

## Population balance modeling of biopolymer production in cellular systems

André Franz\* Robert Dürr\*\* Achim Kienle\*,\*\*

\* Max Planck Institute, Magdeburg, Germany

\*\* Otto-von-Guericke University, Magdeburg, Germany

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**Abstract:** In this contribution we present a population balance modeling approach for the production of the biopolymer poly( $\beta$ -hydroxybutyrate) in *Ralstonia eutropha*. The population balance model is based on a dynamic single cell model, which accounts for cell internal regulation by means of the cybernetic modeling approach. The change of internal coordinates is controlled by cybernetic control variables. Depending on available substrates and internal composition the population balance model is therefore able to switch between growth, synthesis of biopolymer and metabolization of biopolymer. The latter one was neglected in an earlier contribution, but is crucial for overall dynamic behavior.

In a first step we present an extended two-dimensional population balance model which includes metabolization of biopolymer and considers the cell internal biopolymer and residual biomass as internal coordinates. The two-dimensional population balance model includes cell internal regulation by means of cybernetic control variables.

Since concentration of internal biopolymer and amount of residual biomass are costly to determine, we discuss in a second step a reduction of the two-dimensional to a one-dimensional population model by means of correlating cell size with biopolymer concentration.

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### 1. INTRODUCTION

Biopolymers are polymers, which are synthesized by living organisms (e.g. plants, microorganisms) and which are usually biodegradable. A well known biogenic and biodegradable polymer is poly( $\beta$ -hydroxybutyrate) (PHB), which serves as internal energy and carbon reserve material and can be synthesized by many microorganisms, e.g. *Ralstonia eutropha*.

PHB production in *Ralstonia eutropha* has been studied experimentally and also theoretically by few researchers. The majority comes up with non-segregated models that are able to predict average properties of the cells, but neglect cell to cell variance w.r.t. intracellular compounds.

In contrast, heterogeneity can be described within the framework of population balance modeling. First attempts to describe PHB production in *Ralstonia eutropha* by a cell population balance model was recently published (Roussos and Kiparissides [2010]). However, in this publication cell internal regulation is neglected and no experimental data is shown.

In this contribution we will present a population balance model, that includes intracellular regulation mechanisms using a cybernetic modeling approach. The model will be compared to flow cytometry data.

In a first step we present a hybrid cybernetic single cell model. In this single cell model biomass consists of two parts, namely PHB and residual biomass (BIO). The intracellular concentrations of PHB and BIO translate directly into internal coordinates of the population balance equation.

However, it is costly to quantify these parts experimentally, especially on the population level. The intracellular PHB can be stained with a fluorescent dye and measured by flow cytometry. The staining process involves complex steps and is typically toxic for the cells, which makes them unusable afterwards, e.g. for cell sorting and further examinations. In contrast, cell size can be measured easily via forward scatter (FSC) in flow cytometry without staining. In a further step we present therefore an one-dimensional population balance model which is based on cell size, but still describes PHB synthesis and metabolization, and includes regulatory features by means of cybernetic control variables.

### 2. MATERIAL AND METHODS

#### 2.1 Microorganism

The organism used throughout this study, *Ralstonia eutropha* (DSM 428, ATCC 17699, NCIB 10442) was obtained from DSMZ GmbH Braunschweig, Germany, as vacuum dried culture.

Under growth limiting conditions (e.g. lack of essential nutrients, such as nitrogen, phosphorus, sulfur, etc.) *Ralstonia eutropha* is able to synthesize PHB, which is accumulated within the cell. PHB is metabolized again, if limitation is removed. Therefore the organism has to switch between three main processes: growth, PHB accumulation and PHB metabolization, depending on nutritional condition.

The non-PHB biomass (BIO) is the catalytic active component, since it includes all the proteins, lipids and DNA. Growth and cell division depends therefore on BIO and it is convenient to divide total biomass (TBM) into PHB and BIO (see Figure 1).

