and Ulgiati, S. (2007). Less bad is not good enough: approaching zero emissions techniques and systems. *Journal of Cleaner Production* 15 (13, 14): 1185–1189. https://doi.org/10.1016/j.jclepro.2006.08.001.

7 Napper, I.E. and Thompson, R.C. (2016). Release of synthetic microplastic plastic fibres from domestic washing machines: effects of fabric type and washing conditions. *Marine Pollution Bulletin* 112 (1, 2): 39–45. https://doi.org/10.1016/j.marpolbul.2016.09.025.

8 Lei, L., Wu, S., Liu, M. et al. (2018). Microplastic particles cause intestinal damage and other adverse effects in zebrafish *Danio rerio* and nematode *Caenorhabditis elegans*. *Science of the Total Environment* 619: 1–8. https://doi.org/10.1016/j.scitotenv.2017.11.103.

9 Al-Jaibachi, R., Cuthbert, R.N., and Callaghan, A. (2018). Up and away: ontogenic transference as a pathway for aerial dispersal of microplastics. *Biology Letters* 14 (9): 20180479. https://doi.org/10.1098/rsbl.2018.0479.

10 Trotter, B., Ramsperger, A.F.R.M., Raab, P., and Laforsch, C. (2019). Plastic waste interferes with chemical communication in aquatic ecosystems. *Scientific Reports* 9 (1): 5889. https://doi.org/10.1038/s41598-019-41677-1.

11 Eerkes-Medrano, D., Leslie, H.A., and Quinn, B. (2019). Microplastics in drinking water: a review and assessment. *Current Opinion in Environmental Science & Health* 7: 69–75. https://doi.org/10.1038/s41598-019-41677-1.

12 Peixoto, D., Pinheiro, C., Amorim, J. et al. (2019). Microplastic pollution in commercial salt for human consumption: a review. *Estuarine, Coastal and Shelf Science* 219: 161–168. https://doi.org/10.1016/j.ecss.2019.02.018.

13 Liebezeit, G., and Liebezeit, E. (2013). Non-pollen particulates in honey and sugar. *Food Additives & Contaminants: Part A* 30 (12), 2136–2140. https://doi.org/10.1080/19440049.2013.843025.

14 Liebezeit, G., and Liebezeit, E. (2014). Synthetic particles as contaminants in German beers. *Food Additives & Contaminants: Part A* 31 (9): 1574–1578. https://doi.org/10.1080/19440049.2014.945099.

15 Bitencourt, G.R., Mello, P.A., Flores, E.M. et al. (2020). Determination of microplastic content in seafood: an integrated approach combined with the determination of elemental contaminants. *Science of the Total Environment* 749: 142301. https://doi.org/10.1016/j.scifotenv.2020.142301 (accessed 11 November 2021).

16 https://www.wri.org/insights/127-countries-now-regulate-plastic-bags-why-arent-we-seeing-less-pollution. World Resources Institute (accessed 11 November 2021).

17 Barrena, R., Font, X., Gabarrell, X., and Sánchez, A. (2014). Home composting versus industrial composting: influence of composting system on compost quality with focus on compost stability. *Waste Management* 34 (7): 1109–1116. https://doi.org/10.1016/j.wasman.2014.02.008.

18 Kourmentza, C., Plácido, J., Venetsaneas, N. et al. (2017). Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. *Bioengineering* 4: 55. https://doi.org/10.3390/bioengineering4020055.

19 Silva, F.A., Dourado, F., Gama, M., and Poças, F. (2020). Nanocellulose bio-based composites for food packaging. *Nanomaterials* 10 (10): 2041. https://doi.org/10.3390/nano10102041.

20 Koller, M. (2018). Chemical and biochemical engineering approaches in manufacturing polyhydroxyalkanoate (PHA) biopolyesters of tailored structure with focus on the diversity of building blocks. *Chemical and Biochemical Engineering Quarterly* 32 (4): 413–438. https://doi.org/10.15255/CABEQ.2018.1385.

21 Chen, G.Q. and Hajnal, I. (2015). The 'PHAome'. *Trends in Biotechnology* 33 (10): 559–564. https://doi.org/10.1016/j.tibtech.2015.07.006.

22 Doi, Y., Kanesawa, Y., Kunioka, M., and Saito, T. (1990). Biodegradation of microbial copolyesters: poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate). *Macromolecules* 23: 26. https://doi.org/0024-9297/90/2223-0026802.50/0.

23 Koller, M. (2020). Advances in polyhydroxyalkanoate (PHA) production, volume 2. *Bioengineering* 7 (1): 24. https://doi.org/10.3390/bioengineering7010024.

24 Jendrossek, D. (2009). Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes). *Journal of Bacteriology* 191 (10): 3195–3202. https://doi.org/10.1128/JB.01723-08.

25 Koller, M., Maršálek, L., Miranda de Sousa Dias, M., and Braunegg, G. (2017). Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. *New Biotechnology* 37 (A): 24–38. https://doi.org/10.1016/j.nbt.2016.05.001.

26 Obruca, S., Sedlacek, P., Koller, M. et al. (2018). Involvement of polyhydroxyalkanoates in stress resistance of microbial cells: biotechnological consequences and applications. *Biotechnology Advances* 36 (3): 856–870. https://doi.org/10.1016/j.biotechadv.2017.12.006.

27 Obruca, S., Sedlacek, P., Slaninova, E. et al. (2020). Novel unexpected functions of PHA granules. *Applied Microbiology and Biotechnology* 104: 4795–4810. https://doi.org/10.1007/s00253-020-10568-1.

28 Müller-Santos, M., Koskimäki, J.J., Alves, L.P.S. et al. (2020). The protective role of PHB and its degradation products against stress situations in bacteria. *FEMS Microbiology Reviews* fuaa058. https://doi.org/10.1093/femsre/fuaa058.

29 Sedlacek, P., Slaninova, E., Koller, M. et al. (2019). PHA granules help bacterial cells to preserve cell integrity when exposed to sudden osmotic imbalances. *New Biotechnology* 49: 129–136. https://doi.org/10.1016/j.nbt.2018.10.005.

30 Pernicova, I., Novackova, I., Sedlacek, P. et al. (2020). Application of osmotic challenge for enrichment of microbial consortia in polyhydroxyalkanoates producing thermophilic and thermotolerant bacteria and their subsequent isolation. *International Journal of Biological Macromolecules* 144: 698–704. https://doi.org/10.1016/j.ijbiomac.2019.12.128.

31 Obruca, S., Sedlacek, P., Mravec, F. et al. (2016). Evaluation of 3-hydroxybutyrate as an enzyme-protective agent against heating and oxidative damage and its potential role in stress response of poly(3-hydroxybutyrate) accumulating cells. *Applied Microbiology and Biotechnology* 100 (3) https://doi.org/1365-1376: doi: 10.1007/s00253-015-7162-4.

32 Al Rowaihi, I.S., Paillier, A., Rasul, S. et al. (2018). Poly(3-hydroxybutyrate) production in an integrated electromicrobial setup: investigation under stress-inducing conditions. *PLoS One* 13 (4): e0196079. https://doi.org/10.1371/journal.pone.0196079.

33 Ayub, N.D., Tribelli, P.M., and López, N.I. (2009). Polyhydroxyalkanoates are essential for maintenance of redox state in the Antarctic bacterium *Pseudomonas* sp. 14-3 during low temperature adaptation. *Extremophiles* 13 (1): 59–66. https://doi.org/10.1007/s00792-008-0197-z.

34 Obruca, S., Sedlacek, P., Krzyzanek, V. et al. (2016). Accumulation of poly(3-hydroxybutyrate) helps bacterial cells to survive freezing. *PLoS One* 11 (6): e0157778. https://doi.org/10.1371/journal.pone.0157778.

