Advances in polyhydroxyalkanoate (PHA) production by halophilic microorganisms: phylogeny, biosynthesis, and biotechnology for sustainable applications: A review

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Abstract Polyhydroxyalkanoates (PHAs) are biodegradable polymers produced by various microorganisms as intracellular storage compounds. Among other PHA-producing microorganisms, halophiles are considered a promising PHA producer due to their unique metabolic capabilities and adaptability to extreme conditions. Halophiles are also a promising approach to sustainable and cost-effective PHA production, which uses low-cost substrates and simplified bioprocessing conditions. This review explores the phylogenetic diversity of halophiles, the biotechnological advancement in their cultivation, the factors that influence PHA production, and the methods used to detect, extract, and characterize PHAs from halophiles, highlighting opportunities in optimizing PHA production. By studying these aspects, this review focuses on providing a comprehensive understanding of the potential of halophiles in bio-plastic production and their role in reducing plastic waste pollution.

Keywords: Biodegradable polymers, low-cost substrates, PHA extraction methods, Polyhydroxyalkanoates,

Introduction

The global accumulation of synthetic plastics and the carbon emissions during production have posed an environmental crisis. Replacement of petroleum-based plastic with biodegradable polymers is a potential solution for this crisis. Polyhydroxyalkanoates (PHAs) are a promising substitute for synthetic plastics (Meereboer et al., 2020). PHAs are synthesized by various microorganisms under limited nutrient conditions and serve as intracellular carbon and energy reserves (Anderson & Dawes, 1990).

Halophiles can survive in high-salinity environments and are a promising source for PHA production. Due to the ability of halophiles to withstand harsh conditions, halophiles can produce PHA by utilizing low-cost substrates (Mandragutti et al., 2024). Halophiles produce different types of PHAs with varying properties due to their diverse metabolic capabilities (Quillaguamán et al., 2010). Due to the development of biotechnology, halophiles have new opportunities for sustainable PHA production. Furthermore, halophiles have lower energy requirements than non-halophiles during PHA production (Mitra et al., 2020).

This review provides a comprehensive overview of the phylogenetic diversity, biosynthesis pathways, and production strategies of PHA-producing halophiles, highlighting their potential for use in industrial-scale PHA production.

Halophiles

The Greek word from which the word "halophile" is derived means "salt-loving." Halophiles are extremophiles that live in high salt concentrations. Halophiles grow in each of the three life domains: Archaea, Bacteria, and Eukarya (Rathakrishnan & Gopalan, 2022). Halophiles are found in extreme environments such as saline and hypersaline habitats, including ponds, lakes, seas, and saltpans (Vigneshwari et al., 2021). In addition to high salinity conditions, halophilic microorganisms are also exposed to low oxygen concentrations, high or low temperatures, alkaline conditions, low nutrient availability, solar radiation, and pH changes (Manikandan & Senthilkumar, 2017). Halophiles require at least 0.2M NaCl for growth. Depending on their requirement for NaCl, halophiles are classified as slight halophiles, moderate halophiles, and extreme halophiles. Slight halophiles can grow at 0.2-0.85M NaCl (1-5%) concentrations,

moderate halophiles can grow at 0.85-3.4M NaCl (5-20%) concentrations, and extreme halophiles can grow at 3.4-5.1M NaCl (20-30%) concentrations. Halotolerant microorganisms can grow in either a wide range of salinity or in the absence of high concentrations of salt (Purdy et al., 2004; DasSarma & DasSarma, 2015; Mukhtar et al., 2020).

Physiological Adaptation of Halophiles

Halophiles, living in saline environments, face ionic stress and must maintain osmotic balance with their surroundings. Due to membrane permeability to water, they keep their cytoplasm isosmotic with external conditions (Zahran, 1997; Oren, 2002). One strategy for osmoadaptation is the salt-in strategy, where K^-/Cl^- ions accumulate in the cytoplasm to balance osmotic pressure. This is common in Halobacteriales (Archaea), Halanaerobiales, and *Salinibacteria ruber* (Pastor et al., 2013; Vaidya et al., 2018). However, the salt-out strategy, more prevalent in halophiles, involves excluding salts and accumulating compatible solutes like sugars, polyols, amino acids, quaternary amines, and ectoine (Pastor et al., 2013; Weinisch et al., 2018). Halophilic enzymes, adapted to function in high salt concentrations, often have a higher proportion of acidic amino acid residues (Madern et al., 2000.).

Biotechnological Applications of Halophilic Microorganisms

Halophilic microorganisms have numerous biotechnological applications. They produce a wide range of compounds such as pigments, hydrolytic enzymes, bioplastics (PHAs), biofuels, bacteriorhodopsin, biocompatible osmolytes, and bioemulsifiers (Martínez et al., 2022; Al-Daghistani et al., 2024).

Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are bioplastics synthesized by microorganisms in granular form within the cytoplasm for energy storage (Poli et al., 2011). In 1926, Lemogine, a French scientist discovered poly(3-hydroxybutyrate) (PHB) in *Bacillus megaterium* (Zinn et al., 2001). To date, over 150 distinct PHA monomers have been identified, with ongoing advancements in genetically modified microorganisms and chemical alterations to produce PHAs with specific functional groups (Tan et al., 2014). PHAs are insoluble in water, encased in a protein layer, and accumulate as carbon storage when organic carbon is abundant or when growth is limited by nutrients like nitrogen, phosphorus, magnesium, or sulfur (Cristea et al., 2018). In addition to their storage role, PHAs provide bacterial cells with secondary benefits, such as increased robustness and survival under environmental stresses like freezing, osmotic shocks, oxidative pressure, desiccation, H_2O_2, heavy metals, and UV exposure. During starvation, PHAs protect cellular components like RNA and proteins. PHAs also play roles in anoxic photosynthesis, the sulfur cycle in microbial mats, Bacilli sporulation, and nitrogen fixation by diazotrophs under dark conditions, supporting energy production and NADH oxidation (Obruca et al., 2020; Sehgal & Gupta, 2020).

