Polyhydroxyalkanoate (PHA) Biosynthesis from Whey Lactose

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Summary: The potential of three different microbial wild type strains as polyhydroxyalkanoate (PHA) producers from whey lactose is compared. Homopolyester and co-polyester biosynthesis was investigated by the archaeon *Haloferax mediterranei* and the eubacterial strains *Pseudomonas hydrogenovora* and *Hydrogenophaga pseudoflava*. *H. mediterranei* accumulated 50 wt.-% of poly-3-(hydroxybutyrate-*co*-6%-hydroxyvalerate) in cell dry mass from hydrolyzed whey without addition of 3-hydroxyvalerate (3HV) precursors (specific productivity q_p: 2.9 mg/g h). Using *P. hydrogenovora*, the final percentage of poly-3-hydroxybutyrate (PHB) amounted to 12 wt.-% (q_p: 0.03 g/g h); co-feeding of valeric acid resulted in the production of 12 wt.-%. P-3(HB-*co*-21%-HV) (q_p: 0.02 g/g h). With *H. pseudoflava*, it was possible to reach 40 wt.-% P-3 (HB-*co*-5%-HV) on not-hydrolyzed whey lactose plus valeric acid as 3HV precursor (q_p: 9.1 mg/g h); on hydrolyzed whey lactose without addition of valeric acid, the strain produced 30 wt.-% of PHB (q_p: 0.16 g/g h). The characterization of the isolated biopolyesters completes the study.

Keywords: biocompatibility; biopolymers; high-performance polymers; polyhydroxyalkanoates; whey

Introduction

Increasing demands for polymeric materials for the safe distribution of goods is undisputed. Contemporary strategies for disposing of end-of-pipe plastics cause serious global problems like increasing piles of waste. Recycling does not work as effectively as needed for efficiently solving the problem. Incinerating petrol-based polymers generates noxious compounds and elevates the atmospheric CO_2 level, aggravating prevailing problems such as global warming.[1] Further, reserves of fossil feed stocks are limited. In May 2005, the price per barrel of mineral oil amounted to US-$ 55; recently, this value has rocketed up to nearby US-$ 100 (November 2007).

Utilizing alternative polymers like polyhydroxyalkanoates (PHAs) unites two advantages: They are produced from renewable resources, uncoupling them from the availability of fossil feed stocks. Further, when composted, these biopolymers undergo a biodegradation process resulting merely in CO_2 and H_2O, the basics for the photosynthetic regeneration of carbohydrates by green plants. Thus, the carbon in PHA production lines is embedded into a closed circle. This is clearly in contrast to the life cycle of classic polymers, where carbon fixed in the bowels of earth since millions of years is converted to CO_2 which is released in the atmosphere.[2]

Because recent studies state that PHA production from pure sugars has been optimized to a high degree, further

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improvement of the technology by using cheaper carbon feed stocks is urgently needed.[3] This work deals with whey, the major by-product from cheese industry, as feed stock for production of PHA. Whey is not merely inexpensive, but 13 500 000 tons of whey per year containing 620 000 t of lactose (*D*-gluco-pyranose-4-*β*-*D*-galacto-pyranoside) causes a huge disposal problem for the dairy industry in the EU.[2] The utilization of whey lactose for PHA production unites the diminishing of waste and the increase of cost-efficiency in the production of ecologically benign materials.

The study compares kinetic data and polymer characteristics for three microbial strains that turned out to be capable of PHA homo- and copolyester accumulation from whey lactose (the eubacterial species *Pseudomonas hydrogenovora* and the archaeon *Hydrogenophaga pseudoflava* as well as *Haloferax mediterranei*).

Materials and Methods

Microorganism

H. mediterranei DSM 1411, *H. pseudoflava* DSM 1034 and *P. hydrogenovora* DSM 1749 were purchased from DSMZ culture collection, Germany.

