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Review

Techniques for tracing PHA-producing organisms and for qualitative and quantitative analysis of intra- and extracellular PHA

Poly(hydroxyalkanoates) (PHAs) constitute a versatile family of prokaryotic reserve materials that, thanks to their plastic-like properties, displays high potential for application in various fields of the plastic market. Various methods are reported for tracing PHA-producing organisms, and for qualitative and quantitative analysis of both intra- and extracellular PHA. Different techniques are needed first to discover novel powerful PHA-producing microbes, second to identify new PHA-building blocks, and thirdly for structural and conformational PHA analysis on the intramolecular level. Further, as the aspect of utmost industrial significance, methods for fast, reliable, and routine process control during PHA bioproduction are required. This review tracks the development of different methods for PHA detection and determination, such as gravimetry, turbidimetry, optical, fluorescence, or electron microscopic techniques, UV, Raman, and infrared spectrometry, genetic methods (Southern blot hybridization, PCR, fluorescence-labeled in situ hybridization), enzyme-based biosensors, ^{1}H and ^{13}C NMR, and advanced gas and liquid chromatographic approaches. We propose the most suitable techniques for particular challenges of PHA research and production, and critically assess their strengths and limitations. Moreover, we provide suggestions for future developments in order to accelerate the final launch of PHA on the "bioplastic" market as anticipated since decades by the respective scientific community.

Keywords: Biopolymers / Detection / Monomers / Poly(hydroxyalkanoate) (PHA) / Quantification

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1 Introduction

The rapidly increasing need for bio-based materials to replace fossil-based plastics spots the light on poly(hydroxyalkanoates) (PHAs), a group of biodegradable polyoxoesters of hydroxyalkanoates (HAs). PHAs are produced as inert intracellular storage materials by prokaryotic microbes found among numerous genera. The growing interest in these materials is attributed to their manifold applicability in various fields of the plastic market. To a steadily increasing extent, the understanding of PHA's multifacetted roles in different ecosystems, as well as the complex interaction of PHA metabolism and other biological processes calls for deeper investigation [1]. This is coherent with the growing number of techniques developed for tracing PHA-producing

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Abbreviations: **DAPI**, 4′,6-diamidino-2-phenylindole dihydrochloride; **EC**, enzyme commission number; **FID**, flame ionization detection; **FISH**, fluorescence-labeled in situ hybridization; **FTIR**, Fourier transform infrared spectroscopy; **GC-FID**, GC coupled to FID detection; **GC-MS**, GC coupled to MS detection; **GPC**, gel permeation chromatography; **HA**, hydroxyalkanoate; **IR**, infrared; **LC-MS**, LC coupled to MS detection; ***mcl*-PHA**, medium chain length poly(hydroxyalkanoate); **NAD^{+}**, nicotinamide adenine dinucleotide (oxidized form); **NADH**, nicotinamide adenine dinucleotide (reduced form); **PHB**, poly((*R*)-3-hydroxybutyrate); **PHBV**, poly((*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate); **PLA**, poly(lactic acid); **poly-P**, poly(phosphate); ***pyr*-GC**, GC coupled to pyrolysis as pretreatment method; **SBH**, Southern blot hybridization; **SEC-MALS**, SEC coupled to a multiangle light scattering detector; **STEM**, scanning transmission electron microscopy; **TEM**, transmission electron microscopy; ***scl*-PHA**, short chain length PHA; **3HB**, (*R*)-3-hydroxybutyrate; **3HBDH**, (*R*)-3-hydroxybutyrate dehydrogenase; **3HHx**, (*R*)-3-hydroxyhexanoate; **3HV**, (*R*)-3-hydroxyvalerate; **4HB**, 4-hydroxybutyrate

organisms in natural habitats, to quantify PHA in microbial biomass, and to monitor PHA in *statu nascendi* during biosynthesis. Genetic and microscopic techniques to trace such organisms are progressively enhanced; at the same time, quantitative and structural analysis of intracellular and isolated PHA is of increasing importance for kinetic analysis of PHA-formation processes, to develop new functionalized PHA biopolyesters, and for reliable and robust control of large-scale PHA production processes.

Starting from low-tech techniques such as gravimetric determination of PHA laboriously extracted from biomass, the corresponding scientific community soon realized the need for advanced methods to exactly and rapidly determine PHA not only on a quantitative basis, but also regarding its exact composition on the monomeric level. This monomeric composition of PHA became increasingly important by the discovery of various different HAs acting as PHA building blocks. Until 1974, (*R*)-3-hydroxybutyrate (3HB) was the only reported PHA constituent of microbial PHA; this situation changed by the discovery of (*R*)-3-hydroxyvalerate (3HV) by Wallen and Rohwedder in activated sludge bacteria in 1974 [2], and by the identification of 11 different HAs in *Bacillus megaterium* and microbes from marine sediments by Findlay and White in 1983 [3]. In the late 1980s, the first achiral PHA-building block was reported by Doi et al. by the identification of heteropolyesters consisting of 3HB and 4-hydroxybutyrate (4HB) [4]. Soon, it was understood that the exact composition of PHA is pivotal for its physicochemical properties and degradability, hence for its applicability as "green plastic" [5]. Poly((*R*)-3-hydroxybutyrate) (PHB), the most prominent PHA, is a highly brittle material of restricted applicability, whereas its 3HV- or especially 4HB-containing heteropolyesters display advantageous properties such as increased flexibility, degradability, lower melting temperatures, and lower crystallinity. In the case of PHA consisting of HAs with six or more carbon atoms, so-called medium-chain length PHA (*mcl*-PHA), extremely low crystallinities, and glass transition temperatures are reported, giving rise to their classification as "bio-latexes"; such *mcl*-PHA are predominantly produced by *Pseudomonas* sp. [6, 7]. Apart from the average monomeric composition, the molecular structure regarding molar masses, molar mass distribution, and distribution of monomers in the polyester chains is pivotal for the physical and thermal characteristics of PHAs [8, 9]. Such structural and distributional aspects can be elucidated by means of SEC coupled to a multiangle light scattering detector (SEC-MALS); this way it is possible to discriminate between heteropolyesters with randomly distributed monomeric building blocks on the one side, and blends of different homopolyesters on the other side [10].

Until today, the number of described PHA building blocks can be estimated with about 150–200 HAs and hydroxyalkenoates, also encompassing chemically functionalized (unsaturated, halogenated, aromatic etc.) representatives [11]. The identification and quantification of such novel and exotic building blocks requires the implementation of high-tech methodologies such as NMR or MS. From the applicability and production point of view it can be stated that simple short chain length PHA (*scl*-PHA; PHB and its heteropolymers with 3HV and 4HB) are already available on a (semi)industrial scale (quantity of tons), whereas simple saturated *mcl*-PHA is produced on a lower kilogram scale despite the recent announcement of MHG Meridian to start industrial scale production of *mcl*-PHA. Special functional PHA did not exceed laboratory scale production level yet (quantities of grams or even only milligrams) [12]; this is in contrast to the broad prospective of novel, functional biopolyesters to be used in various niches of the plastic market, such as the medical and pharmaceutical field, production of advanced bio-latexes, thermosensitive adhesives, or other "smart biomaterials" [13]. Figure 1 provides the general structure of PHA and indicates the broad range of different compositions.

Fast and reliable detection of PHA in natural environments is of utmost importance to discover novel, powerful PHA-producing organisms adapted to, e.g., extreme environments (temperature, salinity, pH conditions, dryness, etc.) or to exotic substrates (inexpensive carbon sources such as industrial surplus streams, eco-pollutants such as hydrocarbons, etc.). Here, staining methods based on the lipophilic character distinguish PHA-rich from non-PHA-producing cells, but are error-prone by encompassing also various lipophilic non-PHA inclusion bodies. The rapid development of genetic methods and the elucidation of the enzymatic background of PHA biosynthesis resulted in the implementation of Southern blot hybridization (SBH), fluorescence-labeled in situ hybridization (FISH), or PCR to more reliably identify PHA-producing organisms [11].

Qualitative spectroscopic methods such as infrared (IR), RAMAN, and NMR are current techniques of increasing importance for structural characterization of PHA. Whereas IR and RAMAN spectroscopy provides information on functional groups in the polyester and their interaction, NMR enables the identification of different monomeric building blocks.

The exact and rapid determination of quantity and composition of PHA is needed in order to adapt the process regime during the bioproduction in bioreactors by changing the feeding strategy. Several methods, mainly based on GC and, (to an evanescent extent), LC, were continuously developed and improved during the last three to four decades. They were applied both for scientific research (identification of new building blocks, production kinetics of PHA during biosynthesis) and for process monitoring on industrial scale. Especially, the coupling of LC and GC with MS enables the determination of highly complex PHA heteropolyesters, such as produced by *Pseudomonas* sp. based on mixtures of long fatty acids [12].

Triggering polyester composition during biosynthesis involves the adjustment of carbon source supplementation to the microbial cells. The switch between different carbon sources impacts the monomeric composition of the accumulated PHA. As most prominent examples, the species *Cupriavidus necator* switches from the production of PHB based on hexoses [14, 15], glycerol [16], or lipids [17, 18] to heteropolyesters containing 3HV, if structurally related carbon substrates (propionic acid, valeric acid, margaric acid, etc.) are provided as precursors [19], and to heteropolyesters containing 4HB after supplementation of the precursors butyrolactone or 1,4-butandiole [4]. Fast responding analytical techniques reporting the actual amount and composition of PHA in a production process enable the quick adaptation of the feeding regime, hence the production of tailored biopolyesters [12].

