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Review

Linking ecology with economy: Insights into polyhydroxyalkanoate-producing microorganisms

Polyhydroxyalkanoates (PHA) constitute a group of microbial biopolyesters with important ecosystem functions and a high biotechnological potential. During the past decade, the rapid development of new molecular and microscopic techniques resulted in novel insights into the ecology of PHA-producing bacteria in aquatic and terrestrial microenvironments. Ecosystems showing fluctuating availability of carbon or transient limitation of essential nutrients, e.g. the rhizosphere of plants or estuarine sediments, contain a broad number of various PHA producers. PHA-producing microorganisms show a widespread phylogenetic diversity and are often characterized by a symbiotic or syntrophic life style. PHA are already produced commercially in large-scale fermentation. However, they have to compete economically with petrol-based polymers. Hence, the development of low-cost production strategies on the basis of diverse renewable materials is a crucial challenge. Ecological knowledge is required for these developments, which links both parts of the review together. The article highlights how a better understanding of the ecology of PHA-producing microorganisms can lead to a broader application of microbial biopolymers on the basis of sustainable production processes. These processes have to be evaluated by means of life cycle assessment and Cleaner Production studies prior to their industrial implementation.

Keywords: Ecology of PHA producers / PHA / *phaC* / Sustainable production / White biotechnology

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1 Introduction

Prokaryotic microorganisms like bacteria can produce a broad range of extra- and intracellular biopolymers, which fulfill diverse functions and ecosystem services [1]. Polyhydroxyalkanoates (PHA) are polyoxoesters of hydroxyalkanoic acids, and their existence in bacteria was first reported decades ago by Lemoigne [2]. It is well established that PHA are synthesized by bacteria and some archaea as intracellular storage compounds to serve as carbon and energy source under unfavorable conditions [3]. PHA-producing microorganisms can take advantage of their ability to accumulate storage compounds in environments of fluctuating availability and limitation of nutrients. PHA-producing bacteria have been found in both aquatic and terrestrial environments. Figure 1 shows PHA-rich cells of the well-known PHA-producing strain *Cupriavidus necator* cultivated on glucose as the sole carbon source during a continuous cultivation process.

For a long time, the ecology of PHA-producing bacteria was investigated by cultivation-dependent techniques. The latter capture only a small proportion – up to 3% – of the bacterial communities and therefore are restricted to representative results. During the last decade, there was a lot of progress in microbial ecology research, and after structural analysis of the bacterial communities, the focus was on understanding their

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Abbreviations: 3HB, 3-hydroxybutyric acid; **3HV**, 3-hydroxyvaleric acid; **A/O**, anaerobic/aerobic; **FT-IR**, Fourier transform infrared; **LCA**, life cycle assessment; **P(3HB-*co*-5 mol% 3HHx)**, poly(3-hydroxybutyrate-*co*-5 mol% 3-hydroxyhexanoate); **PHA**, polyhydroxyalkanoates; **PHB**, poly-β-hydroxybutyric acid; **PHBISA**, PHB industrial S/A; **SPI**, sustainable process index; **STEM**, scanning transmission electron microscopy

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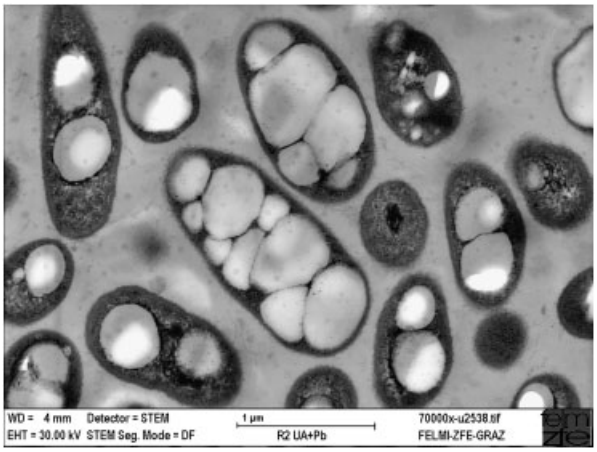

Figure 1. Electron microscopic pictures of PHA-rich *C. necator* DSM 545 cells cultivated in a continuous fermentation process on glucose. Magnification: 1/70 000; 48% of PHB in cell mass. The picture was kindly provided by Dr. Elisabeth Ingolić, FELMI-ZFE-Graz.

natural functions. New techniques like DNA- or RNA-based molecular fingerprinting, metagenomics and environmental proteomics are powerful tools to analyze also PHA-producing bacteria in their environment. This review presents new results on the microbial ecology of PHA-producing microorganisms, mainly based on molecular techniques. Although the genetics and physiology of PHA-producing bacteria are well understood and genetically engineered microorganisms with high yield and special products have been developed, commercialization is still at a first level. Reasons for this are price and sustainability of the production process. Today, the early assessment of the impact of a new industrial process on the ecosphere is a prerequisite for sustainable industrial development. Therefore, in the second part of the review, we discuss the translation of ecological knowledge into production and discuss novel tools for assessing the sustainability of industrial bioprocesses.

2 Microbial ecology of PHA-producing bacteria

2.1 Methods for examination of PHA and PHA-producing organisms

2.1.1 Methods for examination of PHA

Not only for biotechnological but also for ecological and microbiological questions, the quantification of PHA in complex samples is of importance. For example, a high ratio of PHA to phospholipid fatty acids is suggested as a marker for an unbalanced environment [4]. The first analytic methods to determine the PHA content in bacterial cells were based on gravimetric methods. PHA was extracted with chloroform from lyophilized bacterial cells and precipitated with acetone or diethylether [5]. Data about PHA amounts can also be generated by turbidity measurement following lysis of cell material in alkaline sodium hypochlorite [6]. Analysis of PHA contents of environmental samples requires methods of high sensitivity. For analysis using GC and GC-MS, it is necessary to produce derivatives of the polymer, which can be achieved by hydrolysis with sulfuric acid [7], acidic methanolysis [8, 9], acid ethanolysis [10] and propanolysis [11]. By the generation of specific derivatives, an even higher sensitivity of further analysis can be achieved. This has been shown for derivatives produced with pentafluorobenzylbromide [12] and *N-tert*-butyl-dimethylsilyl-*N*-methyltrifluoroacetamide [13]. The products of polymer hydrolysis can also be measured using an enzymatic kit [14]. The methods mentioned above require the breakdown of cell material, but are commonly used for quantitative and qualitative analyses of PHA in environmental samples. Methods that can measure PHA in intact cells are flow cytometry and spectrofluorometry [15, 16], two-dimensional fluorescence spectroscopy and flow cytometry [17], Fourier transform infrared (FT-IR) spectroscopy [18] and Raman spectroscopy [19]. For routine process monitoring in PHA production, methods for rapid quantification at low costs and with rapid sample preparation are necessary. Using FT-IR, prediction models for PHA quantification have been developed [20, 21]. An approach to systematically implement FT-IR for online quantification of PHA production is given by Arcos-Hernandez et al. [22]. Online quantification of PHA in a production process can also be done indirectly using state-of-routine monitoring technologies [23].

