Production of Polyhydroxyalkanoate (PHA) Biopolymers by Extremophiles

Abstract
The article reviews the current state of knowledge of the production of polyhydroxyalkanoate (PHA) biopolymers under extreme environmental conditions.

Although PHA production by extremophiles is not realized yet at industrial scale, significant PHA accumulation under high salinity or extreme pH- or temperature conditions was reported for diverse representatives of the microbial domains of both Archaea and Bacteria. Several mechanisms were proposed to explain the mechanistic role of PHA and their monomers as microbial cell- and enzyme protective chaperons and the factors boosting PHA biosynthesis under environmental stress conditions. The potential of selected extremophile strains, isolated from extreme environments like glaciers, hot springs, saline brines, or from habitats highly polluted with heavy metals or solvents, for efficient future PHA production on an industrially relevant scale is assessed based on the basic data available in the scientific literature. The article reveals that, beside the needed optimization of other cost-decisive factors like inexpensive raw materials or efficient downstream processing, the application of extremophile production strains can drastically save energy costs, are easily accessible towards long-term cultivation in chemostat processes, and therefore might pave the way towards cost-efficient PHA production, even combined with safe disposal of industrial waste streams. However, further challenges have still to be overcome in terms of strain improvement and process engineering aspects.

Keywords: Archaea; Bacteria; Biopolymers; Extremophiles; Halophiles; Polyhydroxyalkanoate; Psychrophiles; Thermophiles

Abbreviations: \( \mu_{\text{max}} \): Maximum Specific Growth Rate; 16S rRNA: 16 Svedberg rRibosomal RNA Sequencing; 3HB: 3-HydroxyButyrate; 3HD: 3-HydroxyDecanoate; 3HDD: 3-HydroxyDodecanoate; 3HHex: 3-HydroxyHexanoate; 3HN: 3-HydroxyNonanoate; 3HO: 3-HydroxyOctanoate; 3HV: 3-HydroxyValerate; 3UD: 3-HydroxyUnDecanoate; 4HB: 4-HydroxyButyrate; AC: Acetyl-CoA synthetase; BOD: Biochemical Oxygen Demand; b-PHA: Blocky Structured PolyhydroxyAlkanoate; CBF: Cyclic Batch Fermentation; CDM: Cell Dry Mass; CFBF: Cyclic Fed-Batch Fermentation; COD: Chemical Oxygen Demand; CT: CoA Transferase; DSC: Differential Scanning Calorimetry; ECS: Extruded Corn Starch; EPS: Extracellular Polysaccharides; ERB: Extruded Rice Bran; FT-IR: Fourier Transform Infrared Spectroscopy; GBL: \( \gamma \)-Butyrolactone; GC-MS: Gas Chromatography coupled to Mass Spectroscopy; H\(_m\): Melting Enthalpy; kDa: 10\(^3\) Dalton; LPS: Lipopolysaccharides; MCL: Medium Chain Length; MW: Weight Average Molecular Mass; NADH: Nicotinamide Adenine Dinucleotide; NADPH: Nicotinamide Adenine Dinucleotide Phosphate; NMR: Nuclear Magnetic Resonance; PCR: Polymerase Chain Reaction; PHA: PolyHydroxyAlkanoate; PHB: Poly (3-HydroxyButyrate); PHBHV: Poly(3-HydroxyButyrate-co-3-HydroxyValerate); PHV: Poly (3-HydroxyValerate); P; Polydispersity; q\(s\): Specific Production rate; scl: Short Chain Length; SDS: Sodium Dodecyl Sulfate; SEM: Scanning Electron Microscopy; SSCAs: Star-Shaped Cell Aggregates; STEM: Scanning Transmission Electron Microscopy; TCA: Citric Acid Cycle; TDS: Total Dissolved Solids; TEM: Transmission Electron Microscopy; \( T_d\): Onset of polymer Decomposition Temperature; \( T_g\): Glass Transition Temperature; \( T_m\): Melting Temperature; X; Degree of Crystallinity

Introduction
Nowadays, we witness innumerable efforts globally to make processes of "White Biotechnology" ever more cost efficient. In this context, we talk about the bioproduction of diverse bulk and niche products based on the conversion of renewable resources by the action of living organisms or by their biocatalytically active components. Bioproduction of several important products, both from the primary or the secondary metabolism of diverse microbial species, e.g., ethanol, acetic acid, citric acid, bacteriocins, lactic acid, bacterial cellulose, xanthan, or fungal antibiotics is considered as state of the art [1]. In contrast, the bioproduction of products with plastic-like properties is still awaiting its ultimate success on the market [2]. In this context, polyhydroxyalkanoates (PHA) are biologically synthesized polymers of hydroxyalkanoic
Production of Polyhydroxyalkanoate (PHA) Biopolymesters by Extremophiles

by the

General chemical structure of PHA biopolymesters. R

Koller M (2017) Production of Polyhydroxyalkanoate (PHA) Biopolymesters by Extremophiles. MOJ Poly Sci 1(2): 00011.

Metalophil production strain

Nevertheless, in order to make PHA production competitive also in Cycle Assessment, such as the Sustainable Process Index [4-10]. Recent scientific studies, mainly based on modern tools of Life Cycle Assessment, such as the Sustainable Process Index [4-10], have evidenced a number of competitors which, in most cases, display highly recalcitrant cycle, evidenced as follows (reviewed by 3):

a. Renewable carbon sources, such as carbohydrates, lipids, alcohols, methane, or CO$_2$ act as feeding stocks for their production

b. Both biosynthesis of PHA monomers (hydroxyalkanoates) and their subsequent polymerization occurs in vivo by the biosynthetic enzyme machinery of the microbial production strain; this displays a major difference to, e.g., chemically polymerized poly (lactic acid), or degradable "bioplastics" of petrochemical origin like, e.g., Ecoflex from the company BASF SE.

c. PHA are biocompatible, thus allowing their in vivo application in the medicinal field

d. During composting, they undergo complete degradation by biological activity, leaving behind only water, biomass, and CO$_2$ as products of their decomposition.

![Figure 1: General chemical structure of PHA biopolymesters. R symbolizes the side chain of individual monomeric building blocks (hydroxyalkanoic acids), n accounts for the number of methylene group in the monomer, representing the "backbone" length of the monomer, whereas x represents the degree of polymerization.](image1)

The ecological advantages of PHA over their petrochemical competitors which, in most cases, display highly recalcitrant full-carbon-backbone polymers, is evidenced by a number of recent scientific studies, mainly based on modern tools of Life Cycle Assessment, such as the Sustainable Process Index [4-10]. Nevertheless, in order to make PHA production competitive also in economic terms, a number of process steps have to be optimized. Efforts in this direction are to an increasing extent devoted to the selection of inexpensive carbon feedstocks, which can replace costly raw materials typically used for biotechnological purposes, such as sugars or edible oils [11]. Among such inexpensive feedstocks, a number of [agro]industrial surplus materials was already investigated for PHA production, such as waste streams from dairy and cheese making industry [12,13], residues of the biodiesel and animal processing industry [14-16], or various abundant lignocellulosics [17], e.g., hydrolyzed bagasse and fruit peels [18], straw [19, 20] or even spent coffee ground [21]. Further, novel processes to recover PHA from microbial biomass are currently in status of development, predominately aiming at the reduction of the input of chemicals and solvents, and at the conservation of the native polymer properties, hence, the PHA’s molecular mass and its quasi-amorphous state [22-24]. In addition, new process engineering strategies to increase productivity and to optimize the polyester composition on the monomeric level are currently in status of development; here, one-, two-, and multistage continuous process-engineering concepts, optimally matching the kinetic characteristics of PHA biosynthesis (microbial growth phase followed by PHA-accumulation phase, the latter provoked by nutritionally unbalanced conditions together with excess feeding of exogenous carbon source), were designed in the recent years [25,26].

Not only during PHA production, but in many biotechnological processes, microbial contamination displays a major risk for running fermentation batches and thus endangers the economic feasibility of new processes in development [27]. In this context, the application of extremophile production strains enables the cultivation under drastically reduced or even without any sterility precautions. In the case of the halophile PHA-producer Haloferax mediterranei [28] or the thermophile Chelatococcus sp. [29], fermentation batches were operated without sterilizing the bioreactor equipment, and were operated monoseptically for extended time periods. Cultivation processes of thermophilic
microorganisms are considered more energy-efficient due to less energy required for cooling. By the high thermal energy generated by the cell’s metabolism, especially during high cell density cultivation, such thermophilic set-ups display “self-heated” systems. Heat input generated by the bioreactor’s stirring system also contributes to the heating of the fermentation process itself. This makes clear that both heating and cooling costs can be reduced and, similar to the above mentioned application of halophiles, sterile conditions may not be essential during a process involving thermophilic microbes. In addition, the use of production strains which thrive well at extreme pH-values, far away from the pH-optimum of potential contaminants, minimizes the risk of microbial contamination; more specifically, the use of alkaliphile production strains avoids the occurrence of fungal infections [30]. In addition to their biotechnological significance, alkaliphile organisms attract interest in bioremediation processes, e.g., in neutralizing alkaline waste and removing contaminations of heavy metals [31].

The potential of both microbial wild-type strains and genetically modified uni- and multicellular organisms (prokaryotes, yeasts, plants, etc.) for PHA production is exhaustively described in the scientific literature. Microbes are characterized by different optimum conditions for their cultivation. In most cases, biotechnology resorts to such organisms best to be farmed under mesophilic conditions, hence with optima for the pH-value near the neutral range, moderate salinities and temperature between approximately 25 and 37 °C, whereas extremophiles thrive best under conditions that would destroy or inactivate most other organism and, in many cases, cannot even survive in normal environments. Extreme environments encompass low [2 to 20 °C] or high [55 to 121 °C] temperature, high salinity (2-5 M NaCl), high acidity (pH-value below 4), or high alkalinity (pH-value exceeding 8) (reviewed by 30). During the last decades, increasing interest is noticed for biotechnological processes to be carried out under extreme conditions. A range of products, such as thermostable “extremozymes”, ectoins, polysaccharides, or pigments can be obtained from extremophile organisms, and be applied for various marketable products [32]. The quest for both Gram-positive and Gram-negative microbial species to be used as extremophile cellular PHA-bio polyester factories started in the middle of the 1980ies by the discovery of the high PHA accumulation potential of the extremely osmophilic Archaeon Hfx. mediterranei, a versatile PHA- [33], pigment-[34], halocin-[35], and polysaccharide [36,37] producer from the haloaarchal group of extremophiles [38]. Pigment- (C50 carotenoids) and extracellular polysaccharide (EPS) production provides cultures of these organisms a typical reddish and mucous character, as illustrated in Figure 3. This high versatility of Hfx. Mediaterranei was the starting point for the global quest for other extremophiles with high biopolymesters production capacity. The subsequent paragraphs invite to a journey into significant R&D activities accomplished in this field in the past and the recent years, and are devoted to elucidate mechanisms, biological functions and perspectives for industrial scale implementation of PHA production by extremophiles in a not too distant future.

**Discussion**

**Halophile PHA producers**

**General aspects about halophiles:** Halophiles are a phylogenetically versatile group of microbes requiring hypersaline environments with NaCl acting as the major salt component. Nature developed different strategies to adapt living organisms to life in highly saline environments. The predominant approach comprises the accumulation of organic compatible osmotic solutes without at the same time requiring special adaptation of intracellular proteins to the high salinity [39]. The manifold strategies of halophile microbes to handle highly saline environments, and the fact that halophilicity occurs throughout the tree of life, suggests that adaptation to high salinity is, from the metabolic point of view, a rather trivial task. Changing pigment patterns in microalgae, which, besides illumination and temperature, is regarded a prime example of the adaptive response to changing environmental conditions [40]. In the case of halophile PHA producers, Soto and colleagues revealed the role of PHA as chaperons, preventing protein agglomeration under combined stress exerted by salt and temperature, by investigating Pseudomonas sp. CT13, a halotolerant bacterium, and it’s not PHA accumulating mutant. PHA was found to be essential to salt stress resistance, and its productivity positively correlated with salt concentration, evidencing its role as a compatible solute in Pseudomonas sp. CT13 [41].