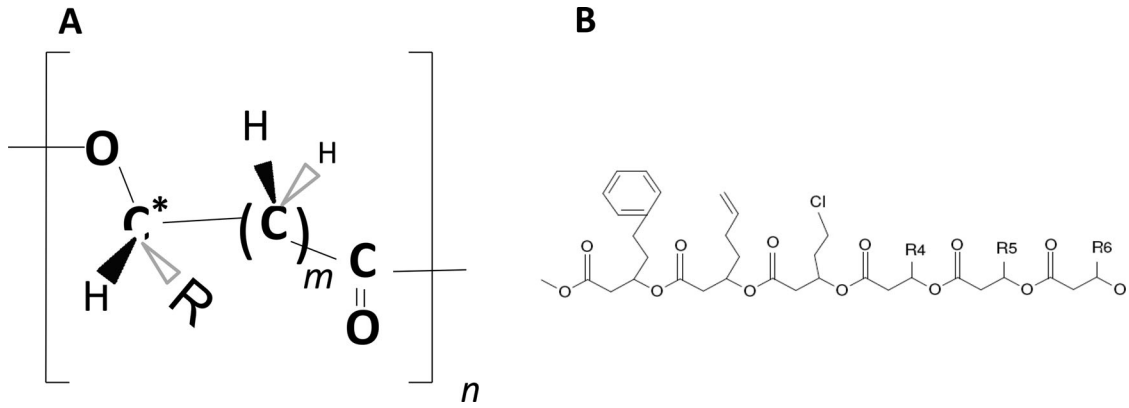

Figure 1. Chemical structures of PHA. (**A**) General structure of PHA (*R*: side group; *m*: number of methylene groups in the monomer's backbone; *n*: number of monomers on the polyester chain). (**B**) Example of functional PHAs containing 5-phenyl-pentane-, hex-6-ene-, and 5-chloro-pentane groups (*R4*, *R5*, and *R6* represent additional monomers).

In addition to chromatographic methods, the application of biosensors for rapid determination of PHA might become of increasing importance for industrial scale PHA production due to a possible permanent and inexpensive online monitoring of PHA production.

The subsequent Table 1 collects PHA detection and determination methods reported in literature, lists their advantages and drawbacks, and reports on their suitability for routine analysis; the methods are subsequently described in details in the core part of this article.

2 Direct observation of PHA

2.1 Optical microscopy

Different intracellular inclusions in prokaryotic cells are often hard to discriminate by simple observation in the light microscope. In phase contrast observation at a typical magnification of 1000×, PHA storage inclusions appear as highly refractive, more or less spherical granules inside the cells. Without applying lipophilic staining methods, it is often difficult, especially for nonexperienced examiners, to distinguish between PHA granules and other intracellular inclusions, such as spores, polyphosphate (poly-P), or glycogen inclusions. Due to the hydrophobic nature of PHA, its differentiation from other inclusions and preliminary identification is usually obtained using lipophilic dyes. This is pivotal especially in the case of such microbes accomplishing PHA accumulation and sporulation at the same time, such as the well-known Gram-positive PHA producers *Bacillus cereus* [20, 21] and *B. megaterium* [22–24].

The Sudan group of dyes is used for well-established histological tests for the presence of diverse lipophilic cellular inclusions [25]. These dyes display higher solubility in lipophilic materials than in the highly polar staining solution (mainly ethanol), and therefore easily migrate into lipophilic environments [26]. Already in 1944, Lemoigne et al. recognized that also PHA inclusions are accessible toward Sudan Black staining [25]; the so-called "sudanophilic character of PHA" is frequently cited in the respective literature [27]. Today, Sudan Black B is extensively used to test the presence of PHA [28, 29]. In order to better discriminate PHA granules from the rest of the cell, a counter-staining applies the diamino phenanzine dye safranin. Here, a black–blue granule embedded into a light pink background indicates the presence of this storage polymer [27]. Although this method provides a simple way to efficiently screen for PHA-producing microbes, it is prone to detection errors; false positives may arise from staining of other lipid storage compounds [30]. Sequential staining with 4′,6-diamidino-2-phenylindole dihydrochloride–poly-P (DAPI–poly-P) and Sudan Black for visualization of both poly-P and PHA in the same cell has been described in combination with FISH (see later). DAPI normally is used to stain DNA (blue–white fluorescence), but, at higher concentrations of the dye, also poly-P and lipid inclusions are stained (yellow fluorescence) [31].

2.2 Fluorescence staining and fluorescence microscopy

Based on fluorescence techniques, a more specific and sensitive imagining of PHA granules (if compared to Sudan Black staining) is attained by applying the fluorescent dye Nile blue [32]. Its oxidized form, also known as either Nile Pink or Nile Red, finally forms in aqueous environment and provides for the bright fluorescence of stained PHA granules due to its high solubility in such neutral lipids. The fluorescence of stained PHA granules is visible using excitation wavelengths of both 460 [32] and 546 nm (emission wavelength 582 nm) [33]. Rees et al. [33] scrutinized the concomitant production of PHA, poly-P, and glycogen in microbial consortia using different filter sets. In contrast to PHA, their inclusions such as glycogen poly-P do not stain at all with this dye [34, 35], whereas cell walls and other lipid-containing cell components apparently do not sufficiently adsorb the dye to provoke a detectable fluorescence [32].

As a rapid and direct screening test to preliminarily discriminate between PHA-positive and PHA-negative cells, colonies can be cultivated on respective solid nutrients containing Nile blue at a concentration of 0.5 μg/mL. By simple irradiation with UV lamps, PHA-rich viable colonies can easily be identified [30].

Table 1. Overview of PHA detection and determination methods

Method	Principle	Time demand	Suitable for routine analysis	Distinguishes between different types of PHA	Quantitative/ qualitative	Inactivation and/or destruction of microbial cells needed	Advantages	Drawbacks	Ref.
Optical microscopy without staining	Microscopic surveillance of living cells in liquid culture or of heat-fixed preparations. Visualization of PHA granules as highly refractive, spherical inclusions	Very low	Yes	No	Qualitative	No (liquid cultures). Yes (heat-fixed preparates)	Easy, inexpensive; fastest method	Limitations in distinguishing PHA granules form other refractive inclusions, e.g. spores. Requires experience of the examiner	
Optical microscopy with Sudan Black staining	Microscopic surveillance of cells on heat-fixed preparations in optical microscope after staining with Sudan Black and counter-staining with safranin	Low	Yes	No	Qualitative	Yes	Easy, fast, inexpensive method	Error-prone by staining also other lipophilic inclusion, e.g. wax esters, oil droplets etc	[25, 27, 29, 31]
Fluorescence microscopy: Nile blue staining of growing colonies	Incubation of colonies on solid medium (agar–agar) containing Nile blue	Low	Yes	No	Qualitative	No	Easy, fast, inexpensive method. Staining of living active cells possible. High-throughput method	Error-prone by staining also other lipophilic inclusions. Gram-positive bacteria tend to give false-positive results	[30]
Fluorescence microscopy: Nile red staining of liquid cultures	Incubation of cell culture with Nile blue excitation at λ = 540–560 nm. Emission at λ = 570–605 nm. Improved sensitivity and specificity by BODIPY 493/503 staining	Low	Yes	Yes (different emission maxima for *scl*- and *mcl*-PHA)	Qualitative and semiquantitative	No	Easy, fast, inexpensive method. Staining of living active cells possible. High-throughput method. Distinguishes between *scl*- and *mcl*-PHA	Error-prone by staining also other lipophilic inclusions. To a high extent restricted to Gram-negative strains	[36, 39–41] [42]
Fluorescence microscopy: Nile Blue staining of heat-fixed cells	Excitation of cells on heat-fixed preparates by fluorescence microscope at excitation wavelength of λ = 460 nm	Low	Yes	No	Qualitative	Yes	Easy, fast, inexpensive. High-throughput method	Error-prone by staining also other lipophilic inclusions. To a high extent restricted to Gram-negative strains	[32]

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Table 1. Continued.

Method	Principle	Time demand	Suitable for routine analysis	Distinguishes between different types of PHA	Quantitative/ qualitative	Inactivation and/or destruction of microbial cells needed	Advantages	Drawbacks	Ref.
Electron microscopy (TEM, STEM)	Fixing with glutaraldehyde, coating with OsO_4	High	No	No	Qualitative	Yes	Information on morphology of PHA granules available	Tedious sample preparation. Cost demanding. Precarious chemicals Solution: "wet-STEM"	[39,47]
Southern blot hybridization	Tracing specific target genes (*phaC* genes encoding for different classes of PHA synthases) by application of oligomers	Medium	Yes	Yes	Qualitative	Yes	Well-established routine method	Specific oligonucleotide probes needed	[52,53]
PCR	Detection and amplification of *phaC* genes encoding for different classes of PHA synthases	Low	Yes	Yes	Qualitative	Yes	Fast,highly sensitive, highly selective, high throughput, small sample quantities sufficient	Only *phaC* target genes with the correct sequence can be detected due to lack of respective primers	[54–64]
FISH and flow cytometry	Application of fluorescence labeled oligonucleotide probes (16 S rRNA) and subsequent fluorescence measurement	Medium	Yes	No (might soon be possible by application of more specific fluorescence probes)	Qualitative	Yes			[31,65–68]
Gravimetry: Lemoigne's chloroform extraction method	Extraction of PHA by chloroform, precipitation, drying, weighting	High	Yes	No	Quantitative	Yes	Low-tech method, minor equipment requirements	Requires considerable sample quantities; underestimation of value likely by losses during extraction, precipitation etc. Displays only sum parameter of entire PHA in sample	[25]
Turbidity measurement	Dissolving non-PHA cellular material; measuring turbidity caused by suspended native PHA granules	Low	Yes	No	Quantitative	Yes	Higher sensitivity than gravimetric approach	Requires standardization for every investigated PHA-producing organism	[69]

(*Continued*)

Table 1. Continued.

Method	Principle	Time demand	Suitable for routine analysis	Distinguishes between different types of PHA	Quantitative/ qualitative	Inactivation and/or destruction of microbial cells needed	Advantages	Drawbacks	Ref.
UV spectroscopy	Transformation of PHB to crotonic acid. Determination of crotonic acid by UV measurement at λ = 235 nm	Low	Yes	No	Quantitative	Yes	Low cost method. Simple and fast method. Specific to PHB	Results tend to overestimate the PHB concentration due to additional matrix component. Extraction with halogenated solvent needed. High purification of PHA needed for reliable results	[71, 72]
Fourier transform infrared spectroscopy (FTIR)	Records the IR-spectrum of PHA a spectral range of 400 to 4000 cm^{-1}	Low	Yes	No	Qualitative and semiquantitative	Yes	Highly sensitive, short analysis time, rapid, noninvasive technique to monitor the intracellular PHB content in biomass. Semiquantitative method. High-throughput method	Low sensitivity; cannot distinguish between heteropolyesters and blends of homopolyesters. Hard to distinguish between *scl*- and *mcl*-PHA	[76–81]

(*Continued*)

Table 1. Continued.

