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Experimental and theoretical analysis of poly(β-hydroxybutyrate) formation and consumption in *Ralstonia eutropha*

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ABSTRACT

In this paper a mathematical model is presented to describe poly(β-hydroxybutyrate) (PHB) formation and consumption in *Ralstonia eutropha*. The model is based on the hybrid cybernetic modeling approach, which was introduced by Kim et al. [1] and which allows a systematic derivation of the model equations from elementary mode analysis. An extension of this approach is presented to allow for non quasi-stationary metabolites, i.e. PHB. The model is shown to be in good agreement with experimental data for PHB formation and consumption. The model is used afterwards to discuss the occurrence of multiple steady states in a continuous bio reactor. It is shown that the multiplicity region predicted by the model is rather small and it is argued that multiple steady states are therefore unlikely to occur in practice for this specific system.

Due to various desirable features such as accounting for cellular regulation at network level and dynamics of intracellular metabolites with a moderate complexity, it is believed that the constructed model is most suitable for control, optimization and monitoring of industrial PHB production processes.

1. Introduction

Poly(β-hydroxybutyrate) (PHB) is an organic polymer, which can be synthesized by many microorganisms and which serves as internal energy and carbon reserve material. *Ralstonia eutropha* is a well known bacterium for producing PHB [2]. It can accumulate PHB to more than 80% of its cell dry weight [3]. *R. eutropha* has been re-classified several times in the past. The history of classification includes the genera *Hydrogenomonas*, *Alcaligenes*, *Ralstonia*, *Wautersia* and recently *Cupriavidus necator*. To avoid confusion, we will use the name *Ralstonia* throughout, since it is still commonly used in the literature, including very recent articles.

Production of PHB is favored under limitation of key nutrients such as nitrogen, phosphate or oxygen. PHB belongs to the group of polyhydroxyalkanoates (PHA) and provides an attractive source of bioplastics that are biodegradable, biocompatible and do not depend on fossil resources.

PHB production in *R. eutropha* has previously been modeled by a few researchers. These models can be divided into two classes: (a) models which do not consider internal regulation, e.g. [4,5] and (b) models which do consider cell internal regulation. Internal regulation can be included into modeling by the cybernetic framework, which was introduced by Ramkrishna and co-workers [6–8]. The cybernetic approach for modeling PHB production in *R. eutropha* was used by Yoo and Kim [9]. They have used a very simple unstructured model and compared their results with unstructured non-cybernetic models of Mulchandani et al. [5] and Asenjo and Suk [4]. Their model could successfully predict PHB production, but did not include the underlying metabolic processes. Gadkar et al. [10] addressed this discrepancy and used a structured cybernetic model to develop a model predictive control for continuous PHB production. Although they considered the metabolic pathways by which the carbon and nitrogen sources are utilized these pathways are still lumped. The cybernetic model by Pinto and Immanuel [11] is also based on a very simplified metabolic network with less complexity than the model of Gadkar et al. [10] and was used for bifurcation analysis. It was shown, that the model structure admits multiple steady states in a continuous bio reactor, depending on the parameter values.

All these models are based on a more or less simplified metabolic network and either neglect PHB consumption or have fitted parameter to experimental data which do not contain any significant PHB consumption. But since PHB is an internal storage material, it is

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appropriate to consider its metabolism as a cycle of synthesis and consumption.

In the current work the PHB consumption is included into a cybernetic model and experiments were performed which show significant consumption to gain more reliable parameters. Additionally an expanded metabolic network is considered to include more internal metabolic information. The model is based on the state of the art hybrid cybernetic approach (HCM) by Kim et al. [1]. The HCM approach allows a systematic derivation of the model equations from elementary mode analysis [12]. It is based on quasi-stationarity of internal metabolites, which are eliminated from the model equations. However, this approach is not suitable for PHB production, since PHB is an internal metabolite, which has to be included explicitly into the model equations. In contrast to this, the more detailed approach by Young et al. [13] takes the dynamics of internal metabolites into account and could also handle synthesis and consumption of PHB. But this approach is computationally more expensive and requires detailed information about internal kinetics, which might be hard to obtain in particular for larger networks. To overcome these limitations this article presents a compromise between the standard HCM and the cybernetic modeling approach by Young et al. [13], by taking the dynamics of a few internal metabolites explicitly into account, while for most of the internal metabolites the quasi-steady state approximation is still applied.

It is shown that the model is in good agreement with experimental observations, where PHB formation and consumption are stimulated separately.

In industrial production usually fed batch or continuous processes are used. While fed batch processes allow for higher biomass and product yield, a continuous process can also be advantageous due to longer periods of operating time, which can reduce production costs. However, in continuous operating mode metabolic reactions will occur simultaneously, which can lead to nonlinear phenomenas, e.g. oscillations [14,15], multiple steady states [16–18], etc.

