The Microbe 8 (2025) 100503

Contents lists available at ScienceDirect

The Microbe

journal homepage: www.sciencedirect.com/journal/the-microbe

Qualitative and quantitative evaluation of bacterial polyhydroxyalkanoates: A critical review of existing methodologies

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ARTICLE INFO

Keywords:
Gas chromatography
High performance liquid chromatography
Nile red
Polyhydroxybutyrate
Nuclear magnetic resonance
Sudan black B

ABSTRACT

Polyhydroxyalkanoates (PHAs) are non-toxic aliphatic polyester biopolymers that are gaining traction as bio-based and biodegradable materials. To support the growing body of research on this subject, scientists and other stakeholders require insight into the synthesis, structure and quantity of PHA produced by bacterial isolates. To address the challenges that researchers face when seeking to evaluate bacterial PHA production, this manuscript presents a cohesive collection and critical evaluation of the methods described in literature, highlighting the applications, advantages, and disadvantages of each. Environmental samples are typically screened for PHA production based on uptake of lipophilic dyes. Thereafter, quantification of polyhydroxybutyrate and/or polyhydroxyvalerate monomers is typically performed using gas chromatography or high-performance liquid chromatography. These methods are robust with good specificity, but their application range is limited by non-availability of commercial standards. Methods based on lipophilic fluorescent dye emissions measured by spectrofluorometry or flow cytometry present simple, cost effective and efficient alternatives, but there are issues related to lack of specificity. For identification and quantification of monomers other than polyhydroxybutyrate and/or polyhydroxyvalerate, and to elucidate monomeric spatial arrangements within the polymer structure requires more sophisticated equipment and skilled analyses including gas chromatography-mass spectrometry, high performance liquid chromatography-mass spectrometry, and finally nuclear magnetic resonance. These methods are presented and discussed, along with other ancillary methods including Raman spectroscopy and Fourier-transform infrared spectroscopy, and methods based on molecular biology principles.

1. Introduction

Polyhydroxyalkanoates (PHAs) are non-toxic aliphatic polyesters that are gaining traction as bio-based and biodegradable polymers that can be processed for a range of applications including bioplastic packaging, agricultural film, medical devices and other medical applications, biofuels, and cosmetics (Fernandez-Bunster and Pavez, 2022; Gao et al., 2023; Guimarães et al., 2022; Pandey et al., 2022). As PHAs are biocompatible, they are used as high-value polymers in the biomedical industry for scaffolds for tissue engineering and controlled drug delivery systems. In agriculture, they act as biodegradable, slow-release carriers for pesticides and fertilizers (Fernandez-Bunster and Pavez, 2022; Gao et al., 2023; Guimarães et al., 2022; Pandey et al., 2022). The packaging industry manufactures completely biodegradable PHA-based food containers that alleviate long-term pollution from non-degradable single--use plastic containers. Furthermore, materials incorporating PHA have been developed for 3-D printing of high-performance bioplastics for consumer electronics and automotive parts, among other applications (Guimarães et al., 2022; Pandey et al., 2022).

Typically, in environments that are unfavorable for growth and reproduction, some bacterial species synthesize PHAs as water insoluble intracellular carbonaceous storage products that are utilized when conditions become more conducive for growth (Guimarães et al., 2022; Alves et al., 2023; Alsaadi et al., 2022; Estévez-Alonso et al., 2021). This usually occurs in the stationary phase when sufficient carbonaceous substrate is present, but other essential nutrients such as oxygen (O_2), and/or nitrogen (N) and/or phosphorus (P) is/are lacking (Guimarães et al., 2022). Conversely, some species accumulate PHAs during the growth phase when substrates and nutrients are abundant (Guimarães et al., 2022). Metabolic pathways of model strains of PHA-synthesizing bacterial species such as *Cupriavidus necator, Pseudomonas putida,* and *Azotobacter vinelandii* have been well elucidated, while ongoing efforts

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https://doi.org/10.1016/j.microb.2025.100503
Received 24 February 2025; Received in revised form 28 July 2025; Accepted 8 August 2025
Available online 8 August 2025

are being directed towards determining regulatory mechanisms in lesser-described strains (Mitra et al., 2022). The large range of PHAs in existence are classified according to their hydroxyalkanoic monomeric composition (Alves et al., 2023; Alsaadi et al., 2022). Short chain length (SCL) PHAs consist of monomers with 3–5 C atoms, medium chain length (MCL) PHAs consist of monomers with 6–14 C atoms, and long chain length (LCL) PHAs consist of monomers with > 15 C atoms (Gao et al., 2023; Alsaadi et al., 2022). The character of heteropolymers can be controlled to some extent by employing substrates which feed into specific metabolic pathways (Gao et al., 2023).

The downstream applications of PHA-based biopolymers are dependent on the physical properties, which are in turn incumbent on the spatial arrangement and types of monomers within the polymeric structures (Min Song et al., 2022). The most widely studied PHA, polyhydroxybutyrate (PHB), is brittle in nature with a low impact strength; however, the structural integrity of this homopolymer can be improved by polymerization with other SCL or MCL monomers to form heteropolymers such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) or 3HB and (R)-3-hydroxy-hexanoate (3HHx), P (3HB-co-3HHx) (Fernandez-Bunster and Pavez, 2022; Guimarães et al., 2022; Alsaadi et al., 2022).

For relatively low value applications such as packaging materials, PHA-based bioplastics are more expensive than their conventional counterparts. Notably, some production processes may be energy intensive, and the use of defined growth substrates can be a significant cost bottleneck (Estévez-Alonso et al., 2021; Kannah et al., 2022). It is therefore important to 'marry' PHA producing strains with the most suitable substrate/s to maximize the volume of the selected biopolymer while keeping production costs to a minimum (Alves et al., 2023). This has led to considerable amounts of research being conducted on high PHA-producing bacterial strains and/or genetically engineered strains using materials such as lignocellulosic biomass and other agri-industrial by-products as growth substrates (Alves et al., 2023; Estévez-Alonso et al., 2021; Andhalkar et al., 2022; Gao et al., 2022). Hundreds of such literature reports on PHA production by a large range of bacterial strains exist (Alves et al., 2023; Estévez-Alonso et al., 2021). Alternatively, to avoid costs associated with maintaining sterile conditions required for monocultures, consortia of PHA-accumulating bacteria can be preferentially selected in complex substrates such as waste activated sludge using a 'feast-famine' strategy (Estévez-Alonso et al., 2021). Using this approach, 3-hydroxybutyrate (3HB) followed by 3-hydroxyvalerate (3HV) generally dominates the PHA monomeric composition, with the 3PB/3HV ratio being dependent on the volatile fatty acid (VFA) composition of the feedstock (Estévez-Alonso et al., 2021).

To support the growing body of PHA-directed research efforts, it is important to gain qualitative and/or quantitative insight into the biopolymers that are formed by bacteria. However, researchers are faced with an array of methods that differ from study to study. Some aspects pertaining to quantification and/or characterization of PHAs have been reviewed previously, including the recovery and purification of PHAs from biomass (Koller et al., 2013a), the use of fluorescent techniques for PHA quantification (Cao et al., 2022), and mass spectrometry (Rizzarelli et al., 2023). A more encompassing review by Koller and Rodríguez-Contreras (2015) requires updating and a recent review by Guimarães et al. (2022) contains limited information on analytical techniques because the scope of the review is wider, mainly addressing the biosynthesis of PHA (Guimarães et al., 2022; Koller and Rodríguez-Contreras, 2015). To address the challenges that researchers face when seeking to evaluate bacterial PHA production, this manuscript presents an up-to-date, cohesive collection and critical evaluation of the methods described in literature, highlighting the applications, advantages, and disadvantages of each.

Material was gathered using the search engines Google Scholar, Web of Science, and Scopus. The most current and relevant literature was selected based on an objective assessment of both quality and relevance. Only articles published in reputable, peer-reviewed journals were included, irrespective of the search platform. Exclusion criteria primarily filtered out non-peer-reviewed content, duplicates, and studies lacking sufficient methodological detail. Additionally, the manuscript includes six summary tables that compare various analytical and staining methods in terms of their benefits, limitations, reproducibility, sensitivity, and potential for standardization. These additions enhance the transparency and practical utility of the review. Polymer production by other microbial species such as yeasts and microalgae are not included in this review per se, although most of the methods are still applicable.

2. Staining procedures (non-fluorescent)

Various methods for staining PHA-containing bacterial cells (Fig. 1) are simple and cost-effective means of screening for taxonomic, morphological and functional characteristics, and have also been used to quantify PHA production, albeit with some shortcomings.

2.1. Gram staining

Gram microscopy differentiates bacteria into two main groups (Gram positive and Gram negative) based on the characteristics of their cell walls. Both Gram-negative and Gram-positive strains have inner cytoplasmic membranes and a periplasmic space surrounded by peptidoglycan. Gram-positive bacteria have a thick cell wall (15–80 nm) comprised mainly of peptidoglycan, while Gram-negative bacteria have a thinner cell wall (≈10 nm) comprised of a single layer of peptidoglycan within a lipo-proteinaceous periplasmic space surrounded by an outer membrane comprised of lipopolysaccharides (LPS) (Choi et al., 2020; Tantray et al., 2023). In the Gram staining process, bacterial cells affixed to a microscope slide are stained with crystal violet followed by an iodine solution, resulting in the formation of a crystal-violet-iodine complex within the cell walls of Gram-positive bacteria. Subsequently, a decolorizing agent such as ethanol or acetone is used to selectively elute the stain from Gram-negative cells. Finally, either safranin or dilute carbol fuchsin is applied as a counterstain, imparting a pink color to Gram-negative cells, while Gram-positive cells retain the crystal violet stain and appear purple (Tantray et al., 2023; Beveridge, 2001). Many commercial PHA-producing bacterial strains are Gram negative, including *Cupriavidus necator*, *Ralstonia eutropha, Alcaligenes eutrophus, Pseudomonas pseudoflava,* and *Pseudomonas putida* (Vicente et al., 2023). Gram staining assists during screening for industrially relevant PHA-producing bacteria according to the intended application. For example, for biopharmaceuticals, Gram-positive strains are preferable because the LPS of Gram-negative cell membranes contain endotoxins that can elicit adverse immune responses in humans and other higher animals (Muhammadi et al., 2015). However, for other applications, Gram-negative strains may be more appropriate because the PHA yields are typically higher (Muhammadi et al., 2015).

