PRAXIS

A Publication by Bioengineering AG

Portrait of the laboratory for biomaterials of Empa (Swiss Federal Laboratories for Materials Testing and Research), St. Gallen: Synthesis, recovery, functionalization, and processing of polyhydroxyalkanoates (PHA).

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Bioengineering –
Experience only specialists can have

St. Gallen. 8.27 p.m.

1 Empa, 3 locations, 5 research and engineering departments – consisting of a total of 27 laboratories – and assisted by a support department.

Empa's Academy offers attractive continuing education and training programs for about 7000 internal and external attendees every year.

The departments are cross-linked within five strategic research programs to foster inter- and transdisciplinary approaches.

What's behind our logo "Empa – Materials Science and Technology"?

First and foremost, the Empa "brand" stands for a modern research and service institution within the ETH Domain covering – as is stated in the byline – the fields of materials science and technology development from nanotechnology to building and environmental technologies.
Empa's R&D activities focus on the needs of industry and society at large. Hence, they bridge the gap from science to engineering, from research to industry and society, and from visions to realization.
Empa operates, and offers its customers, a repository of instrumentation and equipment run by experts in various fields of the macroscopic and microscopic worlds.
As has been known for decades – for more than a century, to be precise – Empa hosts competent groups of experts providing neutral expertise and excellent services to industry and governmental bodies.
To be able to fulfill its obligations in teaching, training, and education, Empa established a Ph. D. program, providing a stimulating educational environment with dedicated supervisors and research projects geared towards practical applications.

Location of the
High Pressure
Bioreactor (HPR)

Location in St. Gallen with around 200 employees; Completion: 1996; Architect: Theo Hotz, Zurich

People

Contents

Empa's Research Programs

Empa is the ETH Domain's (ETH stands for Swiss Federal Institute of Technology) institution for multidisciplinary research in sustainable materials science and systems engineering. As an independent, neutral research institution, Empa solves selected tasks and problems of a scientific and technical nature. Empa combines target-oriented R&D with high quality services. Empa exploits its interdisciplinary skills to ensure an integrated approach.

Empa's most important partners are industry and society, institutes of higher education, universities and public authorities. Empa is an innovative and interdisciplinary participant in global R&D networks.

Nanotechnology

Entering the nanometer scale world allows access to the basic building blocks of our technical materials, leading to new applications and innovative solutions to existing problems. Empa has established itself as the leading Swiss R&D institution in the nanotechnology field, i.e. the applications-oriented exploitation of nanoscale effects. Empa implements its broad-based interdisciplinary know-how in this cutting-edge field between physics, chemistry and biology and uses its experience to develop promising new applications in cooperation with a range of partners. Among other activities, Empa is developing innovative solutions to problems in energy technology, and is working together with the IT industry on new concepts in micro- and nanoelectronics. Other future-oriented projects include investigating the use of the evolutionary building principles found in nature. However, Empa also thoroughly investigates the risks involved in nanotechnology. In cooperation with partner organizations, Empa is researching the interactions of nanoparticles and biological materials.
In projects covering a wide range of disciplines, Empa conducts life-cycle analyses of nanoproducts and attempts to assess the consequences of applying the new technology.

Adaptive Material Systems

This research program deals with the behavior of biological systems which are capable of reacting intelligently to changing environmental conditions. Using textile and fiber based composite materials for macro-scale mechanical components, integrating materials with the ability to act as sensors or actuators, and connecting them by means of adaptive controls or neural networks, Empa develops integrated mechanical systems advanced enough to be of practical use. For example, Empa has created a system which measures and actively suppresses mechanical oscillation in bridge structures.

Materials for Health and Performance

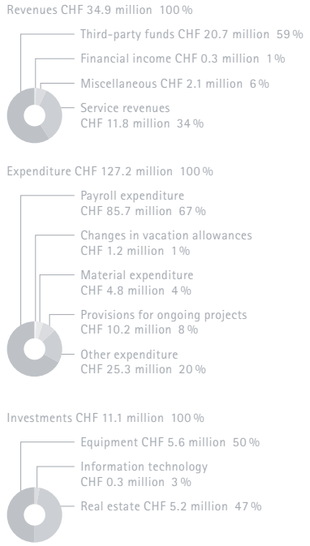
With increasing life expectancy, good health and performance are becoming increasingly important. The Empa research program "Materials for Health and Performance" supports efforts to maintain and restore human health, productivity, and quality of life. We develop novel materials, material combinations, and systems for specific applications. We seek to establish a top international research profile in selected areas, but also aim for results that directly benefit industry and end-users. Our activities focus on biomaterials and implants, health monitoring, medical textiles and protection and sports. With activities in the field of gerontotechnology, we are addressing the needs of the older generation.

Technosphere – Atmosphere

Emissions from human activities in the technosphere find their way into the atmosphere, with varying effects. Greenhouse gases cause climatic changes, toxic chemicals and soot particles affect our health and result, for example, in acid rain and corrosion. Empa's contribution to reducing the effects of this anthropogenic air pollution is to investigate and understand the fundamental physical and chemical processes which are involved, and to develop appropriate, innovative solutions to remediate these problems. For example, in the CLEVER project (Clean and Efficient Vehicle Research), Empa is attempting, in collaboration with industrial partners, to increase the efficiency of the powertrain systems of natural-gas fueled vehicles while simultaneously reducing exhaust gas emissions by a significant degree.

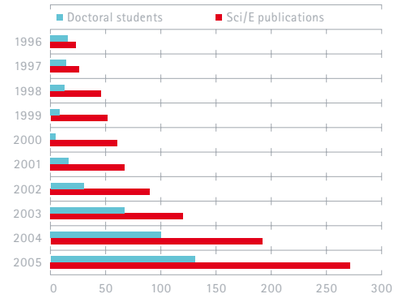
Materials for Energy Technologies

A single person in Switzerland consumes approx. 6000 watts of energy daily: for production of food and other goods, heating/cooling buildings, and mobility. This is much more than the target of the 2000-watt society. So new energy concepts and technologies are urgently needed. Aiming to reduce energy consumption by $^2/_3$, Empa is researching new materials, processes, and systems for energy conversion, storage, and transport. Empa is looking for ways to reduce conversion losses, to develop more efficient energy systems, to increase the use of renewable energy sources, and to minimize the risks associated with new energy concepts.



Scientific output	2005	2004
Sci/E publications	271	191
Patents applied for	18	23
Patents granted	4	7
License agreements	4	4
Spinoffs/startups	3	2
Empa academy		
Events*	114	132
Number of participants	6000	6000
Prizes/distinctions	30	21

*not incl. those in honor of Empa's 125th anniversary



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Statistics for 2005 and development of doctoral student enrolment and publication volume, 1996–2005.

From Bacteria to Biopolyester (PHA)

Culture broth

In general, PHAs are made by fed-batch or continuous fermentation (chemostat). Continuous fermentation reveals a major advantage when two or more distinct carbon sources are employed: In the batch or fed-batch process, the levels of various monomers progressively build up in the accumulated PHA product, ultimately leading to an inhomogeneous biopolymer. In the chemostat, the monomeric composition in the PHA remains constant. This results in a more homogeneous biopolymer with well-defined chemical and physical properties.

Freeze-dried biomass

In order to prevent intracellular depolymerization, the culture broth must be cooled in the collecting tank. The cooled broth is centrifuged and the biomass is frozen (–20 °C) and freeze-dried. The PHA content of the biomass varies widely as a function of bacterial species and carbon source; it ranges between 10 % and 90 %.