For intracellular visualization of PHA granules, staining methods such as with Sudan black B, Nile blue A, or Nile red, followed by light or fluorescent microscopy, are used [24–26]. These dyes are more soluble in PHA and other lipophilic storage materials like wax esters than in the staining solution [27]. Further information about PHA-producing cells can be provided by combining staining methods with the usage of fluorescence-labeled oligonucleotide probes and subsequent fluorescence microscopy [28]. PHA staining can also be combined with staining of other inclusion bodies, such as polyphosphate [29]. For a rapid direct screening of environmental samples, it is possible to include Nile red into solid media at a concentration of 0.5 µg/mL and to detect the presence of PHA-positive strains in viable colonies. Nile red does not restrict the growth of cells, but it is not applicable to Gram-positive bacteria. The intensity of fluorescence can also be used to quantify the amount of poly-β-hydroxybutyric acid (PHB) inside the cells [26]. Nile red dyeing combined with spectrofluorometry was used to monitor PHA production in bacterial isolates [15, 30].

Very recent developments in electron microscopy open the route for imaging PHA granules and other intracellular inclusions within whole living cells. In contrast to traditional electron microscopy characterized by time-consuming steps like drying, fixing, embedding, sectioning and steaming, the so-called "wet scanning transmission" ("wet STEM") electron microscopy features a rapid method to generate images of whole unfixed, hydrated cells in relatively high-pressure environments. The sample preparation for wet STEM is merely restricted to the removal of salts from the cultivation medium by simple washing with water. In addition, wet STEM reduces

the formation of artifacts that normally occur during STEM preparation [31].

Investigating PHA qualitatively or quantitatively in environmental samples with chemical extraction or staining approaches requires conditions that favor the production of PHA at the actual sampling time point. Also, when isolates are screened for PHA production, it is necessary to find cultivation conditions favorable for PHA accumulation when using traditional approaches [32]. This can be avoided using a genotypic approach by confirming the presence of PHA synthase genes [33].

2.1.2 *Methods for examination of PHA-producing organisms*

A broad range of short- and medium-chain length PHA-producing microorganisms can be screened by PCR. PHA synthases (encoded on the *phaC* operon) are key enzymes for producing PHA [34]. López et al. [35] first published the possibility of PCR to detect PHA-producing bacteria in river water. There are primers available for all four classes of PHA synthase genes, and the methods of detection have been reviewed by Solaiman and Ashby [36]. PHA synthase gene-amplifying primers are also useful to differentiate the different classes of PHA synthase genes. Solaiman et al. [37] designed specific primers by multiple sequence alignment in order to detect other class II PHA synthase genes. Sheu et al. [38] developed primers for colony PCR detection of class I and II PHA synthase genes of bacteria isolated from the environment, by aligning highly conserved sequences of class I and II genes of 13 Gram-negative bacteria. For the detection of class IV synthase genes, primers based on class IV *phaC* genes of *Bacillus megaterium* were selected [39]. Hai et al. [40] developed a method to detect class III PHA synthase genes of sulfate-reducing bacteria and cyanobacteria. These primers can also be used successfully in combination with community DNA fingerprint techniques such as single-strand conformation polymorphism (SSCP). Thus, specific patterns showing different *phaC* species for the rhizosphere of sugar beet, oilseed rape and wheat have been revealed [41]. Ciesielski et al. [42] used some of these primers for the construction of a *phaC* clone library from activated sludge samples and monitored *phaC* expression levels in a laboratory-scale plant using reverse transcriptase quantitative PCR (RT-qPCR). Currently, metagenomic approaches have had a high impact on understanding microbial ecosystems: Methods have already been developed for the isolation of clones expressing novel PHA metabolism genes from metagenomic libraries [43].

There is a broad range of currently used cultivation-independent methods for investigating PHA and PHA-producing microorganisms in environmental samples, as shown in Fig. 2. These methods provide detailed insights into single processes correlated with PHA accumulation. To gain further understanding and to achieve deep and encompassing insights into the complex role of PHA-producing microorganisms in different habitats and processes, it is necessary to apply combined approaches covering the measurement of PHA amounts, the investigation of microbial diversity, and the assessment of enzymatic and microbial activities.

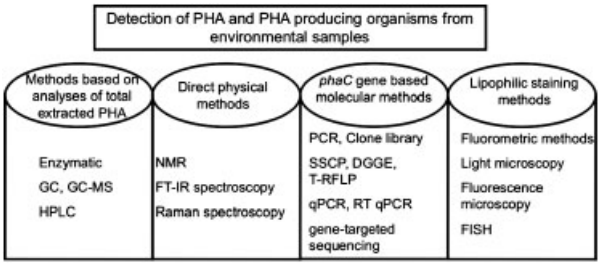

Figure 2. Compilation of currently used methods for the detection of PHA and PHA-producing bacteria in environmental samples. DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence in situ hybridization.

2.2 Occurrence and importance of PHA producers in aquatic ecosystems

2.2.1 *PHA producers in natural aquatic systems*

As fluctuations of available nutrients are strongly pronounced in marine environments, a lot of studies have investigated PHA-producing microorganisms, the amount of PHA present, and the composition of PHA produced by different species. PHA extracted from estuarine sediments showed a high diversity; at least 11 different short-chain PHA were detected in sediments sampled in Florida [10]. In anaerobic layers of marine sediments, PHA were detected only after aeration [44]. As it is a common phenomenon that PHA amounts are increased by perturbation of environments, it has been established that the occurrence of PHA is an indication for unbalanced growth of microorganisms in disturbed environments [45].

Another interesting habitat characterized by fluctuations of carbon availability is given by microbial mats, in which photosynthesis is the most important source of organic carbon. The ability of storing PHA in these environments has several advantages for bacteria. On the one hand, PHA-producing heterotrophic organisms utilize excess organic compounds produced by phototrophic organisms during the day by storing them as PHA and consuming them during the night [46]. This is illustrated in Fig. 3. On the other hand, results suggest that organisms capable of anoxic photosynthesis use stored PHA as electron source for the reduction of sulfate. Thus, PHA metabolism may play an important role for the maintenance of anoxic photosynthesis and the sulfur cycle in these environments [47, 48]. From estuarine microbial mats, PHA-producing strains of *Halomonas* and *Labrenzia* have been isolated [49]. Another aspect of the PHA-producing ability and its ecological advantage in microbial mats is given in a study by Lopez-Cortes et al. [50]. In this study, it was shown that the diversity of PHA-producing bacteria is enriched in microbial mats from a seafood cannery wastewater in comparison to non-polluted microbial mats. The strong connection of photosynthesis and PHA production is not restricted to microbial mats. *Rhodospirillum rubrum*, a photosynthetic bacterium, and several cyanobacteria are known to produce PHA during the light phase [51, 52].

