

Continuous Production Mode as a Viable Process-Engineering Tool for Efficient Poly(hydroxyalkanoate) (PHA) Bio-Production

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Poly(hydroxyalkanoates) (PHAs) constitute promising biomaterials for substituting plastics of fossil origin. Until now, all commercial processes for PHA production were based on discontinuous fed-batch cultivation of prokaryotes. Such processes embody several shortcomings: unpredictable product quality, restricted possibility for supply of toxic carbon substrates, and, most of all, low volumetric productivity. Continuous PHA biosynthesis as a remedy was already investigated on laboratory scale for production of highly crystalline PHA homopolyesters as well as for elastomeric and even functional PHA copolyesters.

Apart from enhanced productivity, chemostat processes are a feasible method to elucidate kinetics of cell growth and PHA formation under constant environmental conditions. In order to adapt the process engineering to the microbial kinetic characteristics for growth and PHA accumulation, continuous single- and multistage approaches are reported.

Key words:

Azohydromonas lata, bioreactor cascade, chemostat, continuous process, copolyester, *Cupriavidus necator*, (Poly)hydroxyalkanoates (PHA), *Pseudomonas*

General aspects of PHAs and aim of this article

Among all types of biopolymers occurring in living cells, poly(hydroxyalkanoates) (PHAs) display the highest potential for applications in diverse fields of the plastic market.^{1,2,3,4} PHAs are biobased, biocompatible and biodegradable polyesters of hydroxyalkanoic acids synthesized by prokaryotic microbes as intracellular carbon and energy storage compounds.^{5,6} Additionally, PHA synthesis by genetically modified plants harboring bacterial PHA synthesis genes is reported.^{7,8} Their material properties are similar to non-biodegradable, highly recalcitrant plastics of fossil origin. Therefore they are expected to substitute them to a significant extent in the not too distant future.⁹ In contrast to plastics of fossil origin, PHAs are produced starting from renewable resources, predominately carbohydrates, lipids or organic acids.¹⁰ Owing to their complete biodegradability, their ecological advantages are

generally acknowledged.^{11,12,13,14,15} At the same time, production price of PHAs are still higher than for “classical” plastics, mainly due to the high cost of raw materials, downstream processing costs and low productivities caused by inappropriate process design.^{16,17,18,19}

PHAs consist of almost 150 different compounds as monomeric building blocks. Based on the chain length of these building blocks, they can be categorized into short chain length PHA (*scl*-PHA) and medium chain length PHA (*mcl*-PHA).²⁰ Monomers of *scl*-PHA consist of 3 to 5 carbon atoms and mainly constitute *R*-configured chiral 3-hydroxyalkanoates. Due to their physical characteristics, *scl*-PHAs mainly exhibit properties of classical thermoplastics to be applied for packaging or everyday plastic commodities.¹⁹ Therefore, they compete on the market with poly(olefines) and, in the field of “green plastics”, also with biobased poly(lactate). Typically, *scl*-PHAs are accessible from sugars like glucose, fructose, sucrose, or lactose. *Cupriavidus necator*, a member of the bacterial group of Burkholderiaceae, constitutes the best investigated microbial *scl*-PHA producer.^{10,21} In contrast, *mcl*-PHAs

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are by far less crystalline than their *scl*-relatives, with monomeric building blocks of mainly *R*-configured chiral 3-hydroxyalkanoates and, to a minor extent, *R*-configured chiral 3-hydroxyalkenoates consisting of 6 to 14 carbon atoms.²² Sometimes, these *mcl*-building blocks possess functionalities (e.g. double bonds or aromatic groups) in their side chain that allow post-synthetic chemical modification for tailoring the material properties.^{23,24} Characteristics of *mcl*-PHA resemble those of rubber-like elastomers, latexes and resinous to sticky materials with potential applications in drug delivery systems, tissue engineering, smart latexes, thermo-sensitive adhesives, or even biofuels.^{3,25,26,27} Due to their low glass transition temperature, *mcl*-PHAs do not become brittle even at temperatures far below the frosting point. Typically, *mcl*-PHAs are produced from structurally related carbon sources like alkanooates or, in the case of *Pseudomonas putida*, also alkanes.^{28,29} As the most prominent microbial *mcl*-PHA producer, *Ps. putida* GPo1 (ATCC 29347; formerly also known as *Pseudomonas oleovorans*) is well-described by the scientific community.^{30,31,32,33,34} Among all known PHAs, the *scl*-PHA homopolymer poly([*R*]-3-hydroxybutyrate) (PHB) is the most widely investigated and best characterized representative.³⁵

PHAs are accumulated as intracellular granules in the cytoplasm of microbial species isolated from various natural locations belonging to more than 200 different genera.^{36,37,38} Determined by the microbial production strain, the process conditions and the applied substrates, intracellular loads up to 90 wt.-% in cell dry mass (CDM) are reported,³⁹ with typical molar masses ranging from 100,000 to 1,000,000,^{40,41} exceeding values of 3,000,000 in exceptional cases.^{42,43} Depending on the production strain and, to a minor extent, the applied carbon substrate, PHA production occurs either strictly separated from the phase of microbial growth after depletion of a growth-essential nutrient (well described for nitrogen, phosphate, or oxygen limited conditions) together with excess carbon source (non-growth associated PHA production), or occurs in parallel with biomass synthesis even at balanced nutritional conditions (growth associated PHA production). Strict non-growth associated PHA production is described e.g. for *Pseudomonas resinovorans* on octanoate⁴⁴ and *Methylomonas extorquens* on glycerol.⁴⁵ Low PHA production under balanced growth conditions followed by increased production under unbalanced conditions is reported dominantly for *C. necator* on glucose⁴⁶ and for *Ps. putida* on alkanooates,⁴⁷ *Azohydromonas lata* on sucrose⁴⁵ or *Azotobacter vinelandii* UWD on glucose⁴⁸ are known for high PHA production already at nutritionally balanced conditions. A special case is pro-

vided by *Pseudomonas* 2F on glucose, where, without limitation of growth-essential nutrients, excessive PHA overproduction is observed after a period of carbon starvation (“carbon overexpression”).⁴⁹

The article integrates metabolic particularities of different PHA producing microorganisms into process engineering considerations, and, based on available experimental results, demonstrates the high potential of continuous PHA production. Finally, additional possibilities to profit from the potential of chemostat continuous PHA production in terms of economic enhancement and product improvement are discussed. To our best knowledge, this is the first detailed review article dealing with the area of continuous PHA production.

