Biomediated production of structurally diverse poly(hydroxyalkanoates) from surplus streams of the animal processing industry*)

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Abstract: For commercial success, enhanced poly(hydroxyalkanoate) (PHA) production must address both material performance and economic aspects. Conventional PHA production consumes expensive feedstocks dedicated to nutrition. Switching to carbon-rich (agro)industrial side-streams alleviates industrial disposal problems, preserves food resources, and can be economically superior. Processes developed in the recently performed EU-FP7 project ANIMPOL resort to lipid-rich surplus streams from slaughterhouses and the rendering industry; these materials undergo chemical transformation to crude glycerol phase (CGP) and biodiesel. The saturated biodiesel share (SFAE) counteracts its applicability as a biofuel but, in addition to CGP, can be converted biotechnologically to PHAs. Depending on the applied microbial production strain and the selected carbon source (SFAE or CGP), thermoplastic short chain length PHA (*scl*-PHA), as well as elastomeric to latex-like medium chain length PHA (*mcl*-PHA), can be produced from these inexpensive feed stocks. The article illustrates the biotechnological conversion of animal-based CGP and SFAE towards poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), respectively, by *Cupriavidus necator* strain DSM 545. SFAE conversion towards *mcl*-PHAs consisting of various saturated and unsaturated building blocks by two pseudomonades, *Ps. citronellolis* DSM 50332 and *Ps. chlororaphis* DSM 50083, are also shown. Together with the kinetics of the bioprocesses, the results from the characterization of isolated samples of these structurally diverse biopolyesters are compared; data demonstrate the high versatility of biopolymer properties making them applicable in various fields of the plastic market. In addition to the need for inexpensive carbon feed stocks, the article points to further hot spots of the PHA-production chain that must be considered in order to lower the overall PHA production costs, and to enhance product quality. The benefits arising from multistage continuous cultivation production set-ups, namely high-throughput production of PHA of predefined composition and constant quality, are especially discussed. Finally, contemporary approaches towards environmentally and ecologically sustainable PHA recovery from biomass are summarized.

Keywords: animal processing, biodiesel, biopolymers, crude glycerol phase, poly(hydroxyalkanoates) (PHA), process design, saturated fatty acid esters, surplus materials.

Bioprodukcja zróżnicowanych strukturalnie poli(hydroksyalkanianów) z produktów ubocznych przemysłu mięsnego

Streszczenie: Artykuł stanowi przegląd literatury dotyczącej biosyntezy poli(hydroksyalkanianów) (PHA) z wykorzystaniem jako surowców odpadów z przemysłu rolno-spożywczego. Omówiono wyniki uzyskane podczas realizacji projektu ANIMPOL (7. PR UE), w którym jako surowiec do syntezy PHA stosowano bogate w tłuszcze produkty uboczne z rzeźni i zakładów utylizacji odpadów zwierzęcych, przekształcone chemicznie w surową fazę glicerynową (CGP) i biodiesel (estry nasyconych kwasów tłuszczowych, SFAE). Zależnie od użytego szczepu bakterii oraz źródła węgla (SFAE lub CGP) otrzymano termoplastyczne krótkołańcuchowe PHA (*scl*-PHA) lub średniołańcuchowe PHA (*mcl*-PHA). Zaprezentowano biotechnologiczną konwersję CGP i SFAE pochodzenia zwierzęcego do poli(3-hydroksymaślanu) (PHB) i poli(3-hydroksymaślanu-co-3-hydroksywalerianu) (PHBV) za pomocą szczepu bakteryjnego *Cupriavidus necator* strain DSM 545, syntezę *mcl*-PHA zawierających nasycone i nienasycone ele-

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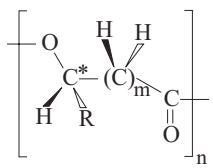
menty strukturalne za pomocą bakterii z rodzaju *Pseudomonas* (*Ps. citronellolis* DSM 50332 i *Ps. chlororaphis* DSM 50083). Omówiono kinetykę bioprocesów oraz charakterystykę otrzymanych biopoliestrów, przedyskutowano elementy cyklu produkcyjnego PHA kluczowe z punktu widzenia zmniejszenia kosztów i poprawy jakości produktów oraz korzyści wynikające z zastosowania układów ciągłej wielostopniowej hodowli w wysokowydajnej produkcji PHA o założonym składzie i stabilnej jakości. Omówiono też nowe metody odzyskiwania PHA z biomasy, zgodne z wymaganiami ochrony środowiska.

Słowa kluczowe: biodiesel, biopolimery, surowa faza glicerynowa, poly(hydroksyalkaniany) (PHA), nasycone kwasy tłuszczowe, produkty uboczne.

Today's society consumes an annual quantity of roughly 300 million tons of different plastic materials. This development shows a clear upward trend, especially in countries with emerging economies [1]. The major part of these plastics is based on the chemical conversion of crude oil. Due to the increasing instability of the political situation of many oil-exporting countries, it appears rather doubtful that, in the near future, a sufficient and safe supply to the global plastics industry from limited fossil resources like crude oil can be ascertained. This underlines the need for alternative feed stocks for the production of polymers, other goods, and energy [2]. As a matter of fact, the scientific community and many innovative and ground breaking companies are aware of this, and, as a consequence, we nowadays witness a tremendously dynamic biopolymer market. So called „bioplastics" are definitely fashionable today [3]. A critical assessment of the performance and entire life cycle of contemporarily commercialized „bioplastics" often reveals severe shortcomings regarding the attributes „biobased", „biodegradable", „compostable" and „biocompatible". These traits are defined by standards and certificates [4, 5]. Prokaryotic poly(hydroxyalkanoates) (PHAs), microbial carbon- and energy reserve materials, conform to all of these four features. They are more and more regarded as promising alternatives to common full-carbon backbone polymers as extensively produced by the chemical conversion of limited fossil resources. Thanks to their highly versatile material properties, PHAs are expected to enter different segments of the plastic market in a not too distant future [6].