The application of halophile microbes in biotechnology opens the door for several options: Firstly, high salinity of cultivation medium.
Gram-negative halophilic PHA producers: The first process for PHA production based on the use of the extremely halophile Archaeon *Hfx. mediterannei*, an organism originally isolated from ponds of a solar saltern near Santa Pola at the Iberian coast of the Mediterranean sea, already underlined the positive effect of the used unusual culture medium, containing more than 20 % NaCl, to prevent contamination by alien microbes [44]. The high osmotic pressure of this strain was also used to release PHA granules from the surrounding cell mass via a simple approach: salt concentration below 10 % already caused partial cell lysis; exposure to hypotonic media (distilled water) completely disrupts the cells due to their high inner-osmotic pressure, and sets free the storage material (PHA granules). Separation of PHA granules from cell debris can easily be accomplished by profiting of the density difference of these two fractions, thus simplifying decantation and centrifugation. This allows designing extremely simple production facilities. In contrast to other extremophiles known at that time, the organism displays fast growth, relatively high specific PHA productivity and excellent product quality in terms of low degrees of crystallinity (X_c), low melting temperature (T_m), high molecular mass, and narrow molecular mass distribution (low polydispersity P_d) [45]. In a continuous chemostat cultivation at a dilution rate of 0.12 1/h, and a temperature of 38 °C, a PHA concentration of 6.5 g/L was obtained using 20 g/L starch as carbon source, which is almost twice the value obtained by using glucose (3.5 g/L) [45]. Astonishingly, the strain produced a high-quality poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) copolyester from simple carbon sources such as glucose or starch; normally, 3-hydroxyvalerate (3HV) synthesis requires the supplementation of structurally related precursor compounds like odd-numbered fatty acids (propionic acid, valeric acid, etc.), which contributes considerably to PHBV´s production cost. This unique metabolic feature is based on particularities of the strain’s PHA-biosynthesis pathway, which accumulates high intracellular pools of the 3HV-precursor propionyl-CoA [46]. In 2006, Don and colleagues fractionated the PHBV by using a chloroform/acetone mixture and revealed that the polymer consisted of two fractions of different monomeric composition; the predominant fraction (about 93 wt.-% of the total PHA) contained about 10.7 % (mol/mol) 3HV, whereas the smaller fraction has a higher 3HV content of about 12.3 % (mol/mol) and a drastically lower molecular mass (78.2 kDa vs. 569.5 kDa, respectively), making it soluble even in the classical ‘PHA-non solvent’ acetone. Both fractions displayed low P_l, and similar T_m and glass transition temperature T_g. Via Differential Scanning Calorimetry (DSC), the authors noticed two overlapping melting peaks at heating rates below 20 °C/min. The relative intensity of these two melting peaks varied by changing the heating rate, hence, it was assumed that the phenomenon is caused by a melt/recrystallization process [47].

Based on these pioneering explorations, *Hfx. mediterannei*-mediated PHA production has been further optimized by researchers all around the world. Whereas some of these groups focused on the application of inexpensive carbon feedstocks to safe substrate costs, such as whey permeate from dairy industry [12,13,48], rice-based ethanol stillage [49], extruded rice bran [50], enzymatically extruded starch [51], crude glycerol phase [28], olive mill waste water [52], vinasse [53], etc., others clarified the enzymatic and genetic background of PHA-synthesis [54-57] and *in vitro* degradation [58] by this organisms. Mathematical models of PHA-production [59], and kinetic studies of PHA and by-product synthesis and degradation [60] were reported for *Hfx. mediterannei*. Only recently, Han and colleagues reported that blocky structured copolymers (6-PHA), consisting of blocks of PHB and poly (3-hydroxyvalerate) (PHV), linked to randomly distributed PHBHIV blocks, can be produced by this organism by optimized co-feeding of glucose and valeric acid. The “blocky” feature and the high 3HV fraction of b-PHA provide this novel copolymer with special material features, encompassing enhanced crystallinity and improved Young’s modulus. Due to the fact that the b-PHA exhibited increased platelet adhesion and accelerated blood clotting compared to random PHBHIV, the material was suggested for medical application [61].

Due to its high robustness and stability of the culture, *Hfx. mediterannei* is currently considered the most promising candidate for whey-based PHA production on an industrial scale [12]. The strain grows excellent on hydrolyzed whey permeate with a maximum specific growth rate (µ_max) of 0.11 1/h, an outstandingly value for halarchaea. PHBHIV was synthesized at a maximum specific production rate (q_P) of 0.08 g/(g.h). By optimizing the process conditions, volumetric and specific productivity were increased to 0.09 g/(L.h) and 0.15 g/(g.h), respectively. Highest biomass concentration amounted to 16.8 g/L, containing 73 % PHBHIV [62]. Using hydrolyzed whey permeate as main carbon substrate and valeric acid and γ-butyrolactone (GBL) as precursors for 3HV and 4-hydroxybutyrate (4HB), respectively, enabled the production of a high-quality poly(3HB-co-21.8%-3HV-co-5.1%-4HB) terpolyester. After recovery of the terpolyester and in-depth polymer characterization [DSC, molecular mass, molecular mass distribution], the authors suggested using the terpolyester for high-performance applications, e.g., in the medical field [48]. Based on the high salinity needed to farm *Hfx. mediterannei* efficiently, the risk of microbial contamination is negligible; sterility precautions can be more or less neglected. Microbial contamination was not evidenced when cultivations were carried out over extended time periods even without any sterilization precautions [28]. Economic assessment of *Hfx. mediterannei*-mediated PHA production on hydrolyzed whey and solvent-free downstream processing, based on experimental data from 200 L scale, estimate the production price below € 3 per kg PHA [12].

*Hfx. mediterannei* also constitutes a promising candidate for production of PHA co- and terpolymers based on crude glycerol phase (GLP), a major by-product of the emerging biodiesel production. Hermann-Krauss et al. [28] reported the production of PHBHIV with 10 mol-% 3HV at a volumetric productivity of 0.12 g/(L.h) and 75 % PHBHIV in biomass. Molecular mass was determined with 150–253 kDa, P_l of 2.1–2.7, and T_m between 130 °C and 140 °C in different production setups. Supplying the 4HB-precursor GBL generated a terpolyester containing 12 mol-% 3HV...
and 5 mol-% 4HB (0.05 mol/mol) with lower Tm (two melting endotherms at 122 and 137 °C) and glass transition temperature (Tg, 2.5 °C) and higher molecular mass (391 kDa).

Huang et al. [50] used extruded rice bran (ERB) and extruded corn starch (ECS) as carbon sources for *Hfx. mediterannei* mediated PHA production without nitrogen limitation. Extrusion was needed due to the strain’s lacking ability to directly convert rice bran or cornstarch in its native form. By employing controlled pH-stat feeding strategy (pH-value between 6.9 and 7.1) in a 5 L bioreactor using ERB/ECS mixtures (1/8 g/g) as main carbon source in a repeated fed-batch feeding regime, a cell dry mass (CDM) concentration of 140 g/L, a PHA concentration of 77.8 g/L and a PHA content in CDM of 56 wt.-% were obtained. Using ECS as sole carbon source, 62.6 g/L CDM, 24.2 g/L PHA concentration and 38.7 wt.-% PHA in CDM were reached. Under the applied highly saline conditions, the authors suggest that it was possible to keep the repeated fed-batch process running for mass production of PHA for extended periods.

Corn starch, pre-treated by an enzymatic reactive extrusion process (single-screw extruder with α-amylase quantities at 1-5 g/100 g wet mass of cornstarch), was used for PHA production by *Hfx. mediterannei* by Chen and colleagues [51]. A mixture of extruded starch and yeast extract was used in a ratio of 1/1.7 g/g in the feeding medium to maintain carbon and nitrogen concentrations constant during the pH-stat fed-batch process. PHA content in CDM reached 50.8 wt.-%. Also in this case, a PHBHV copolyester with 10.4 mol-% 3HV, a Tg of -1.2 °C, and with two melting peaks at 129.1 °C and 144.0 °C was produced.

Independent on the carbon sources, the high salinity of the *Hfx. mediterannei* medium of about 20-25 wt.-% NaCl requires special bioreactor material and measuring sensors sustaining the high salinity; high-quality steel or polymers like PEEK were proposed [63]. For reasons of both economics and sustainability, the possibility to recycle the highly saline side streams of this process was subjected towards investigation. Salt disposal after completion of the fermentation constitutes a problem as valid ecological standards do not permit the discharge of total dissolved solids (TDS) above 2,000 mg/L in waste water. In this context, it was demonstrated that the spent fermentation broth from whey-based PHA-production by *Hfx. mediterannei* can be used to substitute a considerable share of fresh saline cultivation medium in next production cycles. Additionally, about 29 % of yeast extract, an expensive growth additive needed as nitrogen-source, was used to substitute a considerable share of fresh saline cultivation medium in next production cycles. Additionally, about 29 % of yeast extract, an expensive growth additive needed as nitrogen-source, was used to substitute a considerable share of fresh saline cultivation medium in next production cycles. Additionally, about 29 % of yeast extract, an expensive growth additive needed as nitrogen-source, was used to substitute a considerable share of fresh saline cultivation medium in next production cycles.

Singh [67] isolated halotolerant and halophilic organisms from (agro)industrial surroundings, and cultivated the strains on different solid media containing 2 M NaCl. Best growth occurred on solid Luria medium; also on selective media (DSC97), significant growth was observed. After optimizing the culture conditions, the organisms were characterized biochemically and by different staining techniques (Gram, Sudan Black, etc.). Based on 16S rRNA analysis, the isolates with the most promising PHA-production potential were identified as Gram-positive *Bacillus subtilis* ssp. and the Gram-negative *Pseudomonas* sp.

Danis et al. [68] carried out similar experiments with five extremely halophilic archaenal isolates in order to trace the strain with the highest PHA-production capacity. PHA production of each isolate was individually examined on various inexpensive carbon sources such as, e.g., sucrose, whey, corn starch, and waste of apples, melons and tomatoes. Among these feedstocks, corn starch turned out to be a promising substrate for PHA biosynthesis. Among the investigated strains, isolate 1KYS1 displayed the highest PHA biosynthesis capacity. Comparative 16S rRNA gene sequence analysis revealed the close relationship of 1KYS1 to the genus *Natrinema*, especially to the species *Natrinema pallidum* [CM9980]. On starch as sole carbon substrate, 1KYS1 reached a PHA content in CDM of about 53.14 wt.-%. Large and uniform intracellular PHA granules were observed by transmission electron microscopy (TEM), and identified as the copolyester PHBHV. PHBHV produced by 1KYS1 was blended with poly(ethylene glycol) of low molar mass for preparation of biocompatible films which can be used for drug release experiment with Rifampicin as model compound. Most efficient drug delivery was achieved at 37 °C and a pH-value of 7.4.

Cyanobacteria form a group of photosynthetic nitrogen-fixing bacteria, which are present in almost all aquatic or terrestrial habitats, and are increasingly in the spotlight as third generation PHA producers [69,70]. Shrivastav and colleagues [71] studied the marine photoautotroph cyanobacterium *Spirulina subalba*, originally described as an isolated from samples from the Indian coast. This organism turned out to display enhanced PHA production when exposed to elevated salinity levels.

---

**Citation:** Koller M (2017) Production of Polyhydroxyalkanoate (PHA) Biopolymers by Extremophiles. MJ Pol Sci 1(2): 0001 L. DOI: 10.15406/mojps.2017.01.00011
In addition, *Halomonas campaniensis* LS21, a halophile and in parallel alkaliphile eubacterium, was isolated and cultivated in an open, non-sterile, continuously operated PHA production process. This process was based on alkaline seawater and artificial carbohydrate kitchen waste, predominantly consisting of polysaccharides, lipids, and proteins. PHB was selected as model product of the strain during long-term cultivation to assess the general viability of open and long-term cultivation of industrially relevant bacteria, and to investigate genetic engineering of this organism to further enhance the process. Both the wild type strain and a recombinant *H. campaniensis* LS21, additionally equipped with the PHB synthesis genes *phb*CAR, were farmed in a continuous process for 65 days in artificial seawater-based medium. Under extremely saline (27 g/L NaCl) and highly alkaline (pH-value 10) conditions and a moderate temperature of 37 °C according to the strain’s optimum values, the genetically engineered strain achieved about 70 wt.-% PHB in CDM, in contrast to 26 wt.-% achieved by the wild type organism. Despite the open process regime, both cultures remained monoseptic. Extracellular hydrolytic enzymes were excreted during the entire cultivation, which enabled them to convert the mixed substrates. Until the end of the cultivation, the plasmid carrying *phb*CAR genes maintained stable in the recombinant cells. Combined with its expedient susceptibility for genetic improvement, *H. campaniensis* LS21 definitely might constitute a powerful cellular factory for cost- and energy-efficient production of PHA or extremozymes from inexpensive feedstocks [72].

In the context of alkaliphily, as described for *H. campestris*, also cyanobacterial strains were found which preferentially accumulate PHA under elevate pH-values. This is especially the case for *Spirulina platensis*, for this organism, the optimum pH-value for both PHA production and degradation is reported in the rather strong alkaline range of 9 to 11 [73].

The halophile bacterium *Halomonas*TD01, isolated from a Chinese salt lake, was used by Tan and associates [74] for a similar non-sterile, continuous cultivation process. In 56 h fed-batch fermentation set-ups based on glucose as the sole carbon source, the strain reached up to 80 g/L CDM containing 80 wt.-% PHA. In an open, non-sterile, continuous two-stage process operated for two weeks, CDM reached an average concentration of 40 g/L with about 60 wt.-% PHB in CDM in the first stage, which contained a saline medium rich in glucose and nitrogen. The fermentation broth was continuously transferred from the first to the second, nitrogen-free stage. Although this transfer diluted the CDM concentration, a constant PHB fraction in CDM between 65 and 70 wt.-% was reached. In the first stage, between 0.20 and 0.30 g PHB were generated per g converted glucose, but, surprisingly, more than 0.50 g per g in the second stage.