PHA production modes

Discontinuous vs. continuous processes

On an industrial scale, PHA production occurs under controlled conditions in bioreactors, enabling the maintenance of constant process parameters (pH-value, temperature, and dissolved oxygen tension), substrate concentration and operation under monoseptic conditions to avoid infection by microbial competitors.⁵⁰ As mentioned before, for most microbial production strains, the PHA production process encompasses two easily distinguishable phases: first, a desired concentration of catalytically active biomass is produced under balanced growth conditions by providing all substrates required by the microbes for unrestricted growth. In this phase, the production of PHA is minor if compared to biomass formation. In a second phase, the supply of a growth-essential nutrient such as nitrogen, phosphate, oxygen or minor components is restricted.⁵¹ This way, the microbes are subjected to nutritional stress conditions, provoking the deviation of the carbon flux from biomass production towards PHA accumulation.^{52,53,54}

Different operation modes are known for biotechnological PHA production in bioreactors. Among them, discontinuous fed-batch strategies are most widely used on pilot- and industrial scale.⁵⁵ Here, all substrates are re-fed to the system according to their consumption by the production strain. Cell harvest occurs only at the end of the cultivation batch, normally after pasteurizing the cells *in situ* in the bioreactor. Fed-batch processes for PHA production are generally stable and reproducible as soon as reliable and detailed fermentation protocols for the production processes are established. However, discontinuous processes feature restricted productivity (amount of PHA produced

per reactor volume and time – the cost-decisive factor for industrial PHA production) due to the downtime for preparation and post-treatment of bioreactors, as well as product quality fluctuation between different batches.⁴⁶ Furthermore, it is more difficult to obtain high productivity in discontinuous systems in the case of toxic substrates revealing inhibiting effects on the growing cells already at low concentrations. Such substrates are especially needed for *mcl*-PHA production by “fluorescent pseudomonads”.⁵⁶ It has long been known that continuous cultivation regimes in chemostats (from “chemical environment is static”) can guarantee growth of microorganisms under defined nutrient limitations for a longer time period,⁵⁷ and should enable both high productivity and constant product quality^{56,58} provided the long-term genetic stability of the organism.⁵⁹ Here, the concentration of active biomass, PHA and all substrates is kept constant as soon as steady-state conditions are reached. Cell harvest also occurs continuously under these conditions. Apart from enhanced productivity and product quality, chemostat processes are also appropriate for elucidation of the physiological background of bioprocesses, hence kinetics of cell growth and PHA formation under constant environmental conditions.⁵⁸ In addition, the optimization of nutritional media composition can easily be accomplished in chemostat processes by observing the reaction of microbial cultures (growth rate, product formation) if concentrations of single nutrients in the feed stream are changed.⁶⁰

Although not yet widely applied in biotechnological industrial praxis, continuous culture fermentation strategy has already been successfully tested, e.g. for production of acetone and butanol,⁶² ethanol,^{63,64} and lubricant and surfactant formulations.⁶⁵ A broader implementation of chemostats in “white biotechnology” is still hampered by some drawbacks of this mode, such as:

- Continuous culture on commercial scale reveals a high risk of microbial contamination leading to substantial financial loss;

- Coming from the bioreactor’s interior, microbes may reach the reservoir of sterile medium (remedy: interrupting the liquid path by an air barrier in which the medium falls in drops through air, or by thermo-traps);

- Dropping of the media into the bioreactor’s interior results in small pulses of nutrients and thus locally fluctuating concentrations. The same is valid for locally fluctuating pH-values by pulses of acid or hydroxide acting as pH-correctives. This also antagonizes real “steady state” conditions (remedy: reduction of the volume ratio droplets/fermentation broth);

- Very fragile microbial cells can be disrupted by agitation and aeration (remedy: if possible, no excessive agitation and aeration, application of robust microbial strains);

- Cell growth on the walls or other surfaces of the bioreactor (remedy: making the reactor wall hydrophobic by treating e.g. with silane);

- If completely uniform mixing does not occur, true “steady state” conditions are antagonized (remedy: advanced mixing systems);

- Instable reactor volumes by foaming, resulting in an overflow (remedy: application of effective antifoam agents tailored to used combination substrate-strain).

Single-, two-, and multistage continuous processes

Single-, two-, and multistage chemostat process setups for PHA production are feasible. As mentioned before, non-growth associated or only partly growth-associated production strains like *C. necator* do not accumulate excessive amounts of PHA during balanced growth. Therefore, in this case, one-stage continuous production is not a suitable approach because high growth rates and PHA production rates do not occur at the same time. Typically, incomplete conversion of carbon sources is encountered in single-stage continuous processes due to the need for a high molar carbon to nitrogen (C/N) ratio in the feed stream. In case the carbon source cannot easily be recycled to the process, this accounts to a considerable cost factor, firstly due to the direct substrate expenses, and secondly, higher costs for treatment of the spent fermentation broth due to a higher biochemical oxygen demand (BOC).⁶⁶ As a remedy, a two-stage process consisting of two serial continuously stirred tank reactors (CSTRs) is recommendable. In this case, the biomass is produced continuously under balanced nutrient supply in a first vessel and continuously transferred into a second vessel, where carbon source is continuously converted towards PHA without supplementation of the growth-limiting component (e.g. nitrogen source). Thus, in two-stage systems, both growth- and product formation can be optimized separately. One-stage setups are viable, e.g. in the case of *A. lata*, where high PHA productivity can already be observed in parallel with bacterial growth, and the C/N ratio does not have to be as high as in the case of *C. necator*. But, as shown later, even in the case of *A. lata*, a second stage is beneficial to further increase in PHA productivity by providing the cells the required time of exposure (residence time, τ) for complete substrate conversion. Multistage systems are more complicated to handle and, due to the high number of con-

necting devices between the vessels, feature a higher risk of microbial contamination by infiltration of exogenous cells. They provide different cultivation conditions in each stage and thereby approximate the characteristics of a continuous plug flow tubular reactor (CPFTR). It is considered that a cascade with at least five reactors in series can be used as a process-engineering substitute for a CPFTR,⁶⁷ a system even better adapted to the kinetics of PHA accumulation for a number of microorganisms and carbon sources than the second stage of a continuous two-stage system. Enhanced product quality

can be expected in a CPFTR or cascade of CSTRs, respectively, by high uniformity of the cell's physiological state caused by the narrow residence time distribution.⁴⁶ Consequently, this engineering aspect was the background for the recent development of a continuous multistage system of five CSTRs in series (5-SCR) for PHA biosynthesis at high productivities.⁶⁸

Fig. 1 illustrates a schematic of single-, two-, and multistage continuous bioreactor processes for PHA production.

