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A Model Based Analysis of Multiple Steady States in Continuous Cell Cultures

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Abstract. Steady state multiplicity in continuous cell cultures is studied by means of two different types of mathematical models. The first one is a simple cybernetic model, which accounts for intracellular regulatory effects during growth on mixed substrates in a very simplified way. In contrast to this the second model accounts for various regulatory effects in a very detailed way. Due to its simplicity an analytical approach is possible for the simple cybernetic model. Explicit formulae are derived to study the influence of the various parameters on the existence and the size of a multiplicity region. In particular, it is shown that the cybernetic model always predicts multiplicity if the fraction of the preferred substrate in the feed is sufficiently low, no matter which particular substrate or micro-organism is considered. It is further shown for growth of *E. coli* on mixed substrates of glucose-6-phosphate and glucose, that the overall behavior predicted by the cybernetic model is agreeing qualitatively quite well with the detailed model. Quantitative differences are also discussed and conclusions are drawn for future work.

1 Introduction

Metabolic regulation is of fundamental importance for understanding the dynamic behavior of biological systems. While it allows the microbial cells to survive under changing environmental conditions, it can be also the source of intricate nonlinear behavior in biotechnological production processes. As a consequence of the interconnections between metabolism, signal transduction and gene expression, complex nonlinear behavior in the form of steady state multiplicity and bistability may appear in continuous cultures. Depending on the initial conditions, different asymptotic states can be attained with completely different amount of biomass and/or internal metabolite concentrations. Hence suitable start-up strategies may be required to achieve an optimal production rate with high cell density [2]. Suitable mathematical models can contribute to a better understanding and may guide the way to suitable process conditions and operating strategies.

In the present paper, a model based analysis of steady state multiplicity in continuous cell cultures is presented for two different types of models. The first type of model is based on the cybernetic approach, which was proposed by Ramkrishna and co-workers and has been under continuous improvement (see [11] and references there). Cybernetic models are models of moderate complexity, which take into account metabolic regulation by using some cybernetic variables which control enzyme synthesis and activity in order to maximize the growth rate. Cybernetic models were used for the nonlinear analysis of bioreactors in [9, 8, 7] and [10]. In particular it was shown, that the experimental findings in [2] could be predicted theoretically with a cybernetic model [8].

The second model is a detailed mathematical model which was developed by Kremling and co workers and later extended in series of papers (see Bettenbrock et al. (2006) [1] and references therein). The final version of the detailed model used in the present contribution describes the growth of *E. coli* on a mixture of up to six carbon sources and takes into account the regulation (activity as well as gene expression) of the uptake reactions. In particular, the complete signal transduction pathway, starting from the sensory system, the carbohydrate phosphotransferase systems (PTS) to the activation of the global transcription factor Crp is included.

2 Cybernetic model

Here, as a first step, the most simple cybernetic model according to Kompala et al. [5] is considered. The differential equations for the different substrate concentrations s_i, the corresponding enzyme concentrations e_i and the biomass c read

$$\frac{ds_i}{dt} = D(s_{if} - s_i) - \frac{r_i v_i c}{Y_i} \tag{1}$$

$$\frac{de_i}{dt} = r_{ei} u_i - \left(\sum_k r_k v_k + \beta_i \right) e_i \tag{2}$$

$$\frac{dc}{dt} = \left(\sum_k r_k \nu_k - D\right) c. \tag{3}$$

Therein, s_{if} are the substrate concentrations of the feed, β_i are the degradation rate constants of the enzymes, Y_i are the yield coefficients, and D is the dilution rate.

The cybernetic variables u_i and ν_i are given by

$$u_i = \frac{r_i}{\sum_k r_k}, \qquad \nu_i = \frac{r_i}{\max_k\{r_k\}}. \tag{4}$$

The growth rates r_i and the enzyme synthesis rates r_{ei} are calculated using Monod type of kinetics

$$r_i = \frac{\mu_i e_i s_i}{k_i + s_i}, \qquad r_{ei} = \frac{\alpha_i s_i}{k_{ei} + s_i}, \tag{5}$$

where according to Kompala et al. [5] $\mu_i e_i$ replaces the traditional maximum specific growth rate μ_{imax} in the Monod kinetics to bring out the influence of the specific enzyme levels on the growth kinetics.

In the remainder, the focus is on growth on a mixture of two substrates, i.e. $i = 1,2$. The preferred substrate, i.e. the one with the larger individual growth rate, has always index '1'.