Method	Principle	Time demand	Suitable for routine analysis	Distinguishes between different types of PHA	Quantitative/ qualitative	Inactivation and/or destruction of microbial cells needed	Advantages	Drawbacks	Ref.
Raman spectroscopy	Technique to observe vibrational, rotational, and other low-frequency modes in a system. Electromagnetic radiation impinges on a molecule and interacts with the polarizable electron density and the bonds of the molecule in the phase (solid, liquid or gaseous) and environment in which the molecule finds itself. Characteristic Raman bands are evaluated for identification of crystalline and amorphous PHA moieties in pure polyester samples, blends, and composites	Low	Yes	No	Qualitative and semiquantitative	Yes	Highly sensitive. Short analysis time. Rapid, noninvasive technique to monitor the intracellular PHB content in biomass. Useful to investigate blends of different biopolymers	Low sensitivity; cannot distinguish between heteropolyesters and blends of homopolyesters. Restricted to PHB and PHBV. Hard to distinguish between *scl*- and *mcl*-PHA	[83–85]
Biosensors	Enzymatic reactions; co-factor system NAD^+/NADH or consumption of dissolved oxygen is monitored	Low	Yes	No (restricted to few building blocks)	Quantitative	Yes	Fast method for routine application	Restricted to few PHA representatives	[87–91]

(*Continued*)

Table 1. Continued.

Method	Principle	Time demand	Suitable for routine analysis	Distinguishes between different types of PHA	Quantitative/ qualitative	Inactivation and/or destruction of microbial cells needed	Advantages	Drawbacks	Ref.
GC-flame ionization detection (GC-FID): Braunegg's method and its adaptations	Extraction of PHA with chloroform; simultaneous transesterification with alcohols. Separation of building blocks on nonpolar column. Detection by FID	High (original method) Low to Medium (further developments involving pyrolysis or microwave techniques)	Yes	Yes	Quantitative	Yes	Quantitative and qualitative results. Highly sensitive, High separation capacity for different monomers	Laborious sample pretreatment (lyophilization, derivatization etc.) (Solution: microwave heating, pyrolysis-GC). Cannot distinguish between heteropolyesters and blends of homopolyesters. Halogenated solvents needed (solution: method according to [103])	[69,95–108, 110–114]
GC-MS detection (GC-MS)	After derivatization: detection of building blocks by MS	High (could be reduced by applying novel pretreatment techniques as tested for GC-FID)	Yes	Yes	Quantitative	Yes	Tentative identification of unknown building blocks possible	Cannot distinguish between heteropolyesters and blends of homopolyesters. Halogenated solvents needed (solution: method according to [103])	[115–117, 120,121]
LC: ion exchange column	Transformation of PHB to crotonic acid. Detection by UV-detector at $\lambda = 210$ nm	High	Yes	No	Quantitative	Yes	Does not require cell lyophilization. Small sample quantities sufficient. Short pretreatment time	Lower separation performance (difference in the pretreatment). Restricted to PHB. Cannot distinguish between heteropolyesters and blends of homopolyesters	[73,122]
LC: ion chromathography	Conversion of PHA-building blocks to alkenoic acids. Conductivity detection	High	Yes	No	Quantitative	Yes	Higher sensitivity, higher separation performance	Restricted to PHB and PHBV. Cannot distinguish between heteropolyesters and blends of homopolyesters	[87,104]

(*Continued*)

Table 1. Continued.

Method	Principle	Time demand	Suitable for routine analysis	Distinguishes between different types of PHA	Quantitative/ qualitative	Inactivation and/or destruction of microbial cells needed	Advantages	Drawbacks	Ref.
LC: HPLC-MS	Conversion of PHA-building blocks to alkenoic acids. Separation with reversed phase column. MS detection	High	Yes	Yes	Quantitative	Yes	Does not require cell lyophilization. Small sample quantities sufficient. Short pretreatment time. Determination of *mcl*-PHA. Tentative identification of unknown building blocks possible	Cannot distinguish between heteropolyesters and blends of homopolyesters	[12,93,119]
LC: SEC-MALS	Dissolving in chloroform followed by SEC. After coupling to a multiangle light scattering detector	Medium	Yes	No	Qualitative	Yes	Characterization of PHA on a structural level regarding absolute molar masses and conformation	Does not identify different building blocks	[126]
LC: GPC	Dissolving in chloroform followed by separation according to molar masses. Refractive index detection	Medium	Yes	No	Qualitative	Yes	Well-established technique for determination of molar mass distribution	Restricted to relative molar masses. Cannot discriminate between composites and blends. Cannot identify different building blocks	[119, 123–125]
NMR	^{1}H:for *scl*-PHA ^{13}C: for *mcl*-PHA	High	No	Yes	Qualitative and quantitative (in terms of monomer composition)	Yes	No derivatization needed. Differentiates between PHA blends and PHA heteropolymers with random monomer distribution; identification of blocky heteropolymers. Analysis of novel functionalized PHAs for which analytical standards are currently unavailable	For quantification of monomer composition: purified material needed	[127–141]

The fluorescent response of Nile blue increases with increasing PHA concentration in different colonies and cultures, and therefore can be used to monitor fluctuating PHA storage capacity. Intensity of fluorescence can also be used to estimate different amounts of PHB inside the cells [30]. Here, it has to be emphasized that Nile Red does not restrict growth of cells, but it is hardly applicable for Gram-positive bacteria due to the significant adsorption of the dye by the cell wall of such organisms. Wu et al. describe the shift of fluorescence maxima between cells containing *scl*-PHA (590 nm) and such containing *mcl*-PHA (575 nm), thus providing a possibility to rapidly discriminate between these two types of PHA in intact cells [36].

Degelau et al. [37] and Gorenflo et al. [38] proposed the combination of two-dimensional fluorescence spectroscopy and flow cytometry as noninvasive technique for determination of PHA in whole cells. Flow cytometry constitutes a well-known method for cell counting, studying the heterogeneity of a microbial population, and to select subcultures of special metabolic features. Here, Nile blue-stained colonies show a fluorescence maximum of $\lambda = 570–605$ nm when excited with wavelengths of $\lambda = 540–560$ nm. For quantitative application of this method, one can resort to the high correlation between fluorescence intensity and PHA concentration [38]. Unfortunately, this method does not allow for the differentiation of PHA homopolyesters and heteropolyesters [38]. Berlanga et al. measured the relative PHA content in unidentified microbial isolates and in reference organisms (*Escherichia coli, Pseudomonas putida, Pseudomonas oleovorans*). The authors explicitly underline the viability of Nile Red staining for rapid quantification of PHA in such unknown isolates [39].

Based on the different fluorescence maxima of *scl*-PHA and *mcl*-PHA, the coupling of flow cytometry with Nile Red staining was successfully applied by Srienc et al. to differentiate between *scl*-PHA- and *mcl*-PHA-containing cells only a short time after the existence of these two groups of PHA was discovered. [40]. Vidal-Mas et al. reported the use of flow cytometric determination after Nile red staining for monitoring of PHA production from waste frying oil by *Pseudomonas aeruginosa*; the determination of PHA contents in cells was coupled to homogeneity assessment of the microbial culture, and to tracing of subcultures with outstanding PHA production capacity; the authors report an analysis time of less than 30 min [41]. This technique was later improved by comparing the fluorescence of *C. necator* and recombinant *Saccharomyces cerevisiae* with varying PHB content after staining with boron-dipyrromethene (BODIPY) 493/503 and Nile red; automated staining techniques were developed for both cultures. It turned out that BODIPY 493/503 staining had less background staining, and higher sensitivity and specificity to PHB when compared to Nile red staining. The developed automated staining procedure determined the PHB content of a bioreactor sample at regular time intervals with accuracy comparable to GC-FID (where FID is flame ionization detection) analysis. Overall, BODIPY 493/503 resulted in superior PHB staining than Nile Red. When automatized, this technique might provide a new method for the online process monitoring and control of bioreactors [42].

As a drawback, it cannot discriminate PHA of different composition, and therefore appears to be restricted to PHB production processes. Similar to Sudan Black, Nile blue does not stain glycogen, poly-P, or spores [32], but gets adsorbed by other lipophilic storage materials such as wax esters or oil droplets [30, 43], hence, also this method tends to overestimated results. Double PHA and poly-P staining to imagine both types of inclusions in one sample was reported by Rees et al. [33], who used sequential staining with Nile blue and methylene blue on pure *Acinetobacter* sp. cultures, followed by investigation of the stained cells with both bright field (poly-P) and fluorescence (PHA) microscopy. This "Rees double staining" has been applied to active sludge bacteria, sometimes with unsatisfactory results, because this double staining technique causes both a decreased PHA fluorescence and a reduced poly-P response. The reasons for these underestimations are not completely understood yet [44–46].

A further multistaining approach of interest, especially for Gram-positive cells, might be a concomitant spore and PHA staining using malachite green as a dye for spore staining that does not stain PHA inclusions; up to date, such attempts are not reported in literature. This is of interest due to the fact that spore formation causes a loss of the applied carbon source that should be directed toward PHA production.

2.3 Electron microscopy

In order to get reliable structural analysis of cytoplasmic inclusion bodies, one can resort to means of electron microscopy. Transmission electron microscopy (TEM) provides for direct visualization of accumulated PHA granules, thus asserting PHA biosynthesis. Berlanga et al. proposed a representative and viable protocol for sample preparation and PHA visualization via TEM [39]; these authors fixed pellets of PHA-producing microbes using glutaraldehyde, followed by staining with OsO_4 and uranyl acetate; subsequently, the microorganisms were embedded in a low-viscosity resin and sectioned using ultramicrotomes. Finally, the samples were observed by TEM. Examples for TEM visualization of PHA-rich cells of the bacterial strains *C. necator* DSM 545 and *B. megaterium* uyuni S29 are provided in Fig. 2.