Structure and Classification of PHA

3-hydroxy fatty acid monomers are included in PHA. The carboxyl group of one monomer unit and the hydroxyl group of another form an ester bond. Alkyl groups are represented by the R group in the structure. Poly(3-hydroxybutyrate) is the name given to the polymer when the R group is methyl (CH_3) since the monomer unit contains four carbons. Poly (3-hydroxyhexanoate) is the name given to the polymer when the R group is C_3H_7. (Fig. 1.) The diverse size and makeup of the side chain substituents in PHA determine the chemical alteration(Sehgal & Gupta, 2020).

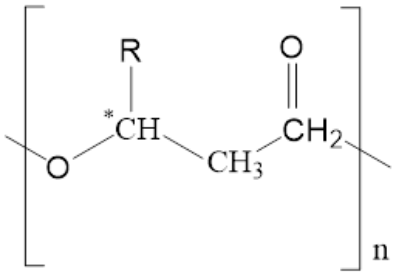

Figure 1. The general structure of PHA, where R can be different groups

PHAs are categorized into three groups based on the length of the carbon chain of the monomeric units. The groups are short chain length (scl) PHAs, medium chain length (mcl) PHAs, and long chain

length (lcl) PHAs. Short-chain-length PHAs include 3 to 5 carbon atoms. P3HB, P3HV, P4HB, and co–polymer of P (3HB–co–3HV) are some short chain length PHAs. *Azohydromonas lata* and *Cupriavidus necator* are some bacteria that synthesize short-chain-length PHAs. Medium chain length PHAs include 6 to 14 carbon atoms. P3HH$_x$, P3HO, and co-polymer of P (3HH$_x$–co–3HO) are some medium chain length PHAs. *Pseudomonas mendocina* and *Pseudomonas putida* are bacteria that produce medium-chain-length PHAs. Long-chain PHAs contain more than 15 carbon atoms. Poly (3-hydroxypentadecanoate) is an example of long-chain length PHAs. *Aureispira marina* and *Shewanella oneidensis* are bacteria that produce long-chain PHAs (Rai et al., 2011; Sehgal & Gupta, 2020; Saratale et al., 2021).

Properties of PHAs

Based on the PHA type, PHAs have different chemical and physical properties. PHAs are insoluble in water but soluble in chloroform and chlorinated solvents. PHAs have resistance to hydrolytic degradation and Ultraviolet radiation. Also, PHAs are biodegradable, biocompatible, and non-toxic. PHAs are sensitive to acids and bases. PHA facilitates anaerobic degradation due to its sinking ability in water (Bugnicourt et al., 2014). PHAs have chiral molecules, and the degradation of PHA depends on the composition and type of PHA, environmental conditions, and the kind of microorganisms (Boyandin et al., 2013; Masood et al., 2014). PHAs with various monomers have distinct melting points, glass transition temperatures, degrees of crystallinity, and hydrophobicity. Usually melting points vary from 40 °C to 180 °C and glass transition temperatures range from -50 °C to 4 °C (Padermshoke et al., 2005; Sehgal & Gupta, 2020).

Biosynthesis of PHA

PHA biosynthetic pathways are linked to bacterial core metabolism, including glycolysis, the Krebs cycle, amino acid catabolism, de novo fatty acid synthesis, β-oxidation, and the serine pathway (Tan et al., 2014). PHA synthase, encoded by phaC, is the key enzyme in PHA production (Sharma et al., 2017). The structural genes for PHA synthesis, *phaC* (PHA synthase), *phaA* (β-ketothiolase), and *phaB* (NADPH-acetoacetyl-CoA reductase) are organized in a single operon (Lee., 1995). The first step in scl-PHA biosynthesis is the condensation of two acetyl-CoA molecules by β-ketothiolase to form acetoacetyl-CoA. NADPH-dependent acetoacetyl-CoA reductase then reduces acetoacetyl-CoA to 3-hydroxybutyryl-CoA, which is polymerized into PHB by PHA synthase (Urtuvia et al., 2018). In mcl-PHA production, β-oxidation pathway intermediates convert into hydroxyalkanoate monomers with enzymes such as enoyl-CoA (*phaJ*), acyl-CoA oxidase, and 3-ketoacyl-CoA reductase. PHA synthase catalyzes monomer polymerization (Fukui et al., 1998; Sharma et al., 2021). If β-oxidation is inactive, acetyl-CoA from carbon source oxidation is redirected to PHA biosynthesis, with transacylase (*phaG*) facilitating intermediate transfer from fatty acid de novo synthesis (Hoffmann et al., 2002).

PHA Production by Halophilic Bacteria

Most PHA-producing halophilic and halotolerant bacteria belong to the family *Halomonadaceae*, with PHA accumulation serving as a phenotypic marker for species differentiation (Mata et al., 2002; Mitra et al., 2020). The genus *Halomonas* has the highest reported PHA accumulation (Table 1). *H. nitroreducens*, an extreme halophile, accumulated 33% PHB using glucose with seawater (25% v/v) under nitrogen limitation (Cervantes-Uc et al., 2014). *H. venusta* KT832796 achieved 88% PHA content using a high-concentration single pulse method (Stanley et al., 2018). *H. daqingensis* produced 68.96% PHA in 3% algal biodiesel waste residue with 5% NaCl (Dubey & Mishra, 2021). *H. halophila* yielded 4.75 g/L PHA using softwood hydrolysate at 66 g/L NaCl, preferring hexose-rich media over pentose-rich media (Kourilova et al., 2021).