Crude polymer

With the conventional method, the water-insoluble PHA is extracted from the cell with an organic solvent and then precipitated again from the solvent. The crude polymer, however, still contains a variety of impurities such as lipids and proteins which must be removed by subsequent recrystallization. Alternative extraction methods (temperature-controlled precipitation) may lead directly to a biopolymer of satisfactory purity.

PHO

Poly(3-hydroxyoctanoate-*co*-3-hydroxyhexanoate) containing 88 % C8, molecular weight (M_w) approx. 200,000 Da, polydispersity (D) approx. 1.8, glass transition temperature (T_g) –40 °C, melting point (T_m) 60–70 °C, low degree of crystallinity (approx. 30 %).

PHB/HV

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) containing 85 % HV, molecular weight (M_w) approx. 1.5 MDa, polydispersity (D) approx. 2, glass transition temperature (T_g) –10 °C, melting point (T_m) 170 °C, high degree of crystallinity.

PHB

Pellets of 100 % poly(3-hydroxybutyrate). Biopolymers must have a sufficiently high degree of crystallinity if they are to be pelletted. The pellets serve as starting product for further processing, such as melt extrusion in a fiber spinning operation.

One goal of Empa's activities is the biotechnological production of new materials. Both the great variety of microorganisms and of starting materials offer a huge potential for newly tailored materials like biopolymers which can further be chemically modified. Additionally, we develop the necessary chemical and biological methods which allow us to extensively characterize the new materials and substances with respect to their chemical, structural and toxicological properties.

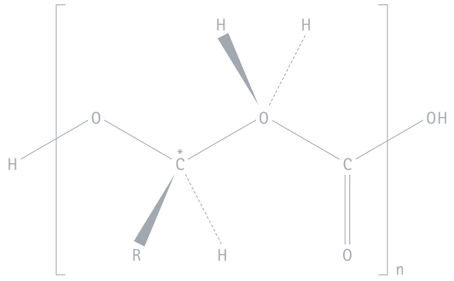
Tailored material properties of polyhydroxyalkanoates through biosynthesis and chemical modification

Introduction

Most research on polyhydroxyalkanoates (PHAs) has been undertaken with the aim of entering the bulk market of synthetic polymers.[1] However, PHAs have additional features rather than just being a material from renewable resources and being biodegradable in the environment. PHAs are potential candidates for applications where biocompatibility and adjustable material properties are a necessity, for example in medicine.[2–4] Here, PHAs could become an interesting material when degradation has to occur by surface erosion and not by bulk erosion as is the case with poly(lactic acid).[5]

PHAs have the general chemical structure depicted in Chart 2. The production of PHA in bacteria guarantees the complete stereospecificity (all chiral carbon atoms are in *R*(-)configuration), which is essential for the biodegradability of PHA. The type of bacterium and growth conditions determine the chemical composition of PHAs, including the molecular weight (M_w) that typically ranges from $2 \cdot 10^5$ to $3 \cdot 10^6$ Da. PHAs can be separated into 3 classes, short-chain-length PHAs (scl-PHA, carbon monomers ranging from C3 to C5), medium-chain-length PHAs, (mcl-PHAs; C6–C14) and long-chain-length PHAs (lcl-PHAs; > C14).[6] Interestingly, there are now PHAs produced by recombinant bacteria that consist of PHB and mcl-PHA.[7–9]

To date, more than 100 different monomers have been reported as PHA constituents but only few of these PHAs have been produced in large quantities and characterized. As a consequence, little is known about the chemical and mechanical properties of the polymers. PHAs with straight, branched, saturated, unsaturated, and also aromatic monomers were found.[10] Of special interest are functionalized groups in the side chain that allow further chemical modification, e.g. halogens, carboxyl, hydroxyl, epoxy, pheonxy, cyanophenoxy, nitrophenoxy, thiophenoxy, and methylester groups.[11–13] The length of the side chain and its functional group considerably influence properties like melting point, glass transition temperature, or crystallinity of the bioplastic, and therefore determine the processing approach and the final application. The average molecular weight and the molecular weight distribution also depend on the carbon source.

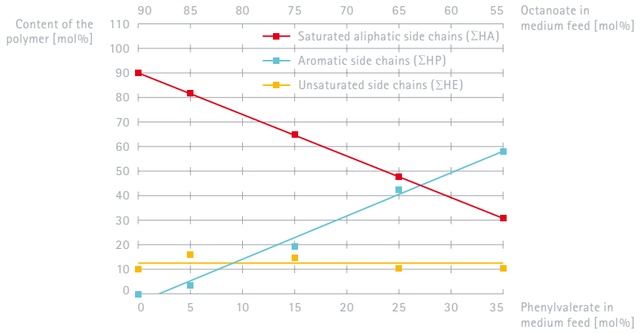


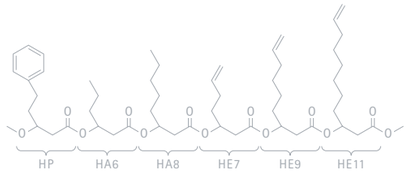
2
General structure of poly(3-hydroxyalkanoate) (PHA).

Experimental section

The cultivation of bacteria in continuous steady-state cultures (chemostat) allows the biosynthesis of PHA in a reproducible way. In such a bioreactor system, the culture broth is continuously exchanged with sterile growth medium. According to the theory of Monod, the specific growth rate of the culture can be defined by the ratio of the medium supply rate versus the volume of the culture in the bioreactor.[14] This allows determining the influence of well-defined growth conditions (e.g. nutrient limitation at a specific growth rate) on PHA accumulation. Thus, growth can be established where carbon (C) and nitrogen (N) limit growth simultaneously. This growth regime offered a new approach for tailoring the PHA composition during biosynthesis, because all carbon substrates are consumed to depletion.[15] Several chemostat experiments have been carried out with *Ralstonia eutropha* (DSM 428) and *Pseudomonas putida* GPo1 (ATTC 29347) that addressed this particular feeding strategy that enabled tailoring of PHA.[15–17]

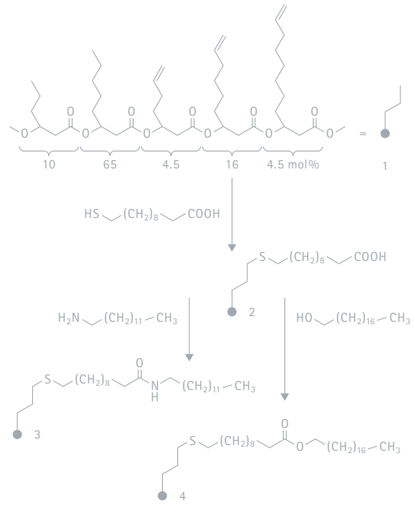
Functional groups in PHA provide sites for further chemical modification, directed to modulate the basic polymer properties or to create functionalities which are impossible to introduce by biosynthesis. The system of olefinic mcl-PHAs was chosen since the olefinic content of PHA could be tailored during biosynthesis by the combination of 10-undecenoic acid and octanoic acid in *P. putida* GPo1.[15] The resulting polymer PHOU enabled free radical addition of ω-mercaptoalkanoic acids or of ω-mercaptoalkanols enabled by AIBN.[18, 19] The products were thus activated for further chemical reactions as described below.





