Sensitivity Function-Based Model Reduction

A Bacterial Gene Expression Case Study

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Abstract: Mathematical models used to predict the behavior of genetically modified organisms require 1) a (rather) large number of state variables, and 2) complicated kinetic expressions containing a large number of parameters. Since these models are hardly identifiable and of limited use in model-based optimization and control strategies, a generic methodology based on sensitivity function analysis is presented to reduce the model complexity at the level of the kinetics, while maintaining high prediction power. As a case study to illustrate the method and results obtained, the influence of the dissolved oxygen concentration on the *cytN* gene expression in the bacterium *Azospirillum brasilense* Sp7 is modeled. As a first modeling approach, available mechanistic knowledge is incorporated into a mass balance equation model with 3 states and 14 parameters. The large differences in order of magnitude of the model parameters identified on the available experimental data indicate 1) possible structural problems in the kinetic model and, associated with this, 2) a possibly too high number of model parameters. A careful sensitivity function analysis reveals that a reduced model with only seven parameters is almost as accurate as the original model. © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* **80**: 195–200, 2002.

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INTRODUCTION

To qualify and quantify the influence of external signals on bacterial gene expression, continuous culture steady-state experiments have been performed throughout the past (Chao et al., 1997; Kasimoglu et al., 1996). These costly, labor-intensive and time-consuming experiments can be reduced to a minimum with the aid of a mathematical model that describes the intrinsic properties of the dynamic bioprocess.

Although the advantages of model-based optimization and control of fermentations (e.g., baker's yeast production processes and biological wastewater treatment systems) are well established, the introduction of mathematical modeling in the field of genetic engineering is fairly recent. The scarce, knowledge-based models that have been developed are usually characterized by complex kinetic expressions involving a large number of parameters.

In this article it is illustrated that sensitivity function analysis is a powerful tool to reduce the complexity of a knowledge-based model. To date, most reported applications of parametric sensitivity analysis are concerned with design and optimal operation of chemical systems (see, e.g., Varma et al., 1999). Also optimal experimental design techniques aimed at obtaining experimental data with a high information content to facilitate parameter identification, rely on sensitivity functions (see, e.g., Bernaerts et al., 2000, in the field of predictive microbiology). To the authors' knowledge, sensitivity function-based methodologies have, however, not yet found widespread appeal in modeling and analysis of biological systems. One exception is the article by Pertev et al. (1997) in the context of modeling the (kinetics) of baker's

yeast metabolism. The bottleneck hypothesis, introduced by Sonnleitner and Käppeli (1986), is incorporated in a primary model. This hypothesis assumes a limited oxygen capacity of yeast, leading to formation of ethanol under conditions of oxygen limitation and/or an excessive glucose concentration. The influence of various parameters on model behavior is determined by sensitivity analysis and inspired by these results only those parameters that strongly affect the biomass production are identified by a parameter estimation technique.

In this article, we adopt a similar approach which will be formalized as a systematic and generic tool to find the least number of parameters required to accurately reduce a multiparameter model. As a vehicle to present the model reduction methodology and the results obtained, a bacterial gene expression case study is considered in which the influence of dissolved oxygen concentration on the expression of the *cytN* gene in *Azospirillum brasilense* Sp7 is modeled. The latter organism's capability of producing poly-3-hydroxybutyrate (PHB) and indole acetic acid clearly justifies the present efforts in unraveling its metabolism. In this respect, the *A. brasilense cytNOQP* operon, encoding a cytochrome cbb_3 terminal oxidase, has been shown to be involved in microaerobic growth and respiration (Marchal et al., 1998). Model-based analysis can indicate the optimal *DO* level for expression of the *cytNOQP* genes. The genetic engineering details, which are beyond the scope of this contribution, are elaborated by Sun et al. (2001).

MATERIALS AND METHODS

Organism Description and Fermentation Strategy

Since the expression of the *cytN* gene cannot be screened directly, a *gusA* reporter gene system, of which the β-glucuronidase activity can be readily monitored, was constructed and integrated in plasmid pFAJ873, details of which can be found in Sun et al. (2001). *A. brasilense* Sp7 containing plasmid pFAJ873 was cultivated in a 2-liter *DO*-stat fermenter in batch mode until the end of the exponential growth phase was reached (after approximately 13 h). Subsequently, a continuous fermentation started in the same fermenter. At regular intervals of about 1.5 h, consecutive small dissolved oxygen shifts were applied before reaching a new steady state. Samples were taken just before each *DO*-shift to determine the β-glucuronidase activity and cell density. A typical dissolved oxygen and dilution rate profile is shown in Figure 1A. During the continuous fermentation, the carbon source malate was assigned as the limiting growth factor.