At steady state Eqs. (1)-(3) can be rewritten as

$$0 = D(s_{if} - s_i) - \frac{r_i \nu_i c}{Y_i} \tag{6}$$

$$0 = r_{ei} u_i - (D + \beta_i) e_i \tag{7}$$

$$0 = \sum_k r_k \nu_k - D. \tag{8}$$

It was shown by Namjoshi and Ramkrishna [9] that this set of equations admits multiple steady state solutions, i.e. depending on the initial conditions (startup strategy) the system can reach different asymptotic values of the state variables for the same set of final operating conditions.

Due to the non differentiability of the maximum function in Eq. (4) different combinatorial cases have to be considered [9], namely

(A) $e_1 = 0, e_2 \neq 0$,
(B) $e_1 \neq 0, e_2 = 0$,
(C-1) $e_1, e_2 \neq 0, r_1 \geq r_2$,
(C-2) $e_1, e_2 \neq 0, r_1 \leq r_2$.

Cases (A) and (B) were treated analytically in [9], whereas for cases (C-1) and (C-2) an iterative solution procedure was devised. In [10] a rigorous numerical bifurcation analysis was given to calculate the multiplicity region for cell cultures of *Klebsiella pneumonia* growing on glucose and xylose. Main bifurcation parameters were the dilution rate D and the feed composition $\gamma = s_{1f}/(s_{1f} + s_{2f})$. The overall concentration of the substrate in the feed was fixed according to $s_{1f} + s_{2f} = 1g/l$. Further, it was shown that solutions of type A and B will vanish if a more detailed model with maintenance is considered. An example with three different steady state solutions of type C is shown in Fig. 1.

In the present paper a new semi-analytical approach for cases C-1 and C-2 is presented. This provides useful insight into the steady state solution structure of the cybernetic model and reveals some surprising features of this type of model. Afterwards a quantitative comparison with a very detailed model is given.

2.1 Analytical treatment

In the first step, Eqs. (6)-(8) are reduced to a single nonlinear equation in r_1 for case C-1 and r_2 for case C-2. For simplicity, the saturation constants of the growth rate k_i and the enzyme synthesis rate k_{ei} are assumed to be equal and hence

$$r_{ei} = \frac{\alpha_i r_i}{\mu_i e_i}. \tag{9}$$

However, it should be noted that the results and procedures presented subsequently can be readily extended to the case $k_i \neq k_{ei}$.

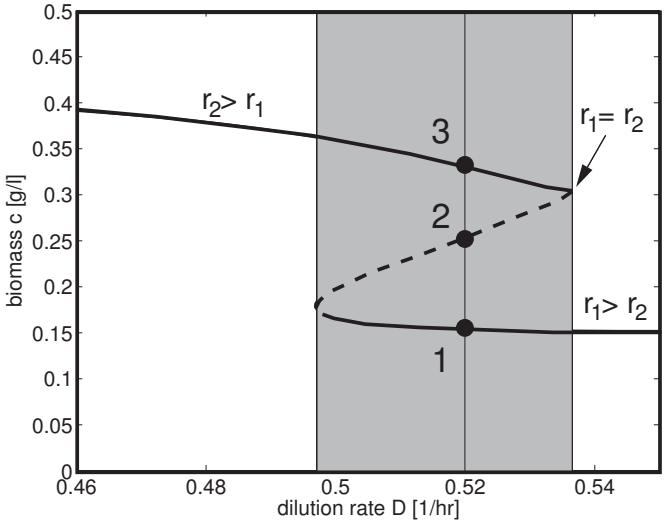

Figure 1: Steady state solutions of type C-1 and C-2 for a continuous culture of *Klebsiella pneumonia* with the parameters of [10], and $s_{1f} = 0.3, s_{2f} = 0.7$.

For $r_1 \geq r_2$ (case C-1) we have

$$\nu_1 = 1, \qquad \nu_2 = r_2/r_1. \tag{10}$$

Upon substituting this into Eq. (8) we find

$$r_2 = \sqrt{r_1(D - r_1)}. \tag{11}$$

From Eqs. (7) we find in view of Eq. (9)

$$e_i = \frac{C_i r_i}{\sqrt{r_1 + r_2}} \qquad \text{with} \quad C_i = \sqrt{\frac{\alpha_i \mu_i}{D + \beta_i}}. \tag{12}$$

Following the model formulation in [10] and using

$$\mu_i = \frac{\mu_{imax}}{e_{imax}} \qquad \text{and} \quad e_{imax} = \frac{\alpha_i}{\beta_i + \mu_{imax}} \tag{13}$$

