



Haloarchaea as emerging big players in future polyhydroxyalkanoate bioproduction: Review of trends and perspectives

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ABSTRACT

Haloarchaea comprise the extremely halophilic branch of the phylum Euryarchaeota and they are members of the prokaryotic domain Archaea. They thrive best in extremely saline habitats with salt concentrations of 2–5 mol L⁻¹ NaCl, and thus under conditions of near salt saturation in water. These ancient organisms are among the oldest species on Earth, and are characterized by ether-linked lipids in the cytoplasmic membrane and murein-free cell walls. Haloarchaea are increasingly receiving attention as microbial cell factories for the bioproduction of diverse marketable products, such as bacterioruberin, bacteriorhodopsin, isoprenoids, and polyhydroxyalkanoates (PHAs). These biopolyesters serve as intracellular storage compounds from the secondary metabolism of haloarchaea and many other prokaryotes. Engineered or wild type PHA-producing haloarchaea that utilize inexpensive raw materials for bioproduction, and which only require clean-in-place procedures to run PHA production bioprocesses, are currently undergoing scale-up within the research and development field of Archaea Biotechnology. In addition, PHAs exhibit high potential as both bio-based and biodegradable plastic-like bulk products on the industrial biotechnology market (“White Biotechnology”) for biopolymers. However, PHA production by haloarchaea has not yet reached industrial maturity. The present review discusses the background, previous research, and biological role of PHA biosynthesis in haloarchaea, as well as current trends, and a critical discussion of its potential for broad industrialization.

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Introduction

“White Biotechnology” is a frequently used synonym for industrial biotechnology and it refers to the large-scale manufacture of products for daily human uses, such as fuels, solvents, enzymes, biopesticides, or polymers in a bio-catalyzed and bio-based manner. Importantly, White Biotechnology starts from renewable resources in order to avoid depleting limited fossil feedstock reserves (Mukherjee and Koller, 2022). Among the polymers produced by living cells, those resembling plastics that are currently manufactured from fossil feedstock, including thermoplastics such as poly(ethene) (PE), poly(propene) (PP), poly(ethylene terephthalate) (PET), polystyrene, and poly(vinyl chloride), or rubber-like elastomers such as poly(siloxanes) (“silicones”), are now produced in excessive quantities exceeding 400 million tons per year (Wang et al., 2021). At present, the production of plastics based on fossil feedstock, especially plastics for single use, is leading to the depletion of limited resources, greenhouse gas emissions, climate change, microplastic release, and pollution of aquatic and terrestrial environments, and thus it is clear that sustainable alternatives to non-renewable resources are urgently needed (Chen et al., 2021).

In order to prevent environmental pollution and preserve limited resources, microbial polyhydroxyalkanoates (PHAs) are attractive as possible sustainable replacements for petrochemistry-derived plastics. This versatile group of biological polyoxoesters of hydroxyalkanoic acids is widely produced by members of both prokaryotic domains, i.e., Bacteria and Archaea. Furthermore, depending on the renewable raw material and microbial production strain selected, the properties of PHAs can emulate those of both thermoplastics and elastomers (Meereboer et al., 2020). PHAs can be further fine-tuned by using chemical, physical, and biological techniques in order to improve their properties for diverse potential applications in different sectors of the plastics market (Sharma et al., 2021).

We are currently witnessing a great increase in the number of established and new companies in different global regions that manufacture PHAs on an industrial scale as part of their product portfolio, and some companies are entirely focused on PHAs as their sole products. These PHA products have many applications, including as replacements for single use petro-plastics (packaging materials, drinking straws, dishes, cutlery, primary microplastic particles in cosmetics, etc.), implants, sutures, and wound dressings in the surgical and medical fields, carriers for active pharmaceutical ingredients or compounds of agricultural relevance, and paper coatings (Koller and Mukherjee, 2022). In this context, it should be stressed that PHAs can be processed into marketable bioplastic items by using established techniques for polymer processing (injection molding, melt extrusion, compression molding, film blowing, solvent casting, melt spinning, etc.), and serve as “inks” in emerging manufacturing techniques (Mehrpouya et al., 2021), such as electrospinning (Puppi et al., 2019) or additive manufacturing (three-dimensional (3D) printing) (Kovalcik, 2021; Kovalcik et al., 2021). The different applications of PHAs require different degrees of purity, which can be achieved by applying the appropriate downstream processing (DSP) method for

recovering intracellular products from microbial biomass. However, DSP is an important cost factor and second highest after the cost of the raw material (Koller, 2020).

Despite numerous efforts worldwide to make PHA production economically competitive, it still cannot compete with petro-plastics in terms of the production costs. In addition to the raw materials and DSP costs, high energy requirements hamper cost-efficient PHA production. In particular, energy (electricity, vapor generation, stirring of bioreactors, etc.) is needed for maintaining the sterility of the production equipment and culture medium in order to allow the monoseptic cultivation of batches (Obruča et al., 2022). In this context, a novel trend in industrial biotechnology is focused on overcoming the obstacle of excessive energy costs. The concept of “next generation industrial biotechnology” (NGIB) introduced by Chen and Jiang (2018) involves the use of robust extremophilic production strains that can survive under “extreme” cultivation conditions, such as high salinity, temperature, or pH-values. These features allow them to be cultivated with limited sterility precautions, or even without any, while avoiding risking microbial contamination, even during long-term continuously operated cultivation processes. Tools for metabolic and/or synthetic biology, such as genetic engineering of NGIB strains, are implemented to optimize the manufacture of desired end-products. An important focus of research is cultivation in inexpensive cultivation media, including using industrial waste and surplus materials as carbon sources, and the application of saline sea water as the aqueous phase for extremely halophilic strains, thereby avoiding the need for fresh water and a salt supply (Yu et al., 2019). Important examples of these NGIB concepts are the processes developed for the strain *Halomonas bluephagenesis*, which is an organism created by using the CRISPR/Cas9 genome editing approach from the Chinese Idyngo Lake isolate *Halomonas* TD01, and its further engineered variants. Among other improvements, this strain was genetically modified for enhanced PHA biosynthesis (Chen et al., 2020). Compared with the wild type *Halomonas* TD01, *H. bluephagenesis* exhibits drastically improved production of 3-hydroxyvalerate (3HV), thereby facilitating the production of high-quality poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (poly(3HB-co-3HV)) copolyesters with enhanced processability compared with the brittle and crystalline poly(3-hydroxybutyrate) (P(3HB)) homopolymer (Qin et al., 2018). At pilot scale (5 m³), *H. bluephagenesis* was also shown to accumulate high quantities of high-performance poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (poly(3HB-co-4HB)) copolyester from inexpensive waste gluconate, corn steep liquor, and γ -butyrolactone (GBL) (Ye et al., 2018). Other studies have described additional genetic engineering approaches with this strain, such as triggering changes in the size of PHA granules to facilitate DSP (Shen et al., 2019), or the enhanced co-production of PHAs and the marketable compatible solute ectoine (Ma et al., 2020). Only recently, this engineered organism was shown to produce highly elastic poly(3HB-co-5-hydroxyvalerate (5HV)) copolyesters and sticky, highly transparent poly(3HB-co-4HB-co-5HV) terpolyesters by feeding different bulk diols as carbon sources in bioreactors with volumes of 7 L. In particular, the copolyesters of 3HB and 5HV are promising for

replacing petrochemically produced poly(ϵ -caprolactone) in biomedical applications where *in vivo* degradable, stretchable materials are needed, e.g., surgical wires (Yan et al., 2022). Clearly, the “programmed” *H. bluephagenesis* is already a suitable work horse for industrial-scale PHA production in PR China, as shown by the activities of young companies such as MedPha and PHABuilder (Koller and Mukherjee, 2022).

However, the strains derived from *Halomonas* sp. are Gram-negative bacteria that generate unwanted by-products, such as lipopolysaccharide (LPS) endotoxins. In addition to bacterial extremophiles, haloarchaea comprise another group of extremely halophilic microbes that are suitable for industrial-scale implementation. Haloarchaea are members of the phylum Euryarchaeota characterized by higher salinity requirements, even as high as 3–5 M NaCl, which makes them more robust in long-term cultivation batches without sterility precautions. These organisms are evolutionarily ancient (the first archaea are considered to have appeared during the first billion years after the Earth's formation) and they exhibit high physiological variability. Due to their polyextremophilic nature characterized by tolerance of salinity near saturation, excessive ultraviolet (UV) and ionizing radiation, anoxic conditions, extremely low and/or high temperatures, low water activity, and heavy metals, they are model organisms for astrobiology based on studies of their survivability on extraterrestrial celestial bodies, such as Mars (Abrevaya et al., 2011; Bayles et al., 2020; DasSarma et al., 2020; Leuko et al., 2014; Oren, 2014). Indeed, studies suggest that a settlement of humans on Mars would be based on microbial biotechnology in order to supply people with food, goods, and energy, thereby enabling independence from permanent replenishment from Earth. In the context of microbial food production to supply humans on other bodies in the Solar System, a hyperthermophilic archaeon with astrobiological relevance was recently reported to excrete amino acids (Taubner et al., 2019). PHAs are viewed as the renewable plastics of choice for biotechnological goods production on Mars (Nangle et al., 2020), and haloarchaea could be the solution to this problem. Finally, bioenergy production by methanogenic archaea could be a viable option to provide the energy required for powering artificial biospheres on Mars. Indeed, the conversion of molecular hydrogen and carbon dioxide into methane is already at an advanced bio-technology readiness level

(B-TRL) (Pfeifer et al., 2021), and hyperthermophilic methanogenic archaea are regarded as particularly promising cell factories for high-rate methane production (Mauerhofer et al., 2021).