H. organivoras produced 90.55% PHB with galactose and 83.41% with fructose (Pernicova et al., 2019). The moderately halophilic *H. alkalicola* produced 45.57% PHBV using galactose and 3% NaCl at pH 10 (Muigano et al., 2024). Under controlled pH and oxygen, *H. boliviensis* produced 88% PHB with sodium acetate and butyric acid and 55% with glucose or sucrose at 4.5% (w/v) NaCl (Quillaguamán et al., 2006). *H. pacifica* produced 71.9% PHB-co-HV using sucrose (El-malek et al., 2020).

Table 1. PHA production by halophilic bacteria using different substrates

Organism Name	Carbon Source	Type of PHA	PHA %	References
Halomonas nitroreducens	Glucose	PHB	33	(Cervantes-Uc et al., 2014)
Halomonas venusta KT832796	Glucose	PHB co HV	88.12	(Stanley et al., 2018)
Halomonas daqingensis	3% Algal biodiesel waste residue	PHB	68.96	(Dubey & Mishra, 2021)
Halomonas halophila	Soft Wood	PHB	73.48	(Kourilova et al., 2021)
Halomonas organivoras	Galactose	PHB	90.55	(Pernicova, Kucera, Novackova, et al., 2019)
Halomonas alkalicola	Galactose	PHBV	45.57	(Muigano et al., 2024)
Halomonas alkalicola	Galactose	PHBV	45.57	(Muigano et al., 2024)
Halomonas boliviensis	Sodium acetate and butyric acid	PHB	88	(Quillaguamán et al., 2006)
Halomonas pacifica	Sucrose	PHB co HV	71.9	(El-malek et al., 2020)
Halomonas sp. (TD01)	Glucose	PHBV	69	(Tan et al., 2011)
Halomonas campisalis	Maltose	PHB co PHV	45-81	(Kulkarni et al., 2010a)
Halomonas hydrothermalis	Jatropha biodiesel byproduct	PHB	75	(Shrivastav et al., 2010)
Halomonas elongata	Glucose	PHB	40	(Cristea et al., 2018b)
Rhodobacter sphaeroides (U7)	Glutamate acetate and valeric acid	PHBV	65.15	(Kemavongse et al., 2008)
Bacillus sonerensis	Jatropha biodiesel byproduct	PHB	71.8	(Shrivastav et al., 2010)
Bacillus megaterium (H16)	Glucose	PHB	40	(Salgaonkar, Mani, & Braganca, 2013a)
Yangia sp. (ND199)	Fructose	PHB co HV	85	(Romero Soto et al., 2021)
Vibrio proteolyticus	Fructose	PHB co HV	54.7	(J. W. Hong et al., 2019)
Salinivibrio sp. (TGB10)	Glucose and propionate	PHB & PHBV	80	
Paracoccus sp. (LL1)	Corn stover hydrolysate sugar	PHB	72.4	(Sawant et al., 2015)

According to Tan et al. (2011), tested *Halomonas* sp. produced 65–70% PHBV via a two-stage, unsterile, continuous fermentation process using glucose. Moderately halophilic *H. campisalis* accumulated 45%-81% PHB-co-PHV in maltose-based media (Kulkarni et al., 2010). *H.*

hydrothermalis and *B. sonorensis* accumulated 75% and 71.8% PHB, respectively, using Jatropha biodiesel byproduct (Shrivastav et al., 2010). The extreme halophile *H. elongata* accumulated 40% PHB using glucose and 10% (w/v) NaCl (Cristea et al., 2018). In a study carried out by Kemavongse et al. (2008), *Rhodobacter sphaeroides* produced 65.15% PHBV in a glutamate–acetate medium with valeric acid.

The moderately halophilic *B. megaterium* H16 accumulated 40% PHB with glucose in the absence of NaCl and 39% with 5% NaCl, showing extended growth in saline conditions (Salgaonkar et al., 2013). The moderate halophile *Yangia* sp. produced 85% PHB-co-HV using fructose (Romero Soto et al., 2021). The marine halophile *Vibrio proteolyticus* reported 54.7% PHA production with fructose and 5% (w/v) NaCl, with the addition of propionic acid leading to PHBV with 15.8 mol% 3HV (Hong et al., 2019). The moderately halophilic *Salinivibrio* sp. TGB10 accumulated 80% PHA (PHB and PHBV) using glucose and propionic acid as a secondary substrate (Tao et al., 2021). *Paracoccus* sp. LL1 produced 72.4% PHB using corn stover hydrolysate sugar (Sawant et al., 2015).

PHA Production by Archaea

PHA accumulation in archaea was first observed in *Halobacterium marismortui* isolated from the Dead Sea (Kirk & Ginzburg., 1972). Since then, numerous haloarchaea species have shown the ability to synthesize PHAs using a wide range of low-cost substrates under high-salinity conditions. Table 02 provides a comprehensive summary of PHA production across various haloarchaea, including the substrates used, types, and yields of PHAs accumulated. Notably, species such as *Natrinema pallidum*, *Haloferax mediterranei*, and *Halopiger aswanensis* have shown high PHA accumulation, particularly when utilizing substrates like glucose, corn starch, or food waste, highlighting their biotechnological potential for cost-effective PHA production.