Possible implementations include, *inter alia*, compostable packaging [7—9], agricultural applications [10], medical and surgical devices [11—13], or biolatexes [14, 15]. The composition of PHA on the molecular level determines their properties and is fixed in *statu nascendi* during biosynthesis. The selection of the microbial production strain, the applied carbon source, process conditions and the feeding regime are responsible for the monomeric composition of PHA homo-, co-, and terpolyesters [16, 17]. Formula (I) shows the general structure of PHA:



(I)

where: m — the number of carbon atoms in the backbone of the PHA building blocks, n — the degree of polymerization (numbers of monomers in polyester chain), R — the side chain of the building blocks.

In the case of 3-hydroxybutyrate (3HB), R = CH_3, in the case of 3-hydroxyvalerate (3HV), R = C_2H_5; in both cases, m = 1. For *mcl*-PHA, R is represented by C_3H_7 or longer side chains. The asterisk indicates the chiral center of most PHA building blocks. For the achiral monomer 4-hydroxybutyrate (4HB), R = H, and m = 2.

In the two major groups, short chain length (*scl*) and medium chain length (*mcl*) PHAs can be distinguished. *scl*-PHA are polyesters mainly of 3- and 4-hydroxyalkanoates that contain 3 to 5 carbon atoms [18, 19]; only recently, research activities have been devoted to 2-hydroxyalkanoate containing PHAs [20]. *scl*-PHAs feature material properties well known from classical thermoplasts [16, 19]. Except the highly elastic and flexible homopolyester poly(4-hydroxybutyrate) [13], *scl*-PHAs display a considerable degree of crystallinity, high glass transition temperature (T_g) and low elasticity; poly(3-hydroxybutyrate) (PHB) constitutes the best described and most widely investigated example of such materials [21]. As the most obvious application, *scl*-PHA can be used to manufacture biodegradable packaging materials [7]. *mcl*-PHAs are by far less crystalline and consist predominantly of different 3-hydroxyalkanoates of 6 to 14 C-atoms [22]. Their characteristics resemble those of elastomers and latexes. Due to their low glass transition temperature (T_g), *mcl*-PHAs do not become brittle even at very low temperatures, making them interesting to be used as rubber-like biomaterials. Sometimes, *mcl*-building blocks possess functionalities allowing post-synthetic polyester modification to tailor material properties, and to use the polymer as carrier matrix to covalently attach bioactive compounds. This way, smart materials can be developed to be used for special niche applications [7].

PHA can further be processed towards composite materials and blends by using other compatible materials of natural origin; this way, material properties, such as density or degradability, can be obtained and the production price of the final composite or blend can be lowered when compared to native PHA [23—27]. Additionally, (bio)functionalized nanoparticles based on PHA are accessible, displaying high-performance materials for tailored *in vivo* applications, *e.g.* for controlled intracellular drug release and especially for designed anti-tumor therapy [28—32].

NEW PROCESS DESIGN FOR PHA PRODUCTION

Apart from the raw material side, we should consider additional factors decisive for the economic viability of PHA production [33]. Classical discontinuous PHA production processes embody several shortcomings: unpredictable product quality by fluctuating process conditions and substrate concentrations, restricted possibility for supply of toxic carbon substrates at required concentrations and, most of all, low volumetric productivity due to unavoidable periods of idle time for arranging and post-treatment of the bioreactor equipment. Continuous PHA biosynthesis as a remedy was already investigated on the laboratory scale for the homopolyesters PHB, as well as for elastomeric and even functional *scl*- and *mcl*-PHA copolyesters. Based on laboratory data, *Azohydromonas* sp., *Cupriavidus* sp., *Delftia* sp., *Haloferax* sp., and *Pseudomonas* sp. are considered to display the highest potential for large-scale continuous PHA production [34]. Based on the fact that the number of new, powerful, stable and robust microbial PHA producers is still greatly increasing [35, 36], it is clear that much research and development work has to be accomplished in order to adapt the process design to the kinetic characteristics of both microbial growth and PHA accumulation. In this context, the continuous cultivation mode is a convenient method to rapidly optimize the medium composition for new microbial strains and to produce adequate amounts of PHA under stable conditions for characterization and processing.

Based on the different rates of growth and PHA accumulation observed by most PHA producers, two-stage continuous processes are generally regarded superior to single-stage processes, where PHA formation is connected to biomass growth [37]. Such single stage processes mainly result in a low mass fraction of PHA in the cells, and high concentrations of unutilized substrate in the effluent flow from the bioreactor. As shown by Zinn and colleagues [38], continuous dual-nutrient-limited (DNL) cultivation mode enables the fine-tuned supply of such substrates and co-substrates that display inhibiting effects to the production strain if fed *via* substrate pulses as is commonly done in batch-fed cultivation mode. Applying tailored mixtures of different substrates under DNL growth conditions enables the production of PHA of pre-determined composition. Co- and terpolyesters of constant composition on the monomeric level can be produced during DNL steady state conditions [38].