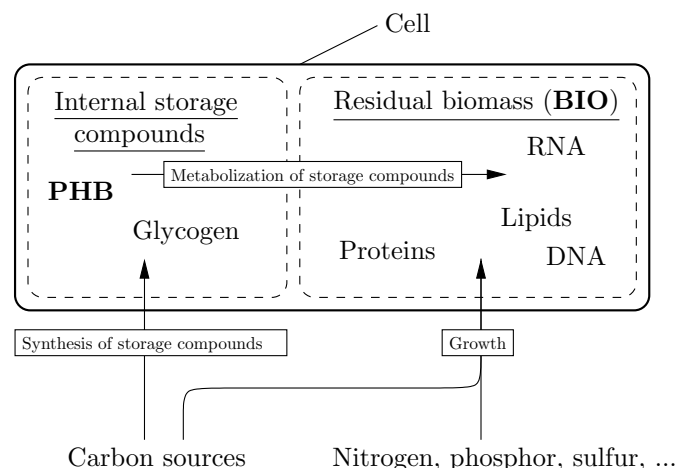


Fig. 1. Cell compartments (PHB and BIO) and main processes (growth, PHB accumulation, PHB metabolization).

## 2.2 Medium

*Ralstonia eutropha* was cultivated with the medium given in Table 2.2 and Table 2.2.  $\text{NH}_4\text{Cl}$  and fructose concentration were varied in each particular experiment.

Table 1. Medium for cultivation.

Ingredient	Concentration
Fructose	3 - 20 g/l
$\text{NH}_4\text{Cl}$	0.1 - 1.5 g/l
$\text{KH}_2\text{PO}_4$	2.30 g/l
$\text{Na}_2\text{HPO}_4 \times 2 \text{H}_2\text{O}$	2.90 g/l
$\text{MgSO}_4 \times 7 \text{H}_2\text{O}$	0.50 g/l
$\text{CaCl}_2 \times 2 \text{H}_2\text{O}$	0.01 g/l
$\text{Fe}(\text{NH}_4)$ citrate	0.05 g/l
Trace element solution (see Table 2.2)	5.00 ml/l

Table 2. Trace element solution.

Ingredient	Concentration
$\text{ZnSO}_4 \times 7 \text{H}_2\text{O}$	0.10 g/l
$\text{MnCl}_2 \times 4 \text{H}_2\text{O}$	0.03 g/l
$\text{H}_3\text{BO}_3$	0.30 g/l
$\text{CoCl} \times 6 \text{H}_2\text{O}$	0.20 g/l
$\text{CuCl}_2 \times 2 \text{H}_2\text{O}$	0.01 g/l
$\text{NiCl}_2 \times 6 \text{H}_2\text{O}$	0.02 g/l
$\text{NaMoO}_4 \times 2 \text{H}_2\text{O}$	0.03 g/l

## 2.3 Analytical procedures

**Substrates** Ammonium chloride concentration in supernatants were determined with an Ammonia test kit from R-Biopharm (Darmstadt, Germany) using the manufacturers recommended procedure.

Concentrations of fructose in supernatants were determined with a D-Glucose/D-Fructose test kit from R-Biopharm (Darmstadt, Germany) using the manufacturers recommended procedure.

**Biomass and PHB** Cell growth was monitored by measuring the optical density (OD) using a Ultrospec 500 spectrophotometer (GE Healthcare, Buckinghamshire, UK). For dilution, NaCl (0.98 % (w/v)) was used when necessary.

$$\text{OD} = -\log\left(\frac{I}{I_0}\right) = \varepsilon \cdot c_{\text{sample}} \cdot l_p \quad (1)$$

Based on the Beer-Lambert law (Equation (1)), which relates the concentration of a sample  $c_{\text{sample}}$  to the attenuation of light, optical density can be proportionally correlated to biomass concentration  $c$ :

$$c = \gamma \cdot \text{OD} \quad (2)$$

However, this is only valid if attenuation coefficient  $\varepsilon$  and path length  $l_p$  are constant.