Table 02. PHA production by halophilic archaea using different substrates

Organism Name	Used substrate	Type of PHA	PHA %	References
Halogeometricum borinquense	Glucose	PHB	14	(Salgaonkar et al., 2013)
Natrinema pallidum	Corn Starch	PHBV	53.14	(Danis et al., 2015)
Haloferax mediterranei	Municipal food waste	PHBV	62-64	(Wang, Chen, et al., 2022)
Natrinema altunense	Glucose	PHB & PHV	25-35	(Abdallah et al., 2020)
Haloterrigena jeotgali	Glucose	PHB & PHV	25-35	(Abdallah et al., 2020)
Haloarcula hispanica	Silkworm excrement	PHB	14.63	(Cai et al., 2021)
Natrinema altunense	Silkworm excrement	PHB	11.29	(Cai et al., 2021)
Halopiger aswanensis	Sodium acetate and n-butyric acid	PHB	53	(Hezayen et al., n.d., 2010)
Halogranum amylolyticum	Glucose	PHBV	20.1 mol	(Zhao et al., 2015)
Halorubrum chaoviator	Starch	PHB	9.25	(Karray et al., 2021)
Natrinema pallidum	Starch	PHB	7.11	(Karray et al., 2021)
Haloarcula tradensis	Starch	PHB	1.42	(Karray et al., 2021)
Halolamina sediminis	Starch	PHB	33.4	(Hagagy et al., 2022)
Natrialba swarupiae	Glucose	PHB	54.4	(Rodge et al., 2023)

PHA production of halophiles using low-cost substrates

PHA production using halophiles offers cost-effective, contamination-free fermentation compared to non-halophiles. Substrate, fermentation process, and PHA recovery are key cost factors (Mitra et al., 2020). *Haloferax mediterranei* produced PHBV using cheese by-products, with enzymatic hydrolysis aiding lactose conversion. With 87% efficiency and a 0.2 g PHBV/g lactose yield, a simulated system projected 9700 MT annual PHBV production, highlighting the potential of dairy-derived feedstocks (Wang, et al., 2022). Another study showed *H. mediterranei* accumulated PHBV using food waste-derived carboxylates, yielding 55% more than glucose, reducing production costs and aiding waste management (Wang & Zhang, 2021). Vinasse, an alcoholic fermentation by-product, supported PHBV accumulation up to 70% in *H. mediterranei* (Bhattacharyya et al., 2012), while rice-based ethanol stillage led to 71% (wt) PHBV production (Bhattacharyya et al., 2014). *Halomonas hydrothermalis* produced up to 62% PHB in 40 g/L NaCl using waste frying oil and inexpensive lipids (Pernicova et al., 2019). *Halomonas halophila* accumulated PHB from carbohydrates, including lignocelluloses, with yields of 38.32%, 64.06%, 61.95%, and 46.85% using cheese whey hydrolysate, molasses, spent coffee grounds, and sawdust hydrolysates, respectively (Kucera et al., 2018). *Halomonas campaniensis* strain LS21, a recombinant halophile, produced ~70% PHB over 65 days in artificial seawater containing kitchen waste and 27 g/L NaCl (Yue et al., 2014). Microalgae biomass serves as an inexpensive substrate for large-scale PHA production. *Haloferax mediterranei* and *Bacillus megaterium* accumulated 55.5% and 29.7% PHA, respectively, using acid-treated defatted *Chlorella* biomass, with *B. megaterium* producing PHB and *H. mediterranei* producing PHBV with a 10.5% 3-hydroxyvalerate fraction (Khomlaem et al., 2021). *Haloarcula marismortui* accumulated up to 30% PHB using pre-treated vinasse (Pramanik et al., 2012). The halophilic archaeon *Halogeometricum borinquense* strain E3 utilized sugarcane bagasse, an agro-industrial waste, to accumulate 45.7% (wt) PHBV (Salgaonkar & Bragança, 2017).

Factors affecting PHA yield

Several factors influence PHA production, including the carbon-nitrogen (C/N) ratio, nutrients, salinity, pH, temperature, fermentation mode, microorganism type, and fermenter characteristics (Yadav et al., 2020). Halophiles utilize various carbon sources, including sugars, fatty acids, and organic acids, for PHA accumulation.

The C/N ratio significantly affects PHA accumulation. Early studies on *Haloferax mediterranei* found that nitrogen limitation with excess carbon led to high PHA accumulation (Fernandez-Castillo et al., 1986). Recent research confirms higher PHA accumulation under nitrogen-deficient conditions, while nitrogen availability favors extracellular polymeric substance (EPS) production. At C/N ratios of 5 and 35, total EPS and PHA yield coefficients were 51.32% and 92.8%, respectively, indicating that nitrogen limitation promotes PHA production (Cui, Shi, et al., 2017). Additionally, phosphorus limitation under excess carbon enhances PHA accumulation (Oliveira-Filho et al., 2020). *Haloferax mediterranei* accumulates poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) without specific precursors, but its composition varies with nitrogen sources. PHBV from ammonium salts contained 16.9 mol% 3-hydroxyvalerate (HV), while nitrate-derived PHBV had 12.5 mol% HV, showing that PHBV composition can be tailored by adjusting the C/N ratio and nitrogen supply (Ferre-Guell & Winterburn, 2019).

Salt concentration impacts PHB accumulation. *Haloferax mediterranei* showed the highest PHB accumulation at 15% salt concentration (Fernandez-Castillo et al., 1986). *Bacillus megaterium* accumulated 40% PHB in the early stationary phase without NaCl, but with 5% NaCl, peak PHB accumulation (39% CDW) occurred at 60 hours (Salgaonkar et al.,2013). Lignocellulose hydrolysis generates high salt content, making halophilic strains like *Halomonas halophila* suitable for PHA production using lignocellulose substrates (Kourilova et al., 2021). Salinity also influences PHA and EPS accumulation in *Haloferax mediterranei*. Increasing NaCl from 7.5% to 25% raised PHA content from 46.7% to 71.1% CDW while reducing EPS from 371.36 to 319.74 mg/g CDW, suggesting high salinity

enhances PHA production but suppresses EPS (Cui, et al., 2017).