Switching from one- or two-stage continuous to multi-stage continuous processes provides a further possibility to fine-tune the polymer properties during biosynthesis. At the moment, controlling the repeating unit composition of PHA copolymers is a hot topic in PHA research [39]. In the case of multi-stage cascades of continuously stirred tank reactors (CSTRs), soft or hard segments can be incorporated into matrices of PHB, resulting in block polyesters (*b*-PHA). Such *b*-PHA was already produced on the laboratory scale and is known to display excellent material properties such as high tensile strength and high elongation to break [40—43]; hence, they are expected to be implemented as high-tech materials in the near future.

The high potential of such multistage processes was recently demonstrated by cultivating *C. necator* in a five-stage CSTR cascade. In this cascade, the first bioreactor vessel acted for high-throughput production of catalytically active biomass under nutritionally balanced

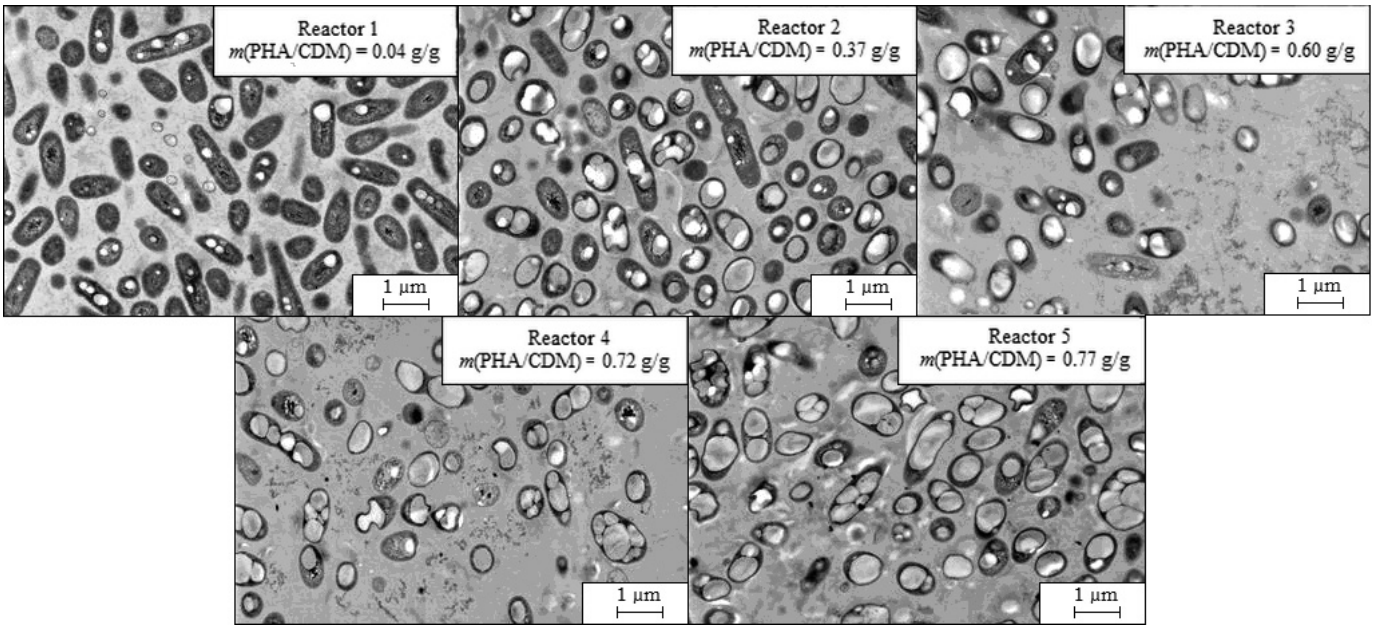


Fig. 1. Electron microscope pictures of *C. necator* cells in a continuously operated 5-stage CSTR cascade; magnification: 30 000× (pictures by E. Ingolić, FELMI-ZFE Graz, with courtesy, according to [34])

conditions, whereas the subsequent four vessels operated under nitrogen-limited, but carbon-rich conditions, hence provoking the accumulation of PHB at high rates. Based on the results obtained by Atlić *et al*. [44], high productivities of up to 2.14 g/dm^{3}h during long-term running of the set-up were experimentally demonstrated. Only an insignificant concentration of unconverted glucose remained in the outflow of the cascade. The intracellular PHA loads increased from a mass fraction *m* of PHA in cell dry mass (CDM) of 0.04 in the first CSTR (dedicated to microbial growth) to 0.77 in the fifth CSTR. Figure 1 provides electron microscopic pictures of the cells in all 5 CSTRs; the increase of the mass fraction in CDM is clear. In addition to the promising kinetic data of this set-up, the polymers were of high uniformity regarding the molar mass distribution, and displayed molar masses sufficiently high for further processing of the materials. The experiments were repeated to generate sufficient datasets for mathematical modeling of multi-stage PHA production. This was accomplished by developing low-structured and formal kinetic models by Horvat *et al.* [45]. The developed models clearly indicate the potential and limitations of multi-stage continuous PHA-production processes, and suggest strategies to further enhance volumetric productivities to up to almost 10 g/dm^{3}h by optimized dilution rates and carbon- and nitrogen concentrations in the feed. Further, high-structured models based on elementary flux modes were established by Lopar and colleagues [46] for this multi-stage process. This work takes into account the reaction of 43 intracellular products and enzymes involved in biomass and/or PHB metabolism and significantly helps to better comprehend the highly complex interactions and dependencies between the single metabolic reactions, especially regarding the transient states during the switch from microbial growth to predominant PHA accumulation.