3

Tailor-made mcl-PHA containing 3-hydroxyalkanoates (HA), 3-hydroxyalkenoates (HE) and 3-hydroxyphenylvalerate (HP) produced in chemostat cultures with 10-undecenoate (10 mol%), 5-phenylvalerate (0–35 mol%), and octanoate (90–55 mol%), respectively.



4

Formation of amid or ester derivatives of PHOU.

Results and discussion

Tailored PHA by biosynthesis

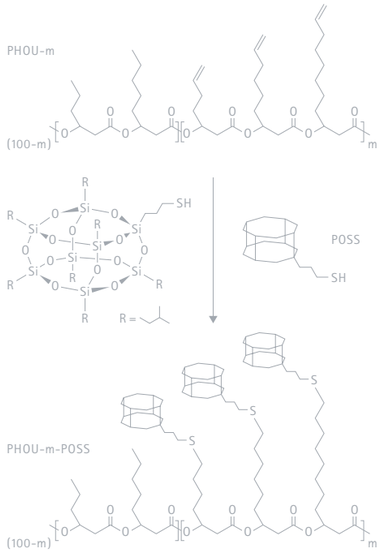
A first test to demonstrate the capability of producing tailor-made PHA using dual (C, N) limited growth conditions was performed with *R. eutropha*. Growth limiting concentrations of ammonium butyric and valeric acid were fed to a chemostat culture. It was found that the composition of the isolated 3-hydroxyvalerate in poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHB/HV) could be controlled reproducibly between 0 and 62 mol%. The polymer composition had a significant influence on the thermal properties and T_m varied from 176 °C for PHB to 85 °C for PHB/HV with a content of about 50 mol% HV.

In another approach dual (C, N) limited growth was used to tailor functionalized mcl-PHA in *Pseudomonas putida* GPo1 using 10-undecenoic acid and octanoic acid as carbon substrates. A given amount of 10-undecenoic acid in the carbon feed resulted in the formation of an identical portion of olefinic (terminally unsaturated) PHA monomers.[15] Further experiments revealed that the integration of an aromatic monomer (3-hydroxyphenylvalerate) increased the glass transition temperature from −38 °C to −6 °C, whereas the functionality could be maintained at 10 mol% olefinic monomers for all *co*-polymer compositions (see Figure 3 and Reference 16). Recently, we have shown that the specific growth rate has a significant influence on the monomeric unit composition and therefore only chemostat cultures can guarantee reproducible PHA synthesis.[15]

Tailored PHA by chemical modification

Comb polymers were produced in a two-step synthesis from a PHA containing 25 mol% terminal side-chain double bonds (see Figure 4). The radical addition reaction of 11-mercaptoundecanoic acid to the side chain alkenes of (1) produced derivative (2) containing thioether bonds with terminal carboxyl functionalities, which were subsequently transformed into the amide (3) or ester (4) derivatives using tridecylamine or octadecanol, respectively. The reactions proceeded to completion with nearly no side reactions, which was confirmed by NMR and GPC experiments. The resulting comb polymers were white crystalline materials. Solid-state NMR spectra and X-ray diffraction results suggested a crystalline textural two-phase organization into polyethylene-like domains and regions characteristic of PHAs. Yields of 2, 3, and 4 were 74, 57, and 69%.

The chemical modification of PHA was slightly altered by the application of 11-mercapto-1-undecanol as linker molecule. Thus, the terminal hydroxy functionalities could be subsequently esterified with cinnamic acid, sulfatized with $ClSO_3H$ or coupled with tert-butyldimethylsilyl-protected coumaric acid, to give, after deprotection with tetrabutylammonium fluoride followed by sulfatization, p-(sulfooxy)cinnamic acid (zosteric acid) labeled poly(3-hydroxyalkenoate). Again these reactions performed with good yields and little side reactions. These PHA derivatives were then used to study the potential of protecting surfaces from biofouling. This antifouling strategy is currently being investigated in a project sponsored by the European Space Agency (ESA). Recently, a polyhedral



5
Free radical addition of mercaptopropyl-isobutyl-POSS (POSS) to the side-chain double bonds of poly(3-hydroxyalkanoate-*co*-3-hydroxyalkenoate) (PHOU).

oligomeric silsesquioxane containing seven isobutyl groups and one mercaptopropyl group (POSS-SH) was linked via a free radical addition reaction to the side-chain double bonds of poly(3-hydroxyalkanoate-*co*-3-hydroxyalkenoate) (PHOU) having 11.5, 55, 78 and 97 mol% of double bonds. The resulting inorganic-organic hybrid materials, PHOU-POSS (Figure 5), changed – with increasing POSS content – their appearance from non-sticky, and elastic to brittle and glass-like. The covalent linking of POSS-SH to PHOU increased the heat stability such as glass transition temperatures, and melting points could be tailored between 48 °C and 120 °C.[20]

Conclusion

Special growth conditions of microorganisms allow the biosynthesis of tailored PHA containing functional groups. Additional functionalities can be added to such PHA after biosynthesis and thus allow further tailoring to suit particular applications. PHA is, as previously mentioned, fully biodegradable and biocompatible. Thus, it is possible to construct devices that permit a release of chemicals by PHA hydrolysis. Potential applications are drug delivery systems in medicine and agriculture (fungicides, fertilizers). Currently, Empa is assessing the usability of PHA as a biopolymer for fiber production. Fabrics of PHA could be applied in medical applications, e.g. for wound treatment. The chemical modification of functionalized PHA enables the integration and design of new properties as we could demonstrate by the covalent linkage of POSS to PHA. Moreover, PHA could be used to form biodegradable block *co*-polymers with new characteristics.

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Biodegradation

Materials and products that come into contact with human beings or the environment are expected to carry out their functions without causing lasting harm to their surroundings. Indeed, it is often desirable to have them "automatically" vanish from the environment after use, for example through biodegradation. Such materials include plastics used in medicine (biodegradable implants) and environment engineering (degradable films) but also such products as detergents, large amounts of which pass into wastewater.
We investigate the biodegradability and toxicity of various materials and products under simulated field conditions and/or well-defined laboratory conditions, using both standardized techniques and test systems devised to address particular problems. Using these test systems, the biodegradability of chemically modified PHAs will be determined, too.

The O_2 probe is generally calibrated in the sterile bioreactor under regular process conditions and atmospheric pressure. A subsequent pressure increase in the reactor boosts the dissolved oxygen content; values of several hundred percent are possible depending on the pressure. The dissolved oxygen falls to the specified level as soon as bacterial growth and the associated oxygen consumption begin.

Display showing dissolved oxygen concentration (other quantities can be selected).