Similar to Nile blue staining, TEM is efficient for providing evidence for PHA production or PHA-producing capacity, but, as a shortcoming, this technique does not provide information about PHA composition. In addition, TEM sample preparation is rather time consuming and involves the use of expensive and often precarious chemicals, thus making it unsuitable for screening purposes. Some of these drawbacks might be overcome by recent developments in electron microscopy, which have opened the route to imaging of PHA granules and other intracellular inclusions within whole living cells. In contrast to traditional electron microscopy, which is characterized by time-consuming steps (drying, fixing, embedding, sectioning, and steaming), the new "wet scanning transmission electron microscopy" (wet STEM) is reported as a rapid method for imaging whole unfixed, hydrated cells at pressure conditions of 800 Pa; with this method, the visualization of internal structures in whole cells became possible. The sample preparation for "wet-STEM" involves only the removal of salts from the cultivation medium by simple washing with water. In addition, fewer artifacts are generated during "wet STEM" than normally reported for STEM

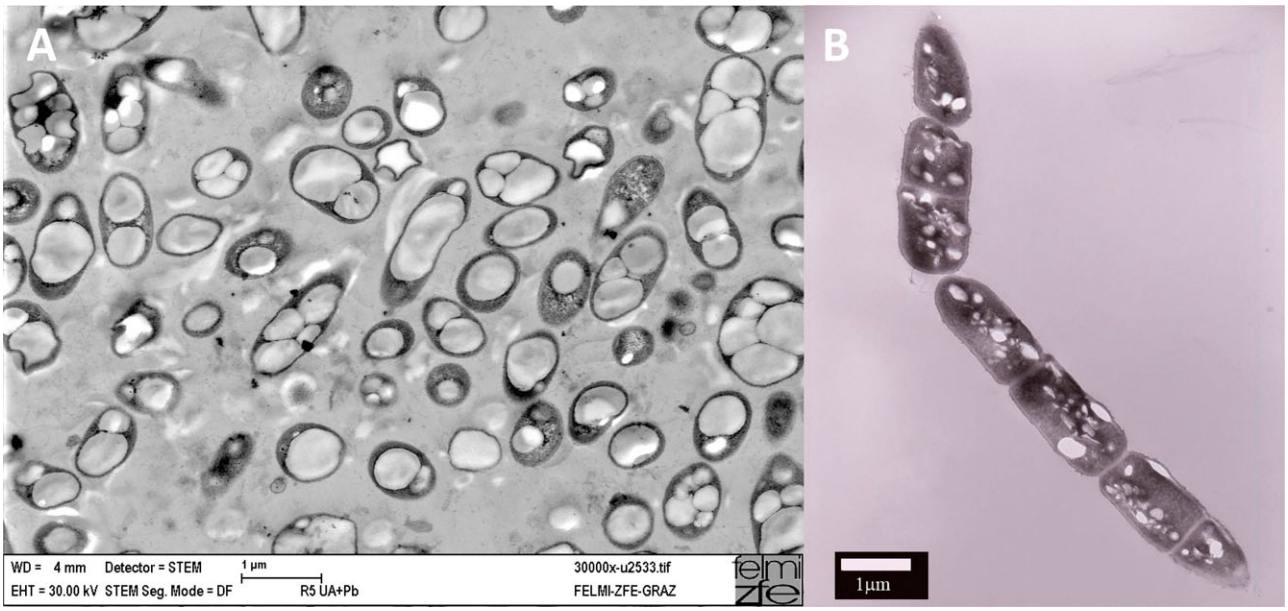

Figure 2. STEM pictures of (**A**) PHA-rich Cupriavidus *necator* cells cultivated in a multistage continuous bioreactor cascade on glucose as carbon source and (**B**) *Bacillus megaterium* uyuni S29 cultivated in fed-batch mode on glucose [23]; image reproduced with permission of *Food Technology and Biotechnology*.

preparation. As a drawback, "wet STEM" is reported to have reduced resolution [47].

3 Molecular genetic methods

Molecular genetic methods described in the next paragraphs (SBH, PCR, and FISH) can detect in principle if a certain microorganism has the potential to produce PHA on the genetic level without indicating if these genes are definitely active or not, and how much PHA can be produced. Quantification of expression of genes responsible for PHA synthesis is possible by techniques like rt-PCR, as reported in the last years [48–50].

3.1 Southern blot hybridization

SBH is a common method to trace diverse organisms by screening for a particular target gene [51]. It was almost self-evident to apply this method also to the screening of microbial samples for PHA producers by searching for specific genes encoding for key enzymes of the PHA metabolisms; especially PHA synthases (enzyme commission (EC) number 2.3.1.x) attracted early attention in this direction. PHA synthases, encoded by the *phaC* operon, are key enzymes for producing PHAs; they catalyze the polymerization of activated HAs; they are grouped in four main classes (I–IV), characterized by their specific substrate spectrum for polymerization (activated *scl*-PHA or *mcl*-HAs building blocks, respectively) [52]. Timm et al. noticed that the synthase gene from *C. necator* was not suitable for detection of all PHA producers; it excludes *mcl*-PHA producers. Therefore, the authors designed an oligonucleotide probe of 30 nucleotides highly specific for PHA synthase of *mcl*-PHA producers, and identified multiple PHA synthases in *Pseudomonas* species [53].

3.2 PCR

In contrast to SBH, PCR provides rapid proof of presence of special genes, thus allowing for screening of a broad range of both *scl*- and *mcl*-PHA-producing microorganisms. The use of PCR in the PHA field based on the detection of the *phaC* gene, and, more specifically, the detection of PHA-producing bacteria in aquatic habitats by using PCR, was first proposed by López et al. [54]. Contemporarily, primers are available to search for all four classes (I–V) of PHA synthase genes; the different applications were comprehensively reviewed before by Solaiman and Ashby [55].

Sheu et al. developed primers for colony PCR detection of class I (encoding for *scl*-PHA synthesis; prototype organism: *C. necator*) and class II PHA synthase genes (encoding for *mcl*-PHA synthesis; prototype organism: *P. putida*) in biomass from different environments. This was accomplished by aligning the highly conserved sequences of class I and II genes of various Gram-negative bacteria. For *C. necator*, the sensitivity limit amounted to merely 1×10^5 viable cells [56]. Solaiman et al. developed a PCR protocol for multiple sequence alignment based on the specific primer pair I-179 L and I-179 R. The authors resorted to the highly conserved coding regions of the class II PHA synthase genes *phaC1* and *phaC2* of *Pseudomonas resinovorans*, *P. putida*, *Pseudomonas citronellolis*, and *Pseudomonas saccharophila*, and used the protocol specifically to detect class II PHA genes encoding for *mcl*-PHA synthesis. Using this method, *Pseudomonas corrugata* was first identified as a potential *mcl*-PHA producer [57]. In order to rapidly trace and classify both *scl*- and *mcl*-PHA producers, Romo et al. designed

three different primers to identify fragments from both class I (*phaC*) and class II (*phaC1* and *phaC2*) synthase genes, plus four additional primers specific for class II synthases [58].

A viable method to detect class III PHA synthase genes of sulfate reducing bacteria and blue algae (cyanobacteria) was developed by Hai et al. [59]. Class III synthases consist of two enzyme subunits encoded by *phaC* and *phaE* genes, and catalyze *scl*-PHA polymerization. Exceptions are provided by *Thiocapsa pfennigi* and *Aeromonas caviae*; here, the catalysis of polymerization of both *scl*- and *mcl*-PHA is reported. By isolating the total *Desulfococcus multivorans* DNA, and deduction of specific primers from highly conserved *phaC* regions, the authors prepared the first PCR product homologous to the central region of a class III PHA synthases [59].

Two sets of primers for class IV *phaC* genes of *B. megaterium*, the prototype organism of this class, were designed to detect class IV synthase encoding genes in different *Bacillus* species [60]. Such class IV synthases require *phaC* genes similar to class III synthase, but one subunit is encoded by *phaR* instead of *phaE* [61]. This work confirmed prior assumptions that PHA synthase in *B. megaterium* constituting a synthase group different from class III synthases. As a further particularity of *B. megaterium* ssp., the authors discovered the production of 3HV-containing heteropolymers from structurally unrelated carbon sources by Fourier transform infrared spectroscopy (FTIR) [61].

All described primers can also be used successfully in combination with community DNA fingerprint techniques such as single-strand conformation polymorphism. This way, specific patterns, indicating species harboring different *phaC* genes, have been revealed for microbial consortia in the rhizosphere of different plants [62]. Ciesielski et al. used some of these primers for construction of a *phaC* clone library from activated sludge samples, and monitored the levels of *phaC* expression by using real time quantitative PCR (RT qPCR) [63].

As detailed, PCR provides a rather simple way to efficiently trace PHA-producing microbes, similar to staining techniques. Nevertheless, also these methods are prone to detection errors. Nonspecific PCR amplification can generate wrong positive results, while false negatives can originate from the use of unsuitable detection primers or inadequate conditions during PCR amplification [30,58,64]. The concept could be correctly applied if RNA is applied for amplification. Realistically, also the combination of PCR and staining methods should rather be conceived as a presumptive test of PHA production potential.

3.3 FISH

Further information about PHA-producing cells can be provided by combining staining methods with the use of fluorescence-labeled oligonucleotide probes (16 S rRNA, based on the rapidly increasing set of bacterial small subunit 16 S rRNA sequences) and subsequent fluorescence microscopy [31]. By FISH analysis, bacteria belonging to a specific taxonomic group (species, genus, class, etc.) can be conveniently identified in situ in mixed cultures. 16 S rRNA fluorescent probes specifically binding to the target bacteria are applied; the target microbes appear as fluorescent cells in the mixed culture. Recently, combinations of FISH analysis and PHA and poly-P double staining have been suggested. Procedures combining FISH with methylene blue [65] or with DAPI–poly-P staining [66] allow for the imaging of poly-P in taxonomically identified cells. Methylene blue staining is carried out after performing the FISH analysis [65]. Detecting poly-P in cells previously identified by FISH occurs as follows: those fields from which FISH images were taken are localized; then, methylene blue staining is carried out, and images of methylene blue stains are recorded. DAPI–poly-P staining may also be performed after FISH analysis [65]. Fluorescent images from probe markers and from DAPI–poly-P staining are collected in the same microscopic field by using different filter sets. However, staining failures and false-negative results have been reported for DAPI staining after FISH [34,67]. For example, a reduction to only 20% of the percentage of poly-P-positive cells was observed when DAPI staining was performed after FISH [67]. Even though the original method [66] has been adapted by different authors, there is not sufficient data available for a critical comparison of the different approaches.