DOWNSTREAM PROCESSING

Economic and environmentally benign downstream processing for PHA recovery is needed. The necessity to develop scalable methods of cell disruption to release diverse intracellular products from the enveloping biomass is generally regarded as a crucial task in biotechnology [47]. In the case of PHA, attempts are mainly devoted towards:

— reducing the need of organic solvents and enhanced solvent recycling [48],

— switching to non-halogenated solvents [49—52],

— using supercritical CO_2 as eco-friendly, residue-free extraction solvent [53, 54],

— disintegrating PHA-rich cells by mechanical methods like continuous flow bead mills, high pressure homogenization [55], ultrasonication [56], or disrupting halophilic cells in hypotonic media [57],

— chemical or enzymatic digestion of non-PHA biomass [58—60],

— separation of released PHA-granules by methods that are managed with low energy demand, *e.g.* dissolved air floatation [61],

— applying combined methods [62].

The final envisaged application of the polyester determines the required degree of purity and therefore the selection of a suitable isolation method. Polymeric materials for food packaging, and especially for *in vivo* applications, necessarily require higher purities; in the rapidly increasing *in vivo* field, isolation and refining methods have to focus on a nearby quantitative removal of lipopolysaccharides (LPS) from the outer cell wall of gram negative bacteria, among which the most frequently applied PHA producers are found. If not properly removed by adequate solvents, these LPS, a.k.a. endotoxins, cause inflammatory reactions [53]. Detailed and comprehensive literature reviews of different methods for the efficient recovery of PHA from biomass, both on the laboratory and industrial scale, are available [63—65].

FOLLOW-UP PRODUCTS OF SPENT PHA

As a rather exotic application, low-quality *mcl*-PHA can be hydrolyzed to use hydrolysis products such as biodiesel-like green energy carriers [66]. Such low-quality *mcl*-PHA is accessible from the conversion of carbon-rich waste water samples by mixed bacterial cultures and this displays insufficient quality to be used as plastic- of rubber-like material [67, 68]. Similar attempts were recently reported by Kang and Yu [69], who chemically converted PHB to produce hydrocarbon oil in one-pot reactions at a mild temperature (around 220 °C) using phosphoric acid as recyclable catalyst. This might be a future possibility to use spent items made of *scl*-PHA as an alternative to their anaerobic digestion to biogas or to composting. Detailed cost estimations of this process will be needed in order to assess its economic viability. In addition to the energetic utilization of spent and low-quality PHA, these materials provide a rich source of chiral hydroxyl acids accessible after hydrolysis; some of them (3HB, 4HB) are well known to display diverse *in vivo* physiological functions [70]. Such compounds are of high significance *e.g.* as chiral starting materials in the chemical, pharmaceutical and medical field, especially for the production of bioactive molecules. Due to the vast number of accessible chiral building blocks and high achievable PHA synthesis rates, this strategy is considered superior to alternative production techniques for chiral 3-hydroxy acids based on the chemical reduction of 3-keto esters or 3-keto acids [71, 72].

RAW MATERIALS FOR PHA PRODUCTION

For commercial success, PHA production must address both excellent material performance and economic aspects. Conventional PHA production consumes expensive feedstocks dedicated to nutrition, provoking the con-

temporary "plate-*vs*.-plastic" controversy. From the economic point of view, such expensive feedstocks (*e.g.* purified sugars, edible oils *etc.*) contribute to up to 50 % of the entire PHA production costs [2]. Switching to locally available carbon-rich (agro)industrial side-streams alleviates industrial disposal problems, preserves food resources, and provides inexpensive feed stocks for biotechnology [2]. Many life cycle assessment (LCA) studies indicate that the entire ecological food print of PHA production can become superior to classical petro-plastics only if PHA production can resort to easily available side-streams, instead of spending energy, fertilizers, and other chemicals to agricultural feed stock production and processing. This is needed in order to minimize greenhouse gas emissions caused by the feedstock production and should go in parallel with the recycling of side streams of the PHA production process itself, such as spent fermentation broth and residual biomass after PHA recovery [73—77]. As an important issue, the feedstock's availability defines the location of an envisioned PHA production plant; hence biopolyesters production from inexpensive raw materials should be integrated into existing production lines, where the raw materials directly accrue at sufficient quantity [2, 78]. Inexpensive raw materials of different industrial origin were already applied at least on small laboratory scale as carbon substrates for PHA production. Predominately, PHA production on laboratory scale based on the conversion of the following surplus material is exhaustively reported in the recent literature:

— carbohydrate-rich waste: whey [79—83], molasses [83—86], celluloses and lignocelluloses and their hydrolysates [87—89], starchy waste [90—93];

— alcohol-rich waste: methanol [94, 95], crude glycerol phase (CGP) [96—101], fusel alcohols [102];

— lipid-rich waste: waste triacylglycerols [103—110], low-quality biodiesel [15, 101, 111, 112].

As reviewed by Khosravi-Darani *et al.* [113], some microbial specialists can even convert gaseous C1-compounds like methane [114, 115] or CO_2 [116] towards PHA. The photosynthetic fixation of CO_2 by phototrophic cyanobacteria to produce PHA is especially attracting more and more attention as demonstrated by the increasing global research activities in this direction [117, 118]. All of the waste materials listed above display potential feedstocks for *scl*-PHA production, whereas, based on the enzymatic background of the cellular polymerase system, lipid-rich materials are the substrate of choice for generation of *mcl*-PHA.