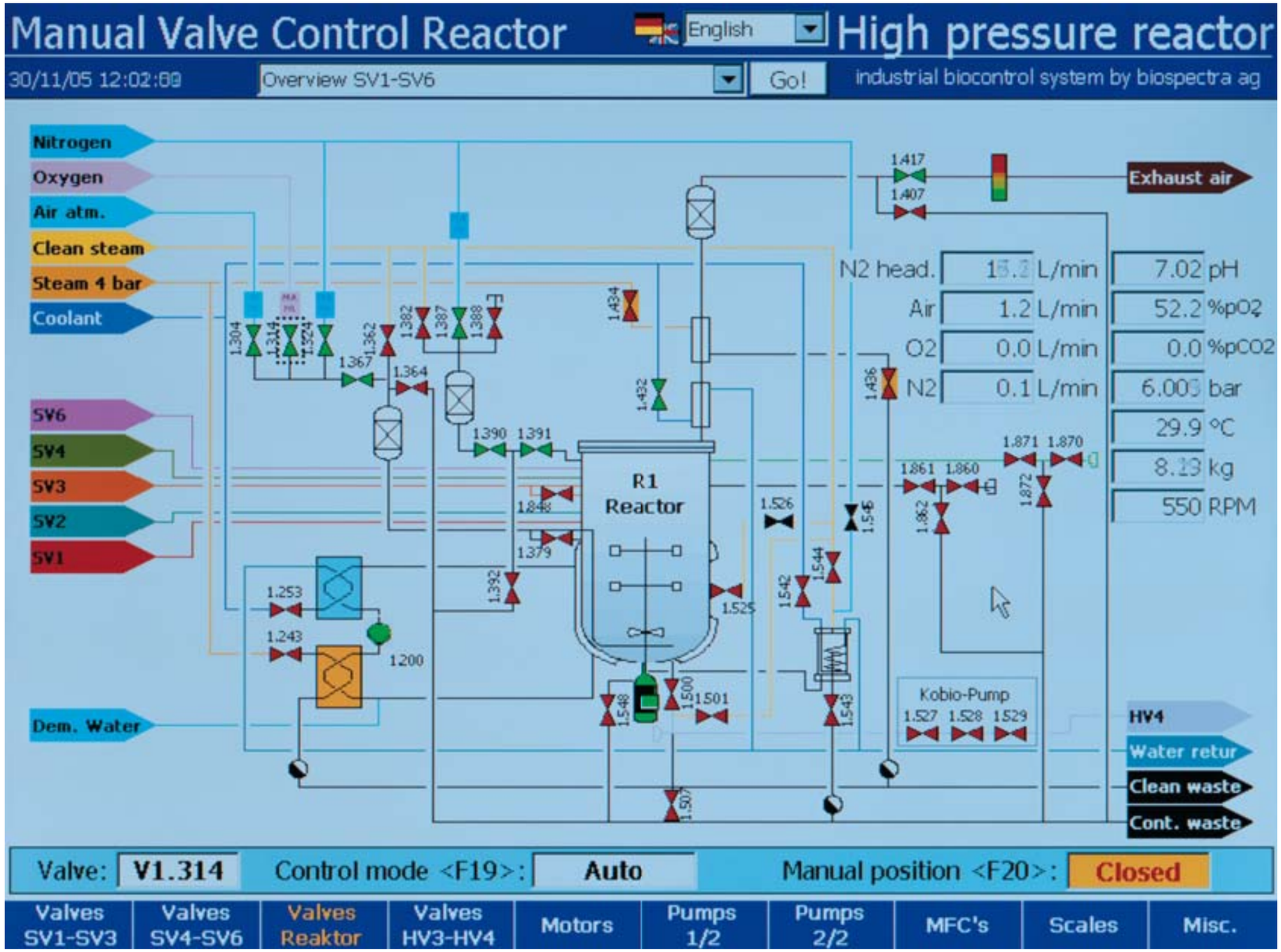
Atmospheric pressure outside the reactor.

High-pressure bioreactor

Bioprocesses can be run in batch, fed-batch and continuous modes under a pressure of up to 1 MPa (= 10 bar) in this reactor. The instrumentation and control features make it possible to develop and study novel bioprocesses. The aim is to achieve a substantial increase in the oxygen transfer rate in high-cell-density microbial cultures by raising the pressure. Both the hardware and the control system are extremely flexible. For example, all tanks can be individually sterilized, refilled, and returned to service even during fermentation. The different pumps can deliver media at precise flow rates from 50 µL min^{-1} to some 100 mL min^{-1}. All medium and gas flow rates are gravimetrically controlled; pressure is regulated with an exhaust valve.

Characteristics:

– Reactor capacity	16 L
– Effective working volume	10 L
– Propeller stirrer	0–1400 rpm
– Flow rate (air)	0–20 L min^{-1}
– Flow rate (O_2)	0–5 L min^{-1}
– Flow rate (N_2)	0–20 L min^{-1}
– Flow rate (N_2, head space)	0–200 L min^{-1}
– Fermentation pressure	0–1 MPa



The process control system not only handles standard operations such as cleaning in place (CIP), pressure test, sterilization and calibration of the probes, but also initiates bioprocesses. Every control variable (pH, feed, foam, etc.) and every tank is assigned an individual role in the process, so that batch, fed-batch and continuous fermentations can be set up in a flexible way. The user can change any setpoint while the process is running. The control panel offers manual access to setpoint devices and actuators at any time. Automation is based on the Industrial Biocontrol System. The Lucullus process information management system (Biospectra AG, Schlieren, Switzerland) is employed for programming more complex sequences (feed ramps, fermentation pattern recognition, changing of control variable dependences, etc.), the process control as well as data acquisition and evaluation.

High-Pressure Bioreactor (HPR)

The HPR by Bioengineering AG, Wald (Switzerland), was originally laid out for two-phase fermentation with highly flammable carbon substrates.

Empa received the opportunity to acquire this reactor, co-financed by the Swiss National Science Foundation. Biospectra AG in Schlieren (Switzerland) was responsible for the modification of the fermentation unit to guarantee the high precision of the carbon substrate feed(s) as well as process automation.

Increase of oxygen transfer rate to cell cultures

High-cell-density cultures (HCDC) are a suitable way to increase the volumetric productivity of PHA in continuous cultivations (chemostat). A major challenge of such aerobic HCDC is the high oxygen demand of the bacterial cells. When insufficient oxygen is transferred to the cells they start to produce side-products such as ethanol and acetate which significantly reduce productivity. Unfortunately, simple solutions such as augmented aeration and stirrer speeds cause excessive foaming and instable culture conditions. Additionally, the increase of the stirrer speed and aeration can improve the oxygen transfer only to a limited extent. Above an aeration rate of 4 vvm, gas bubbles coalesce and inefficient stirring even causes a decrease of the transfer rate. Thus, an obvious and often used approach is the increase of the oxygen content in the air supply. Due to this enrichment, the oxygen partial pressure (pO_2) in the bioreactor is increased and thus, according to Henry's law, the oxygen saturation concentration is significantly enhanced. As a consequence, a higher oxygen concentration gradient is established and therefore the oxygen transfer rate (OTR) is enhanced. The use of enriched air or even pure oxygen is not an option for cells that can become easily intoxicated (oxidative stress). Moreover, the supply of pure oxygen increases the operating cost of the bioreactor.

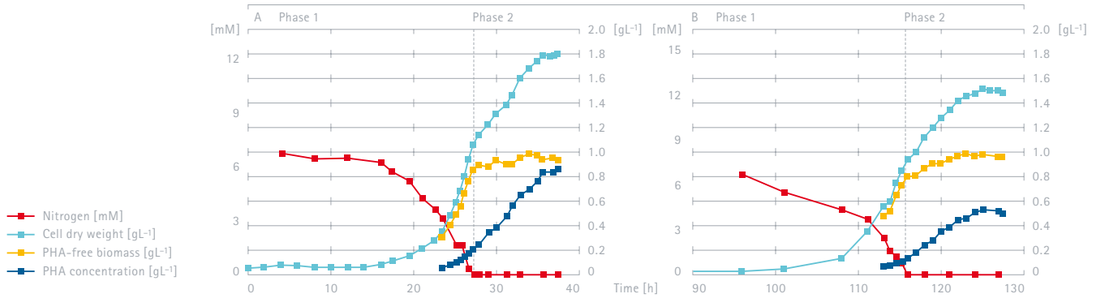
A valid alternative for enhancing the OTR is the increase of the atmospheric pressure in the bioreactor. This method was already proposed at an early stage of industrial fermentation. Under such conditions, the partial pressure of oxygen is increased and as a consequence, the OTR is increased too. While the operating cost is significantly lower, special bioreactors need to be built for safety reasons. A recent study performed at the University of Aachen (B. Maier, Ph. D. thesis No. D82, 2002) revealed that the additional costs for reactors up to 40 m^3 are not significant for the investment of a new fermentation unit.