In aquatic ecosystems, the occurrence of PHA-producing bacteria is not limited to marine environments; also freshwater

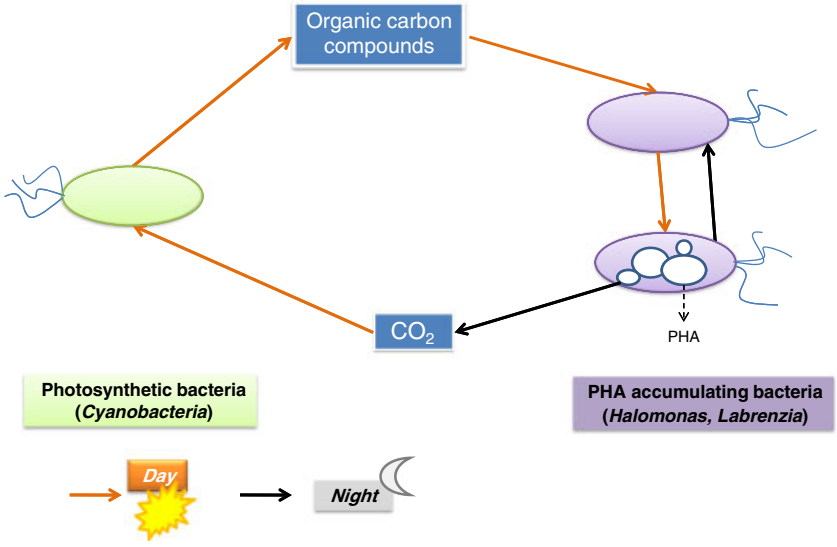

Figure 3. Syntrophic interaction between PHA-producing and photosynthetic microorganisms: Organic carbon compounds are produced in excess by photosynthetic CO_2 fixation during the day by anoxic cyanobacteria; these compounds are stored intracellularly by PHA-producing bacteria (orange arrows). The accumulated PHA is utilized during the night as carbon and energy source (black arrows).

sludge exhibits conditions favorable for PHA-producing microbes. The well-investigated model strain *Ralstonia eutropha* H16 (today: *C. necator*) was isolated from freshwater sludge of the Weende Quelle in Göttingen, Germany [53]. It is used for the industrial production of a copolymer consisting of 3-hydroxybutyric acid (3HB) 3-hydroxyvaleric acid (3HV) [54]. An abundance of PHA synthase genes also has been detected in river water of the Rio de la Plata, Buenos Aires, in Argentina [35]. Another important habitat for PHA-producing bacteria is groundwater sediment where the availability of phosphate and other nutrients is fluctuating with the quality of the percolating water. PHB accumulation is demonstrated for an isolate from these sediments as a response to fluctuation from starving conditions to nutrient-rich conditions [55]. PHA-accumulating bacteria present in groundwater sediment may also be interesting for the bioremediation of pollutants [56].

2.2.2 *PHA producers in engineered systems*

Several anthropogenic aquatic systems offer appropriate conditions for the accumulation of PHA-producing bacteria. From activated sludge, a mixture as Organisms producing PHAS with different building blocks was characterized [57]. Oshiki et al. [58] have found PHA-producing representatives of the genera *Dechloromonas*, *Accumulibacter*, *Thauera*, *Zoogloea*, *Comamonas* and *Competibacter*, and a novel cluster of β-proteobacteria in activated sludge. PHA-producing strains of *Bacillus*, *Pseudomonas*, *Alcaligenes*, *Aeromonas* and *Chromobacterium* have been found in sewage treatment plants [59]. Two novel PHA-producing Gram-positive species, belonging to the high-G+C group, have been isolated from an alternating anaerobic/aerobic (A/O)-activated sludge system [60]. Especially in the A/O-activated sludge systems, PHA-producing bacteria play an important role in nutrient and particularly phosphorous elimination from wastewaters. In the first (anaerobic) stage, polyphosphate-accumulating organisms (PAO) release phosphate from intracellularly stored polyphosphate. Energy from this hydrolysis is used to build PHA as a consequence of oxygen limitation in the presence of sufficient organic carbon. The second stage is characterized by aeration, where these bacteria can utilize the stored carbon source and generate polyphosphate from the soluble phosphate in the wastewater. In this process called enhanced biological phosphorous elimination, it is important to enrich bacteria that are able to undergo both processes, PHA accumulation and polyphosphate synthesis [61]. Depending on the conditions and substrates in such systems, also glycogen-accumulating organisms (GAO) can be enriched, which are also able to produce PHA but compete for carbon sources with polyphosphate-accumulating organisms [62–64]. The mechanism of the enhanced biological phosphorous elimination in A/O-activated sludge system is illustrated in Fig. 4.

Whereas the mentioned habitats require the action of PHA-producing bacteria for the remediation of unwanted substances from different wastewaters, the following habitats should give examples for cheap substrates that can be used as raw material for industrial production of PHA. The finding of cheap raw materials for PHA production is essential in order to keep production costs in the competitive range. For example, palm oil mill effluents, wastewater from olive oil production (alpechìn), wastewater from dairies and wastewater from date syrup production provide both carbon-rich and cheap raw materials, in which PHA-producing bacteria have been found [65, 66]. Also nitrogen-poor pulp and paper wastewaters can be sources of interesting PHA-producing strains. In New Zealand, the strain *Novosphingobium nitrogenifigens* has been isolated from such effluents [67].

2.3 Occurrence and importance of PHA producers in terrestrial ecosystems

Being a well-known source of biotechnologically interesting microorganisms, soil is a promising source of PHA-producing

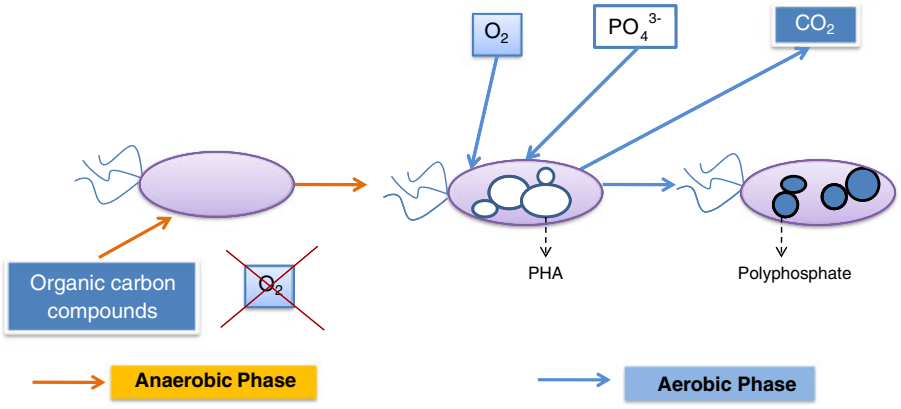

Figure 4. "Enhanced biological phosphorous elimination" in an alternating A/O-activated sludge system for nutrient and phosphate elimination from wastewaters: In the first (anaerobic) phase, phosphate is released from particles of activated sludge. In this stage, PHA-producing bacteria accumulate PHA as a consequence of oxygen limitation in the presence of sufficient organic carbon (orange arrows). The second stage is characterized by aeration where these bacteria can utilize the stored carbon source and generate polyphosphate from the soluble phosphate in the wastewater (blue arrows).

bacteria. Also, in terrestrial ecosystems, the presence of PHA producers is an indicator for unbalanced nutritional conditions: PHA-producing *Bacillus* strains have been isolated from gas field soil [68] or oil-contaminated soil, industrial waste drainage sites, and agricultural soils [69]. Soil from an intensively cultivated hop field had a higher PHA-to-phospholipid fatty acid ratio than soil from grassland and a crop rotation field. This can be traced back to the fact that higher amounts of fungicides and more rigorous farming practices in the hop field led to an unbalanced microbial growth [70].