*Halomonas* TD01 was later subjected towards genetic engineering. By knocking out the 2-methylcitrate synthase encoding gene, propionate-to-3HV conversion efficiency was almost doubled, resulting in an increase of 3HV in random PHBHV copolyester of almost 100 %. In a minimal medium containing glucose and 0.5 g/L propionate, cells accumulated 70 wt.-% PHBHV in CDM with a molar 3HV fraction of 12 %. The effect of deleting the genes encoding for PHA depolymerase in order to prevent intracellular PHA degradation, especially in later stages of large-scale cultivations, was also investigated, but the overall PHA-output was not significantly enhanced. In 500 L pilot-scale cultivation studies, CDM of genetically engineered *Halomonas* TD01 reached 112 g/L, with 70 wt.-% PHB in CDM on glucose as the sole carbon source; in the presence of propionate, 80 g/L CDM with 70 wt.-% PHBHV (8 mol-% 3HV in PHBHV) were obtained. On shaking flask scale, PHB mass fractions of even 92 wt.-% and significantly enhanced glucose-to-PHA conversion were achieved. Further experiments to increase stability of genetically engineered *Halomonas* TD01 encompassed the partial inhibition of the DNA restriction/methylation system, and, in order to induce the expression of multiple pathway genes, the construction of a stable conjugative plasmid pSEVA341. Together with the above described insertion of 2-methylcitrate synthase and knockout of depolymerase, the new construct *Halomonas*TD08 was able to accumulate up to 82 wt.-% PHBHV in CDM [75]. Stability and performance of all these promising genetically engineered halophiles should now be tested for long-term stability under continuous operation.

Han et al. [76] were the first who experimentally investigated the genes responsible for archaeal PHA synthesis. The authors revealed that the halarchaeon *Haloarcula marismortui*, isolated from the Dead Sea, was able to accumulate up to 21 wt.-% PHB in CDM in minimal medium containing excessive glucose, and identified the adjacent genes *phaEHm* and *phaCHm* which encode two subunits of a Class-III PHA synthase and are directed by a single promoter upstream of the transcriptional start site; the genes were constitutively expressed under both nutritionally balanced and nutrient-limited conditions. Remarkably, *PhaCHm* turned out to be, in contrast to *PhaEHm*, strongly connected to the PHA granules. The introduction of *phaEHm* or *phaCHm* into *Haloarcula hispanica*, a strain harboring highly homologous *phaECHh* genes and widely used in halochiral studies, particularly for isolating haloviruses, boosted PHB synthesis in this recombinant organism; coexpression of both genes resulted in highest PHB production. It is worth to mention that the knockout of *phaECHh* genes in *H. hispanica* resulted in a total termination of PHA synthesis. PHA accumulation capability and PHA synthase activity was successfully restored by complementation with *phaECHm* genes. These outcomes demonstrated the importance of *phaEC* genes for PHA accumulation in Archaea. Later, Ding et al. [77] carried out the sequencing of the genome of a *H. hispanica* ssp., and observed significant differences from the published gene sequence of the model strain H. *hispanica* ATCC 33960.

Estuaries and coastal marine sites are examples of saline environments rich in halophilic microbes. Lau et al. [78] reported the gene sequence of a *Yangia* sp. CCB-MM3, a halophilic bacteria isolated from Malaysian Matang Mangrove soil sediments, as the first completely deciphered gene sequence of *Yangia* sp., which are members of the Rhodobacteraceae family. This ecosystem is influenced by both marine conditions and by freshwater flow. The genome consists of two chromosomes plus five plasmids, has a total size of 5.522,061 bp with 65 % GC content in average. Genome sequence analysis confirmed the presence of a propionyl-CoA synthesis pathway and of a gene cluster for PHA production in this strain. The ability of *Yangia* sp. CCB-MM3 for
PHA accumulation was tested and confirmed in vitro. The authors suppose that the genome sequence of this strain will considerably contribute to the understanding of the physiology and metabolism of *Yangia* sp., and should be compared with the genome of other and new members of the Rhodobacteraceae family.

**Gram-positive halophile PHA producers:** *Bacillus megaterium* uyunii S29 represents the still rather scarce number of described Gram-positive organisms which produce significant amounts of PHA in saline environments. This strain, originally isolated from the Uyunii salt lake in Bolivia [79], features high capacity of PHB production in typical nutrient media used in industrial biotechnology [80]. The impact of fluctuating salinity on growth and PHB production by this strain was assessed under different NaCl concentrations selected according to the salinities dominant in the strain’s natural salt lake environment [81]. The strain displayed astounding adaptability to fluctuating salinity, with best results for growth and PHB accumulation at 45 g/L NaCl. The strain still grows well at salinities as high as 100 g/L NaCl, PHB production was detected even at 25 wt.-% NaCl. As another benefit, no sporulation, which typically restricts the application of Gram-positive strains for PHA production due to the loss of the carbon flux, was observed in all experiments. Like all Gram-positive strains, this organism does not produce inflammatory active endotoxins [lipopolysaccharides, LPS], which impedes the in vivo application of its PHA in the medical field. Overall, this strain appears appealing not only for production of PHA for biomedical purposes, but also for other biotechnological process such as saline wastewater treatment.

**Tracing halophile PHA producers:** Numerous techniques for tracing PHA in various different microbes were described in the past [82]. In the case of halophiles, existing PHA detection methods are not precise enough, frequently deliver false positive results, and/or need prior PHA synthesis before the screening. To overcome this shortcoming, Mahansaria et al. [83] developed a new method based on amplifying the conserved gene region phaC, encoding for the Class-III PHA synthase of halophiles. This region is about 280-300 bp in size. For the amplification, the primers codehopCR and codehopCR, developed by Han et al. [84], were used. The new method was tested with in total nine known haloarchaea and halobacterial strains, and 28 own halophilic isolates from the Indian coast. Twenty-nine strains were phaC-positive, eight phaC-negative despite positive results from previous Nile Red staining. 16S rRNA analysis identified nine novel haloarchaea and nine novel halobacteria as PHA producers. Based on multiple sequence alignment of the phaC gene-derived amino acid sequences, it was shown that only seven amino acid residues were conserved within all four synthase classes encoded by phaC, whereas 61 amino acids were identical among the synthase encoded by the phaC specific to the investigated halophiles. All phaC-positive strains produced PHA in nutrient-limited medium, whereas no PHA production was evidenced by the phaC-negative strains. The authors evaluated their new method as highly precise, because it enables eliminating false positive results from Nile Red staining, e.g., due to the accumulation of lipophilic inclusion products different to PHA [83].

**Cryophilic [Psychrophilic] PHA Producers**

**General aspects about cryophilic organisms:** Low temperature is a critical factor in sustaining cell division and survival of organism. Reduced protein synthesis, cold-denaturation of proteins, decreased membrane fluidity, low cross-membrane diffusion rates, formation of ice crystals, and production of noxious highly reactive oxygen species are typical results of the exposure to low temperature. Frequently, microbes living in cold environments are exposed to several stress factors in parallel. Deep-sea organisms, for example, have to endure low temperature and high pressure conditions, while microbes in polar regions usually have to tolerate low temperature, nutrient deficit, and increased UV-radiation. Consequently, microbes resistant to multiple stress factors are to be selected in such extreme environments [85]. A range of different sea-ice bacteria populate small brine channels and have developed a biofilm-like organization, characterized by high cell densities and EPS excretion [86]. Substrate disposal and low temperature act as cooperative growth-restricting factors; nevertheless, some microbes are still able to grow in cold, even frozen water, unless both substrate availability and temperature reach a critical level at the same time [87]. Although sea ice is colder than the seawater underneath, the concentrations of (macro)nutrients and convertible carbon sources can be higher, thus enabling sea-ice microbes to grow and probably to store carbon and energy as PHA granules [87]. Presence of PHA increases the fitness of the microbes under such changing environmental conditions.

**Reports on PHA production by psychrophilic microorganisms:** Ayub et al. [88] have reported that *phaB* genes from *Pseudomonas* sp. 14-3, a Gram-negative, mobile, rod-shaped, non-spore-forming Antarctic bacterium, are located on an isolated gene region, which harbors other genes, which most likely contribute to its adaptability to low temperature. Later, the same researchers studied the effect of PHA accumulation on the adaptability of *Pseudomonas* sp. 14-3 to low temperature by direct comparison to its PHA synthase-minus mutant, obtained by deleting the *phaC* gene encoding for the PHA synthase. The PHA-negative mutant was more prone to freezing than its parent strain, and did not grow at 10 °C. It was assumed that PHA-reserves are pivotal to develop the oxidative stress response provoked by low temperature. It was demonstrated that the cold-sensitive phenotype of the *phaC*-negative mutant can be suppressed by adding reduced compounds [cysteine, glutathione]. Furthermore, a sudden cold shock provoked very rapid PHA degradation and a drastic decrease of the NADH/NAD ratio and NADPH content in the PHA-positive strain. Consequently, lipid peroxidation in the mutant strain was 25-fold higher after decrease of temperature. Hence, PHA metabolism controls the availability of the reducing equivalents NADH and NADPH, thus alleviating the oxidative stress caused by cold temperature, by supplying the reductive power necessary to subdue the oxidative stress induced by cold conditions via PHB degradation [89].

The same organism, *Pseudomonas* sp. 14-3, was characterized by López et al. [90] by 16S rRNA gene sequence analysis as well.
as by physiological and biochemical tests. It was revealed that the strain belongs to the genus *Pseudomonas sensu stricto*, and displays 99.7% sequence similarity to *Pseudomonas veronii* DSM 11331T, although DNA-DNA hybridization experiments between the two strains revealed only modest re-association similarity. The bacterium grew in a temperature range from 4 to 37 °C, but not at moderately thermophile conditions of 42 °C. When cultivated on sodium octanoate, it accumulated the homopolyester PHB. The authors proposed to classify the organism as the type strain of the new species entitled *Pseudomonas extremaustralis* sp. nov. Tribelli et al. [2011] carried out further experiments with this strain and its PHA-negative mutant by investigating the relationship between its PHA accumulation and EPS production at low temperature in shaking flask cultures or in cultures embedded in poly (styrene) biofilm microplates. Microaerobic growth, EPS production and motility were studied. It turned out that, under cold conditions, PHA accumulation goes in parallel with increased motility and supports the survival of planktonic cells in the developed biofilms. Microaerobic conditions, to a certain extent, saved the cold growth deficiency of the PHA-negative mutant. The authors assumed that the capability for PHB accumulation might display an adaptive advantage for the settlement in new, environmentally stressful ecological niches. Later, the same authors sequenced the entire genome of the strain in order to identify all genes responsible for PHA metabolism, adaptability to extreme environments, and degradation of toxic compounds. As a result, the first completely sequenced genome of a psychrophilic organism is reported [91]. Further, they investigated the strain’s potential for bioremediation, specifically for degradation of the eco-pollutant diesel. Such bioremediation requires the use of microbes capable to adapt to extreme environments contaminated with toxic compounds. Different cultivation conditions for *P. extremaustralis*, namely embedding the cells in biofilm (benthic cultivation), or as planktonic cells in agitated shaking flask cultures, were applied. Benthic cultures, hence, those protected by biofilms, displayed better growth, more biosurfactant production, and higher diesel degradation, if compared with the planktonic cultures. PHA accumulation decreased the attachment of biofilm cell and enhanced the production of biosurfactants. Long-chain and branched alkane were degraded in biofilms, while free cells degraded only medium-chain length alkanes. These outcomes suggest the cryophilic PHA-producer *P. extremaustralis* as an excellent candidate for hydrocarbon bioremediation in extreme environments [92].

Giesielski et al. [93] searched for additional cryophilic microbial PHA producers in freshwater reservoirs of the Antarctic Ecology Glacier foreland. 16S rRNA gene sequence of isolated strains enabled their classification as members of the classes of α-, β-, or γ-proteobacteria, followed by detecting the individual PHA synthase genes via PCR. Potential PHA producers were isolated from all investigated sampling sites; predominantly, the organisms belonged to the genera *Pseudomonas* and *Janthinobacterium*. This report on the frequent occurrence of PHA producers in such cold environments was a further evidence that PHA synthesis occurs as a common feature of pioneering microbes. The isolates belonging to the genus *Pseudomonas* had the genetic potential to synthesize medium-chain-length PHA (mcl-PHA; evidenced by the presence of phaC1 genes), whereas some new isolates of *Janthinobacterium* might produce either short-chain-length PHA (scl-PHA) or mcl-PHA (evidenced by presence of both phaC and phaC1 genes) as the first report revealing mcl-PHA production by *Janthinobacterium* sp. The authors also observed the lacking correspondence between the evolutionary history of the 16S rDNA genes and those coding for PHA synthases of the isolated *Janthinobacterium* sp. strains. Based on phylogenetic analyses of phaC and phaC1 the authors assume that these genes could have entered the genomic material of *Janthinobacterium* sp. via horizontal gene transfer, and assumed that presence of these PHA genes may increase bacterial fitness for survival under environmentally harsh conditions.

In order to better understand the role of PHA production in environment characterized by both low temperature and high salinity, a long-term study spanning over a 10-years period was accomplished by Pärnänen et al. [94] to search for PHA producers in the sea ice, the cold seawater, and in the cold estuarine of the northern Baltic Sea, more specifically on the Nord-Eastern coast of Sweden, and the Western and South western Coast of Finland. Samples collected in these extreme environments were scrutinized for PHA producers by Nile Blue A staining of microbial inclusions, and by PCR assays to detect PHA-synthase-encoding genes. Both PHA granules and PHA synthase genes were found in sea water and ice at all sampling sites. By phaC gene sequencing, high similarities of the isolates with the species *Rhodoferax ferrireducens*, *Polaromonas napthalenivorans* and *Polaromonas* sp. [β-proteobacteria], and *Pseudomonas extremaustralis* strain 14-3 [γ-proteobacterium] was evidenced. The study proofed for the first time PHA synthesis by microbes adapted to the environmental conditions of the Baltic Sea, making this region an interesting spot in the search for new PHA producers.