2.2. Sudan black staining

2.2.1. Screening methods

Of the many forms of Sudan dyes, Sudan black B (SBB) is a slightly basic azo dye first introduced by Lison in 1934 to selectively stain phospholipids, neutral triglycerides, and lipoproteins in microbial cells (Lison, 1934; Liu et al., 1998). Staining with SBB improves microscopic visualization of PHA granules, which range from 0.2 to 0.5 mm in diameter (Urtuvia et al., 2014). Different concentrations of SBB, solvents and staining durations have been applied by researchers (Table 1), most commonly 0.3 % SBB in 70 % (v/v) ethanol for 10 min (Bacha et al., 2023; Wei et al., 2011). A counterstain, usually safranin is used for enhanced visualization of the non-lipid components and overall morphology of the organisms (Phanse et al., 2011).

In addition, SBB is commonly used to screen for putative PHA-producing colonies macroscopically using plate assays (Bacha et al.,

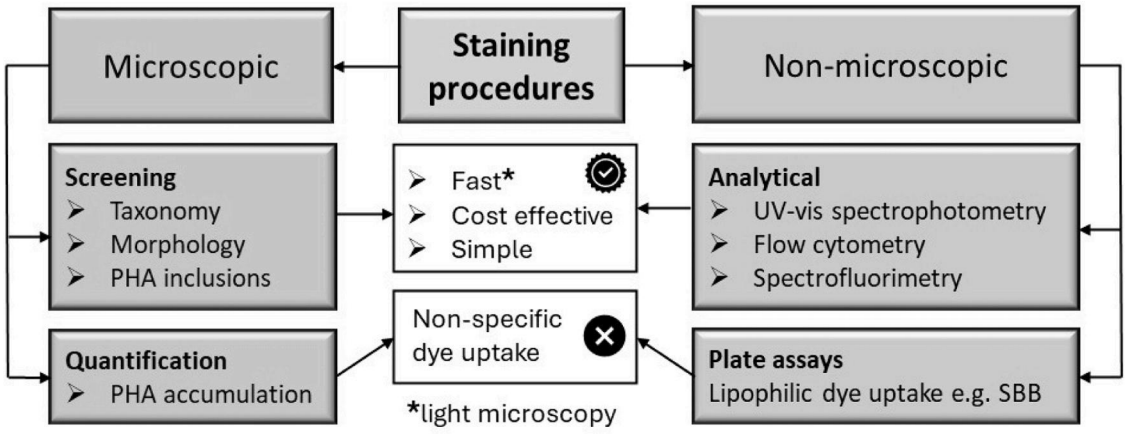

Fig. 1. Flow diagram showing how various staining can be used for assessing polyhydroxybutyrate producing bacteria.

Table 1
Experimental conditions applied for screening bacterial strains for polyhydroxyalkanoate production using Sudan black B staining.

Bacterial species	Culture conditions	Sudan black B plate assays		Sudan black B microscopy		References
		Staining	Washing	Staining	Washing/ Counterstaining	
Gordonia spp. *Micromonospora* sp., *Nocardia gamkensis*, *Streptomyces* spp.	50 % ISP #2 agar, 30°C, 3–7 days	0.02 % (w/v) 30 min	96 % (v/v) ethanol	Not performed	Not performed	(Mabasa et al., 2024)
109 bacterial strains	E2 mineral agar (2.5 % glucose (w/v), 0.35 % (w/v) HN4Y), 30°C, 2 days	0.05 % (w/v) 30 min	96 % (v/v) ethanol	0.3 % (w/v) in 70 % ethanol (v/v), 10 min	5 % (w/v) safranin in xylene, 10 s	(Attapong et al., 2023)
Aquabacterium sp.	HV medium (2 % (w/v) propionate), 30°C, 2–5 days	NG	NG	0.3 % (w/v) in 70 % ethanol (v/v), 10 min	5 % (w/v) safranin in dH_20, 10 s	(Feng et al., 2023)
Bacillus amyloliquefaciens	NLM 37°C 2 days	0.05 % (w/v) 30 min	96 % (v/v) ethanol	0.3 % (w/v) in 70 % ethanol (v/v), 15 min	Ethanol, x% safranin, 300 s	(Bacha et al., 2023)
Bacillus tequilensis	2 % (w/v) lactose agar (C/N = 8), 30°C, 4 days	0.05 % (w/v) 30 min	Sterile saline	NG	NG	(Yasin and Al-Mayaly, 2021)
Bacillus cereus 9 isolates from a polluted lake	Luria agar, 37°C, 2 days	0.05 % (w/v) 30 min	60 % (v/v) ethanol	0.3 % (w/v) in x% ethanol, time NG	NG	(Narayanan et al., 2020a)
73 isolates from agri-industrial food processing	Nutrient agar (1 % glucose (w/v)), 37°C, 48 hr	0.02 % (w/v) 30 min	96 % (v/v) ethanol	Not performed	Not performed	(Susianingsih et al., 2020)
Bacillus aryabhattai 61 isolates from paddy field soils	Basal medium (2 % glucose (w/v), 27°C, 48 hr	0.05 % (w/v) 30 min	Sterile saline	NG	NG	(Balakrishna Pillai et al., 2017)
Mixed microbial cultures	Synthetic WW in SBR 20°C, HRT 12 hr	NA	NA	0.3 % (w/v) in 60 % ethanol (v/v), 10 min	0.5 % (w/v) safranin in dH_20, 10 s	(Mesquita et al., 2015)
Cupraviadus. taiwanensis *Pseudomonas oleovorans*	M9 medium, 30°C, 2 days @ 200 rpm	NA	NA	3 % (w/v) in 70 % ethanol (v/v), 10 min	dH_20, 0.5 % (w/v) safranin in dH_20, 30 sec	(Wei et al., 2011)
Bacillus cereus, *Bacillus megaterium*	Glucose/glycerol supplemented medium, 1–2 day (time/temp NG)	NA	NA	0.3 % (w/v) in 70 % ethanol (v/v), 15–20 min	1 % (w/v) safranin in dH_20, time NG	(Burdon et al., 1942)

ISP = International *Streptomyces* Project; E2 = EnzMet2 formulation; HV = humic acid-vitamin; NLM = nutrient limited medium; HN4Y = 0.35 % (w/v) $NaNH_4HPO_4$ and 0.004 % (w/v) yeast extract; NLM = nitrogen limited medium; HRT = hydraulic retention time; SBR = sequencing batch reactor; rpm = revolutions per minute; M9 = minimal medium #9; NG = not given; NA = not applicable; dH_20 = distilled water

2023; Attapong et al., 2023; Mabasa et al., 2024; Narayanan et al., 2020a; Mohammed et al., 2019). Bacterial strains of interest are cultured on suitable media to promote PHA production. For example, N-deficient medium induces PHA production in some strains (Naser et al., 2021). After an appropriate incubation period, the surfaces of the agar plates are flooded with SBB in a lipid-soluble solvent that is washed off after a suitable staining period (Table 1, Fig. 2).

2.2.2. *Quantification methods*

In addition to screening for putative PHA-producing strains, SBB staining has been proposed for quantification of PHA. Mesquita et al. (2015) explored quantitative image analysis (QIA) tailored for identifying and measuring PHA inclusions in mixed microbial cultures (Mesquita et al., 2015). They utilized bright field microscopy to capture the stained images, which were then analysed using QIA techniques. A partial least squares regression coefficient of 0.90 was obtained between the PHA concentration determined using the gas chromatography (GC)

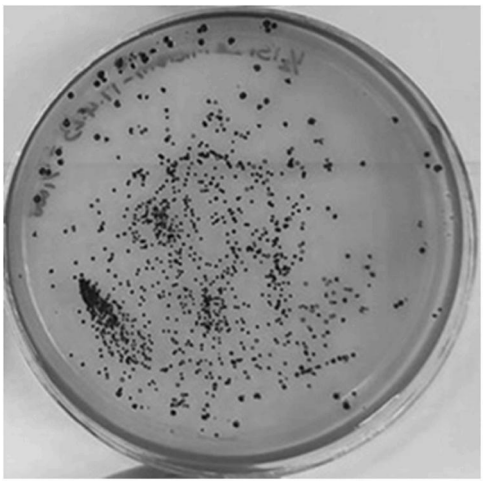

Fig. 2. Colonies of *Gordonia lacunae* BS2T stained with Sudan black (Gao et al., 2023).

method described by Smolders et al. (1994), and the QIA parameters (Smolders et al., 1994). The authors concluded that direct staining methods may offer viable alternatives to conventional PHA quantification methods (Mesquita et al., 2015).

Porras et al. (2017a,b, 2018) found that the PHA concentration could be determined by indirect or direct spectroscopic methods based on the amount of SBB absorbed by *Bacillus megaterium* BBST4 as a model PHA-producing bacterium (Porras et al., 2018, 2017a). The methods were validated by establishing correlations (regression R^2 = 0.786–0.944) between: (i) the amount of PHA (%w/w) measured using gas chromatography-mass spectrometry (GC-MS) (Porras et al., 2017b) extracted from lyophilized cells using a chloroform ($CHCl_3$)-based method (Manna et al., 1999) with (ii) the amount of SBB absorbed (direct method) or not absorbed (indirect method) by wet cell suspensions and determined spectroscopically (Porras et al., 2017a, 2016). The recommended spectroscopic methods that were optimized using response surface methodology (RSM) consisted of staining a washed (dH_2O) centrifuged pellet of the bacterial culture with SBB, mixing (200 rpm) and heating (30°C) for 20 min. Thereafter, the heights of the absorbance peaks obtained from the supernatant (indirect method) or washed and resuspended cells (direct method), were determined by conducting wavelength scans between 500 and 720 nm and 550–800 nm, respectively.

Although promising as cost-effective and simple methods, these SBB-based quantification methods require validation with a wider range of PHA-producing bacteria. In addition, SBB also stains non-PHA lipids such as lipoproteins, triacylglycerols and lipopolysaccharides. Methods based on SBB uptake are therefore questionable, especially if precise quantification of PHAs is required in bacterial cells that contain high concentrations of one or more of these compounds (Mabasa et al., 2024; Frederiks, 1977; Subramaniam and Chaubal, 1990).