INDUSTRIAL WASTE FROM SLAUGHTERHOUSES AS A RAW MATERIAL OF CHOICE FOR PHA PRODUCTION

Processes developed in the recently performed EU-FP7 project ANIMPOL resort to lipid-rich surplus streams from slaughterhouses and the rendering industry that undergo chemical transformation to CGP and biodiesel. Such lipid-rich streams of bovine, porcine, avian *etc.* origin accrue in Europe at estimated annual quantities of 500 000 tons [119]. The saturated share of biodiesel (SFAE) counteracts its' applicability as biofuel but can be converted biotechnologically to PHAs at a theoretical quantity of 35 000 annual tons. Considering the entire amount of biodiesel that is currently produced in the EU — 28 (about 20—30 Mt annually), more than 2 Mt of crude glycerol phase (CGP) are available as the major by-product of the conversion of lipids to biodiesel. This is in considerable excess over the quantities of glycerol needed for its various classical applications. If applied for the production of microbial PHA accumulating biomass, one can expect more than 0.4 gram biomass or PHA per gram of glycerol. Regarding the entire quantity of glycerol from animal lipid transesterification, one can estimate more than 20 000 tons of PHA-rich biomass that can be produced annually thereof [111, 119, 120]. These figures are shown in Fig. 2, illustrating the quantities of lipid-rich waste from the animal processing industry available in the EU, and the theoretically producible amounts of PHA thereof. Figure 3 shows the process cycle of material streams, starting from slaughter waste towards prototype bioplastic items.

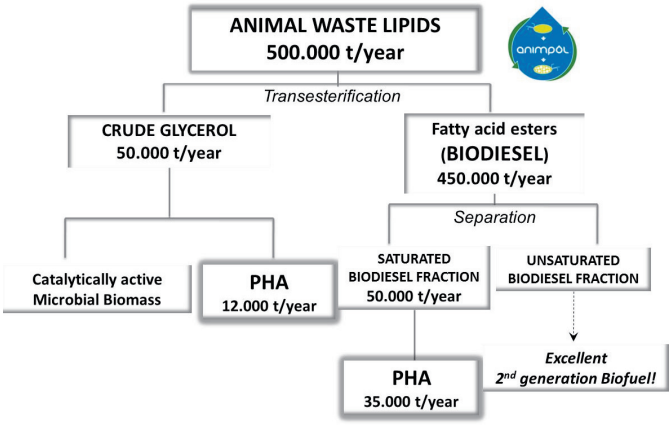


Fig. 2. The ANIMPOL process: available quantities of waste lipids from the animal processing industry, and theoretically producible quantities of PHA thereof after transesterification and the subsequent biotechnological utilization of the saturated biodiesel fraction (SFAE) and the crude glycerol phase (CGP)

To visualize the significance of this novel process for PHA production, one has to face the fact that the contemporary disposal of lipid-rich slaughtering waste amounts to about 1 € per kg of waste material [*personal communication company U. Reistenhofer GesmbH, Austria*]. Converting the lipid fraction of slaughtering waste to CGP and biodiesel is a simple and well-established technique. If optimizing each process step, PHA production based on animal-derived waste lipids could be possible at a price in the range of 2 €/kg PHA. This is superior to cost estima-

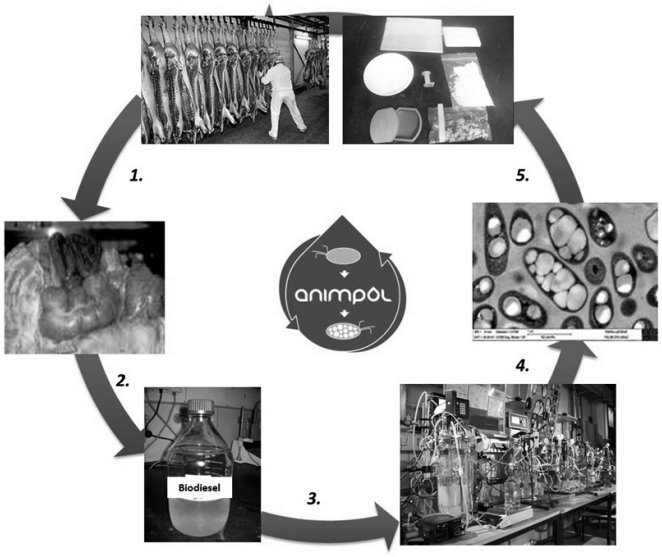


Fig. 3. The ANIMPOL process: 1) cycle of production starting from lipid-rich waste from animal processing, 2) transesterification of the lipids towards convertible carbon sources (SFAE and CGP), 3) microbial conversion of these carbon sources in bioreactors, 4) accumulation of high shares of PHAs in microbial cells, 5) processing of isolated PHA towards prototype items

tions for PHA production on surplus whey (about 2.7 €/kg) [80], or on cane sugar (about 3 €/kg) [50]. As a pre-requisite, PHA production has to be integrated into existing biodiesel facilities, and an efficient energy supply has to be provided [120]. Examples for the autarkic energy supply of PHA production by using in-house waste streams are found in the case of cane-sugar based PHA production at PHB/ISA in Brazil, where bagasse as the major side stream of sugar cane processing is thermally converted, yielding enormous quantities of electric energy (32.4 GW/h per year) and steam (396 000 t per year) [50].