35 Slaninova, E., Sedlacek, P., Mravec, F. et al. (2018). Light scattering on PHA granules protects bacterial cells against the harmful effects of UV radiation. *Applied Microbiology and Biotechnology* 102 (4): 1923–1931. https://doi.org/10.1007/s00253-018-8760-8.

36 Tan, G.-Y.A., Chen, C.L., Li, L. et al. (2014). Start a research on biopolymer polyhydroxyalkanoate (PHA): a review. *Polymers* 6: 706. https://doi.org/10.3390/polym6030706.

37 Chen, G.Q. (2009). A microbial polyhydroxyalkanoates (PHA) based bio-and materials industry. *Chemical Society Reviews* 38 (8): 2434–2446. https://doi.org/10.1039/B812677C.

38 Kucera, D., Novackova, I., Pernicova, I. et al. (2019). Biotechnological production of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate-*co*-3-hydroxyvalerate) terpolymer by *Cupriavidus* sp. DSM 19379. *Bioengineering* 6 (3): 74. https://doi.org/10.3390/bioengineering6030074.

39 Zinn, M. (2010). Biosynthesis of medium-chain-length poly[(*R*)-3-hydroxyalkanoates]. In: *Plastics from Bacteria* (ed. G.Q. Chen), 213–236. Berlin, Heidelberg: Springer https://doi.org/10.1007/978-3-642-03287-5_9.

40 Ramsay, B. (2016). Medium-chain-length Poly-3-hydroxyalkanoates. In: *Recent Advances in BiotechnologyMicrobiology, Feedstocks, and Metabolism*, vol. 1 (ed. M. Koller), 436–473. Sharjah: Bentham Science Publishers, ISSN 2468-5372(online), 2468-5364 (print). https://www.eurekaselect.net/ebook_volume/2006.

41 De Koning, G.J.M., Van Bilsen, H.M.M., Lemstra, P.J., Hazenberg, W., Witholt, B., Preusting, H., van der Galiën, J.G., Schirmer, A., Jendrossek, D. (1994). A biodegradable rubber by crosslinking poly(hydroxyalkanoate) from *Pseudomonas oleovorans*. *Polymer* 35: 2090-2097. https://doi.org/10.1016/0032-3861(94)90233-X.

42 Yamane, T. (1992). Cultivation engineering of microbial bioplastics production. *FEMS Microbiology Reviews* 9 (2–4): 257–264.

43 Yamane, T. (1993). Yield of poly-D(−)-3-hydroxybutyrate from various carbon sources: a theoretical study. *Biotechnology and Bioengineering* 41 (1): 165–170.

44 Pan, C., Ge, L., Lee, P.-H., and Tan, G.-J.A. (2021). An Introduction to the Thermodynamics Calculation of PHA Production in Microbes. In: *The Handbook of Polyhydroxyalkanoates*, Kinetics, Bioengineering, and Industrial Aspects, vol. II (ed. M. Koller), 3–19. CRC Press, Taylor & Francis Group ISBN 9780367275624. https://www.routledge.com/The-Handbook-of-Polyhydroxyalkanoates-Kinetics-Bioengineering-and-Industrial/Koller/p/book/9780367275624.

45 da Cruz Pradella, J.G., Ienczak, J.L., Delgado, C.R., and Taciro, M.K. (2012). Carbon source pulsed feeding to attain high yield and high productivity in poly(3-hydroxybutyrate) (PHB) production from soybean oil using *Cupriavidus necator*. *Biotechnology Letters* 34 (6): 1003–1007. https://doi.org/10.1007/s10529-012-0863-1.

46 Miao, R., Xie, H., Liu, X. et al. (2020). Current processes and future challenges of photoautotrophic production of acetyl-CoA-derived solar fuels and chemicals in cyanobacteria. *Current Opinion in Chemical Biology* 59: 69–76. https://doi.org/10.1016/j.cbpa.2020.04.013.

47 Rehm, B.H. (2003). Polyester synthases: natural catalysts for plastics. *Biochemical Journal* 376 (1): 15–33. https://doi.org/10.1042/bj20031254.

48 Wang, H.H., Zhou, X.R., Liu, Q., and Chen, G.Q. (2011). Biosynthesis of polyhydroxyalkanoate homopolymers by *Pseudomonas putida*. *Applied Microbiology and Biotechnology* 89 (5): 1497–1507. https://doi.org/10.1007/s00253-010-2964-x.

49 Bonilla, M., Olivaro, C., Corona, M. et al. (2005). Production and characterization of a new bioemulsifier from *Pseudomonas putida* ML2. *Journal of Applied Microbiology* 98 (2): 456–463. https://doi.org/10.1111/j.1365-2672.2004.02480.x.

50 Amaro, T.M., Rosa, D., Comi, G., and Iacumin, L. (2019). Prospects for the use of whey for polyhydroxyalkanoate (PHA) production. *Frontiers in Microbiology* 10: 992. https://doi.org/10.3389/fmicb.2019.00992.

51 Basnett, P., Marcello, E., Lukasiewicz, B. et al. (2020). Antimicrobial materials with lime oil and a poly(3-hydroxyalkanoate) produced via valorisation of sugar cane molasses. *Journal of Functional Biomaterials* 11 (2): 24. https://doi.org/10.3390/jfb11020024.

52 Takahashi, R.Y.U., Castilho, N.A.S., Silva, M.A.C.D. et al. (2017). Prospecting for marine bacteria for polyhydroxyalkanoate production on low-cost substrates. *Bioengineering* 4 (3): 60. https://doi.org/10.3390/bioengineering4030060.

53 Obruca, S., Benesova, P., Maršalek, L., and Marova, I. (2015). Use of lignocellulosic materials for PHA production. *Chemical and Biochemical Engineering Quarterly* 29 (2): 135–144. https://doi.org/10.15255/CABEQ.2014.2253.

54 González-García, Y., Grieve, J., Meza-Contreras, J.C. et al. (2019). Tequila agave bagasse hydrolysate for the production of polyhydroxybutyrate by *Burkholderia sacchari*. *Bioengineering* 6 (4): 115. https://doi.org/10.3390/bioengineering6040115.

55 Salgaonkar, B.B. and Bragança, J.M. (2017). Utilization of sugarcane bagasse by *Halogeometricum borinquense* strain E3 for biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate). *Bioengineering* 4 (2): 50. https://doi.org/10.3390/bioengineering4020050.

56 Rebocho, A.T., Pereira, J.R., Freitas, F. et al. (2019). Production of medium-chain length polyhydroxyalkanoates by *Pseudomonas citronellolis* grown in apple pulp waste. *Applied Food Biotechnology* 6 (1): 71–82. https://doi.org/10.22037/afb.v6i1.21793.

57 Kovalcik, A., Pernicova, I., Obruca, S. et al. (2020). Grape winery waste as a promising feedstock for the production of polyhydroxyalkanoates and other value-added products. *Food and Bioproducts Processing* 124: 1–10. https://doi.org/10.1016/j.fbp.2020.08.003.

58 Bhalerao, A., Banerjee, R., and Nogueira, R. (2020). Continuous cultivation strategy for yeast industrial wastewater-based polyhydroxyalkanoate production. *Journal of Bioscience and Bioengineering* 129 (5): 595–602. https://doi.org/10.1016/j.jbiosc.2019.11.006.

59 Gurav, R., Bhatia, S.K., Moon, Y.M. et al. (2019). One-pot exploitation of chitin biomass for simultaneous production of electricity, *N*-acetylglucosamine and polyhydroxyalkanoates in microbial fuel cell using novel marine bacterium *Arenibacter palladensis* YHY2. *Journal of Cleaner Production* 209: 324–332. https://doi.org/10.1016/j.jclepro.2018.10.252.

60 Kumar, P. and Kim, B.S. (2019). *Paracoccus* sp. strain LL1 as a single cell factory for the conversion of waste cooking oil to polyhydroxyalkanoates and carotenoids. *Applied Food Biotechnology* 6 (1): https://doi.org/10.22037/afb.v6i1.21628.