In summary, SBB staining offers several advantages in the identification of PHA-producing microbes. It provides a rapid and cost-effective method for visualizing PHA accumulation within cells from a wide range of microbial samples, including pure cultures, environmental samples, and complex microbial communities (Porras et al., 2018, 2017a; Susianingsih et al., 2020).

However, because SBB also binds to other lipid-rich structures, certain proteins and polysaccharides, false positive results can be obtained (Mabasa et al., 2024; Frederiks, 1977; Subramaniam and Chaubal, 1990). It may therefore be contended that SBB is best suited as a simple, cost-effective PHA screening tool, and other more specific methods described later in this review should be used for validation and more accurate quantification of PHA (Urtuvia et al., 2014; Wei et al., 2011).

3. Fluorescent staining methods

3.1. Introduction

Fluorescent staining with appropriate lipophilic dyes allows microscopic visualization and, in some cases, quantification of PHA within bacterial cells (Cao et al., 2022). Various fluorescent dyes and methods have been used to provide valuable information on the distribution and abundance of PHA-producing bacteria in cultures or within complex communities and environments (Juengert et al., 2018; Ratnaningrum et al., 2019).

3.2. Fluorescent microscopy

Bacterial cultures can be screened for the presence of PHA granules by direct microscopic visualization of fluorescently stained cell inclusions. Microscopy can also offer insights into the intracellular distribution, morphology, and abundance of PHA granules, enabling researchers to study PHA synthesis and accumulation dynamics at a cellular level (Koller and Rodríguez-Contreras, 2015; Koller and Obruča, 2022; Cánovas et al., 2021). Cells are usually fixed, stained, rinsed and transferred to slides or chambers for visualization (Mesquita et al., 2015).

Nile red (NR) has been widely applied to visualize intracellular PHA, while LipidGreen1, LipidGreen2 and BODIPY (4,4′-difluoro-4-bora-3a,4a-diaza-s-indacene) are gaining traction as superior alternatives (Cao et al., 2022). Nile blue A (NBA) and acridine orange have been applied in a similar manner to NR, albeit less frequently, for staining intracellular lipids, imparting yellow to orange fluorescence at 460 nm and 490 nm, respectively (Godbole, 2016; Elain et al., 2015; Narayanan et al., 2020b).

Nile red is the oxazone form of NBA which is soluble in organic solvents such as acetone or dimethyl sulfoxide (DMSO) (Mohammed et al., 2019). Its lipophilic fluorochrome binds to the hydrophobic cores of PHA granules causing a spectral shift to a distinct red fluorescence at excitation wavelengths (λ_{ex}) of 485–550 nm and emission range (λ_{em}) of 560–720 nm (Cao et al., 2022; Elain et al., 2015; Goswami et al., 2023; Spiekermann et al., 1999). Like SBB, NR is often used for screening bacterial isolates for intracellular PHA accumulation (Pham et al., 2024; Yoo et al., 2024). For example, Takahashi et al. (2017) used NR microscopy to identify 30 putative PHA-producing bacterial isolates from 155 marine bacteria grown on starch-supplemented media (Takahashi et al., 2017). Similarly, Sriyapai et al. (2022) selected 6 PHA-producers from 200 soil isolates using NR staining (Sriyapai et al., 2022). However, other lipids like wax esters from thermophilic bacteria also fluoresce, so that techniques such as Fourier-transform infrared (FTIR) spectroscopy are required to confirm PHA production (Pernicova et al., 2020).

The stability and intensity of fluorescence of neutral fats and fat deposits stained with LipidGreen1 surpass those of NR (Choi et al., 2015). The lower hydrophobicity offered by the indoline-3-one structure of this dye ensures more sustained fluorescence at λ_{ex} 450 nm and λ_{em} 510 nm (Cao et al., 2022; Kettner and Griehl, 2020; Lee et al., 2011). BODIPY, with λ_{ex} 493 nm and λ_{em} 503 nm stains neutral lipids with sharp emission peaks and better photostability and brightness than NR (Kettner et al., 2022), while LipidGreen2, the 'upgraded' form of LipidGreen1 has even superior sensitivity and fluorescence over BODIPY (Kettner and Griehl, 2020; Kettner et al., 2022) but still needs to be evaluated for staining PHAs other than PHB (Cao et al., 2022). Overall, it appears that LipidGreen1, LipidGreen2 and BODIPY are more suited to prolonged *in-situ* high throughput measurements than NR (Cao et al., 2022; Kettner and Griehl, 2020; Lee et al., 2011). In addition, although they all stain non-PHA lipids, they do not stain bacterial plasma membranes like NR (Kettner and Griehl, 2020). The conditions and equipment used in a selection of studies using fluorescent staining for microscopic evaluation of PHA are presented in Table 2.

Table 2
Details of fluorescent microscopy methods for evaluating intracellular bacterial polyhydroxyalkanoate production.

Bacterial species	Staining method	Instrument used	Reference
Nile Blue A (NBA)			
Bacillus spp.	Cell suspension + 1 % NBA at 55°C for 10 min -> water wash -> 8 % acetic acid for 1 min. λ_{ex} 361–460 nm	Fluorescent microscope: NDP	(Mohammed et al., 2019)
Azotobacter chroococcum, Bacillus megaterium	Heat fix cell + 1 % NBA @ 55°C for 10 min -> water wash -> 8 % acetic acid for 1 min. λ_{ex} 361–460 nm	Fluorescent microscope: Nikon Labphot® with episcopic fluorescence and fluorimeter	(Ostle and Holt, 1982)
Nile Red (NR)			
Cupriavidus necator	Pellet + 10 mM PBS + 3 µL NR (1 mg/mL in DMSO) -> 30 min -> PBS wash. λ_{ex} 555–590 nm, λ_{em} 600–650 nm	Fluorescent microscope: EVOS™ FL Auto Imaging System	(Li and Wilkins, 2020)
Bacillus spp. (*megaterium, aryabhattai, tequilensis*)	Pellet + 0.1 M PBS wash -> fix in 20 % formaldehyde -> 1 mL+ 100 µL NR (1 µg/mL in acetone) for 10 min. λ_{ex} 561 nm.	Laser scanning confocal spectral microscope: Nikon A1R-Sin®, Yasin: NDP	(Yasin and Al-Mayaly, 2021; Balakrishna Pillai et al., 2017)
Bacterial cultures	2 µg/mL NR + 1 mL of culture, λ_{ex} 540 nm, λ_{em} 570 nm	Fluorescent microscope: Nikon	(Choi et al., 2015)
Acridine orange (AO)			
Escherichia coli	10 mL culture + 50 mL AO -> 37°C for 30 min -> harvest cells -> resuspend in dH2O. λ 490 nm	Fluorescent microscope: NDP	(Narayanan et al., 2020b)
Lipid Green1 (LG1)			
Recombinant *E. coli*	Agar plates 25 µg/mL LG1 @ 37°C for 20 hr -> suspend 100 µl PBS. λ_{ex} 460–500 nm, λ_{em} 510–560 nm	Fluorescent microscope: Nikon	(Choi et al., 2015)
Multiple fluorescent stains			
Cupriavidus necator	*Triple stain: (i) NR (ii) BODIPY 493/503 (iii) LipidGreen2 (LG2)* 500 µLculture + (i) 0.2 µL/mL BODIPY in PBS + 9 % DMSO, 10 % isopropanol, (ii-iii) 1.0 µg/mL LG2 or NR in PBS + 10 % DMSO λ_{ex}: BODIPY 400–440 nm; LG2: 460–490 nm, NR: 460–490 nm	Epifluorescent microscope: Olympus BX41® with Olympus XC50 camera	(Kettner et al., 2022)

NDP = no details provided; DMSO = dimethyl sulfoxide; PBS = phosphate buffered saline

3.3. Non-microscopic fluorescent methods

An agar plate-based method incorporating 0.5 µg NR/mL medium was first described for screening isolates for PHA production potential by Spiekerman et al. in 1999 and has been widely adopted as a screening tool (>650 citations in Scopus). Putative PHA-producing colonies fluoresce under UV light at 312 nm (Spiekermann et al., 1999). The method is more suited to Gram-negative bacteria, with false positive results being more common with Gram-positive isolates.

Uptake of lipophilic fluorescent dyes has also been used to measure intracellular bacterial PHA by flow cytometry or spectrofluorometry (Table 3). Cells are stained, washed and the PHA content is determined by comparing the intensity of fluorescence with concentrations determined using standard methods such as GC (Juengert et al., 2018; Ratnaningrum et al., 2019). However, quantification techniques need to be applied with caution as stains applied are lipophilic and may stain other parts of bacterial cells (Mabasa et al., 2024; Pernicova et al., 2020; Kourilova et al., 2020). For example, lipid components of cell walls of actinobacteria also take up fluorescent dyes as shown in Fig. 3. In addition, it has been suggested that PHA granules with higher densities and mass to volume ratios take up less stain because of the smaller surface area, so the intensity of fluorescence is polymer-specific (Karmann et al., 2016).

Zuriani et al. (2013) developed and optimized a rapid and efficient method for estimating the amount of PHAs produced by *Cupriavidus* USMAA1020 grown in media containing a variety of organic carbon (C) sources and stained with NR. Results were extrapolated from regression analyses generated from GC measurements of known concentrations of PHAs plotted against fluorescence intensity for a range of polymers. They found the method to be applicable for various polymers produced by the test strain, including P(3HB), P(3HB-co-4HB), P(3HB-co-3HV), and P(3HB-co-3HV-co-4HB) PHAs using the same standard graph.

Choi et al. (2015) used linear regression to compare results from fluorescent spectroscopy with LipidGreen1 and GC quantification of PHB produced by a recombinant strain of *Escherichia coli* (Choi et al., 2015). A good linear correlation (R^2=0.96) was noted between the intensity of fluorescence of LipidGreen1 and PHB concentration in the range 0.15–5.0 g/L (Choi et al., 2015). Similarly, Kettner et al. (2022) compared results from fluorescent spectroscopy and high-performance liquid chromatography (HPLC) for quantification of PHB in hydrolyzed biomass from *Cupriavidus necator* cultures, this time comparing BODIPY 493/503, LipidGreen2, and NR (Kettner et al., 2022). The authors found the highest correlation ($R^2 \geq 0.974$) between LipidGreen2 fluorescence intensity and PHB concentration up to 3.5 g/L. However, neither validated their method for more than one isolate or for other PHA monomers or polymers (Choi et al., 2015; Kettner et al., 2022).

