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

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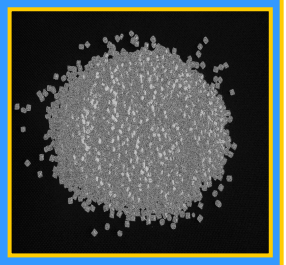
Abstract

Polyhydroxyalkanoates (PHAs) are regarded as future-oriented alternatives for large-scale packaging materials or all-day commodity items. For these purposes, cost effectiveness is the major obstacle in making PHAs competitive with common petrol-based plastic materials. For application in medical and pharmaceutical fields (artificial blood vessels, wound dressing, joints, implants, nerve repair, matrices for controlled drug delivery, dentistry etc.), PHAs are superior to conventional polymers in terms of **in-vivo degradability** and **biocompatibility**. Here, highly purified polyesters with tailor-made properties are demanded. Properties of PHAs are very much dependent on their composition. Compared with pure 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 and leads to polyesters with enhanced physical and thermodynamic properties. The lower degree of crystallinity makes the material more flexible, enables its processability for manufacturing of desired items and enhances its in-vivo degradation rate. Thus, the synthesis of PHA co- and terpolyesters with defined composition provides a wide range of medical and pharmaceutical applications. In this study, the highly osmophilic and robust archaebacterial strain *Haloferax mediterranei* was investigated for the production of two different PHAs. For both cases, the kinetics of the biosynthesis as well as the polymer properties are discussed.



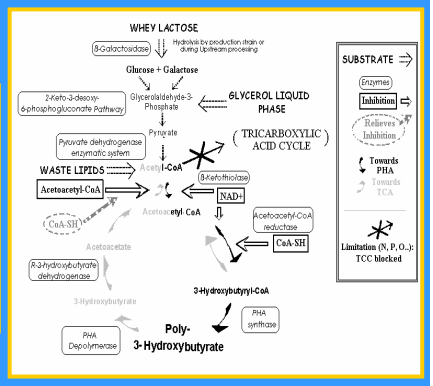


Figure 3: Poly-(3HB-*co*-3HV) in the form of vendible granules (left picture) and metabolic pathway of Poly-3HB production from different waste and surplus materials (right picture [5])

Materials and Methods

- The microorganism *Haloferax mediterranei* DSM 1411 was purchased from DSMZ culture collection
- Whey permeate was provided from Latterie Vicentine, Veneto, Italy and hydrolyzed by addition of Maxilact 2000. *[3]*
- The cultivations were carried out in the following laboratory scale bioreactors: MBR 40 (40 liter total volume; copolyester synthesis) and L1523, Bioengineering, Wald, Switzerland (10 liter volume; terpolyester synthesis).
- Substrate analysis was done via HPLC equipment consisting of a thermostated Aminex HPX 87H column (solvent: H_2SO_4 0.005 M at 0.60 mL/min), an HP 7673 Controller, a JASCO 880-PU intelligent HPLC pump, and a BISCHOFF RI-Detector 8110.
- Content and composition of PHA was determined gas chromatographically after transesterification via acidic methanolysis (HP 5890 Series II gas chromatograph in combination with a Hewlett Packard 7673 Controller; FID). *[1]*
- Molecular weight data were obtained from measurements on a Jasco PU-1580 HPLC connected to Jasco 830-RI detector and equipped with two PLgel 5 µm mixed-C columns. CHCl3 was used as solvent (flow rate 1.0 ml/min). Monodisperse polystyrene standards were used for calibration.
- Thermal analysis characterization was performed on a Mettler TA 4000 System instrument consisting of DSC-30 Differential Scanning Calorimeter, TGA-50 furnace with M3 microbalance, and TA72 GraphWare software. DSC samples of approximately 5 mg were weighed in 40 µl aluminium pans; an empty pan was used as reference. Measurements were carried out under 80 ml/min nitrogen flow rate according to the following protocol: first, second and third heating from -30 to 200 °C at 10 °C/min; first cooling (quenching after the first heating) from 200 to -30 °C at 100 °C/min and the second cooling from 200 to -30 °C at 10 °C/min.

Results

A **PHA copolyester** containing 6% of 3-hydroxyvalerate (3HV) in the poly-3-hydroxybutyrate (P-3HB) matrix was produced from whey sugars. Kinetic process analysis revealed a maximum specific growth rate ($\mu_{max.}$) and a maximum specific PHA synthesis rate ($\pi_{max.}$) of 0.10 1/h and 0.15 1/h, respectively. The cells contained 72.8% (w/w) of the polymer which showed excellent thermal characteristics (low melting points between 150 and 160 °C) and narrow molecular mass distribution (polydispersity P_i = 1.5). The results of this process were compared with the production of a **PHA terpolyester** containing 3HB, 3HV and 4-hydroxybutyrate (4HB) units. The polymer was produced by feeding of whey sugars plus precursors for formation of 3HV and 4HB. The main kinetic data were $\mu_{max.}$ = 0.14 1/h and $\pi_{max.}$ = 0.23 1/h, respectively. The final percentage of P-(3HB-*co*-21.81%-3-HV-*co*-5.14%-4HB) in biomass amounted to 87.5%. Also in this case, the molecular mass distribution was very narrow (P_i = 1.5). The high difference between the two melting endotherms of the material (between 140 and 150 °C) and the onset of decomposition at 236 °C opens a wide window of processability.