The rhizosphere is defined as the volume of soil adjacent to and influenced by the plant root and as a habitat of great microbial diversity [71, 72]. Literature reports about PHA-producing bacteria and PHA amounts associated with the rhizosphere are often inconsistent. On the one hand, early studies based on cultivation-dependent methods report a lower number of PHA-producing microbes in the rhizosphere in comparison to soil [73, 74]. On the other hand, several studies show a high abundance of PHA-producing bacteria or high amounts of PHA in the rhizosphere. For example, in the rhizosphere of rice plants, a higher amount of PHA has been found in comparison to bulk soil [75]. Sugarcane fields are a rich source of PHA-producing microorganisms [76, 77]. On the basis of cultivation-independent, molecular techniques, a statistically significantly higher amount of PHA-producing microorganisms in the rhizosphere of sugar beet, oilseed rape and wheat in comparison to bulk soil was found [41]. The high presence of PHA-producing bacteria in the rhizosphere can be explained by the fact that roots, on the one hand, limit nutrient access for rhizospheric bacteria by consumption of essential inorganic nutrients and, on the other hand, enrich the rhizospheric soil with fluctuating root exudates mainly consisting of organic compounds [78]. Root exudates mainly consist of organic secondary metabolites, and their composition and amount is specific for plant species and fluctuates with the plant's physiological state [79]. Thereby, growing conditions suitable for PHA-producing bacteria (limitation of essential nutrients and concomitant supply of carbon) are generated. Especially carbohydrate and oil-producing crop plants display frequent and intense association with PHA-producing bacteria [41, 76, 77]. Since decades it has been known that PHA is produced by bacteroids of root nodules of legumes and also of free-living nitrogen-fixing bacteria [80], although the role of PHA in the process of nitrogen fixation is not completely understood. Data from the literature suggest that the accumulated PHA may prolong the period of nitrogen fixation in the dark [81] or may support the bacterial cell in maintaining its redox potential by energy production and NADH oxidation [82].

With the aim of keeping production costs for industrial PHA low and thereby developing an attractive alternative to petroleum-based products, the search for new PHA-producing bacterial strains still remains of interest. Important features of such strains could be also the tolerance to extreme conditions regarding cultivation parameters, to salinity, or to high concentrations of growth-inhibiting compounds. As an example, a PHA-producing strain of *Bacillus* isolated from a gas field soil accumulates PHA at temperatures of up to 45°C [68]. In addition to the well-known halophilic PHA-producing strain *Haloferax mediterranei*, PHA-producing strains of the genera *Sphingomonas* and *Bacillus* have been isolated from hypersaline marine mats [49]. Recently, the isolation of a *Burkholderia* F24 strain from Hawaiian soil samples was reported [83]. This strain can be cultivated on hydrolyzed bagasse from the sugarcane production without the need for prior removal of toxins like acids or aromatic constituents stemming from the hydrolysis of the lignocellulosic material bagasse. Such organisms raise considerable interest due to the fact that the conversion of lignocelluloses via "white biotechnology" is of increasing importance for the future. This is due to the fact that lignocellulosic materials, compared to other raw materials, are globally available in large quantities. Photoautotrophic and diazotrophic PHA producers may be helpful in the utilization of certain nutrient-poor substrates for the industrial production of PHA, e.g. from the paper industry [67]. Especially in such cases, the tolerance against different inhibiting compounds is highly desired.

Additionally, reports about microorganisms able to produce PHA from natural gas are found in the literature. Here, a two-step process was developed on a small technical scale, producing PHA in a quasi-continuous mode using four interconnected bioreactors from natural gas. Considering the entire process together with the product isolation, the authors estimate the production cost per kg PHB at €11.50–14 on a

scale of 550 annual tons and emphasize that this price can still be decreased after a scale-up to annually 5000 tons [84].

Environmental risks caused by methane from leaking gas pipes have been identified in many urban areas and are responsible, e.g. for the mortality of trees. On the pilot scale, the production of high amounts of PHA from methane using the methanotroph organism *Methylocystis* sp. GB 25 was demonstrated by Wendlandt et al. [85]. It was possible to run the process in non-sterile mode; applying potassium deficiency as the growth-limiting factor, an ultra-high molecular-mass PHA of 3.1 MDa was obtained. The authors state the possibility to substitute methane with biogas plants where the biogas is obtained from renewable sources. This strategy enables the development of a cost-efficient methane-based production process for high-quality PHA.

2.4 Mixed cultures for PHA production

Engineered ecosystems like certain wastewaters or activated sludge intrinsically harbor PHA-producing microorganisms. In order to achieve lower production costs, these resources are in consideration as cheap substrates for PHA production. A high potential in this field is seen in the use of mixed cultures, meaning the usage of microbial populations of unknown composition, which, by selection via operational conditions, perform specific reactions. An article by Dias et al. [86] reviews current attempts to produce PHA from cheap waste substrates using mixed cultures. Mixed cultures can be adapted to complex feedstock and sterilization of equipment can be omitted, thus lowering production costs. Promising results in terms of yields and specific productivity are given by studies feeding acetate [87], but also by studies using (prefermented) wastewater, like olive oil mill effluents [88], sugarcane molasses [89], food waste [90] and domestic wastewater [91], mainly using the aerobic dynamic feeding strategy where feeding is operated in a pulse-wise mode. Also continuous-feeding strategies enable PHA production with the additional effects of a more stable process and altered copolymer composition [92]. Another interesting aspect and powerful advantage of mixed cultures is identified by the fact that organisms can be selected by functions in order to optimize the process. For example, glycogen-accumulating organisms can be enriched in alternating A/O conditions [62]. Depending on the substrate, PHA is produced under concurrent consumption of glycogen, resulting in higher production rates and different copolymer composition [63].

3 Translation of industrial PHA production to ecological concepts

3.1 General

Nowadays the ecological necessity to switch to alternative raw materials independent of fossil resources is generally undisputed. This is not only valid for polymer production but also for the generation of energy carriers like fuels and for bulk chemicals. Already in 1992, at the Earth summit of the United Nations "Rio Declaration on Environment and Development," the political intention and motivation of most nations to vehemently support the development of biocompatible materials based on renewable resources was explicitly specified. Literally, Principle 4 of the "Rio Declaration" emphasizes that "in order to achieve sustainable development, environmental protection shall constitute an integral part of the development process and cannot be considered in isolation from it." This fact is valid especially in the area of goods that exert considerable ecological pressures, such as bulk chemicals and polymers. In fact, it has to be emphasized that the utilization of renewable resources does not only require novel and innovative technologies but will fundamentally change the global industrial structures [93, 94].