Bioreactor Equipment and Cultivation Conditions

For production of P-3(HB-*co*-6%-HV), *H. mediterranei* was cultivated in a 42 liter bioreactor (MBR Bioreactor AG, CH). 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. Media composition was described before.[4]

For producing PHB and P-3(HB-*co*-5%-HV) by *H. pseudoflava*, a 10 L bioreactor (Bioengineering, CH, type L 1523) was used and stirred with 200–300 rpm depending on the oxygen level in the reactor. Temperature was held at 36 °C and the pH value between 6.8 and 7.0. Details and media composition were described before.[5]

P. hydrogenovora was cultivated for production of PHB and P-3(HB-*co*-21%-HV) in a 2 L bioreactor (Bioengineering, CH, type KLF 2000) as described before.[4] Temperature (30 °C) and pH value (7.0) were controlled automatically. Concentration of dissolved oxygen was controlled by adjustment of the agitation speed.

Preparation of the Main Carbon Source

Hydrolysis of whey lactose was performed enzymatically as previously described.[5]

Determination of Substrates

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

Determination of Protein

After ultrasonic cell disruption, protein was determined according to Lowry's method.[6]

Determination of Quantity and Composition of PHA

Intracellular PHA in lyophilized biomass samples was transesterificated by acidic methanolysis.[7] Analysis was carried out with a HP 5890 Series II gas chromatograph (30 m HP5 column, 5 m HP1 capillary precolumn). Detection: flame ionization detector (FID); carrier gas: helium (split- ratio of 1:10). For calibration of 3HB and 3HV, pure P-3(HB-*co*-19.1%-HV) (BIOPOL^{TM}) was used.

Isolation of PHA

The cells were 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 as well as the completeness of the isolation was determined by gas chromatography.

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 (TA)

TA was performed on a Mettler TA 4000 System instrument (DSC-30 Differential Scanning Calorimeter, TGA-50 furnace, M3 microbalance, TA72 GraphWare software). DSC samples of ca. 5 mg were weighed in 40 μL aluminium pans together with an empty pan as reference. 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 °C/min. Nitrogen flow rate: 80 mL/min. The melting temperature (T_m) was measured during the 2nd heating scan.

Results and Discussion

Haloferax Mediterranei: Production of Poly-3(HB-*co*-6%-HV) from Hydrolyzed whey Lactose Without Feeding of 3HV Precursors

Similar to prior experiments on bioreactor scale, glucose was metabolized faster than galactose.[4] Especially in later phases of the cultivation, after protein formation had stopped, galactose was no longer utilized. During the entire cultivation period, 3HB and 3HV were formed with different rates, but these rates were always nearby constantly proportionate (highest share of 3HV in PHA: 8–9% during growth; approximately 6% in periods without protein formation). Compared to other cultivations with *H. mediterranei*, these values are a little bit lower than expected, but within the same order of magnitude.[4]

The cultivation is 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*.[4] After 76 h, no more protein was formed, but PHA concentration still increased until the end of the process. The production rate of PHA from 31.5 h until the end of the process was $r_{p\ \mathrm{PHA}} = 0.11$ g/L h.

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/L h. $\mu_{\mathrm{max.}}$ (0.10 1/h) was very similar as reported.[4] The highest specific product formation rate ($q_{p\ \mathrm{max.}}$) was calculated with 0.15 g/g h. The yield for PHA from whey sugars was 0.29 g/g.

Molecular weight distribution was determined with a weight average molecular weight $M_w = 1057$ kDa and a polydispersity index PDI = 1.53 at the end of the fermentation, indicating a high degree of polymerization and a narrow distribution of molecular mass. In comparison with data from a first published fermentation on hydrolyzed whey lactose, both values constitute a significant progress in the quality of the polyester.[4] Thermo analysis revealed two melting endotherms Tm_1, Tm_2 at 150.8 and 158.9 °C (Table 2). These data are very similar to those from literature.[4] Compared with 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.[8] According to this analysis, the quality of produced copolyester is certainly sufficient for applications in polymer extrusion technology.

Hydrogenophaga Pseudoflava: Production of PHB from Hydrolyzed whey Lactose

The depletion of the nitrogen source NH_4^+ after 28.5 h provoked the stop of biomass formation (max. biomass concentration 12 g/L) and the start of PHA production. After 35 h, the maximum percentage of PHB in cells (30 wt.-%) was reached. Biomass production was fastest between

20 and 28 h of growth. The highest utilization rate for glucose and galactose was observed after 30 h, accompanied by the highest PHA production rate.