C_i can be rewritten as

$$C_i = \sqrt{\frac{\mu_{imax}(\beta_i + \mu_{imax})}{D + \beta_i}}. \tag{14}$$

From Eqs. (5) we find in view of Eq. (12)

$$s_i = \frac{k_i \sqrt{r_1 + r_2}}{C_i - \sqrt{r_1 + r_2}}. \tag{15}$$

Eliminating the biomass c from Eqs. (6) finally gives a single equation for r_1

$$f(r_1, s_i(r_1, r_2(r_1))) := Y_1(D - r_1)(s_{1f} - s_1) - Y_2 r_1 (s_{2f} - s_2) = 0, \tag{16}$$

where the substrate concentrations have to be replaced by Eq. (15) and r_2 therein has to be replaced by Eq. (11). Note, that an analytical solution of Eq. (16) is not possible. However, all possible solutions are easily constructed graphically by intersecting the linear part of this equation

$$f_{lin} = Y_1(D - r_1)s_{1f} - Y_2 r_1 s_{2f} \tag{17}$$

with its nonlinear counterpart

$$f_{nonlin} = Y_1(D - r_1)s_1 - Y_2 r_1 s_2. \tag{18}$$

An example with two solutions of type C-1 corresponding Fig, 1 is shown in Fig. 2.1a.

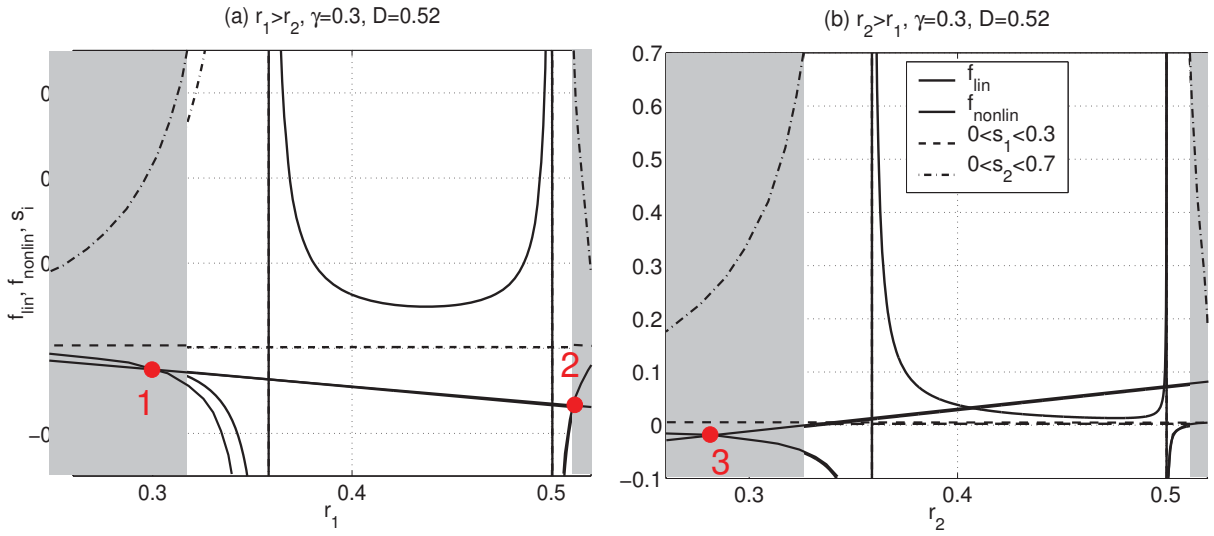

Figure 2: Graphical construction of steady state solutions for the parameters given in [10] and $D = 0.52$, $s_{1f} = 0.3$, $s_{2f} = 0.7$: (a) $r_1 \geq r_2$, (b) $r_1 \leq r_2$.

The vertical lines in Fig. 2.1a are the zeros of the denominator of the substrate concentrations in Eq. (15). For feasibility we have to require in view of Eq. (11)

$$r_1 > r_2 > 0 \quad \Leftrightarrow \quad D/2 < r_1 < D \tag{19}$$

and

$$0 < s_i < s_{if}. \tag{20}$$

The feasible range is indicated by the shaded region in Fig. 2.1a. It follows directly from the curves $s_i(r_1)$ calculated from Eqs. (15), (11) and also shown in Fig. 2.1a.