The used spectrophotometer uses a standardized path length of  $l_p = 1$  cm. Attenuation coefficient depends on wave length  $\lambda$ , cell size and cell morphology. Wave length was set constant to  $\lambda = 600$  nm and cell size and morphology depends on amount of cell internal PHB. Since total biomass can be divided into PHB and BIO, the attenuation constant can be divided in the same way, and OD can be correlated to BIO and PHB:

$$c = (\gamma_{\text{PHB}} + \gamma_{\text{BIO}}) \cdot \text{OD} \quad (3)$$

where  $\gamma_{\text{PHB}}$  and  $\gamma_{\text{BIO}}$  depend on the PHB concentration and BIO concentration, respectively.

Concentration of BIO can be calculated from the uptaken amount of  $\text{NH}_4\text{Cl}$  due to stoichiometric constraints. From optical density and concentration of BIO, PHB concentration can be calculated from Equation (3).

**Flow cytometry** Samples were diluted with NaCl (0.98% (w/v)) to OD 0.1 at 600 nm. 1 ml of diluted samples were centrifuged in 1.5 ml eppendorf cups at 13000 rpm. Supernatant was removed carefully and the remaining pellets were dissolved in 39  $\mu\text{l}$  phosphate buffered saline (PBS) and 1  $\mu\text{l}$  Dapi and 5  $\mu\text{l}$  Bodipy were added. Dapi and Bodipy are fluorescent dyes, where Dapi bounds strongly to DNA in the cells and is therefore used to distinguish between cells and other materials. Bodipy bounds to PHB in the cells and is used for measuring the PHB content via fluorescent light intensity (Kacmar et al. [2006]).

Samples were then incubated in a thermo shaker for 15 min at 37 °C and 450 rpm. For flow cytometry 2  $\mu\text{l}$  of the sample were then dissolved in 2 ml PBS.

Additionally forward scatter (FSC) was measured, which is correlated to the cell volume.

## 3. SINGLE CELL MODEL

The single cell model is based on the hybrid cybernetic modeling approach (Song et al. [2009]) and the model equations for a batch fermentation process are given by (see Franz et al. [2011, 2012]):

$$\frac{d}{dt} \begin{bmatrix} x_{FRU} \\ x_{AMC} \end{bmatrix} = \mathbf{S}_s \mathbf{Z} \mathbf{r}_M c \quad (4)$$

$$\frac{d}{dt} m_{PHB} = \mathbf{S}_{m,s} \mathbf{Z} \mathbf{r}_M - \mathbf{S}_\mu \mathbf{r}_M m_{PHB} \quad (5)$$

$$\frac{dc}{dt} = \mathbf{S}_\mu \mathbf{r}_M c \quad (6)$$

$$\frac{d\mathbf{e}}{dt} = \boldsymbol{\alpha} + \mathbf{r}_{EM} b - \text{diag}(\boldsymbol{\beta}) \mathbf{e} - \mathbf{S}_\mu \mathbf{r}_M \mathbf{e} \quad (7)$$

where  $x_{FRU}$  (g/l) and  $x_{AMC}$  (g/l) are the concentrations of the cell external substrates fructose as carbon source and ammonium chloride as nitrogen source,  $\mathbf{S}_s \mathbf{Z}$ ,  $\mathbf{S}_{m,s} \mathbf{Z}$  and  $\mathbf{S}_\mu$  are stoichiometric matrices,  $m_{PHB}$  (g/g) is the specific PHB concentration,  $c$  (g/l) is the concentration of total biomass (TBM = BIO + PHB) and  $\mathbf{e}$  is the vector of relative enzyme levels, which catalyze the active elementary modes determined by metabolic yield analysis (see Song and Ramkrishna [2009]). The fluxes through elementary modes  $\mathbf{r}_M$  and enzyme synthesis rates  $\mathbf{r}_{EM}$  are controlled by the cybernetic control variables  $\mathbf{u}$  and  $\mathbf{v}$  respectively