The main kinetic data for biomass formation and PHA production of the two fermentations are provided in Table 1, data for polymer characterization are shown in Table 2. The fermentation patterns of the processes for the terpolyester biosynthesis (time courses of substrate consumption and product formation) are depicted in Fig. 2 (time courses of main carbon sources), Fig. 3 (time courses of precursors for 3HV and 4HB formation), Fig. 4 (time courses for 3HV, 4HB and total PHA) and Fig. 5 (composition of the polyester during the process).

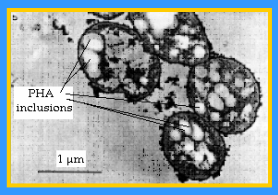


Figure 1: Cells of *Haloferax mediterranei* harbouring PHA inclusions (left picture) *[2]* and culture of *Haloferax mediterranei* grown on solid highly saline medium (right; own picture)

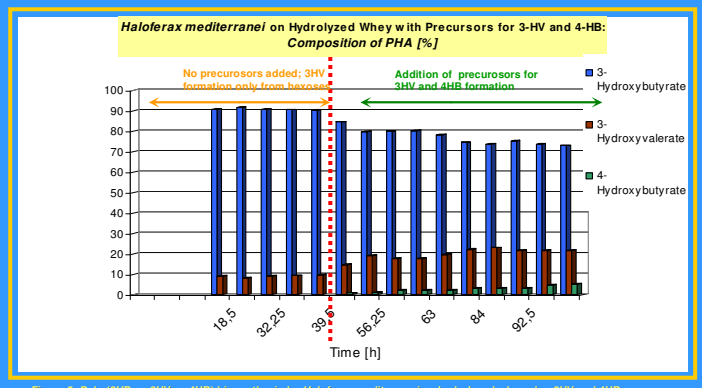


Figure 5: Poly-(3HB-*co*-3HV-*co*-4HB) biosynthesis by *Haloferax mediterranei* on hydrolyzed whey plus 3HV and 4HB precursors: composition of polyester during the production process

Figure 4: MBR 42 liter bioreactor (biosynthesis of copolyester; left picture) and L 1523 Bioengineering bioreactor (biosynthesis of terpolyester; right picture)

Table 1: Kinetic data for the compared cultivations

	Copolyester P-(3HB-*co*-5.6%-3-HV)	*Terpolyester* P-(3HB-*co*-21.81%-3-HV-*co*-5.14%-4HB)
max. μ [1/h]	0.10	0.14
π_{max} [g/g.h]	0.15	0.23
Y (PHA/Whey sugars) [g/g]	0.29	0.20
max. *PHA* concentration [g/L]	12.2	14.7
PHA/CDM [%] (end)	72.8	87.5
Vol. Productivity [g/Lh] (PHA; total duration)	0.09	0.14

Table 2: Polymer characterization

	Copolyester P-(3HB-*co*-5.6%-3-HV)	*Terpolyester* P-(3HB-*co*-21.81%-3-HV-*co*-5.14%-4HB)
1st. Melting endotherm (T_g1), [°C]	150.8 *	139.0 *
2nd. Melting endotherm (T_g2), [°C]	158.9 *	149.0 *
Onset of decomposition (T_d), [°C]	241	236
Cold Crystallization Peak [°C]	62.2	n.d.
Glass Transition Temperature [°C]	6.0	-2.0
3-HV/PHA [% w/w]	6	22
4-HB/PHA [% w/w]	0	5
Weight average molecular mass *Mw* [kD]	1057	987
Polydispersity Index (M*w*/M*n*)	1.5	1.5

* Reported from the second heating scan

Conclusion

The results indicate that *Haloferax mediterranei* constitutes a promising candidate for production of high quality PHA co- and terpolymers starting from surplus materials. This is due to the strains high robustness and stability, the partial conversion of hexoses to 3-hydroxyvalerate units and the excellent polymer characteristics (low melting temperature, high molecular masses with narrow distribution). [3,4] The presented materials feature properties making them interesting for future application i medical and pharamaceutical fields.

Acknowledgement

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References

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(5) Braunegg, Koller, Hesse *et al.*, 2006 (book chapter in press)