Most halophiles optimize PHA production at neutral pH. *Halomonas venusta* (KT832796) exhibited maximum PHA accumulation at pH 7.0, with reduced growth and PHA accumulation at higher pH (Stanley et al., 2018). Similarly, *Halomonas daqingensis* showed 0.191 ± 0.002 g PHA productivity at pH 7.0, which declined at higher pH (Dubey & Mishra, 2021). However, some halophiles accumulate PHA at alkaline pH. *Halomonas alkalicola* accumulated 0.963 g/L PHAs at pH 9.0 (Muigano et al., 2024), and *H. campisalis* and *H. profundus* also produce PHA at pH 9.0 (Simon-Colin et al., 2008; Kulkarni et al., 2010).

Most halophiles are mesophiles, accumulating PHA between 30 °C and 37 °C. Agitation influences PHA production, with low agitation causing cell aggregation and over-agitation reducing biomass and PHA due to imbalanced oxygen and substrate levels (Chavan et al., 2021)

Batch fermentation limits PHA production due to substrate depletion, while fed-batch fermentation improves yield and productivity by controlled substrate addition (Mutturi et al., 2017). *H. venusta* (KT832796) showed 8.65-fold higher PHA productivity under fed-batch fermentation, reaching 88% PHA content (Stanley et al., 2018). *Haloferax mediterranei* accumulated 50.8% PHB (w/w) using a feeding medium with extruded cornstarch and yeast extract at a 1:1.7 ratio (Chen et al., 2006). A novel strategy for PHBV production from volatile fatty acids (VFA) using *Haloferax mediterranei* included Tween 80 as a surfactant, resulting in 59% dry weight PHBV with a productivity of 10.2 mg L^{-1} h^{-1} (Ferre-Guell & Winterburn, 2019). *Halomonas boliviensis* LC1 accumulated 54% PHB in shake flasks with butyric acid and sodium acetate but increased to 88% under controlled pH and oxygen in a fermenter (Quillaguamán et al., 2006).

Methods and techniques for detection of PHA-producing halophiles

Sudan Black staining

Sudan Black B is a lipophilic dye with a strong affinity for lipid-rich inclusions, making it a widely used stain for detecting polyhydroxyalkanoate (PHA) granules in bacterial cells. Since PHA is a class of intracellular storage polymers composed of hydroxyalkanoic acid monomers, its hydrophobic nature allows selective binding of Sudan Black B. Upon staining, PHA granules appear as dark inclusions within the bacterial cytoplasm, while the rest of the cell remains largely unstained, facilitating microscopic identification of PHA-producing bacteria (Wei et al., 2011; Porras et al., 2018).

The staining procedure involves preparing bacterial cell smears on a glass slide, followed by heat fixation. A 3% (w/v) Sudan Black B solution in 70% ethanol is applied to the smear for ten minutes, allowing the dye to permeate the cell and bind to PHA granules. To remove excess dye, the slide is submerged in xylene until complete decolorization is achieved. A counterstain, safranin (5% w/v), is applied for 10 seconds to enhance contrast by staining the cytoplasm, providing a clear distinction between PHA granules and non-lipid cellular components. After air-drying and adding immersion oil, the slide is examined under phase contrast microscopy, where PHA granules appear as distinct dark inclusions within the bacterial cells (Burdon, 1946).

Sudan Black B plate assay

The Sudan Black B plate assay is a qualitative method used for the rapid screening of polyhydroxyalkanoate (PHA)-producing bacteria. This assay exploits the lipophilic nature of Sudan Black B, which has a high affinity for lipid-associated biopolymers, including PHA granules. When applied to bacterial colonies, the dye selectively binds to intracellular PHA, allowing visual differentiation between PHA producers and non-producers. In this method, the colonies on the media plate are covered with an ethanolic solution of (0.05% w/v) Sudan Black B, and the plates are left alone for 30 minutes. To remove excess stain colonies are washed by ethanol (96%). The colonies with the dark blue color are taken as positive for PHA production (Aruna, 2017).

Nile Red staining

Nile Red is a lipophilic fluorescent dye widely used for the detection and quantification of intracellular polyhydroxyalkanoate (PHA) granules in bacteria. Its strong affinity for lipid-based compounds allows

selective staining of PHA within bacterial cells. Upon excitation under ultraviolet (UV) light, Nile Red-bound PHA granules emit a bright fluorescent signal, making this technique highly effective for distinguishing PHA-producing bacteria from non-producers. This staining approach can be utilized both qualitatively, by visualizing fluorescent granules under a fluorescence microscope, and quantitatively, by measuring fluorescence intensity to estimate intracellular PHA accumulation (Spiekermann, 1999; Cánovas et al., 2021)

For qualitative analysis, bacterial cultures are grown in PHA-inducing media supplemented with a 0.25 mg/mL Nile Red solution in DMSO and incubated for 48 hours. Cells are then examined under UV light at 312 nm to observe PHA accumulation (Yoo et al., 2024). Additionally, cell smears are prepared from incubated cultures, washed multiple times with sterile distilled water, and air-dried before staining with a 0.01% Nile Red solution in DMSO for 15–20 minutes. After removing excess stain and washing the smears, fluorescence microscopy using a propidium iodide (PI) filter at 10× magnification is used for visualization. To maintain staining efficiency and prevent photobleaching, all procedures should be conducted in the dark (Salgaonkar et al., 2013a,b).

Nile Blue A staining

Nile Blue A is a lipophilic oxazine dye with a strong affinity for hydrophobic compounds such as polyhydroxyalkanoates (PHAs), making it a valuable tool for detecting intracellular PHA granules in bacteria. When bound to PHA, Nile Blue A fluoresces bright orange under ultraviolet (UV) light, allowing for rapid and efficient visualization of PHA-producing bacteria using fluorescence microscopy (Doi, 1994; Cánovas et al., 2021).