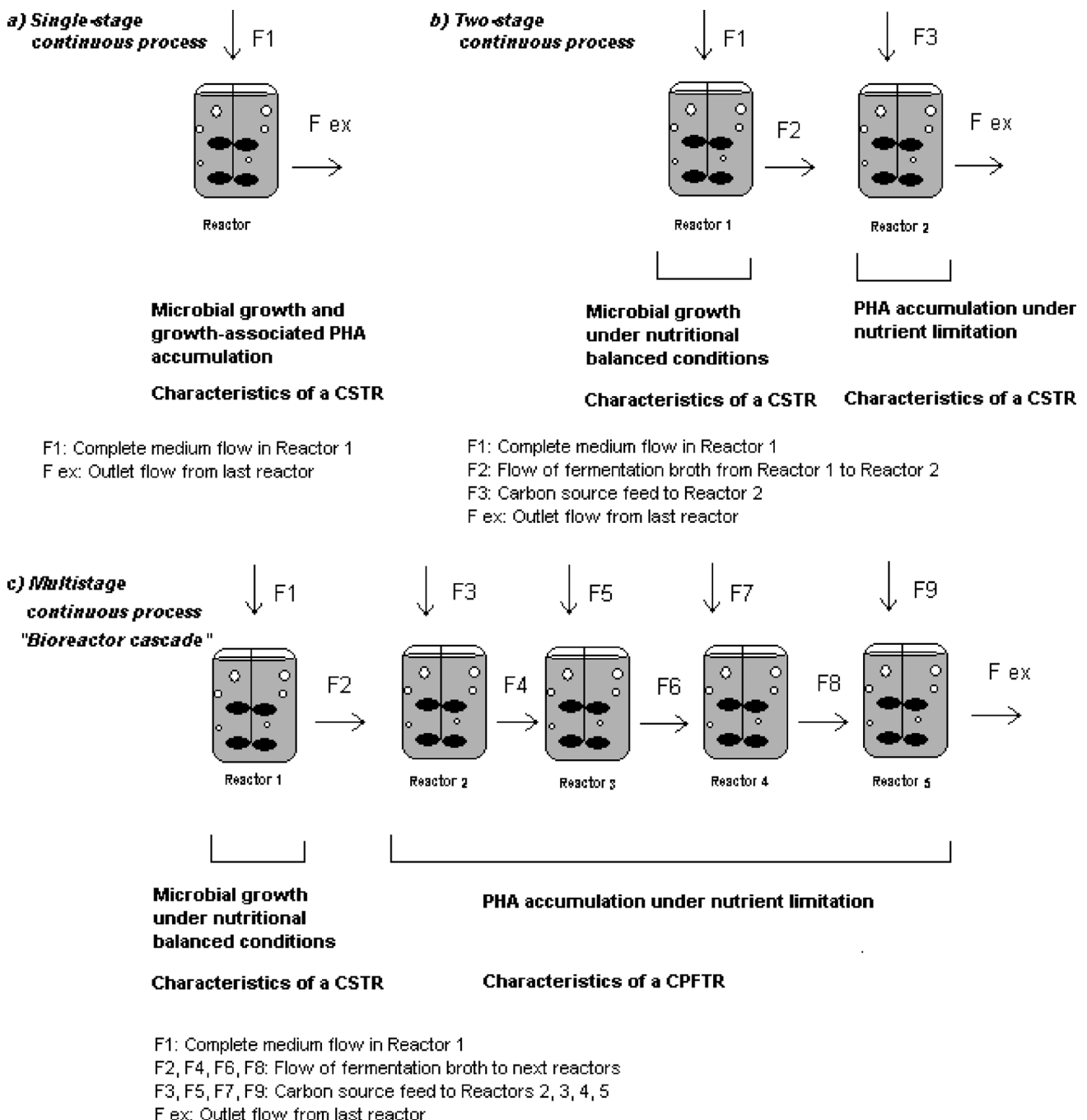


Fig. 1 – Schematic of single-, two-, and multistage continuous PHA production

Experimental achievements in continuous PHA production

Single-stage systems

The first well-grounded report on continuous PHA production was provided by Senior *et al.*⁶⁹ using a non-capsule forming mutant of the diazotrophic (nitrogen-fixing) bacterium *Azotobacter beijerinckii* NCIB 9067. In chemostat cultures using a 2L- Porton type CSTR, the authors investigated the impact of nitrogen, oxygen, and carbon limitation on PHA formation. As the main result, oxygen limitation turned out to be most beneficial for PHA formation. Cultures subjected to carbon- or nitrogen limitation did not accumulate more than 3 % of PHA in CDM, whereas under oxygen-limited conditions, the accumulated PHA amounted to between 20 % at a dilution rate (D) of 0.252 h⁻¹ to 44 % at a D of 0.049 h⁻¹. This indicated that the adaptation of residence time (τ) to the PHA accumulation kinetics was vital to obtaining satisfactory intracellular PHA contents, a decisive factor not only for volumetric PHA productivity, but also for the subsequent downstream processing for PHA recovery from cells. The work could not be perceived as process development for high-throughput PHA production, but was important to elucidate the role of PHA during oxygen-limited conditions. For the first time, it was proposed that PHA do not only display an important function as carbon- and energy reserves, but, during their biosynthesis, they also act as an electron sink to collect excess reducing equivalents (“intracellular pseudo-fermentation”). In addition, the work already describes the major biotechnological risk of continuous PHA production, namely microbial contamination, and proposes safety measures by installing thermo-traps in order to avoid bacterial back-growth from the culture vessel to the sterile medium.

After the works of Senior *et al.*, it took about two decades until studies on continuous PHA production were published by Ramsay *et al.*⁷⁰ These authors were the first to suggest the implementation of continuous process for increasing PHA productivity and the intracellular PHA content, the latter crucial for the economics of PHA isolation. In addition, the work proposed continuous mode to obtain high shares of 3-hydroxyvalerate (3HV) in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV) copolyesters with enhanced material properties. This is an important point due to the fact that in batch- or fed-batch cultivations, odd-numbered fatty acids like propanoate or pentanoate display toxic effects on the production strain already at low concentrations. In addition, the yields for conversion of these precursors to 3HV are cost-decisive due to their rather high prices. One-stage chemostat at a D of

0.15 h⁻¹ with *C. necator* DSM 545 grown on glucose produced 5 g L⁻¹ of biomass with a PHB content of 33 %. Similar results were obtained with *Alcaligenes latus* ATCC 29714 (today known as *A. lata*),⁷¹ grown on sucrose at the same D of 0.15 h⁻¹, biomass concentration and PHB content were 4 g L⁻¹ and 40 wt.-%, respectively. When grown on glucose and various concentrations of propanoic acid in the substrate feed of up to 5 g L⁻¹, *A. latus* produced PHBHV with up to 20 mol-% 3HV in PHA. Substitution of 3 g L⁻¹ propanoic acid by 3 g L⁻¹ pentanoic acid resulted in the same total amount of PHA, but in higher 3HV contents in the copolymer (15 mol-% vs. 38 mol-%, respectively).⁷⁰

Several other studies were conducted to produce PHBHV by *C. necator* in one-stage continuous cultures. Koyama and Doi⁷² produced PHBHV in a 2.5 L jar reactor (working volume 1 L) from fructose and pentanoic acid at concentration of 17.5 and 2.5 g L⁻¹, respectively, in the feed stream, with a maximum PHA productivity of 0.31 g L⁻¹ h⁻¹ at a D of 0.17 h⁻¹. By increasing D from 0.06 h⁻¹ to 0.32 h⁻¹, it was possible to trigger the 3HV content in PHBHV copolymers in a range from 11 mol-% to 79 mol-%, but, at the same time, the biomass concentration and amount of PHA decreased. This indicated on the one hand that the cells needed longer exposure (higher τ) for growth and PHA accumulation, and, on the other hand, the faster consumption rates for pentanoic acid than for fructose. Further, it turned out that the number-average molar mass (M_n) increased in parallel with D .⁷²