Despite the above examples, combinations of FISH and polymer staining have de facto been applied rather rarely, and are restricted to investigations of selected singular examples. A deeper investigation on a range of samples and under different environmental conditions would enable to assess their reliability. The improvement and wide use of the described procedures and others such as FISH microautoradiography [68], combining in situ taxonomic identification with metabolic aspects, would be desirable for identification and investigation of the metabolism of organisms accumulating PHA and other storage compounds such as poly-P. The FISH-DAPI–poly-P double staining has been further improved by introducing a sequential staining of PHA granules with Sudan Black. The images of the PHA granules are taken by locating the same fields from which FISH and poly-P images were collected [31].

4 Gravimetric determination of PHA and turbidimetry

Until the end of the 1950ies, the most commonly applied technique to quantify PHA was direct gravimetric determination of the polyester after its extraction from biomass. This method was developed by Lemoigne (Lemoigne's chloroform extraction method), the discoverer of PHA, and comprised PHB extraction from lyophilized biomass with boiling chloroform followed by precipitation using PHA nonsolvents such as diethylether or acetone [25]. Here, it is of importance to quantitatively remove microbial lipids by applying PHA nonsolvents prior to the PHA extraction step in order not to obtain overestimated results. In addition, both the completeness of extraction (high extraction yield) and the complete reprecipitation have to be guaranteed by optimizing the ratio solvent-to-biomass, the extraction time, and the precipitation conditions. For this method, at least milligram amounts of PHA are needed in the samples; hence, a statistically reliable gravimetric analysis requires the availability of large sample volumes. In total, gravimetric PHA analysis constitutes a rather inexact and time-consuming technique that nowadays disappears from laboratory praxis [69].

In 1958, Williamson and Wilkinson developed a more sensitive and less time-demanding method in order to overcome the above described problems; the authors showed that, under controlled conditions of time and temperature, all non-PHA cellular material gets digested in alkaline NaClO solution. Linear correlation of turbidity of the sample arising from the nondissolved PHA granules with standard gravimetric measurements enables PHA quantification [69]. The accuracy of this method depends on size and shape of PHB granules and on the homogeneity of the suspension [70]; it is only applicable for native, unscratched PHA granules and cannot be applied to determine dissolved PHA. Further the method even requires a separate standardization by the gravimetric method, or by advanced techniques, for every new PHA-producing organism [71].

5 Qualitative spectroscopic techniques

5.1 UV-Spectrometry for PHB quantification

This method can be regarded as a further development of the gravimetric method. Originally, Slepecky and Law detected the simple quantification of alkenoic acids by spectrophotometric determination [72]. In the same article, the authors suggested the conversion of extracted PHB into 2-butenoic acid (crotonic acid) by incubation in concentrated sulfuric acid, resulting in water elimination from 3HB, and the subsequent spectrophotometric determination of crotonic acid [72]. Based on its strong UV-absorption, crotonic acid can conveniently be determined by UV-spectrophotometry at $\lambda = 235$ nm. Soon later, Law and Slepecky detailed and improved their "crotonic acid assay" to detect tiny PHB amounts as low as 5 μg, and to use it to monitor the PHB production kinetics in a *B. megaterium* KM culture [71]. Although this method is an easy technique to quantify PHB, it tends to overestimate PHB content, and is limited to PHB determination; heteropolyesters are not covered [21, 70, 73]. In addition, reliable values are only obtained after extraction of PHB prior to the conversion by sulfuric acid. Due to lacking alternatives, the "crotonic acid assay" was for a long time used as the standard method for quantitative determination of PHB [71, 72, 74]; despite of its limitations, is still used routinely in many laboratories all over the world as a fast and simple method where advanced analytical devices are not accessible [75].

5.2 IR spectroscopy

IR spectroscopy deals with the IR region of the electromagnetic spectrum. It covers a range of techniques, mostly based on absorption spectroscopy. As with all spectroscopic techniques, it can be used to identify and study the structure of chemical compounds. A basic IR spectrum is essentially a graph of IR transmittance on the vertical axis versus frequency or wavelength on the horizontal axis.

Detection of PHA extracted with chloroform by IR spectroscopy, profiting from the strong ester carbonyl absorption at about 1739 cm^{-1}, was first comprehensively studied by Jüttner et al. [76] Although it is advised that lipids should be removed before the analysis in order to avoid interference with PHA quantification [70, 76, 77], this method is reported to require minimal sample pretreatment [55, 77, 78]; detection limits are reported with 50 mg/L [76].

Until today, FTIR has been applied to detect and distinguish between different types of PHA (i.e., *s*cl-PHA, *mcl*-PHA, and *scl-co-mcl*-PHA), both purified [76] or in intact cells [77–79]. Hong et al. first suggested FTIR as a serious method for rapid qualitative PHA determination in intact biomass. These authors reported characteristic ester carbonyl bands for intracellular *scl*-PHA, *mcl*-PHA, and *scl-co-mcl*-PHA at 1732 cm^{-1}, 1744 cm^{-1}, and 1739 cm^{-1}, respectively; the same bands for purified polymer *scl*-PHA, *mcl*-PHA, and *scl-co-mcl*-PHA were shifted to 1728 cm^{-1}, 1740 cm^{-1}, and 1732 cm^{-1}, respectively [78]. Soon after, the method was advanced by Misra et al. who presented a viable protocol for routine FTIR determination of PHA-containing biomass by preparing a thin, semitransparent pellet consisting of lyophilized biomass and KBr; for comparison, the analysis was repeated with extracted PHA films. Different PHA-accumulating microbial strains (*Alcaligenes eutrophus, Methylomonas extorquens, Pseudomonas cepacia*) were investigated. As the most characteristic bands, absorption at 1280 cm^{-1} and 1724 cm^{-1} was reported, representing C-O- and C = O stretching motions of the ester functionality. FTIR was proposed as a suitable method to quickly distinguish between PHA-free, PHA-poor, and PHA-rich biomass [77].

In addition to qualitative and semiquantitative application, FTIR has also been recommended as a tool for *scl*-PHA quantification. Kansiz et al. quantified PHB by FTIR in biomass of recombinant *E. coli* [80]. Arcos-Hernandez et al. correlated the *scl*-PHA content in biomass between 3 and 58% to the respective peak intensities in FTIR spectra, thus providing for a reliable quantitation of the *scl*-PHA content [81]. The solvent-less nature of FTIR eliminates the risk accruing from exposure to hazardous chemicals, whereas the short analysis time rapidly generates results. As a drawback, FTIR-based methods have rather low detection sensitivities, and cannot discriminate between PHA blends and heteropolyesters. In addition, the method needs standardization for each new production strain [78]. Hence, FTIR-based methods should be regarded as suitable for semiquantitative routine monitoring of PHA production for standard bioprocesses with well-known cultures and familiar PHA types.

5.3 Raman spectroscopy

Raman spectroscopy is a method to explore molecular structures and interactions of functional groups, and provides additional information to those obtained by IR spectroscopy. This technique allows the observation of vibrational, rotational, and other low-frequency modes in a system. In principle, electromagnetic radiation impinges on a molecule and interacts with the polarizable electron density and the bonds of the molecule in the phase (solid, liquid, or gaseous) and the environment in which the molecule finds itself. This technique has been applied to biopolymers such as poly(lactic acid) (PLA) to elucidate its crystallinity, conformational aspects, and molecular interactions [82]. In contrast to its detailed investigation by FTIR, PHA has not been exhaustingly studied by Raman spectroscopy.

In order to determine at which Raman shifts a contribution of PHB in bacterial spectra occurs, Raman spectra were established and interpreted by De Gelder et al. from *C. necator* DSM 428, its non-PHB-producing mutant strain *C. necator* DSM 541, and from isolated PHB. The intensity of the representative Raman band at around 1734 cm^{-1} appeared to be appropriate to follow PHB synthesis and consumption in biomass. Peak intensities were shown to be linearly proportional to PHB concentration, as controlled by accompanying chromatographic analysis. The authors suggested Raman spectroscopy as a rapid, noninvasive technique to monitor the intracellular PHB content in biomass [83].

A further article reports a Raman study of films of microbial poly((*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyvalerate) (PHBV;0, 5, 8, and 12 mol% 3HV) for the estimation of sample crystallinity. Based on the spectra, the bands at 1725, 1443, and 1458 cm^{-1} were proposed to be used for crystallinity determination. In addition, the authors noticed similarities between Raman spectra of molten PHA and its solution in $CDCl_3$, evidencing that molten and dissolved PHA display similar conformational behavior. Characteristic bands at 1220, 1402, 1725, 2998, and 3009 cm^{-1} were understood to reflect the crystalline helical PHA structure, whereas bands at 1453, 1740, 2881, 2938, and 2990 cm^{-1} were supposed to represent amorphous domains. Aiming at a quantitation, it turned out that the molar fraction of 3HV in the samples correlated to the ratio of the intensity of the bands at 2938 cm^{-1} and 1740 cm^{-1} in the spectra of polyester solutions, and of bands at 1354 and 1740 cm^{-1} in spectra of molten polyester films, respectively [84].

PHB and PHB/PLA blends were investigated by using Raman microspectroscopy to elucidate structure, dispersibility, and crystallinity. Four different PHB/PLA blends with PLA contents of 20, 40, 60, and 80% were prepared as cast films from chloroform solutions. The study revealed that the PHB component occurs in a crystalline form in the blends, regardless of the blend ratio, and that both components are mixed in nonspherulitic parts of the polymer blend. The authors propose that the crystalline structure of PHB and the nonspherulitic parts of PLA in the blends are characterized by the bands at 3009 and 400 cm^{-1} [85].