61 Talan, A., Kaur, R., Tyagi, R.D., and Drogui, P. (2020). Bioconversion of oily waste to polyhydroxyalkanoates: sustainable technology with circular bioeconomy approach and multidimensional impacts. *Bioresource Technology Reports* 11: 100496. https://doi.org/10.1016/j.biteb.2020.100496.

62 Bustamante, D., Tortajada, M., Ramon, D., and Rojas, A. (2019). Camelina oil as a promising substrate for *mcl*-PHA production in *Pseudomonas* sp. cultures. *Applied Food Biotechnology* 6 (1): 61–70. https://doi.org/10.22037/afb.v6i1.21635.

63 Koller, M., Shahzad, K., and Braunegg, G. (2018). Waste streams of the animal-processing industry as feedstocks to produce polyhydroxyalkanoate biopolyesters. *Applied Food Biotechnology* 5 (4): 193–203. https://doi.org/10.22037/afb.v5i4.18557.

64 Favaro, L., Basaglia, M., and Casella, S. (2019). Improving polyhydroxyalkanoate production from inexpensive carbon sources by genetic approaches: a review. *Biofuels, Bioproducts and Biorefining* 13 (1): 208–227. https://doi.org/10.1002/bbb.1944.

65 Reis, M.A.M., Serafim, L.S., Lemos, P.C. et al. (2003). Production of polyhydroxyalkanoates by mixed microbial cultures. *Bioprocess and Biosystems Engineering* 25 (6): 377–385. https://doi.org/10.1007/s00449-003-0322-4.

66 Oliveira, C.S., Silva, C.E., Carvalho, G., and Reis, M.A.M. (2017). Strategies for efficiently selecting PHA producing mixed microbial cultures using complex feedstocks: feast and famine regime and uncoupled carbon and nitrogen availabilities. *New Biotechnology* 37 (A): 69–79. https://doi.org/10.1016/j.nbt.2016.10.008.

67 Chan, C.M., Johansson, P., Magnusson, P. et al. (2017). Mixed culture polyhydroxyalkanoate-rich biomass assessment and quality control using thermogravimetric measurement methods. *Polymer Degradation and Stability* 144: 110–120. https://doi.org/10.1016/j.polymdegradstab.2017.07.029.

68 Valentino, F., Gottardo, M., Micolucci, F. et al. (2018). Organic fraction of municipal solid waste recovery by conversion into added-value polyhydroxyalkanoates and biogas. *ACS Sustainable Chemistry & Engineering* 6 (12): 16375–16385. https://doi.org/10.1021/acssuschemeng.8b03454.

69 Colombo, B., Sciarria, T.P., Reis, M. et al. (2016). Polyhydroxyalkanoates (PHAs) production from fermented cheese whey by using a mixed microbial culture. *Bioresource Technology* 218: 692–699. https://doi.org/10.1016/j.biortech.2016.07.024.

70 Moretto, G., Russo, I., Bolzonella, D. et al. (2020). An urban biorefinery for food waste and biological sludge conversion into polyhydroxyalkanoates and biogas. *Water Research* 170: 115371. https://doi.org/10.1016/j.watres.2019.115371.

71 Moita, R., Freches, A., and Lemos, P.C. (2014). Crude glycerol as feedstock for polyhydroxyalkanoates production by mixed microbial cultures. *Water Research* 58: 9–20. https://doi.org/10.1016/j.watres.2014.03.066.

72 Meixner, K., Kovalcik, A., Sykacek, E. et al. (2018). Cyanobacteria biorefinery – production of poly(3-hydroxybutyrate) with *Synechocystis salina* and utilisation of residual biomass. *Journal of Biotechnology* 265: 46–53. https://doi.org/10.1016/j.jbiotec.2017.10.020.

73 Kamravamanesh, D., Lackner, M., and Herwig, C. (2018). Bioprocess engineering aspects of sustainable polyhydroxyalkanoate production in cyanobacteria. *Bioengineering* 5 (4): 111. https://doi.org/10.3390/bioengineering5040111.

74 Price, S., Kuzhiumparambil, U., Pernice, M., and Ralph, P.J. (2020). Cyanobacterial polyhydroxybutyrate for sustainable bioplastic production: critical review and perspectives. *Journal of Environmental Chemical Engineering* 8 (4): 104007. https://doi.org/10.1016/j.jece.2020.104007.

75 Troschl, C., Meixner, K., and Drosg, B. (2017). Cyanobacterial PHA production – review of recent advances and a summary of three years' working experience running a pilot plant. *Bioengineering* 4 (2): 26. https://doi.org/10.3390/bioengineering4020026.

76 Gomes Gradíssimo, D., Pereira Xavier, L., and Valadares Santos, A. (2020). Cyanobacterial polyhydroxyalkanoates: a sustainable alternative in circular economy. *Molecules* 25 (18): 4331. https://doi.org/10.3390/molecules25184331.

77 da Silva, C.K., Costa, J.A.V., and de Morais, M.G. (2018). Polyhydroxybutyrate (PHB) synthesis by *Spirulina* sp. LEB 18 using biopolymer extraction waste. *Applied Biochemistry and Biotechnology* 185 (3): 822–833. https://doi.org/10.1007/s12010-017-2687-x.

78 Bhati, R. and Mallick, N. (2016). Carbon dioxide and poultry waste utilization for production of polyhydroxyalkanoate biopolymers by *Nostoc muscorum* Agardh: a sustainable approach. *Journal of Applied Phycology* 28: https://doi.org/161–168. doi: 10.1007/s10811-015-0573-x.

79 Tarawat, S., Incharoensakdi, A., and Monshupanee, T. (2020). Cyanobacterial production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) from carbon dioxide or a single organic substrate: improved polymer elongation with an extremely high 3-hydroxyvalerate mole proportion. *Journal of Applied Phycology* 32: 1095–1102. https://doi.org/10.1007/s10811-020-02040-4.

80 Amstutz, V. and Zinn, M. (2020). Syngas as a sustainable carbon source for PHA production. In: *The Handbook of Polyhydroxyalkanoates*, Microbial Biosynthesis and Feedstocks, vol. I (ed. M. Koller), 377–416. CRC Press, Taylor & Francis Group ISBN 9780367275594.

81 Karmann, S., Panke, S., and Zinn, M. (2019). Fed-batch cultivations of *Rhodospirillum rubrum* under multiple nutrient-limited growth conditions on syngas as a novel option to produce poly(3-hydroxybutyrate) (PHB). *Frontiers in Bioengineering and Biotechnology* 7: 59. https://doi.org/10.3389/fbioe.2019.00059.

82 Heinrich, D., Raberg, M., Fricke, P. et al. (2016). Synthesis gas (syngas)-derived medium-chain-length polyhydroxyalkanoate synthesis in engineered *Rhodospirillum rubrum*. *Applied and Environmental Microbiology* 82 (20): 6132–6140. https://doi.org/10.1128/AEM.01744-16.

83 Revelles, O., Beneroso, D., Menendez, J.A. et al. (2017). Syngas obtained by microwave pyrolysis of household wastes as feedstock for polyhydroxyalkanoate production in *Rhodospirillum rubrum*. *Microbial Biotechnology* 10 (6): 1412–1417. https://doi.org/10.1111/1751-7915.12411.

84 Sadykov, V., Bobrova, L., Pavlova, S. et al. (2012). *Syngas Generation from Hydrocarbons and Oxygenates with Structured Catalysts* (ed. A. Kurucz and I. Bencik), 53–140. New York: Nova Science Publishers, Inc. ISBN-13: 978-1608763238, ISBN-10: 1608763234.