Karmann et al. (2016) employed a dual staining protocol with BODIPY and SYTO 62 to rapidly quantify PHA from *Pseudomonas putida* KT2440 and *Rhodospirillum rubrum* S1 using flow cytometry. The nucleic acid stain SYTO 62 was used to distinguish viable cells from background noise. The authors demonstrated a strong linear correlation ($R^2 = 0.95$) between BODIPY 493/503 fluorescence intensity and MCL PHA content of *P. putida* within 2–43 % dry cell weight measured by GC. Background fluorescence due to uptake of stain by cell membranes and auto-fluorescence was noted (Karmann et al., 2016). In addition, the fluorescence was lower for SCL than the same mass of MCL PHAs, suggesting that PHA granules with higher densities and mass to volume ratios take up less stain because of the smaller surface area (Karmann et al., 2016). In contrast to Zuriani et al. (2013), the authors recommended using separate standard graphs for different bacterial strains and types of PHAs (Karmann et al., 2016; Zuriani et al., 2013). Alve et al. (2017) quantified PHB in *Herbaspirillum seropedicae* and *Azospirillum brasilense* using flow cytometry with NR staining and GC measurement of PHB. The authors found a strong correlation ($R^2 = 0.99$) between fluorescence intensity and PHB (% dry cell weight) (Alve et al., 2017).

In summary, spectrofluorimetry and flow cytometry using fluorescent dyes may have a place in PHB quantification as fast, efficient and cost-effective methods. There are some inconsistencies and methods still require further development. For example, Karmann et al. (2016) and Alve et al. (2017) did not report on the correlation between the actual PHA concentrations and fluorescence intensity, only the yield (% dry weight) (Karmann et al., 2016; Alve et al., 2017). To the best of our knowledge, only one group of researchers quantified PHA's other than PHB (Karmann et al., 2016). In addition, while these quantification methods show promise for measuring PHB from Gram-negative isolates, they have not been tested using Gram-positive isolates. It is likely that

Table 3
Non-microscopic fluorescent quantification of bacterial polyhydroxyalkanoates.

Microorganism	Staining method	Instrument used	Validation	Reference
Nile Red (NR)				
Herbaspirillum seropedicae, Azospirillum brasilens	Pellet + 1 mL permeabilization solution (30 % ethanol or 0.1 % Triton X−100) for 1 min –> 9.42 mM NR for 30 min. λ_{ex} 488 nm	Flow cytometer: BD Accuri® C5s	GC: $R^2 = 0.99$ PHB range: 0–25 % DCW	(Alve et al., 2017)
Cupriavidus sp.	3.1 μg/mL NRA in cell suspension. λ_{ex} 535 nm, λ_{em} 605 nm	Spectrophotometer: Perkin Elmer EnVision®	GC: "good" correlation P(3HB-co−4HB) range: 0–1.5 5.0 mg/mL	(Zuriani et al., 2013)
Lipid Green1 (LG1)				
Recombinant *E. coli*	Cell pellet –> PBS to OD 2.0 (600 nm) –> 2 μg/mL LG1 in 1 mL suspension –> 0.5 and 2 hr (dark). λ_{ex} 450 nm, λ_{em} 510 nm	Micro-fluorospectrometer: TECAN®	GC: $R^2 = 0.96$ PHB range: 0.15–5 g/L	(Choi et al., 2015)
Pseudomonas putida, Rhodospirillum rubrum	Culture in 50 % PBS with 5 mM EDTA –> 10 μg/mL –> SYTO 62* + BODIPY 493/503 in DMSO –> 4–6 min @ 30°C. λ_{ex} 488 nm, λ_{em} 530 nm	Flow cytometer: BD Accuri® C6	GC: $R^2 = 0.95$ *P. putida* MCL range: 2 %−43 % DCW	(Karmann et al., 2016)
Multiple lipophilic stains				
Cupriavidus necator	500 μL culture + (i) 0.2 μg/mL BODIPY in PBS with 9 % DMSO and 10 % Isopropanol, or 1.0 μg/mL LG2 or NRA with 10 % DMSO. λ_{ex}: BODIPY 400–440 nm; LG2: 460–490 nm, NR: 460–490 nm	Spectrophotometer: PerkinElmer LS45	HPLC: $R^2 = 0.974$ (LG2), 0.922 (BODIPY), 0.926 (NRA) PHB range: NDP	(Kettner et al., 2022)

* nucleic acid stain; DCW = dry cell weight; PBS = phosphate buffered saline: OD = optical density; NDP = no details provided

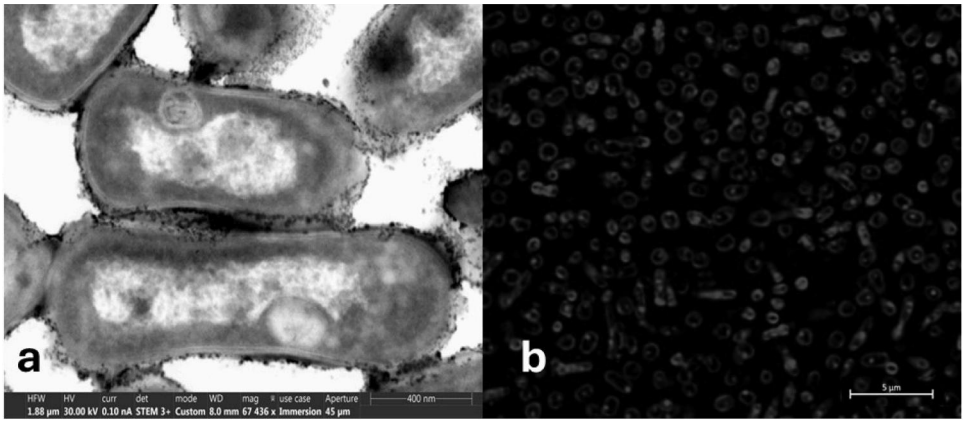

Fig. 3. Transmission electron microscopic image of Gordonia lacunae BST2 showing intracellular polyhydroxyalkanoate inclusions (a), and confocal images of the same organism stained with Nile red showing dye uptake by both polyhydroxyalkanoate inclusions and cell wall lipids (b).

correlation between PHA and/or PHB concentration and fluorescence intensity may be reduced in the latter, and possibly in Gram negative isolates other than those already tested due to non-specific binding of dyes to non-PHA lipids. From the studies that have been conducted, it appears that separate standard graphs may be required for each test strain and type of polymer.

4. Electron microscopy

Transmission electron microscopy (TEM) is commonly used to generate high quality images to visualize and sometimes measure PHA granules within bacterial cells (Fig. 3a). Cryo-Scanning electron microscopy (Cryo-SEM) can be employed as an alternative (Hrubanova et al., 2023). With TEM, the bacterial cells are pre-prepared, fixed, embedded and stained. Scanning electron microscopy (SEM) is usually applied with staining such as NR to visualize PHA granules in bacterial cells as already discussed. It can also be used to visualize the surfaces of PHA films, for example, to study the effects of enzyme hydrolysis, environmental degradation or mechanical fracturing, or to assess the structure of bioplastic layers after 3D printing (Rydz et al., 2019).

5. Analytical methods for identification and quantification of polyhydroxyalkanoates

5.1. Introduction

The successful commercialization of PHAs necessitates reliable methods for their identification and quantification using analytical instrumentation. A variety of such methods are currently available, including spectrophotometric analysis, FTIR, HPLC, GC, TEM, and nuclear magnetic resonance (NMR) spectroscopy. In most instances, the PHA needs to be extracted from the bacterial cells that have been harvested by sedimentation, filtration, or centrifugation before identification and quantification. Thereafter, requirements for separating the PHA molecules from lipids and cell debris depend on the application and the type of bacteria and include physical, mechanical and/or chemical disruption of the cells, for example, by freeze-drying or with sodium hypochlorite (NaOCl) (Koller et al., 2013a).

To protect the environment and human health, there is a need for laboratories to transition from using hazardous chemicals to greener alternatives. The most common solvents used during PHA extraction are environmentally hazardous halogenated chemicals, namely $CHCl_3$, NaOCl and dichloromethane (CH_2Cl_2). Studies have explored the use of green solvents that are more degradable and less toxic. These include alcohols (ethanol, 1-propanol, 1-butanol), acetone, cyclohexanone, methyl ethyl ketone, ethylene carbonate and dimethyl carbonate (DMC).

It has been reported that DMC can achieve PHA recovery yields exceeding 85 % and purities of up to 95 %, making it a sustainable alternative to commonly used solvents such as $CHCl_3$ (Samorì et al., 2015).

Laboratory research methods usually differ to those for extraction of PHA for commercial applications of PHA, although there may be similarities in some methods. Table 4 presents a comparison of various analytical methods commonly used in the qualitative and quantitative analysis of PHAs, highlighting their advantages and disadvantages. Thereafter, each method is reviewed in more detail.

5.2. Ultraviolet-visible spectrophotometry

The ultraviolet-visible (UV-Vis) spectrophotometry method for PHB quantification, initially reported by Law and Slepecky, has been widely applied due to its simplicity (Law and Slepecky, 1961). Biopolymers are first extracted using $CHCl_3$, after which the $CHCl_3$ is evaporated and the PHB is converted to (*E*)-but-2-enoic acid (BE) by treatment in concentrated sulfuric acid (H_2SO_4) at 100°C for one hour (Fig. 4). The concentration of PHB in the extracted sample is estimated by measuring the absorbance of BE at 235 nm using a UV–Vis spectrophotometer. It must be noted that (*E*)-but-2-enoic acid (BE) is the stipulated nomenclature of the International Union of Pure and Analytical Chemistry (IUPAC), but the common term crotonic acid is widely used in literature as an alternative. Some researchers use standard graphs prepared from BE and assume 100 % conversion from PHB (e.g., (Kag et al., 2024)) while others more correctly use standard graphs prepared from acidified PHB standard dilutions which account for conversion losses (e.g., (Mabasa et al., 2024; Rodge et al., 2023)). This method tends to overestimate the PHB content due to various sources of interference. For example, non-PHB PHAs such as 3HV can thermally degrade into products such as 2-pentenoic acid that absorbs light at similar wavelengths to BE (Duvigneau et al., 2021a). Interference by carbohydrates may occur due to dehydration reactions and the subsequent formation of conjugated double bond systems during acid hydrolysis with H_2SO_4, further contributing to absorbance in the same spectral region (Law and Slepecky, 1961; Slepecky and Law, 1960). In addition, if NaOCl is used for cell disruption, a white precipitate can form that interferes with spectrophotometric analyses (Slepecky and Law, 1960).