Cryo scanning electron microscopy (cryo-SEM) provides a modern tool to investigate the influence of intracellular PHA granules on the cell’s mechanical properties, mobility of cytoplasmic solutes, and the cell’s reaction to temperature changes, e.g., during freezing, thawing, or drying [95]. Using this tool, Obruca and associates [96] revealed the cryo-protective effect of PHB in bacterial survival under freezing conditions on a mechanistic basis. PHB’s monomer 3HB was recognized as a powerful cryo-protectant for lipase, yeasts, and the PHB-producing bacterium *C. necator* during freezing-thawing cycles. Viability of frozen–thawed PHB-positive *C. necator* cultures was drastically increased if compared to that of the PHB-negative mutant cultures. Presence of intracellular PHB granules featured a significant advantage during freezing; the authors assumed that this effect is partly due to the high levels of 3HB in strains capable of simultaneous PHB synthesis and mobilization, as it is the case in *C. necator* cyclic PHB metabolism. Apart from the beneficial role of 3HB, the cryo-protective mechanisms of PHB granules seem to be more complex, as detected by cryo-SEM observations: even at extremely low temperatures, intracellular PHB granules preserve their high flexibility, which make it likely that they prevent cellular damage by intracellular ice crystals, such as physical membrane damage, gas bubble formation, or disruption of organelles. The presence of PHB granules seems to change the adhesive forces between water molecules and cellular components; water in PHB-containing cells obviously can be more easily released from the
cell during drying or freezing by a higher rate of transmembrane water transport, which prevents ice formation.

**Thermophile PHA producers**

**General aspects about thermophiles:** Cultivations in bioreactors carried out with thermophilic microorganisms are considered energy-efficient, because only little cooling efforts are required. In addition, thermophilic fermentations are "self-heating" due to the heat energy generated by the microbes’ metabolic activity, as especially noticed at cultivations with high biomass concentration. In addition, the bioreactor’s stirring system generates heat energy, which can also be provided to the fermentation process. These multiple effects enable the lowering of both heating and cooling costs. As an additional benefit in contrast to the application of mesophilic bacteria, sterility precautions may be saved when culturing thermophilic organisms, which is similar to the advantages described above for halophilic organisms [29].

Mechanistic consideration about the interrelation between high temperature and the metabolic PHB cycle were recently carried out by Obruca and colleagues [97], who revealed the role of the monomer 3HB as a chemical chaperone protecting several enzymes like lipase and lysozyme from denaturation by high temperature and oxidation. High temperature-mediated lipase denaturation in the presence or absence of 3HB was monitored, and showed a significant protective effect of PHB; this effect was even higher than displayed by common chaperones like trehalose and hydroxyectoine. PHB was able to protect lipase not only against heat-mediated denaturation but also against oxidative damage by Cu²⁺ and H₂O₂; its protection was higher than that of trehalose and comparable to that of hydroxyectoine. These results confirm previous reports by Soto et al. [41], who suggested the role of PHA in preventing enzyme denaturation under extreme salinity and temperature conditions.

**Gram-negative thermophile PHA producers:** Pantazaki et al. [98] were the first to study PHA biosynthesis by the thermophilic bacterium *Thermus thermophilus*. At outstandingly high temperatures up to 75 °C, the authors used sodium gluconate or sodium octanoate as sole carbon sources, and reached PHA contents in CDM of about 35-40 wt-%. The two substrates resulted in completely different types of PHA in terms of their monomeric composition. Whereas cultivation on gluconate resulted in a copolyester consisting mainly (about 64 mol-%) of 3-hydroxydecanoate (3HD) and smaller fractions of 3-hydroxyoctanoate (3HO), 3HV, and 3HB, whereas analysis of the PHA obtained by cultivation on octanoate revealed the composition of this copolyester as follows: 3HB 24.5 mol-%, 3HO 5.4 mol-%, 3-hydroxyoctanoate (3HN) 12.3 mol-%, 3HD 14.6 mol-%, 3-hydroxydecanoate (3HDD) 35.4 mol-%, and 3-hydroxyoctanoate (3HDD) 7.8 mol-%. The first copolyester (based on gluconate) had a weight average molecular mass of Mw = 480,000 g/mol, whereas Mw of the second polymer (based on octanoate) amounted to 391,000 g/mol. Activities of enzymes pivotal for PHA biosynthesis, namely 3-ketothiolase, NADPH-dependent reductase, and PHA synthase were measured in the soluble cytosolic fraction obtained from *T. thermophilus* cells grown on gluconate. PHA synthase from gluconate-grown cells was isolated, purified, and characterized, revealing temperature and pH optimum for its enzymatic activity of about 70 °C and pH 7.3, respectively. Free CoA and alkaline phosphatase completely inhibited PHA synthase activity. In contrary to other known PHA synthases, kinetics of this enzyme did not exhibit any lag phase. The authors provide further predicted an important role of cysteine in the catalytic mechanisms of the synthase as the first purified and characterized thermophilic PHA synthase. In a later study, the authors used PCR-based methods to identify the PHA biosynthesis genes in the strain’s genome. The experiments revealed that *T. thermophilus* HB8 belongs to PHA producers harboring Class-II PHA synthases [99].

In the field of phototrophic thermophiles, Hai et al. [100] screened 11 different cyanobacteria for their capacity of PHA production, and determined the type of involved PHA synthase. For the investigations, Southern blot analysis using a *phaC*-specific DNA probe, Western blot analysis using specific polyclonal anti-PhaE antibodies raised in this study against PhaE of *Synechocystis* sp. strain PCC 6803, PCR sequence analysis, and sequence analysis of PHA synthase structural genes, were used. These methods revealed the presence of a Class-III PHA synthase in the *cyanobacterial species Anaabaena cylindrica* SAG 1403-2, *Chlorogloeopsis fritschii* PCC 6912, *Cyanothece* sp. strains PCC 7424, PCC 8303 and PCC 8801, *Gloeocapsa* sp. strain PCC 7428, and *Synechococcus* sp. strains MA19 and PCC 6715, whereas no Class-III synthase genes were found in *Cyanothece* sp. strain PCC 8955, *Gloeocapsa* sp. strain PCC 6501, and *Stanieria* sp. strain PCC 7437. Protein extracts and chromosomal DNA of *Synechocystis* sp. strain PCC 6803, the first cyanobacterium with completely available PHA synthase genes, acted as positive control. The entire PHA synthase structural genes of *Chlorogloeopsis fritschii* PCC 6912 and *Synechococcus* sp. strain MA19 and a central region of the *Cyanothece* sp. strain PCC 8303 *phaC* gene of, were cloned, sequenced, and transferred into *Escherichia coli* for heterologous expression.

*Synechococcus* sp. MA19, a thermophilic cyanobacterium known for photoautotrophic production of high amounts of PHB, was isolated from the surface of a Japanese volcanic rock, as originally described by Miyake and colleagues [101]. This strain grows best at 50 °C and, when cultivated photoautotrophically in simple bottles by a submerged aeration stream enriched with 2 % CO₂, can accumulate up to 21 wt-% PHB in CDM. This was the first report suggesting a cyanobacterial strain as serious candidate for PHA production on larger scale. Careful fine-tuning of CO₂ supply and illumination are decisive to improve PHA productivity by this strain, requiring an optimized process engineering for large-scale implementation. In nitrogen-free dark cultivation setups, it was possible to boost the PHB content in CDM to even 27 wt-%; this was based on the degradation of a second storage compound of this strain, glycogen, under dark conditions. Once accumulated, intracellular PHB was only degraded in nitrogen-rich conditions under illumination; no PHB degradation occurred in the dark, neither with nor without exogenous nitrogen.

In addition to nitrogen-limited cultivation conditions, further efforts to boost PHA production in thermophile cyanobacteria were undertaken by Nishioka et al. [102], who cultivated the organism under phosphate-deprivation. Under autotrophic conditions and phosphate limitation at 50 °C, PHB production...
at intracellular phosphate levels between 0.043 and 0.076 mmol per g CDM was investigated in *Spirulina* sp. MA19. 55 wt.-% PHB in CDM, 2.4 g/L PHB and 4.4 g/L CDM were achieved in a 260 h old culture containing Ca$_3$(PO$_4$)$_2$ as insoluble phosphate source, which was almost the two-fold output in comparison with setups well supplied with phosphate. Based on the results, the authors suggested to operate fedbatch cultures with this organisms in such a way to prevent phosphate concentration to exceed the intracellular phosphate level.

In the field of chemoorganothroph extremophile PHA producers, Chen et al. [103] isolated an amylose-excreting strain from a hot spring located in Pingtung, Taiwan, and labeled it as isolate On1T. Its cells were Gram-negative, monotrich flagellated rods. Temperature optimum for growth was determined with about 55 °C, together with a neutral pH optimum, whereas 16S rRNA gene sequence analysis classified it as a member of the phylogenetic class of β-proteobacteria. Based on 16S rRNA sequences, DNA-DNA similarity, physiological and biochemical particularities, and its fatty acid pattern, the strain was determined as a new species in the genus *Caldimonas*. The authors proposed its classification as *Caldimonas taiwanensis* sp. nov. Later, Sheu et al. [104] detected that, under nitrogen-limited conditions, *C. taiwanensis* produces PHB at 55 °C from common carbon sources such as fructose, gluconate, glycerol, and maltose, but not from fatty acids if provided as sole carbon source. PHBHV copolyester production occurred by co-feeding a mix of gluconate and valerate. Depending on the valerate concentration, the molar HV fraction was triggered from 10 to 95 mol-%. These outcomes are coherent with the strain *Caldimonas manganoxidans* previously described as PHA producer at a temperature optimum of about 50 °C by Takeda et al. [105]. Similar to other PHA producing strains such as *Burkholderia fungorum* (previously *Pseudomonas hydrogenovora*) [106], valerate substantially inhibited growth and PHA synthesis already at levels as low as 5 mM in Sheu’s experiments with *C. taiwanensis*. Yeast extract supplementation elevated the inhibiting effect, and enhanced both biomass yield and PHA productivity. In vivo tests with isolated *C. taiwanensis* PHA synthase showed its substrate specificity for 3HB, 3HV, and 3-hydroxyhexanoate (3HHx). Based on the strain’s amylose activity, cultivations were also performed on mixtures of soluble starch from cassava, corn, potato, sweet potato or wheat as main carbon source and valerate as 3HV precursor, again resulting in the production of PHBHV copolymers with different 3HV fraction in dependence on the starch/valerate ratio in the substrate feed. Based on the conversion of inexpensive starch as carbon source and the reduced cooling costs by the thermophilic process regime, the authors underline the promise of this strain for cost-efficient PHBHV production.

Ibrahim et al. [107] isolated and screened a range of thermophilic microbes able to convert inexpensive carbon feedstocks to PHA under elevated temperature conditions. The microbes were enriched from a German aerobic waste treatment plant, and from Egyptian hot springs. Nile Red staining of colonies grown on solid agar plates with different sole carbon sources resulted in the isolation of six Gram-negative bacteria. During growth, five of these strains, labeled MW9, MW11, MW12, MW13 and MW14, were organized in stable star-shaped cell aggregates [SSCs], whereas strain MW10 occurred as a consortium of individual planktonic rods. 16S rRNA gene sequence analysis revealed all of them as members of the class of α-proteobacteria. All isolates revealed 16S rRNA gene sequence similarity of more than 99 %, and highest similarities between 93 to 99 % with *Bosea minititlanensis*, *Bosea thiioxidans*, *Chelatococcus asachavorans*, *Chelatococcus daeguensis*, *Chelatococcus sambhunathii*, and *Methylobacterium lusitanum*. MW9, MW10, MW13 and MW14 preferred glucose as carbon source, whereas MW11 and MW12 grew best and produced most PHB on glycerol. Highest attained values for CDM concentration and PHB fraction in CDM were 4.8 g/L and 73 wt.-%, respectively. All strains grew between 37 and 55 °C, with 50 °C as the optimum growth temperature. The authors highlighted their study as the first report on SCA formation as well by thermophiles as by PHA-accumulating strains, and emphasize again the high economic potential of PHA-producing thermophiles in terms of process cost reduction. The authors characterized strain MW9 by 16S rRNA gene sequence analysis and suggested its relationship to the genus *Chelatococcus*. Later, rep-PCR genomic fingerprints, fatty acid pattern, G+C content of DNA, and partial dnaK gene sequence confirmed this assumption, but also revealed that these isolates differ from other described *Chelatococcus* sp. DNA-DNA hybridization with other *Chelatococcus* sp. revealed degrees of relation between 11.0 and 47.7 %. The authors therefore suggested the classification of the strain MW9 as a novel *Chelatococcus* sp., and proposed the name *Chelatococcus thermostellatus* for this powerful candidate for PHA production at elevated temperature.