The yield for PHA from lactose was 0.12 g/g; the yield for biomass (0.43 g/g) from sugar was typical as for other PHA producing strains. Maximum specific rates for biomass and PHA formation were calculated with $\mu_{max.} = 0.17$ 1/h and $q_{p\ max.} = 0.16$ 1/h; volumetric productivity for PHA amounted to 0.12 g/L h.

Table 2 presents all polymer data for comparison with the biomaterials obtained by the other fermentations presented in this study. The high molecular weight ($M_w = 827$ kD) and the PDI (2.7) indicate the high quality of the this polymer. The high melting point T_m of 178.9 °C constitutes a typical value for PHB homopolymers.[8]

Hydrogenophaga Pseudoflava: Production of Poly-3(HB-*co*-5%-HV) from Non-Hydrolyzed whey Lactose with Co-Feeding of the 3HV Precursor Valeric Acid

The depletion of the nitrogen source NH_4^+ after 40 h provoked the stop of biomass formation and the start of PHA production. Production of biomass and 3HB stopped after about 45 h, only production of 3HV augmented by addition of valerate after 50 h. The fraction of polymer in the cells increased after about 10 h until 22 h. Biomass production was fastest between 20 and 30 h of growth. The highest lactose utilization rate was observed after 30 hours, accompanied by the highest PHA production rate.

The yield for PHA from lactose (0.20 g/g); was in a similar low range as in the prior fermentation on hydrolyzed whey permeate; the yield for biomass from sugar (0.44 g/g) was typical as for other PHA producing strains. Maximum rates for biomass and PHA formation (0.08 1/h and 0.05 1/h) were lower than on hydrolyzed whey permeate; the same goes for the volumetric productivity (0.05 g/L h on non-hydrolyzed whey lactose vs. 0.12 g/L h on hydrolyzed whey lactose) as well as for the maximum specific growth rate (0.08 1/h vs. 0.12 1/h) and the specific productivities 12.5 mg/g h vs. 15.0 mg/g h).

Table 2 presents all polymer data for comparison with the biomaterials from *H. mediterranei* and *P. hydrogenovora*. The high molecular weight ($M_w = 859$ kDa) and the PDI (3.3) indicate the high quality of the polymer produced by *H. pseudoflava* on whey lactose and the 3HV precursor valerate, although the melting point T_m (176.7 °C) is still rather high. This might be overcome by increasing the share of 3HV in the polyester.

Pseudomonas Hydrogenovora: Production of PHB from Hydrolyzed whey Lactose

For the period of biomass production (until 16 h), $\mu_{max.}$ was calculated with 0.29 1/h. When the conversion of inorganic nitrogen source was completed, production of PHB started. This was immediately followed by higher consumption rates for the sugars, which are co-metabolized. After 24 h, production rates for PHB decreased, although the consumption rates of sugars stayed constant. Because of the cyclic nature of production and degradation of biopolymers in living cells, this is very likely caused by degradation of one or more of the enzymes involved in PHB synthesis, without a possibility to renew them in this phase of nitrogen starvation. The ongoing consumption of sugars is a consequence of diverting the carbon towards excretion of metabolites, especially TCA cycle intermediates (the one with the highest quantity was identified as 2-oxoglutarate with a final concentration of 9 g/L). Hence, in the late stationary phase, carbon is directed towards three routes: maintenance energy, PHB production, and excretion of metabolic intermediates. At the end of the fermentation (41 h), 1.3 g/L PHA was achieved (Table 1)

The polymer was analyzed for molecular weight distribution ($M_w = 353.5$ kDa, PDI = 3.8). This data are typical for PHB. Thermal analysis characterization is collected in Table 2. The melting temperature T_m (169.3 °C) is lower than expected for PHB.[8]

Table 1.
Kinetic data for the compared cultivations.

