Biosynthesis of High Quality Polyhydroxyalkanoate Co- and Terpolyesters for Potential Medical Application by the Archaeon *Haloferax mediterranei*

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Summary: *Haloferax mediterranei* was investigated for the production of two different high-performance polyhydroxyalkanoates (PHAs). A copolyester containing 6 mol-% 3-hydroxyvalerate (3HV) was produced from whey sugars as sole carbon source. The maximum specific growth rate ($\mu_{max.}$) and the maximum specific PHA production rate ($q_{p\ max.}$) were determined with 0.10 1/h and 0.15 1/h, respectively. The cells contained 72.8 wt.-% of P-(3HB-*co*-6%-3HV) which featured low melting points between 150 and 160 °C and narrow molecular mass distribution (polydispersity PDI = 1.5). Further, a PHA terpolyester with an increased 3HV fraction as well as 4-hydroxybutyrate (4HB) building blocks was accumulated by feeding of whey sugars plus 3HV - and 4HB precursors. Kinetic analysis of the process reveals a $\mu_{max.}$ of 0.14 1/h and a $q_{p\ max.}$ of 0.23 1/h, respectively. The final percentage of P-(3HB-*co*-21.8%-3HV-*co*-5.1%-4HB) in biomass amounted to 87.5 wt.-%. Also this material showed a narrow molecular mass distribution (PDI = 1.5) and a high difference between the two melting endotherms of the material (between 140 and 150 °C) and the onset of decomposition at 236 °C. The accomplished work provides viable strategies to obtain different high-quality PHAs which might be potential candidates for application in the medical and pharmaceutical field.

Keywords: biocompatibility; biopolymers; *Haloferax mediterranei*; high-performance polymers; polyhydroxyalkanoates

Introduction

Starting from the conversion of different renewable resources, numerous microbial strains posses the metabolic requirements to redirect the intracellular carbon flux from formation of biomass components towards polyhydroxyalkanoate (PHA) accumulation. These polyoxoesters of hydroxycarboxylic acids serve as carbon and energy storage materials thus providing the microbe an advantage for survival under conditions of starvation. Based on their integration into nature's closed carbon cycle, PHAs are more and more regarded as future-oriented alternatives for a broad range of mineral oil – based plastics. Replacing those materials which accrue in large amounts mainly for packaging by ''green plastics'' like PHAs or poly-lactic acid features a bio-inspired approach towards a reduction of the growing piles of waste. Here, enhanced cost efficiency in biopolymer production is the crucial

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factor in fostering the switch from petrochemical end-of-pipe plastics to materials being embedded into the patterns of sustainability. Nowadays, minimizing costs in PHA production constitutes a topic of major interest in biopolymer research.[1]

In addition to the application as bulk materials, PHAs can act as high-performance polymers in special areas. In particular in the medical and pharmaceutical field, the number of potential "target areas" is versatile. Here, PHAs could be used for manufacture of artificial blood vessels, scaffolds for reconstruction of skin, joints, implants for bone regeneration, wound dressings, or surgical pins, sutures, stables and swabs. They are also of interest for nerve repair, intraocular lenses and for dentistry purposes. Additionally, they can be applied as matrices for controlled delivery of pharmaceutically active substances, e.g. release of drugs for promoting wound healing, anti-thrombosis, anti-infection and anti-tumor.[2,3,4] In all these fields, PHAs are superior to conventional polymers in terms of in-vivo degradability and biocompatibility and do not have to be produced as cost-efficient as for application as bulk materials. The ideal biocompatibility of PHAs is underlined by the natural occurrence of 3-hydroxybutyric acid (3HB) and its low molecular weight oligo- and polymers in human blood and tissue.[2,5]

For in-vivo applications, polyesters with tailor-made properties (e.g. a demanded strength of sutures) are required. Properties of PHAs are very much determined by their composition. Compared with the most frequently occurring and best scrutinized homopolyester poly-3-hydroxybutyrate (P-3HB), the incorporation of building blocks such as 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB) interrupts the highly crystalline P-3HB lattice creating polyesters with enhanced physical and thermodynamic properties. The lower degree of crystallinity makes the material more flexible, thus resulting in better strength properties and enhances its in-vivo degradation rate; a higher distance between the melting temperature and the decomposition temperature enables its processability for manufacturing of desired items.[6] A recent study reveals the excellent properties of P-3(HB-*co*-HV) sutures for healing of muscular-fascial wounds without adverse effects on the physiological state of the investigated animals (rats).[7]

