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Formal- and high-structured kinetic process modelling and footprint area analysis of binary imaged cells: Tools to understand and optimize multistage-continuous PHA biosynthesis

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Abstract

Competitive polyhydroxyalkanoate (PHAs) production requires progress in microbial strain performance, feedstock selection, downstream processing, and more importantly according to the process design with process kinetics of the microbial growth phase and the phase of product formation. The multistage continuous production in a bioreactor cascade was described for the first time in a continuously operated, flexible five-stage bioreactor cascade that mimics the characteristics involved in the engineering process of tubular plug flow reactors. This process was developed and used for *Cupriavidus necator*-mediated PHA production at high volumetric and specific PHA productivity (up to 2.31 g/(Lh) and 0.105 g/(gh), respectively). Based on the experimental data, formal kinetic and high structured kinetic models were established, accompanied by footprint area analysis of binary imaged cells.

As a result of the study, there has been an enhanced understanding of the long-term continuous PHA production under balanced, transient, and nutrient-deficient conditions that was achieved on both the micro and the macro kinetic level. It can also be concluded that there were novel insights into the complex metabolic occurrences that developed during the multi-stage-continuous production of PHA as a secondary metabolite. This development was essential in paving the way for further process improvement. At the same time, a new method of specific growth rate and specific production rate based on footprint area analysis was established by using the electron microscope.

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Introduction

Polyhydroxyalkanoates (PHAs), a group of prokaryotic intracellular storage compounds, display high potential to replace conventional plastic materials in defined areas of application, such as the compostable packaging market, the medical and pharmaceutical field, or nanoparticles (1-3), and even act beneficial during bioremediation processes (4,5). Therefore, PHAs have attracted increasing attention by both scientists and industrialists, as manifested in the considerable devotion currently devoted to them both on lab-scale and in white biotechnology. Based on renewable resources, PHAs are accumulated by various microbial production strains as granules ("carbonosomes") predominantly under conditions characterized by excess carbon source availability and parallel limitation of a growth-essential component of the nutritional medium, e.g., depletion of the nitrogen- or the phosphate source (6). Hence, in most described production strains, PHA production occurs as an outcome of the secondary metabolism, enabling cells to survive periods of insufficient availability of exogenous carbon sources; further, PHA biosynthesis provides further protection mechanisms to the cells against various stress factors such as extreme temperature, oxidative damage or UV-radiation (7,8).

From the chemical point of view, PHAs are polyoxoesters mainly of hydroxyalkanoic and, rather rarely, of hydroxyalkenoic acids; an almost infinite number of different homo-

and heteropolyesters of different molecular mass, molecular mass distribution, and thermomechanical properties (melting point, glass transition temperature, onset of decomposition temperature, degree of crystallinity, etc.) can be found in living cells. These properties are determined by the monomers present in a PHA sample, and by the distribution of the monomers in the PHA chains, the latter discriminating PHAs with randomly distributed monomers from blocky structured representatives. Whereas the molecular mass of PHA is determined by the activity of the polymerizing enzyme PHA synthetase (determined by process conditions and substrate availability), the applied carbon source, and by the production strain, the monomeric composition of PHA results from the combination of main carbon source and additional co-substrates with a chemical structure related to defined monomers (9-11). To address the complex variety of different PHA molecules present in a biological system regarding chain length and composition, the terminus "PHAome" was recently coined by leading scientist in the steadily emerging field of PHA research (12). **Fig. 1** shows the general chemical structure of PHAs.

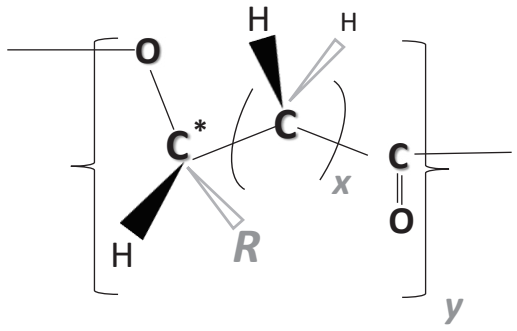

Figure 1. General structure of polyhydroxyalkanoates (PHA). ***R*** indicates the side chain of the monomers, ***x*** reflects the number of methylene groups in the monomers´ backbones, and ***y*** indicates the degree of polymerization (number of monomers in polyester chain). The asterisk indicates the asymmetric carbon atom found in most PHA building blocks (important exception: 4-hydroxybutyrate).