Finally, continuous cultivation under elevated pressure is new and has the potential to become a useful method for other biotechnological processes (e.g., oxidation of aromatic compounds, protein and polysaccharid synthesis) since reduced foam formation could be found in our experiments.

First experiments

Preliminary cultivation experiments in the HPR showed a significant difference in the fermentation performance of a batch culture when *P. putida* GPo1 was grown on an equimolar mixture of octanoic acid and 10-undecenoic acid under atmospheric (Figure 6A) and elevated pressure (0.6 MPa) (Figure 6B). The relative concentrations of nitrogen (NH_4-N) and of carbon were adjusted such that ammonia was consumed to depletion, thereby terminating the exponential growth phase, whereas the carbon source remained in excess. The dissolved oxygen during both fermentations was kept constant at a saturation of 50 % and was automatically controlled by the air flow rates as well as by stirring speed.

Figure 6 shows a significant difference in terms of fermentation performance. For both fermentations, two different phases could clearly be identified. During phase 1, *P. putida* GPo1 grew exponentially and only little PHA formation was observed. Phase 2 began after all ammonia was utilized. In this phase, the PHA-free biomass remained more or less constant but the total biomass increased further. This increase was exclusively due to the intracellular accumulation of PHA.



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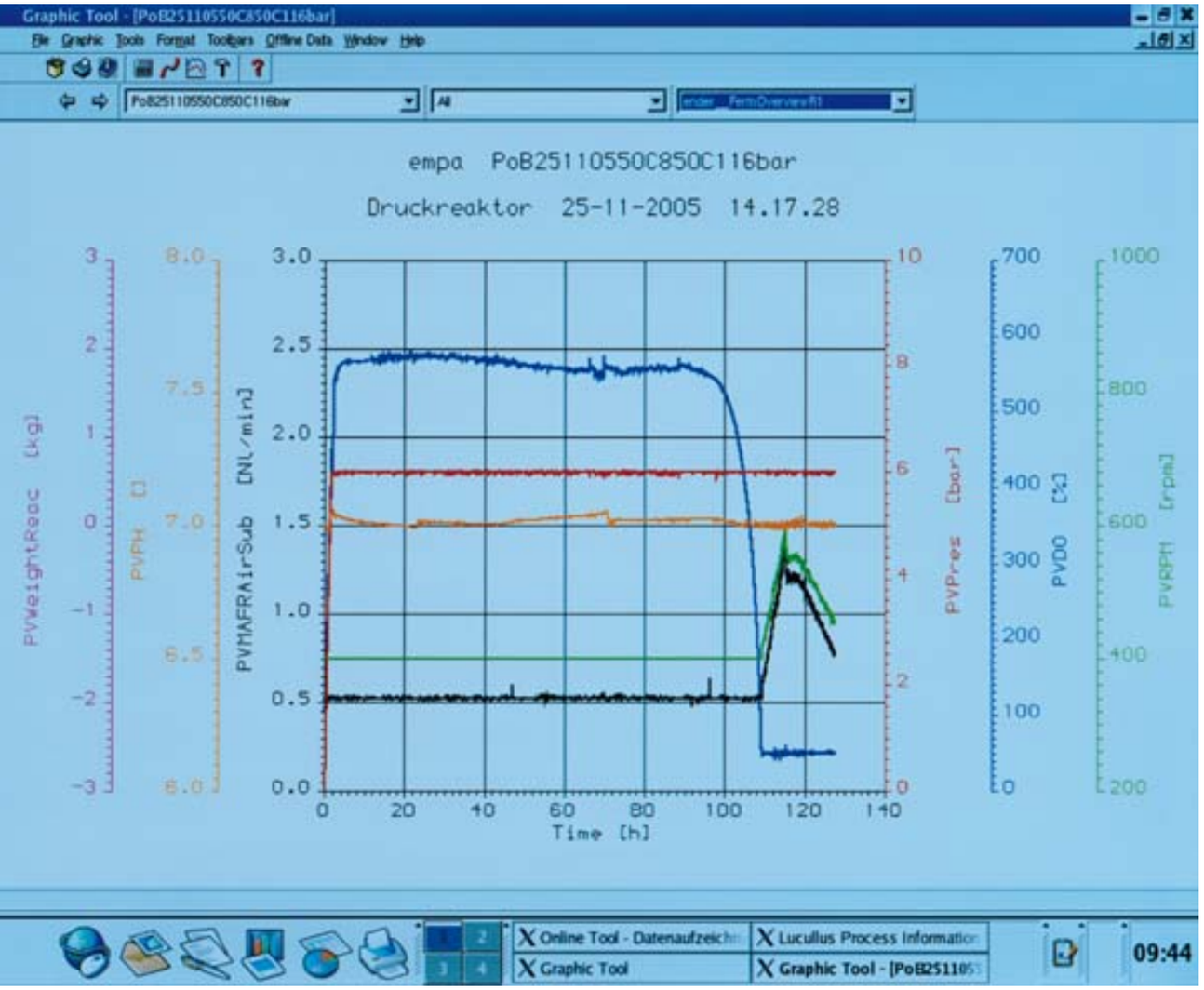
Cell growth and PHA accumulation of *P. putida* GPo1 under atmospheric (Figure 6A) and under elevated pressure (0.6 MPa) (Figure 6B).

A noticeable phenomenon is the significant extension of the lag phase, when the cells were grown under elevated pressure, particularly since the precultures were treated in the same way. It might be that under elevated pressure, cells in lag phase are stressed and develop special response. However, after entering the exponential growth phase, the performance of the batch culture under elevated pressure was comparable to that under atmospheric pressure. In a next experiment, where the pressure was gradually elevated to 0.2, 0.4 and 0.6 MPa during exponential growth of *P. putida* GPo1, the batch cultures showed comparable performance rates as under atmospheric pressure (data not shown). There were no significant differences in maximum specific growth rate (μ_{max}), cellular PHA content, monomeric composition of the olefinic mclPHAs and yield.