Analytical Procedures

Quantitative β-glucuronidase activity (GUS activity) was measured as described in Vande Broek et al. (1992)

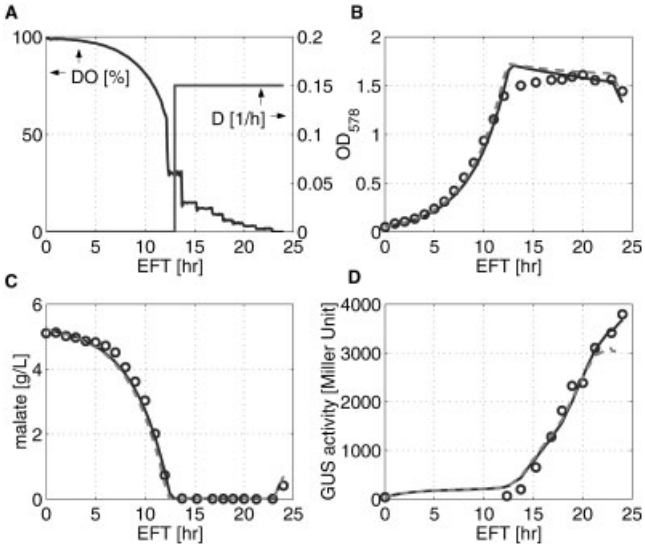

Figure 1. Effect of *DO* concentration on fusion protein expression in nonsteady-state continuous operation: performance of primary model vs. reduced model. **A:** The profile of on-line measured values of *DO* and the applied dilution rate profile *D*. In **B**, **C**, and **D**, open symbols: experimental data; solid lines: simulation primary model (calibration); dashed lines: simulation reduced model. **B:** Evolution of biomass concentration with respect to elapsed fermentation time EFT. **C:** Evolution of malate concentration with respect to elapsed fermentation time EFT. **D:** Evolution of GUS activity (reflecting the amount of fusion protein) with respect to elapsed fermentation time EFT.

and expressed in Miller units (Miller, 1972), but calculated per hour instead of per minute. Cell growth was monitored by measuring the optical density at 578 nm wavelength with a Perkin-Lambda 2 UV-spectrum spectrophotometer. L-malate concentration in the culture broth was determined with the test kit from Boehringer Mannheim (Mannheim, Germany). All the data shown in this article are average values of at least two replicates.

PRIMARY MODEL DEVELOPMENT

Model Description

Changes in β-glucuronidase activity of a hybrid gene reporter in the presence of an altering external signal indicate that this external signal initiates transcriptional activation. However, in the experimental set-up under study, not only alterations in transcriptional activation of the plasmid encoding *cytN-gusA* fusion induced by the *DO*-shifts, but also accumulation and turnover of the fusion protein (i.e., the *cytN-gusA* gene product, which is a cytoplasmic protein) accounts for the measured β-glucuronidase activity. Therefore, only the *specific* expression rate of the fusion protein (i.e., the amount of fusion proteins expressed per cell and per hour), being independent of the experimental design, can reflect the influence of dissolved oxygen on the

expression of the target gene. In order to derive the specific expression rate of the fusion protein from the measured β-glucuronidase activity, the following general dynamic mathematical model based on mass balances was applied (Van Impe and Bastin, 1995):

$$\frac{dX}{dt} = \mu X - DX \tag{1}$$

$$\frac{dS}{dt} = -\sigma X - DS + DS_{in} \tag{2}$$

$$\frac{dP}{dt} = \pi X - DP - kP \tag{3}$$

where X (g cells/L) denotes the concentration of the biomass, S (g malate/L) is the concentration of the carbon source, and P (g protein/L) is the concentration of the fusion protein. D (1/h) is the dilution rate, μ (1/h) is the specific growth rate of cells, σ (g malate/g cells/h) is the specific consumption rate of the carbon source, π (g protein/g cells/h) is the specific expression rate of the fusion protein, and k (1/h) is the in vivo degradation rate of the fusion protein. According to the definition of GUS activity (Miller, 1972), the value of β-glucuronidase activity is assumed to be proportional to the amount of the fusion protein per cell:

$$P = \alpha UX \tag{4}$$

where U [Miller Unit] is the β-glucuronidase activity [Miller Unit stands for GUS enzyme activity/g cells/h] and α (g protein/g cells/Miller Unit) is a proportionality constant. By combining Equation (4) with Equations (1) and (3), the following equation can be deduced:

$$\frac{dU}{dt} = \beta - \mu U - kU \tag{5}$$

where β equals π/α [Miller Unit/h] and is defined as the apparent specific expression rate of the fusion protein, reflecting the direct influence of the external signal on the transcriptional activation of the hybrid gene fusion.