6 Enzymatic methods and biosensors

3HB, the most important PHA building block, is one of the ketone bodies; high 3HB levels in body fluids are known as ketosis, an indicator, e.g. for type 1 diabetes [86]. Therefore, the development of reliable and fast tools for 3HB analysis was pivotal for the pharmaceutical industry. Quantification of PHA using an enzyme kit developed for 3HB determination in biological samples such as blood, serum, urine, milk for clinical or veterinary purposes, or other foodstuffs (Roche Molecular Biochemicals, USA, No. 127833) was described by Hesselmann et al. PHA determination with this method follows propanolytic digestion of biomass by acid catalyzed transesterification with propanol. The generated 3HB gets enzymatically oxidized by the enzyme (*R*)-3-hydroxybutyrate dehydrogenase (3HBDH; EC 1.1.1.30), which catalyzes the oxidation of 3HB with nicotinamide adenine dinucleotide (NAD^+), yielding acetoacetic acid and nicotinamide adenine dinucleotide (NADH). The generated NADH is reoxidized in the presence of iodonitro-tetrazoliumchloride to produce formazan, which is spectrophotometrically measured at $\lambda = 492$ nm. Although the enzyme kit was commercialized for 3HB detection, also the conversion of C5 (3HV) and C6 ((*R*)-3-hydroxyhexanoate, 3HHx) HAs to formazan was demonstrated; hence, the method is not specific for PHB, but can be used to quantify the sum of 3HB, 3HV, and 3HHx shares in PHA [87]. A more simple and convenient method involves the direct determination of the consumed NAD^+ by UV-spectrophotometric determination at $\lambda = 340$ nm; also in this case, although only after a lag phase, 3HV and 3HHx are detected due to their conversion by 3HBDH [88]. This method was improved by Parry et al., who developed a rapid technique for the enzymatic determination of 3HB in whole liquid egg using 3HBDH, reporting a detection limit as low as 20 μmol 3HB/L in 18-day-old reject eggs [89].

An advanced kinetic method was later developed for simultaneous determination of 3HB and 3HV based on the above described different rates of the 3HBDH-catalyzed reactions of these compounds with NAD^+. A flow injection system with two reactors of immobilized 3HBDH was applied. NADH produced after two different reaction times was fluorimetrically or spectrophotometrically quantified. Low concentrations of 3HB and 3HV between 1×10^{-6} and 1×10^{-4} M can be determined at sampling intervals of 3 min. In contrast to alternative methods, this approach enables the discrimination of 3HB and 3HV without previous extraction or chromatographic separation. The method can provide information on the degradation rate and mechanism and composition of PHA. Its applicability was proved by determination of 3HB and 3HV after chemical degradation of different commercially available PHAs [90].

Cui et al. developed an amperometric enzyme-driven biosensor based on a Clark electrode. This biosensor worked as combination of alkaline hydrolysis of PHB to 3HB, and the subsequent two-step enzymatic determination of the hydrolysis products. The first enzymatic step involves the 3HBDH-catalyzed reaction; the generated NADH participates in the subsequent conversion of salicylic acid catalyzed by salicylate hydroxylase (SHL, EC 1.1.4.13.11). Dissolved oxygen consumption for salicylate conversion is directly proportional to 3HB conversion; hence, the actual dissolved oxygen level constitutes a parameter for 3HB quantification. According to the authors, this method is highly sensitive (detection limit of 0–3 mg PHB/L, linear range of 1–160 mg PHB/L), simple, reproducible, and fast [91].

7 Chromatographic methods

7.1 General

Most of the above described methods are time consuming or/and not accurate, especially at low PHA concentrations. Large sample quantities and numerous analytical repeats are required to get reliable results. Furthermore, various cell components potentially interfere with PHA extraction, and the methods are generally restricted to determination of PHB homopolyesters or the entire amount of PHA, without providing the possibility to discriminate between different PHA building blocks and/or

types of PHA (*scl*- and *mcl*-PHA) [70]. Therefore, contemporarily LC and especially GC chromatography based methods are the most frequently used analytical techniques for exact PHA determination. This is due to the nowadays high automation, and the accurate quantitative and qualitative information on the monomeric composition. Compared to all other methods, chromatography-based methods have higher detection sensitivities, ranging 0.014–14 μg for LC and 0.05 pg–15 mg for GC depending on the type of detectors (FID or MS) and the applied pretreatment methods [92]. Despite these obvious advantages, prior to analysis, chromatography-based methods require depolymerization of PHA by conversion into acids, diols, or esters, accounting for the high time demand of the analytical process [73–94]. Independent from the pretreatment method, a cocktail of derivatives of all different building blocks originally present in the sample is obtained; this implements that chromatography-based methods cannot discriminate if different PHA monomers are part of blends of different PHA homopolyesters, or if they derive from PHA heteropolyesters.

7.2 Gas chromatographic determination of PHA

7.2.1 GC-FID

At present, GC-FID constitutes the globally favored method for analysis of PHA monomers both quantitatively and regarding the composition on the monomeric level; this is due to the high separation power of applied GC columns and the excellent detector sensitivity. The use of GC for the identification and quantification of PHA components was first proposed in 1978 [70]. The motivation to develop this well-known "Braunegg's method" was the urgent need to identify PHA building blocks others than 3HB, and to establish a quick, reliable routine technology to monitor PHA synthesis and degradation during running bioprocesses. The method is based on the fact that the polymeric PHA has to be fragmented into small volatile units to enable the determination of the monomers via GC. "Braunegg's method" involves simultaneous extraction and methanolysis of PHA, in mild acid or, less often, alkaline conditions, to generate methyl esters of HAs volatile enough to subsequently undergo GC-FID analysis [70]. In details, isolated PHA or lyophilized PHA-containing biomass (original experiments were carried out with *A. eutrophus* H16) is incubated in tightly sealed screw-capped glass tubes together with chloroform that displays high solubility for PHA, and the same volume of a "transesterification mixture" consisting of methanol, sulfuric acid, and an internal standard, most commonly hexanoic acid [95] or benzoic acid [70]. The incubation is performed at temperatures of 100–105°C, hence well above the boiling points of chloroform and methanol. Classically, the incubation is performed in block heaters, or in hot air cabinets. The method requires about 4 h with repeated shaking the tubes for 1–2 min in 1 h intervals. After incubation and cooling down, water is added to the mixture and, after vigorously mixing, the heavy chloroform-rich phase settles down simply by gravity [70] or by centrifugation [96]. This chloroform phase contains the respective methyl esters of HAs and is injected into the GC. As most beneficial aspect, "Braunegg's method" is highly reproducible, requires only small sample quantities, and displays a determination limit as low as 10 μg/L; reaction and extraction take place as "one-pot operation" in the same screw-capped tube [70].

Originally, based on the restricted knowledge about the variety of PHA building blocks, this method was developed for determination of 3HB and 3HV in PHB and PHBV [97, 98]. By the increasing interest in 4HB-containing PHAs since the end of the 1980s, the method was also applied to determine 4HB-containg heteropolyesters. Here, it is worth noting that under the applied conditions only part of 4HB undergoes transesterification toward 4-HB methylester; the major part undergoes the ring-formation toward γ-butyrolactone (GBL); a minor part is additionally converted to methoxy-4-hydroxybutyrate. The peak representing GBL is the best reproducible one, and most frequently used to quantify 4HB [99]. By the increasing interest in *mcl*-PHA, the transesterification parameters were adapted by Lageveen et al. by increasing the amount of the acidic catalyst to 15% v/v and by shortening the incubation time to 140 min; this specifically aimed at the determination of *mcl*-PHA building blocks produced by *P. oleovorans* [100]. Later, Huijberts et al. further modified this method for *mcl*-PHA production by *P. putida* KT2442 by extending the transesterification time to 4 h both in the case of *scl*- and *mcl*-PHA; the detection limit of this method amounted to 0.018 mg for PHB, but only to 0.304 mg for *mcl*-PHA [96]. Oehmen et al. reported the use of different acid concentrations for transesterification of PHBV, and heteropolyesters harboring also 3-hydroxy-2-methylvalerate. For PHBV, the authors suggest the application of 20% sulfuric acid and an incubation time of 2–20 h, whereas for 3-hydroxy-2-methylvalerate containing PHA, 10% sulfuric acid and 20 h of incubation are proposed [101]. Further improvements of GC-FID based *mcl*-PHA determination were accomplished by Furrer et al. [102] who realized that reaction kinetics for transesterification are much slower in the case of *mcl*-PHA than for *scl*-PHA, and side-product formation occurs especially in case of functionalized side chains. This implies that the transesterification of *mcl*-PHA catalyzed by protic acids such as H_2SO_4 and HCl cannot be complete for *mcl*-PHA under common conditions [102]. In addition, losses of the target compounds to be analyzed are likely when using H_2SO_4 due to acid-catalyzed elimination reactions, resulting in underestimated results. Therefore, these authors replaced protic acids by boron trifluoride (BF_3), a catalyst that is also used for routine analysis of the fatty acid pattern in lipid samples such as oils or biodiesel [6, 7], and highly suitable both for saturated and unsaturated fatty acids. The authors report highly quantitative methanolysis obtained by this method and propose its suitability for quantitative determination both of extracted and for intracellular *mcl*-PHA [102].

Beside the high time demand, well-known limitations of "Braunegg's method" are: PHA recovery is often incomplete; it is ineffective in the presence of water, hence, lyophilization is needed. Further, it causes rapid GC column deterioration because of the high amount of acid and cell fragments in the sample. Finally, the incubation of the transesterification mixture in heating blocks or air drying cabinets at temperatures exceeding 100°C causes a significant risk of explosion, especially during manual shaking of tubes. For all these drawbacks, solutions were successfully elaborated: PHA recovery can be increased by using acidic propanolysis or butanolysis with HCl acting as acidic catalyst instead of acidic methanolysis in H_2SO_4.

The application of the longer alcohols propanol or butanol instead of methanol results in a more complete transfer of the resulting HA esters into the organic phase, whereas the change to HCl lowers the degree of 3HB decomposition to crotonic acid. In addition, 1,2-dichlorethane or trichloroethylene were used instead of chloroform; this enables the incubation at temperature conditions below the solvents boiling points, contributing to a minimized risk of explosion; even the operation in open glass tubes is suggested [103]. Comeau et al. proposed sample lyophilization and reextraction prior to the GC injection in order to minimize the influence of compounds present in the bacterial matrix on the analysis result [104]. Contemporarily, modifications of the "Comeau method" exist involving the change of alcohols (methanol to propanol), the catalyst (H_2SO_4 to HCl), the internal standard (hexanoic acid to benzoic acid) and in the type of PHA solvent used for the extraction (chloroform, dichloromethane, or 1,2-dichloroethane). Regarding the rapid column deterioration, a solution was discovered decades after the report of the original method by replacing water after extraction and transesterification by a 10% $NaHCO_3$ solution, thus reducing the high acidity of the mixture [105]. Similar attempts were described even later by Furrer et al. who used a Na_2CO_3 solution for neutralizing harsh acidic conditions [102]. A higher degree of safety can be obtained by replacing the incubation in block heaters or dry air cabinets by incubation at 90°C under continuous shaking in a thermostated water bath. Due to the continuous shaking, no manual shaking of the sample tubes is required; in addition, the incubation time can be lowered to 3.5 h [105].