$$\mathbf{r}_M = \text{diag}(\mathbf{v}) \text{diag}(\mathbf{e}) \text{diag}(\mathbf{k}_r) \mathbf{r}^{core} \quad (8)$$

$$\mathbf{r}_{EM} = \text{diag}(\mathbf{u}) \text{diag}(\mathbf{k}_e) \mathbf{r}^{core} \quad (9)$$

where the core rates are Monod type

$$r_1^{core} = \frac{x_{FRU}}{K_{FRU} + x_{FRU}} \quad (10)$$

$$r_i^{core} = \frac{x_{FRU}}{K_{FRU} + x_{FRU}} \frac{x_{AMC}}{K_{AMC} + x_{AMC}}, \quad i = 2, 3, 4 \quad (11)$$

$$r_5^{core} = \frac{x_{FRU}}{K_{FRU} + x_{FRU}} \frac{x_{AMC}}{K_{AMC} + x_{AMC}} \frac{m_{PHB}}{K_{PHB} + m_{PHB}} \quad (12)$$

The cybernetic control laws are given by

$$\mathbf{u} = \frac{\mathbf{p}}{\|\mathbf{p}\|_1}, \quad \mathbf{v} = \frac{\mathbf{p}}{\|\mathbf{p}\|_\infty} \quad (13)$$

where  $\mathbf{p}$  is the return on investment (ROI), which can be calculated from a metabolic objective function. In this study it is assumed that the organism maximizes carbon source uptake and  $\mathbf{p}$  is therefore defined as (Song et al. [2009]):

$$\mathbf{p} = \text{diag}(\mathbf{f}_c) \text{diag}(\mathbf{e}) \text{diag}(\mathbf{k}_r) \mathbf{r}^{core} \quad (14)$$

where  $\mathbf{f}_c$  is the vector of uptaken carbon units.

In Figure 2 four independent data sets and model prediction (solid lines) of the single cell model are shown. Synthesis and metabolization of PHB are stimulated via  $\text{NH}_4\text{Cl}$  and fructose pulses.

For population balance modeling the states  $c$ ,  $m_{PHB}$  and  $\mathbf{e}$  will translate into internal coordinates. Since this is computationally challenging, the model can be reduced (see Franz et al. [2012]).

For reduction enzyme levels are approximated by  $\mathbf{e} = \mathbf{u}$  (Baloo and Ramkrishna [1991]). Equation (7) is then omitted. Additionally the core rates  $r_i^{core}$  ( $i = 2, 3, 4$ ) are

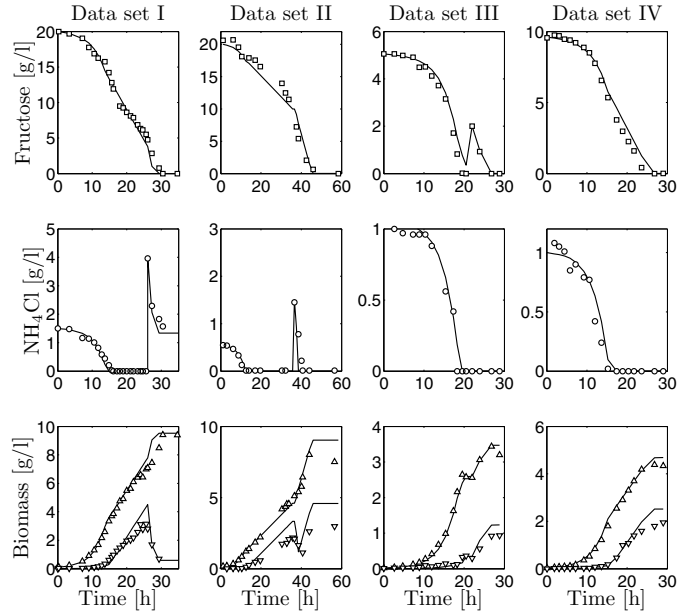


Fig. 2. Four sets of independent experimental data (Fructose:  $\square$ ,  $\text{NH}_4\text{Cl}$ :  $\circ$ , TBM:  $\triangle$ , PHB:  $\nabla$ ) and model prediction (solid lines) of the single cell model.

lumped into one single core rate and  $r_1^{core}$  is supplemented with a term, which inhibits PHB synthesis:

$$r_1^{core} = \frac{x_{FRU}}{K_{FRU} + x_{FRU} + K_I m_{PHB}^2} \quad (15)$$

From the resulting reduced model only the states  $c$  and  $m_{PHB}$  will translate into internal coordinates of the population balance model and the resulting rates will translate into three fluxes through space of internal coordinates.