85 Sharmila, G., Muthukumaran, C., Kumar, N.M. et al. (2020). Food waste valorization for biopolymer production. In: *Current Developments in Biotechnology and Bioengineering* (ed. S. Varjani, A. Pandey, E. Gnansounou, et al.), 233–249. Amsterdam: Elsevier B.V https://doi.org/10.1016/B978-0-444-64321-6.00012-4.

86 Flüchter, S., Follonier, S., Schiel-Bengelsdorf, B. et al. (2019). Anaerobic production of poly(3-hydroxybutyrate) and its precursor 3-hydroxybutyrate from synthesis gas by autotrophic clostridia. *Biomacromolecules* 20 (9): 3271–3282. https://doi.org/10.1021/acs.biomac.9b00342.

87 Moradi, M., Rashedi, H., Mofradnia, S.R. et al. (2019). Polyhydroxybutyrate production from natural gas in a bubble column bioreactor: simulation using COMSOL. *Bioengineering* 6 (3): 84. https://doi.org/10.3390/bioengineering6030084.

88 Rahnama, F., Vasheghani-Farahani, E., Yazdian, F., and Shojaosadati, S.A. (2012). PHB production by *Methylocystis hirsuta* from natural gas in a bubble column and a vertical loop bioreactor. *Biochemical Engineering Journal* 65: 51–56. https://doi.org/10.1016/j.bej.2012.03.014.

89 Ghoddosi, F., Golzar, H., Yazdian, F. et al. (2019). Effect of carbon sources for PHB production in bubble column bioreactor: emphasis on improvement of methane uptake. *Journal of Environmental Chemical Engineering* 7 (2): 102978. https://doi.org/10.1016/j.jece.2019.102978.

90 Levett, I., Birkett, G., Davies, N. et al. (2016). Techno-economic assessment of poly-3-hydroxybutyrate (PHB) production from methane – the case for thermophilic bioprocessing. *Journal of Environmental Chemical Engineering* 4 (4): 3724–3733. https://doi.org/10.1016/j.jece.2016.07.033.

91 https://www.calysta.com/our-technology. Calysta, Inc. (accessed 11 November 2021).

92 Jane Byrne. Single cell producer Unibio signs commercial deal with Chilean distribution giant. https://www.feednavigator.com/Article/2020/12/14/Single-cell-producer-Unibio-signs-commercial-deal-with-Chilean-distribution-giant (accessed 11 November 2021).

93 Mike Wither This company harvest CO_2 and methane to make carbon-negative plastics. https://nowthisnews.com/earth/this-company-harvest-co2-methane-to-make-carbon-negative-plastics (accessed 11 November 2021).

94 Mango materials moves on methane gas for bioplastics. https://www.mangomaterials.com/news-press/mango-materials-moves-on-methane-gas-for-bioplastics. Mango Materials (accessed 11 November 2021).

95 Liu, L.Y., Xie, G.J., Xing, D.F. et al. (2020). Biological conversion of methane to polyhydroxyalkaonate: current advances, challenges, and perspectives. *Environmental Science and Ecotechnology* 2: 100029. https://doi.org/10.1016/j.ese.2020.100029.

96 Khosravi-Darani, K., Yazdian, F., Babapour, F., and Amirsadeghi, A.R. (2019). Poly(3-hydroxybutyrate) production from natural gas by a methanotroph native bacterium in a bubble column bioreactor. *Chemical and Biochemical Engineering Quarterly* 33 (1): 69–77. https://doi.org/10.15255/CABEQ.2017.1263.

97 Orita, I., Nishikawa, K., Nakamura, S., and Fukui, T. (2014). Biosynthesis of polyhydroxyalkanoate copolymers from methanol by *Methylobacterium extorquens* AM1 and the engineered strains under cobalt-deficient conditions. *Applied Microbiology and Biotechnology* 98 (8): 3715–3725. https://doi.org/10.1007/s00253-013-5490-9.

98 Koller, M. (2020). Production, properties, and processing of microbial polyhydroxyalkanoate (PHA) biopolyesters. In: *Microbial and Natural Macromolecules – Synthesis and Applications* (ed. S. Das and H.R. Dash), 3–55. Amsterdam: Elsevier Inc ISBN: 978-0-12-820084-1. https://doi.org/10.1016/C2019-0-00320-2.

99 Litchfield, C.D. (2011). Potential for industrial products from the halophilic archaea. *Journal of Industrial Microbiology & Biotechnology* 38 (10): 1635. https://doi.org/10.1007/s10295-011-1021-9.

100 Koller, M., Salerno, A., Dias, M. et al. (2010). Modern biotechnological polymer synthesis: a review. *Food Technol Biotechnology* 48 (3): 255–269.

101 Poirier, Y., Dennis, D.E., Klomparens, K., and Somerville, C. (1992). Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. *Science* 256 (5056): 520–523. https://doi.org/10.1126/science.256.5056.520.

102 Poirier, Y., Nawrath, C., and Somerville, C. (1995). Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. *Bio/Technology* 13 (2): 142–150. https://doi.org/10.1038/nbt0295-142.

103 Poirier, Y. and Brumbley, S.M. (2010). Metabolic engineering of plants for the synthesis of polyhydroxyalkanaotes. In: *Plastics from Bacteria* (ed. G.-Q. Chen), 187–211. Berlin, Heidelberg: Springer https://doi.org/10.1007/978-3-642-03287-5_8. ISBN: 978-3-642-03287-5 (online), 978-3-642-03286-8 (print).

104 van Beilen, J.B. and Poirier, Y. (2012). Plants as factories for bioplastics and other novel biomaterials. In: *Plant Biotechnology and Agriculture* (ed. A. Altman and P.M. Hasegawa), 481–494. Amsterdam: Elsevier Inc. ISBN: 978-0-12-381466-1. https://doi.org/10.1016/C2009-0-61282-4.

105 Robert, J., Marchesini, S., Delessert, S., and Poirier, Y. (2005). Analysis of the β-oxidation of trans-unsaturated fatty acid in recombinant *Saccharomyces cerevisiae* expressing a peroxisomal PHA synthase reveals the involvement of a reductase-dependent pathway. *Biochimica et Biophysica Acta (BBA) – Molecular and Cell Biology of Lipids* 1734 (2): 169–177. https://doi.org/10.1016/j.bbalip.2005.02.010.

106 Poirier, Y., Erard, N., and MacDonald-Comber Petétot, J. (2002). Synthesis of polyhydroxyalkanoate in the peroxisome of *Pichia pastoris*. *FEMS Microbiology Letters* 207 (1): 97–102. https://doi.org/10.1111/j.1574-6968.2002.tb11035.x.

107 Haddouche, R., Poirier, Y., Delessert, S. et al. (2011). Engineering polyhydroxyalkanoate content and monomer composition in the oleaginous yeast *Yarrowia lipolytica* by modifying the ß-oxidation multifunctional protein. *Applied Microbiology and Biotechnology* 91 (5): 1327–1340. https://doi.org/10.1007/s00253-011-3331-2.

108 Gao, C., Qi, Q., Madzak, C., and Lin, C.S.K. (2015). Exploring medium-chain-length polyhydroxyalkanoates production in the engineered yeast *Yarrowia lipolytica*. *Journal of Industrial Microbiology & Biotechnology* 42 (9): 1255–1262. https://doi.org/10.1007/s10295-015-1649-y.

109 Abd-El-Haleem, D.A. (2009). Biosynthesis of polyhydroxyalkanotes in wild type yeasts. *Polish Journal of Microbiology* 58 (1): 37–41.

110 Williams, M.D., Rahn, J.A., and Sherman, D.H. (1996). Production of a polyhydroxyalkanoate biopolymer in insect cells with a modified eucaryotic fatty acid synthase. *Applied and Environmental Microbiology* 62 (7): 2540–2546. 0099-2240/96/$04.0010.