A second isolate of Ibrahim’s series [29], *Chelatococcus* sp. strain MW10, was subjected by the same group of scientists towards experiments aiming at increasing CDM by advanced cultivation techniques. In a fedbatch fermentation on a 2 L bioreactor scale, excess glucose above 20 g/L was provided by pulse feedings to boost both biomass and PHB production, and to avoid intracellular PHB degradation. Maximum CDM and PHB concentration was obtained after 53 h with 5.2 ± 0.6 g/L and 2.9 ± 0.7 g/L, respectively. Unfortunately, in the later phase of the process, PHB content drastically decreased, despite the permanent availability of carbon source. Therefore, a cyclic batch fermentation (CBF) was carried out on a 42 L bioreactor scale at 50 °C and 50 g/L glucose. Cycle time (50 h cultivation batches) was chosen according to the results from the the 2 L fedbatch setup. The cultivation process was initiated with a volume of 25 L; pO$_2$ was controlled permanently at 20 % saturation by adjusting the stirrer speed and the aeration rate. During the first cycle, high growth ($\mu_{max} = 0.125$/h) and significantly increased CDM up to 12.7 ± 0.9 g/L was reached, whereas PHB concentration (55.0 ± 5.7 wt.-%) was comparable to the value from the fedbatch setup. Now, 23 L of the fermentation broth were withdrawn, and replaced by the same volume of fresh, not sterilized medium. Remaining fermentation broth in the bioreactor acted as inoculum for the next cultivation cycle. Similar CDM of about 11 g/L, but lower PHB contents in CDM (38.5 ± 6.4 wt.-% and 32.5 ± 3.0 wt.-%) were reached after the second and third cycle run, respectively, despite the permanent presence of glucose. As further process improvement, cyclic fed-batch fermentation (CFFB) was carried out as a modified semi-continuous culture technique, again on
a 42 L scale. Here, different volumes of the fermentation broth were replaced by fresh medium, in that way partly recycling 20 to 40 % of the fermentation broth’s volume. Culture cycling was done at different intervals, considering the volume increase due to the semicontinuous feeding, and also the reduced pH level which occurred during the high cell density fedbatch process. In principle, adequate concentrations of glucose and ammonium were chosen to reach high CDM concentration and high PHB fractions in biomass. The CBF was started as a batch process with 30 g/L glucose. Feeding of fresh medium was initiated after 21 h of growth. The first cycling was performed after 44 h of cultivation, when the cells grew relatively fast with a μ of 0.070 1/h and well before PHB degradation had to be expected. Five L of the fermentation broth was replaced by the same volume of fresh medium. Subsequently, continuous feeding was carried out, followed by subsequent medium replacement cycles according to the need to minimize the volume increase. In order to prevent excessive dilution of the culture broth, the second cycle was completed by withdrawing 10 L of culture volume and replacing by only 5 L of fresh medium. After 14 h in the third cycle, a final refilling of 5 L of fresh medium was accomplished. Highest PHB contents exceeding 50 wt.-% were obtained between 82 h and 143 h during cycle 2. After finishing this cycle after 181 h, 43.0 ± 1.4 g/L CDM, a polymer fraction in CDM of 39.0 ± 8.5 wt.-%, and the maximal PHB productivity of 16.8 ± 4.2 g/L were achieved. Cell growth increased drastically at the end of cycle 3, when a cell concentration of 1150 ± 4.3 g/L was monitored. Although lower PHB fraction in CDM of 11.8 ± 3.8 wt.-% was noticed at the end of this process, a promising volumetric PHB productivity of 13.7 ± 4.9 g/L was achieved.

In 2014, the thermophile Chelatococcus daeguensis TAD1, a strain closely related to Chelatococcus sp. strain MW10, was isolated by Xu et al. [108] from a biological mat of a biotrickling filter designated for NO removal, and extensively investigated and compared to other PHB-producing microbes. This organism is a thermophilic PHB-producing bacterium. C. daeguensis TAD1 produced PHA in a growth-associated way without nutrient deprivation; within only 24 h of cultivation at 45 °C, the strain accumulated PHB up to 83.6 wt.-% of CDM from glucose as the sole carbon source. As a remarkable outcome, C. daeguensis TAD1 displayed high tolerance to heat stress as well as to high nitrogen loads during PHB accumulation. On glucose, the highest PHB concentration, 3.44 ± 0.3 g/L, occurred at 50 °C and a molar C/N-ratio of 1/30. Further, the strain could efficiently convert various inexpensive carbon sources to PHB, such as glycerol and non-hydrolyzed starch. Using glycerol, the highest product yield (0.26 g PHB per gram converted glycerol) as well as PHB fraction in CDM (80.4 wt.-%) were obtained. As a consequence, the authors suggest also C. daeguensis TAD1 as a viable production strain for industrial-scale PHB production on expense feedstocks. These results were further improved by the same research group, who reported on the possibility of further increasing C. daeguensis TAD1-mediated PHB productivity on glycerol by a fed-batch cultivation regime. The authors discovered that PHB accumulation is inhibited by excess glycerol, but found out the beneficial effect of organic nitrogen sources like tryptone or yeast extract to boost growth of this strain. In batch fermentation setups, the application of low glycerol concentrations together with the supply of a nitrogen source cocktail consisting of NH₄Cl, tryptone, and yeast extract, significantly promoted PHB biosynthesis by C. daeguensis TAD1. Based on these batch-results, fedbatch and two-stage fedbatch cultivation techniques were developed. In the two-stage fedbatch process, best results for PHB concentration (17.4 g/L), corresponding to a volumetric productivity of 0.434 g/(Lh), which is the currently highest PHA productivity reported for extremophiles, were obtained [109].

**Gram-positive thermophilic PHA producers:** In contrast to the huge number of reports on PHA production by Gram-negative bacteria and archaea, investigation of Gram-positive microbes able to produce PHA efficiently is still manageable, especially regarding the small number of reports on PHA production by extremophile Gram-positive strains [81]. However, PHA produced by Gram-positives is characterized by low levels of LPS causing immunogenic reactions, making them interesting for biomedical applications. The first report on a thermophilic Gram-positive PHA producer was provided by Liu et al. [110], who describe the PHA accumulation by the bacterial isolate K5, which displayed excellent growth and PHB-accumulating capacity. Strain K5 has been isolated from the biofilm of a biotrickling filter designed for the removal of NOX from flue gas of a Chinese coal-fired power plant. Based on 16S rRNA gene sequence analysis and physiological and biochemical characterization, strain K5 has has turned out as a subspecies of Bacillus shackletonii, a species only scarcely found in literature. B. shackletonii K5 accepted glucose as carbon source for PHB synthesis at meso- and thermophilic temperatures between 35 to 50 °C, with the temperature optimum determined at 45 °C. Using glucose on shaking flask scale, the strain accumulated up to 69.9 wt.-% PHB in 22.8 g/L CDM. Employing succinate or glycerol as carbon source, 56.8 and 52.3 wt.-%, respectively, of PHB in CDM were obtained. In batch-mode cultivations at 45 °C and pH 7.0, B. shackletonii K5 produced PHB of up to 72.6 wt.-% of CDM, which amounted to 9.76 g/L on glucose as sole carbon source.

A further screening of thermophilic, contamination-resistant PHA producers was carried out by Xiao et al. [111]. During this study, the strain XH2, isolated from an oilfield, was characterized from a morphological, physiological, and biochemical perspective, and, supported by 16S rDNA analysis, classified as Aeranubibacillus sp. Nile red staining and transmission electron microscopy (TEM) revealed PHA granule accumulation by this strain, which was the first evidence for PHA production by a thermophilic representative of the phylum Firmicutes. By batch cultivation on glucose, peptone, and yeast extract at 55 °C, 111.6 mg/L PHA were produced. The PHA output was more than doubled in peptone-free medium. The produced PHA product mainly consisted of 3HB and 3HV, which were mainly found in literature. B. shackletonii K5 produced PHB of up to 72.6 wt.-% of CDM, which amounted to 9.76 g/L on glucose as sole carbon source.

Giedraitytė et al. [112] detected PHB production by the thermophilic organism Geobacillus sp. AT 946034, which can be cultivated at temperatures up to 60 °C. Under phosphate-limited conditions and glucose acting as the sole carbon source, the highest PHB fraction of 68.9 wt.-% of CDM was observed. Based on the high monitored activities of 3-ketoacyl-CoA reductase...
and PHA synthase, hence, the enzymes responsible for PHB biosynthesis, the authors assumed that this organism uses the classical PHB biosynthesis pathway. FT-IR (characterization of chemical composition), DSC (thermoanalysis) and viscometry (molecular mass determination) were carried out by PHB characterization. FT-IR analysis identified the polyester as the homopolyester PHB. DSC revealed the semi-crystalline character of the polymer (X of 42 %), an onset temperature of thermal decomposition (T_D) of 280 °C, T_m of 169 °C, and a melting enthalpy (H_m) of about 63 J/g. Molecular mass determination revealed a Mw of 556 kDa.

For rapid and efficient in vitro PHA production, thermostable enzymes are required. Tajima et al. [113] reported PHA in vitro synthesis by the engineered thermostable PHA synthase PhaC1SG(STQK). In this system, however, also non-thermostable enzymes from mesophilic organisms, namely acetyl-CoA synthetase (ACS) and CoA transferase (CT), were used to biosynthesize the monomers. Therefore, the authors cloned ACS from Pelotomaculum thermophorum (CM10971 and CT from Thermus thermophilus (CM10941), both thermophilic organisms, in order to design a completely thermostable in vitro PHA synthesis system. Both enzymes displayed high thermostability, with temperature optima around 60 °C and 75 °C, respectively. In vitro PHA synthesis at 45 °C was successfully accomplished by the collaborative action of ACSPT, CTIT, and PhaC1SG(STQK). In addition, yields of PHB and poly(lactate-co-3HB) were even higher at 37 °C than those obtained in in vitro synthesis system resorting to non-thermostable ACS and CT from mesophilic organisms.

Only recently, a comprehensive study by Levett et al. [114] addresses the lack of abundantly available, inexpensive feedstocks as the major obstacle impeding large-scale PHA production [114]. Methane’s abundant availability and its rather low market price qualifies this simple alkane it as a potential substrate for PHA production [115]. Therefore, Levett and colleagues [114] presented a comprehensive techno-economic analysis of methane-based PHA production using the process optimization software ASPEN Plus for process design and simulation. An annual production of 100,000 t PHB by methanotrophic fermentation, and extractive polymer recovery using a system of acetone and water, was considered. Production costs per kg PHA were tentatively calculated with US-$ 4.1-6.8, which is in the same range as the production price obtained by other studies. Compared with sugar-based PHA production, raw material costs are reduced from 30-50 % of the entire production costs [116] to only 22 % when using methane as feedstock. Importantly, the authors revealed that heat removal from the two-stage bioreactor process contributes 28 % of total operating cost. Thermophilic methanotrophs could enable using cooling water instead of refrigerants, which further reduces production costs per kg PHA to US-$ 3.2-5.4, which already approximates the optimistic estimations for whey-based PHA production by halophiles [12]. Because energy needed for air compression and drying of biomass prior to PHA extraction were also spotted as significant cost factors, it is evident that bioreactor geometry and operation conditions, as well as biomass drying techniques should be optimized. Further understanding the mass transfer of methane at high biomass concentration and elevated pressure is needed to support the economic optimization, together with a reduction of the acetone loss during polymer extraction. Most importantly, it has to be emphasized that these are in silico calculated estimations based on fictive model organisms; the quest for thermophilic methanotrophs with high PHA production potential is still in progress.

PHA production under heavy metal contamination and other stress factors

Pioneering work in the investigation of diverse stress factors like oxidants, organic solvents or freezing in triggering PHA synthesis, and in elucidating the protective role of PHA to sustain these stress factors were done in the recent years by a research group in Brno, Czech Republic. In the first of their experiments, Obruca et al. [117] added diverse alcohols to cultivations of C. necator H16 on waste rapeseed oil as main carbon source for PHA accumulation. The authors noticed that especially the supplementation of 1-propanol after 24 h fedbatch cultivation increased both biomass growth (from ca. 11 to ca. 15 g/L CDMA) and PHA biosynthesis, which doubled from about 6 g/L in control experiments without alcohol addition to about 12 g/L after supplementation of a constant concentration of 1% 1-propanol in fed-batch mode. A PHBV copolyester with 8 mol-% 3HV in PHA was obtained. Later, the same group of authors revealed that exposure of C. necator to ethanol or H_2O_2 at the early stationary phase increases PHB production by about 30 %. Mechanistically, the authors assumed that H_2O_2 enhances the pentose phosphate pathway, which increases the intracellular pool of reduction equivalents (higher ratio of NADPH/NADP^+). This slightly inhibits the citric acid cycle (TCA), and shifts the acetyl-CoA flux to PHB biosynthesis and boosts the enzymatic activities of 3-ketothiolase and acetoacetyl-CoA reductase, while PHA synthase activity remains unaffected. The beneficial role of ethanol addition was also explained by the increased pool of reduction equivalents NAD[P]H, which occur due to the ethanol oxidation catalyzed by alcohol dehydrogenase. In addition to PHA overproduction provoked by these stressors, molecular mass was significantly higher under stress conditions than in control cultivation. In particular, molecular mass values were dependent on stress factor concentrations [118]. Application of exogenous stress, provoked by oxidants and/or solvents thus displays a simple approach to significantly boost PHB biosynthesis in C. necator.