The influence of pressure on the OTR was considerable: pO_2 increased significantly in a batch culture of *P. putida* GPo1 on octanoic acid when aeration rate (5 L min^{-1}) and stirrer speed (1200 rpm) were kept constant:

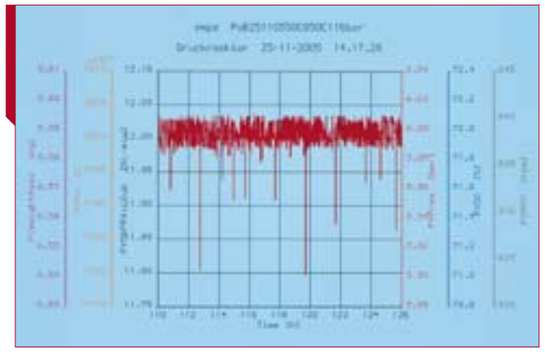
Pressure	pO_2
0 MPa (atmospheric)	5 %
0.2 MPa	150 %
0.4 MPa	260 %
0.6 MPa	360 %

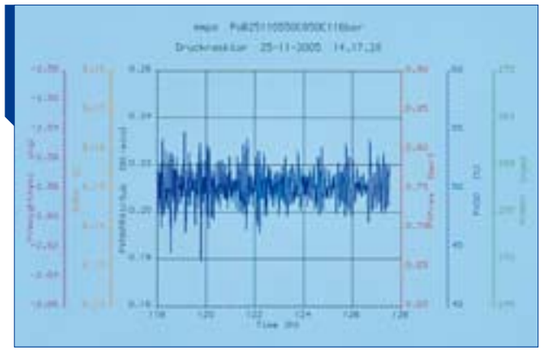


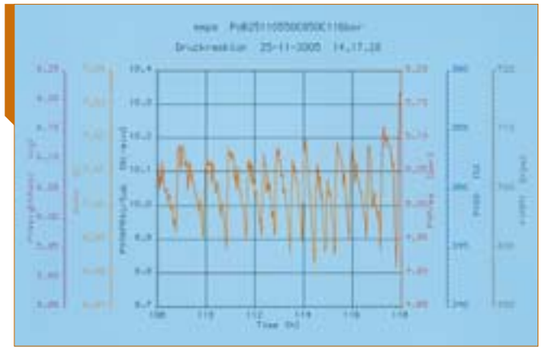
Example of process logging in a batch experiment

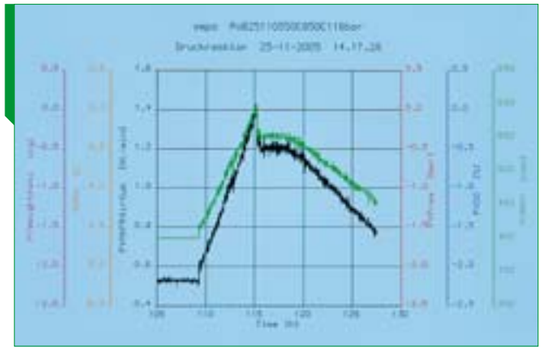
This process log is from the batch experiment of Fig. 6, with *P. putida* GPo1 at 30 °C, pH 7 and pressure 0.6 MPa gage. A minimal medium was employed; the carbon source was an equimolar mixture of octanoic acid and 10-undecenoic acid with a resulting C/N ratio of 26 gC (gN)$^{-1}$. Dissolved oxygen (pO_2) was held constant at 50% saturation (relative to atmospheric pressure). With the gas flow rate at its lower limit of 0.5 L min^{-1}, it took about 2 hours after the start of the trial (inoculation) for the pressure to reach 0.6 MPa (gage). The signal from the pO_2 probe then rose to just under 600%. The bacteria were in a lag phase for about 95 hours.

The subsequent exponential growth is reflected by the rise in oxygen consumption. After 109 hours, pO_2 had fallen to the specified 50% saturation and was now held constant through the stirrer speed and aeration rate. The exponential phase came to an end after roughly 115 hours when the nitrogen source of the medium was fully exhausted, and *P. putida* GPo1 began storing PHA. Growth was completed at the transition to the stationary phase starting at 125 hours (see Fig. 6).









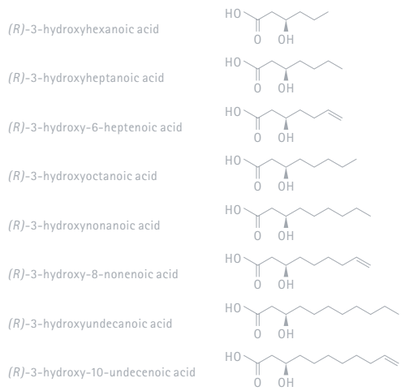
Pressure control
Pressure is controlled with an exhaust valve to an accuracy of about ± 0.0005 MPa, virtually independent of aeration rate. The signal peaks with the somewhat greater pressure drop are due to periodic sampling for offline measurements.

pH control
Stability of the pH signals is also ensured under pressure. Control of pH is achieved with an accuracy of approx. ± 0.02 pH units. Only rapid pressure changes exceeding 0.1 MPa cause a transient fluctuation of approx. ± 0,1 pH units, but this can be corrected via the software (not used in the experiment shown here).

pO_2 control
In this experiment, pO_2 was controlled to 50% through the air flow and stirrer speed in parallel. The accuracy of pO_2 control was approx. ± 5%.

Aeration rate and motor speed
The aeration rate and motor speed, like all other control variables, can be handled by a variety of controllers as needed. In the present experiment, this was achieved exclusively via the pO_2 controller. The air mass flow rate and motor speed controllers show an almost continuously variable output. Different control variable dependences are conceivable, however. If foaming is heavy, for example, the foam controller can drive (i.e. reduce) the motor speed and/or aeration rate; pO_2 could then be controlled via the O_2 aeration rate or the pressure.

Production of *(R)*-3-hydroxycarboxylic acids



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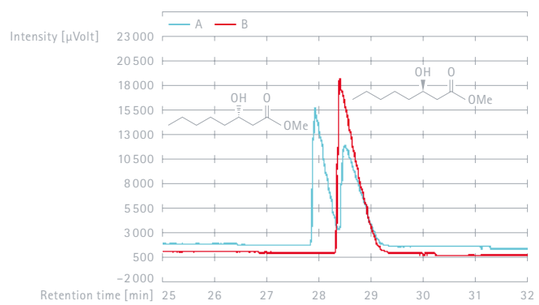
PHA monomers were recently produced by biotechnological conversion of PHA. The method can be applied for the isolation of further *(R)*-3-hydroxycarboxylic acids.

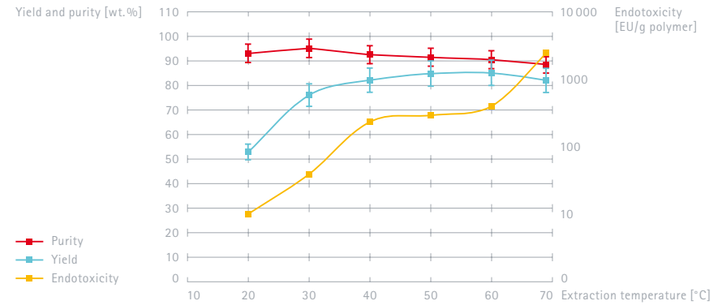


8

Chirality of the obtained monomers: Monomers produced were analyzed by chiral gas chromatography. A: commercially racemic *(R, S)*-3-hydroxyoctanoic acid methylesters. B: identification of the *R* enantiomer of methylesters of the 3-hydroxyoctanoic acid that was biotechnologically produced and purified.

An efficient method was developed to prepare enantiomerically pure *(R)*-3-hydroxycarboxylic acids from PHA. In a first step, PHA was accumulated by bacterial cells such as *P. putida* GPo1. In a second step, *(R)*-3-hydroxycarboxylic acids, which are monomers of PHA, were obtained from whole cells when conditions were provided to promote *in vivo* depolymerization of intracellular PHA. The monomers were secreted into the extracellular environment. The type of monomers produced depends on carbon feed during polymer accumulation and on the bacterial strain in use. In a third step, the monomers were separated and purified by acidic precipitation, preparative reversed-phase column chromatography, and subsequent solvent extraction. Many different *(R)*-3-hydroxycarboxylic acids were isolated (Figure 7). The overall yield based on released monomers was around 80 % by weight. All obtained monomers had a purity of over 95 % by weight and showed exclusively *(R)*-configuration (Figure 8).
The method developed here is easy to handle, environmentally friendly and more efficient than what has been reported so far.