The improvement of large-scale productivity and biochemical/genetic properties of producing strains requires optimization procedures of wild type and genetically modified microbes, process design adapted to the kinetic characteristics of both the microbial growth phase and the PHA production phase, mathematical modeling of the bioprocess based on experimentally obtained kinetic data, and efficient and environmentally friendly methods for PHA recovery from microbial biomass. These are the prerequisites to make PHA production one the one hand ecologically feasible and, on the other hand, environmentally sustainable (13-15). The review at hands summarizes our efforts dedicated to the development of a multistage bioreactor cascade for continuous PHA production, and different approaches to describe and assess this novel process by formal kinetic, metabolic, and footprint area analysis models.

PHA biosynthesis in multistage continuous mode

Continuous and discontinuous PHA production

Letting alone the vast number of PHA production process found in literature, which are carried out at tiny shaking flask scale without major control of the process conditions, PHA production under controlled conditions is predominately carried out in stirred tank bioreactors operated in classical batch or fed batch mode (16). Some attempts are described to use more sophisticated solutions using e.g., membrane techniques for cell recycling to increase productivity (17), airlift reactors for photoautotrophic PHA production by cyanobacteria (18), or sequenced batch process to convert highly polluted wastewater into PHA-rich mixed microbial cultures (19). The number of reported processes for continuous PHA production is definitely manageable; these processes are operated either in one-stage continuous mode, which makes it difficult to adapt the nutrient composition in such a way enabling high PHA contents in biomass and low non-utilized carbon source in the reactor´s effluent; such one-stage processes are only effective in the case of growth-associated product formation. Two-stage continuous PHA production divides the process in a first stage characterized by biomass growth under nutritionally balanced conditions, and a second stage dedicated to PHA accumulation by the cells provoked by nutritional stress such as nitrogen- or phosphate depletion (20-22).

The review at hands is dedicated to a novel process engineering approach to produce PHA biopolyesters more efficiently in terms of volumetric and specific productivity, aiming at obtaining constant product quality, and at providing an option to fine-tune the process conditions at different metabolic stages of the process. This novel process is based on theoretical considerations which suggest that continuous PHA biosynthesis at high throughput is best achieved in a combination of a continuously operated stirred tank bioreactor (CSTR) for formation of catalytically active biomass and a tubular plug flow reactor (TPFR) for formation of the secondary storage product PHA under progressing nitrogen- or phosphate limitation from the entrance of the TPFR until its exit (22). As known from basic chemical engineering considerations, the flow characteristics of the TPFR can be mimicked by a cascade of CSTRs (23). In contrast to a TPFR, a CSTR-cascade provides the additional benefit to adapt the process conditions (pH-value, dissolved oxygen concentration, co-substrate supply) in each individual stage of the cascade, which enables the production of PHA biopolyesters of pre-defined composition and molecular mass (22).

The multistage continuous production process in a bioreactor cascade

We described for the first time the performance of a five-stage bioreactor cascade for PHA production in the case of a series of cultivations of the well-known eubacterial PHA producer *Cupriavidus necator* DSM545, a strain known for its high PHA accumulation efficiency (24). Glucose was selected a model substrate for this process. Based on the metabolic tools of