An analogous equation can be obtained for case C-2 with $r_2 \geq r_1$ as a function of r_2 by a change of the indices in Eq. (16). The corresponding graphical construction for the parameters in Fig. 1 is shown in Fig. 2.1b.

The corresponding multiplicity regions in the parameter plane of the adjustable operating parameters D and γ can be calculated numerically using a two parameter continuation for given kinetic parameters corresponding to a given substrate and a given microorganism. The results for the model organism *Klebsiella pneumonia* corresponding to Figs. 1, 2.1 are shown in Fig. 3.

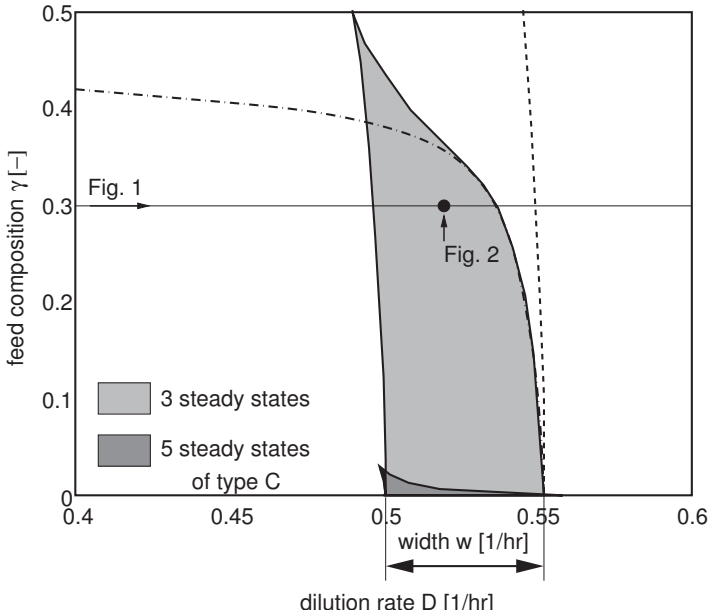

Figure 3: Multiplicity regions according to [10] corresponding to Figs. 1, 2.1. Solid lines - limit point curves, dashed dotted line - catch up point curve, dashed line - transcritical bifurcation.

	glucose	glucose-6-phosphate
μ_{imax}	0.76	0.66
$k_i = k_{ei}$	5e-03	5e-03
Y_i	0.5	0.5
α_i	1.e-03	1.e-03
β_i	0.05	0.05

Table 1: Parameters of the simple cybernetic model for growth of *E. coli* on glucose-6-phosphate and glucose.

From the practical point of view, we are especially interested in the width of the multiplicity region as illustrated in Fig. 3 and how this is influenced by the various other parameters of the model. In other words, we want to find out how the multiplicity region will change for different substrates and/or different microorganisms.

Due to the simplicity of the cybernetic model an analytical approach is possible. The right boundary of the multiplicity region in Fig. 3 corresponds to the so-called catch up point (see also Fig. 1) where

$$r_1 = r_2 = D/2 \qquad \text{and hence} \quad u = \sqrt{r_1 + r_2} = \sqrt{D}. \tag{21}$$

This boundary coincides with the wash out of substrate '2' for $\gamma \to 0$ (vanishing concentration of substrate '1'). The value of the dilution rate D at this point follows from Eq. (15) for substrate '2'

$$s_2 = s_{2f} = \frac{k_2 u}{C_2 - u} \tag{22}$$

which is approximately $\mathbf{D = \mu_{2max}}$ if the enzyme degradation rate constant β_2 is small in Eq. (14).

The left boundary is the turning point for $r_1 \geq r_2$ (see also Fig. 1), which follows from Eq. (16) and the corresponding singularity condition according to

$$f(r_1, s_i(r_1, r_2(r_1))) = \frac{df}{dr_1}(r_1, s_i(r_1, r_2(r_1))) = 0 \tag{23}$$

For $\gamma \to 0$ we have $s_{1f}, s_1 \to 0$ and hence $r_1 \to 0$. Since, by definition, $r_1 \geq r_2$ it follows that r_2 also has to be equal to zero and hence $s_2 = s_{2f}$. Further, we find that the derivative in Eq. (23) will vanish for $\gamma \to 0$ if and only if

$$\frac{d}{dr_1}\left(\sqrt{r_1 + r_2(r_1)}\right) = 0 \tag{24}$$

which yields

$$u = \sqrt{r_1 + r_2(r_1)} = \sqrt{\frac{1+\sqrt{2}}{2} D} \approx 1.1\sqrt{D} \tag{25}$$

With this, the value of the dilution rate D at the critical point follows again from Eq. (22). It is approximately $\mathbf{D = \mu_{2max}/1.1}$, if the enzyme degradation rate constant β_2 is small.