	Haloferax mediterranei[a)]	Hydrogenophaga pseudoflava[a)]	Hydrogenophaga pseudoflava[b)]	Pseudomonas hydrogenovora[a)]	Pseudomonas hydrogenovora[b)]
$\mu_{max.}$ [1/h]	0.10	0.17	0.08	0.29	0.20
q_p [g/g h]	0.15	0.16	0.05	0.03	0.02
$Y_{(PHA/Whey\ sugars)}$	0.29	0.12	0.20	0.08	0.07
$Y_{(3HV/Valerate)}$	–	–	0.19	–	0.09
max. PHA concentration [g/L]	12.2	4.1	2.7	1.3	1.4
PHA/CDM [wt.-%]	73	30	40	12	12
Vol. productivity PHA [g/L h]	0.09	0.14	0.05	0.03	0.05

$\mu_{max.}$ – max. specific growth rate; q_p – specific production rate for PHA; $Y_{(PHA/Whey\ sugars)}$ – yield coefficient for PHA from whey sugars; $Y_{(3HV/Valerate)}$ – yield coefficient for PHA from whey sugars; CDM – cell dry mass.
a) No addition of 3HV – precursor;
b) Addition of 3HV – precursor.

Pseudomonas Hydrogenovora: Production of Poly-3(HB-*co*-21%-HV) from Hydrolyzed whey Lactose with Co-Feeding of the 3HV Precursor Valeric Acid

$\mu_{max.}$ (0.20 1/h) was lower than calculated for the prior experiment. Accompanied by an increased PHA production, biomass formation continued until nitrogen sources were depleted. After 25 h, production rates for PHB decreased, although high consumption rates of sugars were monitored. From this time, high amounts of 2-oxoglutarate (final concentration ca. 6 g/L) were produced, leading to low yields for PHB formation. Similar to the prior fermentation, the final share of PHA in cells was determined with 12 wt.-%; 1.44 g/L PHA were obtained at the end of the process. Formation of 3HV from the precursor valerate mainly occurred between 14 and 21 h of cultivation. The final mass fraction of 3HV building blocks in the polymer amounted to 21% (Table 2); the average yield for 3HV production from the precursor was 0.09 g/g (Table 1).

Data for molecular weight distribution are in a similar range like the value for PHB obtained in the prior experiment (weight average molecular weight $M_w = 299\,200$, PDI of 4.3). The melting temperature T_m (165.3 °C) is significantly lower than for the homopolymer PHB (Table 2). According to this analysis, product quality might be sufficient for applications in melt extrusion technology, and probably also for film blowing. Data for thermal analysis characterization are collected in Table 2.

Table 2.
Thermal properties, molecular weights and polyester composition of polyhydroxyalkanoates produced from whey by different strains.

	Haloferax mediterranei[a)]	Hydrogenophaga pseudoflava[a)]	Hydrogenophaga pseudoflava[b)]	Pseudomonas hydrogenovora[a)]	Pseudomonas hydrogenovora[b)]
Melting endotherm T_m [°C]	150.8[c)]/158.9[d)]	178.9	176.7	169.3	165.3
3HV/PHA [%]	6	–	5	–	21
M_w [kDa]	1057	827	859	353.5	353.5
PDI [M_w/M_n]	1.5	2.7		3.8	4.3

M_w – Weight average molecular mass, M_n – Number average molecular mass.
a) No addition of 3HV – precursor;
b) Addition of 3HV – precursor;
c) First melting endotherm;
d) Second melting endotherm.

Conclusion

According to the results, *H. mediterranei* constitutes the most promising candidate for industrial scale PHA production starting from the surplus feed stock whey among the investigated microbial strains. This is due to the strain's high robustness and stability; the risk of microbial contamination during cultivation is restricted to an absolute minimum, thus a lot of energy is saved by lower sterility demands. The formation of 3HV units from whey sugars to and the excellent polymer characteristics (low melting temperature, high molecular masses with narrow distribution) together with a viable cheap and simple downstream processing make the strain of special interest. Recycling of the highly saline side streams has to be tested and optimized. Although also producing homo- and co-polyesters with interesting properties, *P. hydrogenovora* features the disadvantage of low final polymer contents, low productivities and product yields due to redirection of the carbon flux towards unwanted by-products. Here, strain improvement by genetic engineering should be considered. Nevertheless, depending on the substrate supply, the strain is able to accumulate PHB and P-3(HB-*co*-HV). *H. pseudoflava* produces PHA homo- and co-polyesters of rather good quality directly from non-hydrolyzed whey lactose at acceptable production rates and yields, but is not competitive with *H. mediterranei* in terms of strain stability and robustness.

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