Investigation of Bacterial Growth on Mixed Substrates: Experimental Evaluation of Cybernetic Models

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Cybernetic models, developed earlier by the authors, have been evaluated experimentally for the growth of *Klebsiella oxytoca* in batch cultures using mixed substrates from glucose, xylose, arabinose, lactose, and fructose. Based entirely on information procured from batch growth on single substrates, the models accurately predict without further parameter fitting, diauxic growth on mixed substrates, automatically predicting the order in which the substrates are consumed. Even triauxic growth on a mixture of glucose, xylose, and lactose is predicted by the model based on single substrate data. Growth on glucose–fructose mixtures appears to need a slightly modified strategy for cybernetic variables.

INTRODUCTION

In previous articles,[1–4] we have introduced the concept of a cybernetic framework for modeling the growth of microorganisms on multiple substrates. This approach differs from kinetic modeling efforts by incorporation of the optimal nature of microbial regulatory processes. It was established therein that cybernetic models can describe most of the available experimental observations of microbial growth on mixed substrates with quantitative accuracy. Thus, both batch and continuous culture data could be predicted by suitable choice of model parameters. However, the basic premise, the most important attribute of our modeling effort, that information obtained from growth on single substrate experiments on each of the substrates will yield all of the information required for predicting growth in mixed substrates had remained to be verified. This was because experimental results published in the literature pertained to different organisms, different substrate mixtures, different experimental conditions and so on. The objective of this article is to put to test the fundamental hypotheses in the model by performing systematic experiments with the same organism (*Klebsiella oxytoca*) grown on different carbon substrates both singly and in mixtures.

The diauxie phenomenon, discovered by Monod,[5] is a striking indication of the optimal nature of microbial regulatory processes. When an organism is presented with two substrates for carbon and energy source, it first consumes only that substrate which supports a faster growth rate. Only after the faster growth-supporting substrate is virtually exhausted, does it begin to synthesize the enzymes necessary for the utilization of the second substrate. This usually results in a lag period of reduced or no growth towards the end of growth on the first substrate and before growth on the second substrate reaches its maximum rate. The intermediate lag has been termed the diauxic lag period. In many cases of heterotrophic bacteria, glucose is the substrate supporting the fastest growth rate. Hence, these bacteria grow first on glucose and only after glucose gets consumed, they consume the other substrates. This has often caused the mislabeling of the diauxie phenomenon as "the glucose effect." When glucose happens to be the slower growth-supporting substrate, the organism's preference is for the faster growth-supporting substrate. The labeling of this phenomenon as "reverse diauxie" merely perpetuates the prior misinterpretation of the diauxie phenomenon as the "glucose effect." Within the framework of our cybernetic models both the "glucose effect" and "reverse diauxie" can be seen as manifestations of the same strategy which is to optimize the cellular growth rate.

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The growth of yeasts like *Saccharomyces cerevisiae* presents another clear indication of optimal nature of microbial regulatory processes. In batch cultures of yeast on glucose, the yeast prefers the faster growth-supporting fermentative pathway over the slower growth-supporting oxidative pathway for glucose assimilation. While in continuous cultures at low dilution rates, as the growth rate is held at the low values, the yeast prefers the oxidative pathway, maximizing the ATP production.

Our previous article discussed the efforts of several workers at modeling the diauxie phenomenon. All of these were designed to simulate their particular experimental observations and depended on prior knowledge of the actual order of preference made by the organism. Thus their predictive value has been limited. The cybernetic framework which we have proposed[1–4] broadly aims at describing the results of microbial regulatory processes as arising from optimal strategies. In this framework, in addition to the usual concentration variables, we incorporate *cybernetic variables* as the instruments of optimization (control variables). The modelling effort in this framework would consist in identifying the kinetics of enzyme syntheses and growth processes both incorporating the relevant concentration and cybernetic variables, and an optimality criterion which will assign optimal values for the cybernetic variables at each instant.