In summary we find, that multiplicity is predicted by the simple cybernetic model for any substrate combination and any microorganism. The width of the multiplicity region close to $\gamma = 0$ is always about 10 % of the maximum growth rate of the less preferred substrate '2'. This result was checked numerically for various parameter combinations.

Remark: This calculation refers to the light shaded region in Fig. 3 with three different steady state solutions of type C. Additional solutions and singularities arise for the dark shaded region for $r_2 \geq r_1$ and $s_2 \neq s_{2f}$. These are mainly due to single substrate effects and are therefore of minor interest for the mixed substrate case considered here.

In view of the subsequent discussion, a second example for the growth of *E. coli* on mixed substrates of glucose-6-phosphate (preferred substrate) and glucose is illustrated in Fig. 4. The parameters of the cybernetic model for this case are summarized in Table 1. The yield coefficients were taken from the detailed model to be described subsequently. For the α's and β's some standard values were applied, since these parameters have not much effect on the biomass and the substrate concentrations but only on the relative enzyme levels. The growth rate constant were fitted to experimental data from batch experiments.

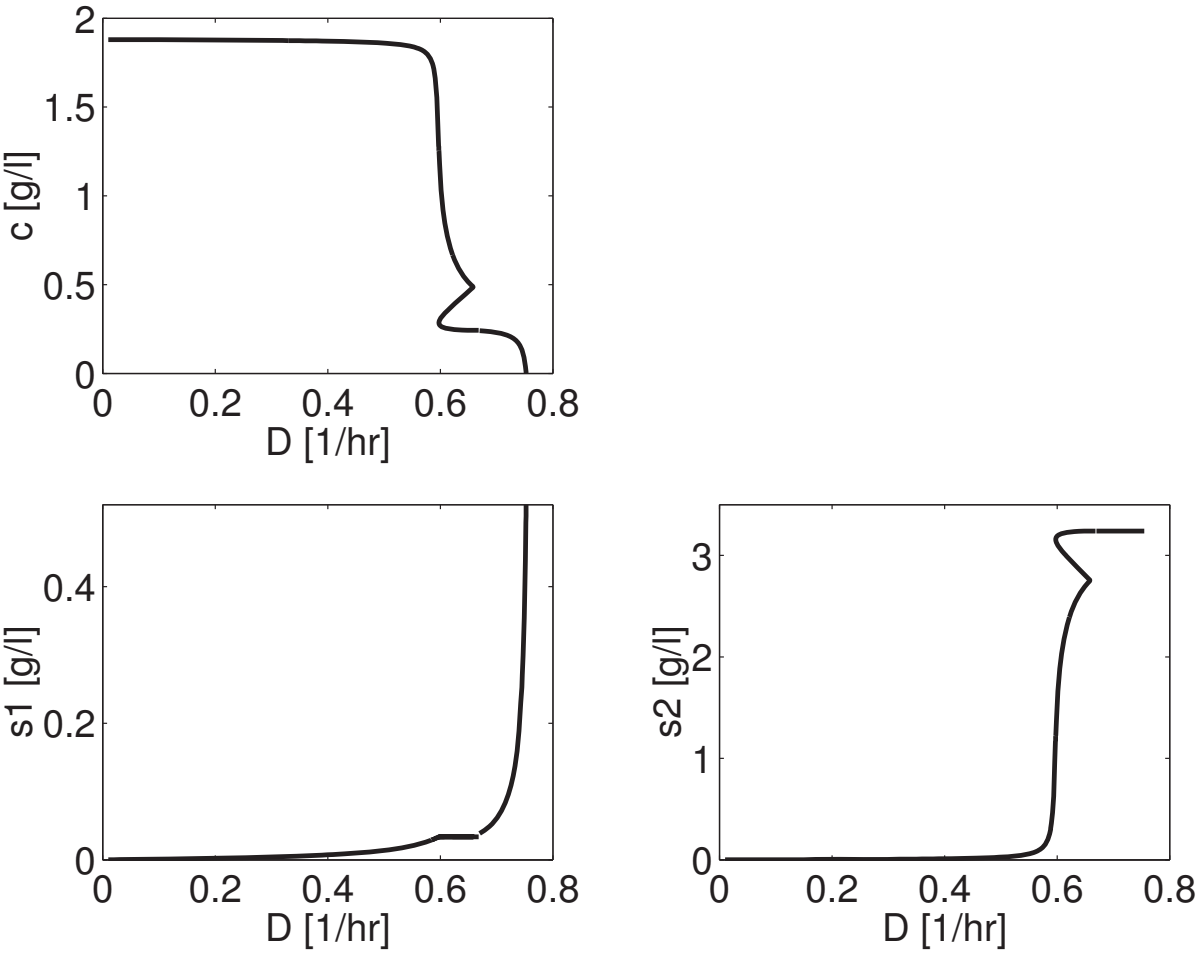

Figure 4: Bifurcation diagram for a continuous culture of *E. coli* growing on glucose-6-phosphate (preferred substrate '1') and glucose (substrate '2') with feed concentrations $s_{1f} = 0.52$ g/l, $s_{2f} = 3.24$ g/l. Results for the simple cybernetic model with parameters from Table 1.

3 Detailed model

Next, a very detailed model for the growth of *E. coli* on mixed substrates, which was introduced in [1] is discussed. The original model describes uptake, metabolism, gene expression and signal transduction of *E. coli* during growth on five carbohydrates (glucose, lactose, glycerol, galactose, sucrose), see Figure 5. For the present