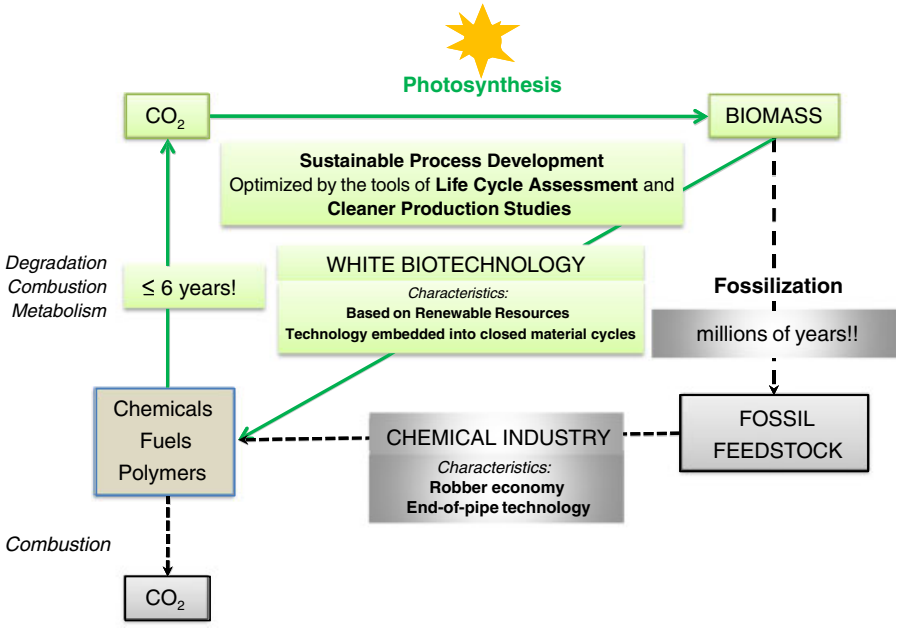

Figure 5. Scheme of the global carbon cycle. Full arrows: Biotechnological production of chemicals, fuels and polymers; the mass stream for carbon is balanced. Dashed arrows: Utilization of fossil feedstocks; carbon fixed in the bowels of the Earth is finally converted to surplus CO_2 causing a balancing problem.

3.2 The embedding of PHA into the patterns of sustainability

PHA biopolyesters represent examples for biobased, biocompatible and biodegradable materials that can be processed to create a broad range of commercial plastic products. For PHA-harboring prokaryotic cells, these inclusions mainly serve as storage materials for carbon and energy; this provides them an advantage for survival under starvation conditions. Besides this, PHA play crucial metabolic roles for different microbial species in sporulation, cyst formation, enzymatic activities, germination, and the control of excretion of extracellular polysaccharides and enhance their endurance under environmental stress conditions. Under conditions of starvation, these reserve materials are catabolized again by the cells. If items made of PHA are composted, they are completely degraded to water and CO_2 as the final products of their oxidative breakdown. Here, it has to be emphasized that water and CO_2 are the starting materials for the photosynthetic regeneration of carbohydrates by green plants. This demonstrates that, in contrast to petrol-based plastics, PHA are perfectly embedded into the natural closed cycle of carbon.

The integration of biobased materials like PHA, biofuels and biochemicals into the global carbon cycle is illustrated in Fig. 5. Here, it is well visible that the carbon balance for the creation and conversion of biobased materials is in contrast to the application of fossil resources, where carbon that was fixed in the Earth's interior is released as additional CO_2 within a very short time frame.

3.3 Modern tools for assessment of the ecological impact of PHA production

Using the tools of life cycle assessment (LCA) and Cleaner Production studies, a lot of effort is contemporarily made to quantify the environmental impact and feasibility of processes for the production of polymeric materials [95]. An LCA, also known as "ecobalance" or "cradle-to-grave analysis," is the investigation and evaluation of the environmental impacts of a given product or service caused or necessitated by its existence. The term "life cycle" refers to the notion that a fair, holistic assessment requires the assessment of raw material production, manufacture, distribution, use and disposal, including all intervening transportation steps necessary or caused by the product's existence. The sum of all these steps constitutes the life cycle of the product. The concept also can be used to optimize the environmental performance of a single product (ecodesign) or to optimize the environmental performance of a company.

The sustainable process index (SPI) developed by Krotscheck and Narodoslawsky [96] is based on the assumption that a sustainable economy has its fundaments on solar energy. Solar energy can be directly used via the techniques of photovoltaics and thermal solar energy or by the indirect utilization of solar energy via conversion of biomass [94]. Surface area is needed for the conversion of energy into products and services. The available surface area is the limiting factor in a sustainable economy because the Earth has a finite surface. Area is the underlying dimension of the SPI; the more area a process needs to fulfill a service, the more it costs from a sustainable point of view. The SPI as a tool looks at the whole product – service chain of PHA production and provides concrete hints about the environmental impacts of the processes in question.

Cleaner Production is a preventive, company-specific environmental protection initiative. It is intended as a tool to minimize waste and emissions and to maximize product output. By analyzing the flow of materials and energy in a company, one tries to identify options to minimize waste and emissions out of industrial processes through source reduction strategies [97, 98]. Improvements of organization and technology help to reduce or suggest better choices in the use of materials and energy (especially solar energy), and to avoid the formation of waste streams, wastewater generation, gaseous emissions and also surplus heat and noise. According to the sustainability of the product, the production process has to meet several requirements. Up to now, there is not too much experience available about the applications of Cleaner Production principles to biotechnology but some of the principles of "green chemistry" have considerable relevance. The concept of green chemistry was introduced in the early 1990s by the US Environmental Protection Agency (www.epa.gov), in order "to promote chemical technologies that reduce or eliminate the use or generation of hazardous substances in the design, manufacture and use of chemical products." Its principle emphasizes that prevention of ecological damage has to be aspired rather than its cure. Green chemistry is currently closely associated with the 12 principles formulated by Anastas and Warner in 1998 [99], which advocate a decrease in the environmental impact of a chemical product by considering aspects of its entire life cycle – from the raw material to product use until its final fate.

From the chemical engineering point of view, the production of biodegradable polymers has to be performed in zero-emission processes. Zero emission in this context means: no wastewater, no emission of global warming gases, no solid waste to landfill or simple incineration.

The application of Cleaner Production possibilities has up to date not been studied in the field of PHA production. This has to be done for future PHA production processes that can be optimized far beyond the state-of-the art, saving process energy and minimizing waste from the process. Together with the tools of LCA, Cleaner Production studies constitute a precious tool to indicate the embedding of the biopolymer production process into the patterns of sustainability. Figure 6 illustrates the connections between the strategies of Cleaner Production, the tools of LCA, and the quantitative measure SPI for achieving ecological sustainable development (ESD).

3.4 Ecological "hot spots" in the PHA production chain

In order to put the complete PHA production process into the patterns of sustainability, the entire process chain has to be taken into account. Here, PHA must outperform synthetic plastics regarding the overall environmental impact [100].