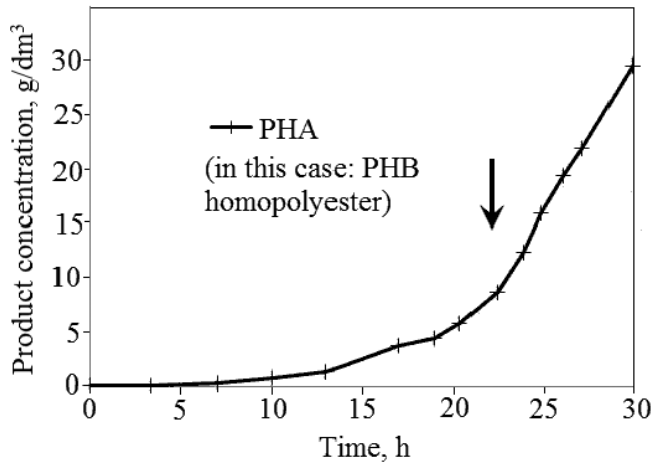


Fig. 4. Product patterns of 4 fermentations on animal-derived waste streams: fermentation F1 (*C. necator* on CGP), arrow indicate the stop of nitrogen supply

The work at hand demonstrates poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) production by *Cupriavidus necator* strain DSM 545 using SFAE as the sole carbon substrate; the same microbial strain was cultivated on CGP for the production of poly(3-hydroxybutyrate) (PHB) of low molecular mass. Two different pseudomonad strains, namely *Ps. citronellolis* and *Ps. chlororaphis*, were applied for *mcl*-PHA production from SFAE. Using these different production strains and the two different waste fractions from animal-based biodiesel production (SFAE and GLP, respectively), polyesters of completely different quality were obtained, providing new basic polymers for different applications. Details of the production process and analytical methods were reported elsewhere; readers are kindly referred to the original articles [15, 100, 111, 112] together with the subsequent description of the four fermentation processes.

PHA PRODUCTION FROM SIDE-STREAMS OF THE ANIMAL-PROCESSING INDUSTRY: BIOTECHNOLOGICAL CASE STUDIES

Fermentation F1: PHB production by *Cupriavidus necator* from CGP

Glycerol was utilized by *Cupriavidus necator* DSM 545 for the production of poly-3-hydroxybutyrate (PHB) in aerobic fed-batch mode as described before in detail by Vrana-Spoljarić *et al*. [100]. The maximal specific growth rate, μ_{max}, and maximal specific PHA production rate were determined as 0.11 1/h and 0.16 g/gh, respectively. The nitrogen supply was stopped after 23 h in order to provoke nutritional stress conditions resulting in the stop of microbial growth and favoring PHA formation. After 30 h of cultivation, a final mass fraction of PHA in CDM of 0.65 g/g was determined. The volumetric productivity for PHA for the entire process (30 h) amounted to 0.98 g/dm^{3}h. The yield of glycerol conversion to cell dry mass amounted to 0.29 g/g. During the entire cultivation, the polyester consisted of 3-HB as the sole building block; hence, the homopolyester PHB was produced. The time curve of PHB during this cultivation is shown in Fig. 4. Regarding the results from polyester characterization, data were in a range typical for this type of PHA (melting temperature T_m = 173.0 °C; T_g = 5.6 °C, degree of crystallinity X_c = 71.2 %, $\overline{M}_w$ = 380 180, $\overline{M}_n$ = 296 150, dispersity index $Đ$ = 1.28). These values are shown in Table 1.

Fermentation F2: PHBV production by *Cupriavidus necator* from SFAE

Figure 5 illustrates the fermentation pattern of the laboratory bioreactor-scale production of PHB by *C. necator* on SFAE as the main carbon source. This fermentation was accomplished using SFAE as the sole carbon source both for biomass growth and PHA accumulation in a fed-batch cultivation mode (repeated supply of SFAE

T a b l e 1. Fermentation results and data from polymer characterization [11, 15, 100, 112]

Production strain	Fermentation F1 *Cupriavidus necator* DSM 545	Fermentation F2 *Cupriavidus necator* DSM 545	Fermentation F3 *Ps. citronellolis* DSM 50332	Fermentation F4 *Ps. chlororaphis* DSM 50083
Carbon source	CGP	SFAE	SFAE	SFAE
Type of PHA produced	PHB	PHBV	*mcl*-PHA	*mcl*-PHA
μ_{max}, 1/h	0.11	0.17	0.08	0.13
q_p, g/g h	0.16	0.19	0.003	0.006
m PHA / CDM*), g/g	65.0	80.3	26.6	29.4
Max. concentration PHA, g/dm^3	29.5	28.0	3.56	6.44
Volumetric productivity, g/dm^{3}h	0.98	0.94	0.05	0.14
Yield biomass / C-source, g/g	0.29	0.60	0.59	0.62
$\overline{M}_n$	296 000	204 000	35 000	38 000
$\overline{M}_w$	380 000	306 000	66 000	74 000
$Đ$ ($\overline{M}_w/\overline{M}_n$)	1.28	1.50	1.88	1.93
T_m, °C	173.0	169.0	48.6	broad melting range
T_g, °C	5.6	4.6	-46.9	-47.0
X_c, %	71.2	30.8	12.3	completely amorphous material

μ_{max} — max. specific growth rate, q_P — specific PHA production rate, m — mass fraction PHA in CDM, $\overline{M}_n$ — number averaged molar mass, $\overline{M}_w$ — weight averaged molar mass, $Đ$ — polydispersity (dispersity index), T_m — melting temperature, T_g — glass transition temperature, X_c — degree of crystallinity.