To complete the model, the following kinetic expressions are proposed, inspired by available knowledge.

$$\mu = \mu_{\max} \frac{S}{(KM_{XS} + S + \frac{S^2}{KI_{XS}})} \frac{DO}{(KM_{XG} + DO + \frac{DO^2}{KI_{XG}})} \tag{6}$$

$$\beta = \beta_{\max} \frac{S}{(KM_{PS} + S)} \frac{DO + KB_{PG}}{(KM_{PG} + DO + DO^2/KI_{PG})} \tag{7}$$

$$k = k_{\max} \frac{DO}{K_k + DO} \tag{8}$$

$$\sigma = \frac{\mu}{Y_{XS}} + \frac{\beta}{Y_{US}} \tag{9}$$

A double Haldane type model (Eq. [6]) is applied to describe the specific growth rate of cells, μ, as a function of two substrates: malate S and dissolved oxygen DO (%). KM_{XS} (g malate/L)] and KI_{XS} (g malate/L) are the saturation constant and inhibition constant of malate to cell growth, respectively, while KM_{XG} (%) and KI_{XG} (%) are the saturation constant and inhibition constant of DO to cell growth, respectively. $\mu_{\max}$ (1/h) represents the maximum growth rate of cells. The apparent specific expression rate of the fusion protein, β (Eq. [7]), is described as function of the carbon substrate malate with a Monod type model and as function of dissolved oxygen with a Haldane-like model. KM_{PS} (g malate/L) is the saturation constant of malate to the fusion protein expression, while KM_{PG} (%) and KI_{PG} (%) are the saturation constant and inhibition constant of DO to the fusion protein expression, respectively. KB_{PG} (%) was introduced into the model since constitutive back ground expression of the fusion protein has been observed under early anaerobic conditions during preliminary experiments. $\beta_{\max}$ (Miller Unit/h) reflects the maximum apparent expression rate of the fusion protein. Since the dependency of the degradation of the fusion protein on DO has been observed in the experiments carried out in test tubes (data not shown), the degradation rate k of the fusion protein (Eq. [8]) is expressed as function of DO in the frame of the Monod model. K_k (%) is the saturation constant of DO to decay of the fusion protein and $k_{\max}$ [1/h] represents the maximum degradation rate of the fusion protein. The correlation between the three specific reaction rates is expressed by the specific substrate consumption rate σ (Eq. [9]) via the yield coefficients Y_{XS} (biomass with respect to substrate) and Y_{US} (GUS activity with respect to substrate).

In order to assess the influence of DO on the specific expression level of the *cytN-gusA* fusion, the measured experimental values were fed to the model to identify the appropriate parameters. Once identified, the complete model (parameters and model structure) will be used to predict the behavior of the hybrid fusion protein as a function of the external variables.

Model Calibration

The profile of the dissolved oxygen concentration for the parameter identification experiment is presented in Figure 1A. The 14 parameters in the mathematical model were identified by minimizing the following cost function $\mathcal{J}$:

$$\mathcal{J} = \sum_{j=1}^{n} \frac{\sum_{i=1}^{m} \left(\frac{Y_{s,ij} - Y_{e,ij}}{Y_{e,j}} \right)^2}{\sigma_{sj}^2} \tag{10}$$

where i denotes the sampling time and j denotes the components. $Y_{s,ij}$ is the data set of the simulation results, $Y_{e,ij}$ is the dataset of the experimental results, and $\tilde{Y}_{e,j}$ is

Table I. Parameter values of the primary model.