Only recently, this group revealed that 3HB protects the enzyme lipase not only against heat-mediated denaturation but also against oxidative damage if exposed to metal ions [Cu^2+] and H_2O_2: its chaperon effect was higher than that of trehalose and similar to that of hydroxectoine. Considering the fact that the PHB-producing C. necator H16 displays 16.5-fold higher intracellular 3HB level which improves stress resistance of bacteria capable of parallel PHB synthesis and degradation. As a potential technological application, the authors suggest the use of 3HB as an efficient stabilizer in enzyme formulations [97].

C. necator’s tolerance to elevated Cu^{2+} concentrations, by the way, gave rise to the name of this metalophile bacterium; “Cutrapia necator” is the Latin expression for a “killer enduring high copper concentrations” [119]. This initiated the search for
additional metalophile PHA producers. In this context, Chien et al. [120] found that the tolerance of microbes to heavy metals might be linked to its PHB synthesis. *Cupriavidus taiwanensis* EJ0, a strain phylogenetically related to *C. necator*, was investigated in their study in the presence of Cd²⁺ ions. *C. taiwanensis* EJ0 tolerated up to 5 mM Cd²⁺ ions when grown in complex media, which is about the five-fold concentration compared to PHA-negative *C. taiwanensis* strains. Resistance towards Cd²⁺ ions by *C. taiwanensis* EJ0, was further increased up to 7 mM after supplementing defined carbon sources, which boost PHB biosynthesis in this organism. These outcomes go in parallel to studies revealing the high potential of diverse microalgal strains, which can efficiently be cultured in environments highly polluted by heavy metals like Cd⁴⁺-Pb⁴⁺, or even hexavalent Cr, or other severe toxins like formaldehyde. Analogous to increased PHA biopolymer synthesis by *C. taiwanensis*, such microalgae generate diverse valued products like pigments or valued lipids under these environmentally stressful conditions [reviewed by 121 and 122].

### Conclusion

The study presents a number of promising outcomes from novel PHA production process based on a number of phylogenetically highly variable extremophiles. Some of these concepts were already tested under controlled conditions in laboratory bioreactors of different size and operational regimes, ranging from simple shaking flask setups, discontinuous bioreactor processes in batch- and fedbatch mode, until more sophisticated solutions like cyclic batch, cyclic fedbatch and continuous chemostat processes. Many of these processes delivered enough polymeric products to demonstrate that the quality of PHA from extremophiles is at least competitive with PHA from well-known mesophilic production strains. Mainly those presented extremophile processes which are based on the combination of robust, extremophile strains, abundantly available feedstocks, and continuous operation mode hold promise for a high-throughput industrial-scale application in a not too distant future. Considering the fact that the research community already has more or less clear ideas about feasible inexpensive feedstocks for "White Biotechnology", such as for PHA-production, further process optimization has to focus on higher energy efficiency of the processes. In this context, the application of extremophiles provides the change to safe costs for the price, cooling, or heating.

Nevertheless, the route in the direction of routinely implementing extremophile PHA producers is still cumbersome. What is needed are further screening studies to explore novel, even more powerful extremophile production strains which display growth and PHA accumulation kinetics comparable to the performance of mesophilic strains, and, at the same time, are well adapted to the conversion of inexpensive raw materials without the need for excessive upstream procedures. In addition to the wealth of wild type strains provided by nature, genetic engineering might be reasonable, as the case arises, to fine tune selected production strains. This encompasses the disclosure of metabolic bottle necks to enhance the conversion of defined feedstocks, which, for example, could be a viable tool to facilitate the conversion of galactose from hydrolyzed whey by *Hfx. mediterranei*. Successful attempts in the direction of enhanced substrate conversion by halophiles were presented in this review for the halophile *Halomonas* sp. The knockout of genes responsible for by-product formation, as successfully demonstrated by the development of EPS-negative mutants of haloarchaean PHA producers, would contribute to achieve higher product yields. Further, deletion of depolymerase genes offers a chance to further improve process productivity by impeding the intracellular product degradation in later stages of the process, as evident in the case of thermophilic PHA production by *Chelatococcus* sp., or as reported for halophile *Halomonas* sp.

As the downside of the medal, extremophile cultivation conditions request special requirements to the bioreactor equipment and its surroundings, such as improved reactor materials, resistant sensors, and smart sealing systems. This opens the door for new engineering developments, as well as investigations to improve materials and electronic devices.

Despite all these challenges, data from bench-scale experiments and current R&D efforts on pilot scale give rise to optimism that the application of extremophiles for production of PHA and other marketable bioproducts by no way constitutes an academic shenanigan, but is among the top-objectives towards an efficient and sustainable bio-based industry of the future. Table 1 summarizes the most promising outcomes discussed in this review, collecting the production strains, the extremophile conditions and carbon sources applied, the type of PHA produced, productivity and production scale.

Table 1: Overview on most promising PHA production processes by extremophiles.

| Production strain (Archaea) | Extremophile conditions and carbon source | Type of PHA produced | Production scale and PHA Productivity | Ref. |
|-----------------------------|------------------------------------------|----------------------|--------------------------------------|------|
| *Hfx. mediterranei*         | 25 % marine salts [halophile]; Starch (20g/L) Glucose (10 g/L) | PHBV [in original literature: "PHB"] | Stable continuous cultivation over 3 months in 1.5 L bioreactor; T = 38 °C 6.5 g/L PHA on starch 3.5 g/L on glucose | [45] |
| *Hfx. mediterranei*         | 15 % NaCl [halophile]; Starch (20g/L) Glucose (10 g/L) | PHBV | Fedbatch fermentation in 10 L bioreactor; 0.21 g/L/h | [60] |
| *Hfx. mediterranei*         | 20 % NaCl [halophile]; Hydrolyzed whey permeate Hydrolyzed whey permeate plus GDL | PHBV, PH3HV (3HV-co-3HV-co-4HB) | 42 L bioreactor; 0.09 g/(L·h), 12.2 g/L PHBV; 0.14 g/(L·h), 14.7 g/L | [48], [64] |

Citation: Koller M (2017) Production of Polyhydroxyalkanoate (PHA) Biopolymers by Extremophiles. MO J Poly Sci 1(2): 00011. DOI: 10.15406/mojps.2017.01.00011
| Organism/Strain                                      | NaCl, Temperature, pH (Environment) | Carbon Source          | PHA/PHAHV Composition | PHA Production Parameters | References |
|-----------------------------------------------------|-------------------------------------|------------------------|-----------------------|---------------------------|------------|
| *Hfx. mediterranei* (ArCHAe)                        | 15% NaCl, 15°C, pH 8               | Crude glycerol phase   | PHBHV                 | 42 L / 10 L bioreactor; 0.12 g/(L·h), 16.2 g/L PHA | [28]       |
| *Hfx. mediterranei* (ArCHAe)                        | 24% NaCl, 15°C, pH 8               | Extruded rice bran     | PHBHV                 | pH-stat feeding strategy in 5 L bioreactor; 24.2 g/L PHA | [50]       |
| *Hfx. mediterranei* (ArCHAe)                        | 23.4% NaCl, 15°C, pH 8             | Extruded corn starch   | PHBHV                 | pH-stat feeding strategy in 5 L bioreactor; 24.2 g/L PHA | [51]       |
| *Natrinema pallidum* ICM 8980 (ArCHAe)              | 25% NaCl, 15°C, pH 8               | Starch                 | PHBHV                 | Shaking flask scale; PHA content in CDM of about 53.14 wt.-% | [68]       |
| *Halomonas campaniensis* LS21 (BACtERIA; Gram-negative) | 27% NaCl, 15°C, pH 8               | Extruded rice bran     | PHBHV                 | Open cultivation for 65 days 26% PHA in CDM [wild type strain], 70% PHA in CDM [engineered strain] | [72]       |
| *HalomonasTD01* (BACtERIA; Gram-negative)           | 60% NaCl, 15°C, pH 8               | Glucose                | PHBHV                 | Open continuous cultivation process for 56 h; 64 g/L PHA | [74]       |
| *HalomonasTD01* (genetically engineered) (BACtERIA) | 60% NaCl, 15°C, pH 8               | Glucose, propionic acid| PHBHV                 | Open continuous cultivation process for 56 h; 60 g/L PHB, 56 g/L PHBHV | [75]       |
| *Bacillus megaterium* uyuni S29 (BACtERIA; Gram-positive) | 45% NaCl, 15°C, pH 8               | Glucose                | PHBHV                 | Shaking flask scale; 2.2 g/L PHB, 0.10 g/(L·h) | [81]       |
| *Pseudomonas extremaustralis* (BACtERIA; Gram-negative) | 28°C, 15°C, pH 8                   | Contaminated soil samples | PHBHV                 | Static cultivation on poly(styrene) biofilms biofilm vs. shaking flask cultures PHA productivity not reported | [91], [92]|
| *Thermus thermophiles* (BACtERIA; Gram-negative)    | 75°C, 15°C, pH 8                   | Gluonate Octanoate     | PHBHV                 | Shaking flask scale; Up to 40% PHA in CDM | [98]       |
| *Synechococcus* sp. MA19 (CYNanobacteria; Gram-negative) | 50°C, 15°C, pH 8                   | 2% CO₂                 | PHBHV                 | Photoautotrophic cultivation in simple bottles; 20-27% PHA in CDM | [101]      |
| *Spirulina* sp. MA19 (CYNanobacteria; Gram-negative) | 50°C, 15°C, pH 8                   | CO₂                    | PHBHV                 | Autotrophic cultivation 2.4 g/L PHB | [102]      |
| *Chelatococcus* sp. strain MW10 (BACtERIA; Gram-negative) | 55°C, 15°C, pH 8                   | Glucose                | PHBHV                 | 2 L fedbatch fermentation [ca. 3 g/L PHB]; 42 L cyclic batch fermentation [ca. 4 g/L PHB]; 42 L cyclic fed-batch fermentation [ca. 16.8 g/L PHB] | [29]       |
| *Chelatococcus daeguensis* TAD1 (BACtERIA; Gram-negative) | 50°C, 15°C, pH 8                   | Glucose, Glycerol      | PHBHV                 | Shaking flask scale; 3.44 g/L PHB; 80% PHB in CDM | [108]      |
| *Bacillus shackletonii* KS (BACtERIA; Gram-positive)  | 45°C, 15°C, pH 8                   | Glucose                | PHBHV                 | Two-stage fedbatch process; 17.4 g/L PHB; 0.434 g/(L·h) | [109]      |

**Table Abbreviations**: CDM: Cell dry mass; GBL: γ-Butyrolactone; Hf: Haloferax; mcl: medium chain length; PHA: Polyhydroxyalkanoate; PHB: Poly (3-hydroxybutyrate); scl: short chain length
References

1. Hermann BG, Patel M (2007) Today’s and tomorrow’s bio-based bulk chemicals from white biotechnology. Applied biochemistry and biotechnology 136(3): 361-386.

2. Iles A, Martin AN (2013) Expanding bioplastics production: sustainable business innovation in the chemical industry. Journal of Cleaner Production 45: 38-49.

3. Koller M, Maršíšek L, de Sousa Dias MM, Braunegg G (2017) Producing microbial polyhydroxyalkanoate (PHA) biopolymesters in a sustainable manner. N Biotechnol 37(Pt A): 24-38.

4. Narodoslawsky M, Shazad K, Kollmann R, Schnitzer H (2015) LCA of PHA Production—Identifying the Ecological Potential of Bio-plastic. Chemical and Biochemical Engineering Quarterly 29(2): 299-305.

5. Piemonte V, Gironi F (2011) Land-use change emissions: How green are the bioplastics? Environmental Progress & Sustainable Energy, 30(4): 685-691.

6. Koller M, Sandholzer D, Salerno A, Braunegg G, Narodoslawsky M (2013) Biopolymer from industrial residues: Life cycle assessment of poly(hydroxyalkanoates) from whey. Resources, conservation and recycling 73: 64-71.

7. Harding KG, Dennis JS, von Blottnitz H, Harrison ST (2007) Environmental analysis of plastic production processes: Comparing petroleum-based polypropylene and polyethylene with biologically-based poly-β-hydroxybutyric acid in life cycle analysis. J Biotechnol 130(1): 57-66.

8. Shazad K, Kettl KH, Titiz M, Koller M, Schnitzer H, et al. (2013) Comparison of ecological footprint for bio-based PHA production from animal residues utilizing different energy resources. Clean Technologies and Environmental Policy 15(3): 525-536.

9. Pietrini M, Roes L, Patel MK, Chielhini E (2007) Comparative life cycle studies on poly (3-hydroxybutyrate)-based composites as potential replacement for conventional petrochemical plastics. Biomacromolecules 8(7): 2210-2218.

10. Yates MR, Barlow CY (2013) Life cycle assessments of biodegradable, commercial biopolymers—a critical review. Resources, Conservation and Recycling 78: 54-66.

11. Jiang G, Hill DJ, Kowalczyk M, Johnston B, Adamus G et al. (2016). carbon sources for polyhydroxyalkanoates and an integrated biorefinery. Int J Mol Sci 17(7): E1157.

12. Koller M, Hesse P, Bona R, Kutschera C, Atli šálek L, de Sousa Dias MM, Braunegg G, Narodoslawsky M, Shazad K, Kollmann R, Schnitzer H (2015) LCA of PHA Production–Identifying the Ecological Potential of Bio-plastic. Chemical and Biochemical Engineering Quarterly 29(2): 299-305.

13. Piemonte V, Gironi F (2011) Land-use change emissions: How green are the bioplastics? Environmental Progress & Sustainable Energy, 30(4): 685-691.