Features of haloarchaeal PHA production

The advantages and disadvantages of using haloarchaea as microbial cell factories for PHA production have already been comprehensively reviewed (Abrevaya et al., 2011; Koller, 2019; Mitra et al., 2020; Simó-Cabrera et al., 2021). Many haloarchaea can utilize inexpensive raw materials for cell growth and product formation, such as cheese whey permeate (Koller et al., 2005), crude glycerol phase (CGP) from biodiesel industry (Hermann-Krauss et al., 2013), extruded starch (Chen et al., 2006), and hydrolyzed algae biomass (Ghosh et al., 2019). Recently, the formation of haloextremozymes (lipases and proteases) by *Haloferax* (*Hfx.*) *lucentensis* GUBF-2 MG076078 using inexpensive raw materials such as shrimp waste and coconut oil cake was demonstrated by Gaonkar and Furtado, who determined the optimum salinity for enzyme excretion as 300 g L⁻¹, which is in the same range as the salt concentration in the Dead Sea (Gaonkar and Furtado, 2021). Moreover, due to the high intracellular osmotic pressure, which is a strategy employed by these organisms to adapt to very high salinity (“salt-in” strategy where salt mainly comprising KCl is accumulated inside the cells in amounts equimolar to the salinity (NaCl) outside the cells), these organisms are highly prone to lysis in hypoosmotic media (distilled water), which considerably simplifies the DSP of intracellular products such as PHAs (Koller, 2019; Koller, 2020). Indeed, as recently summarized by Pfeifer et al., various interesting materials are produced by different archaea, including methane, biohydrogen, surface layer (glyco)proteins, gas vesicles, bacteriorhodopsin, isoprenoids, carotenoid pigments, high-value extracellular polysaccharides (EPS) for the food industry, haloextremozymes, and halocins. (Pfeifer et al., 2021). Fig. 1 provides an overview of the marketable products produced by the haloarchaeon *Haloferax* (*Hfx.*) *mediterranei*, which is the best studied representative of haloarchaea in terms of bioproduct formation.

Among these archaeal products, only squalene, bacteriorhodopsin, bacterioruberin, and diether/tetraether lipids have already reached

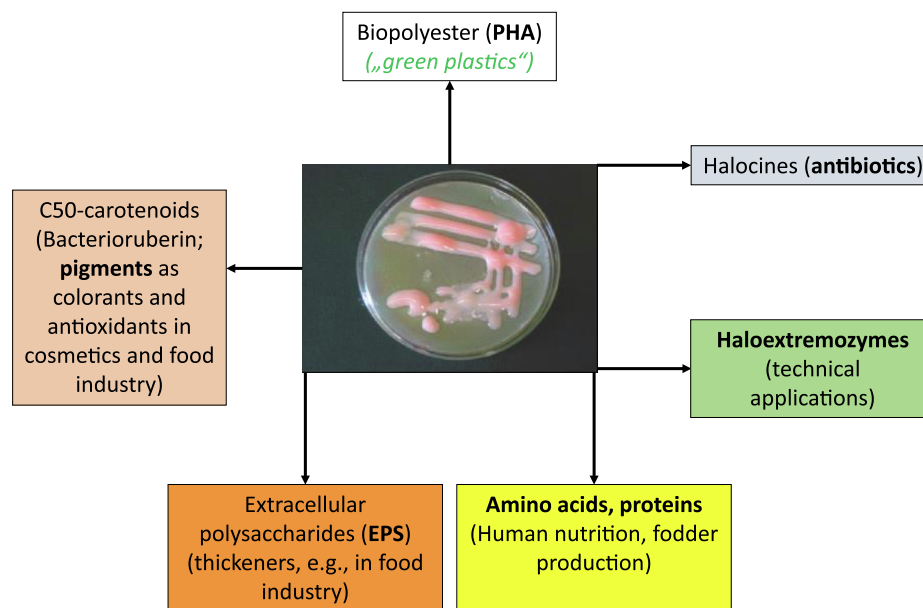


Fig. 1. Overview of marketable products produced by the haloarchaeon *Hfx. mediterranei*, which is a potential standard organism for NGIB. Center: Agar plate with colonies of the strain, where the pinkish coloration is due to pigment (bacterioruberin) formation and the mucous character is associated with EPS excretion (picture by the author, M.K.).

Table 1

Selected PHA-accumulating species, locations where they were isolated, and salinity preferences (based on Koller, 2019; the three-letter abbreviations for haloarchaeal genera are as suggested by Oren (2012) and Arahall et al. (2017)).

Strain	Isolation site	Optimum salinity (NaCl) according to original study
Genus <i>Haloferax</i> (Hfx.)		
<i>Hfx. mediterranei</i>	Salt pond at the coast near Alicante, Spain	150–220 g L ⁻¹
<i>Hfx. volcanii</i>	Dead Sea	200–250 g L ⁻¹
<i>Hfx. gibbonsii</i>	Salt pond at the coast near Alicante, Spain	250 g L ⁻¹
Genus <i>Haloarcula</i> (Har.)		
<i>Har. marismortui</i>	Dead Sea	200 g L ⁻¹
<i>Har. hispanica</i>	Salt pond at the coast near Alicante, Spain	200–250 g L ⁻¹
<i>Har. sp. IRU1</i>	Hypersaline Urmia Lake, Iran	250 g L ⁻¹
<i>Har. japonica</i>	Solar salterns at Ribandar in Goa, India	200 g L ⁻¹
Genus <i>Haloquadratum</i> (Hqm.)		
<i>Hqm. borinquense</i>	Solar salterns at Marakkanam in Tamil Nadu, India	200 g L ⁻¹
Genus <i>Halobacterium</i> (Hbt.)		
<i>Hbt. noricense</i>	Bore core of an Austrian Permian salt deposit	200 g L ⁻¹
Genus <i>Halococcus</i> (Hcc.)		
<i>Hcc. dombrowskii</i>	Dry rock salt from Austrian alpine salt mine	200 g L ⁻¹
<i>Hcc. hamelinensis</i>	Stromatolites from the Hamelin pool in Australian Shark Bay	200 g L ⁻¹
<i>Hcc. morrhuae</i>	Dead Sea	200 g L ⁻¹
<i>Hcc. qingdaonensis</i>	Crude sea-salt sample collected near Qingdao, PR China	200 g L ⁻¹
<i>Hcc. saccharolyticus</i>	Salt; Cadiz, Spain	200 g L ⁻¹
<i>Hcc. salifodinae</i>	Austrian alpine rock salt	200 g L ⁻¹
Genus <i>Halorubrum</i> (Hrr.)		
<i>Hrr. chaviator</i>	Sea salt from Baja California, Mexico, Western Australia and Greece	200 g L ⁻¹
<i>Hrr. coriense</i>	Dead Sea	200 g L ⁻¹
Genus <i>Halobiforma</i> (Hbf.)		
<i>Hbf. haloterrestis</i>	Surface of hypersaline soil collected in Aswan, Egypt	220 g L ⁻¹
Genus <i>Natrinema</i> (Nnm.)		
<i>Nnm. ajinwuensis</i> (= <i>altunense</i>)	Indian salt production pans	200 g L ⁻¹
<i>Nnm. palladium</i>	Kayacik saltern, Turkey	250 g L ⁻¹
Genus <i>Natronobacter</i> (Nbt.)		
<i>Nbt. gregoryi</i>	Soda salt lake liquors from the East African Magadi soda lake, Kenya	200 g L ⁻¹
Genus <i>Natronococcus</i> (Ncc.)		
<i>Ncc. occultus</i>	Magadi salt lake, Kenya	200 g L ⁻¹
Genus <i>Halorhabdus</i> (Hrd.)		
<i>Hrd. utahensis</i>	Sediments from the Great Salt Lake in Utah	270 g L ⁻¹
<i>Hrd. tiamatea</i>	Hypersaline, anoxic deep-sea brine–sediment interface in the Red Sea	270 g L ⁻¹
<i>Hrd. rudnickae</i>	Borehole in Polish salt mine	200 g L ⁻¹
Genus <i>Haloquadratum</i> (Hqr.)		
<i>Hqr. walsbyi</i>	Sinai Peninsula and saltern crystallizers in Australia and Spain	greater than 180 g L ⁻¹
Genus <i>Halopiger</i> (Hpg.)		
<i>Hpg. aswanensis</i>	Surface of hypersaline soil collected in Aswan, Egypt	250 g L ⁻¹
Genus <i>Halogramma</i> (Hgn.)		
<i>Hgn. amylolyticum</i>	Tainan marine solar saltern near Lianyungang, PR China	200 g L ⁻¹
Genus <i>Haloterrigena</i> (Htg.)		
<i>Htg. hispanica</i>	Saltern crystallizer pond at Fuente de Piedra saline lake, Malaga, Spain	200 g L ⁻¹

commercial production scale (Pfeifer et al., 2021). Interestingly, haloarchaea are used as production strains for all of these commercial archaeal products. However, many more archaea have high biotechnological potential, such as methanogenic archaea that produce methane at volumetric production rates beyond 900 mmol L⁻¹ h⁻¹ (Rittmann et al., 2018), or that are applicable as high-pressure cell factories (Mauerhofer et al., 2021), and biohydrogen producing archaea that generate molecular hydrogen via the water–gas shift reaction or from formate (Rittmann et al., 2015).

Among the class Haloarchaea, several have been used as expedient PHA biopolyester producers. A considerable advantage of haloarchaea is that they do not produce LPS, which are endotoxins found in the cell walls of Gram-negative bacteria. This problem affects some of the most widely used PHA production strains, such as the Gram-negative species *Cupriavidus necator* (Tan et al., 2014). These endotoxins are typically co-extracted with PHAs during DSP and they pollute the product obtained. Endotoxins are problematic because LPS causes inflammatory reactions when present in implants or other polymeric products used *in vivo* (Zinn et al., 2001). Hence, PHAs obtained from Gram-negative bacteria need to be highly purified after recovery when high-end applications are required, which increases the PHA manufacturing costs (Koller, 2018a). Thus, haloarchaea are advantageous for the production of PHAs for biomedical applications (Koller et al.,

2007), particularly because there are no known pathogenic archaea (Borrel et al., 2020). Table 1 provides an overview of the PHA-accumulating haloarchaeal species described in this review, including the locations where they were isolated and salinity ranges.