The staining procedure involves preparing bacterial smears on glass slides, heat-fixing them, and staining with a 1% (w/v) Nile Blue A solution at 55 °C for 10 minutes. After staining, the slides are rinsed with tap water and then treated with 8% acetic acid for one minute to remove excess dye and enhance specificity. The slides are subsequently washed, air-dried, and examined under a fluorescence microscope at an excitation wavelength of approximately 460 nm. The bright orange fluorescence emitted by Nile Blue A-PHA complexes facilitates the identification of PHA-containing cells, distinguishing them from non-producers (Ostle & Holt, 1982).

Transmission electron microscopy (TEM)

TEM is used as a simple method to visualize PHA granules. Transmission Electron Microscopy (TEM) is a powerful tool for examining the ultrastructure of bacterial cells, including the intracellular accumulation of polyhydroxyalkanoate (PHA) granules. The technique relies on the interaction of a high-voltage electron beam with an ultra-thin biological specimen, where different cellular components absorb or scatter electrons to varying degrees. PHA granules, being lipid-rich, exhibit lower electron density than other cellular structures, appearing as bright, round, or irregular inclusions within the cytoplasm. Contrast in TEM imaging is enhanced using heavy metal-based stains, such as osmium tetroxide, which selectively binds to lipophilic regions, improving the visualization of PHA granules against the cellular background. The transmitted electrons generate an image that is magnified by electromagnetic lenses and captured on a fluorescent screen or a digital detector (Beeby et al., 2012; Jendrossek & Pfeiffer, 2014).

In a typical TEM preparation, PHA-producing bacterial cells are harvested by centrifugation at 10,000 rpm at room temperature. The resulting pellets are washed with phosphate-buffered saline (PBS, pH 7.0) and fixed using glutaraldehyde to preserve cellular morphology. Samples are subsequently stained with osmium tetroxide and uranyl acetate to enhance contrast (Berlanga et al., 2006). Alternatively, cells may be fixed in 4% (v/v) glutaraldehyde in 0.1 mol/L sodium cacodylate (pH 7.1) with 0.1% (w/v) Brij 35 (Polyoxyethylene lauryl ether) followed by post-fixation in 2% osmium tetroxide and staining with 2% uranyl acetate in 10% ethanol. The dehydration process is performed using a graded ethanol series, followed by embedding in epoxy or araldite resin. Ultra-thin sections (~50 nm) are cut using a diamond knife, placed on Formvar-coated copper grids, and contrasted with an aqueous uranyl acetate solution before TEM analysis (Van-Thuoc et al., 2012).

Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) is a powerful imaging technique that utilizes a focused electron beam to scan the surface of a specimen, resulting in the emission of secondary electrons which are detected to form high-resolution images.

In typical SEM preparation, PHA-producing bacterial cells are harvested and washed with phosphate-buffered saline. Then harvested cells are fixed in a 3% glutaraldehyde solution overnight. Excess glutaraldehyde in fixed cells is removed by washing with PBS and dehydrated in a series of ethanol (30%, 50%, 70%, 80%, and 100%). The cell suspension is coated with gold and then observed using a Scanning Microscope (Pillai et al., 2017).

However, SEM may not capture internal structures such as PHA granules effectively. To overcome this, methods like freeze-fracturing are employed, where cells are rapidly frozen and fractured, exposing internal components like PHA granules. This allows for their visualization of the fractured surface under cryo-SEM (Hrubanova et al., 2023).

Polymerase Chain Reaction (PCR)

PCR is a molecular biological technique used to amplify specific DNA sequences, enabling the detection and identification of microorganisms. In the context of PHA-producing bacteria, PCR can be employed to detect the presence of genes involved in PHA biosynthesis. PHA biosynthesis in bacteria is primarily controlled by genes encoding PHA synthase enzymes, such as *phaC*, which is responsible for polymerizing hydroxyalkanoates into PHA granules. Specific primers are designed to target the *phaC* gene or other PHA-related genes in the bacterial genome, allowing the detection of PHA-producing bacteria through amplification (Dalal et al., 2010).

PHA Extraction methods

PHA extraction is considered the major cost-determining factor of PHA production. Chemical, mechanical, enzymatic, and solvent-based methods are developed to extract PHA from cells. PHA recovery methods should be feasible for the industry. Also, these methods must be sustainable by avoiding the use of excessive hazardous chemicals. Recently, new techniques have been used to release PHA granules under pressure using "green solvents," such as ionic liquids, supercritical solvents, hypotonic cell disintegration, and switchable anionic surfactants (Koller, 2020). Some methods are discussed here.

Sodium hypochlorite extraction

Sodium hypochlorite extraction is widely used for PHA extraction with different modifications. Cells are harvested by centrifugation, and the pellet is washed by cold water and cold hexane. After the drying step to get a constant weight dried pellet is treated with 30% sodium hypochlorite and incubated at 30 ˚C for 1 hour. The pellet obtained after centrifugation is washed with water, cold acetone, and ethanol. Extracted PHA was dissolved in chloroform and boiled at 100 °C for 20 minutes (Darshan & Nishith, 2014). In one of the modified methods, the cell pellet is suspended in a 0.01M Tri-HCl at 7.0 pH, and then samples are freeze-dried. Freeze-dried samples are digested with a solution of hypochlorite and chloroform (1:1) at 200 rpm for 1 hour. The organic phase is taken after the centrifugation, and PHA was precipitated with cold methanol and dried at 20˚C (Blandón et al., 2020).

Acetone extraction of PHA

Medium-chain-length poly-3-hydroxyalkanoates (mcl-PHA) were extracted from *Pseudomonas putida* using acetone extraction. The biomass was pre-treated using methanol, and then acetone was added. The mixture was incubated for 24 hours at 170 rpm at 22±1 °C. Solubilized PHA in acetone was filtered and residual biomass was washed with fresh acetone. Both acetone volumes were combined and rotary evaporated at 50 ˚C. The combined samples were added to cold methanol dropwise with vigorous stirring, and the precipitate was air-dried after filtration (Jiang et al., 2006).