111 Koller, M. (2018). A review on established and emerging fermentation schemes for microbial production of polyhydroxyalkanoate (PHA) biopolyesters. *Fermentation* 4 (2): 30. https://doi.org/10.3390/fermentation4020030.

112 Blunt, W., Levin, D., and Cicek, N. (2018). Bioreactor operating strategies for improved polyhydroxyalkanoate (PHA) productivity. *Polymers* 10 (11): 1197. https://doi.org/10.3390/polym10111197.

113 Sindhu, R., Silviya, N., Binod, P., and Pandey, A. (2013). Pentose-rich hydrolysate from acid pretreated rice straw as a carbon source for the production of poly-3-hydroxybutyrate. *Biochemical Engineering Journal* 78: 67–72. https://doi.org/10.1016/j.bej.2012.12.015.

114 Gahlawat, G. and Srivastava, A.K. (2018). Enhancing the production of polyhydroxyalkanoate biopolymer by *Azohydromonas australica* using a simple empty and fill bioreactor cultivation strategy. *Chemical and Biochemical Engineering Quarterly* 31 (4): 479–485. https://doi.org/10.15255/CABEQ.2017.1148.

115 Penloglou, G., Vasileiadou, A., Chatzidoukas, C., and Kiparissides, C. (2017). Model-based intensification of a fed-batch microbial process for the maximization of polyhydroxybutyrate (PHB) production rate. *Bioprocess and Biosystems Engineering* 40: 1247–1260. https://doi.org/10.1007/s00449-017-1784-0.

116 Ibrahim, M.H. and Steinbüchel, A. (2010). High-cell-density cyclic fed-batch fermentation of a poly(3-hydroxybutyrate)-accumulating thermophile, *Chelatococcus* sp. strain MW10. *Applied and Environmental Microbiology* 76 (23): 7890–7895. https://doi.org/10.1128/AEM.01488-10.

117 Haas, C., El-Najjar, T., Virgolini, N. et al. High cell-density production of poly(3-hydroxybutyrate) in a membrane bioreactor. *New Biotechnology* 37 (A): 117–122. https://doi.org/10.1016/j.nbt.2016.06.1461.

118 Koller, M. and Muhr, A. (2014). Continuous production mode as a viable process-engineering tool for efficient poly(hydroxyalkanoate) (PHA)

bio-production. *Chemical and Biochemical Engineering Quarterly* 28 (1): 65–77. https://hrcak.srce.hr/117989.

119 Atlić, A., Koller, M., Scherzer, D. et al. (2011). Continuous production of poly([*R*]-3-hydroxybutyrate) by *Cupriavidus necator* in a multistage bioreactor cascade. *Applied Microbiology and Biotechnology* 91 (2): 295–304. https://doi.org/10.1007/s00253-011-3260-0.

120 Horvat, P., Špoljarić, I.V., Lopar, M. et al. (2013). Mathematical modelling and process optimization of a continuous 5-stage bioreactor cascade for production of poly [−(*R*)-3-hydroxybutyrate] by *Cupriavidus necator*. *Bioprocess and Biosystems Engineering* 36 (9): 1235–1250. https://doi.org/10.1007/s00449-012-0852-8.

121 Lopar, M., Špoljarić, I.V., Atlić, A. et al. (2013). Five-step continuous production of PHB analyzed by elementary flux, modes, yield space analysis and high structured metabolic model. *Biochemical Engineering Journal* 79: 57–70. https://doi.org/10.1016/j.bej.2013.07.003.

122 Vadlja, D., Koller, M., Novak, M. et al. (2016). Footprint area analysis of binary imaged *Cupriavidus necator* cells to study PHB production at balanced, transient, and limited growth conditions in a cascade process. *Applied Microbiology and Biotechnology* 100 (23): 10065–10080. https://doi.org/10.1007/s00253-016-7844-6.

123 Koller, M., Vadlja, D., Braunegg, G. et al. (2017). Formal-and high-structured kinetic process modelling and footprint area analysis of binary imaged cells: tools to understand and optimize multistage-continuous PHA biosynthesis. *The EuroBiotech Journal* 1 (3): 203–211. https://doi.org/10.24190/ISSN2564-615X/2017/03.01.

124 Strong, P., Laycock, B., Mahamud, S. et al. (2016). The opportunity for high-performance biomaterials from methane. *Microorganisms* 4 (1): 11. https://doi.org/10.3390/microorganisms4010011.

125 Karmann, S., Follonier, S., Egger, D. et al. (2017). Tailor-made PAT platform for safe syngas fermentations in batch, fed-batch and chemostat mode with *Rhodospirillum rubrum*. *Microbial Biotechnology* 10 (6): 1365–1375. https://doi.org/10.1111/1751-7915.12727.

126 Singh, A.K. and Mallick, N. (2017). Advances in cyanobacterial polyhydroxyalkanoates production. *FEMS Microbiology Letters* 364 (20): fnx189. https://doi.org/10.1093/femsle/fnx189.

127 Koller, M., Khosravi-Darani, K., and Braunegg, G. (2017). Advanced photobioreactor systems for the efficient cultivation of cyanobacteria. In: *Photobioreactors Advancements, Applications and Research* (ed. Y.F. Tsang), 35–90. New York: Nova Science Publishers, Inc ISBN: 1536123544, 9781536123548.

128 Costa, J.A.V., Moreira, J.B., Lucas, B.F. et al. (2018). Recent advances and future perspectives of PHB production by cyanobacteria. *Industrial Biotechnology* 14 (5): 249–256. https://doi.org/10.1089/ind.2018.0017.

129 Drosg, B., Fritz, I., Gattermayr, F., and Silvestrini, L. (2015). Photo-autotrophic production of poly(hydroxyalkanoates) in cyanobacteria. *Chemical and Biochemical Engineering Quarterly* 29 (2): 145–156. https://doi.org/10.15255/CABEQ.2014.2254.

130 Koller, M. (2020). Established and advanced approaches for recovery of microbial polyhydroxyalkanoate (PHA) biopolyesters from surrounding microbial biomass. *The EuroBiotech Journal* 4 (3): 113–126. https://doi.org/10.2478/ebtj-2020-0013.

131 Gonzalez, K., Navia, R., Liu, S., and Cea, M. (2020). Biological approaches in polyhydroxyalkanoates recovery. *Current Microbiology* online ahead of print. https://doi.org/10.1007/s00284-020-02263-1.

132 Koller, M., Niebelschütz, H., and Braunegg, G. (2013). Strategies for recovery and purification of poly[(*R*)-3-hydroxyalkanoates] (PHA) biopolyesters from surrounding biomass. *Engineering in Life Sciences* 13 (6): 549–562. https://doi.org/10.1002/elsc.201300021.

133 Nonato, R., Mantelatto, P., and Rossell, C. (2001). Integrated production of biodegradable plastic, sugar and ethanol. *Applied Microbiology and Biotechnology* 57 (1, 2): 1–5. https://doi.org/10.1007/s002530100732.

134 Koller, M., Bona, R., Chiellini, E., and Braunegg, G. (2013). Extraction of short-chain-length poly-[(*R*)-hydroxyalkanoates] (*scl*-PHA) by the "anti-solvent" acetone under elevated temperature and pressure. *Biotechnology Letters* 35 (7): 1023–1028. https://doi.org/10.1007/s10529-013-1185-7.

135 Cerrone, F., Radivojevic, J., Nikodinovic-Runic, J. et al. (2019). Novel sodium alkyl-1, 3-disulfates, anionic biosurfactants produced from microbial polyesters. *Colloids and Surfaces B: Biointerfaces* 182: 110333. https://doi.org/10.1016/j.colsurfb.2019.06.062.

136 Jiang, G., Johnston, B., Townrow, D. et al. (2018). Biomass extraction using non-chlorinated solvents for biocompatibility improvement of polyhydroxyalkanoates. *Polymers* 10 (7): 731. https://doi.org/10.3390/polym10070731.