## 4. POPULATION BALANCE MODEL

### 4.1 Two-dimensional population balance model

Based on the reduced single cell model the following population balance model can be formulated

$$\frac{\partial n(t, \mathbf{x}_c)}{\partial t} + \nabla_{\mathbf{x}_c} \{ \Pi \mathbf{r}_L n(t, \mathbf{x}_c) \} = a(\mathbf{x}_c, \mathbf{x}_s) n(t, \mathbf{x}_c) \quad (16)$$

Here  $n$  is the number density distribution with respect to the intracellular concentrations of PHB and BIO ( $\mathbf{x}_c = [x_{BIO} \ x_{PHB}]^T$ ). The rate  $a(\mathbf{x}_c, \mathbf{x}_s)$  describes sources and sinks as a result of cell division and cell death. It is assumed that this rate depends on  $\mathbf{x}_c$  and the substrates in the reactor  $\mathbf{x}_s = [x_{FRU} \ x_{AMC}]^T$ .

The reaction rates translates into the fluxes  $\mathbf{r}_L = [r_L^{BIO} \ r_L^{PHB+} \ r_L^{PHB-}]^T$  with:

- $r_L^{BIO}$ : flux in direction of BIO synthesis
- $r_L^{PHB+}$ : flux in direction of PHB synthesis
- $r_L^{PHB-}$ : flux in direction of PHB utilization and BIO biomass synthesis

The fluxes are regulated via cybernetic control variables:

$$\mathbf{r}_L = \text{diag}(\mathbf{v}) \text{diag}(\mathbf{u}) \text{diag}(\mathbf{k}_{L,r}) \mathbf{r}_L^{\text{core}} \quad (17)$$

For low ammonium chloride concentration and high fructose concentration synthesis of PHB is favored, while BIO is increasing and PHB is decreasing when there is sufficient ammonium chloride available.

As pointed out in the introduction of this contribution the direct flow cytometric measurement of the intracellular PHB content in *Ralstonia eutropha* has crucial disadvantages, e.g. a complex staining procedure and the cells may not be usable afterwards for additional analysis steps. In contrast, measurements of the FSC which is correlated to cell size are relatively simple and have little effect on the cells viability. For this reason we will present a reduced population balance model by correlating size and intracellular PHB-content.

#### 4.2 One-dimensional population balance model

In our experiments the intracellular amount of PHB is measured using flow cytometric analysis of Bodipy stained cells. When plotting the mean values of Bodipy and FSC distributions, a direct correlation can be observed (see Fig. 3, top row).

Furthermore, the mean values of the FSC distribution show the same course as experimental PHB data (Figure 3, bottom row).

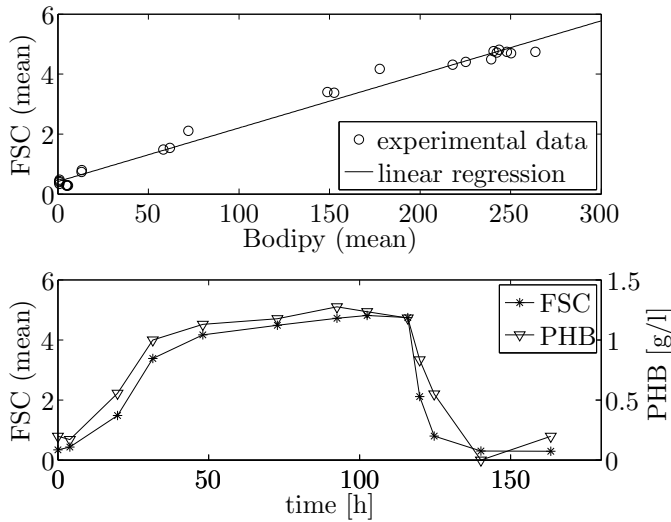


Fig. 3. Mean values of FSC and Bodipy distributions show linear correlation (top row) and mean values of the FSC distribution show same course as experimental PHB data (bottom row).