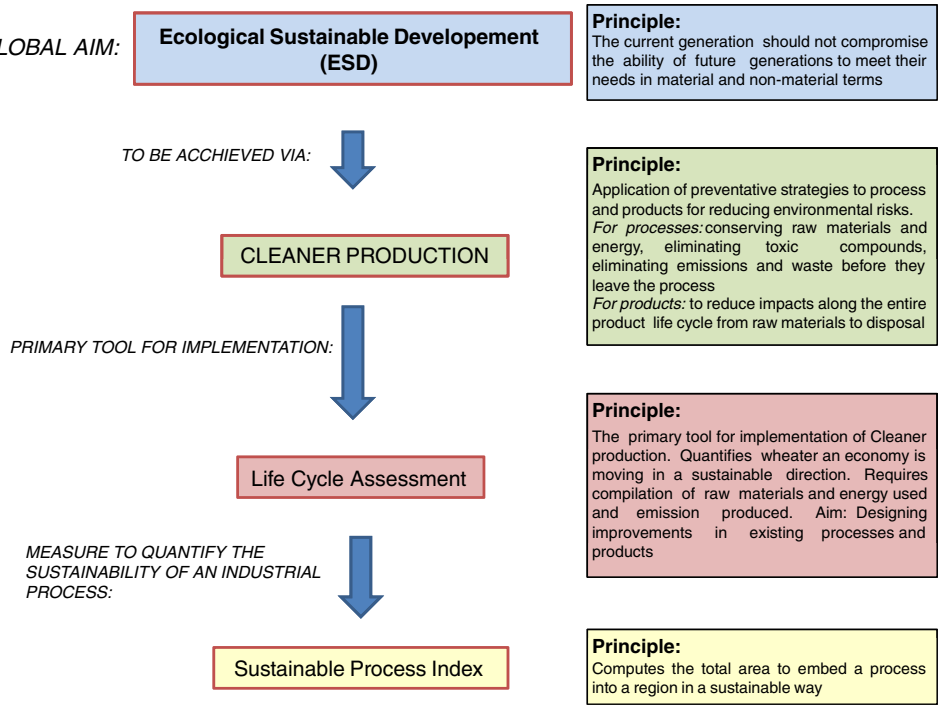

Figure 6. The connections between the strategies of Cleaner Production, the tools of LCA and the quantitative measure SPI for achieving ecological sustainable development.

Some authors have concluded that PHA production from pure glucose using monoseptic cultures does not have a real environmental advantage over conventional polymer production from crude oil [101, 102]. This is explained by the fact that high levels of energy consumption throughout the production process (from cradle to grave) are the most important contributor processes. The identification of ecological "hot spots" in the PHA production chain is performed by ecological appraisal using the SPI. This tool shows that process yield, energy consumption and release of CO_2 are of vital significance to the ecological pressure of PHA production. Energy consumption, on the one hand, is by far the main contributor of ecological pressure in the whole process; process yield, on the other hand, is the factor that defines the amount of product on which the pressure caused by the process is burdened.

3.4.1 *Selection of the raw materials*

The selection of the proper raw material for biopolymer production has an additional impact on the ecological pressure of the entire process. It has to be emphasized that the production of glucose from corn as a substrate for PHA production negatively contributes to the entire eco-balance due to photochemical smog, acidification and eutrophication as the environmental burdens associated with corn cultivation [100]. The utilization of carbon-rich waste streams upgraded to the role of starting materials for PHA biosynthesis constitutes a feasible strategy for cost-efficient biopolymer production and helps the industry to overcome their disposal problems. Examples for such carbon-rich waste and surplus streams are found in agriculture and such industrial branches that are closely related to agriculture, such as the dairy industry, the sugar industry, forestry, biodiesel production [103] and other food industries [104]. Choosing the most suitable substrate first of all depends on the intended location of the PHA production plant, and on the quantities of available resources. This is of major importance for minimizing fuel requirements and CO_2 emissions by shorter distances for transportation of the raw materials [105, 106]. Also, in the case of cheap raw materials, the usage of open mixed cultures instead of monoseptic cultures can additionally help in lowering PHA production costs as the energy for sterilization of equipment can be saved [23, 100].

3.4.2 *Downstream processing and product refining*

Downstream processing constitutes a key part of the entire PHA production process. After biosynthesis of the polyester and separation of the bacterial biomass (normally via centrifugation, sedimentation or filtration), the needed process for PHA recovery constitutes a non-negligible cost factor, especially in large-scale production. Typical halogenated PHA extration solvents like chloroform, dichloromethane or 1,2-dichloroethane show excellent performance in the isolation of short and medium chain length PHA in terms of extraction yields and product purity [107]. But, in addition to the costs, the applied method for PHA isolation and purification has a significant impact on the ecological footprint of the entire production process [108]. The said halogenated solvents, especially chloroform, reveal a high risk not only for the environment but also for the personnel working with them. In order to avoid leaving the patterns of sustainability in biopolymer production, it is crucial to concentrate the development of new extraction

processes on easily recyclable solvents with environmentally benign properties, such as lactic acid esters [109].

Several attempts are described in the literature to break the surrounding microbial cell mechanically, without the excessive need of solvents. The application of ultrasonic disruption and enzymatic digestion of the cell mass are examples for solvent-free isolation of unscathed, native PHA granules [110]. These strategies are often very feasible regarding the quantitative release of native PHA granules. Nevertheless, these granules are still covered with membranes that have to be removed if high polymer purities are required, e.g. for application in the medical field. Here, the quantitative removal of lipopolysaccharides (endotoxines) has to be guaranteed in order to avoid inflammatory in vivo reactions [111]. These additional purification steps severely increase the entire costs for downstream processing. Further, enzymatic methods, especially the application of free enzymes, are highly cost demanding.

The selection of the adequate method for separating PHA from residual biomass is determined by several factors: the production strain, the required product purity, the availability of isolation agents and the acceptable impact on the molecular mass of the biopolymer. Research in this field is quite advanced in terms of minimizing the required amounts of solvents and other cost-intensive and/or hazardous compounds as well as in minimizing the needed energy input. What is missing in most cases are feasibility studies for the application on the industrial scale [112].

3.5 Case studies for the ecological impact of PHA production

Due to the fact that not too many commercial data are available from large-scale PHA production, complete and well-grounded LCA studies encompassing the entire environmental benefit of PHA are rather rare. Despite this fact, some attempts to quantify the environmental impact of PHA production via the tools of LCA are found in the literature, mainly focusing on isolated aspects of the production like merely the polymer production itself or only CO_2 emissions or energy requirements [101, 113]. In addition, the conclusions of these studies are often not in agreement or even in contradiction to each other.