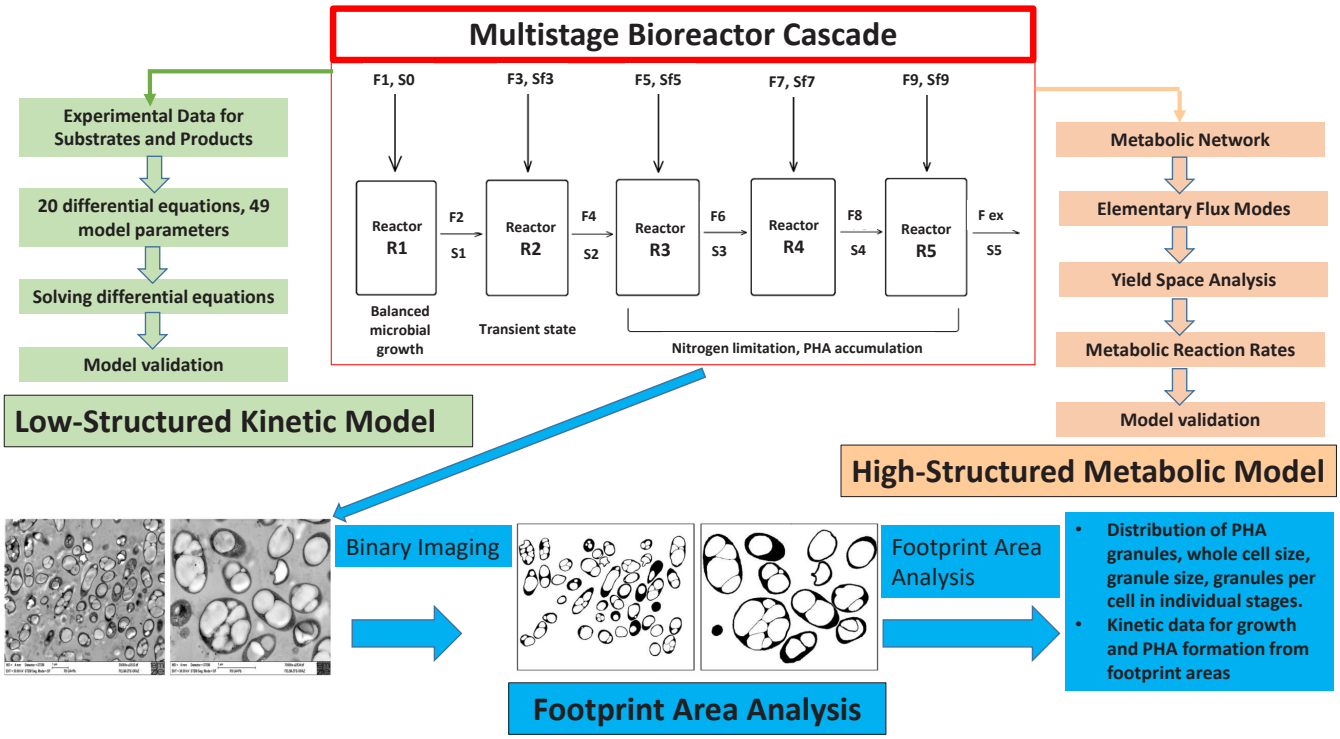

Figure 2. Schematic of the multistage continuous bioreactor cascade for PHA production and three modelling approaches for process description discussed in the review (low-structured kinetic model, high-structured metabolic model, footprint area analysis). R1-R5 indicate the individual bioreactors (cascade stages), F1-Fex indicate the flows into and from the reactors, S1-S5 represent the substrate concentration in the corresponding reactors, S0 signifies the substrate concentration from the storage tank, and S3-Sf9 the substrate concentration in the feeds.

the strain, glucose was used as an easily convertible substrate for biomass growth in the first stage of the cascade (R1), and for PHA accumulation in the subsequent stages (R2-R5). The process was started by "batching" the catalytically active, PHA-poor biomass for all five bioreactors in the first stage (R1) to a concentration of 27 g/L and subsequently distributing equal volumes to R2-R5 before switching to continuous operation mode. The cultivations were carried out at a temperature of 30°C and a pH-value of maintained at 6.8; pH-value was kept constant by automatic supply of aqueous ammonia solution acting also as nitrogen source for microbial growth in R1 (parallel acidification of fermentation broth and biomass growth!), or by NaOH solution and sulfuric acid in R2-R5. Dissolved oxygen concentration was kept constant at 40% of air saturation in R1 and at 20% in R2-R5 by automatic adjustment of aeration rate and stirrer agitation in the individual stages. Complete nutrition medium was continuously supplied from a steam sterilized agitated 120 L storage tank. Feed solutions added to R2-R5 contained each 500 g/L of glucose. The volume of each stage was kept constant by using immersion tubes at the desired height; transfer of the fermentation broth between the reactors was accomplished via silicone tubes and peristaltic pumps. The dilution rate for the entire process amounted to 0.139 1/h, for R5 to 0.130 1/h (24). **Fig. 2** shows a schematic of the five-stage cascade process.