PHAs in biomedical fields necessarily require a high degree of purity; commercially available PHAs often contain significant amounts of endotoxins (lipopolysaccharides) and other impurities such as lipids, carbohydrates and proteins. Together with the shape of the materials and the surface structure, inflammatory reactions of cells and tissue to biomaterials are mainly determined by the degree of purity. After isolation of PHAs from microbial biomass, the product should therefore by re-dissolved and precipitated several times to ensure a sufficient removal of impurities.[2]

The archeon *Haloferax mediterranei* belongs to the extremely halophilic class of *halobacteria* and requires 2-5 M NaCl for growth.[8] The high intracellular salinity makes halobacteria sensitive to exposure to hypotonic media; in distilled water, cells lyse immediately. This property enables a simple isolation procedure of PHA.[9] The genus *Haloferax* is of special interest due to the faster growth compared to related organisms and a wider substrate spectrum.[10,11] Because of the highly saline medium, the risk of microbial contamination is negligible; a continuous culture of *H. mediterranei* was maintained running for 3 months with minimal sterility precautions.[11] At University of Technology Graz, *H. mediterranei* was cultivated for several days without any sterilization step; until cell harvest, the culture stayed monoseptic (unpublished data). Using extremophilic organisms for biotechnological purposes was for a long time restricted to thermophilic strains. Rodriguez-Valera and Lillo first proposed the application of *H. mediterranei* for contamination-free industrial biotechnology.[11] Despite extensive research, there are only few industrial applications of archaeal biomass.[12]

Materials and Methods

Microorganism

H. mediterranei DSM 1411 was purchased from DSMZ culture collection, Germany.

Bioreactor Equipment and Experimental Set-up

For production of P-(3HB-*co*-3HV), *H. mediterranei* was cultivated in a 42 liter bioreactor (MBR Bioreactor AG, CH). The medium was supplemented with 10 g/L of sugars from hydrolyzed whey; nitrogen and phosphate were supplied by adding 5 g/L yeast extract. Further substrate pulses were supplied if necessary. Inoculation was done with 5 vol-% of a preculture from the late exponential phase. The cells were cultivated under controlled conditions of pH (7.0), temperature (37 °C) and oxygen tension (50% of air saturation during balanced growth, 30–40% of air saturation during predominant PHA formation; control by adjustment of the agitation speed at constant aeration of 10 mL/min).

For production of P-(3HB-*co*-3HV-*co*-4HB), a 10 L bioreactor (type L 1523, Bioengineering, Switzerland) was used. The nutrient medium was inoculated with overnight cultures from the late exponential phase with an inoculum concentration of 12 vol.-% of the working volume. Temperature (37 °C) and pH value at (7.0) were held constant. The cultivation was carried out at an oxygen partial pressure (control by agitation speed) of about 50% of air saturation. The strain was cultivated on hydrolyzed whey as the sole carbon source at a sugar concentration of 10 g/L; nitrogen and phosphate were supplied by yeast extract (5 g/L); further additions of substrates were done if necessary. 3HV and 4HB precursors were first added after 34.25 h.

Media Compositions

H. mediterranei was cultivated on a highly saline medium containing (g/L): NaCl 150, $MgCl_2 \cdot 6\ H_2O$ 13, $MgSO_4 \cdot 7\ H_2O$ 20, $CaCl_2 \cdot 2\ H_2O$ 0.67, KCl 4, $NaHCO_3$ 0.2, NaBr 0.5, yeast extract 5, NH_4^+-Fe(III)-citrate 0.05 and (mL/L): hydrolyzed whey permeate 50 and trace element solution SL6 1).[1]

Determination of substrates

A HPLC equipment (thermostated Aminex HPX 87H column, HP 7673 Controller, JASCO 880-PU HPLC pump, BISCHOFF RI-Detector 8110, SIC Chromatocorder 12) was used. Elution was done isocratically with H_2SO_4 (0.005 M; flow rate 0.60 mL/min).

Determination of Protein

After ultrasonic cell disruption, protein was measured according to Lowry's method.[14]

Determination of Quantity and Composition of PHA

PHA in lyophilized biomass samples was transesterificated by acidic methanolysis. Equipment: HP 5890 Series II gas chromatograph (30 m HP5 column, protected by a 5 m HP 1 capillary pre-column). The methyl esters of PHA constituents were detected by a flame ionization detector; carrier gas: helium (split- ratio of 1:10).[15] P-(3HB-co-19.1%-3HV) (Biopol; Imperial Chemical Industries) was used for 3HB and 3HV calibration, Na^+-4-hydroxybutyrate (Fluka) for calibration of 4HB (internal standard: hexanoic acid). The PHA content (wt.-%) was defined as the percentage of PHA concentration to dry cell mass (CDM). CDM (g/L) was defined as the sum of protein (g/L) and PHB (g/L).