*) For *mcl*-PHA, this value refers to the PHA content in the sum of PHA + cellular protein.

according to its conversion by cells) as reported before by Koller *et al*. [111]. A maximum of 28.0 g/dm^3 PHA were obtained, corresponding to a PHA mass fraction in CDM of 0.80 g/g. The specific growth rate μ_{max} amounted to 0.17 1/h. The high yield for biomass production from SFAE of 0.6 g CDM per g SFAE is exceptionally high compared to well-known PHA substrates like sugars where the theoretical yield does not exceed 0.48 g/g, or the prior fermentation with a yield biomass from glycerol of 0.29 g/g. This is due to the metabolic background of fatty acid catabolism by the cells. Considering the early stage of process development, also the high volumetric productivity for PHA of 0.94 g/dm^{3}h for the entire process can be considered very promising compared to available data for industrial PHA production from expensive substrates. Considering only the phase of predominant PHA accumulation after nitrogen limitation (stop of nitrogen supply after t = 7 h), volumetric productivity was as high as 1.36 g/dm^{3}h. Regarding the specific volumetric productivity q_p during nitrogen limited conditions, a value of 0.19 g/gh was calculated. During this phase, residual biomass (NPCM) concentration remained constant at about 7 g/dm^3. For the entire process, q_p amounted to 0.14 g/gh. In addition, the produced PHA was a poly(3-HB-co--0.84%-3-HV) copolyester; here, odd-numbered fatty acids in the SFAE acted as 3-HV-related precursor substrates. It is clear from Fig. 5 that, due to the identical composition of the added SFAE during the entire process, the share of 3HV in PHA (about 0.8 %) is constant during the phase of nitrogen limitation. Regarding the results from polyester characterization, most data were in a range typical for this type of PHA (melting temperature T_m = 169.0 °C; T_g = 4.6 °C, $\overline{M}_w$ = 305 700, $\overline{M}_n$ = 203 740, dispersity index $Đ$ = 1.50). The only exception is found regarding the degree of crystallinity X_c that amounted to only 30.8 %. These values are shown in Table 1.

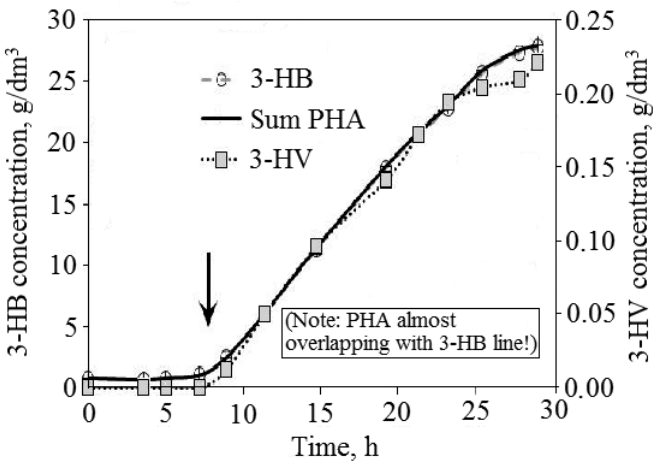


Fig. 5. Product patterns of 4 fermentations on animal-derived waste streams: fermentation F2 (*C. necator* on SFAE), arrow indicate the stop of nitrogen supply

Fermentation F3: *mcl*-PHA production by *Ps. citronellolis* from SFAE

Using the strain *Ps. citronellolis* DSM 50332, a *mcl*-PHA biolatex was produced in fed-batch cultivation with

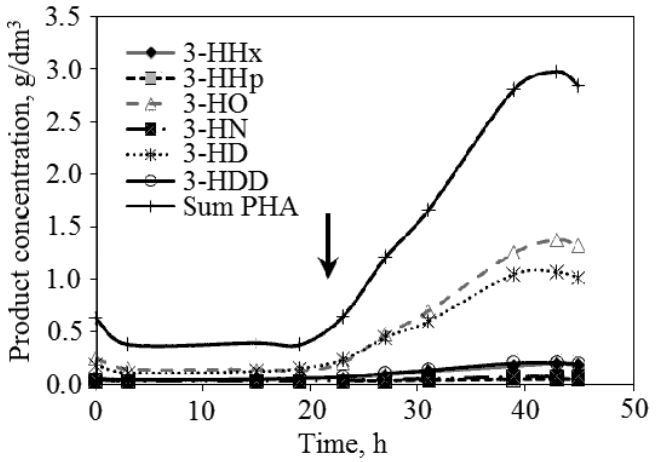


Fig. 6. Product patterns of 4 fermentations on animal-derived waste streams: fermentation F3 (*Ps. citronellolis* on SFAE), arrow indicate the stop of nitrogen supply