Under steady state conditions, characterized by constant concentrations of substrates (ammonia, glucose) and products (PHA, biomass), samples were taken at regular intervals from all reactors and analyzed regarding substrate, biomass and PHA concentration, and properties of the produced biopolyester in terms of molecular mass, polydispersity (D_i), and thermoanalytical data (melting point, crystallinity, glass transition temperature). After optimizing the operational conditions, the results of the experimental work with the reactor cascade demonstrated its potential in terms of volumetric (1.85 (g/Lh)) and specific PHA productivity (0.100 g/(gh)) for the entire cascade process, high intracellular polymer fraction (77 wt.-% in R5), and polymer properties (weight average molecular mass Mw 665 kDa; D_i 2.6; R5). R1, dedicated to nutritionally balanced biomass growth, reached its steady state after 68 h with a biomass concentration of 26 g/L and a low intracellular PHA fraction of 2-4 wt.-%. With a certain time delay, R2-R5 also reached steady state conditions after 68 h (R2), 98 h (R3), 116 h (R4), and 139 h (R5). Biomass concentration and PHA fractions in the individual reactors had steady state concentrations of 42 g/L and 37 wt.-% (R2), 59 g/L and 60 wt.-% (R3), 71 g/L and 72 wt.-% (R4) and 81 g/L and 77 wt.-% (R5). Steady state conditions remained until 261 h of running the cascade. Among the individual stages, R3 displayed the highest volumetric PHA productivity (3.27 g/L h), which decreased to 1.50 g/(Lh) in R5. The same trend was observed for the specific PHB production rate with the highest value measured for R3 with 0.139 g/(gh). For the entire process, yields for formation of biomass and PHA from the substrate glucose amounted to 0.37 g/g

and 0.29 g/g, respectively. The actual concentration of nitrogen source was 0 in all stages (immediate conversion by the cells in R1); the concentration of glucose was as low as 2.0 g/L in the outflow from R5. Residual biomass concentration dropped from 25 g/L (R1) to 19 g/L (R5) caused by cell death under long-term nitrogen starvation (24).

This process was repeated with some adaptations of the feeding strategy regarding the feeding rate, number of stages continuously supplied with fresh glucose solution, and the dilution rates in individual cascade stages. It was experimentally demonstrated that higher retention time (lower dilution rate) in stage R5 (0.102 1/h) increases the mass fraction of PHA in biomass to almost 79 wt.-%, and the PHA concentration to 73 g/L, whereas shorter retention time (higher dilution rate of 0.193 1/h) in R5 decreases both PHA fraction in biomass and PHA concentration to 71 wt.-% and 56 g/L, respectively (24).

Mathematical modelling of the multistage cascade process

General features of mathematical modelling of PHA biosynthesis

Mathematical models are useful tools for optimizing and controlling microbial product formation and microbial metab-

Table 1. Principles, utilized software packages and major outcomes of formal kinetic modelling, metabolic modelling and footprint area analysis of the multistage continuous cascade process for PHA production

Modelling Approach (Ref.)	Principle	Applied Software Tools	Major outcomes
Formal Kinetic Model (29)	• Partially growth-associated PHA production under N-limited growth: Luedeking-Piret's model of partial growth-associated product synthesis. • Megee *et al.* and Mankad-Bunkay relations tested to reflect μ. • Basic formal kinetic model with growth-associated and non-growth-associated PHB synthesis under nitrogen limitation adopted from Koller *et al.* (2006)	• Berkeley-Madonna quick solver using four-step Runge–Kutta numerical integration method for solving differential equations.	• Model predicts well the cascade system if glucose is added in all reactors • PHB productivity of the whole system could be significantly increased (from experimentally achieved 2.14 g/(L h) to 9.95 g/(L h) if certain experimental conditions would have been optimized (dilution rate, C- and N-source feed concentration) • No difference in the simulated end-state values if "Megee *et al.*" or "Mankad–Bungay" relation for μ are applied. • Mankad-Bungay relation predicts better the increased glucose concentration after switching from batch to continuous mode
Metabolic Model (34)	• Calculation of Elementary Flux Modes; Two-dimension Yield Space Analysis ($Y_{BIO/GLU}$, $Y_{PHA/GLU}$)	• Metatool, version 5.1 based on scripts written for Matlab software package (calculation of stoichiometric matrix and elementary modes) • Matlab (metabolic yield analysis). • Matlab function fmincon (calculation of weighting factors)	• Model predicts well the cascade system if glucose is added in all reactors • All possible metabolic states of cells in different cascade stages were illustrated by applying experimental yields and metabolic flux calculations • Glucose feeding in all five reactors turned out as the most suitable strategy to perform multistage continuous PHB synthesis. • Suitable option for detailed optimization of such production systems concerning biomass degradation under long-term nitrogen starvation.
Footprint Area Analysis (28)	• Binary Imaging of cells to visualize whole cell and PHA granule areas; analysis of obtained areas for whole cells and carbonosomes	• Image J software (inaging of picture) • STATISTICA software version 8.0 (analysis of sizes of cells and granules and distribution of sizes)	• Model describes well long-term continuous PHA production under balanced, transient, and nutrient-deficient conditions and their impact on the granules size, granules number and cell structure on the microscopic level. • Reactor R1 (balanced biomass synthesis) predominately contains cells with rather small PHA granules • With increasing residence time (R2-R5), maximum and average granule sizes by trend increase, approaching an upper limit. • Number of granules per cell decreases along the cascade. Data for μ and specific productivity correlate well with the experimental results