Based on all existing observations known to us on the behavior of microbial growth on mixed substrates we present below as *basic postulates*, certain characteristics which all models for growth in a multisubstrate environment must accommodate. For economizing on words we will loosely refer to slower (or faster) growth-supporting substrates as slower (or faster) substrates:

1) Given multiple substrates, on which growth rates are distinctly different the microbes prefer to grow on the fastest substrate with the growth curve showing multiauxial behavior.

2) More generally, the growth behavior ranges from simultaneous utilization of multiple substrates (which occurs when the growth rates are very nearly the same) to sequential utilization with intermediate lag periods.

3) The growth rate on a mixture of substrates is never greater than the maximum of the growth rates on individual substrates.

4) While growing on a slower substrate, if a faster substrate is added, the microbes inhibit the activity of the already available enzymes for the slower substrates.

While the above postulates are firm guidelines for the present, no sight must be lost of the fact that they are built only on existing observations and that further experimentation could enforce their revision. The cybernetic *framework* must derive its veracity from such revision requiring only a modification of the *model* without having to discard the framework itself.

CYBERNETIC MODEL

We have presented previously[4] a cybernetic model which satisfies the requirements listed above. Let us now recall the salient features of the model briefly. The assimilation of ith substrate S_i by the biomass, B, is assumed to be catalyzed by a key enzyme E_i, representing the whole set of enzymes catalyzing the metabolic pathways of growth on S_i:

$$B + S_i \xrightarrow{E_i} (1 + Y_i)B + \cdots \tag{1}$$

The key enzyme E_i, required for utilization of S_i, is induced in the presence of S_i according to

$$B \xrightarrow{E_i} E_i + B' \tag{2}$$

where B' is the biomass excluding the key enzyme E_i.

The rate equations for these two reaction sequences can be written as

$$r_i = \frac{\mu_i e_i s_i c}{K_i + s_i} \tag{3}$$

$$r_{E_i} = \frac{\alpha_i s_i c}{K_i' + s_i} \tag{4}$$

where c is the biomass concentration; s_i is the concentration of S_i; e_i is the specific level of E_i such that $e_i c$ is the concentration of E_i; and α_i is the enzyme synthesis rate constant; $\mu_i e_i$ replaces the traditional maximum specific growth rate $\mu_{\max,i}$ in the unstructured Monod kinetics to bring out the influence of specific enzyme levels on the growth kinetics.

When multiple substrates are present, the cellular regulatory processes of inhibition/activation and repression/induction affect rate equations (3) and (4) respectively. The actual rate of synthesis of enzyme E_i may be written as

$$r_{E_i} u_i \qquad (0 < u_i < 1;\ \Sigma_i u_i = 1) \tag{5}$$

where u_i is the fractional allocation of a critical resource for the synthesis of E_i. This fractional resource is not necessarily material in nature. For example, it could even be the fractional time allotted to the RNA–polymerase for transcription of the key enzyme concerned. Incorporating the effect of dilution of the specific enzyme level due to cell growth and the constant protein decay in the cells, the rate equation for e_i can be written as:

$$\frac{de_i}{dt} = \frac{\alpha_i s_i}{K_i' + s_i} u_i - \frac{d}{dt}(\ln c)\, e_i - \beta_i e_i \tag{6}$$

where α_i and β_i are the enzyme synthesis and decay rate constants. The cybernetic variable, u_i, represents the control actions of the cellular regulatory mechanisms of catabolite repression and induction. Similarly, the mechanisms of catabolite inhibition and activation controlling the activity of the existing enzymes are

represented with another cybernetic variable v_i. The actual rate of substrate utilization may be written as:

$$\frac{ds_i}{dt} = -\frac{1}{Y_i} r_i v_i \quad (0 \leq v_i \leq 1) \tag{7}$$

and the total growth rate

$$\frac{dc}{dt} = \Sigma_i r_i v_i \tag{8}$$

In our earlier formulation of this model we had an additional constraint on the v_i's: $\Sigma_i v_i = 1$. The cybernetic variables u_i, v_i are then determined by the matching law strategy, which has been shown[2] to be the solution of a constrained optimization problem. The constraint on the enzyme synthesis rate may, for example, be identified as the limited time of the RNA–polymerase for the transcription of the catabolic key enzymes. For the control of enzyme activity such a clear constraint on the summation of v_i's has not been identified. In a subsequent section, we will relax this constraint on $\Sigma_i v_i$ as it leads to an unrealistic simulation.