Moreover, there are mechanistic differences in PHA biosynthesis and intracellular degradation (mobilization) between bacteria and haloarchaea. In bacteria, the PHA reserves are utilized rather rapidly by cells after exogenous carbon sources are depleted (Kadouri et al., 2005), while the PHA reserves in cells are degraded very slowly under conditions that normally favor intracellular PHA degradation in the haloarchaeon *Hfx. mediterranei*, i.e., carbon source limitation and the presence of convertible nitrogen and phosphate sources (Koller et al., 2015). Comprehensive reviews indicate that PHA formation can generally be understood as an “SOS response” of cells to diverse stressors (Obruca et al., 2018; Obruca et al., 2021), such as osmotic imbalances (Sedlacek et al., 2019). In particular, it is assumed that the primary role of PHAs in haloarchaea is for protection against hyperosmotic conditions. According to Saponetti et al., the major function of PHA granules in cells of the halobacterium *Haloquadratum* (*Hqr.*) *walsbyi* might be to reduce the cytosol volume in order to minimize the energy demand of cells when maintaining osmotic homeostasis, and thus they are considered to play an essential role in managing high salinity (Sublimi Saponetti et al., 2011). Moreover, haloarchaea

often thrive in habitats characterized by excessive solar UV irradiation, such as saline brines at coasts (Matarredona et al., 2021). According to Huovinen et al., UV-A radiation penetrates more deeply in saline water than salt-poor water. In addition, some salt lakes where many haloarchaea have been isolated are located at high altitudes, and thus they are exposed to greater UV exposure. Therefore, haloarchaea are particularly prone to UV damage (Huovinen et al., 2003). As shown experimentally for bacteria (Slaninova et al., 2018; Tribelli et al., 2020), PHAs have a light-scattering effect to minimize cell-damaging UV radiation, which would otherwise lead to the formation of lethal reactive oxygen species. Zhao et al. studied the impact of PHAs on the resistance of *Aeromonas hydrophila* CQ4 to UV radiation and other stressors, and suggested that enhanced UV resistance may be due to the formation of higher quantities of UV damage recovery products, such as exonuclease III and DNA-glycosylases, which could be related to the presence of PHA granules (Zhao et al., 2007). Similarly, Slaninova et al. described enhanced UV resistance in PHA-harboring *C. necator* cultures compared with cultures of PHA-negative mutants, and they suggested that this resistance was due to the light scattering effect of PHA granules (Slaninova et al., 2018). Similar effects might be found in haloarchaea where PHAs seem to provide an additional UV protection effect to that due to the presence of efficient pigments (Jones and Baxter, 2017). This light scattering effect of PHA granules could have technological applications, and PHA nanoparticles are already used in sun screen cosmetics to replace primary microplastic particles derived from petrochemistry (Online resource 1).

However, haloarchaeal PHA production is currently at a B-TRL of no more than 5, which can be understood as the “technology demonstration” stage located between “proof of concept” (B-TRL 4) and “technology validation” (B-TRL 6) (Pfeifer et al., 2021). The highest production scale reported for PHA production by haloarchaea was a fed-batch bioreactor setup with a working volume of 300 L (Koller et al., 2013a, 2013b). In this process, *Hfx. mediterranei* DSM 1411, a versatile producer of PHAs, bacterioruberin (a C50 carotenoid with high antioxidative capacity) (Giani and Martínez-Espinosa, 2020; Giani et al., 2021), a xanthan-like extracellular polysaccharide (EPS) with potential food technology applications (Antón et al., 1988), and antimicrobial halocins (Atanasova et al., 2013; Kumar et al., 2021; Mhandi, 2022), was cultivated on the industrial surplus material whey (Koller et al., 2013a, 2013b). It should be noted that the previously reported bioprocesses for haloarchaeal PHA biosynthesis under controlled conditions in bioreactors are restricted to only a few production strains: *Hfx. mediterranei* on native corn starch (Chen et al., 2006), extruded rice bran (Huang et al., 2006), hydrolyzed whey permeate (Koller et al., 2013a), CGP from biodiesel production (Hermann-Krauss et al., 2013), stillage based on waste rice (Bhattacharyya et al., 2015), sweet date waste (Alsafadi et al., 2020), glucose (Koller et al., 2015), and mixtures of butyric and valeric acid (Ferre-Güell and Winterburn, 2019); *Halogranum* (*Hgn.*) *amylolyticum* on glucose (Zhao et al., 2015), *Halopiger* (*Hpg.*) *aswanensis* on sodium acetate plus butyric acid (Parroquin Gonzalez and Winterburn, 2022), and *Haloterrigena* (*Htg.*) *hispanica* on complex carrot-waste medium with very low productivity (Di Donato et al., 2011).

The current review describes the basic features and current trends in PHA biosynthesis by haloarchaea. The benefits and obstacles that arise when using these organisms for biopolyester production are discussed, and the prospects of this technology are highlighted.

Current trends in using inexpensive raw materials for haloarchaeal PHA biosynthesis

Surplus whey from dairy industry

Whey permeate is an abundantly available surplus material from the cheese making industry, which is generated by the ultrafiltration

of whey, the surplus product that remains after separating curd cheese during the cheese making process and subsequently skimming the remaining liquid. This technological process generates a lactose-rich whey permeate and protein (predominantly albumin)-rich retentate fraction. Whey permeate is a saline material and it constitutes the first second-generation feedstock ever tested for PHA biosynthesis by haloarchaea, specifically by the strain *Hfx. mediterranei* DSM 1411. Due to the lack of β -galactosidase (EC 3.2.1.23) activity in this strain, lactose comprising the main carbon source in whey permeate needs to be hydrolyzed to an equimolar mixture of the monosaccharide sugars glucose and galactose as an indispensable part of the upstream processing (USP). Enzymatic and acidic hydrolysis are viable methods for preparing an accessible substrate for growth and PHA biosynthesis by this strain. Together with the mineral salt content originally present in the whey permeate (12–15 % of the total whey solids are minerals; Ryan and Walsh, 2016), acidic hydrolysis with hydrochloric acid and subsequent neutralization with NaOH provides a considerable part of the salt required for preparing the highly saline cultivation medium (Koller et al., 2016). Initially, *Hfx. mediterranei* was cultivated in a 42 L stirred tank bioreactor made of stainless steel (MBR Bioreactor AG, Switzerland) in a medium containing enzymatically hydrolyzed whey permeate and yeast extract, as well as casamino acids as an organic nitrogen source. Under these conditions, a cell dry mass (CDM) up to 11 g L⁻¹ was achieved containing 5.5 g L⁻¹ PHAs as intracellular storage products. Surprisingly, the produced PHAs comprised a high-quality (low melting temperature, low crystallinity, high molecular mass, and low polydispersity) copolyester with about 90 % 3HB and 10 % 3HV building blocks (Koller et al., 2005). For established PHA production strains such as *C. necator*, feeding PHA producers simple carbon sources like glucose and galactose leads to the biosynthesis of a poly(3-hydroxybutyrate) (P (3HB)) homopolyester with high crystallinity, brittleness, and restricted processability, whereas PHA copolyester production requires co-feeding with precursor substrates that are structurally related to 3HV, such as propionic, valeric, or levulinic acid (Koller et al., 2017). This process using *Hfx. mediterranei* was elucidated by Han et al. who discovered multiple propionyl-CoA delivering pathways in this strain. Propionyl-CoA couples in a condensation reaction with acetyl-CoA, the degradation product of sugars, to yield the desired PHA-building block valeryl-CoA (Han et al., 2013). This process was scaled up to a stainless-steel bioreactor with a working volume of 300 L, where 7.2 g L⁻¹ P(3HB-co-3HV) was obtained in the fed-batch feeding mode, corresponding to a CDM of 0.66 g g⁻¹ P (3HB-co-3HV) and volumetric P(3HB-co-3HV) productivity of 0.11 g L⁻¹ h⁻¹. A techno-economic evaluation conducted for this almost pilot-scale process (B-TRL 5) indicated that haloarchaea-mediated P (3HB-co-3HV) production using this process could outperform competing techniques for whey utilization (i.e., in terms of the preparation and commercialization of dry whey powder), and it could deliver P(3HB-co-3HV) at a competitive production price of less than 3 € kg⁻¹ (Koller et al., 2013a). An added benefit is that the highly saline spent fermentation broth can be re-used as a salt and water source for subsequent cultivation setups, and the salt-rich cell debris that remains after the release of PHAs from cells during DSP can be recycled as a complex nitrogen source in follow-up cultivations without major impacts on the growth and product formation kinetics (Koller, 2015). However, these initial processes for the conversion of hydrolyzed whey permeate by *Hfx. mediterranei* have one major disadvantage, where glucose as the preferred monosaccharide for this strain is converted considerably faster than galactose, which was quantitatively demonstrated based on a formal kinetic model of this process (Koller et al., 2006). This disadvantage leads to the accumulation of galactose in the fermentation broth, thereby resulting in the economic loss of part of the carbon source, and the spent fermentation broth has a high biochemical oxygen demand. Several years later, Pais et al. showed that elevated concentrations of trace ele-

ments in the cultivation medium are beneficial for enhanced galactose conversion by *Hfx. mediterranei*, which considerably improves the carbon source to P(3HB-co-3HV) conversion yield, as shown in a 2 L borosilicate glass bioreactor (BioStat® B-Plus, Sartorius, Germany) operated in the batch cultivation mode (Pais et al., 2016). In addition to P(3HB-co-3HV) formation, the production of high-quality P(3HB-co-3HV-co-4HB) terpolyesters for potential biomedical applications was achieved by co-feeding the 4HB-related precursor compound GBL together with hydrolyzed whey permeate as the main carbon source (Koller et al., 2007). Subsequently, Raho et al. tested the use of enzymatically hydrolyzed whey lactose obtained by the ultrafiltration and subsequent nanofiltration of whey from ricotta cheese production for producing P(3HB-co-3HV) by *Hfx. mediterranei* in a 3 L borosilicate glass bioreactor. However, in this experiment conducted for 72 h, no more than 1.27 g L⁻¹ P(3HB-co-3HV) was obtained, corresponding to a rather modest volumetric productivity of 0.018 g L⁻¹ h⁻¹ (Raho et al., 2020).