Motivated by these observations a linear relationship is assumed, which make direct computation of the PHB distribution from measurement of the size distribution possible. A corresponding one-dimensional PBE can be constructed:

$$\begin{aligned} \frac{\partial n(l,t)}{\partial t} + \frac{\partial}{\partial l} \left\{ r_l^{\text{PHB}+} n(l,t) \right\} = \\ - (r_l^{\text{BIO}} + r_l^{\text{PHB}-}) S(l) n(l,t) \\ + \int_{l_{\min}}^{l_{\max}} (r_l^{\text{BIO}} + r_l^{\text{PHB}-}) S(l) p(l,l^*) n(t,l^*) dl^* \end{aligned} \quad (18)$$

Here it is assumed that each cell grows by PHB production in absence of ammonium chloride ( $r_l^{\text{PHB}+}$ ). In addition the cells divide in presence of ammonium chloride by consuming either fructose and ammonium chloride ( $r_l^{\text{BIO}}$ ) or PHB and ammonium chloride ( $r_l^{\text{PHB}-}$ ).

The overall system is numerically solved using a finite volume scheme for discretization of the population balance equations (see Mantzaris et al. [2001]).

The reaction rates  $\mathbf{r}_1$  are again regulated by cybernetic control variables:

$$\mathbf{r}_1 = \text{diag}(\mathbf{v}) \text{diag}(\mathbf{u}) \text{diag}(\mathbf{k}_{1,r}) \mathbf{r}_1^{\text{core}} \quad (19)$$

with core rates characterizing the following subprocesses:

- Cell division by utilizing fructose and ammonium chloride

$$r_{l,\text{BIO}}^{\text{core}} = k_{l,\text{BIO}}^{\text{max}} \frac{x_{\text{FRU}}}{K_{\text{FRU}} + x_{\text{FRU}}} \frac{x_{\text{AMC}}}{K_{\text{AMC}} + x_{\text{AMC}}} \quad (20)$$

- Growth by PHB synthesis

$$r_{l,\text{PHB}+}^{\text{core}} = k_{l,\text{PHB}+}^{\text{max}} \frac{l x_{\text{FRU}}}{K_{\text{FRU}} + x_{\text{FRU}} + K_I l^2} \quad (21)$$

- Cell division by utilization of PHB

$$r_{l,\text{PHB}-}^{\text{core}} = k_{l,\text{PHB}-}^{\text{max}} \frac{x_{\text{AMC}}}{K_{\text{AMC}} + x_{\text{AMC}}} (l - l_{\min})^{1.5} \quad (22)$$

The PBE describing the size distribution of the cell culture is coupled to the concentrated dynamics of the external substrates.

Utilization of external substrates are described by:

$$\frac{dx_{\text{FRU}}}{dt} = - \int_{l_{\min}}^{l_{\max}} \Pi_{\text{FRU}} \mathbf{r}_1 n(l,t) dl \quad (23)$$

for fructose and

$$\frac{dx_{\text{AMC}}}{dt} = - \int_{l_{\min}}^{l_{\max}} \Pi_{\text{AMC}} \mathbf{r}_1 n(l,t) dl \quad (24)$$

for ammonium chloride, with  $\Pi = [\Pi_{\text{FRU}} \Pi_{\text{AMC}}]^T$  being the stoichiometric matrix.