paper it has been extended to also account for glucose-6-phosphate substrate in the medium. The model is structured in such a way that pathways well known from biochemical text books are represented as modules: Glycolysis represents the central pathway where all the individual carbohydrates enter. The fluxes from the glycolysis into the TCA and to the monomers are lumped into a single reaction.

The pathways for the individual carbohydrates include also a description of protein synthesis. Gene expression is considered on different levels. The individual modules include a description of the specific control by a repressor ("regulon level"). In general, repressors like LacI have a small number of binding sites on the genome. Furthermore, all pathways considered in the model are under control of the global transcription factor Crp ("modulon level"). To calculate the influence of the two transcription factors, a method introduced in [6] was applied. The method defines a hierarchy where the signal is transduced from the upper level, here the modulon level to the regulon level, but not vice versa.

The signal transduction pathway leading to the activation of Crp represents a further module of the system. It consists of the phosphotransferase system (PTS), the synthesis of cAMP by adenylate cyclase (enzyme Cya) and the binding of cAMP to Crp. Cya as well as Crp are under control of the cAMP·Crp complex as well. The PTS is a sensory and an uptake system at the same time. It consists of two common cytoplasmatic proteins, EI (enzymeI) and HPr (histidine containing protein), as well as of an array of carbohydrate-specific EII (enzymeII) complexes. The PTS is connected to glycolysis via PEP and pyruvate. Since all components of the PTS, depending on their phosphorylation status, can interact with various key regulator proteins, the output of the PTS is represented by the degree of phosphorylation of the proteins. Protein EIIA in its phosphorylated state is able to activate Cya and in this way, cAMP is synthesized.