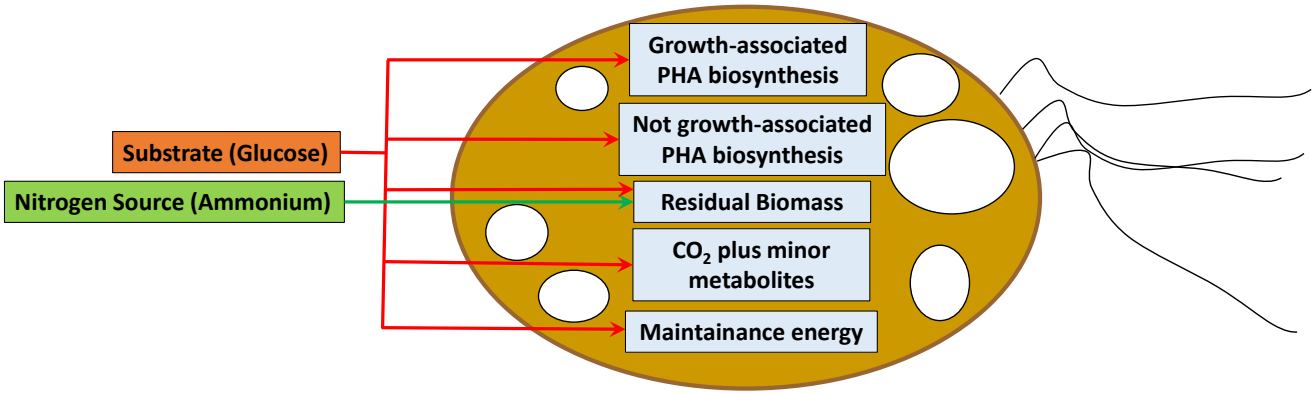

Figure 3. Schematic of the partially growth associated PHA biosynthesis in C. necator (sketched as a flagellated cell containing white PHA granules) based on nitrogen flow (towards residual biomass production) and glucose (towards growth-associated and not growth-associated PHA production, residual biomass formation, production of CO2 and minor metabolites, and maintenance energy).

olism, encompassing the modeling of cultivation techniques, the design of single cell metabolic models, or the modelling of whole cell populations. The segregated nature of biological systems and the complexity of cell reactions are burdensome for mathematical illustration of bioengineering processes. In addition, linking experimental data with mathematical modeling can reveal new aspects of microbial physiology, providing reasonable interpretations of results from experimental work. This enables the broadening of knowledge and the design of more call-oriented experiments (15).

Currently available models dealing with structurally diversified PHAs, of both structured and unstructured nature, can be divided in formal kinetic, low-structured, dynamic, metabolic (high-structured), cybernetic, neural networks and hybrid models, as comprehensively summarized before (15, 25). Characteristic properties of specific groups of models are underlined in light of their benefit to better understand PHA biosynthesis, and their applicability for enhanced productivity, respectively. This is especially valid in the case of complex multi-compound substrates, e.g., surplus materials from (agro)industrial processes like cheese manufacturing (26) or biodiesel production from tallow (27).

Unfortunately, there is no unique model type able to expresses all the characteristics of combinations of microbial production strains and applied substrates. Moreover, modelling of PHA production by pure or mixed microbial cultures have different requirements. Therefore, it is crucial to adapt, as the case arises, the modelling strategy in accordance to the particularities of a given process. For most "simple" standard cases, formal-kinetic and low-structured models will be sufficient to describe kinetics of a PHA production process in a satisfying way. These models are relatively simple and of manageable computational requirements. In contrast, metabolic mathematical models reflect the real biochemical metabolic situation of living microbes. They refer either to simple cases where the metabolic pathway with at least two or three enzymatic reactions is investigated, or to metabolic networks representing the major catabolic and anabolic pathways (15). As a new approach in modelling PHA biosynthesis, foot print areas of whole cells and the included PHA granules from binary imaged electron microscope pictures can be analyzed to describe the distribution of PHA granules in cells, cell size, granule size and number of PHA granules per cell at different environmental conditions. As shown later, this approach is especially useful for describing PHA biosynthesis on a microscopic level under balanced, transient, and limited cultivation conditions, as it is the case in the multistage cascade process (28). **Table 1** summarizes the individual principles, utilized software packages and major outcomes of formal kinetic modelling, metabolic modelling and footprint area analysis of the multistage continuous cascade process for PHA production.