Despite its ease of use for PHB quantification, this method cannot distinguish between PHB copolymers and has associated hazards due to the use of concentrated H_2SO_4 and heat. In addition, high dilutions are required to obtain absorbance readings in the recommended 0.1–1.0 range which can diminish the accuracy (Mabasa et al., 2024). Despite its limitations, many laboratories still employ this method regularly owing to its simplicity.

5.3. Gas chromatography

Gas chromatography coupled with a flame ionization detector (GC-FID) stands out as one of the most common methods used for PHA quantification and determination of monomeric composition. This method is used extensively for its accuracy and reproducibility. It was first described for analysis of PHB in bacterial cells by Braunegg et al. in 1978 and continues to be used for quantification of methanolized PHB (Juengert et al., 2018; Braunegg et al., 1978). *Sample pretreatment includes conversion of P*HAs to volatile methyl esters derivatization in $CHCl_3$ and acidified methanol (15–20 % H_2SO_4) solution at 100 °C

Table 4
Comparison of principles, advantages and disadvantages of analytical methods for polyhydroxyalkanoate analyses.

Technique	Principle	Advantages	Disadvantages	References
UV-Vis spectroscopy	PHB converted to BE in hot conc. H_2SO_4 followed by spectrophotometric measurement of BE @ 235 nm	- Simple and rapid	- Limited to PHB estimation - Cannot distinguish between PHB copolymers - Tends to overestimate PHB - Hot acid is hazardous	(Darshan and Nishith, 2014)
GC	Methanolysis of sample in Methanol/H_2SO_4 followed by column separation and PHA detection (FID or MS)	- Accurate and reproducible measurements - Qualitative & quantitative	- Hazardous solvents required. - Labor-intensive sample pretreatment - Large sample sizes required	(Bano et al., 2024; Shahid et al., 2021; Huang et al., 2018a)
HPLC	PHB & PHV converted to BE and PE in hot conc. H_2SO_4 followed by column separation and detection of acid products	- Short analysis time - Simple sample pretreatment	- Unable to distinguish between homogenous PHA and PHA copolymers - Standards required - Hot acid is hazardous	(Duvigneau et al., 2021b; Ishii-Hyakutake et al., 2021; Watanabe et al., 2012)
FTIR	Used to confirm the presence of specific PHB functional groups	- Minimal sample preparation - Rapid & non-destructive - Can screen bacterial cells - Can distinguish between different types of PHAs - Qualitative & quantitative	- Cannot distinguish between PHB copolymers - Cannot detect low PHA content in cells	(Guimarães et al., 2022; Deng et al., 2024; Oliveira et al., 2022; K. Shah et al., 2012)
Raman spectroscopy	It measures fingerprint-like information about vibrational modes of a sample	- Rapid and non-invasive - No solvents required for sample preparation - Qualitative & quantitative	- Cannot distinguish between different types of PHAs - Low sensitivity	(Alfano et al., 2023; Samek et al., 2016; Jawhari, 2012; De Gelder et al., 2008)
NMR	Pure PHA samples are dissolved in deuterated $CHCl_3$ for measurement	- No polymer hydrolysis required - Can be applied to novel functionalized PHA - No standards required - Qualitative & quantitative	- Requires pure product - Time consuming - Not suited to routine analyses	(Bano et al., 2024; Ge et al., 2016; Etxabide et al., 2022; Shrivastav et al., 2010)

BE = (*E*)-but-2-enoic acid (crotonic acid): PE = (E)—pent-2-enoic acid; FTIR = Fourier-transmission infrared spectroscopy; GC = gas chromatography; HPLC = high performance liquid chromatography; NMR = nuclear magnetic resonance spectroscopy; H_2SO_4 = sulfuric acid; $CHCl_3$=chloroform; FID = flame ionization detection; MS = mass spectrometry

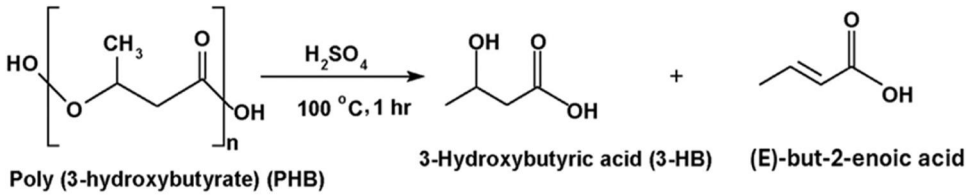

Fig. 4. Conversion of polyhydroxybutyrate to (*E*)-but-2-enoic acid in hot sulfuric acid (Shrivastav et al., 2010; García-Chumillas et al., 2024).

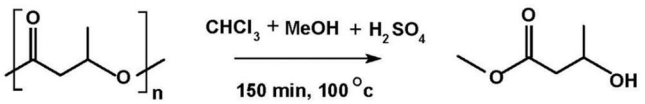

Fig. 5. Methanolysis of polyhydroxyalkanoates (Juengert et al., 2018).

(Fig. 5). Benzoic acid is commonly used as an internal standard (Duvigneau et al., 2021b; Saito et al., 2023). Braunegg's method was later modified by Riis and Mai, 1988, who developed an approach involving the hydrolysis and transesterification of PHB using dichloroethane($C_2H_4Cl_2$), propanol, and hydrochloric acid (HCl) (Braunegg et al., 1978; Riis and Mai, 1988).

The pretreatment method employs chlorinated solvents such as $CHCl_3$, CH_2Cl_2 and 1,2-dichloroethane, which, although effective in achieving high PHA recovery, are all known for their toxicity and environmental persistence (Pérez-Rivero et al., 2019; Koller et al., 2013b; Ruder, 2006). Thus, non-halogenated solvents have been explored as more environmentally friendly alternatives. Yang et al. (2015) investigated using butan-2-one (methyl ethyl ketone) as a potential replacement for $CHCl_3$ in the extraction process (Yang et al., 2015). The solvent enhanced the recovery of P(HB-co-HV) but was less efficient than $CHCl_3$ in extracting PHB. Several other solvents have been investigated including anisole, cyclohexanone, and phenetole and Werker et al. (2008) proposed hydrolysis and derivatization using a combination of butanol and HCl (Rosengart et al., 2015; Werker et al., 2008). The application of less toxic solvents in pretreatment methods supports the principles of green chemistry in reducing environmental impact and promoting sustainability. Modifications to the methanolysis procedure have been reported. For example, Cui et al. (2007) proposed a method based on the combination of alkaline hydrolysis of PHB to its monomer, 3HB, and subsequent acid esterification of the monomer to form methyl 3HB. The results showed that while alkaline conditions promote the hydrolysis of PHB, acidic conditions favor esterification, resulting in improved derivatization efficiency (Cui et al., 2007).

The robustness of the GC method relies on using available PHA analytical standards against which the retention times (RTs) of the polymers are compared. The main drawback of the method is that high purity standards are required, and the commercially available range is limited. To identify less common PHA monomers, GC coupled with mass spectrometry (GC-MS) is used, where mass spectral patterns are compared against the National Institute of Standards and Technology (NIST) reference library. For example, Balakrishna Pillai et al. (2020) detected three peaks in a chromatogram at 3.630, 19.224, and 21.667 min which were identified using the NIST library as methyl esters of 3-HB, pentadecanoic acid and hexadecenoic acid, respectively (Balakrishna Pillai et al., 2020). The GC-MS technique is also a valuable tool that is used to obtain structural information on the chain length, monomeric composition and the position of double bonds in unsaturated PHA monomers. However, further derivatization steps are required, which include thiomethylation and trimethylsilylation (TMSi). The latter is performed using silylation reagents such as bis(trimethylsilyl) acetamide (BSA), N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) and bis(trimethylsilyl)trifluoroacetamide (BSTFA). It is based on α-cleavage of derivatized hydroxyl (-OH) groups, providing information about their position, while fragmentation patterns and *m/z* values provide information about the monomer's carbon chain length (Huang et al., 2018a; Yeol and Choi, 1995; Ashby et al., 2009; Simon-Colin et al., 2012a, 2012b; Lee and Choi, 1995). Thiomethylation is commonly performed using dimethyldisulfide (DMDS) in the presence of iodine. It involves the addition of thiomethyl groups (–SCH_3) across carbon–carbon double bonds, thus allowing the precise localization of double bonds in unsaturated hydroxyalkanoic acid methyl esters to be identified (Simon-Colin et al., 2012b; Murata et al., 1978).

While GC combined with acidic alcoholysis is a common quantification method for PHA, it has drawbacks such as the use of hazardous solvents, labor-intensive sample pretreatment, and the need for a large sample size (Huang et al., 2018a; Khang et al., 2021; Arcos-Hernandez et al., 20110). For convenience, the extraction, pre-treatment, equipment, operation and relevant retention times for PHA analysis by GC-FID and GC-MS from previous studies are presented in Table 5. For more detailed information on the use of MS for PHA identification, readers are referred to a review on the subject by Rizzarelli et al. (2023).