14. Titiz M, Kettl KH, Shazad K, Koller M, Schnitzer H, et al. (2012) Process optimization for efficient biomediated PHA production from animal-based waste streams. Clean technologies and environmental policy 14(3): 495-503.

15. Obrusa S, Benesova P, Marsalek, Maroval (2015) Use of lignocellulosic materials for PHA production. Chemical and Biochemical Engineering Quarterly 29(2): 135-144.

16. Kulkarni SO, Kanekar PP, Jogi JP, Sarnaik SS, Nilegaonkar SS (2015) Production of copolymer, poly (hydroxybutyrate-co-hydroxyvalerate) by Halomonas campisalis MCM B-1027 using agro-wastes. Int J Biol Macromol 72: 784-789.

17. Ahn J, Jho EH, Kim M, Nam K (2016) Increased 3HV Concentration in the Bacterial Production of 3-Hydroxybutyrate (3HB) and 3-Hydroxyvalerate (3HV) Copolymer with Acid-Digested Rice Straw Waste. Journal of Polymers and the Environment 24(2): 98-103.

18. Cesário MT, Raposo RS, de Almeida MC, van Keulen F, Ferreira BS (2014) Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. N Biotechnol 31(1): 104-113.

19. Obrusa S, Benesova P, Petrik S, Oborna J, Prikyrl R, et al. (2014) Production of polyhydroxyalkanoates using hydrolysates of spent coffee grounds. Process Biochemistry 49(9): 1409-1414.

20. Koller M, Niebelschütz H, Braunegg G (2013) Strategies for recovery and purification of poly ([R]-3-hydroxyalkanoates)(PHA) biopolymesters from surrounding biomass. Engineering in Life Sciences 13(6): 549-562.

21. Madkour MH, Heinrich D, Alghamdi MA, Shabbaj II, Steinbüchel A (2013) PHA recovery from biomass. Biomacromolecules 14(9): 2963-2972.

22. Jacquel N, Lo CW, Wei YH, Wu HS, Wang SS (2008) Isolation and purification of bacterial poly(3-hydroxyalkanoates). Biochemical Engineering Journal 39(1): 15-27.

23. Koller M, Muhr A (2014) Continuous production mode as a viable process-engineering tool for efficient poly(hydroxyalkanoate)(PHA) bio-production. Chemical and Biochemical Engineering Quarterly 28(1): 65-77.

24. Koller M, Braunegg G (2015) Potential and prospects of continuous polyhydroxyalkanoate (PHA) production. Bioengineering 2(2): 94-121.

25. Pirttijärvi TS, Ahonen LM, Maunuksela LM, Salkinoja-Salonen MS (1998) Bacillus cereus in a whey process. Int J Food Microbiol 44(1): 31-41.

26. Hermann-Krauss C, Koller M, Muhr A, Fahl, Stelzer F (2013) Archaeal production of poly(3-hydroxyalkanoates)(PHA) co-and terpolymesters from biodiesel industry-derived by-products. Archaea 2013: 129268.

27. Ibrahim MH, Steinbüchel A (2010) High-cell-density cyclic fed-batch fermentation of a poly (3-hydroxybutyrate)-accumulating thermophile, Chernatococcus sp. strain MW10. Appl Environ Microbiol 76(23): 7890-7895.

28. Gomes J, Steiner W (2004) The biocatalytic potential of extremophiles. In: Rampelotto Pabulo H (Ed.), Biotechnology of Extremophiles. Springer International Publishing, pp. 243-272.
32. Yin J, Chen JC, Wu Q, Chen GQ (2015) Halophiles, coming stars for industrial biotechnology. Biotechnol adv 33(7): 1433-1442.

33. Xiang H (2016) PHBV Biosynthesis by Haloferax mediterranei: from Genetics, Metabolism, and Engineering to Economical Production. In: Koller M (Ed.), Recent Advances in Biotechnology, Vol I: Microbial Bioproduction, Performance, and Processing-Microbiology, Feedstocks, and Metabolism, Bentham Science Publishers, UAE, pp. 348-379.

34. Chen CW, Hsu SH, Lin MT, Hsu YH (2015) Mass production of C50 carotenoids by Haloferax mediterranei in using extruded rice bran and starch under optimal conductivity of brined medium. Bioprocess Biosyst Eng 38(12): 2361-2367.

35. Cheung J, Danna KJ, O’Connor EM, Price LB, Sand RF (1997) Isolation, sequence, and expression of the gene encoding halocin H4, a bacteriocin from the halophilic archaeon Haloferax mediterranei R4. J Bacteriol 179(2): 548-551.

36. Antón J, Meseguer I, Rodriguez-Valera F (1988) Production of an extracellular polysaccharide by Haloferax mediterranei. Appl Environ Microbiol 54(10): 2381-2386.

37. Parolis H, Parolis LA, Boim IF, Rodriguez-Valera F, Widmalm G, et al. (1996) The structure of the exopolysaccharide produced by the halophilic Archaeon Haloferax mediterranei strain R4 (ATCC 33500). Carbohydr Res 295: 147-156.

38. Fernandez-Castillo R, Rodriguez-Valera F, Gonzalez-Ramos J, Ruiz-Bernaqueo F (1986) Accumulation of poly (β-hydroxybutyrate) by halobacteria. Appl Environ Microbiol 51(1): 214-216.

39. Wood JM, Bremer E, Csonka LN, Kraemer R, Poolman B, et al. (2001) Osmosensing and osmoregulatory compatible solute accumulation by bacteria. Comp Biochem Physiol A Mol Integr Physiol 130(3): 437-460.

40. Borowitzka MA, Borowitzka LJ, Kessly D (1990) Effects of salinity increase on carotenoid accumulation in the green alga Dunaliella salina. J Appl Phycol 2(1): 111-119.

41. Soto G, Setten L, Lisi C, Maurelis C, Mozckafreddo M, et al. (2012) Hydroxybutyrate prevents protein aggregation in the halotolerant bacterium Pseudomonas sp. CT13 under abiotic stress. Extremophiles 16(3): 455-462.

42. Koller M, Puppi D, Chiellini E, Braunegg G (2016) Comparing chemical and enzymatic hydrolysis of wheet lase to generate feedstocks for holoarchaeal poly(3-hydroxybutyrate-co-3-hydroxyvalerate) biopolyester synthesis. International Journal of Pharmaceutical Sciences Research 3(1): IJPSR-112.

43. Koller M, Dias MMD, Rodriguez-Contreras A, Kanaver M, Žagar E, et al. (2015) Liquefied wood as inexpensive precursor-feedstock for bio-mediated incorporation of βH-3-hydroxyvalerate into polyhydroxyalkanoates. Materials 8(9): 6543-6557.

44. Rodriguez-Valera F, Lillo JG (1992) Halobacteria as producers of polyhydroxyalkanoates. FEBS Microbiology Letters 103(2-4): 181-186.

45. Lillo JG, Rodriguez-Valera F (1990). Effects of culture conditions on poly (β-hydroxybutyric acid) production by Haloferax mediterranei. Appl Environ Microbiol 56(8): 2517-2521.

46. Han J, Hou J, Zhang F, Ai G, Li M (2013) Multiple propionyl coenzyme A-supplying pathways for production of the bioplastic poly (3-hydroxybutyrate-co-3-hydroxyvalerate) in Haloferax mediterranei. Appl Environ Microbiol 79(9): 2922-2931.

47. Don TM, Chen CW, Chan TH (2006) Preparation and characterization of poly (hydroxyalkanoate) from the fermentation of Haloferax mediterranei. J Biomater Sci Polym Ed 17(12): 1425-1438.

48. Koller M, Hesse P, Bona R, Kutschera C, Atli A, et al. (2007) Biosynthesis of high quality polyhydroxyalkanoate co- and terpolymers for potential medical application by the archaeon Haloferax mediterranei. Macromolecular symposia 253(1): 33-39.

49. Bhattacharyya A, Jana K, Haldar S, Bhowmic A, Mukhopadhyay UK, et al. (2015) Integration of poly-3-( hydroxybutyrate-co- hydroxyvalerate) production by Haloferax mediterranei through utilization of stillage from rice-based ethanol manufacture in India and its techno-economic analysis. World J Microbiol Biotechnol 31(5): 717-727.

50. Huang TY, Duan KJ, Huang SY, Chen CW (2006) Production of polyhydroxyalkanoates from inexpensive extruded rice bran and starch by Haloferax mediterranei. J Ind Microbiol Biotechnol 33(8): 701-706.

51. Chen CW, Don TM, Yen HF (2006) Enzymatic extruded starch as a carbon source for the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by Haloferax mediterranei. Process Biochemistry 41(11): 2289-2296.

52. Alsaifi D, Al-Mashaqbeh O (2017) A one-stage cultivation process for the production of poly(3-hydroxybutyrate-co-hydroxyvalerate) from olive mill wastewater by Haloferax mediterranei. N Biotechnol 34: 47-53.

53. Bhattacharyya A, Pramanik A, Maji SK, Haldar S, Mukhopadhyay UK, et al. (2012) Utilization of vinasse for production of poly-3-(hydroxybutyrate-co-hydroxyvalerate) by Haloferax mediterranei. AMB Express 2(1): 34.

54. Lu Q, Han J, Zhou L, Zhou J, Xiang H (2008) Genetic and biochemical characterization of the poly (3-hydroxybutyrate-co-3-hydroxyvalerate) synthase in Haloferax mediterranei. J Bacteriol 190(12): 4173-4180.

55. Cai S, Cai L, Liu H, Liu X, Han J, et al. (2012) Identification of the haloarchaeal phasin (PhaP) that functions in polyhydroxyalkanoate accumulation and granule formation in Haloferax mediterranei. Appl Environ Microbiol 78(6): 1946-1952.

56. Feng B, Cai S, Han J, Liu H, Zhou J, et al. (2010) Identification of the phaB genes and analysis of the PHBV precursor supplying pathway in Haloferax mediterranei. Wei sheng wu xue bao 50(10): 1305-1312.

57. Han J, Zhang F, Hou J, Liu X, Li M, et al. (2012) Complete genome sequence of the metabolically versatile halophilic archaeon Haloferax mediterranei, a poly (3-hydroxybutyrate-co-3-hydroxyvalerate) producer. J Bacteriol 194(16): 4463-4464.

58. Liu G, Hou J, Cai S, Zhao D, Cai L, et al. (2015) A patatin-like protein associated with the polyhydroxyalkanoate (PHA) granules of Haloferax mediterranei acts as an efficient depolymerase in the degradation of native PHA. Appl Environ Microbiol 81(9): 3029-3038.

59. Koller M, Horvat P, Hesse P, Bona R, Kutschera C, et al. (2006) Assessment of formal and low structured kinetic modeling of polyhydroxyalkanoate synthesis from complex substrates. Bioprocess Biosyst Eng 29(5-6): 367-377.

60. Koller M, Chiellini E, Braunegg G (2015) Study on the production and re-use of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and extracellular polysaccharide by the archaeon Haloferax mediterranei strain DSM 1411. Chem Biochem Eng Q 29(2): 87-98.
Production of Polyhydroxyalkanoate (PHA) Biopolymesters by Extremophiles

Han J, Wu LP, Hou J, Zhao D, Xiang H (2015) Biosynthesis, characterization, and hemostasis potential of tailor-made poly (3-hydroxybutyrate-co-3-hydroxyvalerate) produced by Haloferax mediterranei. Biomacromolecules 16(2): 578-588.

Koller M, Attié A, Gonzalez-Garcia Y, Kutscher C, Braunegg G (2008) Polyhydroxyalkanoate (PHA) biosynthesis from whey lactose. Macromolecular Symposia 272(1): 87-92.

Hezyen FF, Rehm BH, Eberhardt R, Steinbüchel A (2000) Polymer production by two newly isolated extremely halophilic archaea: application of a novel corrosion-resistant bioreactor. Appl Microbiol Biotechnol 54(3): 319-325.

Koller M (2015) Recycling of waste streams of the biotechnological poly (hydroxyalkanoate) production by Halomonas mediterranei on whey. International Journal of Polymer Science 2015: 370164.

Bhattacharyya A, Saha J, Haldar S, Bhownic A, Mukhopadhyay UK et al. (2014) Production of poly-3-(hydroxybutyrate-co-hydroxyvalerate) by Haloferax mediterranei using rice-based ethanol stillage with simultaneous recovery and re-use of medium salts. Extremophiles 18(2): 463-470.

Salgaonkar BB, Mani K, Braganca JM (2013) Characterization of polyhydroxyalkanoates accumulated by a moderately halophilic salt pan isolate Bacillus megaterium strain H16. J Appl Microbiol 114(5): 1347-1356.

Singh R (2014) Isolation and characterization of efficient poly--hydroxybutyrate (PHB) synthesizing bacteria from agricultural and industrial land. International Journal of Current Microbiology and Applied Sciences 3(6): 304-308.

Danis O, Ogan A, Cakmakci E, et al. (2015) Preparation of poly (3-hydroxybutyrate-co-hydroxyvalerate) films from halophilic archaea and their potential use in drug delivery. Extremophiles 19(2): 515-524.

Drosog B, Fritz I, Gattermayr F, Silvestrini L (2015) Photo-autotrophic production of poly(hydroxyalkanoates) in cyanobacteria. Chem Biochem Eng Q 29(2): 145-156.