Yu *et al.*⁷³ tested different mixtures of glucose and sodium propanoate as well as different D for continuous PHBHV production, demonstrating the direct correlation of the molar 3HV ratio and the amount of propanoic acid in the feed stream. PHBHV with 3HV contents of 40–60 mol-% were produced using mixed feeds of glucose (10 g L⁻¹) and propanoate (7 to 15 g L⁻¹). At sodium propanoate concentrations below 5 g L⁻¹, stable steady states were reached at D up to 0.15 h⁻¹ with maximum PHA productivities at $D = 0.10$ h⁻¹. Employing sodium propanoate at concentrations exceeding 7 g L⁻¹, the continuous culture systems did not reach significant steady states, at $D = 0.028$ h⁻¹ it was possible to run the process stable if sodium propanoate concentrations did not exceed 7 g L⁻¹.⁷³

Ramsay *et al.*⁷⁴ where the first authors describing continuous *mcl*-PHA (in text: “long-side-chain poly- β -hydroxyalkanoates”) biosynthesis by *Ps. putida* GP01 (in text “*Ps. oleovorans* ATCC 29347”) in a Multigen F-2000 2L bioreactor. The background of this study was the highly inhibiting effect of most structurally related substrates needed for *mcl*-PHA biosynthesis by this strain like, in the discussed case, sodium octanoate. The authors recognized continu-

ous cultivation mode as a viable strategy to provide the cells with sufficient substrate without reaching inhibiting substrate concentrations in the culture broth. At a D of 0.24 h^{-1} , a *mcl*-PHA copolyester with constant proportions of the building blocks C4, C6, C8, and C10 was produced under nitrogen-limited conditions at intracellular polyester content of 13 %. Surprisingly, nitrogen limitation did not increase productivity to an expected degree using octanoate as substrate. In addition to octanoate, the authors investigated the application of octane as carbon source with modest results due to the high hydrophobicity of this substrate, limiting its availability for the cells.

Using the carbon sources hexanoic acid and octanoate for *mcl*-PHA biosynthesis in continuous cultures, the same group of researchers investigated another representative of the “fluorescent pseudomonads”, namely *Pseudomonas resinovorans*. The same bioreactor equipment was used as for the prior cultivations of *Ps. putida* GPo1 on octanoate. At a fixed D of 0.25 h^{-1} in the case of octanoate and 0.125 h^{-1} in the case of hexanoic acid, different C/N ratios in the feed stream were investigated. It turned out that, under conditions of nitrogen limitation, the composition of the produced *mcl*-PHA consisting of C4 (only traces in the case of octanoate), C6, C8 and C10 building blocks remained constant. Similar to the outcomes with *Ps. putida* GPo1, the predominant building block represented the chain length of the substrate, i.e. C6 in the case of hexanoic acid, and C8 in the case of octanoate. For application of hexanoic acid, no data were reported for productivity, PHA loads in cells or PHA concentration, obviously due to the low amounts of produced polyester. In contrast to the results from *Ps. putida* GPo1 on octanoate, nitrogen limitation boosted *mcl*-PHA productivity enormously in the case of *Ps. resinovorans*. Nevertheless, the calculated specific *mcl*-PHA productivity on octanoate under comparable conditions (D , substrate concentration) was still considerably lower for *Ps. resinovorans* than for *Ps. putida* GPo1 ($0.50 \text{ g g}^{-1} \text{ h}^{-1}$ vs. $0.74 \text{ g g}^{-1} \text{ h}^{-1}$, respectively).⁴⁴

One-stage continuous cultivation for production of *mcl*-PHA was also accomplished by the group of B. Witholt,⁷⁵ again using *Ps. putida* GPo1 as production strain. This was done after a detailed optimization of the medium composition regarding ammonium, phosphate, iron and magnesium concentration. Due to the rare ability of this strain to convert alkanes towards *mcl*-PHA, gaseous octane was used as carbon source and provided to the cultivation broth by pre-saturation of the aeration stream with octane in a thermostated bubble-column. Under nitrogen-limited conditions, *mcl*-PHA consisting of C6 and C8 building blocks were produced with maximum productivities of $0.17 \text{ g L}^{-1} \text{ h}^{-1}$

mcl-PHA at a D of 0.2 h^{-1} . Increasing the cell density by higher nitrogen supply, the productivity was increased to $0.58 \text{ g L}^{-1} \text{ h}^{-1}$ at the same D . With only 23 % in CDM, the intracellular PHA content remained rather low, but the process operated under stable steady-state conditions for about one month.⁷⁶ By further improvement of the process, especially regarding the media composition and mixing performance of the bioreactor, it was possible to increase volumetric productivity to $0.76 \text{ g L}^{-1} \text{ h}^{-1}$.⁷⁵

Investigating different C/N ratios in single-stage chemostat cultures of *Ps. putida* GPo1 at fixed D of 0.2 h^{-1} , Durner and co-workers⁷⁷ detected the existence of dual-nutrient-limited growth conditions (DNL), hence an intermediate growth regime of simultaneous nitrogen and carbon limitation, and the use of DNL for efficient *mcl*-PHA production. In contrast to nitrogen-limited cultures, in dual-nutrient-limited cultures, neither the nitrogen nor carbon source is actually detectable in the supernatant of the fermentation broth. The authors concluded that DNL continuous conditions provide the best strategy to produce PHA from toxic substrates by providing the cells with the adequate amount of the often toxic substrate without compromising the cell activity by surplus substrate. As carbon sources for detailed investigations, hexanoate, heptanoate, octanoate, and nonanoate were applied, resulting in copolyesters in which the chain length of the predominant monomer corresponds to the substrate, the rest to its products of β -oxidation. In the case of octanoate, 42 % of *mcl*-PHA were found in the cells cultivated under only nitrogen-limited conditions, this is higher than the values obtained for heptanoate and nonanoate (around 30 %), and especially hexanoate (0.6 %). Under carbon-limited conditions, minor amounts (about 2 %) of *mcl*-PHA in CDM were detected in the case of heptanoate and octanoate, and around 19 % in the case of nonanoate. No PHA was detected using hexanoate. For the octanoate case, it clearly turned out that when using DNL conditions, the C/N ratio has to approximate the boundaries to nitrogen limitation, but not exceeding a value that results in detectable substrate concentrations in the supernatant.⁴⁷ Due to the fact that all carbon sources are completely converted during dual-limited cultivation, the scientists soon realized that this opens the door for production of structurally tailored PHA copolyesters by using multiple carbon substrates. Once the ratio between the applied carbon sources is adapted to a desired polyester composition, copolyesters of constant composition will be produced during the dual-nutrient-limited steady-state.^{27,78}