By-products of biodiesel production

A second inexpensive carbon source tested for *Hfx. mediterranei* that is also available in large quantities is CGP, which is the major by-product from biodiesel production. This material contains an average of 85 wt% glycerol and it also has considerable salinity (2–3 wt% salts, primarily sodium and potassium as cations) (Yang et al., 2012), which makes it advantageous for helping halophilic organisms thrive. After removing toxic impurities such as methanol (derived by transesterification to convert lipids into biodiesel and CGP), CGP was used for the first time in a 10 L bioreactor (L 1523, Bioengineering, Switzerland) made of stainless steel in fed-batch cultivations, where it yielded similar PHA concentrations to those when hydrolyzed whey permeate was used. However, the molecular masses were lower when using CGP compared with hydrolyzed whey permeate (Hermann-Krauss et al., 2013), as also observed with other PHA production strains. Feeding with glycerol or other polyols stops PHA chain propagation due to the so-called “endcapping effect” where glycerol couples to growing PHA chains to prevent further polyester elongation (Ashby et al., 2011). Similar to the application of hydrolyzed whey permeate, a P(3HB-co-3HV) copolyester was also obtained when using CGP as carbon source instead of P(3HB) homopolyester, which is the case when supplying established PHA production strains with glycerol as the sole carbon source. A P(3HB-co-3HV-co-4HB) terpolyester was again obtained when co-feeding CGP plus GBL (Hermann-Krauss et al., 2013) in a similar manner to the setups described above with whey permeate plus GBL (Koller et al., 2007).

Starch-based materials as feedstock for haloarchaeal PHA production

Other experiments aimed to convert native corn starch pre-treated by enzymatic (α -amylase) extrusion by using *Hfx. mediterranei*. In a 6 L glass bioreactor (Firstek, Taiwan), a P(3HB-co-3HV) copolyester was produced in the fed-batch pH-stat feeding mode in a similar manner to the setups based on whey permeate or CGP. 20 g L⁻¹ copolyester. When grown on inexpensive raw materials in the fed-batch cultivation mode, the product contained 10.4 mol% 3HV, which was obtained at a volumetric productivity of 0.28 g L⁻¹ h⁻¹, and this is the highest PHA productivity reported to date for this strain. The biomass contained more than 50 wt% P(3HB-co-3HV) (Chen et al., 2006). A P(3HB-co-3HV) copolyester with a very similar monomeric composition was obtained when using α -amylase co-extruded rice bran and corn starch. In particular, 77.8 g L⁻¹ copolyester was obtained at 56 wt% biomass in a 5 L glass bioreactor (Firstek, Taiwan) with a pH-stat repeated fed-batch feeding strategy using *Hfx. mediterranei* ATCC 33500. This study obtained the highest PHA concentration and volumetric productivity (77.8 g L⁻¹ and 0.66 g L⁻¹ h⁻¹, respectively) ever measured for haloarchaea to date (Huang et al., 2006).

Rice-based stillage as feedstock

A P(3HB-co-3HV) copolyester with about 17 mol% 3HV was produced when using stillage from Indian waste rice-based bioethanol production as the carbon source at a volumetric productivity of 0.14 g L⁻¹ h⁻¹ in a simple plug-flow bioreactor (Bhattacharyya et al., 2015). This process is important because high quantities of rice are lost in India as a food resource due to the climatic conditions, problematic transportation and storage infrastructure, and the milling process applied. However, this waste rice is a primary source for bioethanol production in India and other Asian countries, and the remaining carbon-rich stillage is available for follow-up bioprocesses such as PHA production (Suresh et al., 1999). A techno-economic analysis of this process when coupled to a new desalination process procedure for spent fermentation broth obtained a calculated production price per kg of P(3HB-co-3HV) of about 2 US \$ for industrial scale (1890 annual tons capacity) (Huang et al., 2006), which is considerably lower than that for the process based on hydrolyzed whey permeate process discussed above (Koller et al., 2013a, 2013b). Importantly, it should be noted that in this process, PHA production is accompanied by a drastic reduction (by 90 %) in the biochemical oxygen demand of the substrate stream used, i.e., stillage as a waste product of the bioethanol industry. Thus, PHA production is again combined with mitigation of an eco-pollutant (Huang et al., 2006).

Additional inexpensive feedstock

Recently, Alsafadi et al. tested date (*Phoenix dactylifera* L.) waste, which is an abundantly available surplus stream in many global regions, especially Saudi Arabia, as the raw material for PHA biosynthesis by *Hfx. mediterranei*. This material is rich in sugars (glucose, fructose, and sucrose) and the minerals needed for cultivating the strain. A P(3HB-co-3HV) copolyester with 18 mol% 3HV was obtained in fed-batch 5 L bioreactor cultivation at a volumetric productivity of 0.014 g L⁻¹ h⁻¹, corresponding to a CDM of 18 g L⁻¹ with 25 wt% P(3HB-co-3HV). The bioreactor (BioStat A, Sartorius, Germany) consisted of borosilicate glass (Alsafadi et al., 2020). More recently, Gonzalez and Winterburn studied P(3HB-co-3HV) biosynthesis using *Hfx. mediterranei* by continuous feeding with the volatile fatty acids butyric acid and valeric acid in a 3 L cylindrical glass bioreactor (Applikon Biotechnology, NL). Using this strategy, polymer production was doubled to about 5 g L⁻¹ P(3HB-co-3HV) compared with pulse-feeding fed-batch cultivation. The volumetric productivity of P(3HB-co-3HV) increased to 0.13 g L⁻¹ h⁻¹, which was four times the overall PHA productivity obtained in the fed-batch cultivation mode. The composition of P(3HB-co-3HV) was controlled by the feed composition (ratio of butyric to valeric acid) and maintained at a constant level of around 40 mol% 3HV (Gonzalez and Winterburn, 2022). Under co-feeding with valerate and glucose in a 7.5 L glass bioreactor (Biotech-7BG-3, PR China), the EPS-negative mutant strain *Hfx. mediterranei* ES1 was shown to produce different types of P(3HB-co-3HV) copolymers with various 3HV fractions that depended on the glucose-to-valerate ratio in the feed, which ranged from 10 to 57.5 mol%. Randomly distributed and higher-order (consisting of short covalently linked P(3HB) and P(3HV) segments plus random P(3HB-co-3HV) segments) P(3HB-co-3HV) copolymers were obtained depending on the feeding strategy. These polyesters differed in terms of the degree of crystallinity and improved Young's modulus (Han et al., 2015).

Apart from *Hfx. mediterranei*, only one other haloarchaeon has been reported as a PHA producer at controlled bioreactor scale using inexpensive second-generation raw materials, i.e., *Htg. hispanica*, which is a thermo-halophilic organism isolated from a saltern crystallizer pond at Fuente de Piedra saline lake near Malaga, Spain. This strain was cultivated in batch setups on carrot waste medium. However, the PHA (a P(3HB-co-3HV-co-4HB) terpolyester) content only reached a modest 0.125 wt% (Di Donato et al., 2011). The same 1 L glass vessel bioreac-

tor was also applied by this research group for producing biohydrogen, ethanol, and hemicellulolytic enzymes from vegetable waste by the strictly anaerobic thermophile *Thermoanaerobacterium thermotolerans* (Finore et al., 2021). *Halogranum* (Hgr.) *amylolyticum* isolated from a Tainan marine solar saltern near Lianyungang, PR China was cultivated in a 7.5 L glass bioreactor (Biotech-7BG-3, PR China) with purified expensive first-generation raw materials and operated in the fed-batch cultivation mode. In this process, 14 g L⁻¹ of P(3HB-co-3HV) with more than 20 mol% 3HV was produced using glucose at a volumetric productivity of 0.074 g L⁻¹ h⁻¹ (Zhao et al., 2015). Moreover, *Hpg. aswanensis* (“strain 56” in the original study) grew and produced 4.6 g L⁻¹ of P(3HB) homopolymer from acetate and butyrate in an 8 L composite material bioreactor, with a volumetric productivity of 0.018 g L⁻¹ h⁻¹ and CDM of 53 wt% P(3HB) (Hezayen et al., 2000).

Cultivation setups for diverse inexpensive feedstocks at the shaking flask scale

In addition to cultivation under bioreactor conditions, several inexpensive raw materials were tested as substrates for haloarchaeal PHA biosynthesis at the scale of shaking flasks or simple aerated and/or stirred flasks. Small-scale experiments using *Hfx. mediterranei* were conducted for P(3HB-co-3HV) production based on molasses wastewater with a volumetric productivity of 0.62 g L⁻¹ h⁻¹ in 2.5 L stirred and aerated glass flasks. This process appears promising as the basis for large-scale cultivation trials (Cui et al., 2017a). At the shaking flask scale, the same strain achieved a volumetric productivity of 0.21 g L⁻¹ h⁻¹ for P(3HB-co-3HV) when supplied with pre-treated vinasse (Bhattacharyya et al., 2012). In addition, de-phenolized and untreated waste water from olive oil production were tested for P(3HB-co-3HV) production by *Hfx. mediterranei*, although the descriptions of the productivity were unclear in the original study (Alsafadi and Al-Mashaqbeh, 2017). Furthermore, *Hfx. mediterranei* was grown with the alkaline hydrolysate of macroalgae (*Ulva* sp.), although the volumetric productivity was relatively low (0.035 g L⁻¹ h⁻¹) for this process at the shaking flasks scale (Ghosh et al., 2019). Subsequently, the conditions for co-producing PHAs and biochar using biomass of this seaweed with *Hfx. mediterranei* were optimized by the same group. They treated *Ulva* sp. biomass by applying subcritical water hydrolysis and optimized the effects of temperature, hydrolysis time, salinity, and inoculum density on the biomass and PHA formation (Ghosh et al., 2021).