Koller M, Marsalek L (2015) Cyanobacterial Polyhydroxyalkanoate Production: Status Quo and Quo Vadis? Current Biotechnology 6(4): 464-480.

Shrivastav A, Mishra SK, Mishra S (2010) Polyhydroxyalkanoate (PHA) synthesis by Spirulina subsalsa from Gujarat coast of India. Int J Biol Macromol 42(2): 255-260.

Yue H, Ling C, Yang T, Chen X, Chen Y, et al. (2014) A seawater-based open and continuous process for polyhydroxyalkanoates production by recombinant Halomonas campaniensis LS21 grown in mixed substrates. Biotechnology for biofuels 7(1): 308.

Jau MH, Yew SP, Toh PS (2005) Biosynthesis and mobilization of poly (3-hydroxybutyrate) (P (3HB)) by Spirulina platensis. International journal of Biological Macromolecules 36(3): 144-151.

Tan D, Xue YS, Aibadiula G, Chen QQ (2011) Unsterile and continuous production of polyhydroxybutyrate by Halomonas TD01. Bioresour Technol 102(17): 8130-8136.

Tan D, Wu Q, Chen JC, Chen QQ (2014) Engineering Halomonas TD01 for the low-cost production of polyhydroxyalkanoates. Metab Eng 26: 34-47.

Han J, Lu Q, Zhou L, Zhou J, Xiang H (2007) Molecular characterization of the phaECHm genes, required for biosynthesis of poly (3-hydroxybutyrate) in the extremely halophilic archaean Haloarcula marismortui. Appl Environ Microbiol 73(19): 6058-6065.

Ding JY, Chiang PW, Hong MJ, Dyall-Smith M, Tang SL (2014) Complete genome sequence of the extremely halophilic archaean Haloarcula hispanica strain N601. Genome Announc 2(2): e00178-e00214.

Lau NS, Sam KK, Amirul AA (2017) Genome features of moderately halophilic polyhydroxyalkanoate-producing Yangia sp. CCB-MM3. Stand Genomic Sci 12: 12.

Rodríguez-Contreras A, Koller M, Braunegg G, Marqués-Calvo MS (2016) Poly ((R)-3-hydroxybutyrate) production under different salinity conditions by a novel Bacillus megaterium strain. N Biotechnol 33(1): 73-77.

Rodríguez-Contreras A, Koller M, de Sousa Dias MM, Calafell M, Braunegg G, et al. (2013) Novel Poly((R)-3-hydroxybutyrate)-producing bacterium isolated from a Bolivian hypersaline lake. Food Technology and Biotechnology 51(1): 123-130.

Rodríguez-Contreras A, Koller M, Miranda-de Sousa Dias M, Calafell-Monfort M, Braunegg G, et al. (2013) High production of poly(3-hydroxybutyrate) from a wild Bacillus megaterium Bolivian strain. J Appl Microbiol 115(5): 1378-1387.

Koller M, Rodríguez-Contreras A (2015) Techniques for tracing PHA-producing organisms and for qualitative and quantitative analysis of intra- and extracellular PHA. Engineering in Life Sciences 15(6): 558-581.

Mahansaria R, Choudhury JD, Mukherjee J (2015) Polymerase chain reaction-based screening method applicable universally to environmental halarchaeae and halobacteria for identifying polyhydroxyalkanoate producers among them. Extremophiles 19(5): 1041-1054.

Han J, Hou J, Liu H, Cai S, Feng B, et al. (2010) Wide distribution among halophilic archaea of a novel polyhydroxyalkanoate synthase subtype with homology to bacterial type III synthases. Appl Environ Microbiol 76(23): 7811-7819.

D’Amico S, Collins T, Marx JC, Feller G, Gerday C (2006) Psychrophilic microorganisms: challenges for life. EMBO rep 7(4): 385-389.

Krembs C, Eicken H, Deming JW (2011) Exopolymer alteration of physical properties of sea ice and implications for ice habitability and biogeochemistry in a warmer Arctic. Proceedings of the National Academy of Sciences 108(9): 3653-3658.

Pomeroy LR, Wiebe WJ (2001) Temperature and substrates as interactive limiting factors for marine heterotrophic bacteria. AMB 23(2): 187-204.

Ayub ND, Pettinari MJ, Méndez BS, López NI (2007) The Polyhydroxyalkanoate biosynthesis from whey lactose. Applied Sciences 3(6): 304-308.

Mahansaria R, Choudhury JD, Mukherjee J (2015) Polymerase chain reaction-based screening method applicable universally to environmental halarchaeae and halobacteria for identifying polyhydroxyalkanoate producers among them. Extremophiles 19(5): 1041-1054.
91. Tribelli PM, López NI (2011) Poly (3-hydroxybutyrate) influences biofilm formation and motility in the novel Antarctic species *Pseudomonas extremaustralis* under cold conditions. Extremophiles 15(5): 541-547.

92. Tribelli PM, Di Martino C, López NI, Raiger Lustman LJ (2012) Biofilm lifestyle enhances diesel bioremediation and bio surfactant production in the Antarctic polyhydroxyalkanoate producer *Pseudomonas extremaustralis*. Biodegradation 23(5): 645-651.

93. Cesielksi S, Górniak D, Możęjko J, Świątecki A, Grzesiak J, et al. (2014) The diversity of bacteria isolated from Antarctic freshwater reservoirs possessing the ability to produce polyhydroxyalkanoates. Curr Microbiol 69(5): 594-603.

94. Pärnänen K, Karkkanen A, Virta M, Eronen-Rasimus E, Kaartokallio H (2015) Discovery of bacterial polyhydroxyalkanoate synthesis (PhaC)-encoding genes from seasonal Baltic Sea ice and cold estuarine waters. Extremophiles 19(1): 197-206.

95. Krzyzanek V, Hrubanova K, Samek O, Obruca S, Marova I, et al. (2015) Cryo-SEM and Raman Spectroscopy study of the involvement of polyhydroxyalkanoates in stress response of bacteria. Microscopy and Microanalysis 21(S3): 183-184.

96. Obruca S, Sedlacek P, Krzyzanek V, Mravec F, Hrubanova K, et al. (2016) Accumulation of poly(3-hydroxybutyrate) helps cells to survive freezing. PLos One 11(6): e0157778.

97. Obruca S, Sedlacek P, Mravec F, Samek O, Marova I, et al. (2016) Evaluation of 3-hydroxybutyrate as an enzyme-protective agent against heating and oxidative damage and its potential role in stress response of poly(3-hydroxybutyrate) accumulating cells. Appl Microbiol Biotechnol 100(3): 1365-1376.

98. Pantazaki AA, Tambaka MG, Langlois V, Guerin P, Kyriakidis DA (2003) Polyhydroxyalkanoate (PHA) biosynthesis in and molecular characterization of the PHA synthases from two thermophilic cyanobacteria: Chlorogloeopsis fritschii PCC 6912 and Thermus thermophilus: a tale of lost and found. International journal of systematic and evolutionary microbiology 54(6): 2285-2289.

99. Tribelli PM, López NI (2011) Poly (3-hydroxybutyrate) influences biofilm formation and motility in the novel Antarctic species *Pseudomonas extremaustralis* under cold conditions. Extremophiles 15(5): 541-547.

100. Obruca S, Sedlacek P, Mravec F, Samek O, Marova I, et al. (2016) Evaluation of 3-hydroxybutyrate as an enzyme-protective agent against heating and oxidative damage and its potential role in stress response of poly(3-hydroxybutyrate) accumulating cells. Appl Microbiol Biotechnol 100(3): 1365-1376.

101. Miyake M, Eonta M, Asada Y (1996) A thermophilic cyanobacterium, *Synechococcus sp.* strain MA19. Microbiology 147(pt 11): 3047-3060.

102. Miyake M, Eonta M, Asada Y (1996) A thermophilic cyanobacterium, *Synechococcus sp.* strain MA19. Microbiology 147(pt 11): 3047-3060.

103. Chen WM, Chang JS, Chiu CH, Chang SC, Chen WC, et al. (2005) *Caldimonas taiwanensis* sp. nov., a amylase producing bacterium isolated from a hot spring. Syst Appl Microbiol 28(5): 415-420.

104. Sheu DS, Chen WM, Yang YJ, Chang RC (2009) Thermophilic bacterium *Caldimonas taiwanensis* produces poly (3-hydroxybutyrate-co-3-hydroxyvalerate) from starch and valerate as carbon sources. Enzyme and Microbial Technology 44(5): 289-294.

105. Takeda M, Kamagata Y, Ghiurse WC, Hanada S, Koizumi J (2002) *Caldivilis manganoxidans* gen. nov., sp. nov., a poly (3-hydroxybutyrate)-degrading, manganese-oxidizing thermophile. Int J Syst Evol Microbiol 52(3): 895-900.

106. Koller M, Bona R, Chiellini E, Fernandes EG, Horvat P, et al. (2008) *Polyhydroxyalkanoate* production from whey by *Pseudomonas megazosovora*. Bioreour Technol 99(11): 4854-4863.

107. Ibrahim MH, Willem's A, Steinbüchel A (2010) Isolation and characterization of new poly (3HB)-accumulating star-shaped cell-aggregates-forming thermophilic bacteria. J Appl Microbiol 109(5): 1579-1590.

108. Xu F, Huang S, Liu Y, Zhang Y, Chen S (2014) Comparative study on the production of poly (3-hydroxybutyrate) by thermophilic *Chelatococcus daeguensis* TAD1: a good candidate for large-scale production. Appl Microbiol Biotechnol 90(9): 3965-3974.

109. Cui B, Huang S, Xu F, Zhang R, Zhang Y (2015) Improved productivity of poly (3-hydroxybutyrate) (PHB) in thermophilic *Chelatococcus daeguensis* TAD1 using glycerol as the growth substrate in a fed-batch culture. Appl Microbiol Biotechnol 99(14): 6009-6019.

110. Liu Y, Huang S, Zhang Y, Xu F (2014) Isolation and characterization of a thermophilic *Bacillus shackletonii* K5 from a biotrickling filter for the production of polyhydroxybutyrate. J Environ Sci China 26(7): 1453-1462.

111. Xiao Z, Zhang Y, Xi L, Huo F, Zhao Y (2015) Thermophilic production of polyhydroxyalkanoates by a novel *Aerimonas daeguensis* strain isolated from Guidao oilfield, China. J Basic Microbiol 55(9): 1125-1133.

112. Giedraitytė G, Kalėdienė L (2015) Purification and characterization of polyhydroxyalkanoate produced from thermophilic *Geobacillus sp.* AF 946034 strain. Chemija 26(1): 38-45.

113. Tajima K, Han X, Hashimoto Y, Sato Y, Sato T, et al. (2016) *In vitro* synthesis of polyhydroxyalkanoates using thermostable acetyl-CoA synthetase, CoA transferase, and PHA synthase from thermotolerant bacteria. J Biosci Bioeng 122(6): 660-665.

114. Levett I, Birkett G, Davies N, Bell A, Langford A, et al. (2016) Techno-economic assessment of poly-3-hydroxybutyrate (PHB) production from methane-The case for thermophilic bioprocessing. Journal of Environmental Chemical Engineering 4(4): 3724-3733.

115. Khosravi-Barani K, Mokhtari ZA, Amal T, Tanaka K (2013) Microbial production of poly (hydroxybutyrate) from C1 carbon sources. Appl Microbiol Biotechnol 97(4): 1407-1424.

116. Nonato R, Mantelatto P, Rossell C (2015) Integrated production of biodegradable plastic, sugar and ethanol. Applied Microbiology and Biotechnology 82(5): 512-514.

117. Nishioka M, Nakai K, Miyake M, Asada Y, Taya M (2001) Production of poly-β-hydroxybutyrate by thermophilic cyanobacterium, *Synecococcus sp.* MA19, capable of accumulating poly-β-hydroxybutyrate. Journal of fermentation and bioengineering 85(4): 512-514.

118. Chen WM, Chang JS, Chiu CH, Chang SC, Chen WC, et al. (2005) *Caldimonas taiwanensis* sp. nov., a amylase producing bacterium isolated from a hot spring. Syst Appl Microbiol 28(5): 415-420.

119. Sheu DS, Chen WM, Yang YJ, Chang RC (2009) Thermophilic bacterium *Caldimonas taiwanensis* produces poly (3-hydroxybutyrate-co-3-hydroxyvalerate) from starch and valerate as carbon sources. Enzyme and Microbial Technology 44(5): 289-294.

120. Koller M, Bona R, Chiellini E, Fernandes EG, Horvat P, et al. (2008) *Polyhydroxyalkanoate* production from whey by *Pseudomonas megazosovora*. Bioreour Technol 99(11): 4854-4863.
120. Chien CC, Wang LJ, Lin WR (2014) Polyhydroxybutyrate accumulation by a cadmium-resistant strain of *Cupriavidus taiwanensis*. Journal of the Taiwan Institute of Chemical Engineers 45(4): 1164-1169.

121. Koller M, Salerno A, Tuffner P, Koinigg M, Böchelt H, et al. (2012) Characteristics and potential of micro algal cultivation strategies: a review. Journal of Cleaner Production 37: 377-388.

122. Koller M, Muhr A, Braunegg G (2014) Microalgae as versatile cellular factories for valued products. Algal research 6: 52-63.