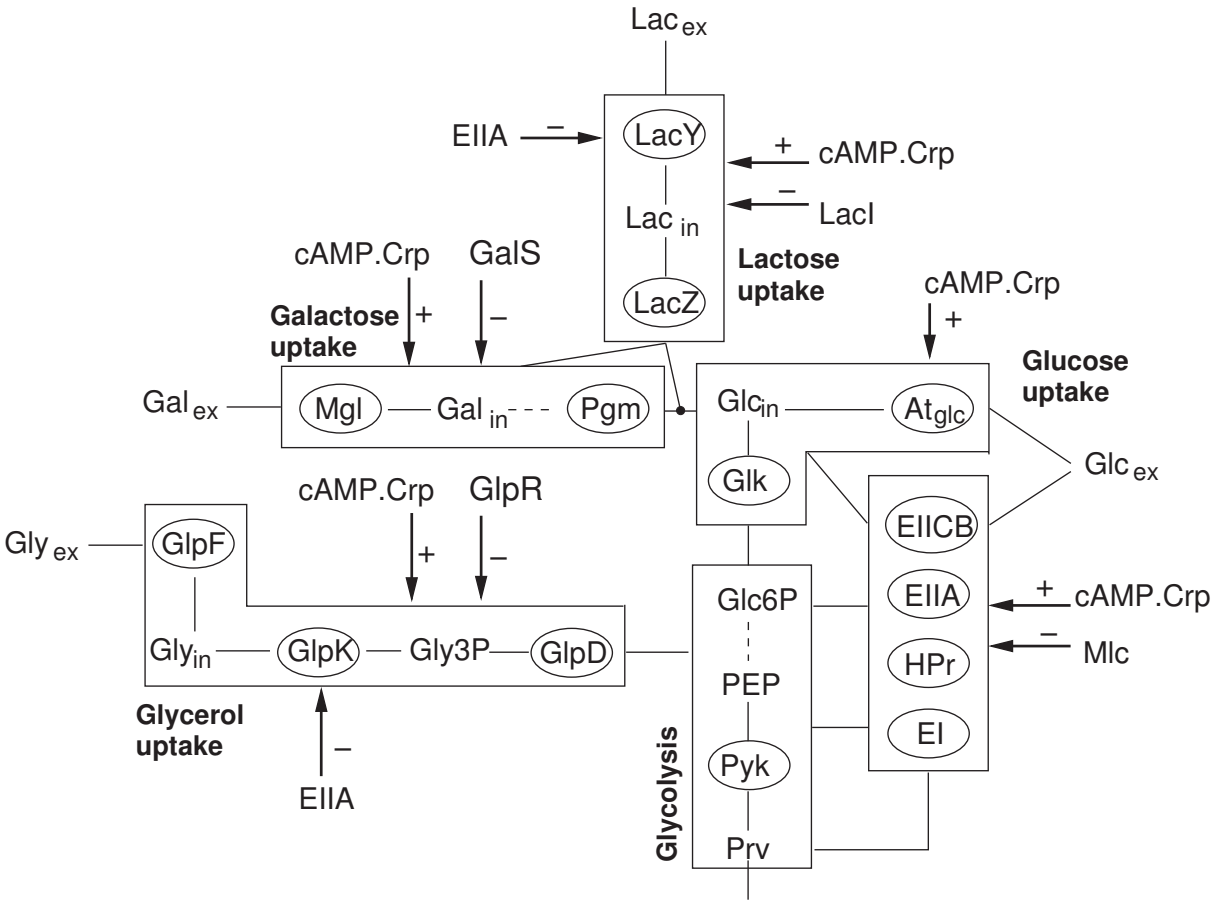

Figure 5: Rough scheme of the Bettenbrock model. Shown are the individual uptake systems for lactose, glucose, glycerol and galactose.

To simulate the dynamics of cellular systems it is desirable to determine kinetic parameters from experimental data. Since in most cases a direct measurement is not possible, the parameters are estimated during a parameter identification procedure [1]. This comprises the check of identifiability and the estimation of the parameters. With the Bettenbrock model [1], kinetic parameters for a detailed dynamic model of carbohydrate uptake were estimated. Model predictions were verified by measuring time courses of several extra- and intracellular components such as glycolytic intermediates (in a pulse experiment), EIIA phosphorylation level, both LacZ and PtsG concentrations and total cAMP concentrations under various growth conditions. The entire database consists of 18 experiments performed with 9 different strains (wild type and mutant strains). The model describes expression of 17 key enzymes, 38 enzymatic reactions and the dynamic behavior of more than 50 metabolites. Based on the experiments and with help of the ProMoT/Diva environment [3] with highly sophisticated methods for sensitivity analysis, parameter analysis and parameter estimation, 50 parameters (34 %) could be estimated. Especially the analysis of mutant strains offers the possibility to check if the control structures are reproduced well. In addition, pulse experiments, “disturbed” batch experiments and continuous culture allow to determine and analyze the dynamics in different time windows.

For comparison with the simple cybernetic model, a bifurcation diagram was calculated for the growth on a mixture of glucose-6-phosphate and glucose for the conditions corresponding to Fig. 4 from the previous section. The result is shown in Fig. 6. It turned out that a direct continuation of steady state solutions was not possible with the available algorithms due to numerical problems that arise from the various scales included in this model. Instead, dynamic simulation was used to trace out the branches of stable steady state solutions by changing the dilution rate step by step. The black curve with plus signs corresponds to a step by step increase of the dilution rate. The blue curve with circles corresponds to a step by step decrease of the dilution rate. In the figures sharp transitions occur from the low biomass production branch to the high biomass production branch with strong hysteresis. This behavior is qualitatively very similar to the behavior predicted by the simple cybernetic model shown in Fig. 4. However, the range of hysteresis predicted by the detailed model is much larger: about 50 % of μ_{2max} compared to about 10 % of μ_{2max} for the simple cybernetic model. The intermediate unstable steady states represent the separatrix between the domains of attraction of the stable steady states. They, however, could not be calculated for the detailed model with the computational procedure applied in Fig. 6.