IDENTIFICATION OF PARAMETERS

All the model parameters are determined solely from growth on single substrates. Associated with growth on any substrate S_i are one set of parameters $(\mu_i, K_i, Y_i, \alpha_i, \beta_i)$. We have tacitly assumed here that $K_i = K_i'$ although they are quite likely to be different. However, until accurate experiments including measurement of enzyme levels are possible the distinction between K_i and K_i' cannot be realized. In our model development, the experimentally determinable parameter $\mu_{\max,i}$, the maximum specific growth rate, has been rewritten as

$$\mu_{\max,i} = \mu_i e_{i,\max} \tag{9}$$

where $e_{i,\max}$ is the maximum specific enzyme level. From the rate equation (6) for e_i, the maximum specific enzyme level can be written as

$$e_{i,\max} = \frac{\alpha_i}{\mu_{\max,i} + \beta_i} \tag{10}$$

For any dynamic simulation of the model equations, the actual value of $e_{i,\max}$ is not so much important as the relative level $e_i/e_{i,\max}$ as

$$\mu_i e_i = \mu_{\max,i} \left[\frac{e_i}{e_{i,\max}}\right] \tag{11}$$

Protein decay is relatively slow and has been found[6] to reach its maximum rate of ca. 5%/h under nongrowing conditions. Hence β_i can be fixed at 0.05 h^{-1} for all enzymes E_i's. The maximum specific growth rate $\mu_{\max,i}$ is determined directly from the growth data on a single substrate S_i and has been found to be in the range 0.8–1.1 h^{-1} for the substrates and the experimental conditions used below. The numerical estimate[7] for the maximum specific level of a single enzyme, on average, is of the order of 1.0×10^{-3} for the kind of heterotrophic bacteria used here. Hence, α_i can be fixed at 1.0×10^{-3} for an average enzyme E_i. The values of the true parameters α_i and β_i are assumed to be the same for all key enzymes. The remaining parameters $\mu_{\max,i}$, K_i, and Y_i are same as that of the Monod model for growth on a single substrate and can be determined readily with the established procedures.

The maximum specific growth rate constants $\mu_{\max,i}$ and Michaelis constants K_i are determined by the initial growth rate data experiments[8] on single substrates and by fitting the model simulations with the batch growth data. The yield coefficients are determined from initial substrate concentrations and final cell concentrations. The growth rate parameter μ_i in eq. (3) is determined from eqs. (9) and (10) as:

$$\mu_i = \frac{\mu_{\max,i}(\mu_{\max,i} + \beta_i)}{\alpha_i} \tag{12}$$

Thus, all the parameters in the cybernetic model equations shown above are determined solely from the growth on single substrates. We have, in this discussion, tacitly implied that it is unnecessary to identify precisely the key enzyme for each substrate and make measurements of their levels during growth. In this regard, it is important to note that one is able to find the quantitative consequences of a model without being stultified by the very difficult task of having not only to identify suitable key enzymes but also make dynamic measurements of their levels. Equation (11) shows that the *absolute* enzyme levels are not required. The model simulation requires only the *initial values* of the relative enzyme levels (that is enzyme levels relative to the maximum during balanced exponential growth). Such initial values can be used to characterize the state of the inoculum and can be determined by fitting the batch growth curve including the initial acceleration to exponential growth.

As pointed out by Ramkrishna and co-workers,[2] the set of kinetic constants connected with describing growth on a particular substrate *identifies* the substrate *and* the organism. Thus the "quality" of the substrate is contained in the numerical values of the constants.