Extraction using DMSO

Dimethyl sulfoxide (DMSO) is a non-toxic and aprotic solvent that can dissolve lipophilic molecules such as PHA. DMSO is used to extract PHA from *Cupriavidus necator*. This method does not require halogenated solvents. Cells are resuspended in PBS. Aliquots of this cell

suspension are added to 50 mL of DMSO and incubated at 70 °C with agitation. Aliquots of cell suspension are added regularly until maximal production is achieved. Solubilized PHB can be precipitated by adding cold ethanol for 160 minutes at 4 °C (Vizcaino-Caston et al., 2016).

Osmotic cell lysis method

Osmotic cell lysis is a non-chemical approach used for the extraction of polyhydroxyalkanoates (PHA) from bacterial cells. The principle relies on exposing PHA-producing bacterial cells to a hypotonic environment, typically distilled water, leading to an influx of water due to osmotic pressure differences. This causes the cells to swell and eventually rupture, releasing intracellular PHA granules. The process is advantageous as it minimizes the use of harsh chemicals and reduces environmental impact while maintaining polymer purity.

In a typical procedure, bacterial cells containing PHA are harvested through centrifugation and suspended in distilled water at 5°C for 24 hours. The prolonged exposure to hypotonic conditions facilitates cell lysis. The lysed cells are then centrifuged at 5000 rpm for 30 minutes, and the resulting pellet, primarily consisting of PHA granules, is washed multiple times (5–10) with distilled water to remove cell debris. The purified pellet is dried at 80°C until a constant weight is obtained. To further purify the extracted PHA, the dried sample is dissolved in chloroform, and undissolved impurities are removed via filtration. Finally, the chloroform is evaporated at room temperature, yielding purified PHA (Selvakumar K et al., 2011).

Extraction using ionic liquids

Ionic liquids selectively dissolve non-PHA cellular components while preserving the integrity of PHA granules. Unlike conventional organic solvents, ionic liquids offer advantages such as low volatility, tunable solubility, and reduced environmental toxicity, making them an attractive alternative for sustainable PHA recovery. Ionic liquids function by disrupting bacterial cell membranes through ionic interactions, leading to the release of intracellular PHA granules. Depending on the type of ionic liquid used, the extraction process can be optimized to achieve high PHA purity with minimal degradation (Koller, 2020) 1-ethyl-3-methylimidazolium diethylphosphate ([C2mim]-[$(C_2)_2OPO_3$]) has been used for extracting PHA from *Halomonas hydrothermalis* (Dubey et al., 2018).

Chemical Digestion for PHA Extraction

Digestion is a safer option for extracting PHA from cell biomass compared to solvent extraction, which can be hazardous to the environment when employed on a large scale. When a cell is lysed, the membrane-bound PHA granules are loosened, and the hydrophilic non-PHA cell mass is changed to form a water-soluble substance through chemical or enzymatic activity (Koller, 2020). Chemical Digestion involves the solubilization of non-PHA cell components using chemicals like cyclic carbonates, SDS, and hypochlorite. Hypochlorite is an efficient but not eco-friendly solvent for PHA extraction. Surfactants like Triton X-100 were used to treat PHA-containing biomass while using hypochlorite (Kurian & Das, 2021). Cyclic carbonic esters (ethylene carbonate and 1,2-propylene carbonate) are better alternatives for hypochlorite and can extract PHB moist microbial biomass of *Alcaligenes eutrophus* H16. Reusability is a huge advantage of this solvent (Koller et al., 2013). Also, Sodium Dodecyl sulfate (SDS) can solubilize non-PHA cell components. 0.025%–0.2% of SDS concentration is generally used for PHA extraction. However, beyond the optimum concentration of SDS, solubilization of the PHA is caused (Kurian & Das, 2021). In some studies, alkaline treatment was used with SDS for the purification of PHA (Jiang et al., 2015).

Enzymatic Digestion for PHA Extraction

Enzymatic digestion is used as another alternative option for solvent extraction. However, enzyme digestion of non-PHA cell components is not yet cost-efficient due to extended reaction times and low product purity (Koller, 2020). Proteolytic enzymes such as alcalase, and cell wall degrading enzymes like lysozyme are involved in enzymatic digestion. Heat treatment was given to biological material before the enzyme digestion, and chemicals such as SDS and EDTA were used to complete the digestion (Kathiraser et al., 2007).

Mechanical disruption of cell

Mechanical methods such as high-pressure homogenization (HPH), Ultrasonic cell disruption, bead mills, and the use of air classifiers break the cell wall by mechanical forces and recover the intracellular components like PHA (Kurian & Das, 2021). Intracellular medium chain length PHA produced by *Pseudomonas putida* was extracted at a high rate of extraction using an ultrasound-assisted process. This method uses a mixture of acetone as a solvent and heptane as a marginal non-solvent in the medium for extraction with a sonication frequency of 37 kHz (Ishak et al., 2016).

Supercritical (SC) Fluids

Supercritical (SC) fluids are good solvents that have low viscosity and high densities. CO_2 is widely used as a supercritical fluid due to its low toxicity and reactivity. Using supercritical CO_2, 89% recovery of PHB was reported using *Ralstonia eutropha* as the PHA producer (Hejazi et al., 2003).

Characterization of PHA

UV-Visible Spectroscopy (UV-Vis)

The extracted PHAs were dissolved in chloroform and UV-Vis scanned from 200-800 nm. It showed a prominent peak and absorbance at 240 nm. But in the solvent control, chloroform there was no absorption at 240 nm. This clearly showed the existence of the PHA compound in the isolated sample (Selvakumar K et al., 2011). Crotonic acid assay is another quantification method of PHA. Crotonic acid forms as a result of a reaction between PHA and concentrated H_2SO_4 at 100 $^{\circ}$C. The presence of crotonic acid is confirmed by the presence of a peak at 235 nm against sulfuric blank in UV-Vis spectroscopy (Senthilkumar et al., 2017; Law & Slepecky, 2024).