Formal kinetic modelling of the multistage cascade process

Formal kinetic modelling of the above described five-stage cascade process was based on assuming partially growth-associated PHA biosynthesis under nitrogen limited cultivation conditions (29), applying a well-established equation originally used by Luedeking and Piret to describe lactic acid production in batch mode under pH-controlled conditions (30) as working hypothesis; the basic mass flows of nitrogen- (ammonium) and carbon source (glucose) on the multistage-continuous PHA production process are shown in **Fig. 3**.

In order to reflect specific growth rate (μ) as realistic as possible, both relations adjusted for dual (nitrogen and carbon) limited growth according to Megee *et al.* (31) or according to Mankad-Bungay (32) were tested. Hence, the first cascade stage (R1) was modelled as nutritionally balanced continuous biomass production system. R2 was considered a two-substrate (carbon, nitrogen) controlled process, whereas stages R3-R5 were dedicated to produce PHA under continuous carbon fed, but permanent nitrogen deficiency. The developed model consisted of 20 differential equations and 49 model parameters: 20 initial values for PHA, biomass, glucose, and nitrogen concentration, 10 flow parameters for in- and outflow for R1-R5, 7 kinetic constants for substrate saturation and inhibition, 5 parameters related to tank volumes R1-R5, 4 conversion factors, 3 inlet stream substrate concentrations. For fine-tuning

the parameters and for model validation, three experimental runs were carried out in the cascade, differing in the feeding strategy (supply of glucose to all stages or not) and dilution rate in individual stages (29).

Simulated results, obtained by the applied models and computational optimization, predicted well the experimental data for glucose, biomass, and PHA when glucose is added in all five stages. Importantly, the *in silico* obtained values signify that the system´s productivity and the maximum specific growth rate $\mu_{max.}$ could be further drastically increased from 2.14 g/(Lh) (experimental) to 9.95 g/(Lh) (simulated) and 0.25 1/h (experimental) to 0.85 1/h (simulated), respectively, provided the optimization of certain experimental conditions, such as the overall dilution rate, the substrate inflow rate, and substrate (nitrogen and carbon source) feed concentration in individual stages. Under these modelled conditions, up to 164 g/L biomass and 123 g/L PHA could be obtained. Regarding the two tested relations for μ, it turned out that both relations result in the same *in silico* final values of PHA and biomass concentrations. Remarkably, the Mankad and Bungay relation better predicts the sudden increase of glucose concentration occurring after switching from batch to continuous cultivation mode, when cells have to adapt to this new process regime. In addition, it should be emphasized that the oxygen transfer rate of the cascade constitutes another limiting factor for both growth and the PHA production rate. If biomass concentrations as high as calculated *in silico* can be definitely achieved *in realitas*, the required oxygen consumption is definitely higher than the oxygen transfer rate, which negatively affects the cell metabolism by oxygen limitation. To overcome this problem, technical adaptation of the production system will be needed, e.g., using oxygen-enriched air for aerating the cascade (29).

Apart from describing the multistage-continuous process, a similar low-structured (formal kinetic) model was established for a fed-batch cultivation of *C. necator* on glycerol as an inexpensive raw material accruing as surplus stream from the biodiesel production. Five relations for specific growth rate μ were tested using mathematical models. *In silico* performed optimization procedures based on varied glycerol/nitrogen ratios and feeding strategies show the way to achieve a high PHA content of 71%, shorter cultivation time reduced to 23 h, and increased PHA yield of 0.347 g/g. It was shown that an initial concentration of biomass of 16.8 g/L, and a permanent glycerol concentration in the cultivation medium between 3 and 5 g/L were the optimum parameters to increase the biopolyester productivity (33).