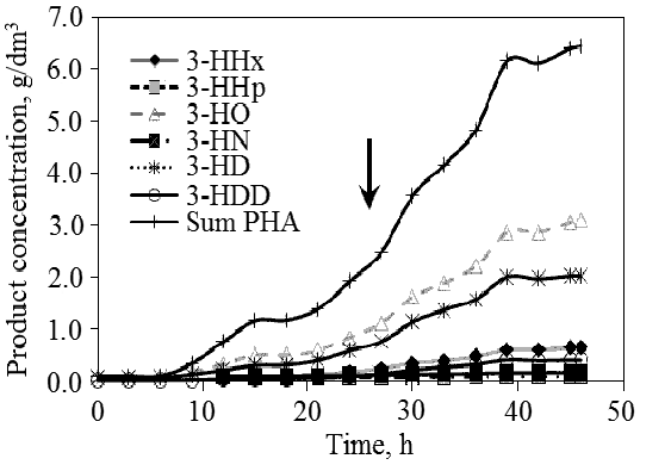


Fig. 7. Product patterns of 4 fermentations on animal-derived waste streams: fermentation F4 (*Ps. chlororaphis* on SFAE), arrow indicate the stop of nitrogen supply

SFAE as the only carbon source. Details of this bioprocess are described by Muhr *et al*. [15]. A maximum specific growth rate μ_{max} of 0.10 1/h and a specific productivity q_P of 0.003 g/gh was achieved; the volumetric productivity for *mcl*-PHA during the entire process amounted to 0.050 g/dm^{3}h, the intracellular PHA mass fraction was 0.027 g/g. The nitrogen supply was stopped after 23 h in order to provoke nutritional stress conditions favoring PHA formation. The yield for SFAE conversion biomass (sum of protein plus *mcl*-PHA) amounted to 0.59 g/g, hence almost the same as observed in fermentation F2. The obtained biopolyester predominantly consisted of 3-hydroxyoctanoate (3-HO) and 3-hydroxydecanoate (3-HD) and, to a minor extent, 3-hydroxydodecanoate (3-DD), 3-hydroxynonanoate (3-HN), 3-hydroxyhexanoate (3-HHx) and 3-hydroxyheptanoate (3-HHp) monomers plus traces of unsaturated building blocks (see Fig. 6; unsaturated building blocks are not shown due to the low quantities). Regarding the results from polyester characterization, data were in a range typical for this type of latex-like PHA (peak of melting range T_m = 48.6 °C; T_g = -46.9 °C, degree of crystallinity X_c = 12.3 %, $\overline{M}_w$ = 66 000, $\overline{M}_n$ = 35 000, dispersity index $Đ$ = 1.9). These values are shown in Table 1.

Fermentation F4: *mcl*-PHA production by *Ps. chlororaphis* from SFAE

Using *Ps. chlororaphis* DSM 50083 for *mcl*-PHA production from animal-based biodiesel, a μ_{max} of 0.13 1/h and a specific productivity q_P of 0.006 g/gh was achieved, together with a volumetric productivity of 0.14 g/dm^3 h and a final intracellular PHA mass fraction of 0.23 g/g. The nitrogen supply was stopped after 27 h in order to provoke nutritional stress conditions favoring PHA formation. Also, in this case, the yield for SFAE conversion towards biomass (protein plus PHA) was exceptionally high (0.62 g/g). Details of this bioprocess are described by Muhr *et al.* [112]. The biopolyester predominantly consisted of 3-HO and 3-HD and, to a minor extent, 3-HDD, 3-HN, 3-HHx, and 3-HHp monomers (see Fig. 7). In this case, it was not easy to determine a sharp melting endotherm peak or the degree of crystallinity due to the resin-like behavior of the materials; similar to the product obtained in fermentation F3, the value for T_g was determined as -47.0 °C. These findings indicate the production of a completely amorphous material by *Ps. chlororaphis* from SFAE. Data for the molar mass distribution were slightly higher ($\overline{M}_w$ = 74 000, $\overline{M}_n$ = 38 000) than in the case of the *Ps. citronellolis* polyester with slightly higher polydispersity ($Đ$ = 1.93). Also, these data are collected in Table 1.

CONCLUSIONS

As illustrated by the article at hand, carbon-rich surplus streams from diverse industrial sectors are available as feed stocks for the bio-production of different types of PHA. Doubtless, the required quantities of biopolyesters will strongly increase in the near future. If successfully implementing the presented strategies of using locally available carbon-rich side streams on an industrial scale, PHAs will not have to be allocated from such manufacturers in diverse global regions, where production competes with nutrition, and frequently lacks environmental considerations and tolerable working conditions.

These measures related to raw materials must go in parallel with the implementation of sustainable and efficient strategies for product recovery, autarkic energy supply, and the application of fermentative process designs adapted to the kinetic particularities of different PHA producing microbes. Only the synopsis of these single process steps can realize sustainable biopolyester production in the future, regarding both ecological and environmental aspects. This way, PHA can definitely become a "biopolymer of the future".

In addition to the already mentioned organic carbon-rich surplus materials from diverse industrial branches, a future perspective to produce high-quality *mcl*-PHA with functional building blocks can be identified in the utilization of algal oils rich in polyunsaturated fatty acids (PUFAs). The accumulation of such PUFA-rich algal oils also starts from the photoautotrophic conversion of CO_2, *e.g.* stemming from industrial effluent gas. Thanks to the high versatility of cyanobacteria as multi-product cell factories, the large-scale production of PHA and additional valued products (proteins, phycobilins, bioactive molecules) from CO_2 can be expected to launch in a not too distant future.

In any case, resourcing locally available carbon feed stocks requires the integration of PHA-production facilities directly into the production lines, where the carbon-rich waste stream accrues. This is needed to avoid transportation costs, thus further lowering the ecological foot print of biopolyester production.

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