Based on these considerations, *C. necator* DSM 428 was grown in a chemostat culture under DNL conditions regarding carbon (mixtures of butanoic and pentanoic acid) and nitrogen source for *scl*-PHA

production. Only by changing the ratio of butanoic and pentanoic acid, the authors kept the total C/N ratio constant at 17/1 to provide metabolic conditions beneficial for high PHA synthesis. For all experiments, a D of 0.1 h^{-1} was applied. As expected, butanoic acid as the sole carbon source resulted in synthesis of PHB homopolyester. With increasing pentanoate content in the carbon feed, PHBHV copolyesters with increasing 3HV contents were produced. In the case of using pentanoic acid as the sole carbon source, the share of 3HV in PHBHV amounted to 62 mol-%. The isolated PHAs showed high molar mass at low dispersity indices (D_i) with sharply decreasing melting points (T_m between $178 \text{ }^\circ\text{C}$ for PHB and $80 \text{ }^\circ\text{C}$ for PHBHV with 62 mol-% 3HV) and glass transition temperatures (T_g between $5.9 \text{ }^\circ\text{C}$ for PHB and $-4.1 \text{ }^\circ\text{C}$ for PHBHV with 62 mol-% 3HV) at increasing 3HV contents. As a major outcome, the authors suggested the DNL continuous process as a more accurate tool to trigger the composition of PHBHV copolyesters than possible in simply nitrogen-limited continuous cultures. Similar to the findings with *Ps. putida* GPO1,⁴⁷ also in the case of these rather toxic compounds, dual-limited growth is beneficial because microbial metabolism is not negatively affected when the substrate is completely converted, or at least, its concentration is kept below a threshold level of inhibition.⁵⁶ For further investigations, the impact of different D values on the resulting polyester composition, especially in the case of high pentanoic acid contents in the feed stream.

Tailored synthesis of PHA by application of multiple carbon substrates under dual-nutrient limitation in one-stage chemostats was also demonstrated for *mcl*-PHA production by *Ps. putida* GPO1. Here, a functionalized *mcl*-PHA harbouring unsaturated side chains was produced by co-feeding of octanoic and 10-undecenoic acid as carbon sources. The applied D amounted to 0.1 h^{-1} , and the applied C/N ratio of 16/1 corresponded to both nitrogen and carbon limitation under steady-state conditions. A share of 10 % 10-undecenoic acid in the feed mix resulted in *mcl*-PHAs harbouring 10 % monomers with unsaturated units. 25 % of unsaturated building blocks were detected using 25 % of 10-undecenoic acid in the feed mix. Due to the breakdown of the substrates by one or more acetyl-CoA units during β -oxidation, the resulting polymers consisted of C6, C8, C11:1, C9:1, and C7:1 units. For the polymer containing 10 % unsaturated building blocks, M_n is reported with 113000 at a D_i of 2.02. The functional *mcl*-PHA turned out to be very suitable for post-synthetic chemical modification to produce coatings against bio-fouling by linking the functional PHA to bioactive zoosteric acid.³³

Even structurally more enhanced functionalization of *mcl*-PHA was achieved by applying multiple

carbon substrates to DNL continuous cultures of *Ps. putida* GPO1 in a KLF 2000 bioreactor. At a D of 0.1 h^{-1} and molar C/N ratio in the feed stream of 15/1, different mixtures of 5-phenylpentanoate, octanoate, and 10-undecenoate were used for production of poly(3-hydroxy-5-phenylvalerate-co-3-hydroxyalkanoate-co-3-hydroxy- ω -alkenoate) with a share of monomers with aromatic side chains ranging from 0 to 52 %. With increasing 3-hydroxy-5-phenylpentanoate content, the glass transition temperature T_g linearly increased from $-37.6 \text{ }^\circ\text{C}$ to $-6 \text{ }^\circ\text{C}$.³⁴

The application of extremophilic organisms for continuous cultivation can be regarded as a feasible tool due to the low risk in microbial contamination. This was studied already in 1990 by Lillo and Rodriguez-Valera⁵⁹ using the highly halophilic archaeon *Haloferax mediterranei*, a member of the class of halobacteria. This organism requires a minimum of 1.5 M NaCl for growth and tolerates up to 5 M (about 300 g L^{-1}). Continuous production in one-stage setups was possible due to the fact that the strain accumulates considerable amounts of PHA already during exponential growth. In fed-batch cultures, intracellular PHA loads exceeding 80 % by far were reported.⁷⁹ As a particularity, the strain produces 3HV-containing copolyesters from structurally unrelated carbon sources like glucose, fructose, sucrose,⁸⁰ starch,⁸¹ or maltose (Koller, unpublished data). Further advantages of this organism for industrial PHA production (convenient solvent-free downstream processing, application of inexpensive waste streams as carbon source etc.) as well as the drawbacks (high salinity of the nutritional medium demands high requirements for bioreactor equipment and disposal of spent fermentation broth) are discussed in details elsewhere.³⁸ Based on batch-experiments conducted by Lillo and Rodriguez-Valera, it turned out that, unlike phosphate limitation, nitrogen or oxygen limitation does not benefit the boosting of PHA biosynthesis in *H. mediterranei*. In the continuous experiments of the same study, in a Braun Biostat M bioreactor of 1.5 L working volume, the authors used glucose as carbon source at two different phosphate concentrations. Five different values for D between 0.02 and 0.10 h^{-1} were studied for two different phosphate concentrations. Best results were obtained at $D = 0.02 \text{ h}^{-1}$ with intracellular PHA contents of 42 wt.-% and 1.5 g L^{-1} PHA, corresponding to a productivity of $0.03 \text{ g L}^{-1} \text{ h}^{-1}$, or a specific productivity of $0.014 \text{ g g}^{-1} \text{ h}^{-1}$, respectively. Due to the rather simple analytical method, the authors were unable to report the exact composition of the produced PHA. In order to test the robustness and long-term stability of the strain, the continuous process was repeated by taking only minimal sterility precautions. At $D = 0.12 \text{ h}^{-1}$, the process maintained monoseptic for 3 months.⁵⁹

Two-stage systems

Together with incomplete carbon source conversion, all single-stage experiments with the most powerful strains *C. necator* or *Ps. putida* resulted in rather low concentrations of active biomass and low intracellular PHA contents, as shown previously. For high-throughput production of catalytically active biomass as a pre-requisite for high productivity of the entire process, high D approximating μ_{\max} is needed. However, at the same time, increasing D in the described cases results in decreasing intracellular PHA content. These are expected outcomes, since these organisms produce PHA predominately in a non-growth-associated mode. Here, applying single-stage setups always requires a compromise between productivity, concentration and intracellular content of PHA.⁵⁸ This means that the phase of formation of active biomass under balanced nutritional conditions can be clearly distinguished from the subsequent phase of predominant diverting of the intracellular carbon flux towards PHA formation.⁸² For such organisms, optimal conditions for both cell growth and PHA accumulation cannot be maintained in a single-stage continuous system. Therefore, a multistage system should be more suitable for continuous PHA production, and in fact, Ramsay *et al.*⁷⁰ demonstrated their results for PHA production from a two-stage chemostat superior to those from one-stage experiments. These authors also describe the first two-stage chemostat process using *A. lata* on sucrose and propanoic acid for PHBHV production. In the first stage, propanoic acid was completely converted, but sucrose remained at detectable concentrations. In this first stage, 48 % PHA in biomass with 18.5 mol-% 3HV were produced continuously. By continuously transferring the fermentation broth from the first bioreactor into the second, the excess sucrose from the first reactor was metabolized in the second vessel, and 58 % of copolymer in biomass with 11 mol-% of 3HV was obtained. The experiment also showed that uptake of sucrose was inhibited when propanoic acid concentrations exceeding 8.5 g L⁻¹ were applied in the feed.⁷⁰