Downstream Processing

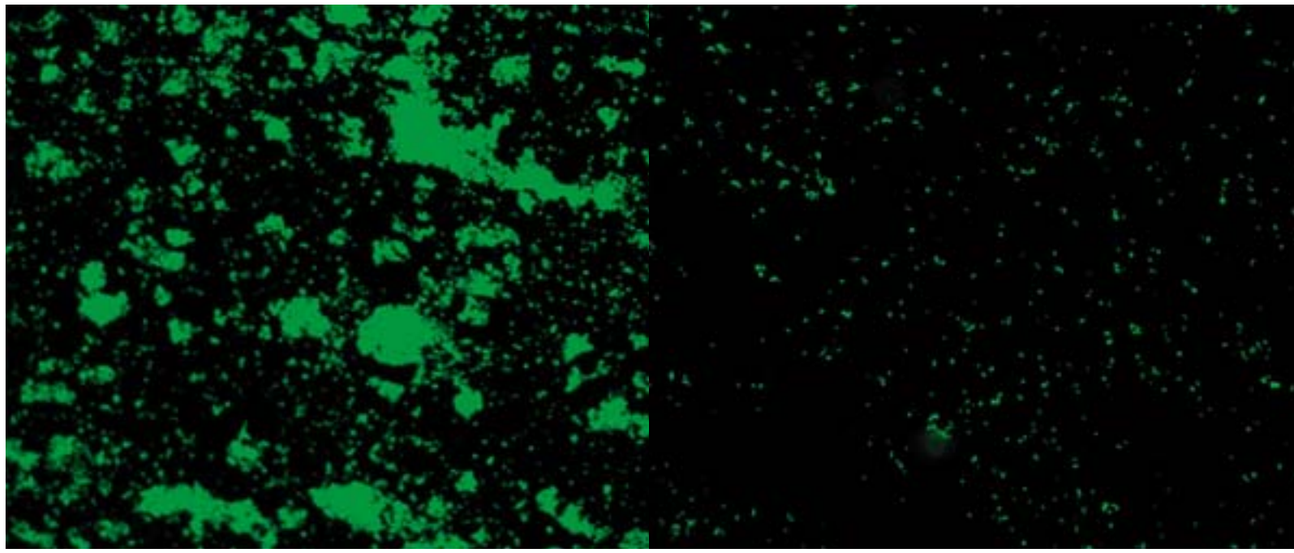


9

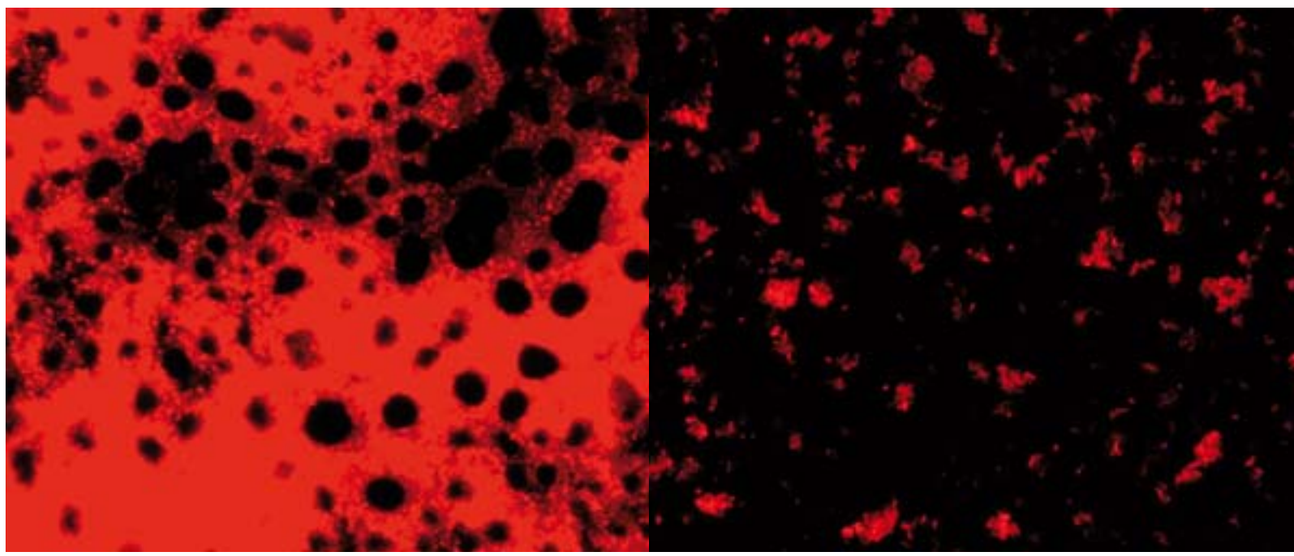
Example with poly(3-hydroxyoctanoate-*co*-3-hydroxyhexanoate), PHO: yield, purity and endotoxicity in temperature-controlled extraction with hexane. The purity was determined by gas chromatography, the endotoxicity by the Limulus amoebocyte lysate test. The FDA-recommended limit on endotoxins for implants is 20 EU.

Medium-chain-length mcl-PHA is stored in the bacteria in granule form. There are several methods for isolating the plastic. Usually the biomass is dried and extracted with an organic solvent. Filtration or centrifugation separates the polymer solution from the biomass, and the polymer is precipitated by the addition of an alcohol. This technique, however, produces large amounts of solvent mixtures that have to be expensively recycled. For this reason, Empa has developed an alternative method that does not require precipitants.
The biopolymer is extracted with a suitable organic solvent at elevated temperature. After filtration, it is precipitated by cooling the solution. Parameters such as extraction yield, polymer purity, endotoxicity, and molecular weight can be controlled via the extraction and precipitation temperatures. Extraction yield is higher at high temperatures, but endotoxicity is higher as well. Purity falls off slightly at high temperatures as more impurities are extracted along with the desired product. Moreover, the average molecular weight can be increased and low-molecular-weight polymers separated through the choice of the proper precipitation temperature. This can be important for certain downstream processes and for biodegradation of the polymer. Temperature-controlled extraction and precipitation makes it easy to obtain PHA of high purity and low endotoxicity.

Most bacteria can attach to solid surfaces and form biofilms, in which different bacterial populations are enclosed in a complex polymeric matrix and adhere to an abiotic surface. Bacterial biofilms are able to colonize every surface that is in contact with naturally occurring fluids. For instance, biofilms are found to grow on catheters as well as in industrial pipes or water distribution systems and eventually cause detrimental effects such as biocorrosion of materials and potential risks for human health. Our aim is to prevent biofilm formation using novel coatings and antifoulants based on tailor-made PHA.
Antimicrobial chemicals, enzymes which destroy the polysaccharide matrix, as well as quorum sensing inhibitors can be covalently linked to PHA by polymer analogous reactions under mild conditions. Biofilm formation and antimicrobial effects of resulting polymers are examined using fluorescence microscopy. In addition, the influence of such modified PHA on microorganisms will be examined on a molecular level. These studies are made as a part of the European Space Agency ESA project "Molecular tools for monitoring and control of pathogenic microorganisms in advanced life support systems".