p_{01}	Y_{XS}	0.3196	(OD_{578}/g malate)
p_{02}	Y_{US}	2.7957×10^4	(Miller Unit/g cells)
p_{03}	μ_{max}	0.2766	(1/hr)
p_{04}	β_{max}	9.3150×10^3	(Miller Unit/hr)
p_{05}	k_{max}	0.1194	(1/hr)
p_{06}	KM_{XS}	1.2531×10^{-2}	(g malate/L)
p_{07}	KI_{XS}	1.0483×10^3	(g malate/L)
p_{08}	KM_{XG}	5.2341×10^{-2}	(%)
p_{09}	KI_{XG}	1.0219×10^4	(%)
p_{10}	KM_{PS}	1.2056×10^{-3}	(g malate/L)
p_{11}	KB_{PG}	0.2766	(%)
p_{12}	KM_{PG}	11.6485	(%)
p_{13}	KI_{PG}	0.8135	(%)
p_{14}	K_k	0.8941	(%)

the average value of the components. σ_{sj} is the standard deviation of the experimental data. The continuous fermentation started at 13 h with a dilution rate of 0.15 1/h. The identified initial values (t = 0) are: S_0 = 5.2087 g malate/L; X_0 = 0.0554 OD_{578}, and U_0 = 41.9123 Miller Unit. The carbon source concentration in the feed flow is equal to S_{in} = 5.0075 g malate/L. The results are shown in Figure 1B–D (solid lines), while the values of the identified parameters are summarized in Table I. Because cell density rather than dry weight of cells was used to monitor the cell growth, the yield coefficient Y_{XS} as defined in Equation [9] is expressed in [OD_{578}/g malate] instead of [g cells/g malate]. The agreement between the simulation results of this primary model and the experimental data is remarkable.

Model Validation

In order to further validate the applicable range of the model, a continuous fermentation with a totally different *DO* profile (Fig. 2A) was performed. The *DO* concentration was kept at 10% during the batch fermentation and subsequently shifted from low to high values during the continuous fermentation. The continuous fermentation started at 12 h with a dilution rate of 0.1193 1/h. The initial values for validation are based on the first experimental measurements: S_0 = 5.3812 g malate/L; X_0 = 0.038 OD_{578}; U_0 = 126.84 Miller Unit. For the carbon source concentration in the feed flow, the measured value was used: S_{in} = 5.308 g malate/L. The simulation results (solid line) and the experimental data (open symbols) are shown in Figure 2B–D. The agreement between simulated and experimental results corroborates the generality of the model.

RESULTS AND DISCUSSION

Sensitivity functions

Sensitivity functions reflect the sensitivity of the system outputs (denoted by y_i) to (small) variations in the model parameters (denoted by p_j), given certain inputs (denoted by u_k). In this study the dilution rate *D* and the dissolved oxygen signal *DO* are defined as the system inputs u_1 and u_2, respectively, and the biomass concentration *X*, the malate concentration *S*, and the GUS activity *U* are defined as the system outputs y_1, y_2, and y_3, respectively. The parameters are denoted p_j with *j* ranging from 1–14 (see also Table I). The time evolution of the 3×14 sensitivity functions $\frac{\partial y_i}{\partial p_j}(t)$ is then computed as follows:

$$\frac{d}{dt}\left(\frac{\partial y_i}{\partial p_j}\right) = \frac{\partial}{\partial p_j}\left(\frac{dy_i}{dt}\right) \quad \text{for } i = 1 \text{ to } 3 \text{ and } j = 1 \text{ to } 4$$

with $\frac{dy_i}{dt}$ represented by Equations [1], [2], and [5] in which the kinetic expression for μ, β, *k*, and σ are as proposed in Equations [6]–[9]. The initial sensitivity of the system (i.e., $(\partial y_i/\partial p_j)$ (t = 0)) is set equal to zero.

The simulation and parameter identification of the model described above and the calculation of the sensitivity functions were performed using Matlab 5.3 (MathWorks, Natick, MA) on a Linux platform.