Du *et al.*⁸³ used two CSTRs in series for cultivation of *Ralstonia eutropha* WSH3, with 1.2 L volume of the first stage and 1.4 L of the second one. Under nitrogen-rich and carbon-limited conditions, the first stage produced mainly active cell mass, resulting in the maximal CDM of 27.1 g L⁻¹ at a D of 0.21 h⁻¹. By applying nitrogen-limitation and sufficient carbon source (glucose), the conditions in the second stage were favorable for PHB production, resulting in a maximal PHA concentration of 47.6 g L⁻¹ at D of 0.14 h⁻¹. The maximal PHA productivity reached 1.43 g L⁻¹ h⁻¹ at a D of 0.12 h⁻¹, but with relatively low PHB content of around

48 %. Highest yields for PHA production from glucose (0.36 g g⁻¹) together with satisfying PHA productivity (1.23 g L⁻¹ h⁻¹) and PHA content in biomass (72.1 %) were obtained at D of 0.075 h⁻¹.⁸³ Therefore, the authors obtained fairly good results for CDM, PHB concentration and productivity, but not simultaneously, i.e. at different D , and hence could not fully benefit from the potential of the equipment. After the experimental work, a detailed kinetic analysis of the described two-stage continuous culture was carried out⁸⁴ as the first holistic attempt to explicitly study the bio-kinetics of both microbial growth and PHA formation under steady-state conditions. The analysis revealed that microbial growth in the first stage can be well described by the Monod kinetic model, and washout by excessive feed stream in the first vessel has to be expected at D above 0.40 h⁻¹. Maximum specific growth rate (μ_{\max}), glucose affinity constant (K_S), theoretical yield coefficient of cell mass ($Y_{X/S}$) and maintenance coefficient (m_s) amounted to 0.41 h⁻¹, 0.43 g L⁻¹, 0.57 g g⁻¹ and 0.04 g L⁻¹ h⁻¹, respectively. Also the specific PHA synthesis rate in the second vessel, highly dependent of the C/N ratio, closely approximated the Monod model. The maximum specific PHA production rate for nitrogen-limited conditions ($q_{p\max}$) amounted to 0.18 g g⁻¹ h⁻¹.⁸⁴

Few other studies in a two-stage continuous system for *scl*-PHA production were conducted with other microorganisms. Mothes and Ackermann cultivated *Delftia acidovorans* P4a (DSM 10474) on mixtures of acetic acid and γ -butyrolactone (GBL) in two serial CSTRs, and produced a copolymer poly(3HB-co-4HB) with a fraction of 2.7–19 mol-% of 4HB, dependent on the ratio of acetic acid and GBL in the feed stream. D amounted to 0.2 h⁻¹ for the first and 0.06 h⁻¹ for the second bioreactor. It was demonstrated that the optimum PHA productivity of the bioreactor and target 4HB contents in the copolyester can be triggered by the ratio between the two substrates. Similar to the findings by Zinn *et al.*^{56,85} for application of butanoate and pentanoate, this example of toxic substrates (acetic acid and GBL), continuous cultivation strategy is especially convenient for supplying the cells with the required amounts of substrates without exceeding an inhibiting concentration.⁸⁶

mcl-PHAs were produced with *Pseudomonas oleovorans* ATCC 29347 from gaseous octane in a continuous system of two 3L-CSTRs in series. Also here, the separation of the process in a first stage for biomass production at high specific growth rates (D and $\mu_{\max} = 0.21$ h⁻¹) and a second stage for PHA accumulation at long exposure time ($D = 0.16$ h⁻¹) turned out to meet the requirements of the kinetic situation. After optimizing all process parameters (D , temperature, pH value, selecting nitrogen as most

beneficial limiting factor for *mcl*-PHA accumulation by this strain, and carbon source), a volumetric PHA productivity of $1.06 \text{ g L}^{-1} \text{ h}^{-1}$ and PHA content of 63 % in the second fermenter were obtained. The authors emphasize that especially in the case of *mcl*-PHA, the application of a two-stage process is even more superior to a single-stage setup due to the completely different kinetics of biomass growth and *mcl*-PHA accumulation.⁸⁷ This can be visualized by the fact that, using octanoate as carbon source for *Ps. oleovorans*, *mcl*-PHA accumulation is maximal when the cells grow at a μ of 0.21 h^{-1} , whereas μ_{max} of *Ps. oleovorans* amounts to 0.48 h^{-1} as demonstrated in single-stage chemostat cultures.⁸⁸

Multistage process

An unique approach to efficient PHA production was chosen by Atlíć *et al.*⁶⁸ by using a bioreactor cascade system consisting of five serial CSTRs, which can, as mentioned previously, be considered a process engineering substitute of a CPFR.⁶⁷ The first stage of the reactor cascade (Labfors 3 type bioreactor, 7.5 L, operated with 1 L under continuous conditions) is dedicated to balanced bacterial growth aiming at production of high concentration of catalytically active biomass (ca. 20 g L^{-1}) under nutritionally balanced conditions. Thereafter, the fermentation broth is continuously fed from the first reactor into the subsequent reactors (Labfors 3 type 3.6 L, each operated with 1 L under continuous conditions), where PHA accumulation takes place under nitrogen-limiting conditions, but under continuous supply of glucose as carbon source to each vessel. *C. necator* DSM 545 was used as microbial production strain.

The focus of this work was devoted to the development of a PHA production process characterized by high productivity and high intracellular polymer content. The results obtained by the 5-stage bioreactor cascade demonstrated its potential in terms of both high volumetric and specific productivity ($1.85 \text{ g L}^{-1} \text{ h}^{-1}$ and $0.100 \text{ g g}^{-1} \text{ h}^{-1}$, respectively), high polymer content (77 %) and concentration (63 g L^{-1}), and excellent polymer properties ($M_w = 665000$, $D_i = 2.6$). The cascade operated under stable steady-state conditions for more than 200 h. By the careful adaptation of the glucose supply to the substrate conversion rate, only minor glucose concentrations were detected in the outlet stream from the last cascade reactor, resulting in a lower BOC of the spent fermentation broth. Based on the results, the authors concluded that implementing the technology for multistage continuous PHA production might result in an economically viable process. Further, the authors suggest that, due to the flexibility of the system, the properties of the final product can be fine-tuned, e.g. by triggering the polymer composition with co-substrate additions in each vessel.⁶⁸ Thus, block-copolymers consisting of alternating soft- and hard segments, e.g. 3HB and 3HV blocks,⁸⁹ 3HB and 4HB blocks,⁹⁰ 3HB and HHx blocks⁹¹ or 3-hydroxypropionate (3HP) and 4HB blocks⁹² with superior properties, especially regarding tensile strength and elongation to break, should finally be accessible on a relevant scale.