Fourier Transform-Infrared Spectroscopy (FT-IR)

FT-IR technique provides information on the chemical structure of the PHA and its monomeric units. This method, which requires minimal sample preparation, can detect the functional groups of PHA molecules and differentiate between different types due to its strong carbonyl absorption. In this method, about 1 mg extracted sample of PHB was dissolved in chloroform. After the pellet formed, KBr was added, and spectra were recorded at 4000–400 cm^{-1} range with a resolution of 4 cm^{-1}. Absorbance peaks at 1728 cm and 1741 cm are considered PHA maker bands allocated to carbonyl C=O stretches of the ester groups (Getachew & Woldesenbet, 2016). In pure polymers, intracellular scl-PHA, mcl-PHA, and scl-mcl-PHA have a characteristic ester carbonyl band at 1728 cm^{-1}, 1740 cm^{-1}, and 1732 cm^{-1}, respectively (Hong et al., 1999).

Nuclear Magnetic Resonance Spectroscopy (NMR)

The chemical composition of an intact PHA can be studied by NMR. Also, NMR distinguishes between PHA blends and PHA copolymers based on their structure and functional groups. The combination of ^{1}H-NMR and ^{13}C-NMR allows for a more comprehensive investigation of polymers. NMR is widely used for analyzing saturated and unsaturated PHAs. NMR spectra may identify functional groups like methane, methylene, and -CH=CH-, whereas ^{1}H-NMR and ^{13}C-NMR spectra can reveal microstructures like 3-hydroxypropionate (3HP) and 4-hydroxybutyrate - (4HB) (Tan et al., 2014b).

Raman spectroscopy

Raman spectroscopy provides more information than IR spectroscopy by examining molecular structures and interactions of functional groups (Koller & Rodríguez-Contreras, 2015). A study suggests Raman spectroscopy as a rapid method to monitor PHA production and Raman band intensities at 1734 cm^{-1} to use as a relative value for the PHB content in bacteria (De Gelder et al., 2008).

Gas Chromatography-Mass Spectrometry (GC–MS)

Gas Chromatography is an accurate and highly automated method. Hence, this method is widely used for PHA detection. Also, it can provide precise information about the monomeric composition of polyester with great sensitivity. GC-MS involves depolymerization of PHA into acids, diols, or esters before analysis. In early studies, to induce methanolysis, cells were treated with a solution of chloroform, methanol, and concentrated sulfuric

acid before being detected using gas chromatography (Braunegg et al., 1978). In the present day, modified methods are used. A recent study reports pyrolysis gas chromatography combined with mass spectrometry (Py GC/MS), a rapid analysis method for the determination of PHA (polyhydroxyalkanoates) contents and their monomer composition in microbial cells. Using this method, monomer compositions of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) in *Ralstonia eutropha* were correctly determined based on the peaks of 2-butanoic acid and 2-pentanoic acid, which correspond to 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) in PHBV. According to this study, Py GC/MS will be a reliable and rapid method for screening PHA-producing strains (Khang et al., 2021).

High-Performance Liquid Chromatography (HPLC)

HPLC is an initial assay method, Sulfuric acid is applied to P(3HB)-accumulating cells at 100°C to convert P(3HB) to crotonic acid (trans-2-butenoic acid). The treated samples are then run by HPLC using a UV detector to quantify absorption at 210 nm because of the bonds of unsaturated crotonic acid. This approach is highly P(3HB) concentration measurement is convenient, although it is not relevant to different PHAs due to PHA monomers lasting longer than 3HB not transformed into matching unsaturated fatty acids (Watanabe et al., 2012). However, modified and simple HPLC techniques are currently being developed. One study, alkaline digestion, was done before HPLC. Due to the alkaline digestion, 3-hydroxybutyrate (3-HB) units were converted into crotonic acid and 3-hydroxyvalerate (3-HV) units into 2-pentanoate. After the treatment of sulphuric acid, HPLC was conducted. This method is reported as a cost-efficient, simple, and environmentally friendly method (Duvigneau et al., 2021).

Conclusion

Polyhydroxyalkanoates (PHAs) produced by halophiles offer significant potential as biodegradable and eco-friendly alternatives to petroleum-based plastics. The phylogenetic diversity of halophiles, extending both bacteria and archaea, presents a prime resource for sustainable PHA production. Halophiles exhibit unique metabolic pathways that make them more suited for industrial biotechnology applications. Moreover, halophiles show a promising yield of PHA utilizing low-cost substrates, which could be economically beneficial for large-scale PHA production. Also, the development of efficient detection, extraction, and characterization techniques for PHA production is critical for cost reduction and optimization of PHA yield. Further research should focus on novel organisms from untouched environments for efficient PHA production, improving the scalability of PHA production by presenting halophiles, understanding the regulatory mechanisms to control PHA biosynthesis, and exploring novel substrates for more cost-effective processes. PHA-producing halophiles can pave the way for innovative solutions to the global plastic waste crisis.

Abbreviations

CDW- Cell Dry Weight
C/N - Carbon Nitrogen ratio
EPS - Extracellular polymeric substance
PHB - Polyhydroxybutyrate
PHBV – Poly (3-hydroxybutyrate-co-3-hydrox yvalerate)

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Author Contributions

K.V.D.K.R. Vithana contributed to the planning, gathering of data and information, and writing of the original draft and editing.
I.V.N. Rathnayake contributed to conceptualization, selecting the review topic, planning, funding acquisition, project administration, review, and editing.

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