Preparation of the main carbon source

Lactose in sweet whey permeate (dairy company *Latterine Vincentine,* Italy) was hydrolyzed enzymatically as described before.[16]

Isolation of PHA

Biomass was in situ pasteurized, centrifuged, frozen and lyophilized for 24 h. After degreasing the biomass by overnight Soxleth extraction with C_2H_5OH, PHA was isolated by overnight Soxleth extraction with $CHCl_3$. Product purity and completeness of the isolation were determined by GC.

Determination of Molecular Mass and Molecular Mass Distribution

Molecular mass was determined with a JASCO PU-1580 HPLC (JASCO 830-RI detector; two PLgel 5 μm mixed-C columns). Solvent: $CHCl_3$ at 1.0 mL/min; calibration was done using monodisperse polystyrene standards.

Thermal Analysis Characterization

Equipment: Mettler TA 4000 System instrument (DSC-30 Differential Scanning Calorimeter, TGA-50 furnace with M3 microbalance, and TA72 GraphWare software). Samples (5 mg) were weighed in 40 μL aluminium pans with an empty pan as reference. Operating protocol: 1st, 2nd and 3rd heating from −30 to 200 °C at 10 °C/min; quenching after the 1st heating from 200 to −30 °C at 100 °C/min and the 2nd cooling from 200 to −30 °C at 10 °Cmin. Nitrogen flow rate: 80 mL/min. Glass transition temperatures (T_g) and melting temperatures (T_m) were reported from the 2nd heating scan.

Results and Discussion

Production of Poly-3-(hydroxybutyrate-co-6%-hydroxyvalerate)

Similar to prior experiments on bioreactor scale,[16] glucose was metabolized faster than galactose by the strain. Especially in later phases of the cultivation, after protein formation had stopped, galactose was not any more utilized. During the entire cultivation period, 3HB and 3HV were formed with different rates, but these rates were always nearby constantly proportionate (highest percentage of 3HV in total PHA: 8-9 % during growth phase; approximately 6 % in the periods, where no more protein is formed, see Figure 1a). In direct comparison with other cultivations with *H. mediterranei*, these percentages are somewhat lower than expected, but within the same order of magnitude.[16]

The cultivation can be divided into two phases. From inoculation to 76 h, protein formation was accompanied by PHA production. This growth-associated PHA accumulation corresponds well to prior experiments with *H. mediterranei*.[16] After 76 h, no more protein was formed, but PHA concentration still increased until the end of the process. The production rate of PHA obeys a linear function from 31.5 h until the end of the fermentation with a rate of $r_{p\ PHA} = 0.11$ g/Lh. The maximum concentrations for protein and PHA amounted to 4.6 g/L and 12.2 g/L, respectively. The final content of PHA in biomass was 72.8 wt.-%, the volumetric productivity for the entire process was determined with 0.09 g/Lh. The maximum specific growth rate μ_{max} (0.10 1/h) was very similar as reported in literature.[16] The highest specific product formation rate ($q_{p\ max.}$, PHA) was calculated with 0.15 g/gh. The yield for PHA from whey sugars was 0.29 g/g.

Molecular weight was determined with a weight average molecular weight $M_w =$ 1057 kDa and a polydispersity index of PDI = 1.5 at the end of the fermentation, indicating an extremely high degree of polymerization and a very narrow distribution of molecular mass. In direct comparison with data from a first published fermentation on hydrolyzed whey lactose,[16] both values constitute a significant progress in the quality of the produced polyester. Thermo analysis revealed a glass transition point T_g of 6.0 °C, a cold crystallization point T_c at 62.2 °C, and two melting endotherms $T_{m\,1}$, $T_{m\,2}$ at 150.8 and 158.9 °C (Table 2). These data are very similar to those from literature.[16] Compared with homopolymer PHB, the low melting temperatures are advantageous for further processing of the material. The low melting points are probably caused by the presence of 3HV units, leading to a disruption of the high crystalline PHB matrix.[6] This polyester shows more than one melting endotherm, which indicates a not-random distribution of 3HV in the PHB matrix, i.e. the material is of heterogeneous nature. A formation of blocks of 3HV or/and the formation of polymer blends might have taken place. The decomposition temperature was determined with $T_d = 241$ °C.