Fig. 2 provides STEM pictures of the cells at steady-state conditions in the 5 stages.

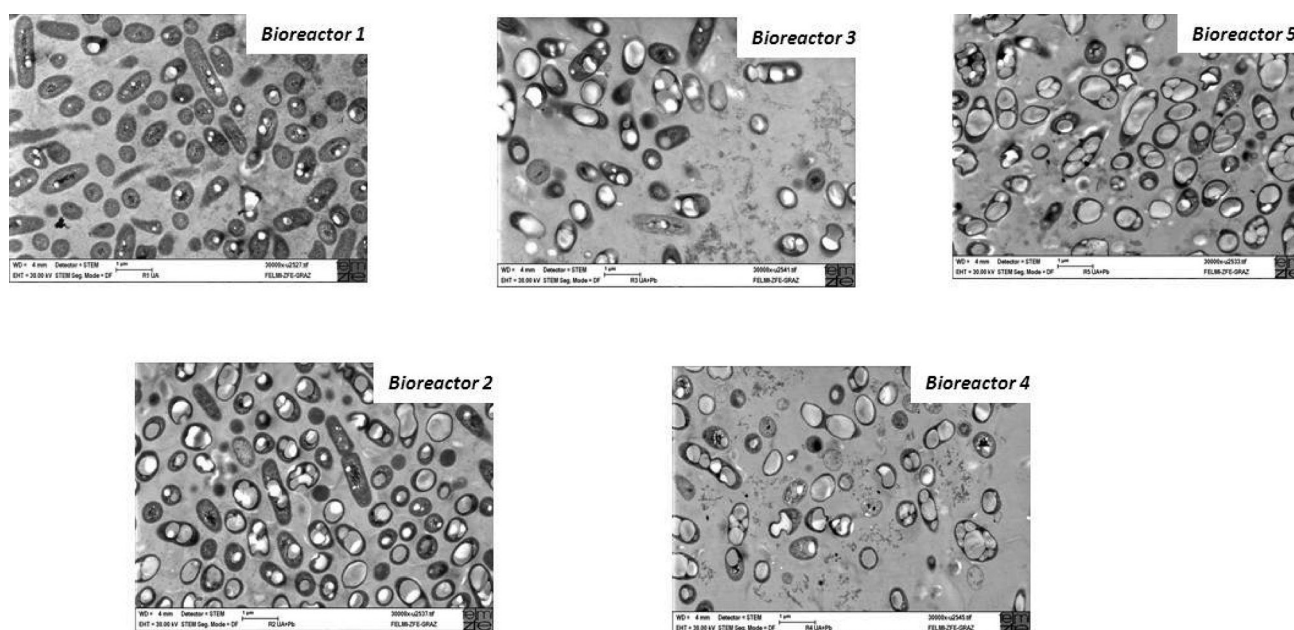


Fig. 2 – STEM pictures of *C. necator* cultivated in a 5-stage continuous bioreactor cascade on glucose. The intracellular polymer content amounts to 4 % (bioreactor 1), 37 % (bioreactor 2), 60 % (bioreactor 3), 72 % (bioreactor 4), and 77 % (bioreactor 5)

Table 1 – Continuous PHA production on laboratory scale in comparison to the most relevant semi-industrial scale PHA production in fed-batch mode at PHB/ISA, Brazil¹⁵⁵

Production strain	Number of stages	Type of PHA produced	Vol. prod [g L ⁻¹ h ⁻¹]	<i>D</i> [h ⁻¹]	PHA / CDM [%]	Carbon source(s)	Growth-limiting factor	Ref.
<i>Azotobacter beijerinckii</i> NCIB 9067	1	PHB	n.r.	0.049–0.252	19.6 – 44.6	Glucose	Oxygen	69
<i>Cupriavidus necator</i> DSM 545	1	PHB	0.25	0.15	33	Glucose	Nitrogen	70
<i>Azohydromonas lata</i> ATCC 29174	1	PHB	0.24	0.15	40	Sucrose	Nitrogen	70
<i>Azohydromonas lata</i> ATCC 29174	1	PHBHV	0.3	0.15	43	Sucrose plus propanoic acid	Nitrogen	70
<i>Cupriavidus necator</i>	1	PHBHV	0.31	0.17	42	fructose and propanoic acid	Nitrogen	72
<i>Cupriavidus necator</i> ATCC 17699	1	PHBHV	0.02	0.10 (optimum)	41	Glucose and sodium propanoate	Nitrogen	73
<i>Pseudomonas putida</i> GPo1	1	<i>mcl</i> -PHA (C4, C6, C8, C10)	0.14	0.24	13	Octanoate	Nitrogen	74
<i>Pseudomonas resinovorans</i> ATCC 14235	1	<i>mcl</i> -PHA (C4, C6, C8, C10)	n.r.	0.125	n.r.	Hexanoic acid	Nitrogen	44
<i>Pseudomonas resinovorans</i> ATCC 14235	1	<i>mcl</i> -PHA (C4 [traces], C6, C8, C10)	0.4	0.25	9	Octanoate	Nitrogen	44
<i>Pseudomonas putida</i> GPo1	1	<i>mcl</i> -PHA (C6, C8)	0.56	0.2	25	Octane	Nitrogen	76
<i>Pseudomonas putida</i> GPo1	1	<i>mcl</i> -PHA (C6, C8)	0.76	0.2	30	Octane	Nitrogen	75
<i>Pseudomonas putida</i> GPo1	1	<i>mcl</i> -PHA (C6, C8) (on octanoate)	0.8 (on octanoate)	0.2 (on octanoate)	42 (on octanoate)	Octanoate; Hexanoate, heptanoate, nonanoate	Dual nutrient limitation (C and N)	47,77
<i>Cupriavidus necator</i> DSM 428	1	PHBHV	n.r.	0.10	40	mixtures of butanoic and pentanoic acid	Dual nutrient limitation (C and N)	56,85
<i>Pseudomonas putida</i> GPo1	1	<i>mcl</i> -PHA with saturated and unsaturated side chains	n.r.	n.r.	n.r.	Octanoate and 10-undecenoate	Dual nutrient limitation (C and N)	33
<i>Pseudomonas putida</i> GPo1	1	<i>mcl</i> -PHA with saturated, unsaturated, and aromatic side chains	0.017–0.030	0.1	16–29	5-phenylpentanoate, octanoate, and 10-undecenoate	Dual nutrient limitation (C and N)	34
<i>Haloferax mediterranei</i> DSM 1411	1	“PHB” (according to today’s knowledge: PHBHV)	0.03	0.02	42	Glucose	Phosphate	59
<i>Pseudomonas putida</i> KT2442	1	<i>mcl</i> -PHA (C6, C8, C10, C12, C12:1, C14, C14:1)	0.69	0.1	23	Oleic acid	Nitrogen	94
<i>Azohydromonas lata</i> 29174	2	PHBHV	0.28	0.15	58	Sucrose plus propanoic acid	Nitrogen	70
<i>Azohydromonas lata</i> 29174	2	PHBHV	0.28	0.15	58	Sucrose plus pentanoic acid	Nitrogen	70
<i>C. necator</i> (<i>Ralstonia eutropha</i> WSH3)	2	PHB	1.43 (at <i>D</i> = 0.12 h ⁻¹)	1. stage 0.21 2. stage 0.14	72.1 (at <i>D</i> = 0.075 h ⁻¹)	Glucose	Nitrogen	83,84
<i>Ps. oleovorans</i> ATCC 29347	2	<i>mcl</i> -PHA (C6, C8)	1.06	1. stage 0.21 2. stage 0.16	63	Octane	Nitrogen	87
<i>C. necator</i> DSM 545	5	PHB	1.43 2. stage 2.13 3. stage 3.46 4. stage 2.98 5. stage 1.97 Entire process: 1.85	1. stage 0.14 2. stage 0.14 3. stage 0.15 4. stage 0.16 5. stage 0.13	1. stage 4 2. stage 37 3. stage 60 4. stage 72 5. stage 77	Glucose	Nitrogen	68,94, 95
<i>C. necator</i> DSM 545 discontinuous	discontinuous	PHB, PHBHV	1.44	–	65–70	Hydrolyzed sucrose from sugar cane	Nitrogen, phosphate Carbon	55
<i>Delftia acidovorans</i> P4a (DSM 10474)	2	Poly(3HB-co-4HB)	0.15	1. stage 0.2 2. stage 0.06	51	Acetic acid plus GBL	(1. stage) Nitrogen (2. stage)	86