In Figure 4 the comparison of simulation results with experimental data is shown. Initial conditions for the simulation are the experimental data at  $t = 19.8$  h. In the first 116 hours fructose concentration was high and ammonium chloride concentration was low (see Figure 5). During this phase PHB content increases and FSC distribution shifts to higher values as seen in Figure 4. At  $t = 116$  h ammonium chloride was added and the organisms switches to PHB metabolization, which results in a shift of FSC distribution to lower values as can be observed in Figure 4.

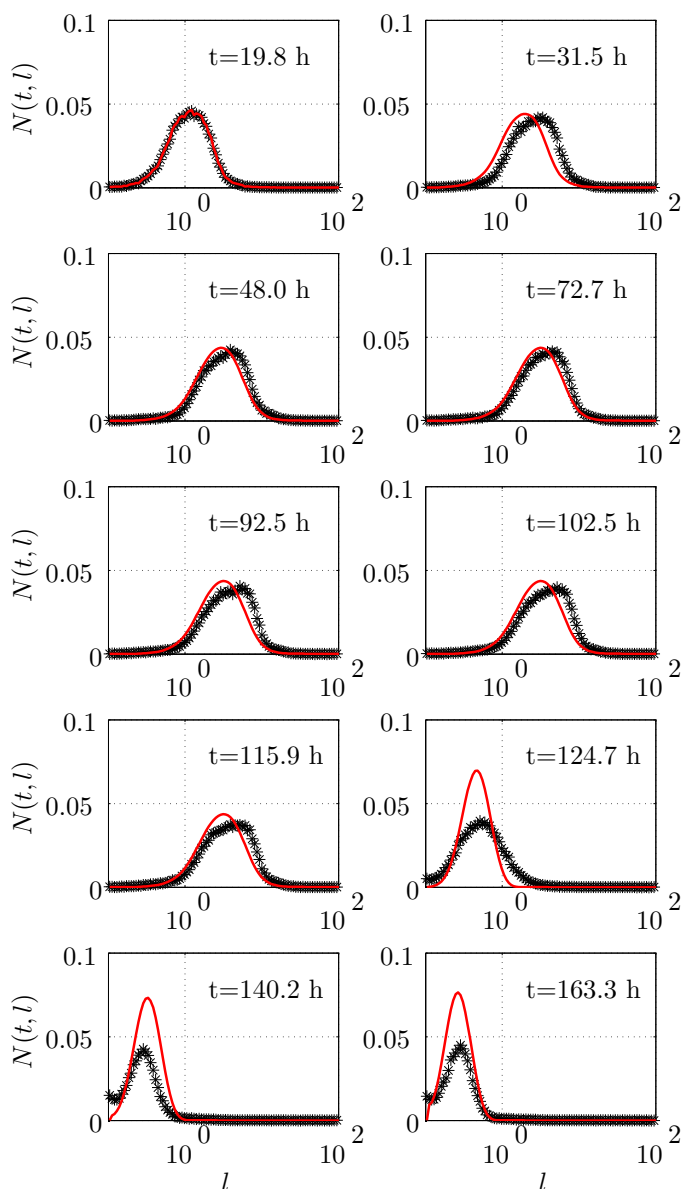


Fig. 4. Simulation results (red line) of one-dimensional population balance model and experimental FSC distribution data (black \*).

## 5. CONCLUSION AND FUTURE WORK

We have presented two population balance models for the synthesis and also metabolization of PHB in *Ralstonia eutropha*. The population balance models are coupled with the cybernetic modeling framework to capture cell internal regulation.

Since total biomass can be divided into PHB and BIO, it is obvious to formulate a two-dimensional population balance model with PHB and BIO as internal coordinates. However, experimental determination of BIO and PHB especially on population level is not trivial. Furthermore, staining is typically toxic and cells might not be usable afterwards.