5.4. High performance liquid chromatography

High performance liquid chromatography is based on separation of the PHA's or PHA conversion products through a column, which are then measured by a diode array detector (DAD). The HPLC method was first used by Karr et al. (1983) to quantify PHB produced by *Rhizobium japonicum* using an ion-exclusion column. The original method involved preparing samples for quantification by treating bacterial cells or extracts with conc. H_2SO_4 at 100 °C to convert PHB to BE which is measured in the UV range (210 nm). The method can detect as little as 0.01 μg of PHB but it appears to be limited to detection of PHB and BE (Satoh et al., 2016). More recently, methods based on alkaline sodium hydroxide (NaOH) pretreatment have been developed that enable detection of additional copolymers such as PHV and 3-HHx, which are not always found in acid pre-treated samples (Watanabe et al., 2012). When acidified, PHV is converted into (*E*)-pent-2-enoic acid (PE) in a similar fashion to the conversion of PHB to BE (Satoh et al., 2016). For alkaline pretreatment, the samples or PHA extracts are hydrolyzed with 1 M NaOH at temperatures $\geq$ 100 °C for 30 min to 5 hr (Table 6). Thereafter, samples are usually H_2SO_4 acidified and PHA conversion products are detected after separation in C18 or ion-exclusion columns using dilute acid as solvents.

Ion-exclusion chromatography is commonly used for the separation of organic acids based on differences in pKa values, which lead to variations in elution times during HPLC analysis (Głód, 1997). In ion-exclusion columns, the stationary phase consists of an ion-exchange resin that acts as a semi-permeable membrane. This membrane allows non-ionic species to pass through while largely excluding charged species (Morales et al., 1998). Monomers with lower pKa values tend to interact more strongly with the resin and elute later, whereas compounds with higher pKa values, which are less ionized, elute earlier (Głód, 1997; Morales et al., 1998). Ion-exclusion columns often use sulfonated polystyrene divinylbenzene as the ion-exchange resin, which is maintained in the hydrogen (H^+) form using dilute acids such as sulfuric acid (H_2SO_4) at concentrations ranging from 0.005 to 0.01 N as eluents (Głód, 1997; Morales et al., 1998).

Alternative methods have also been applied, such as detection of PHB from cells washed in acetone and ethanol and extracted with hot

Table 5
Details of gas chromatographic methods for analyzing bacterial polyhydroxyalkanoates.

Method	Extraction	Pre-treatment	Column	Carrier gas	Operation	Retention times	Reference
GC-FID	No extraction	*Hydrolyzed & trans esterified*: 4 mL 1,2-DCE + 2 mL 4:1 (v/v) propanol:H_2SO_4 + 20 g/L BA* 4 hr @ 120°C	**Stabilwax** 30 m (L) x 0.25 mm (ID)	He 1.44 mL/min	Column temp: Gradient from 120°C (3 min) to 240°C @ 3 °C/min to 140°C to 230°C @ 50 °C/min to 240°C @ 10 °C/min	BE: 16 min	(Duvigneau et al., 2021b)
GC-FID	Soxhlet $CHCl_3$	*Alkaline decomposition*: 5 M NaOH 30 min @ 120 °C *Methanolysis*: 3 % H_2SO_4 in 100 mL CH_3OH + 2 mL $CHCl_3$ + BA* 3.5 hr @ 100°C.	**Zebron ZB-FFAP** 30 m (L) x 0.32 mm (ID)	He 2.36 mL/min	Injector temp: 180°C Column temp: 145°C Detector temp: 260°C Injection: 1:48 ratio split	3HB- CH_3 ester: 1.8 min, 3HV-CH_3 ester: 2.1 min, BA*: 2.5 min	(Saito et al., 2023)
GC-FID	$CHCl_3$	*Methanolysis:* 20 % (v/v) CH_3OH in H_2SO_4 7 hr @ 100°C	**InertCap WAX-HT** 30 m (L) x 0.25 mm (ID)	He 1.0 mL/min	Injector temp: 230°C Column temp: Gradient 4 min @ 80°C to 200°C @ 12 °C/min, 6 min @ 200°C for 6 min. Injection: 1:20 ratio split	0.60–0.83 C-mol PHA/C-mol substrate	(Ren et al., 2024)
GC-FID, GC/MS	Soxhlet $CHCl_3$	*Methanoloysis*: 2.8 M H_2SO_4 in 50 % (v/v) $CHCl_3$ & CH_3OH 2 hr @ 100°C	**DB-WAX capillary** 30 m (L) x 0.32 mm (ID)	He Rate NG	Injector temp: 250°C Column temp: Gradient from 50°C to 200°C @ 15 °C/min Injection: 1:18 split ratio MS ionizing energy: 70 eV	4HB: 4.20 & 6.89 min, 117 *m/z*, 4HV: 4.44 min & 6.68 min, 131 *m/z*, 4HHx: 5.18 & 7.50 min, 145 *m/z*.	(Stauffer, 2013)
GC-MS	7.3 % (v/vl) $CHCl_3$ 4 % aq. NaOCl	*Methanolysis*: 10 mg sample to 2:2 (v/v) $CHCl_3$ & 15 % H_2SO/CH_3OH 2 hr @ 100°	**HP−5 ms Ultra Inert** 30 m (L) × 0.25 mm (ID)	He 1.2 mL/min.	Column temp: Gradient from 35°C to 280°C @ 15 °C/min, 2 min hold Total-ion scan mass-to-charge ratio: 40–600 (*m/z*).	MCL-PHA monomers with C4: 6.506 min, C8: 9.559 min, C10: 12.206 min. C12: 15.607 min	(Yasin and Al-Mayaly, 2021)
GC-MS	No extraction	*Acidic alcoholysis*: 3:1 butanol & 37 % HCl 4 hr @ 100 °C	**Varian VF−1ms** 30 m (L) x 0.25 mm (ID)	He 0.7 mL/min	Injector temp: 250°C FID detector temp: 300°C Column temp: Gradient 5 min @ 70°C to 290°C @ 10 °C/min, 18 min @ 290°C Injection: 1:20 (FID) 1:25 (MS) ratio split	BE: 7.17 min 3HB: 8.08 min 3HV: 9.09 min	(Werker et al., 2008)
GC-MS	SDS 1 hr @ 50°C $CHCl_3$	*Methanolysis:* Dissolved in $CHCl_3$, CH_3OH/ H_2SO_4 (85:15 v/v) 210 min @ 105°C	**Restek RTX−5MS WCOT** 30 m (L) × 0.25 mm (ID)	He 1 mL/min	Injector temp: 250°C Column temp: Gradient 1 min @ 40°C to 120°C @ 15 °C/min, 2 min @ 120°C to 300°C @ 10 °C/min EI 70 eV @ 0.2 scan/sec, range: 50–600 m/	3HB: 4.1 min CH_3 ester: $m/z = 74$	(Pagliano et al., 2020)
GC-MS	$CHCl_3$	*Methanolysis:* $CHCl_3$ & CH_3OH/H_2SO_4 15 % (v/v) 140 min @ 100 °C	**HP-INNOWax** 30 m (L) x 0.25 mm (ID)	He 3 mL/min	Injector temp: 230°C Column temp: Gradient from 40°C to 240 °C @ 20 °C/min, 10 min @ 240°C Scanning range: 40–360 *m/z* Splitless mode	3-hydroxy: -octanoate, -decanoate, -tetradecanoate, -hexadecanoic acid, -octadecanoic acid	(Choonut et al., 2020)

* benzoic acid (BA) internal standard; BE = (*E*)-but-2-enoic acid; $CHCl_3$ = chloroform; NaOCl = sodium hypochlorite; SDS = sodium dodecyl sulfate; DCE = dichloroethane; H_2SO_4 = sulfuric acid; NaOH = sodium hydroxide; CH_3OH = methanol; HCl = hydrochloric acid; CH_3 = methyl group; L = length; ID = internal diameter; He = helium; MS = mass spectrometry; FID = flame ionization detector; EI = impact ionization HB = hydroxybutyrate; HV = hydroxyvalerate; HHx = hydroxyhexanoate; MCl = medium chain length

$CHCl_3$, where PHB was measured at 306 nm using a C18 column (Hagagy et al., 2021). While some methods include acetonitrile (CH_3CN) in their solvent mixtures, less toxic alternatives are available that achieve comparable results (Table 6).

Like GC, HPLC has been coupled with MS to provide more detailed information about the eluted compounds. Overall, studies suggest that the results obtained by HPLC are comparable to those obtained by GC, but sample preparation is less time consuming, and less organic solvents are used (Satoh et al., 2016). For convenience, the extraction, pre-treatment, equipment, operation and relevant retention times for PHA analysis by HPLC and HPLC-MS from previous studies are presented in Table 6. All methods were based on measurement of peaks from chromatograms obtained at 210 nm unless otherwise stated in the table.

5.5. Fourier-transform infrared spectroscopy

Fourier-transform infrared spectroscopy can be used to detect the different functional groups of PHA molecules by recording a spectrum in the 400–4000 cm^{-1} range. It was first reported for analysis of bacterial PHA by Hong et al. (1999), who used it to differentiate between SCL, MCL and LCL PHAs. Samples are usually prepared by mixing with FTIR-grade potassium bromide (KBr) to form a pellet (Shamala et al., 2009). Alternatively, samples can be dissolved, and a single drop applied from a glass Pasteur pipette onto the attenuated total reflection (ATR) element of the FTIR instrument, which provides sufficient spectral absorption for effective analysis (Kansiz et al., 2007). Studies have shown good correlations between GC quantification and normalized FTIR spectral intensities (Alfano et al., 2023; Kansiz et al., 2000), but such methods require robust development of calibration and predictive models using FTIR and GC-FID and are not yet widely applied (Christensen et al., 2023).

Table 6
Details of high-performance liquid chromatography methods for analyzing bacterial polyhydroxyalkanoates.