n.r.: not reported

The complex kinetics ongoing in this multi-stage-continuous process encouraged Horvat *et al.*⁹³ to establish a formal kinetic mathematic model of the process, aiming at identifying possibilities to further profit from the system in terms of productivity and PHA content. Partially growth-associated PHA production under nitrogen-limited growth was chosen as the modeling strategy, applying the Luedeking-Piret's model of partial growth-associated product synthesis. Specific growth rate relations, adjusted for double substrate (C and N source) limited growth according to Megee *et al.* and Mankad-Bungay relation, were tested. The first stage of the reactor cascade was modeled according to the principle of a nutritionally balanced continuous biomass production system, the second as a dual-substrate controlled process, while the three subsequent reactors were adjusted to produce PHB under carbon-rich and nitrogen-limited conditions. Simulated results for production optimization, obtained by the applied mathematical models and computational optimization, indicate that PHB productivity of the whole system could be significantly increased further to 9.95 g L⁻¹ h⁻¹ if experimental conditions regarding the overall *D*, C and N source feed concentration were optimized.⁹³

This work was further intensified by Lopar and colleagues who developed a high-structured mathematic model of the five-stage cascade process. The metabolic stages of the cells in the five distinct vessels were analysed by elementary flux mode and two-dimensional yield space analysis. The authors emphasize that this strategy displays a precious tool to optimize multistage continuous PHA production systems, especially regarding the optimum carbon feeding strategy.⁹⁵

Conclusions and outlook

The article demonstrates the high potential of chemostat continuous processes for PHA biosynthesis by various microbial strains. Exactly adjusting the process design to the kinetic particularities of selected microbes on applied substrates is decisive to achieve an economically efficient process by high productivity and complete substrate conversion. Table 1 compares the results from chemostat culture published in literature and discussed in the text at hand. In the last row of Table 1, characteristic data for fed-batch production of PHA at PHB/ISA, Brazil, are provided as a relevant example of discontinuous PHA production on semi-industrial scale.

High genetic stability of the whole-cell biocatalysts is the pre-requisite for long-term stability of the continuous process. In this context, exploring

novel, powerful PHA producers especially suitable for long-term cultivation, should forcefully be aspired. These strains should display unaltered metabolic performance in long-lasting continuous cultivations and growth rates superior to those of eventual microbial contaminants under the established environmental conditions. In terms of sustainability, technical feasibility, and public acceptance, this approach might be more advantageous than increasing stability of existing production strains by means of genetic engineering or protecting cultivation setups by continuous supply of antibiotics.

In order to further minimize PHA production costs, inexpensive complex carbon substrates should be tested under continuous conditions.

In future, development should also be dedicated to fully benefit from the possibilities of continuous PHA production for designing of biopolymers with exactly tailored composition on the monomeric level for fine-tuning of material characteristics. The production of block-copolyesters using a multi-stage bioreactor cascade is an important example of possible developments of biopolymers displaying novel properties. This might constitute another step towards a broader market penetration of biopolymers by exactly meeting the customer's demands regarding product performance.

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List of Abbreviations and Symbols

ATCC	– American type- and culture collection
BOC	– Biochemical oxygen demand
CDM	– Cell dry mass
CPFTR	– Continuous plug flow tubular reactor
CSTR	– Continuously stirred tank reactor
C4	– 3-hydroxybutyrate
C6	– 3-hydroxyhexanoate
C7	– 3-hydroxyheptanoate
C7:1	– 3-hydroxy- <i>ω</i> -heptenoate
C8	– 3-hydroxyoctanoate
C9	– 3-hydroxynonanoate
C9:1	– 3-hydroxy- <i>ω</i> -nonenoate
C10	– 3-hydroxydecanoate

C11:1 – 3-hydroxyundecanoate
 C12 – 3-hydroxydodecanoate
 C12:1 – 3-hydroxy- ω -dodecanoate
 C14 – 3-hydroxytetradecanoate
 C14:1 – 3-hydroxy- ω -tetradecanoate
 C/N ratio – Molar carbon-to-nitrogen ratio
 D_i – Dispersity index
 D – Dilution rate
 DNL – Dual nutrient limitation
 DSM – Deutsche Stammsammlung für Mikroorganismen und Zellkulturen
 GBL – γ -butyrolactone
 K_s – Substrate affinity constant
 M – Molarity
 mcl-PHA – medium chain length Poly(hydroxyalkanoate)
 M_n – Number average molar mass
 M_w – Weight average molar mass
 μ – specific growth rate
 μ_{max} – Maximum specific growth rate
 NCIB – National Center for Biotechnology Information
 P – Volumetric productivity
 PHA – Poly(hydroxyalkanoate)
 PHB – Poly([R]-3-hydroxybutyrate)
 PHBHV – Poly([R]-3-hydroxybutyrate-co-[R]-3-hydroxyvalerate)
 Q_p – Specific PHA productivity
 q_{Pmax} – Maximum specific PHA production rate
 scl-PHA – short chain length Poly(hydroxyalkanoate)
 τ – Residence time
 T_g – Glass transition temperature
 T_m – Melting temperature
 X – PHA-free microbial biomass
 X_e – Biomass concentration at end of process
 X_0 – Biomass concentration at beginning of process
 Y_{XS} – Theoretical yield coefficient of cell mass
 3HP – 3-hydroxypropionate
 3HB – 3-(R)-hydroxybutyrate
 4HB – 4-hydroxybutyrate

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