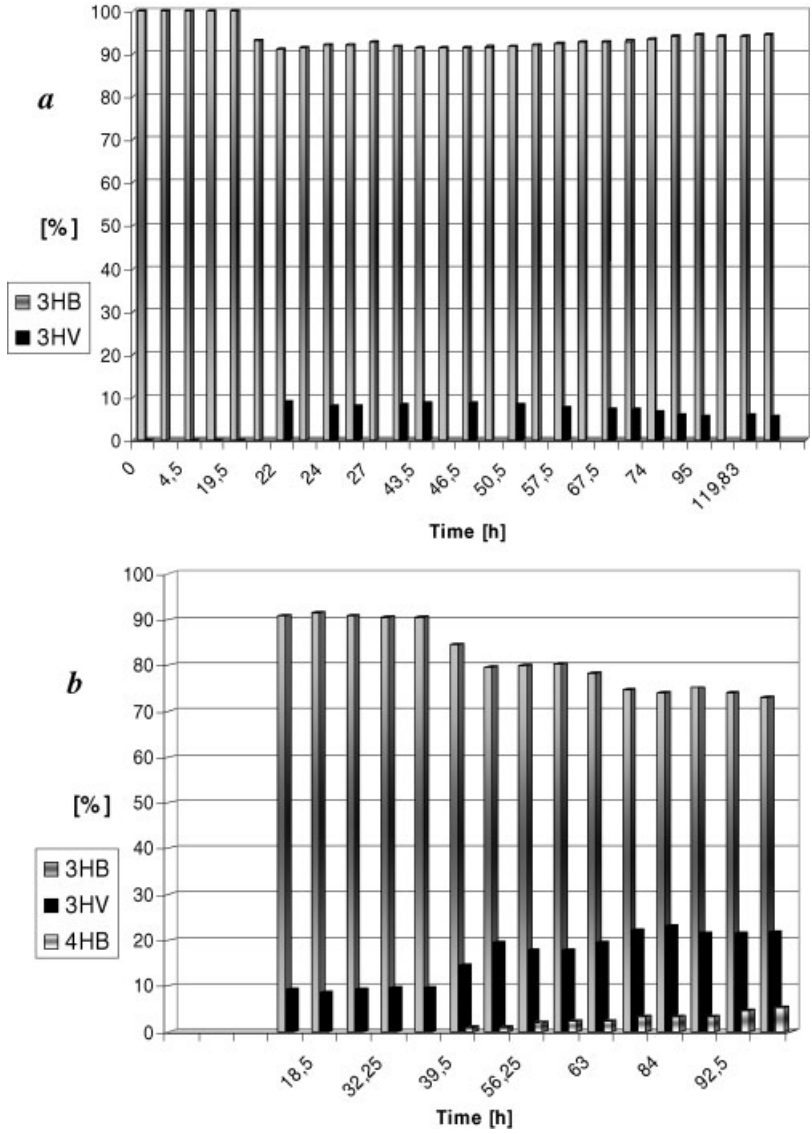

Figure 1.
Time courses of PHA building blocks during biosynthesis of P-(3HB-*co*-6%-3HV) (*a*) and P-(3HB-*co*-21.8%-3-HV-*co*-5.1%-4HB) (*b*).

According to this analysis, the quality of produced copolyester is certainly sufficient for applications in polymer extrusion technology.

Production of Poly-(3-hydroxybutyrate-co-21.8%-3-hydroxyvalerate-co-5.1%-4-hydroxybutyrate)

Starting from the 3HV content formed by *Haloferax mediterranei* from hexoses (see prior experiment), it was tried to trigger the content of 3HV to a desired level of approximately 20% by additions of sodium valerate (3HV precursor). No information exists in literature dealing with the production of polymers containing 4HB monomers by *H. mediterranei*. Therefore the cultivation broth was also supplied with γ-butyrolactone, a known 4HB precursor for other PHA producing strains.[15]

Table 1.
Kinetic data for the compared cultivations.

	P-(3HB-co-6%-3HV)	P-(3HB-co-21.8%-3-HV-co-5.1%-4HB)
$\mu_{max.}$ [1/h]	0.10	0.14
q_p [g/gh]	0.15	0.23
$Y_{(PHA/Whey\ sugars)}$	0.29	0.20
max. PHA concentration [g/L]	12.2	14.7
PHA/CDM [wt.-%]	72.8	87.5
Vol. productivity PHA [g/Lh]	0.09	0.14

μ specific growth rate; q_p specific production rate for PHA; $Y_{(PHA/Whey\ sugars)}$ yield coefficient for PHA from whey sugars; CDM cell dry mass.

Precursor addition started after 34.25 h of cultivation. At this time, a mixture consisting of valerate and γ-butyrolactone was added to achieve a concentration of approximately 1 g/L sodium valerate and 0.5 g/L γ-butyrolactone in the medium. The mixture was added after 34.25; 58; 63 and 87.5 h.