Extraction	Pre-treatment	Column	Mobile phase	Operation	Retention	Reference
HPLC						
$CHCl_3$ x 3 10 min @ 100 °C	1 M NaOH I hr @ 105°C	**Inertsil® 100 A ODS−3 C18** 250 × 4.6 mm (L x ID)	92 % ®dil. H_2SO_4 + 8 % CH_3CN	Column temp: 60°C Flow rate: 1 mL/min	BE: 17 min PE: 77 min	(Duvigneau et al., 2021b)
Soxhlet $CHCl_3$ 48 hr @ 80°C	5 M NaOH 30 min @ 120 °C 6 M HCl to pH 3	**Shim-pak® SCR-102H ion exclusion** 300 × 8.0 mm (L x ID)	5 mmol/L $HClO_4$	Column temp: 45 °C Flow rate: 1.5 mL/min	2BE: 11.1 min 2PE: 15.0 min 3HB: 6.3 min	(Saito et al., 2023)
$CHCl_3$	2 M NaOH 1 hr @ 105°C Acidified 2 N H_2SO_4	**Shim-pak® SCR-101H ion exclusion** 300 × 7.9 mm (L x ID)	0.025 % H_2SO_4	Column temp: 60°C Flow rate: of 1 mL/ min	2BE: 17.1 min 2PE: 24.0 min	(Satoh et al., 2016)
6 % NaOCl + $CHCl_3$	Wash pellet: Acetone, ethanol ⟶ hot $CHCl_3$ ⟶ filter ⟶ $CHCl_3$	**Sciex Exion® C18** 150 × 4.6 mm (L x ID)	$CHCl_3$ + PO_4^{2-} buffer (0.1 M, pH 4.5)	Column temp: NG Flow rate: 0.3 mL/min Wavelength: 306 nm	PHB: 7.5 min	(Hagagy et al., 2021)
No extraction	1 M NaOH 3 hr @ 100 °C	**Aminex® HPX-87H ion exclusion** 300 × 7.8 mm (L x ID)	0.014 N H_2SO_4 ± 20 % (v/v) CH_3CN	Column temp: 60°C Flow rate: 0.7 mL/min	3HB: 12.5 min	(Watanabe et al., 2012)
$CHCl_3$	1 M NaOH 3 hr @ 100 °C Neutralized 1 N H_2SO_4	**Aminex® Fast Acid Analysis** 100 × 7.8 mm (L x ID)	0.014 N H_2SO_4 + 20 % (v/v) CH_3CN	Column temp: 60°C Flow rate: 0/7 mL/min	BE: 4.5 min 3HB: 3.4 min	(Ishii-Hyakutake et al., 2021)
HPLC-MS & HPLC-MS-MS						
$CHCl_3$	96 % H_2SO_4 1 hr @ 100°C	**Hamilton® PRP-X300 ion exclusion** 250 × 4 mm (L x ID)	3 Gradients: 10–70 % 1 mM H_2SO_4 + 30–90 % CH_3CN	Column temp: 25 °C Flow rate: 1 mL/min MS ESI + signal @ 400°C, max pressure: 70 eV.	BE: 2.4 min, 83 *m/z*	(Stoica et al., 2018)
Soxhlet 10 % (w/v) CH_2Cl_2 8 hr @ 55°C	1 M NaOH 5 hr @ 100°C	**Zorbax Eclipse plus® C18** 150 × 4.6 mm (L x ID)	0.1 % (v/v) CH_2O_2 (A) 100 % CH_3OH (B) Gradient (v/v)	Column temp: 25°C Flow rate: 1.2 mL/min MS ESI - signal @ 350°C, scan range: 50–350 *m/z*, 3500 V	3HB: 5.30 min, 130 *m/z*) Other C6-C16 monomers*	(Ge et al., 2016)
$CHCl_3$	1 M NaOH 1 hr @ 105°C	**Thermo Scientific™ Hypersil™ ODS (C18)** 100 × 4.6 mm (L x ID)	0.001 M $CHCl_3$	Column temp: 40°C Injection rate: 5.0 µL/s Flow rate: 0.003 mL/min Triple Quadrupole MS	BE = 8.1 min	(Hagagy et al., 2022)

* Refer to manuscript for details; HPLC = high performance liquid chromatography; MS = mass spectrometry; NaOH = sodium hydroxide; $CHCl_3$ = chloroform; HCl = hydrochloric acid; CH_2Cl_2 = dichloromethane; NaOCl = sodium hypochlorite; $HClO_4$ = perchloric acid; CH_2O_2 = methanoic (formic) acid; CH_3OH = methanol; RT = room temperature; RP = reversed phase; L = length; ID = internal diameter; H_2SO_4 = sulfuric acid; CH_3CN = acetonitrile; BE = (*E*)-but-2-enoic acid (crotonic acid), PE = (*E*)-pent-2-enoic acid; NG = not given; ESI = electrospray ionization.

The presence of a carbonyl (C═O) band in the region of 1724–1740 cm^{-1} is suggestive of PHA accumulation (Shamala et al., 2009), but the exact location of the C═O peak depends on the crystallinity and composition of the PHA (Christensen et al., 2023). For example, Randriamahefa et al. (2003) used the location of the C═O peaks to distinguish between PHA (1724 cm^{-1}) and poly-3--hydroxyoctanoate (1742 cm^{-1}) (Randriamahefa et al., 2003). Sharpening and shifts in the C═O bands are associated with changes in the degree of PHA crystallization (Alfano et al., 2023; Shamala et al., 2009).

The IR spectrum of a commercial PHB standard (Sigma-Aldrich, St Louis, USA cat no. 363501) has been provided as a typical example of how FTIR is used to investigate the structural characteristics of PHAs (Fig. 6). The strong band at 1732 cm^{-1} corresponds to the stretching vibration of the C═O group, characteristic for ester bonding in PHAs (Biradar et al., 2018). A very weak absorption band is present around 3440 cm^{-1} of the stretching -OH group. The peak around 2981 cm^{-1} represents stretching of the CH_3 group, while the peak at 2920 cm^{-1} represents methylene (CH_2) groups. Bands at 1454 and 1373 cm^{-1} can be attributed to the asymmetric and symmetric deformation of the CH_3 groups, respectively, while those within 1272–1054 cm^{-1} are characteristic of the asymmetric and symmetric stretching vibrations of the ester (C–O–C═O) groups (Kamnev et al., 2018).

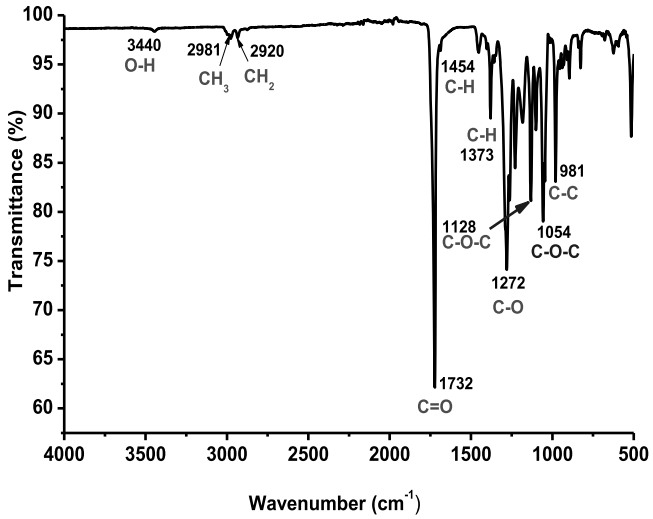

Fig. 6. Representative Fourier-transform infrared spectrum of polyhydroxybutyrate.

5.6. Raman spectroscopy

Raman spectroscopy measures the energy of different vibrations in molecules and is slowly becoming a recognized analytical technique for PHA identification and quantification. For example, De Gelder et al. (2008) found that the intensity of the Raman band at 1734 cm^{-1} appeared to be suitable for the monitoring of PHB production and consumption when compared with HPLC. Among other findings, Izumi and Temperini (2010) found that the structure of helical PHB and PHBV from *Cupraviadus necator* could be predicted by Raman bands at 1220, 1402, 1725, 2998 and 3009 cm^{-1} while the bands of amorphous PHB and PHBV appeared at 1453, 1740, 2881, 2938 and 2990 cm^{-1}. Furthermore, they found that the ratio of the intensity of the bands at 2938 cm^{-1} and 1740 cm^{-1} in the spectra of solutions, and 1354 cm^{-1}

and 1740 cm^{-1} in the spectra of molten polymers could be used to estimate the composition of PHBV samples (Izumi and Temperini, 2010).

5.7. Nuclear magnetic resonance

Nuclear magnetic resonance is a spectroscopic technique that detects the energy (electromagnetic radiation) absorbed by changes in the nuclear spin state of atoms (Godbole, 2016). This technique has been used by researchers to characterize the structure of PHAs. One advantage of NMR is that no prior hydrolysis is required. However, NMR cannot be applied for rapid structural determination of intracellular PHA since complex patterns of chemical shifts of C atoms from other cell components and PHAs overlap. Hence, NMR requires highly pure PHA extracts usually obtained by an intensive extraction procedure consisting of lyophilization, fine grinding of lyophilized cells, solvent extraction, precipitation, and separation of PHA (Samek et al., 2016). The NMR procedure itself can be rapid because the high number of protons in PHA polymers promote proton NMR (^{1}H NMR) sensitivity, typically keeping analyses times to under an hour (Kunasundari and Sudesh, 2011). However, carbon-13 (^{13}C NMR) analysis takes longer due to the reduced sensitivity associated with low natural abundance of ^{13}C in PHA (Clendinen et al., 2014).

A number of studies have incorporated NMR to analyze PHAs. For example, Etxabide et al. (2022) used ^{1}H NMR to study the chemical structure of PHB produced from *Cupriavidus necator* H1, which was confirmed by signals corresponding to CH_3, CH_2 and methine (CH) at 1.25, 2.50, and 5.25 ppm, respectively (Etxabide et al., 2022). Montiel-Jarillo et al. (2022) used both ^{1}H NMR and ^{13}C NMR to study the structure of PHB and PHBV. The ^{1}H NMR of PHB showed characteristic signals at 1.28 ppm (CH_3), 2.50–2.58 ppm (CH_2) and 5.25 ppm (CH), while PHBV gave characteristic signals for CH_3 for HV and HB at 0.90 and 1.28 ppm, respectively. The signals for the proton of CH were observed at 5.15 and 5.25 ppm for HV and HB, respectively. The ^{13}C NMR signals for CH_2 of HB and HV monomers were detected at 26 and 41 ppm, respectively (Montiel-Jarillo et al., 2022).