A B

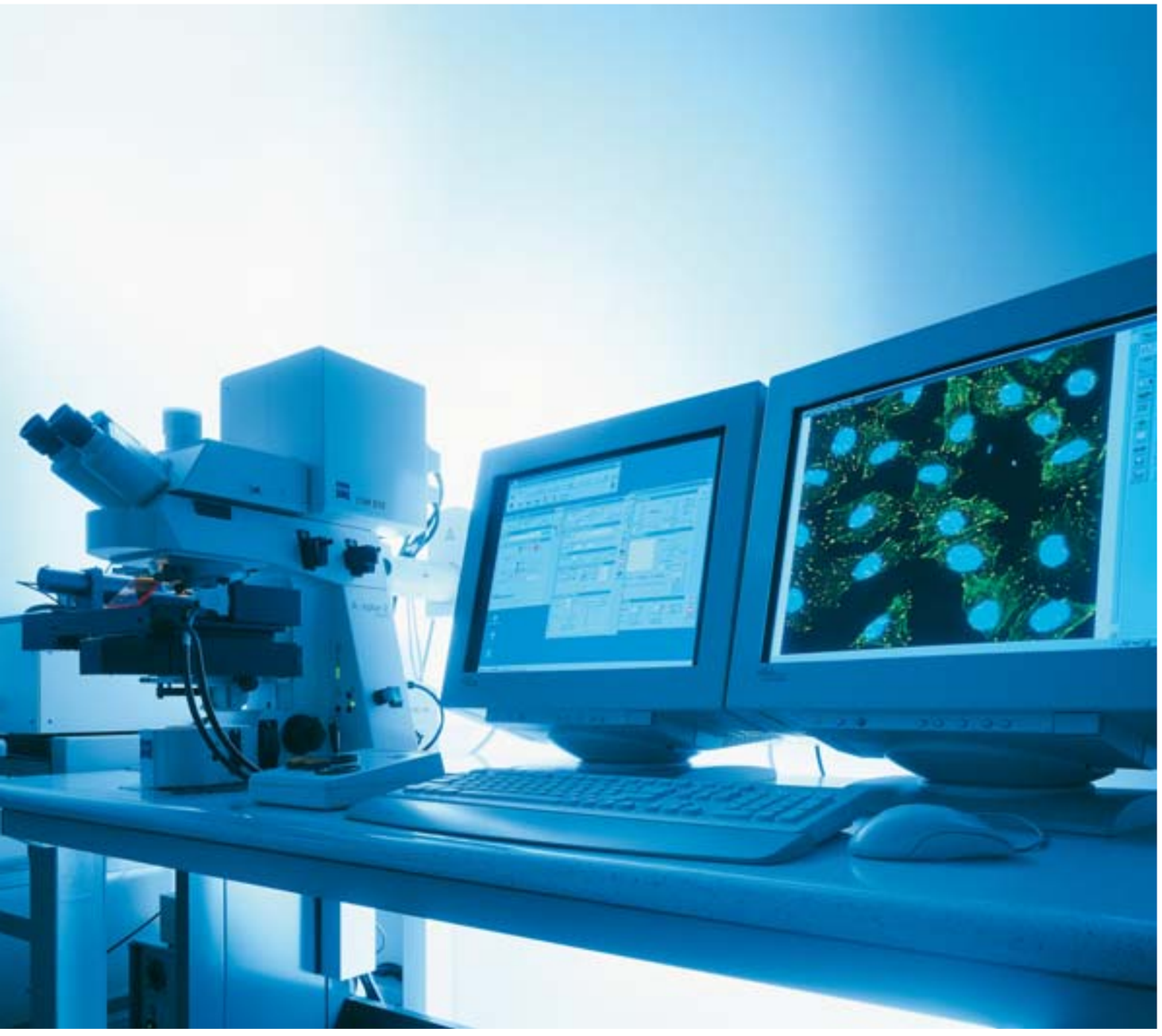


A B

Top row:
Biofilm formation of green fluorescent *Staphylococcus aureus* grown on poly(3-hydroxyundecanoate-*co*-3-hydroxynonanoate-*co*-3-hydroxyheptanoate), PHUA, (A) and on a blend of PHUA and 2% tenside (B).

Bottom row:
Reduction of *Escherichia coli* (red fluorescent) biofilm formation by silver plasma-coated PHUA (B), compared to untreated PHUA (A).

Biocompatibility of PHAs



The team *Materials-Biology Interactions* at Empa investigates and optimizes materials for medical applications. The goal of many research projects is to define material surfaces in such a way that cell migration, proliferation and differentiation is controlled according to the final function an implant has to fulfill in the human body. For this purpose, we model the *in vivo* situation as closely as possible using cell lines and primary cells of different species, preferentially human cells. Numerous external stimuli like applied forces or chemicals, protein-factors and nano materials in the medium have an influence on cell behavior. In order to be able to draw comprehensive conclusions about these effects we characterize cells and cultivation medium before, during and at the end of an experiment. Since cellular behavior at surfaces depends on the cell type as well as cell differentiation status, we investigate cellular differentiation as precisely as possible in addition to studying cell migration and proliferation. Analytical methods used for studying cellular parameters range from non-specific like cell number or vitality to very specific for certain cell types (like expression of a certain gene or production of a certain protein). Sophisticated microscopy systems are employed to examine cellular behavior on a single cell level, and life imaging of cells is used to monitor dynamic processes at the material surface.

Example: Cell tests with PHAs

For biocompatibility analysis of different PHAs human bone cells were cultivated on thin films of polymer that had been applied onto carrier samples. Cells were cultivated for three days, fixed and stained for structural proteins that give information on cell adhesion, cell spreading and vitality. Distinct differences in cell behavior could be identified between cells that had been cultivated on PHAs of different types.

Confocal laser scanning microscopy is used to analyze cell behavior on surfaces that are coated with a biomaterial such as PHA, PLLA or PEEK. To analyze cell behavior on non-transparent surfaces, cell proteins and DNA are stained with fluorescent dyes.

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2 Thomas Ramsauer, Technical staff member, Downstream processing and biodegradation
3 Eva Brombacher, Microbiologist, Biofilms
4 Patrick Furrer, Doctoral student, Downstream processing
5 Sabrina Hasler, Apprentice
6 Manfred Schmid, Polymer chemist, Analysis and functionalization
7 Manfred Zinn, Biotechnologist, Group leader
8 Dominik Noger, Chemist, Fermentations
9 Ernst Pletscher, Technical staff member, Fermentations and biodegradation
10 Andreas Grubelnik, Biochemist, Analysis and functionalization
11 Karl Kehl, Chemical laboratory technician, Downstream processing and analysis
12 Katinka Ruth, Doctoral student, *R*-3-hydroxycarboxylic acids
13 Sergio Rezzonico, Chemist, Downstream processing and analysis
14 René Hartmann, Microbiologist, Fermentations

Biotechnology R&D at Empa in St. Gallen focuses on the biosynthesis, recovery, chemical functionalization, and downstream processing of polyhydroxyalkanoates (PHAs) for industrial and medical applications. With the new Bioengineering AG high-pressure bioreactor, the only one of its kind, it is now possible to perform continuous cultivations (chemostat) under a pressure of up to 1 MPa. The extraordinary flexibility of the equipment and control system makes it an ideal platform for further R&D activities in bioprocess engineering. The special strength of the bioreactor is in general bioprocesses which pose problems with aeration at high cell densities or at the lowest possible shear forces and where excessive foaming must be controlled. Empa, a part of the ETH Domain, is a materials research institute with 125 years of experience in collaborating with partners from industry and higher education. Our aim is to continue this tradition and to further strengthen it in the field of biotechnology.

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