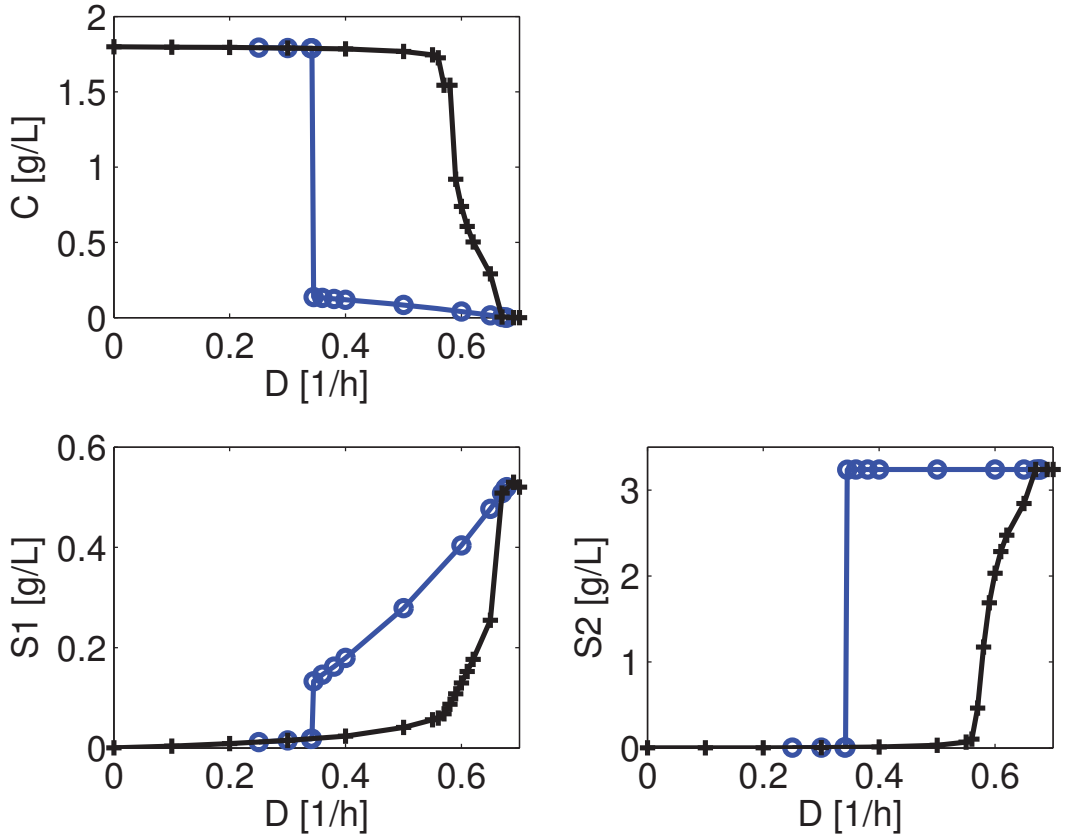

Figure 6: Bifurcation diagram for a continuous culture of *E. coli* growing on a mixed substrate of glucose-6-phosphate (preferred substrate '1') and glucose (substrate '2') with feed concentrations $s_{1f} = 0.52$ g/l, $s_{2f} = 3.24$ g/l. Results for the detailed model.

4 Conclusions

In this paper a model based analysis of steady state multiplicity in continuous cell cultures was presented for two different types of models. The first is a simple cybernetic model, which accounts for regulatory effects during growth on mixed substrates with a very simplified approach. In contrast, the second model accounts for various regulatory effects in a very detailed way. Due to its simplicity an analytical approach was possible for the cybernetic model. In particular, the extension of the multiplicity region and its dependency on the various model parameters was studied. The analysis reveals, that the cybernetic model always predicts multiplicity if the fraction of the preferred substrate in the feed is sufficiently low, no matter which particular substrate or micro-organism is considered. It was further shown that the overall behavior predicted by the cybernetic model was agreeing qualitatively quite well with the detailed model for growth of *E. coli* on mixed substrates of glucose-6-phosphate and glucose. However, the size of the multiplicity region predicted by the detailed model was much larger. To overcome this deficiency, the use of more advanced cybernetic models as proposed recently by Ramkrishna and co-workers [11, 4] is recommended.

Experimental validation of the theoretical findings is in progress.

5 Acknowledgement

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