^{1}H/^{13}C heteronuclear single quantum coherence (HSQC) uses NMR to provide two-dimensional (2D) correlations between ^{13}C and ^{1}H, where only one peak is obtained for the pair of coupled atoms. Using this method to study PHA from a mixed microbial culture from a sequencing bath reactor, Montiel-Jarillo et al. (2022) validated the results of the individual NMR (^{1}H and ^{13}C) which showed that the PHA was comprised of a PHBV co-polymer containing 41–42 % HV and 58–59 % HB (Montiel-Jarillo et al., 2022). Bossu et al. (2020) also reported on the composition of PHBV from mixed microbial cultures, this time using a combination of one-dimensional ^{1}H and ^{13}C and 2D dimensional diffusion-ordered spectroscopy (DOSY) and HSQC to determine the exact composition of the co-polymer and the compositional distribution of HB and HV monomers.

6. Molecular biology methodologies

6.1. Introduction

Depending on the type of substrates available and the enzymes expressed by different bacterial strains, there are hundreds of intracellular metabolic reactions that can take place related to the synthesis of PHA molecules (Attapong et al., 2023; Steinbüchel and Lütke-Eversloh, 2003; Baltacı et al., 2024). Methods based on detection and/or quantification of genes or ribonucleic acid (RNA) transcripts responsible for encoding for enzymes involved in PHA synthesis have been used to screen for putative PHA producing bacteria and to elucidate PHA metabolic pathways (Attapong et al., 2023; Catone et al., 2014).

6.2. Elucidation of metabolic pathways and genetic engineering

Three main metabolic pathways for PHA synthesis exist, all ultimately relying on PHA synthases (phaC) to polymerize the alkanoate monomers (Fig. 7). The first major metabolic pathway (Pathway I, Fig. 7) involves the conversion to acetyl-coenzyme A (CoA) derived from carbohydrates through intermediates to 3HB-CoA catalyzed by phaA (β-ketothiolase) and phaB (acetoacetyl-CoA reductase) (Guimarães et al., 2022; Alves et al., 2023; Gao et al., 2022). The second (Pathway II, Fig. 7) involves β-oxidation of fatty acids, while the third (Pathway III, Fig. 7) involves *de-novo* synthesis of fatty acids, with the penultimate formation of 3-hydroxyacyl-CoA monomers. To date, four classes of pha synthases have been defined (Neoh et al., 2022; Yanagawa et al., 2023), but it is possible that others exist (Mabasa et al., 2024). Class I phaC are involved in the classical pathway for PHB synthesis (Pathway I, Fig. 7), while Class II phaC are associated with synthesis of MCL polymers (Neoh et al., 2022; Chek et al., 2019). Class III and IV pha synthases each require another enzyme subunit (phaE and phaR, respectively) in addition to the catalytic (phaC) unit to be functional (Neoh et al., 2022).

Real-time polymerase chain reaction (RT-PCR) using bacterial ribonucleic acid (RNA) can be used to elucidate PHA metabolism in individual or multiple bacterial strains or within different environments. For example, Huang et al. (2018b) extracted RNA from mixed microbial communities in a sequential batch reactor operated in a feast-famine mode to enhance PHA production. Using primers encoding for class I and II phaC, they found that although the bacterial community structure was highly dynamic, the level of *phaC* expression was relatively stable *viz*. functional and compositional stability were independent from one another (Huang et al., 2018b). In a study examining a single bacterial strain, Baltaci et al. (2024) found the highest expression of fabD (malonyl-ACP transacylase), phaG (acyl-ACP-CoA transacylase) and phaC in *Pseudomonas neustonica* NGB15 grown with waste banana peel powder (Baltacı et al., 2024). As fabD catalyzes the conversion of malonyl-ACP to malonyl co-A and phaG catalyzes the conversion of 3-hydroxyacyl-ACP to 3-hydroxyacyl-coA (Fig. 7), the authors were able to deduce that the test strain utilized the *de-novo* fatty acid synthesis pathway for PHA production (Baltacı et al., 2024). In a similar study, Rueda et al. (2022) found a positive correlation between the expression of genes codifying for glycogen phosphorylase, acetyl-CoA reductase and phaC in a strain of *Synechocystis* sp., demonstrating that PHB synthesis and glycogen catabolism were strongly related (Rueda et al., 2022).

Metabolic studies are also required to genetically engineer strains to increase PHA production. For example, Song et al. (2023) showed that MCL-PHA production by *Pseudomonas* sp. SG4502 from sodium octanoate increased by 54 % when the PHA-depolymerase *phaZ* gene from the Class II PHA synthase gene cluster was knocked out (Song et al., 2023). Similar results were obtained by Zhao et al. (2019) with *Pseudomonas mendocina* NK-01 and *phaZ* gene knock out. In this study, increased *phaC* gene expression and improvement in PHA production was also achieved by inserting promotors upstream of the *phaC* operon (Zhao et al., 2019).

6.3. Screening for polyhydroxyalkanoate producing bacterial strains

Screening methods for putative PHA producers have been based on PCR detection of *phaC* amplicons in DNA extracted from cultures or environmental samples. Mahansaria et al. (2015) were able to amplify the conserved region of the Class III *phaC* gene of 29 halophiles that produced PHA but did not detect the amplicon in non-PHA producers that appeared positive using conventional NR staining. Tan et al. (2019) detected Class I *phaC* amplicons in 12 of 23 isolates from marine environments that were positive on NR plate assays. They deduced that the remaining 11 isolates were false NR positives, but did not confirm this biochemically (Tan et al., 2019). Chou et al. (2022) used GC analysis of bacterial extracts for PHA quantification to confirm screening results and found positivity rates of 39 % for NR plate screening of 33 strains, and 40 % for detection of *phaC* amplicons from 10 of the NR positive strains (Chou et al., 2022). Possible reasons for the 'false' positives

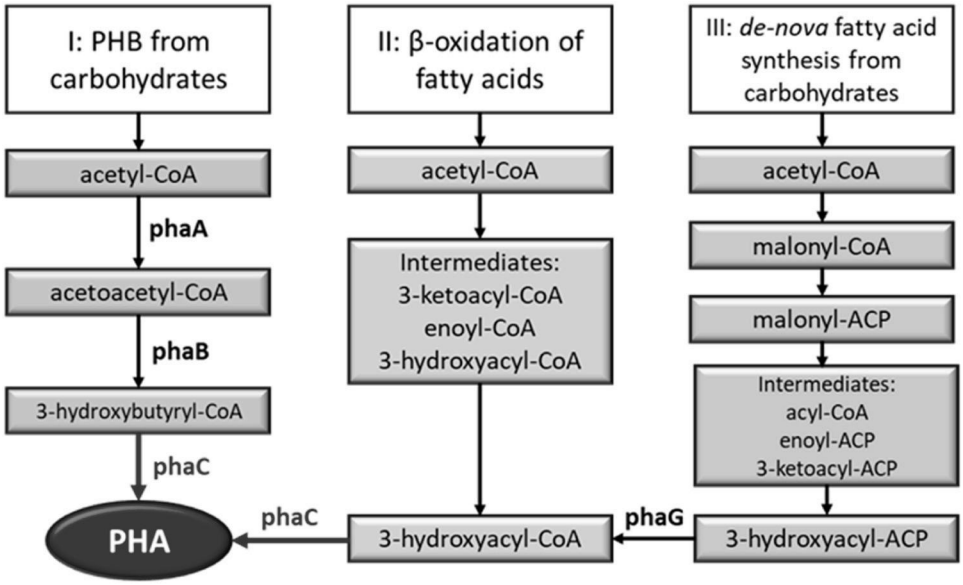

Fig. 7. Simplified schematic of the three major metabolic pathways for polyhydroxyalkanoate synthesis (adapted Baltaci et al., 2024; Steinbüchel & Lütke-Eversloh, 2003; Bossu et al., 2020).

obtained from *phaC* amplicon screening include: (i) although present, the genes were not expressed because the environmental conditions under which the isolates were grown were not conducive for PHA production, and/or (ii) complementary genes required for PHA production were not present or non-functional and/or, (iii) there was insufficient PHA production for detection using the extraction and detection methods employed. In a later study, the authors used NR plate screening followed by *phaC* amplicon detection as a strategy to screen for putative PHA producers from activated sludge samples. Of the 136 NR positive colonies tested, 61 (45 %) harbored the test *phaC* amplicon. Similar results were found by Chou et al. (2023) who used GC and NMR to detect PHA. They found that 50 % of the strains in samples from industrial wastewater were able to produce PHA when grown aerobically with glucose as a sole source of C under N-limited conditions (Chou et al., 2023).

6.4. Gene expression for quantification of polyhydroxyalkanoates

A few studies have been conducted to ascertain whether *phaC* gene expression correlates with PHA production, i.e., as an indirect means of quantifying PHA. Paganelli et al. (2011) designed primers for the *phbC* genes of *Sinorhizobirum* and *Rhizobium.* They found that the level of gene expression was compatible with the biochemical data and concluded that RT-PCR was reliable for quantification of PHB. Of interest is that expression of *phbC* was low during the lag phase of bacterial growth, increasing during the log phase, while PHB production itself only increased well into the log phase *viz.* a delay between gene expression and PHB production (Paganelli et al., 2011). In another study, Wang and Nomura (2010) grew *Pseudomonas putida* KT2440 using different C sources and quantified expression of genes involved in the Entner–Doudoroff pathway, glycerol metabolism, the tricarbocylic acid (TCA) cycle, β-oxidation and PHA synthesis. They found that all of the RNA transcripts of the genes involved in PHA metabolism (*phaC1, phaC2, phaZ, phaG, phaJ, phaJ3, phaJ4*) and β-oxidation were present in the highest amounts when the bacterium was grown with laureate, and that this correlated with the highest PHA production (18 % w/w) compared with when grown with other substrates (glucose, glycerol, citrate all <2 % (w/w) (Wang and Nomura, 2010).

7. Conclusion

Plastic pollution is escalating and significant negative effects of conventional micro- meso- and macro-plastics on biota, including humans, are being uncovered. This has led to a concurrent growth in the amount of research to find alternatives to harmful plastics. Polyhydroxyalkanaotes have been purported as safe and biodegradable alternatives. Analytical and screening methods are required to assess the quality and quantity of PHA polymers. This manuscript provides a 'one-stop' avenue for researchers to select the type/s of methods relevant to their research.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or non-profit sectors.

CRediT authorship contribution statement

Amrita Ranjan: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Data curation, Conceptualization. **Thandekile Mthethwa:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Welz Pamela:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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