In addition to *Hfx. mediterranei*, other studies used strains such as *Hfx. volcanii* BBK2 and *Haloarcula japonica* BS2, which were both isolated from solar salterns at Ribandar in Goa, India. These strains produced PHAs from hydrolyzed sugarcane bagasse. Unfortunately, no data regarding the productivity or PHA composition were reported in this study (Salgaonkar and Bragança, 2017). More data were provided regarding P(3HB-co-3HV) production by *Halogeometricum* (*Hgm.*) *borinquense* E3, a carotenoid and PHA co-producer, which was also isolated from Indian solar salterns. This strain utilized inexpensive cassava bagasse for PHA production and achieved production of 1.52 g L⁻¹ P(3HB-co-3HV) with about 20 mol% 3HV in shaking flask setups (Salgaonkar et al., 2019). The same strain was also cultivated with acidically hydrolyzed sugar cane bagasse, where around 2 g L⁻¹ of P(3HB-co-3HV) was obtained at a volumetric productivity of about 0.01 g L⁻¹ h⁻¹. In parallel cultivation setups presented in the same study, *Haloarcula* (*Har.*) *marismortui* (isolated from the Dead Sea) produced P(3HB) homopolymer from untreated and charcoal-treated vinasse derived from bioethanol production. Up to 4.5 g L⁻¹ of P(3HB) was obtained at a volumetric productivity of 0.02 g L⁻¹ h⁻¹ and a CDM of up to 30 wt% P(3HB) using vinasse detoxified with charcoal (Salgaonkar and Bragança, 2017). *Har.* sp. IRU1 (isolated from the hypersaline Iranian Urmia Lake) accumulated P(3HB) when cultivated with petrochemical wastewater (Taran, 2011a) and even with crude oil, which makes this strain of interest for bioremediation (e.g., after

oil tanker accidents) (Taran, 2011b). Using both substrates, the P(3HB) content amounted to a CDM of about 40–50 wt% (Taran, 2011a, 2011b). Recently, Kurt-Kızıldoğan et al. reported P(3HB) biosynthesis using different agricultural waste materials (sugar beet pulp, corn cobs, and hazelnut husks) as inexpensive carbon sources by the Turkish Tuz Salt Lake isolate *Haloarcula* sp. TG₁. The highest P(3HB) content by biomass (45.6 wt%) was obtained using enzyme-pretreated sugar beet pulp, whereas biomass of 17.8 wt% P(3HB) was obtained using acid-hydrolyzed sugar beet pulp. Shaking flask scale cultivation experiments were conducted at a salinity of 5 M (-Kurt-Kızıldoğan et al., 2021).

PHA production by other haloarchaea from purified substrates

A limited number of other PHA-producing haloarchaeal strains have been used for PHA production, but only with expensive purified substrates such as sugars or fatty acids. Examples include *Halobiforma* (*Hbf.*) *haloterrestis* isolated from hypersaline soil collected in Aswan, Egypt, which accumulated up to 40 wt% P(3HB) using butyric acid as the sole carbon source (Hezayen et al., 2002), and *Natrinema* *ajinwuensis* (*altunense*) isolated from salt production pans in India, which produced more than 60 wt% P(3HB-co-3HV) copolyester with about 20 mol% 3HV using glucose as the sole carbon source (Mahansaria et al., 2018).

The salinities used to prepare the culture media in these processes justify the designation “haloarchaea”. *Hfx. mediterranei* was typically cultivated at concentrations of 150–220 g L⁻¹ (2.5–3.8 M) NaCl (exception: 110 g L⁻¹ (1.9 M) used by Han et al., 2013), which is similar to the salt concentrations used for *Har. marismortui*, *Har. japonica*, and *Hgm. borinquense*. *Hbf. haloterrestis* was cultivated with 220 g L⁻¹ (3.8 M) NaCl, and *Hfx. volcanii*, *Har. hispanica*, *Har.* sp. IRU1, *Hpg. aswanensis*, *Nnm. ajinwuensis* (= *altunense*), and *Nnm. palladium* required up to 250 g L⁻¹ (4.3 M) NaCl (Koller, 2019). The highest salinity used for PHA production by haloarchaea was applied in processes with *Haloarabidus* (*Hrd.*) *utahensis* isolated from the Great Salt Lake in Utah (Waino et al., 2000) and *Hrd. tiamaatea* (Antunes et al., 2008) isolated from the hypersaline, anoxic deep-sea brine-sediment interface in the Red Sea. These two strains were reported to thrive and produce PHAs at 270 g L⁻¹ (4.6 M) NaCl, although quantitative data for PHA formation were not provided.

Search for novel haloarchaeal PHA production strains

Screening for novel PHA-producing haloarchaea is ongoing at present. In this context, Nile Red/Sudan Black staining is typically conducted to obtain preliminary identifications of PHA-positive isolates, and the exact compositions of the accumulated PHAs can be determined subsequently (e.g., based on nuclear magnetic resonance measurements) (Legat et al., 2010). More recent screening studies have confirmed the results obtained by staining by PCR amplification of the *phaC* and/or *phaE* genes encoding PHA polymerase (Ben Abdallah et al., 2020; Karray et al., 2021; Mahansaria et al., 2015). Some novel methods have also been developed for the rapid screening for PHA inclusions in new haloarchaeal isolates, such as double-fluorescence staining with Nile red and SYBR Green, followed by visualizing the stained PHA granules by confocal fluorescence microscopy. This approach overcomes the challenges of staining haloarchaeal cells due to the high ionic strength of the medium, which is unsuitable for most established dyes, and due to the low permeability to dyes of the S-layer in haloarchaea (Cánovas et al., 2021). Screening for novel PHA producers can also be performed using bioinformatics approaches, as shown by Wang et al. (2019). The disadvantage of the bioinformatics screening approach is that the type of biopolymer and physiological and biotechnological characteristics of the strain cannot be predicted. When novel putative PHA-producing haloarchaea are isolated, they can directly undergo microbiological characterization as well as

screening to determine the polymer type and assessments of the basic physiological and biotechnological properties.

Trends in advanced bioengineering for haloarchaeal PHA biosynthesis

The production of bioproducts requires readily accessible, inexpensive, and abundant raw materials, powerful microbial cell factories (production strains), bioprocess development, and appropriate bioengineering.

USP

Bioengineering starts with UPS to convert the raw materials into a form that can be converted by the production strain (e.g., the hydrolysis of whey lactose to generate its monomers, de-methanolization of CGP, or the detoxification and de-phenolization of bagasse hydrolysates or olive oil waste water), inoculum preparation, and sterilizing the media and bioreactor equipment. Sterilization can even be omitted when using haloarchaea. Thus, clean-in-place procedures might be sufficient in most cases. Due to the reported excessive halophilic characteristics of these strains, the cultivation processes can even be operated in a non-sterile mode without the risk of cultivating alien microbes. For example, this was demonstrated using *Hfx. mediterranei*, which was cultivated for three months in a continuous setup without any microbial infection according to microscopic observation (Lillo and Rodriguez-Valera, 1990).

Bioreactors and cultivation regimes used for haloarchaeal PHA production

Adequate bioreactor facilities are needed for the bioprocess itself and they should be adapted to the kinetics of cell growth and product (PHA) formation. Haloarchaea exhibit Monod kinetics when cultivated on defined, sole carbon sources (e.g., glycerol) and diauxic growth on substrate mixtures (e.g., hydrolyzed whey permeate) (Lorantfy et al., 2014a). Typically, PHA biosynthesis by haloarchaea on a bioreactor scale is carried out in stirred tank reactors. PHA production is a secondary metabolic process, so the cultivation process needs to be separated into two phases. In the first stage, adequate availability of all the nutrient components is necessary, where micronutrients (trace elements) are needed in addition to the carbon source, and the optimum concentrations of convertible nitrogen and phosphate sources. After reaching the desired concentration of PHA-poor biomass, the process conditions need to be changed when feeding the carbon source and an essential nutrient (typically nitrogen or phosphate source) becomes limiting, and cell metabolism switches from biomass growth toward PHA biosynthesis (Braunegg et al., 1995). This process was demonstrated in 1990 for the haloarchaeon *Hfx. mediterranei* by Lillo and Rodriguez-Valera who reported enhanced PHA accumulation by this strain at suboptimal phosphate concentrations (Lillo and Rodriguez-Valera, 1990). It should be noted that when cultivating PHA-rich cells in stirred tank reactors, it is beneficial to design the bioreactor so the stirring system is located at the top of the device because bioreactor stirring systems are typically closed to the outside by a double mechanical seal containing a rotating and static ring. When growing PHA-rich cells, there is always a risk that the cells could enter between the two rings where they would be ground and form a thin film of PHAs, thereby causing the rings to separate, and thus the contents of the bioreactor could flow out when stirred from below. This effect is even more evident when using highly saline cultivation media, where salt crystal formation between the two rings can also result in bioreactor leakage through the seals (Koller, 2018b).