137 Samorì, C., Basaglia, M., Casella, S. et al. (2015). Dimethyl carbonate and switchable anionic surfactants: two effective tools for the extraction of polyhydroxyalkanoates from microbial biomass. *Green Chemistry* 17 (2): 1047–1056. https://doi.org/10.1039/C4GC01821D.

138 Riedel, S.L., Brigham, C.J., Budde, C.F. et al. (2013). Recovery of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from *Ralstonia eutropha* cultures with non-halogenated solvents. *Biotechnology and Bioengineering* 110 (2): 461–470. https://doi.org/10.1002/bit.24713.

139 Reverchon, E. (1997). Supercritical fluid extraction and fractionation of essential oils and related products. *Journal of Supercritical Fluids* 10 (1): 1–37. https://doi.org/10.1016/S0896-8446(97)00014-4.

140 Williams, J.R., Clifford, A.A., and Al-Saidi, S.H. (2002). Supercritical fluids and their applications in biotechnology and related areas. *Molecular Biotechnology* 22 (3): 263. https://doi.org/10.1385/MB:22:3:263.

141 Hampson, J.W. and Ashby, R.D. (1999). Extraction of lipid-grown bacterial cells by supercritical fluid and organic solvent to obtain pure medium chain-length polyhydroxyalkanoates. *Journal of American Oil Chemistry Society* 76 (11): 1371–1374. https://doi.org/10.1007/s11746-999-0152-x.

142 Hejazi, P., Vasheghani-Farahani, E., and Yamini, Y. (2003). Supercritical fluid disruption of *Ralstonia eutropha* for poly(β-hydroxybutyrate) recovery. *Biotechnology Progress* 19 (5): 1519–1523. https://doi.org/10.1021/bp034010q.

143 Khosravi-Darani, K., Vasheghani-Farahani, E., Shojaosadati, S.A., and Yamini, Y. (2004). Effect of process variables on supercritical fluid disruption of *Ralstonia eutropha* cells for poly(*R*-hydroxybutyrate) recovery. *Biotechnology Progress* 20 (6): 1757–1765. https://doi.org/10.1021/bp0498037.

144 Marudkla, J., Patjawit, A., Chuensangjun, C., and Sirisansaneeyakul, S. (2018). Optimization of poly(3-hydroxybutyrate) extraction from *Cupriavidus necator* DSM 545 using sodium dodecyl sulfate and sodium hypochlorite. *Agriculture and Natural Resources* 52 (3): 266–273. https://doi.org/10.1016/j.anres.2018.09.009.

145 Fujita, K., Kobayashi, D., Nakamura, N., and Ohno, H. (2013). Direct dissolution of wet and saliferous marine microalgae by polar ionic liquids without heating. *Enzyme and Microbial Technology* 52 (3): 199–202. https://doi.org/10.1016/j.enzmictec.2012.12.004.

146 Kobayashi, D., Fujita, K., Nakamura, N., and Ohno, H. (2015). A simple recovery process for biodegradable plastics accumulated in cyanobacteria treated with ionic liquids. *Applied Microbiology and Biotechnology* 99 (4): 1647–1653. https://doi.org/10.1007/s00253-014-6234-1.

147 Koller, M. (2019). Polyhydroxyalkanoate biosynthesis at the edge of water activity-haloarchaea as biopolyester factories. *Bioengineering* 6 (2): 34. https://doi.org/10.3390/bioengineering6020034.

148 Choi, J.I. and Lee, S.Y. (2000). Efficient and economical recovery of poly(3-hydroxybutyrate) from recombinant *Escherichia coli* by simple digestion with chemicals. *Biotechnology and Bioengineering* 62: 546–553. https://doi.org/10.1002/(SICI)1097-0290(19990305)62:5<546::AID-BIT6>3.0.CO;2-0.

149 Ghatnekar, M.S., Pai, J.S., and Ganesh, M. (2003). Production and recovery of poly-3-hydroxybutyrate from *Methylobacterium* sp V49. *Journal of Chemical Technology and Biotechnology* 77: 444–448. https://doi.org/10.1002/jctb.570.

150 Holmes, P.A. and Lim, G.B. (1990). Separation process. US Patent 4910145-A, filed 30 June 1989 and issued 20 March 1990.

151 Kachrimanidou, V., Kopsahelis, N., Vlysidis, A. et al. (2016). Downstream separation of poly(hydroxyalkanoates) using crude enzyme consortia produced via solid state fermentation integrated in a biorefinery concept. *Foods and Bioproducts Processing* 100: 323–334. https://doi.org/10.1016/j.fbp.2016.08.002.

152 Rodríguez Gamero, J.E., Favaro, L., Pizzocchero, V. et al. (2018). Nuclease expression in efficient polyhydroxyalkanoates-producing bacteria could yield cost reduction during downstream processing. *Bioresource Technology* 261: 176–181. https://doi.org/10.1016/j.biortech.2018.04.021.

153 Murugan, P., Han, L., Gan, C.Y. et al. (2016). A new biological recovery approach for PHA using mealworm, *Tenebrio molitor*. *Journal of Biotechnology* 239: 98–105. https://doi.org/10.1016/j.jbiotec.2016.10.012.

154 Chee, J.Y., Lakshmanan, M., Jeepery, I.F. et al. (2019). The potential application of *Cupriavidus necator* as polyhydroxyalkanoates producer and single cell protein: a review on scientific, cultural and religious perspectives. *Applied Food Biotechnology* 6 (1): 19–34. https://doi.org/10.22037/afb.v6i1.22234.

155 Koller, M. (2019). Switching from fossil plastics to microbial polyhydroxyalkanoates (PHA): the biotechnological escape route of choice out of the plastic predicament? *The Euro-Biotech Journal* 3 (1): 32–44. https://doi.org/10.2478/ebtj-2019-0004.

156 Yutaka, T. and Buenaventurada, C. (2004). *Review* Degradation of microbial polyesters. *Biotechnology Letters* 26: 1181–1189. https://doi.org/10.1023/B:BILE.0000036599.15302.e5.

157 Li, Z., Lin, H., Ishii, N. et al. (2007). Study of enzymatic degradation of microbial copolyesters consisting of 3-hydroxybutyrate and medium-chain-length 3-hydroxyalkanoates. *Polymer Degradation and Stability* 92: 1708–1714. https://doi.org/10.1016/j.polymdegradstab.2007.06.001.

158 Renard, E., Walls, M., Guérin, P., and Langlois, V. (2004). Hydrolytic degradation of blends of polyhydroxyalkanoates and functionalized polyhydroxyalkanoates. *Polymer Degradation and Stability* 85: 779–787. https://doi.org/10.1016/j.polymdegradstab.2003.11.019.

159 Vu, D.H., Åkesson, D., Taherzadeh, M.J., and Ferreira, J.A. (2020). Recycling strategies for polyhydroxyalkanoate-based waste materials: an overview. *Bioresource Technology* 298: 122393. https://doi.org/10.1016/j.biortech.2019.122393.

160 Zaverl, M., Seydibeyoğlu, M.Ö., Misra, M., and Mohanty, A. (2012). Studies on recyclability of polyhydroxybutyrate-co-valerate bioplastic: multiple melt processing and performance evaluations. *Journal of Applied Polymer Science* 125: E324–E331. https://doi.org/10.1002/app.36840.

161 Zembouai, I., Bruzaud, S., Kaci, M. et al. (2014). Mechanical recycling of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)/polylactide based blends. *Journal of Polymers and the Environment* 22: 449–459. https://doi.org/10.1007/s10924-014-0684-5.

162 Rivas, L.F., Casarin, S.A., Nepomuceno, N.C. et al. (2017). Reprocessability of PHB in extrusion: ATR-FTIR, tensile tests and thermal studies. *Polímeros* 27: 122–128. http://dx.doi.org/10.1590/0104-1428.2406.