High-Structured metabolic model of the multistage cascade process

In order to get deeper insights into the metabolic fluxes of *C. necator* cells cultivated in this multistage cascade process for PHA production, a high structured metabolic model with excellent predictive power for growth and PHA synthesis was established (34). This model constitutes the first metabolic model for a multistage production process, and describes a metabolic network consisting of 43 mass balance equations representing 43 intracellular compounds. Metabolic states of cells in the individual cascade stages were analyzed via elementary flux modes, which were projected to a two-dimensional yield space, composed of the yields for biomass and PHA, respectively, from glucose. The elementary flux modes represented the minimal subset of metabolic network reactions that can operate at steady state; such subsets cannot be decomposed/reduced anymore without destroying the functionality of the system to operate in steady state. From each elementary mode, an overall reaction containing substrates (glucose, ammonia, oxygen) and products (biomass, PHA, CO_2) with the related stoichiometric coefficients was obtained. These stoichiometric coefficients were normalized with respect to glucose, which delivers the amount of products per unit of this substrate. Normalized stoichiometric coefficients were represented in yield spaces: Unbounded polyhedral convex cone from the flux space were projected onto the yield space resulting in a bounded convex hull. Because glucose acts as reference substrate, the elementary modes are represented in a two-dimensional yield space ($Y_{BIO/GLU}$, $Y_{PHB/GLU}$); experimental yields located inside the convex hull were represented in the yield space as linear combination of elementary modes. This approach favors those modes whose yield data in the yield space are closer to experimental yield data. Calculation of the stoichiometric matrix and elementary modes was done using the program Metatool, whereas Matlab, equipped with the "fmincon" function, delivered metabolic yield analysis and the essential weighting factors. Hence, all possible metabolic states of cells in different cascade stages were illustrated by applying experimental yields and metabolic flux calculations performed by the Metatool software (34).

Henceforward, all calculations were based on experimental PHA and biomass yields by applying the quadratic programming approach, which minimizes the sum of squared weighting factors. Two different carbon source-feeding strategies were performed. Concerning PHB and biomass yields, values of the more efficient strategy were used as the data source for elementary modes and metabolic flux calculations, respectively. The high structured metabolic model was validated by comparison of experimental data from 24 h batch cultivation and simulated results. Excellent agreement of the metabolic model with experimental results was achieved for the growth-associated PHA synthesis phase of cultivation. Concerning biomass and PHA yields, *in silico* calculated yield space data reflect well the experimental results in all cascade stages. Most of all, this high-structured model provides additional hints to optimally operate the cascade by further optimizing the feeding strategy. It provides an especially suitable option for detailed optimization of such production systems, which are confronted with biomass degradation under long-term nitrogen starvation, as it is needed to boost PHA productivity. Consistent with the outcomes from the formal kinetic modelling, glucose feeding in all five stages turned out as the most suitable strategy to perform multistage continuous PHB synthesis (34).

In order to get additional information on metabolic fluxes in the network which are currently not unambiguously clarified when consulting the available literature, two different metabolic scenarios for glucose-6-phosphate isomerase´s (G6PI) function and catalytic mechanism in this process were simulated. This enzyme is responsible for the catalytic step from glucose-6-phosphate (G6P) to fructose-6-phosphate (F6P), and is pivotal for the maintenance of the NADH/NADPH ratio, which is decisive for PHA biosynthesis. On the one hand, G6PI was considered bidirectional (G6P to F6P and opposite direction), on the other hand, the unidirectional function from G6P to F6P was modelled. Dependent on the situation (uni- or bidirectional reaction), the metabolic fluxes in the network change tremendously their rates, or even change the direction. Nevertheless, the *in silico* results indicate that both situations are theoretically possible. Which of the scenarios *de facto* occurs in living cultures can unambiguously only be clarified after experimental determination of the impacted fluxes (34).