The occurrence of multiple steady states in a PHB production process with *R. eutropha* was discussed by Pinto and Immanuel [11] based on a simplified model. Following their idea, the model is therefore afterwards used to investigate the possibility of multiple steady states in a continuous bio reactor. It is shown that multiple steady states are unlikely to occur in practice for this specific system. Furthermore, the influence of PHB consumption is analyzed, which is usually of minor importance in a batch process, since there is usually sufficient carbon source available and PHB will not be consumed. But in continuous processes PHB consumption can have crucial influence, as shown in this study. Even in a fed batch process PHB consumption can be of great importance if other growth essential nutrients than carbon are fed to the fermenter and PHB consumption will be stimulated. It is therefore necessary to include PHB consumption into modeling, if a fed batch or continuous process is used.

2. Material and methods

2.1. Experimental section

2.1.1. Microorganisms and medium

The organism used throughout this study, *R. eutropha* (DSM 428, ATCC 17699, NCIB 10442) was obtained from DSMZ GmbH Braunschweig, Germany, as vacuum dried culture. The strain was cultivated with the medium given in Table 1. All chemicals were from Carl Roth GmbH (Karlsruhe, Germany).

Table 1
Used medium for cultivation.

Ingredient	Concentration
Fructose	20.0 g/L
NH_4Cl	1.50 g/L
KH_2PO_4	2.30 g/L
$Na_2HPO_4 \cdot 2\,H_2O$	2.90 g/L
$MgSO_4 \cdot 7\,H_2O$	0.50 g/L
$CaCl_2 \cdot 2\,H_2O$	0.01 g/L
$Fe(NH_4)$ citrate	0.05 g/L
Trace element solution [a]	5.00 ml/L

[a] Trace element solution (g/L): $ZnSO_4 \cdot 7\,H_2O$ 0.10, $MnCl_2 \cdot 4\,H_2O$ 0.03, H_3BO_3 0.30, $CoCl \cdot 6\,H_2O$ 0.20, $CuCl_2 \cdot 2\,H_2O$ 0.01, $NiCl_2 \cdot 6\,H_2O$ 0.02, $NaMoO_4 \cdot 2\,H_2O$ 0.03.

2.1.2. Cultivation conditions

R. eutropha was grown heterotrophically in a 7 L fermenter (Biostat C, Sartorius, BBI Systems, Melsungen, Germany) with a 5 L working volume. The temperature was kept constant at 30 ° C and the pH was automatically maintained at pH 6.8 by adding 2 M NaOH as corrective agent. The culture broth was agitated at 400 rpm and dissolved oxygen was maintained above 50 % air saturation by changing the oxygen and nitrogen flow rate mixed with an air stream. Total flow rate was 1.5 L/min.

2.1.3. Analytical procedures

Cell growth was monitored by measuring the optical density at 600 nm using a Ultrospec 500 spectrophotometer (GE Healthcare, Buckinghamshire, UK). For dilution, NaCl (0.98 % (w/v)) was used when necessary.

For the determination of the cell dry weight, 3×10 ml of the culture broth were centrifuged in pre-weighted glass tubes for 15 min at $3000 \times g$. The pellets were washed in 0.98 % NaCl and subsequently dried in a freeze-dryer (Christ, Osterode am Harz, Germany).

PHB content was measured as crotonic acid, formed by acid depolymerization of PHB according to Law and Slepecky [19]. Cell pellets, harvested by centrifugation, were dissolved in methylene chloride by rapid mixing and afterwards boiled for 10 min. After the samples were cooled down, they were centrifuged at $3000 \times g$ for 15 min and the supernatants were carefully removed and collected in glass tubes. This procedure was repeated three times. The supernatants were then evaporated and the remaining PHB-containing samples were digested in 2 ml H_2SO_4 (96 %) at 100 ° C for 30 min and subsequently diluted with concentrated H_2SO_4. UV absorbance spectra were measured with an UV–vis spectrophotometer V-560 (Jasco, Gross-Umstadt, Germany). The concentration of crotonic acid was calculated from a set of reference standards.

Ammonium chloride concentration in culture supernatants broth was measured by determining NH_3 with a VITROS DT60 II Chemistry System and VITROS NH3 MicroSlide from Ortho-Clinical Diagnostics (Neckargemünd, Germany) using the manufacturers instructions. 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.

2.2. Hybrid cybernetic model

2.2.1. Basic formulation

The state **y** of a metabolic system can be described by the vector of external biochemical species concentrations **x**, the vector of specific internal biochemical species concentrations **m** and the biomass concentration c

$$\mathbf{y} = \begin{bmatrix} \mathbf{x} \\ \mathbf{m} \\ c \end{bmatrix} \quad (1)$$