163 Ferreira, J.A. and Åkesson, D. (2020). Aerobic and anaerobic degradation pathways of PHA. In: *The Handbook of Polyhydroxyalkanoates - Postsynthetic Treatment, Processing and Application*, vol. III (ed. M. Koller), 317–338. CRC press Taylor & Francis Group ISBN 9780367275594.

164 Morikawa, H. and Marchessault, R.H. (1981). Pyrolysis of bacterial polyalkanoates. *Canadian Journal of Chemistry* 59: 2306–2313. https://doi.org/10.1139/v81-334.

165 Sato, S., Ishii, N., Hamada, Y. et al. (2012). Utilization of 2-alkenoic acids for biosynthesis of medium-chain length polyhydroxyalkanoates in metabolically engineered *Escherichia coli* to construct a novel chemical recycling system. *Polymer Degradation and Stability* 97: 329–336. https://doi.org/10.1016/j.polymdegradstab.2011.12.007.

166 Yang, X., Odelius, K., and Hakkarainen, M. (2014). Microwave-assisted reaction in green solvents recycles PHB to functional chemicals. *ACS Sustainable Chemistry and Engineering* 2: 2198–2203. https://doi.org/10.1021/sc500397h.

167 Niaounakis, M. (2013). *Biopolymers: Reuse, Recycling, and Disposal*, PDL Handbook Series. Elsevier.

168 Van Walsem, J., Licata, J., Anderson, E. A., Sparks, K. A., et al. (2014). U.S. Patent Application No. 14/237, 808.

169 Yañez, L., Conejeros, R., Vergara-Fernández, A., and Scott, F. (2020). Beyond intracellular accumulation of polyhydroxyalkanoates: chiral hydroxyalkanoic acids and polymer secretion. *Frontiers in Bioengineering and Biotechnology* 8: 248. https://doi.org/10.3389/fbioe.2020.00248.

170 Jendrossek, D. (1998). Microbial degradation of polyesters: a review on extracellular poly(hydroxyalkanoic acid) depolymerases. *Polymer Degradation and Stability* 59: 317–325. https://doi.org/10.1016/S0141-3910(97)00190-0.

171 Bano, K., Kuddus, M., and Zaheer, M.R. (2018). A novel, thermotolerant, extracellular PHB depolymerase producer *Paenibacillus alvei* PHB28 for bioremediation of biodegradable plastics. *Turkish Journal of Biochemistry* 44 (3): 344–353. https://doi.org/10.1515/tjb-2018-0207.

172 Gao, D., Maehara, A., Yamane, T., and Ueda, S. (2001). Identification of the intracellular polyhydroxyalkanoate depolymerase gene of *Paracoccus denitrificans* and some properties of the gene product. *FEMS Microbiology Letters* 196 (2): 159–164. https://doi.org/10.1111/j.1574-6968.2001.tb10558.x.

173 Grigore, M.E., Grigorescu, R.M., Iancu, L. et al. (2019). Methods of synthesis, properties and biomedical applications of polyhydroxyalkanoates: a review. *Journal of Biomaterials Science, Polymer Edition* 30: 695–712. https://doi.org/10.1080/09205063.2019.1605866.

174 Ong, S.Y., Chee, J.Y., and Sudesh, K. (2017). Degradation of polyhydroxyalkanoate (PHA): a review. *Journal of Siberian Federal University. Biology* 10: 211–225. http://elib.sfu-kras.ru/handle/2311/33371.

175 Urbanek, A.K., Mirończuk, A.M., García-Martín, A. et al. (2020). Biochemical properties and biotechnological applications of microbial enzymes involved in the degradation of polyester-type plastics. *Biochimica et Biophysica Acta (BBA), Proteins and Proteomics* 1868 (2): 140315. https://doi.org/10.1016/j.bbapap.2019.140315.

176 Jendrossek, D., Schirmer, A., and Schlegel, H.G. (1996). Biodegradation of polyhydroxyalkanoic acids. *Applied Microbiology and Biotechnology* 46: 451–463. https://doi.org/10.1007/s002530050844.

177 Knoll, M., Hamm, T.M., Wagner, F. et al. (2009). The PHA depolymerase engineering database: a systematic analysis tool for the diverse family of polyhydroxyalkanoate (PHA) depolymerases. *BMC Bioinformatics* 10 (1): 89. https://doi.org/10.1186/1471-2105-10-89.

178 Shah, A.A., Hasan, F., Hameed, A., and Ahmed, S. (2008). Biological degradation of plastics: a comprehensive review. *Biotechnology Advances* 26: 246–265. https://doi.org/10.1016/j.biotechadv.2007.12.005.

179 Aneja, P. and Charles, T.C. (1999). Poly-3-hydroxybutyrate degradation in *Rhizobium* (*Sinorhizobium*) *meliloti*: isolation and characterization of a gene encoding 3-hydroxybutyrate dehydrogenase. *Journal of Bacteriology* 181: 849–857. https://doi.org/10.1128/JB.181.3.849-857.1999.

180 Lee, S.Y. and Choi, J.I. (1999). Production and degradation of polyhydroxyalkanoates in waste environment. *Waste Management* 19: 133–139. https://doi.org/10.1016/S0956-053X(99)00005-7.

181 Doi, Y., Segawa, A., Kawaguchi, Y., and Kunioka, M. (1990). Cyclic nature of poly(3-hydroxyalkanoate) metabolism in *Alcaligenes eutrophus*. *FEMS Microbiology Letters* 67: 165–169. https://doi.org/10.1111/j.1574-6968.1990.tb13856.x.

182 Kusaka, S., Iwata, T., and Doi, Y. (1999). Properties and biodegradability of ultra-high-molecular-weight poly[(*R*)-3-hydroxybutyrate] produced by a recombinant *Escherichia coli*. *International Journal of Biological Macromolecules* 25: 87–94. https://doi.org/10.1016/S0141-8130(99)00019-7.

183 Adaya, L., Millán, M., Peña, C. et al. (2018). Inactivation of an intracellular poly-3-hydroxybutyrate depolymerase of *Azotobacter vinelandii* allows to obtain a polymer of uniform high molecular mass. *Applied Microbiology and Biotechnology* 102: 2693–2707. https://doi.org/10.1007/s00253-018-8806-y.

184 Choi, G.G., Kim, H.W., and Rhee, Y.H. (2004). Enzymatic and nonenzymatic degradation of poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolyesters produced by *Alcaligenes* sp. MT-16. *Journal of Microbiology* 42: 346–352. PubMed ID: 15650693.

185 Steinbüchel, A. (2005). Non-biodegradable biopolymers from renewable resources: perspectives and impacts. *Current Opinion in Biotechnology* 16: 607–613. https://doi.org/10.1016/j.copbio.2005.10.011.

186 Kourilova, X., Pernicova, I., Sedlar, K. et al. (2020). Production of polyhydroxyalkanoates (PHA) by a thermophilic strain of *Schlegelella thermodepolymerans* from xylose rich substrates. *Bioresource Technology* 315: 123885. https://doi.org/10.1016/j.biortech.2020.123885.

187 Koller, M. (2014). Poly(hydroxyalkanoates) for food packaging: application and attempts towards implementation. *Applied Food Biotechnology* 1 (1): 3–15. https://doi.org/10.22037/afb.v1i1.7127.

188 Kovalcik, A., Machovsky, M., Kozakova, Z., and Koller, M. (2015). Designing packaging materials with viscoelastic and gas barrier properties by optimized processing of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) with lignin. *Reactive and Functional Polymers* 94: 25–34. https://doi.org/10.1016/j.reactfunctpolym.2015.07.001.

189 Plackett, D. and Siró, I. (2011). Polyhydroxyalkanoates (PHAs) for food packaging. In: *Multifunctional and Nanoreinforced Polymers for Food Packaging* (ed. J.M. Lagarón), 498–526. Woodhead Publishing https://doi.org/10.1533/9780857092786.4.498.