Later, a similar approach was used for metabolic network analysis for glycerol-based fed batch PHA biosynthesis by *C. necator*. Also here, a two-dimensional yield space was created based on elementary flux mode calculations (35). The major question to be clarified for this process was, if *C. necator´*s metabolic network was capable to reach the same specific growth rate μ and the same PHA productivity rate on the inexpensive substrate glycerol as reached on glucose. Further, it was if interest to learn wheatear the Entner–Doudoroff pathway (ED, KDPG) or the Embden–Meyerhof–Parnas pathway (glycolytic pathway) are dominant in the glycerol metabolism, if the pair NAD/NADH or FAD/FADH2 contribute to the reaction of glycerol-3-phosphate dehydrogenase (GLY-3-P DH), and if the crucial enzyme 6-phosphogluconate dehydrogenase (6-PG DH) was present in the metabolic pathway or not. The analyzed metabolic network consisted of 48 individual reactions. Four sets of elementary modes were obtained, depending on whether NAD/NADH or FAD/FADH2 contributes to the GLY-3-P DH reaction, and whether 6-PG DH is present or not. As major outcome, experimentally determined yields for biomass and PHB (with respect to glycerol) fit well to the *in silico* obtained values both when the ED dominates over the glycolytic pathway and if the glycolytic pathway dominates over the ED; this outcome signifies the high metabolic adaptability of this organism. Further, it was revealed that not the intracellular metabolic network constitutes the rate-determining step for glycerol uptake, but the cellular glycerol import system. Both pairs of coenzymes (NAD/NADH or $FAD/FADH_2$, respectively) can contribute in the GLY-3-P DH reaction: the same final yields were obtained for both scenarios. Finally, some fluxes of the pentose phosphate pathway have changed their direction when 6-PG DH is considered absent (35).

Footprint area analysis to describe the multistage cascade process

Microscopic observations of cells and their inclusion bodies, e.g., PHA, provides a possibility to directly study the morphological changes of cells during fluctuating environmental conditions (36). Mravec *et al.* used confocal fluorescence microscopy analysis of *C. necator* cells to study changes in the diameters of cells as well as PHA granules ("carbonosomes") during growth and PHA accumulation in shaking flask cultivations. These authors came to the conclusion that bacterial cells increased their length during growth and PHA accumulation, although the width of the cells remained constant. The volume fraction of PHA granules in cells increased during PHA accumulation, but did never exceed 40 vol. % independent on the PHA mass fraction (37).

As a follow-up, we investigated statistical distribution of cell- and PHA granule size and granules number per cell microscopically for all individual stages R1-R5 in the multistage cascade process (28). These investigations provide in insight into the cellular morphology and PHA granules formation under balanced (R1), transient (R2), and nutrient-limited (R3-R5) conditions in the cascade process. For this purpose, electron microscopic pictures of cells were converted to binary images using the Image J software tool, thus visualizing PHA (white) and non-PHA biomass (black) footprint areas (see also **Fig. 1**). For each cascade stage, results for μ and specific PHA production rate correlated well with experimentally determined kinetics. Log-normal and gamma distribution best describe granule size distribution in the individual cascade stages. In R1, predominately cells with rather small granules were found; with increasing residence time, granule sizes gradually increased, approaching an upper limit, which seems to be determined by steric hindrance factors when the ratio of carbonosomes/cell volume comes near to its biochemical maximum. These larger PHA granules are of practical importance due to their ease to be separated from cell debris during downstream processing, a benefit originating from their lower density in comparison to the non-PHA (residual) biomass (38). Generally, granule-to-cell area ratios increase along the cascade until a value of 64% is reached in R5 (28), which is considerably higher than reported by Mravec *et al.* (40%) (37). In addition, it was noticed that the increase of the intracellular PHA content and the granule-to-cell area ratio slow down along the cascade from R1 to R5, and also the number of granules per cell decreases with increasing retention time. These data assist in optimizing the cascade set-up, mainly by evaluating the optimum number of stages.

Table 1 summarizes the principles, utilized software packages and major outcomes of formal kinetic modelling, metabolic modelling and footprint area analysis of the multistage continuous cascade process for PHA production.

Conclusions

It is demonstrated that the multistage bioreactor cascade provides a powerful process-engineering tool for high-throughput continuous PHA production regarding the product formation rates and the constant product quality, together with the additional benefit of having the possibility to adapt the process conditions at each individual process stage. Formal kinetic modelling shows the route to optimize the process in terms of reactor

volumes, dilution rate, and feed concentration, whereas metabolic modelling based on elementary flux modes calculations and yield space analysis reflects individual reactions and metabolic states of the cells at different potential scenarios, such as changing enzymatic activity or direction, or preferences of defined enzymes for different co-factors. Thus, the high-structured metabolic network contributes to the identification of metabolic bottlenecks to be overcome in order to enhance the performance of the cascade. Footprint area analysis serves for an understanding of the morphological changes of the cells and their PHA inclusions under fluctuating environmental conditions, as it is the case along the continuous cascade process; this facilitates the design of the cascade on larger scale, e.g., regarding the optimum number of stages.

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Conflict of interest statement

The authors declare no conflict of interest.

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