3.5.1 *Integration of PHA production into the sugarcane and bioethanol industry*

Recently, a comprehensive and complete "cradle-to-grave" LCA study was elaborated for a PHA production process by the company PHB Industrial S/A (PHBISA) in the Brazilian state of São Paulo, in comparison with the petrochemical plastics polyethylene (PE) and polypropylene (PP) [114]. At PHBISA, PHA production is embedded into the sugar and bioethanol industry. Starting from sugarcane, the company produces saccharose and ethanol. The waste streams from the sugar production (the lignocellulosic material bagasse that remains after sugar extraction from the crushed sugarcane plant) and the bioethanol production (fusel alcohols from the distillation process) are used for running the PHA production and making it economically competitive. About 3 kg of sucrose is needed to produce 1 kg PHB using *C. necator* as production strain. The electrical power needed is generated by high-pressure steam from burning bagasse, the major by-product of the sugar production. Low-pressure steam that is additionally required for heating and sterilization is also provided from bagasse combustion [115, 116]. Due to the fact that the generation of energy is based on the renewable resource bagasse, the independence from fossil fuels is valid for the entire PHA production process. The avoidance of typically halogenated solvents for the isolation of the biopolymer from the cells by using fusel alcohols from the distillative bioethanol production additionally lowers the ecological footprint of this process. The LCA presented in the study encompasses the net CO_2 production and all major categories of the production cycles. As a conclusion, PHB from the "Brazilian process" turned out to be superior to polypropylene and polyethylene in all major LCA categories [114]. Figure 7 provides a scheme indicating the closed cycles for material streams for the production of sucrose, bioethanol and PHA at PHBISA. This illustration also indicates the potential utilization of different waste streams of the process. For example, the recycling of residual PHA-free biomass after hydrolysis as a carbon and nitrogen source should be assessed and compared to alternative applications like the utilization as fertilizer for sugarcane cultivation or the generation of the energy carrier methane via anaerobic digestion of the biomass hydrolyzate in biogas plants.

3.5.2 *Application of osmophilic organisms for PHA production on waste materials*

As the major risk factor in large-scale biotechnology, microbial contamination may occur in all PHA production processes, thus endangering expensive fermentation batches. Especially when non-purified complex raw materials like whey are used as substrates, the risk of microbial contamination increases considerably [117]. Thermal processing, which is frequently used to prevent microbial contamination, is an expensive, time- and energy-consuming process step [118], without guaranteeing the deactivation of frequently occurring thermo-resistant spore-forming bacteria [119]. A way of minimizing the risk of microbial contamination is the use of, e.g. highly osmophilic strains like the archaeon *H. mediterranei* or the genus *Halomonas* for PHA production [120, 121]. Under the highly saline conditions used for cultivation of *H. mediterranei* (typically 120–200 g/L NaCl), growth of contaminants like, e.g. *Bacillus cereus* is totally inhibited. Laboratory experiments confirmed the possibility to run *H. mediterranei* cultivations monoseptically under continuous conditions without major sterilization requirements. As a drawback, the high salt concentration in the medium increases the costs for the nutritional broth [122] and makes the medium highly corrosive for common steel materials used for bioreactor equipment, thus shortening the shelf life of the production plant. This demands the utilization of steel of highest quality. Here, it has to be decided as the case arises if the low risk of contamination compensates the higher costs and the possibly higher environmental impact for the production of high-quality steel. This problem could be overcome by the utilization of corrosion-resistant materials like PEEK [120, 123].

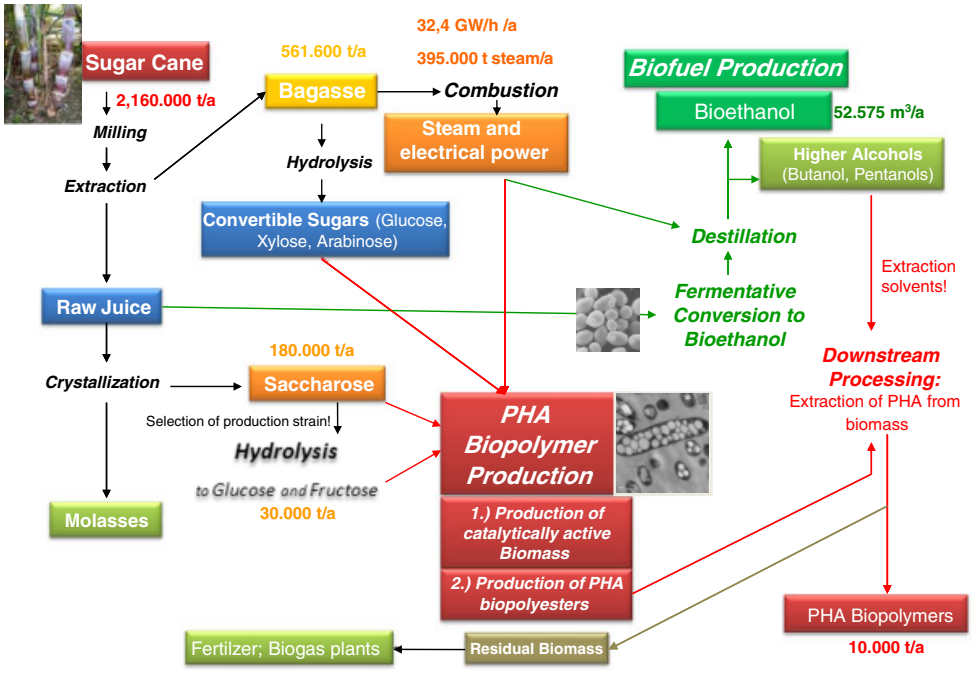

Figure 7. Embedding of PHA production into the sugarcane and bioethanol industry at PHBISA, Brazil.

Concerning the product quality, *H. mediterranei* raises considerable interest due to the fact that the strain produces PHA copolyesters consisting of 3HB and 3HV building blocks from simple carbon sources like carbohydrates [122, 124–126]. For the formation of the 3HV building blocks, no supplementation of expensive odd-numbered fatty acids like propionate or valerate is required using this organism. This behavior is very rare among PHA-producing wild-type species. In addition, the copolyester composition is constant during the entire production phase. At the moment, the elucidation of the genetic and metabolic background of 3HV production from unrelated carbon sources by *H. mediterranei* and other representatives of the extreme halophilic branch of archaea constitutes a research field with increasing attendance [126].

When highly osmophilic PHA production strains like *H. mediterranei* are exposed to hypotonic media (distilled water), their cells become fragile. Under these conditions, the osmophilic cells burst, releasing all the cell components into the medium. Hence, cell disruption of the PHA-loaded biomass can be carried out without the use of chemicals, only by input of mechanical energy. Also in this step, recycled water will be used instead of fresh water, reducing the overall water consumption of the PHA production process. Because of the considerable size and density of the PHA granules, they can easily be recovered by low-speed centrifugation, sedimentation or filtration (see Fig. 8). If higher purity is demanded, the obtained whitish crude sediment of PHA granules has to be further washed several times with detergents (e.g. SDS) that can break down impurities consisting of proteins and lipids. After drying, a fine powder made up of PHA with a degree of purity sufficient for many applications is obtained, which can be directly used for polymer processing.

The recycling of the major side streams from PHA production with *H. mediterranei* (mainly the disposal of the highly saline supernatant) is not only a crucial cost factor but also of major importance for the minimization of the ecological risks stemming from the enormous salty lots. Experiments focusing on the impact of reutilizing those side streams (salty supernatant and saline cell debris after cell disruption) should constitute the core part of future research activities with this strain, which is considered to have a high potential for industrial-scale PHA production [120]. In addition, the application of extremophilic organisms opens the route for novel PHA production strategies. Figure 8 illustrates the process scheme for the production of biopolymers from complex waste materials using *H. mediterranei*. The recycling of different material streams (water, salts and biomass) is indicated in the scheme.