190 Haugaard, V.K., Udsen, A.M., Hoegh, L. et al. (2001). Potential food applications of biobased materials – an EU-concerted action project. *Starch* 53: 189–2000. https://doi.org/10.1002/1521-379X(200105)53:53.0.CO;2-3.

191 https://www.bacardilimited.com/media/news-archive/bacardi-first-in-fight-against-plastic-pollution-with-100-biodegradable-spirits-bottle (accessed 11 November 2021).

192 Triantafillopoulos, N. and Koukoulas, A.A. (2020). The future of single-use paper coffee cups: current progress and outlook. *BioResources* 15 (3): 7260–7287. https://doi.org/10.15376/biores.15.3.

193 Wu, B., Zhou, M., Song, L. et al. (2021). Mechanism insights into polyhydroxyalkanoate-regulated denitrification from the perspective of peri-cytoplasmic nitrate reductase expression. *Science of the Total Environment* 754: 142083. https://doi.org/10.1016/j.scitotenv.2020.142083.

194 Cyras, V.P., Commisso, M.S., Mauri, A.N., and Vázquez, A. (2007). Biodegradable double-layer films based on biological resources: polyhydroxybutyrate and cellulose. *Journal of Applied Polymer Science* 106: 749–756. https://doi.org/10.1002/app.26663.

195 Rastogi, V.K. and Samyn, P. (2020). Compression molding of polyhydroxybutyrate nano-composite films as coating on paper substrates. *Proceedings of The 2nd Coatings and Interfaces Web Conference* (*CIWC 2020*). MDPI: Basel, Switzerland. 7 p.

196 Online resource: Kemira Announces Partnership with Danimer Scientific to Develop Biodegradable Coating for Paper and Board Industry, Business Wire (7.1.21)

197 Amelia, T.S.M., Govindasamy, S., Tamothran, A.M. et al. (2019). Applications of PHA in agriculture. In: *Biotechnological Applications of Polyhydroxyalkanoates* (ed. V. Kalia), 347–361. Singapore: Springer.

198 Chen, L. (2009). Next generation of electrospun textiles for chemical and biological protection and air filtration. Massachusetts Inst of Tech Cambridge.

199 Online resource NAFIGATE Corporation Natural polymer Vyvíjíme technologie pro lepší svět.https://www.nafigate.com/ (accessed 11 November 2021).

200 Online resource. Bio-on | Turn Off Pollution (bio-on.it). http://www.bio-on.it/?lin=inglese (accessed 11 November 2021).

201 Rodriguez-Contreras, A. (2019). Recent advances in the use of polyhydroyalkanoates in biomedicine. *Bioengineering* 6 (3): 82. https://doi.org/10.3390/bioengineering6030082.

202 Rodríguez-Contreras, A., Calafell-Monfort, M., and Marqués-Calvo, M.S. (2012). Enzymatic degradation of poly(3-hydroxybutyrate) by a commercial lipase. *Polymer Degradation and Stability* 97: 2473–2476. https://doi.org/10.1016/j.polymdegradstab.2012.07.042.

203 Wu, L.P., Wang, D., Parhamifar, L. et al. (2014). Poly(3-hydroxybutyrate-*co*-*R*-3-hydroxyhexanoate) nanoparticles with polyethylenimine coat as simple, safe, and versatile vehicles for cell targeting: population characteristics, cell uptake, and intracellular trafficking. *Advanced Healthcare Materials* 3: 817–824. https://doi.org/10.1002/adhm.201300533.

204 Mukheem, A., Muthoosamy, K., Manickam, S. et al. (2018). Fabrication and characterization of an electrospun PHA/graphene silver nanocomposite scaffold

for antibacterial applications. *Materials* 11 (9): 1673. https://doi.org/10.3390/ma11091673.

205 Chai, J.M., Amelia, T.S.M., Mouriya, G.K. et al. (2021). Surface-modified highly biocompatible bacterial-poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate): a review on the promising next-generation biomaterial. *Polymers* 13 (1): 51. https://doi.org/10.3390/polym13010051.

206 Pereira, J.R., Araújo, D., Marques, A.C. et al. (2019). Demonstration of the adhesive properties of the medium-chain-length polyhydroxyalkanoate produced by *Pseudomonas chlororaphis* subsp. *aurantiaca* from glycerol. *International Journal of Biological Macromolecules* 122: 1144–1151. https://doi.org/10.1016/j.ijbiomac.2018.09.064.

207 Bhardwaj, A., Alam, T., Sharma, V. et al. (2020). Lignocellulosic agricultural biomass as a biodegradable and eco-friendly alternative for polymer-based food packaging. *Journal of Packaging Technology and Research* 4: 205–216. https://doi.org/10.1007/s41783-020-00089-7.

208 Giubilini, A., Siqueira, G., Clemens, F.J. et al. (2020). 3D-printing nanocellulose-poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) biodegradable composites by fused deposition modeling. *ACS Sustainable Chemistry & Engineering* 8 (27): 10292–10302. https://doi.org/10.1021/acssuschemeng.0c03385.

209 Meischel, M., Eichler, J., Martinelli, E. et al. (2016). Adhesive strength of bone-implant interfaces and *in-vivo* degradation of PHB composites for load-bearing applications. *Journal of the Mechanical Behavior of Biomedical Materials* 53: 104–118. https://doi.org/10.1016/j.jmbbm.2015.08.004.

210 Elmowafy, E., Abdal-Hay, A., Skouras, A. et al. (2019). Polyhydroxyalkanoate (PHA): applications in drug delivery and tissue engineering. *Expert Review of Medical Devices* 16 (6): 467–482. https://doi.org/10.1080/17434440.2019.1615439.

211 Sosa-Hernández, J.E., Villalba-Rodríguez, A.M., Romero-Castillo, K.D. et al. (2020). Poly-3-hydroxybutyrate-based constructs with novel characteristics for drug delivery and tissue engineering applications – a review. *Polymer Engineering & Science* 60 (8): 1760–1772. https://doi.org/10.1002/pen.25470.

212 Wu, Q., Wang, Y., and Chen, G.Q. (2009). Medical application of microbial biopolyesters polyhydroxyalkanoates. *Artificial Cells, Blood Substitutes, and Biotechnology* 37 (1): 1–12. https://doi.org/10.1080/10731190802664429.

213 Puppi, D., Pecorini, G., and Chiellini, F. (2019). Biomedical processing of polyhydroxyalkanoates. *Bioengineering* 6 (4): 108. https://doi.org/10.3390/bioengineering6040108.

214 Hablot, E., Dharmalingam, S., Hayes, D.G. et al. (2014). Effect of simulated weathering on physicochemical properties and inherent biodegradation of PLA/PHA nonwoven mulches. *Journal of Polymers and the Environment.* 22 (4): 417–429. https://doi.org/10.1007/s10924-014-0697-0.

215 Online resource. http://www.bio-on.it/minerv-supertoys.php (accessed 11 November 2021).

216 Online resource Restore Foodware – Products (accessed 11 November 2021).

217 Online resource Covalent. covalentfashion.com (accessed 11 November 2021).

218 Zhang, X., Luo, R., Wang, Z. et al. (2009). Application of (*R*)-3-hydroxyalkanoate methyl esters derived from microbial polyhydroxyalkanoates as novel biofuels. *Biomacromolecules* 10 (4): 707–711. https://doi.org/10.1021/bm801424e.

219 Wang, S.Y., Wang, Z., Liu, M.M. et al. (2010). Properties of a new gasoline oxygenate blend component: 3-hydroxybutyrate methyl ester produced from bacterial poly-3-hydroxybutyrate. *Biomass and Bioenergy* 34 (8): 1216–1222. https://doi.org/10.1016/j.biombioe.2010.03.02.