MODIFICATION TO THE MODEL

According to the matching law, the optimal allocation of resources to an alternative is proportional to the amount of returns obtained from that alternative. Therefore the fractional allocation of the limited resources, u_i to the ith alternative, that optimizes the total returns, is given by

$$u_i = \frac{r_i}{\Sigma_j r_j} \tag{13}$$

where r_i is the returns from the ith alternative. We have, in our previous work,[4] applied this matching law twice for optimizing the cell growth rate and the energy production rate. However, this double matching law model produces an unrealistic simulation of a hypothetical situation described below:

Consider the model simulation of a culture growing in a medium containing two substrates, with identical values for each of the model parameters (μ_{max}, K_s, Y). For such a situation, the model predicts a growth rate one-half of that on either of the substrates, i.e., $\mu_{mixed} = 0.5\mu_{max}$. This is because all the cybernetic variables take the value of 0.5; e_i levels are at $0.5e_{max}$ for both key enzymes; this alone will not cause the halving of the total growth rates which is the summation of the growth rate on each substrate. Since the v_i values are also 0.5 for both key enzymes, each (e_i, v_i) corresponds to that of $\frac{1}{4}$ μ_{max} and the total growth thus amounts to only $0.5\mu_{max}$. In actual experimental observations, however, the presence of a second substrate does not cause any reduction from the maximum growth rate possible on the preferred substrate alone. Hence the double matching law model needs to be modified.

As we have noted above, a physical constraint for the control actions of enzyme inhibition and activation is not clear, even though many observations of catabolite inhibition have been made. These observations are only for the activity of the enzymes for the less preferred substrate, the growth rate on which is less than the growth rate on the preferred substrate. Based on these observations, we will develop a heuristic optimal strategy for v_i.

Let r_i be the maximum possible growth rate on S_i with the existing levels of E_i. The actual growth rate will be $\Sigma_i r_i v_i$, where v_i represents the control actions of catabolite inhibition and activation and $0 \leq v_i \leq 1$. If there were no other constraints, then the maximum growth will occur when v_i's are equal to 1. However, such an additive growth has not been observed. Hence, there is some constraint that imposes inhibition on the slower growth pathways. Even if we are not able to identify this constraint precisely, from the experimental measurements of catabolite inhibition, we may model the result of the constraint as $v_i \propto r_i$, or

$$v_i = \lambda r_i \tag{14}$$

This proportionality combined with the constraints on each v_i determines the bounds on λ as shown below:

$$0 \leq v_i \leq 1 \Rightarrow 0 \leq \lambda \leq \frac{1}{r_i} \text{ or } \lambda \leq \frac{1}{\max_j (r_j)} \tag{15}$$

The actual growth rate (including the catabolite inhibitions) is given by

$$\Sigma_i r_i v_i = \lambda \Sigma_i r_i^2 \leq \frac{1}{\max_j (r_j)} \Sigma_j r_i^2$$

Therefore, for the maximum of the actual growth rate $\Sigma_i r_i v_i$, we must have $\lambda = \frac{1}{\max_j (r_j)}$. Hence,

$$v_i = \frac{r_i}{\max_j (r_j)} \tag{17}$$

This proportional expression for v_i and the matching law expression, eq. (13), for u_i, coupled with the basic cybernetic model equations, eqs. (6)–(8), constitute the modified cybernetic model for microbial growth on multiple substrates.

Let us now consider with this modified model, the hypothetical situation of growth on two substrates with identical parameters discussed previously. As the u_i's are still determined with the matching law expression, eq. (13), their values remain at 0.5 for both substrates. The v_i's are determined by eq. (15) in this modified model. From this expression for $r_1 = r_2$, both v_i's are equal to 1.0. Hence, the total growth amounts to μ_{max}, which is the expected growth rate for this situation. Thus, the modification brings about an improved performance of the model.

In this work, the cybernetic model is tested with consistent experimentation using the same organism growing on the same set of substrates supplied singly or in mixtures, in same batch growth conditions. The material and methods are described below. All the parameter values, that characterize the growth of the bacteria on any single substrate, are determined from the batch growth experimental data on single substrate growth. The simulations of the cybernetic model for growth on multiple substrate uses only these parameter values, determined previously from single substrate rate growth experiments, without any other information as to the order of preference of these substrates or any interactions in the kinetics of substrate utilization.