3.5.3 *Utilization of recombinant organisms for PHA biosynthesis*

Akiyama et al. calculated the production costs and cradle-to-factory energy input and CO_2 emission for the large-scale fermentative production of poly(3-hydroxybutyrate-*co*-5 mol% 3-hydroxyhexanoate) [P(3HB-*co*-5 mol% 3HHx)] from soybean oil by a recombinant strain of *R. eutropha* harboring *Aeromonas caviae* PHA synthesis genes. The results were compared with the production of PHB from glucose using the same organism and the production of petrochemical plastics [108].

As a result, the authors report production costs for P(3HB-*co*-5 mol% 3HHx) of US-$ 3.53 and 4.77 per kg, taking into account an annual production of 5000 tons. This is similar to the reported price for PHB from glucose where the authors calculate a price of US-$ 3.88–4.24 per kg. Here, it has to be emphasized that the utilization of soybean oil results in lower raw material costs due to the fact that this substrate

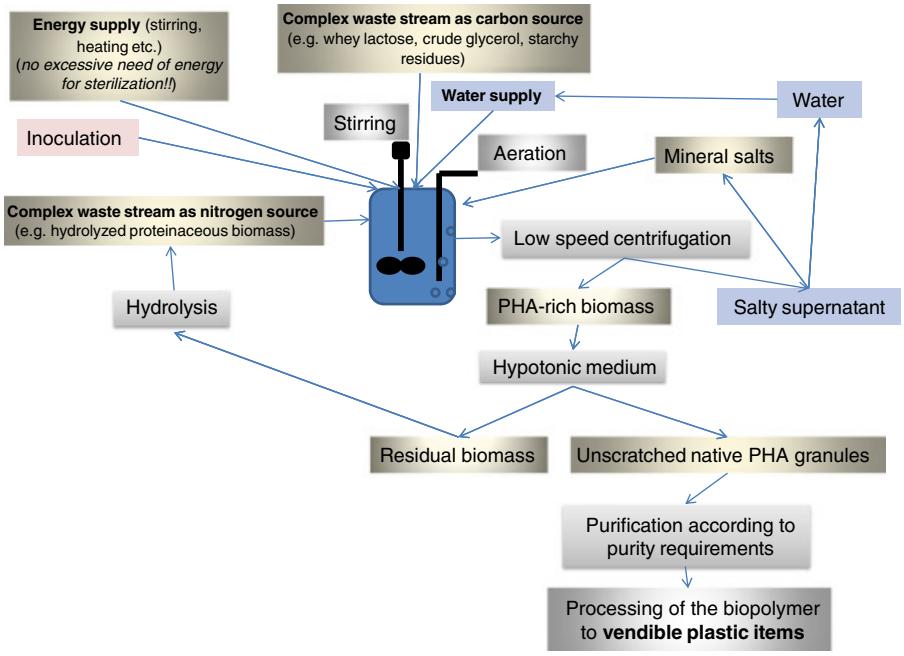

Figure 8. Process scheme for the production of biopolymers from complex waste materials using the osmophilic organism *H. mediterranei*. The recycling of materials (water, salts, and biomass) is indicated in the scheme.

is converted to P(3HB-*co*-5 mol% 3HHx) at a yield of 0.7–0.8 g/g, which is much higher than in the case of PHB where 1 g of substrates yields only 0.3–0.37 g of biopolymer. However, due to the fact that in the case of soybean oil lower cell concentrations and lower volumetric productivities are achieved, the equipment-related costs are significantly higher as in the case of glucose [108].

Comparing the cumulative energy use and CO_2 emissions of a range of petrochemical plastics and the biopolymers PHB and P(3HB-*co*-5 mol% 3HHx), the group of petrochemical polymers has much higher values in both categories than PHA. This can be clearly understood by the fact that feedstock energy accounts for about 50% of the cumulative energy use for petrochemical polymers but for 0% for PHA, due to different origins of the feedstock. On the contrary, process energy shares nearly all of the cumulative energy used for PHA production. As for cumulative CO_2 emissions, absorption of CO_2 from the air by the green plants has mainly contributed to reducing the CO_2 emissions for PHA production [108].

3.5.4 *LCA of PHA production using mixed cultures*

An LCA together with a cost analysis of PHA and biogas production using mixed cultures was performed by Gurieff and Lant based on industrial wastewater treatment [100]. The internal rate of return of materials and CO_2 emissions were used to quantify the economic viability and the environmental impact. The authors conclude that the production of PHA from mixed cultures was preferable to biogas production for treating the specified industrial effluent. In addition, the PHA production was also economically more attractive if compared to monoseptic PHA production. Both PHA production processes had similar environmental impacts that were significantly lower than the production of petrochemical polymers. The authors indicate a large potential for environmental and economic optimization for the PHA production process, mainly due to the energy use for downstream processing. Energy has been shown to be the largest contributor to process costs and environmental impact. If cheap, reliable sources of renewable energy were made available through the help of legislation and subsidies, PHA production from mixed cultures would become both economically and environmentally attractive and would provide a viable strategy for effective industrial wastewater treatment [100].

4 Concluding remarks

The article demonstrates the considerable importance of PHA for numerous microbial functions in the ecosphere. Advanced methods for rapid and accurate detection and characterization of PHA already in the biologically active environment are described and compared; in future, some of these methods are expected to constitute precious tools to facilitate a deeper understanding of the versatile roles of PHA in nature. In addition, the work at hand combines the said ecological functions of PHA with the explication of the high potential of these materials for sustainable development of the polymer industry. Especially the utilization of materials with a negative impact on the environment as substrates for PHA biosynthesis provides the industry with a possibility to overcome disposal problems and is beneficial for cost efficiency in PHA production. Further, the polymer industry can get precious impulses for future-oriented product and process development. Regarding the results of research in the field of PHA accomplished all over the world, especially during the last few years, one can easily realize that the development of a biopolymer production process that deserves to be classified as "sustainable" both in the economic and environmental aspect needs the narrow cooperation of experts from different

scientific fields. Experts in the areas of chemical engineering, microbiology, enzymology, polymer chemistry and genetic engineering are invoiced to combine their knowledge in order to close the still existing gaps between promising data from laboratories and the industrial implementation of the research results. In order to assure ecological soundness and support economical success of bioproducts like PHA, the early and critical assessment during process development helps to identify and avoid ecological "hot spots" that can easily endanger the sustainable embedding of a bioproduct into nature's closed cycles. The work at hand teaches that also in the case of biopolymers, such LCA can efficiently be accomplished by using members of the ecological footprint family such as the SPI. In combination with Cleaner Production studies that aim at the creation of closed "Zero Emission" production cycles, these modern tools provide a plain and straightforward comparison of PHA production with the manufacturing of plastics from petrochemistry. As a prerequisite, this must not only take into account single aspects like the CO_2 balance, but has to consider the holistic impacts on the eco- and the sociosphere. As a conclusion, it has to be emphasized that such comparisons have to encompass the entire "cradle-to-grave" polymer production chains starting from the raw materials until the final treatment of utilized plastics, considering each side stream of all single process steps.

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