Motivated by this and the observation, that cell size of *Ralstonia eutropha* correlates linear with PHB content, we

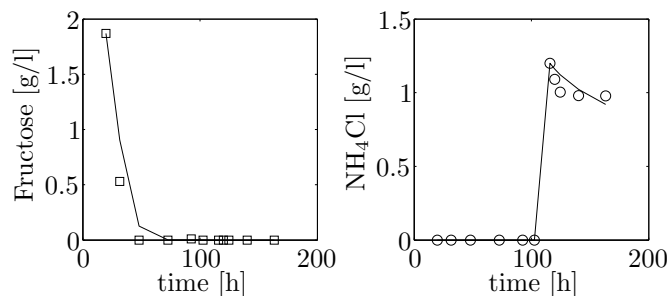


Fig. 5. Simulation results (black solid line) of one-dimensional population balance model and experimental data (Fructose:  $\square$ ,  $\text{NH}_4\text{Cl}$ :  $\circ$ ). Substrate concentration in medium at  $t = 0$  h: Fructose: 3 g/l,  $\text{NH}_4\text{Cl}$ : 0.1 g/l.  $\text{NH}_4\text{Cl}$  pulse at  $t = 116$  h:  $\approx 1.3$  g/l

could show, that staining of PHB might not be necessary and the easy to measure FSC is sufficient to describe PHB synthesis and metabolization in *Ralstonia eutropha*.

In future additional experiments with FSC data from flow cytometry are necessary to validate the one-dimensional population balance model.

## ACKNOWLEDGEMENTS

The authors appreciate the financial support of the German Federal Ministry for Education and Research (BMBF) under the FORSYS program.

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## Appendix B. ABBREVIATIONS

AMC	ammonium chloride
BIO	residual biomass, non-PHB biomass
EM	elementary mode
FRU	fructose
FSC	forward scatter
OD	optical density
PBS	phosphate buffered saline
PHB	poly( $\beta$ -hydroxybutyrate)
ROI	return on investment
TBM	total biomass (BIO+PHB)

## Appendix A. NOMENCLATURE

$b$	fraction of catalytical active parts in biomass
$c$	biomass concentration
$\mathbf{e}$	vector of relative enzyme levels
$\mathbf{f}_c$	vector of uptaken carbon units
$\mathbf{k}_e$	vector of enzyme synthesis rate constants
$\mathbf{k}_r$	vector of rate constants
$K_{AMC}$	saturation constant for ammonium chloride
$K_{FRU}$	saturation constant for fructose
$K_{PHB}$	saturation constant for PHB
$K_I$	inhibition constant
$l$	characteristic length
$l_{min}$	minimal characteristic length
$l_p$	path length
$m_{PHB}$	specific concentration of PHB
$N$	number of cells w.r.t. internal coordinates
$n$	number density of cells w.r.t. internal coordinates
$p$	partition probability density function
$\mathbf{p}$	vector of ROI
$r^{BIO}$	flux in direction of non-PHB biomass synthesis
$r^{PHB+}$	flux in direction of PHB synthesis
$r^{PHB-}$	flux in direction of PHB utilization
$\mathbf{r}_{EM}$	vector of regulated enzyme synthesis rates of EMs
$\mathbf{r}_M$	vector of regulated fluxes through EMs
$\mathbf{r}^{core}$	vector of core rates
$\mathbf{S}_{m,s}\mathbf{Z}$	stoichiometry matrix for internal metabolites
$\mathbf{S}_s\mathbf{Z}$	stoichiometry matrix for external metabolites (e.g. substrates)
$\mathbf{S}_\mu$	stoichiometry matrix for biomass
$\mathbf{u}$	vector of cybernetic variables which control enzyme synthesis
$\mathbf{v}$	vector of cybernetic variables which control enzyme activity
$x_{AMC}$	concentration of ammonium chloride
$x_{FRU}$	concentration of fructose
$\mathbf{x}_c$	state vector of biomass concentration
$\mathbf{x}_s$	state vector of substrate concentration
$\boldsymbol{\alpha}$	vector of constitutive enzyme synthesis rates
$\boldsymbol{\beta}$	vector of enzyme consumption constants
$\varepsilon$	attenuation coefficient
$\mu$	growth rate
$\Gamma$	division rate
$\Pi$	stoichiometry matrix