Due to the highly saline cultivation media used for growing haloarchaea, it is necessary to employ highly corrosion-resistant bioreactor materials. In many studies, stainless steel was used as the bioreactor

material in applications. However, even stainless steel is prone to corrosion by Cl^- ions under long-term use, which is considered one of the main obstacles that hinder the biotechnological use of extremely halophiles (Schiraldi and De Rosa, 2002). Therefore, several studies employed corrosion-resistant polymeric materials for bioreactor development. The plug flow-type reactor operated by Bhattacharyya et al. for P(3HB-co-3HV) production by *Hfx. mediterranei* using waste rice-based stillage comprised the polymer poly(methyl methacrylate) (PMMA). The tank had a total volume of 50 L and it was originally used as a model “activated sludge processor unit” to study and optimize wastewater treatment. In this process, 7 L of inoculum culture prepared in 7 L stillage in a shaking flask was used to inoculate 14 L of fresh, non-sterile stillage with other medium components in the 50 L tank. The tank was aerated and the cultivation process proceeded for 4 days (Bhattacharyya et al., 2015).

A corrosion-resistant bioreactor for cultivating extreme halophiles was constructed by Hezayen et al. This bioreactor was manufactured by the company FairMen Tec and it comprised a composite material made of poly(ether ether ketone) (PEEK), silicon nitride ceramics, and tech glass. Two new extremophilic haloarchaeal isolates comprising “strain 56” and *Natrialba* (*Nab.*) sp. isolated from hypersaline soil samples in Aswan, Egypt, were cultivated in this new device. “Strain 56” was later classified as *Hpg. aswanensis* DSM 13151, and it was investigated for P(3HB) biosynthesis using a cultivation medium with a salinity of more than 200 g L^{-1} NaCl. At the same salinity, *Natrialba* sp. was studied to assess its potential for synthesizing the water-soluble, biodegradable polypeptide poly(γ -glutamic acid), which is an important material for packaging pharmaceutical products or as a humectant in the food and cosmetic industries. The promising biosynthesis of P(3HB) by *Hpg. aswanensis* was achieved with mixtures of acetate and n-butyric acid, which yielded 4.6 g L^{-1} of the biopolyester at a volumetric productivity of 0.016 g $\text{L}^{-1} \text{h}^{-1}$ (Hezayen et al., 2000). An advanced version of this bioreactor was used by Lorantfy et al. for cultivating *Hfx. mediterranei* by adapting a fully instrumented corrosion-resistant 2 L laboratory borosilicate/PEEK bioreactor (Labfors bioreactor; Infors, Switzerland). This bioreactor comprised a borosilicate glass vessel, and all parts and devices in the bioreactor that made contact with the saline cultivation broth were made of corrosion-free glass and PEEK. Moreover, silicone tubing was used for all connections, and the ports and connections to the bioreactor setup were made of either glass tubing or PEEK (Lorantfy et al., 2014a).

Mahler et al. reported the development of a custom-made cylindrical bubble-column bioreactor (diameter = 13.4 cm, height = 110 cm; Möstl Anlagenbau, Austria) with a 15 L working volume for the continuous cultivation of extreme halophiles. This bubble-column bioreactor was made of Hastelloy™, which is a highly corrosion-resistant nickel-molybdenum alloy, and it was equipped with an external cell retention unit containing a tangential flow polysulfone filter module to allow the process to run at dilution rates (D) far beyond the specific growth rates of organisms, thereby resulting in higher volumetric productivities. Mahler et al. indicated that this bubble-column design was three times more efficient than a continuously stirred tank reactor (CSTR) operated in parallel setups in terms of the energy input required for optimum molecular oxygen (O_2) transfer. The CSTR was made of glass (vessel) and PEEK (lid and stirrer), and equipped with a cell retention module. Continuous cultivation of *Hfx. mediterranei* on glycerol was performed in this novel reactor at a dilution rate (D) of 0.37 h^{-1} and cell retention rate of 0.93 (Mahler et al., 2018). Indeed, cell retention systems might be viable solutions to overcome the typical problems associated with PHA production by haloarchaea using inexpensive substrate streams. The frequent high dilution of these feed streams (e.g., hydrolyzed whey permeate and molasses) dilutes the bioreactor’s working volume in fed-batch feeding processing, thereby reducing the volumetric productivity. Retaining the active cell biomass in the system while

Table 2

Bioreactor materials and scales used for producing different types of PHA biopolyesters with various carbon substrates by different haloarchaea.

Bioreactor material	Type of bioreactor/ Bioreactor volume	Production strain	Salinity (NaCl [g/L])	Main carbon source	Type of PHA produced	Ref.
Stainless steel	CSTR/42 L	<i>Hfx. mediterranei</i>	156	Hydrolyzed whey permeate	P(3HB-co-3HV)	Koller et al., 2005
Glass	CSTR/6 L	<i>Hfx. mediterranei</i>	234	Enzymatically extruded corn starch	P(3HB-co-3HV)	Chen et al., 2006
Glass	CSTR/5 L	<i>Hfx. mediterranei</i>	234	Extruded rice bran and extruded cornstarch	P(3HB-co-3HV)	Huang et al., 2006
Stainless steel	CSTR/300 L	<i>Hfx. mediterranei</i>	156	Hydrolyzed whey permeate	P(3HB-co-3HV)	Koller et al., 2013a
Stainless steel	CSTR/10 L	<i>Hfx. mediterranei</i>	150	Crude glycerol phase	P(3HB-co-3HV)	Hermann-Krauss et al., 2013
Borosilicate glass and PEEK	CSTR/2 L	<i>Hfx. mediterranei</i>	156	Various (acetate, glycerol, lactate, and ethanol)	P(3HB-co-3HV)	Lorantfy et al., 2014
PMMA	Plug flow bioreactor/50 L	<i>Hfx. mediterranei</i>	200	Waste rice-based stillage	P(3HB-co-3HV)	Bhattacharyya et al., 2015
Glass	CSTR/7.5 L	<i>Hfx. mediterranei</i>	110	Glucose plus valeric acid	P(3HB-co-3HV) (random and high-order copolymers)	Han et al., 2015
Borosilicate glass	CSTR/2 L (working volume)	<i>Hfx. mediterranei</i>	156	Hydrolyzed whey permeate	P(3HB-co-3HV)	Pais et al., 2016
PMMA	1.2 L “airlift fermenter” according to authors (<i>de facto</i> : bubble column)	<i>Hfx. mediterranei</i>	75–250	Glucose	P(3HB-co-3HV)	Cui et al., 2017b
Hastelloy™ (nickel-molybdenum alloy)	Bubble column/62 L (15 L working volume)	<i>Hfx. mediterranei</i>	150	Glycerol	P(3HB-co-3HV)	Mahler et al., 2018
Borosilicate glass	CSTR/2 L (working volume)	<i>Hfx. mediterranei</i>	200	Hydrolyzed whey permeate	P(3HB-co-3HV)	Raho et al., 2020
Borosilicate glass	CSTR/5 L	<i>Hfx. mediterranei</i>	150	Date waste	P(3HB-co-3HV)	Alsafadi et al., 2020
PE	Cylindrical pneumatically agitated sleeves (bubble column-like)/40 L	<i>Hfx. mediterranei</i>	144 (saline water plus salinity due to hydrolyzed seaweed)	Hydrolyzed seaweed biomass		Ghosh et al., 2022
Glass	CSTR/3 L	<i>Hfx. mediterranei</i>	156	Volatile fatty acids	P(3HB-co-3HV)	Parroquin Gonzalez and Winterbrun, 2022
PET	Bubble columns (bottles)/1 L	<i>Hfx. mediterranei</i>	144 (saline water plus salinity due to hydrolyzed seaweed)	Hydrolyzed seaweed biomass	P(3HB-co-3HV)	Ghosh et al., 2022
Glass	1 L (working volume)	<i>Htg. hispanica</i>	200	Carrot waste	P(3HB-co-3HV-co-4HB)	Di Donato et al., 2011; Finore et al., 2021
Glass	CSTR/7.5 L	<i>Hgr. amylolyticum</i>	200	Glucose	P(3HB-co-3HV)	Zhao et al., 2015
Composite material: PEEK, silicon nitride ceramics, and tech glass	CSTR/8 L	<i>Hpg. aswanensis</i>	greater than 200	Mixture of acetate and butyric acid	P(3HB)	Hezayen et al., 2000

CSTR: continuously stirred tank bioreactor; PE: poly(ethene); PEEK: poly(ether ether ketone); PET: poly(ethylene terephthalate); PMMA: poly(methyl methacrylate).

discharging the spent fermentation broth can significantly boost overall PHA formation, as demonstrated for PHA production processes with bacteria in waste feed streams, such as *Bacillus megaterium* with diluted volatile fatty acids (Kacanski et al., 2022), recombinant *Escherichia coli* with whey (Ahn et al., 2001), and *C. necator* with diluted glucose solution (Haas et al., 2017). For haloarchaea, Lorantfy et al. operated a bioreactor system with external cell retention at the optimal cross flow rate for cultivating *Hfx. mediterranei* using a synthetic medium with lactate, glycerol, acetate, and/or ethanol as carbon sources. They explicitly developed this system as a strategy to overcome the physiological limitations of haloarchaea, such as generally lower specific growth rates than those observed for many industrially implemented microorganisms. A cell-free harvest stream that was almost free of total organic carbon was obtained with a 10-fold increase in biomass productivity compared with continuous chemostat cultures. Unfortunately, PHA formation was not monitored in this study (Lorantfy et al., 2014b).