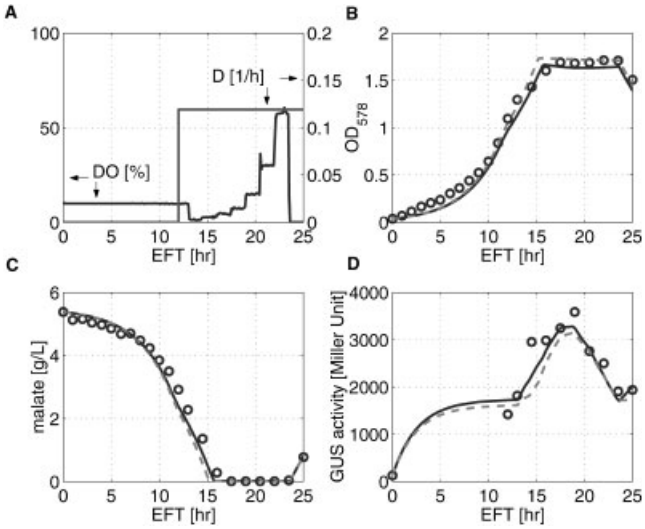

Figure 2. Effect of *DO* concentration on fusion protein expression in nonsteady-state continuous operation: performance of primary model vs. reduced model. **A:** the profile of on-line measured values of *DO* and the applied dilution rate profile *D*. In **B**, **C**, and **D**, open symbols: experimental data; solid lines: simulation general model (validation); dashed lines: simulation reduced model. **B:** Evolution of biomass concentration with respect to elapsed fermentation time EFT. **C:** Evolution of malate concentration with respect to elapsed fermentation time EFT. **D:** Evolution of GUS activity (reflecting the amount of fusion protein) with respect to elapsed fermentation time EFT.

Sensitivity Function-Based Model Reduction

Given the high number of parameters to be identified and the large range in order of magnitude, a legitimate question to ask is whether a similar high-quality fit of the experimental data can be obtained with a simplified model including fewer parameters. In order to mathe-

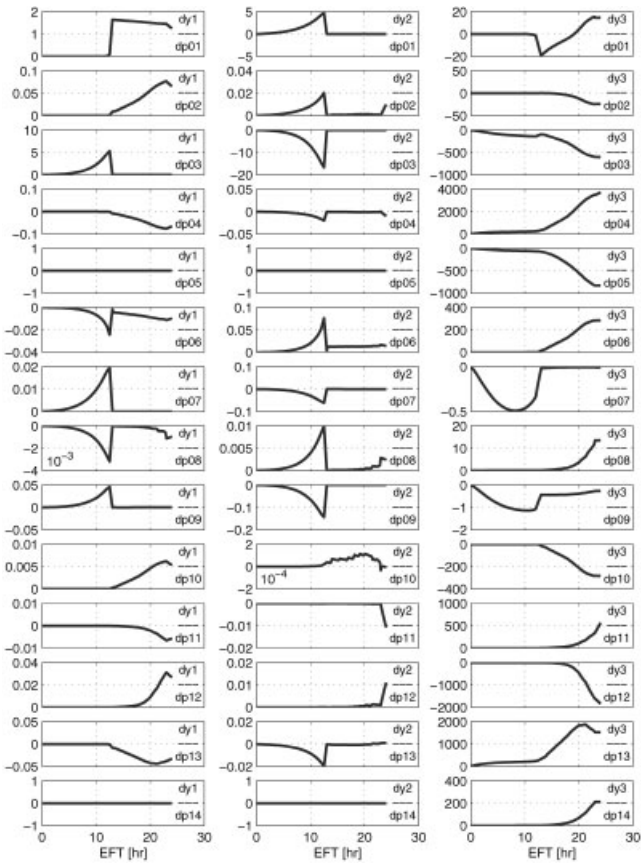

Figure 3. Evolution of the 3 × 14 (rescaled) sensitivity functions with respect to elapsed fermentation time EFT. Left column: sensitivity of biomass concentration (i.e., output y_1), towards changes in the 14 parameters. Middle column: sensitivity of malate concentration (i.e., output y_2), towards changes in the 14 parameters. Right column: sensitivity of GUS activity (i.e., output y_3), towards changes in the 14 parameters.

matically investigate this statement, a thorough sensitivity analysis was performed. As mentioned above, sensitivity functions will reveal the sensitivity of each output y_i to (small) variations in each model parameter p_j. To allow for a proper comparison between all sensitivities related to a specific output y_i, each sensitivity function is rescaled by multiplying with the parameter value under study resulting in *semirelative* sensitivity functions (see Fig. 3) The system (or at least one of the outputs) is said to be sensitive to a certain parameter if a change in the parameter's value significantly affects the predictive quality of the model. In other words, the fit of the experimental data becomes worse if that parameter value is changed, and the parameter can, accordingly, be classified as *essential*. Hence, how can the *essential* parameters be selected on the basis of the sensitivity functions plotted in Figure 3? Hereto, each column of subplots, i.e., the sensitivity of one output with respect to all 14 parameters, is to be considered separately and the different orders of magnitude have to be compared. As mentioned before, due to the rescaling this comparison is justified. If the order of magnitude of a certain sensitivity function is substantially larger than the average order of magnitude, then the corresponding parameter is retained. Following this line of reasoning parameters p_{01} and p_{03} i.e., Y_{XS} and μ_{max}, respectively, are considered essential for the biomass concentration output y_1, since the order of magnitude ($\mathcal{O}(10^1)$) is significantly larger than the average value of $\mathcal{O}(10^{-2})$. As for the malate concentration output y_2, the same parameters are selected. Finally, with respect to the GUS activity output y_3, following parameters with $\mathcal{O}(10^3)$ (compared to $\mathcal{O}(10^1)$ or $\mathcal{O}(10^2)$) will be retained: p_{04}, p_{05}, p_{12}, and p_{13}, i.e., β_{max}, k_{max}, KM_{PG}, and KI_{PG}, respectively.