In addition to acid-catalyzed transesterification, also alkaline catalysis was investigated. According to Wallen and Rohwedder, alkaline hydrolysis of PHA leads to a mixture of 3-HA methyl esters and 2-alkenoic acid methyl esters, whereas the acidic transesterification results in most cases (important exception: 4HB) in only one methyl ester per component. In addition, acid catalysis is considerably faster because of the simultaneous one-pot extraction and transesterification [2]. A new pretreatment method for the GC-FID determination of PHB was developed based on a combination of alkaline hydrolysis with acid esterification. The determination involves alkaline PHB hydrolysis to 3HB, followed by the acidic catalyzed esterification with methanol to generate the methyl ester of 3HB, which is detected by GC. Alkali is superior for hydrolysis, while acid is superior for esterification [106].

As mentioned before, "Braunegg's method" takes about 4 h only for the incubation step, together with the need for regular manual shaking. This can be overcome by the application of microwave-assisted heating that radically reduces the time effort needed for the transesterification process. As reported by Betancourt et al., a complete transesterification of PHB can be accomplished within 10 min by microwave heating at 10% of the maximum performance of the device; higher performance results in the formation of byproducts such as crotonic acid. The authors report that their method not only saves time, but also energy [107]. Unfortunately, the application of this method is only reported for determination of PHB; it would especially be of interest to apply this method for a reproducible determination of 4HB-containing heteropolyesters, and to investigate its performance for analysis of functional *mcl*-PHA.

In spite of all the modifications described in the prior paragraphs that master the original shortcomings of "Braunegg's method," GC-FID determination of PHA still requires the handling and disposal of volatile chlorinated solvents. This is in drastic contrast to the idea of PHA production as a sustainable technique. To overcome this, an extraction and derivatization method was developed by Werker et al. for environmentally benign routine GC quantification of PHAs in activated sludge biomass without chlorinated solvents. This method can be further applied to follow changes in carbohydrate concentrations in biomass, predominantly relating to glycogen or extracellular polysaccharides. Further, the coextracted major membrane fatty acids are also covered analytically, acting as additional indicators for the metabolic ongoings in active biomass. Hence, this method can provide a set of relevant data reflecting the actual stage of a bioprocess. Methodologically, acidic alcoholysis of dried microbial biomass using 3:1 butanol to concentrated HCl takes place at 100°C for 8 h. Intracellular PHA, carbohydrates, and membrane lipids get hydrolyzed and derivatized. Esters stemming from the PHA-building blocks, levulinic acid from the acidic conversion of carbohydrates, and long chain microbial fatty acids mainly from membrane lipids are subsequently extracted into hexane; this organic phase is used for GC-FID analysis. Calibration can be achieved with benzoic, 2-hydroxyvaleric, or 2-hydroxycaproic acids as standard materials [108].

To overcome the problems connected to the application of halogenated solvents, a very recent paper proposes two novel protocols for extracting PHAs from *C. necator*, which are effective and sustainable. The first method makes use of dimethyl carbonate, a completely biodegradable green solvent, less harmful to humans and the environment than most solvents. The second protocol uses fatty acid carboxylates as surfactants. The recovery and purity of the obtained PHAs are very high, the use of toxic chemicals is avoided, and the recycling of various solvents/surfactants used in the processes is optimal [109].

As the sole drawbacks of "Braunegg's method" that is not solved until today, the dependency on the availability of adequate analytical standards, namely PHA heteropolyesters with all building blocks of interest has to be noticed. As shown later, this can be overcome by supplementary methods such as NMR or MS.

Despite all discussed modifications, the original "Braunegg's method" is widely utilized as a well-established routine technique for determining PHA production and degradation kinetics, and to establish carbon mass balances during the bioprocess due to its high sensitivity and reproducibility. From today's point of view, the "optimized" GC-FID method encompasses the combination of microwave technique together with the application of a halogen-free solvent.

7.2.2 Pyrolysis-GC and solid-phase microextraction-GC

An innovative pretreatment method for GC-FID analysis was proposed at Graz University of Technology [110]. This approach has been recently developed as fast and solventless tool for quantitative and qualitative determination of PHAs from mixed and/or pure cultures [111]. The precision of the method was comparable to that of conventional methanolysis within a broad PHA content in CDM from 4.6 to 70% w/w. From

prior studies [112], it was known that pyrolysis of isolated PHA or PHA-containing biomass results in the formation of pyrolysis products characteristic for specific PHA-building blocks; here, 3HB results in the formation of crotonic acid, whereas 3HV is converted to 2-pentenoic acid and terminally unsaturated oligomers of 3HV; 4HB undergoes ring formation toward GBL (unpublished own data). All these pyrolysis products are subsequently subjected toward GC-FID analysis. As the major advantage of this method, pyrolysis can be carried out with microquantities of wet biomass; hence, no laborious drying step is needed, in addition to the complete saving of the time for incubation as normally needed for concomitant extraction and transesterification. The heteropolyesters composition can be estimated simply by comparing the sum of the products arising from 3HB and that from 3HV observed in the pyrogram. Hence, during synthesis in bioreactors, the operator gets a very quick response within a reasonable time of about 20 to 30 min about the actual relative shares of the PHA-building blocks; this enables the quick adaptation of the process conditions by changing the feeding rates for main carbon source and cosubstrates [110,111].

Recently, *pyr*-GC in the presence of a strong organic alkali such as tetramethylammonium hydroxide ($(CH_3)_4NOH$) was reported as a useful technique for structural characterization of polyesters. Sato et al. investigated the composition of heteropolyesters of 3HB and 3HV with a broad range of 3HV from 4 to 23%. Nine characteristic peaks of methylated products, five of which were attributed to the products from 3HV unit and four to the 3HB unit, occurred in the pyrograms. The heteropolymer compositions estimation was based on the relative peak intensities; the results were consistent with those of ^{1}H NMR determination. Hence, *pyr*-GC in the presence of tetramethylammonium hydroxide provides for accurate and highly sensitive compositional analysis of PHBV using trace amounts of the samples below 0.1 mg; the method appears to be more sensitive than the original GC-FID techniques. 3HV shares below 1% can be detected by this method in PHA samples that are commercialized as pure PHB homopolyesters [113].

No experiments are reported about the applicability of *pyr*-GC for *mcl*-PHA determination. Considering the fact that pyrolysis of *mcl*-PHA, especially with functional building blocks, is expected to create a vast number of pyrolysis products, provoking a great challenge for effective chromatographic separation, it is very likely that its application is restricted to analysis of *scl*-PHA.

An alternative, intriguing method resorts to solid-phase microextraction (SPME) as pretreatment prior to GC-FID measurement for PHB quantification in cells of *Azohydromonas lata*. PHB is depolymerized either via acid-catalyzed methanolysis to methyl 3HB (internal standard: benzoic acid, solvent: chloroform), or by acid-catalyzed hydrolysis and dehydration to crotonic acid (internal standard: *trans*-hex-2-enoic acid). These compounds subsequently are subjected to headspace SPME/GC-FID analysis. The authors compared the results with those obtained from "Braunegg's method" after sample digestion by acidic methanolysis and parallel chloroform extraction of the methyl esters (internal standard: benzoic acid); the chromatograms are provided in Fig. 4. Exceptional agreements between the different methods are reported; as an additional positive aspect, SPME-based methods are supposed to be ecologically more benign, and more convenient to be accomplished [114]. As a drawback, slightly lower precision is reported if compared to the conventional technique.

7.2.3 GC coupled to mass spectroscopy (GC-MS)

Coupling GC to MS detector (GC-MS) ensures a more advanced and reliable detection, identification, and quantification method for diverse PHA monomers; most of all, it enables tentative identification of novel PHA monomers in the absence of analytical standards [115, 116], providing a possibility to overcome the only left limitation of the GC-FID method. Already in 1995, Lee and Choi used trimethylsilyation of both saturated and unsaturated *mcl*-PHA building blocks for structural analysis of *mcl*-PHA [115]. Later, MS detection was also used to identify the building blocks of PHA produced by transgenic plants. Here, herbal samples were pretreated by PHA extraction in chloroform, subsequent methanolysis generated methyl esters of the different saturated and unsaturated monomers; the esters were finally separated on a 30-m-long HP-5MS column, and detected using a HP 5972 mass spectrometer [117]. Another study showed that PHA monomers with carbon numbers between 4 and 16 have strong linear correlations with their retention times in GC-MS chromatograms, which enabled the prediction of retention times of other PHA building blocks. This method enables a wide series of *scl*-PHA and *mcl*-PHA to be reliably detected and quantified without the need for reference standards. The authors expected further improvements in sensitivity and specificity for determination of PHA monomers by coupling of GC to tandem MS (GC-MS/MS) [118].

Fast atom bombardment (FAB-MS) is a relatively soft MS ionization technique where a high-energy beam of atoms strikes a surface to create ions. The ionization mechanism is similar to ESI and MALDI [119].

MALDI-TOF-MS offers a cost-effective, straightforward, and high-throughput alternative to traditional GC-MS methods [118]. Saeed et al. reported for the first time the use of high resolution MALDI-TOF-MS for characterization of bacterial PHA [120]. Compared with GC-MS, MALDI-TOF-MS does not require chemical derivatization during sample preparation. As the differences in monomer composition and detailed PHA structural information, e.g. end-group identification, can easily be accomplished by MALDI-TOF-MS, it constitutes a convenient complementary technique to NMR [121]. The total amount of needed sample can be restricted to the pico- to femtomole range, making MALDI-TOF-MS particularly sensitive. Further, it determines absolute molar masses in contrast to apparent molar masses as obtained by gel permeation chromatography (GPC). These apparent advantages of MALDI-TOF-MS might enable the precise, simple, and holistic routine analysis of PHA [122].