The lab-scale process for PHA production developed by Ghosh et al. (2019, 2021) using *Hfx. mediterranei* with seaweed (*Ulva* sp.) biomass hydrolyzed with supercritical water was recently upscaled using pneumatically agitated reactors for outdoor cultivation. A volumetric PHA productivity of 0.05 g L⁻¹ h⁻¹ was achieved at 40 L scale, where the maximum PHA fraction in the biomass was 0.56 g g⁻¹. The cultivation process was first performed under non-sterile conditions in a set of 15 PET bottles with a volume of 1 L to optimize the process conditions (aeration rate and time of fermentation). In the second step, cylindrical sleeve-like 40 L macroalgae photo-bioreactors (1.55 m in length, 0.02 m in thickness, and 0.4 m in width) made of PE and filled with 10 L cultivation broth were operated as bubble column reactors. They also investigated how to improve biomass separation. Ultrasonication favored settling of the *Hfx. mediterranei* biomass to facilitate cell harvesting (Ghosh et al., 2022). The authors described this process as a “halophyte biorefinery” or “seagiculture,” and this is an important example of processes in the emerging area of marine biotechnology

(“blue biotechnology”) (Chemodanov et al., 2017; Ghosh et al., 2022). As a technologically advanced version of bubble columns, airlift bioreactors can be applied for cultivating aerobic organisms such as haloarchaea, as shown by Cui et al. who produced PHAs and EPS from glucose as the sole carbon source by using *Hfx. mediterranei* (Cui et al., 2017b).

Table 2 shows the diverse bioreactor materials and bioreactor scales used for PHA production by haloarchaea with different raw materials.

DSP for product recovery

Finally, DSP for product recovery and refining constitutes the last important part of bioprocessing (Koller, 2020). In this stage, haloarchaea have particularly advantageous features due to their adaptability to extreme salinity (“salt-in-strategy”). In 1986, Fernandez-Castillo et al. pioneered haloarchaeal PHA research and showed that *Hfx. mediterranei* cells readily lyse in the absence of salt (hypotonic media like distilled water) and release PHA granules into the aqueous phase (Fernandez-Castillo et al., 1986), thereby facilitating the remarkably simple DSP of the product. PHA granules have a lower density than water and any cell debris, so they can be separated by simple centrifugation to form a cream on the surface, which can then be removed (“skimming of PHA granules”). However, the PHA granules obtained in this manner are still covered by the PHA granule membrane. Thus, further purification of crude PHAs is required when highly pure PHAs are needed (e.g., for biomedical applications).

In their PHA production process based on waste rice stillage using *Hfx. mediterranei*, Bhattacharyya et al. showed that the cultivation broth could be collected in a settling vessel after the production phase. The cell mass was separated after settling for 24 h. The spent medium containing 222 g L⁻¹ NaCl was desalinated by mixing with hot decanoic acid, which resulted in the precipitation of the pure salts. The desalted stillage and decanoic acid were separated into two phases, where the decanoic acid phase was used

for subsequent desalination cycles. The recovered salts were used for follow-up cultivations. PHA separation from the cell mass in this process occurred in a cell lysis tank under stirring in an aqueous sodium dodecyl sulfate solution. The crude PHAs obtained were further purified by using an organic solvent (acetone/ethanol mixture), followed by dissolving in hot chloroform, and recycling the chloroform after cooling down and precipitating the polymer (Bhattacharyya et al., 2015).

An efficient process for recovering high purity P(3HB-co-3HV) produced by *Hfx. mediterranei* was developed based on the application of acetone as the sole extraction solvent (Koller et al., 2013b). Acetone can be produced from renewable materials by anaerobic fermentation with solventogenic *Clostridia*, and it is generally more sustainable than the widely used halogenated PHA solvent chloroform (Liew et al., 2022). However, the extraction of relatively crystalline PHAs produced by *Hfx. mediterranei* is not possible using acetone at room temperature and ambient pressure because the polymer is not soluble in acetone under these benign conditions. By contrast, under elevated temperature (121 °C) and pressure (7 bar) conditions in closed vessels, PHAs was readily dissolved from the *Hfx. mediterranei* biomass and it precipitated from the solution in a highly pure form after cooling down to room temperature. Cell debris was separated from the hot solution by simple pressure-driven transfer through a filtration unit, and lipids co-extracted with PHA remained in the acetone solution after precipitating the PHAs. The acetone was readily re-used in further extraction cycles after distillation to remove the extracted lipids (Koller et al., 2013b). These lipids could be used as raw materials for biodiesel synthesis in a similar manner to the process reported by Siow et al. who used *Tenebrio molitor* (mealworm) lipids as biodiesel raw material (Siow et al., 2021). In this process, *T. molitor* was used to digest PHA-rich *C. necator* biomass. The larvae only absorbed the non-PHA components of the biomass and excreted intact PHA granules as white feces in a highly pure form (Zainab-L and Sudesh, 2019). This is an excellent example of the combination of different fields of biotechnology for USP and DSP.

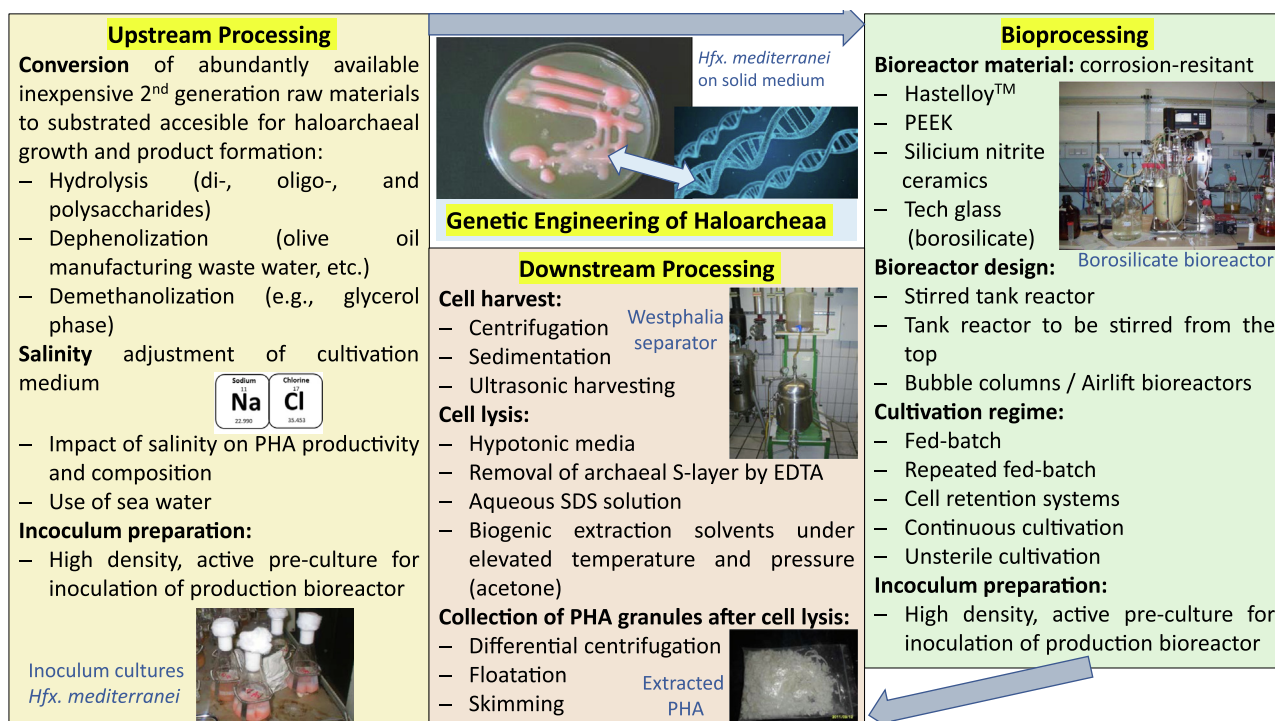


Fig. 2. Bioengineering stages that need to be optimized for improved industrial-scale haloarchaeal PHA production. *Hfx. mediterranei* is shown as an example as the prototype PHA-producing haloarchaeon.

Some haloarchaea such as *Hfx. volcanii* (a strain named after B.E. Volcani who first demonstrated the diversity of microbial life under the extreme saline conditions in the Dead Sea; reviewed by Arahall et al., 2000) have been studied for PHA recovery by using novel methods. In addition to its potential for PHA accumulation (Fernandez-Castillo et al., 1986), *Hfx. volcanii* was also described as an exceptional pigment (the antioxidative C50 isoprenoid bacterioruberin; Zalazar et al., 2019) producer. *Hfx. volcanii* also lacks a rigid murein-containing cell wall, where the cells are only protected by a glycoprotein single layer called the surface layer (S-layer). This S-layer is stabilized by magnesium ions, and thus it can be readily removed by treating cells with the chelating agent EDTA, and thus intracellular products such as PHAs and pigments can be recovered (Rodrigues-Oliveira et al., 2019). Isolated S-layers are of interest for applications in the field of nanobiotechnology, e.g., as isoporous ultrafiltration membranes and immobilization matrices for binding functional molecules (Madhurantakam et al., 2014; Pfeifer et al., 2021).

Fig. 2 illustrates the process steps needed for optimizing haloarchaeal PHA manufacturing.