Based on the sensitivity analysis and given the experimental data, only six parameters have to be retained, which, however, does not imply that the remaining eight parameters can be omitted without further investigation. As for the inhibition constants (KI_{XS} and KI_{XG}), the yield coefficient Y_{XS}, and the background expression KB_{PG}, they all can be neglected without any loss of accuracy, the former by setting the value equal to infinity whereas the latter is set equal to zero. Note, however, that, from a mechanistic point of view it cannot be claimed that, e.g., the growth of this species is *not* inhibited by high substrate or dissolved oxygen concentration. It is merely concluded that an inhibition effect cannot be inferred from the available experimental data. Likewise, the yield coefficient Y_{US} is assumed to be infinitely large as to reflect the negligible contribution of the product formation to the substrate consumption rate σ. As for the saturation constants, according to the definition they have to ensure a switch from the maximum to a lower specific rate when the concentration of malate or *DO* drops to low values. Therefore, each of the Monod-like expressions is tested separately to see whether loss of the switching characteristic by setting the saturation constant equal to zero affects the prediction quality based on the available data. Following this strategy, the Monod-like dependence of the specific apparent expression rate of the fusion protein β_{max} on the malate concentration is clearly of negligible importance as compared to the influence of *DO* concentration. Therefore, KM_{PS} can be omitted. The remaining saturation constants, i.e., KM_{XS}, KM_{XG}, KM_{PS}, do have an effect and are set equal to a small positive nonzero constant ϵ, which has to be estimated. Hence, the total number of model parameters has been reduced to seven and the reduced kinetic expressions of the above introduced primary model can be written as follows.

$$\mu = \mu_{max} \frac{S}{\epsilon + S} \cdot \frac{DO}{\epsilon + DO} \tag{11}$$

$$\beta = \beta_{max} \frac{DO}{KM_{PG} + DO + DO^2/KI_{PG}} \tag{12}$$

Table II. Parameter values of the reduced model.

p_{01}	Y_{XS}	0.3196	(OD_{578}/g malate)
p_{03}	μ_{max}	0.2766	(1/hr)
p_{04}	β_{max}	9.3150×10^3	(Miller Unit/hr)
p_{05}	k_{max}	0.1194	(1/hr)
p_{12}	KM_{PG}	11.6485	(%)
p_{13}	KI_{PG}	0.8135	(%)
	ϵ	0.0062	(g malate/L) or (%)

$$k = k_{max} \frac{DO}{\epsilon + DO} \tag{13}$$

$$\sigma = \frac{\mu}{Y_{XS}} \tag{14}$$

Figures 1 and 2 illustrate that the descriptive quality of this simplified model (dashed line) is as good as the descriptive quality of the original 14 parameter model (full line). A value of 0.0062 has been identified for ϵ. Observe from Table II that, apart from the ε value, there was apparently no need to reoptimize the parameters.

Further, in the simplified model the background GUS activity (represented by KB_{PG}), is neglected. This explains why the simplified model predicts a stagnation instead of a persisting increase of GUS activity at the end of the experiments, i.e., under early anaerobic conditions. One might argue that, merely from inspecting the values of, for example, the inhibition parameters KI_{XS} and KI_{XG}, the negligible effect could be inferred directly. However, such *qualitative* reasoning will not reveal all *nonessential* parameters. The power of the proposed method is that a *quantitative* basis is provided which enables a straightforward classification of all parameters.

A detailed exploration of the significance of model parameters in the general as well as in the reduced model are the subject of ongoing research. Hereto, optimal experiments (complemented with parameter uncertainty analysis) will be designed to check whether the model features present in the general model, but omitted in the simplified model (e.g., inhibition of the specific growth rate at high substrate or dissolved oxygen concentrations or the presence of background gene expression in early anaerobic conditions), are truly needed or not.

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