7.3 LC

HPLC techniques are characterized by higher operational pressure and small diameter particles as column packing for separation, coupled to different detection devices as detailed below.

7.3.1 LC coupled to UV detection (LC-UV)

The rapid development of automated LC devices has tremendously increased the progress in LC-based *scl*-PHA analysis. Improvement in the accuracy of crotonic acid determination originating from PHB was made possible with ion-exchange HPLC coupled with UV detection [122]. Following biomass hydrolysis with concentrated H_2SO_4, 3HB gets converted to crotonic acid, which is determined by HPLC using a UV detector at $\lambda = 210$ nm. Recovery of PHB by this method amounts to 84%; the method is restricted to determination of the homopolyester PHB [73].

7.3.2 Conductivity detection

Simultaneous analysis and quantification of 3HB and 3HV monomers can be achieved using ion chromatography equipped with an anion trap column and a conductivity detector [77]. PHA detection by ion chromatography is based on the conversion of PHA-building blocks to alkenoic acids. The determination is performed after biomass digestion by acidic propanolysis followed by an alkaline conversion with $Ca(OH)_2$ or acidic treatment with concentrated H_2SO_4; the method does without extraction using chlorinated solvents. After centrifugation, the sample is separated using an anionic column; detection is accomplished by conductivity measurement. The separating capacity achieved between PHB and PHV is comparable to the "Comeau method" [104]; the reported detection limit for PHB and PHBV amounts to 0.1% of cell dry mass and with recovery yields exceeding 97% [77].

7.3.3 LC coupled to mass spectroscopy (LC-MS)

To date, LC-MS technique has been applied only to a limited extent to analyze specific PHA monomers, mainly as a complementary technique to well-established methods [12, 92, 121]. Grubelnik et al. presented a protocol for the reductive depolymerization of *mcl*-PHAs, leading to monomeric diols that can be separated and quantified by LC-MS. The reduction is carried out at RT within a few minutes. The authors underline that this method is fast, simple, and quantitative. The obtained results are consistent with those from control measurements with ^{1}H NMR [92]. For sure, there remains considerable potential to enlarge the capabilities of LC platforms beyond the quantitative analysis of simple *scl*-PHA by applying LC-MS techniques for quantification of other PHA monomers; efforts in this direction should be forcefully encouraged.

7.3.4 GPC

Masses of PHA (weight average molecular weight and number average molecular weight) and molar mass distribution (polydispersity) are routinely determined by GPC, as special variant of LC. This is accomplished by applying refractive index detection with monodisperse polymers, e.g. poly(styrene), as reference materials. GPC provides a valuable tool to compare molar masses and their distribution, e.g. before and after processing of PHA; it can be considered as one of the most important routine characterization techniques for PHA [122–125].

7.3.5 SEC with multiangle light scattering detector (SEC-MALS)

To overcome the restrictions of GPC in obtaining reliable data for absolute molar masses, characterization of chloroform solutions of PHB and PHBV using SEC-MALS was performed and reported by Žagar and Kržan. Using this method, it was possible to determine absolute molar mass averages, molar mass distribution, and even the radius of gyration of the PHA helix, hence, even conformational information is provided by SEC-MALS [126].

8 NMR

NMR provides a technique to study the chemical composition of intact PHA polyesters. This method differentiates between PHA blends and PHA heteropolymers, and provides details on the topology and presence of functional groups in the polymers. The importance of NMR in the PHA field became first obvious in the elucidation of 3HV, the first discovered PHA building block different from 3HB, by Wallen and Rohwedder. These authors used ^{1}H-NMR for structural identification of the heteropolymer PHBV in activated sludge bacteria [2]; at that time, all available methods for PHA identification were restricted to the total quantity of polymer, or the quantification of 3HB.

Another widely applied technique is ^{13}C-NMR. The high number of protons in natural compounds suggests the higher sensitivity of ^{1}H-NMR, enabling analytical times below 1 h. Due to the lower sensitivity and restricted natural occurrence of ^{13}C, recording a proper ^{13}C-NMR spectrum takes up to 24 h. Nevertheless, ^{13}C-NMR performs superior to ^{1}H-NMR in analysis both of macromolecules and of monomers with longer carbon chains. Several studies suggest that generally ^{1}H NMR measurements are sufficient to expose the composition of *scl*-PHA, whereas for *mcl*-PHA, one should resort to time-consuming ^{13}C NMR techniques [102]. By using ^{13}C-NMR, Fritzsche et al. confirmed the production of "untraditional" *mcl*-PHA building blocks by *Pseudomonas* sp. on structurally related, rather exotic, carbon sources in a series of studies. For the first time, unknown saturated and unsaturated [127], aromatic [128], and even branched [129] monomers were unambiguously identified.

As PHA contain both hydrogen and carbon, ^{1}H-NMR and ^{13}C-NMR without chemical derivatization of the polymer are usually applied in parallel to provide a holistic and more comprehensive analysis of the polyester. NMR is broadly used for analysis of both saturated and unsaturated PHA. Methane and methylene protons can be identified from both NMR spectra, while microstructures such as 3-hydroxypropionate (3HP) and 4HB needs both ^{1}H-NMR and ^{13}C-NMR spectra [130, 131]. Also quantitative estimation of PHA monomers by NMR can be performed using the intensity ratio of the signals, although for quantitative measurements [130], NMR is limited to isolated PHA. As a powerful nondestructive tool, NMR was also applied to the analysis of novel functionalized PHAs for which analytical standards are currently unavailable [132, 133]; hence, it provides a valuable solution to overcome the limitations of the well-established GC-FID methods regarding the frequently lack of suitable reference materials.

Additional tools of interest to characterize functional PHA are found among two-dimensional (2D) NMR methodologies that provide information about the environment where each carbon/hydrogen is positioned. Examples for 2D-NMR are correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear multiple-quantum correlation (HMQC), or heteronuclear multiple bond coherence (HMBC) [134–137]. In addition, 2D homonuclear or heteronuclear NMR techniques also reveal the exact position of double bonds and the *cis/trans* configuration of unsaturated monomers. Using a combination of ^{1}H-NMR and 2D-COSY, Huijberts et al. elucidated for the first time the *cis/trans* configuration of unsaturated *mcl*-PHA building blocks produced by *P. putida* [138]. Deeper insights into these results were later provided by Eggink et al. who exactly identified the building blocks by using ^{13}C-NMR [139]. Later, ^{1}H-NMR and 2D homonuclear NMR were used to unambiguously identify not only the positions of the double bonds in *mcl*-PHA, but also the position of the hydroxyl group [140]; as an example, 2D heteronuclear COSY NMR was successfully applied by Valentin et al. to confirm for the first time the incorporation of 4-hydroxyhexanoic acid in *scl*-PHA terpolyesters [134].

2D HMBC NMR was used to characterize PHA produced by *P. putida* KTQQ20 on hexanoic and dodecanoic acid as a novel P(3HHx)-*b*-P(3HD-*co*-3HDD) diblock PHA [140]. More simple ^{1}H NMR techniques were used by the same group for characterization of the structure of different P(3HP)-*b*-P(4HB) diblock PHAs produced by recombinant *E. coli* [141]. This is of special significance due to the expected high interest in blocky-structured PHA in the next future [142].

In addition, the microstructure of bacterial PHBV heteropolyesters as well as a mixture of two PHBV heteropolyesters of different 3HV content and sequence distribution was studied by two different NMR-based approaches: ^{13}C NMR based on dyad and triad analysis was combined with multistage ESI MS fragmentation (ESI-MS^{n}). The obtained results evidenced the excellent agreement of both methods. Based on the melting behavior obtained by differential scanning calorimetry, the effect of the polyester's microstructure on the thermal properties was investigated. A PHBV heteropolyester with randomly distributed 3HV (12.0 mol% 3HV in polyester) showed a single melting peak, whereas samples with nonrandom distribution showed multiple melting peaks in their thermograms. Based on this complex melting behavior, the authors assumed that the PHBV samples with 12.9 and 27.1 mol% 3HV were de facto blends of different heteropolymers with completely different composition on the monomeric level [10].

9 Concluding remarks

This review discusses the applicability of different methodological approaches to trace, quantify, and characterize PHA in biological samples and as isolated polymeric material. Further, molecular genetic methods are discussed tracing the ability of microbial consortia to produce PHA.

To discover novel powerful PHA-producing microbes, one can resort to traditional or enhanced staining methods, most preferably in combination with application of genetic tools. Currently, metagenomic approaches are of increasing importance for the deeper understanding of diverse microbial ecosystems; methods are now available for the isolation of clones expressing novel PHA metabolism genes from metagenomic libraries, providing for a more detailed search for specific microbes. Such methods can be supported by the rapid identification of significant PHA functionalities by using FTIR techniques, applicable also to intact biomass. Even deeper insights into such biopolyesters, especially regarding conformational aspects, molecular interactions, crystallinity, etc., can be gained by Raman spectroscopy.

Reliable quantitation of both *scl*- and *mcl*-PHA should most preferably be accomplished by chromatography equipped with well-established detector systems. Here, one should profit from the recently developed pretreatment methods using microwave heating or pyrolysis, together with optimized protocols for analysis of the different types of PHA. The unambiguous identification of the myriad of new and exotic functional PHA-building blocks preferably resorts to, as alternative to MS detection, means of NMR. Advanced NMR methods appear indispensable at the moment to elucidate special features of the polyesters, such as identification of blocky structures, or end group analysis.

In addition, SEC-MALS appears to be a powerful, still underestimated method that can provide for new insights into PHA-based composited and blends. Regarding the need for a quick and reliable monitoring of PHA accumulation kinetics in running production processes, online measurements using enzyme-based biosensors or online fluorescence monitoring might be the methods of choice in a not too distant future; unfortunately, until now, such endeavors are restricted to rather "simple" PHA representatives, and the current methods are not suitable for online measurement as they also require the depolymerization of PHA. Especially in this field, considerable R&D efforts should be devoted in order to provide online control possibilities for production of "smart," functional PHAs to be used in special niches.

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10 References

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