Trends in elucidating the genetic and enzymatic background for haloarchaeal PHA biosynthesis: Identifying the targets for genetic engineering

Hfx. volcanii is the major haloarchaeal model organism used for studying expression vectors and gene deletions, including by CRISPR. A broad range of genetic, molecular, and biochemical tools have been developed for this fast-growing and easy-to-cultivate organism. It is very likely that the genetic engineering of other industrially relevant haloarchaea will become more frequent in the near future (Pohlschroder and Schulze, 2019). The genome sequence of the most important haloarchaeal PHA producer *Hfx. mediterranei* has been available for a decade, thereby allowing the application of synthetic biotechnology tools for tailored product formation (Han et al., 2012). The same applies to the identification and characterization of the genes involved in the biosynthesis of the key enzymes for PHA production by this strain, including the haloarchaeal 3-ketothiolase, which links acetyl-CoA to acetoacetyl-CoA or acetyl-CoA and propionyl-CoA to 3-oxoketovaleryl-CoA. In addition, acetoacetyl-CoA reductase catalyzes the “pseudofermentative” reduction of ketoacetyl-CoA to (R)-3-hydroxyacyl-CoA, as well as (R)-3-hydroxybutyryl-CoA and (R)-3-hydroxyvaleryl-CoA. Finally, PHA synthases polymerize (R)-hydroxyacyl-CoA monomers to form polymeric PHA chains. These haloarchaeal enzymes are considerably different from their bacterial counterparts. Eight putative 3-ketothiolase-encoding genes were found in *Hfx. mediterranei* but only one of them is involved in 3-hydroxyvaleryl-CoA formation via the condensation of acetyl-CoA and propionyl-CoA for 3HV biosynthesis. The other identified 3-ketothiolases can only catalyze the condensation of acetyl-CoA to acetoacetyl-CoA (Hou et al., 2013). The first report of a haloarchaeal PHA synthase was by Hezayen et al. who described a PHA granule-associated PHA synthase in *Hpg. aswanensis* (“strain 56”) when granules were released by hypertonic disintegration and collected by differential centrifugation. This enzyme is a homologue of the Class III PHA synthases in bacteria, and it exhibits a higher activity at elevated temperature and salinity compared with the bacterial PHA synthases described previously at that time (Hezayen et al., 2002). By contrast, most halophilic bacterial PHA producers such as *Halomonas* sp. are Class I PHA synthases (Mittra et al., 2020). Similar types of Class III PHA synthases have also been found in other haloarchaea, such as *Har. marismortui* (Han et al., 2007), *Hgn. amylolyticum* (Zhao et al., 2015), and *Hqr. walsbyi* (Lu et al., 2008). Han et al. showed that the PHA synthase in *Har. marismortui* comprises two subunits encoded in the *phaEC_{Hm}* operon. The subunit encoding the *phaC_{Hm}* synthase gene binds strongly to PHA granules but the *phaE_{Hm}*-encoded subunit is not

granule-associated. Both subunits are constitutively expressed under both, nutritionally balanced and limited culture conditions (Han et al., 2010a). A similar constitutively expressed gene cluster called *phaEC_{Hme}* was identified in *Hfx. mediterranei* (Lu et al., 2008). Thus, the Class III synthase complex encoded by the *phaEC* gene cluster is a typical feature of PHA-producing haloarchaea, and thus its detection by PCR is used to identify new PHA-positive haloarchaea isolates (Ben Abdallah et al., 2020; Karray et al., 2021; Mahansaria et al., 2015). In contrast to bacterial Class III PHA synthase enzymes, the haloarchaeal counterparts differ in terms of their molecular mass and different conserved motives. Thus, the Class III PHA synthases are categorized as “subgroup IIIA” (“archaeal”) and “subgroup IIIB” (“bacterial”) (Han et al., 2010a). Interestingly, three additional “cryptic” PHA synthesis genes (*phaC₁*, *phaC₂*, and *phaC₃*) were identified in *Hfx. mediterranei*, which are distributed throughout the genome and not clustered together with *phaEC_{Hme}*. The expression levels of these cryptic genes determine the 3HV fraction in P(3HB-co-3HV), and thus genetic engineering of these targets could contribute to tailoring *Hfx. mediterranei* biopolyesters at the monomeric level. At present, *Hfx. mediterranei* has the largest number of known *phaC* genes among haloarchaea. Interestingly, *PhaC_{Hme}* synthase shares higher homology with *phaC* genes in other haloarchaea compared with its own three “cryptic” PHA synthesis genes. Therefore, it seems likely that *phaC₁*, *phaC₂*, and *phaC₃* were acquired by horizontal gene transfer from other haloarchaea during evolution (Han et al., 2010b). In addition to haloarchaea, multiple *phaC* genes in a single genome have been described in members of the domain Bacteria. For example, in *C. necator*, a second *phaC* gene that does not cluster with the *phaCAB* operon was discovered, but its function has not yet been elucidated (Pohlmann et al., 2006).

In order to genetically engineer haloarchaea for enhanced PHA biosynthesis, Zhao et al. knocked out the gene cluster encoding EPS formation by *Hfx. mediterranei* and improved the yields of poly(3HB-co-3HV) with less by-product (EPS) formation, thereby decreasing the cost of the carbon source used in the process and reducing the viscosity of the fermentation broth due to less EPS production, and favoring the transfer of O₂ to the cells (Zhao et al., 2013).

Trends in adapting culture conditions to trigger PHA productivity and properties of haloarchaea

Triggering the intracellular carbon flux toward biosynthesis of PHAs or EPS

During PHAs and EPS coproduction in haloarchaea, Pacholak et al. established three pathways with key responses to extreme salinity in terms of PHAs and EPS production in *Hfx. mediterranei*. The enzymes 3-oxoacyl-ACP reductase and 3-hydroxyacyl-CoA dehydrogenase were overexpressed at high salinity, thereby resulting in enhanced PHA biosynthesis. Moreover, the enzymes serine-pyruvate transaminase and serine-glyoxylate transaminase were upregulated under these conditions, which enhanced the glucose-to-PHA conversion yield. By contrast, the expression levels of the enzymes sulfate-adenylyl transferase and adenylyl-sulfate kinase were downregulated under high salinity, thereby reducing EPS formation (Pacholak et al., 2021). These proteome studies support previous reports by Cui et al. who also suggested altering the salinity as a tool for directing the carbon flux toward predominant PHA or EPS biosynthesis based on their cultivation in 1.2 L airlift bioreactors made of PMMA polymer at salinities between 75 and 250 g L⁻¹. They also found that high salinity (250 g L⁻¹ NaCl) inhibited EPS excretion but it was beneficial for PHA biosynthesis (Cui et al., 2017b). These studies showed that the salinity of the cultivation medium can be used as a control to obtain higher PHA concentrations, as well as reducing losses of the carbon source in EPS formation during the industrial-scale cultivation of *Hfx. mediterranei*, and without the need to genetically engineer the strain.

Triggering haloarchaeal PHA copolyester composition at the monomer level

The bioprocess conditions can also influence the composition of the PHAs produced by haloarchaea at the monomer level, as shown by Ferre-Güell and Winterburn who used ammonium ions as a nitrogen source to obtain 16.9 mol% 3HV in P(3HB-co-3HV) produced by *Hfx. mediterranei* on a medium containing glucose and yeast extract as organic substrates. By contrast, replacing ammonium with nitrate as the inorganic nitrogen source reduced the 3HV fraction in P(3HB-co-3HV) to only 12.5 %. Moreover, the C/N ratio in the medium can affect the 3HV fraction in P(3HB-co-3HV), where lower C/N ratios typically favor the 3HV content of copolyesters, probably by influencing the propionyl-CoA generating pathways (Ferre-Güell and Winterburn, 2017). Similarly, Melanie *et al.* found that higher fractions of the phosphate source (KH_2PO_4) yielded higher 3HV fractions in P(3HB-co-3HV) produced by *Hfx. mediterranei* as well as lower overall productivity (Melanie *et al.*, 2018). By contrast, the cultivation temperature determined overall PHA productivity by this strain but not its composition at the monomeric level, as shown by Cui *et al.* who cultivated this haloarchaeon on a medium that mimicked molasses wastewater (Cui *et al.*, 2017a). Recently, Sato *et al.* reported that selecting an appropriate cultivation medium drastically affected the composition of P(3HB-co-3HV) produced by *Hfx. mediterranei* and its molecular mass. They demonstrated the production of ultra-high molecular mass P(3HB-co-3HV) with an average molecular mass (M_n) of more than $3.0 \times 10^6 \text{ g mol}^{-1}$ and weight-average molecular mass of more than $5.0 \times 10^6 \text{ g mol}^{-1}$ by using a nutrient medium deficient in complex nutrients (e.g., yeast extract or casamino acids) (Sato *et al.*, 2021). The ultra-high molecular mass P(3HB-co-3HV) is considered beneficial for polymer processing, such as for cold drawing polymer films (Ino *et al.*, 2020). Moreover, using these media resulted in the production of the highest 3HV fractions in P(3HB-co-3HV) of more than 26 mol% (Sato *et al.*, 2021).

Conclusions

The production of PHAs by haloarchaea has not yet reached industrial maturity within the research and development field of Archaea Biotechnology. However, the biotechnological potential of haloarchaea for PHA production under non-sterile conditions from rather inexpensive feedstocks could overcome the drawbacks associated with low substrate concentrations or slow growth rates for established PHA-producing organisms. These advantages could be enhanced further by using process analytical technology and specially designed bioreactors. Comparatively cost-efficient DSP operations and the non-pathogenic nature of haloarchaea make the application of haloarchaea in PHA production attractive for industrial development to obtain high-quality PHA. The most widely researched haloarchaeal cell factory for PHA production is *Hfx. mediterranei* because of its broad substrate range and well understood genetic features.

The main obstacle that needs to be overcome to facilitate the successful implementation of haloarchaea as microbial cell factories for the individual or combined production of PHA, EPS, bacterioruberin, bacteriorhodopsin, and isoprenoids is making it economically competitive with the well-established fossil fuel utilizing industries. The cost-efficient production of compounds synthesized by archaea will be possible when biorefineries have been established at industrial scale. Further steps for bioprocess enhancement might involve developing novel tools for metabolic engineering to allow carbon flux fine tuning and synthetic biology to make haloarchaea even more efficient in the high-throughput conversion of inexpensive raw materials into tailored bioproducts (e.g., by expanding the substrate scope or further facilitating DSP). Haloarchaeal PHA production will then become an established branch of industrial biotechnology.

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Martin Koller: Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Simon K.M.R. Rittmann:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: S.K.-M.R.R. declares a competing interest due to his employment at Arkeon GmbH.

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