Table 1 shows the calculated kinetic data and yields. Similar to other experiments with *H. mediterranei* on hydrolyzed whey (see prior experiment), glucose was converted faster than galactose. At 24; 32.25; 42; 56.25 h, glucose was depleted, but high concentrations of galactose were still present in the medium. The 3HV content was successfully triggered to 22% by the feeding strategy what is close to the desired value of 20% (see Figure 1b). After the first addition of valerate, 3HV content increased from below 10% (typical for 3HV production without precursor, see prior experiment) to approximately 14% 5 h later. After 42 h, the polyester already contained around 19% 3HV. It was possible to maintain this value in a narrow range until the end of the cultivation (final 3HV concentration: 3.22 g/L). 5 h after the first feeding of precursors, three different polyester building blocks (3HB, 3HV and 4HB) were detected, confirming that it was possible to produce a P-(3HB-*co*-3HV-*co*-4HB) terpolymer. The 4HB share was increased during the process from a level of 1 mol-% until approximately 5 mol-%; the finally isolated terpolyester was identified as P-(3HB-*co*-21.8%-3HV-*co*-5.1%-4HB). The final PHA concentration amounted to 14.7 g/L, corresponding to a polyester content in cells of 87.5 wt.-% which is significantly higher than in the prior fermentation (see Table 1). The value for the maximum specific growth rate $\mu_{max.}$ (0.14 1/h) is the highest from all described fermentations with this strain on hydrolyzed whey permeate (prior experiment: 0.10 1/h; literature: 0.11 1/h).[16] The same is valid for the maximum specific production rate $q_{p\ max.}$ (here: 0.23 g/g h; prior

Table 2.
Thermal properties, molecular weights and polyester composition of polyhydroxyalkanoates produced by *H. mediterranei*.

	P-(3HB-co-6%-3HV)	P-(3HB-co-21.8%-3-HV-co-5.1%-4HB)
Melting endotherm T_m [°C]	150.8[a)]/158.9[b)]	139.0[a)]/140.0[b)]
Glass transition temperature T_g [°C]	6.0	−2.0
Decomposition temperature T_d [°C]	241	236
3HV/PHA [mol-%]	6.0	21.8
4HB/PHA [mol-%]	0.0	5.1
M_w [kD]	1057	987
Polydispersity index PDI [M_w/M_n]	1.5	1.5

[a)] First melting endotherm $T_{m\ 1}$.
[b)] Second melting endotherm $T_{m\ 2}$.
M_w Weight average molecular mass; M_n Number average molecular mass.

experiment: 0.15 g/g h; literature data: 0.08 g/g h).[16] Volumetric productivity (0.14 g PHA /L h) was significantly higher than in the prior cultivation (0.05 g/L h), but lower than in a control experiment on pure glucose (0.21 g/L h).

Table 2 compares data for molecular mass and molecular mass distribution of the isolated polyesters as well as results for thermoanalysis with the data from the prior experiment. Molecular mass is in a similar high range like measured for the copolyester ($M_w = 986$ kDa versus 1057 kDa), PDI values (1.5) are identical. DSC analysis of the polymer traces two melting endotherms in the melting diagram (1st melting point: 139 °C, 2nd melting point: 140 °C) which are significantly lower than obtained for the P-(3HB-*co*-6%-3HV) copolyester. The decomposition temperature was determined with $T_d = 236$ °C (copolyester: 241 °C), meaning that the product exhibits a broad window of processability due to the big difference between T_m and T_d.

According to the results, a polyester with interesting properties was produced by *H. mediterranei* from whey and precursors for 3HV and 4HB formation. It was possible to trigger the content of 3HV to a desired level (approximately 20%). Additionally, the possibility to produce PHA containing 4HB by this strain under controlled conditions was demonstrated. Kinetic data and yields show typical values for the strain.

Conclusion

The results indicate that *Haloferax mediterranei* constitutes a promising candidate for production of high-performance PHA co- and terpolymers starting from whey sugars. This is due to the strains high robustness and stability, the partial conversion of hexoses to 3-hydroxyvalerate units and the excellent polymer characteristics. For both products, the big difference between melting temperature and decomposition temperature might open the way for an easy processability by melt extrusion. Additionally it is very likely that this materials are also suitable for film blowing techniques. To assess the performance of the polyesters in biomedical application, future activities will focus on a more detailed investigation of decisive mechanical properties (e.g. tensile strength) and on the results from degradation studies. Based on these data, a decision can be made for a defined niche of application in a medical and/or pharmaceutical area.

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