MATERIALS AND METHODS

Organism

Klebsiella oxytoca B199 (ATCC 8724), obtained from U.S. Department of Agriculture (Peoria, IL), was used in all the experiments.

Medium

The carbon-free salts medium used in all experiments has been described elsewhere.[9] The medium was prepared and autoclaved in two separate parts, namely, a concentrated solution of trace metals and EDTA and a solution containing the phosphate and ammonium

salts. Concentrated sugar solutions are made containing only glucose, fructose, arabinose, xylose, or lactose. After autoclaving separately, these components are mixed together to give the required concentrations. The total sugar concentration was not allowed to exceed 10 g/L, so that the carbon and energy source is the only limiting substrate.

Inoculum Preparation

The organism was stored in small vials in a biofreezer at −75°C in a glycerol-rich medium. Inocula were prepared in 250-mL shake flasks containing 50 mL salts medium and 10 g/L glucose. Flasks were incubated for 24 h at 37°C and the organism was transferred to another flask with an identical medium. At the stationary phase of this batch culture, 5 mL was injected into the fermentor in a sterile manner.

Fermentor Description

Experiments were conducted in a 2-L New Brunswick fermentor with an initial culture volume of 1.5 L. Temperature was controlled at 37°C. The pH of the medium was 7.1. As the medium was adequately buffered for the low cell concentrations used, it was not necessary to control pH. Oxygen was supplied by sparging the fermentor with air.

Growth Measurement

Cell dry weight was estimated from absorbance, measured at 540 nm. One unit of absorbance was determined[9] to be equivalent to 0.35 g/L cell dry weight linear up to the absorbance value of 0.2.

RESULTS AND DISCUSSION

Single Substrate Data and Simulation

Fermentations were conducted with each of sugars mentioned below as the single carbon and energy substrate. Simulations were carried out with the model equations for growth on single substrate. The initial values c_0, and s_0 were specified by the experimental conditions. The initial value for enzyme level, e_0, was specified by the preculturing or the past history of the inoculum. Figure 1 shows the experimental data and simulation of model equations for growth on glucose. As the inoculum was cultured on glucose, the initial value for specific enzyme level was assumed to be 8.0 $\times 10^{-4}$ or 90% of the maximum specific enzyme level. The model parameters μ_{max}, K_s, and Y were determined by fitting the experimental data with model simulations.

Figure 2 shows the data and simulation for growth on xylose with the inoculum precultured on glucose.

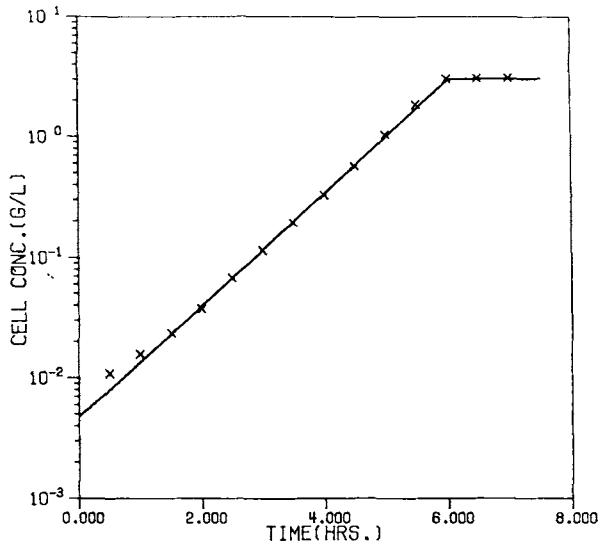

Figure 1. Estimation of model parameters for growth of *Klebsiella oxytoca* on glucose, with inoculum precultured on glucose: $e_{1,0}$ = 8.0×10^{-4} = 90% $e_{1,max}$.

With c_0 and s_0 specified by the experimental conditions, the initial specific enzyme level was fixed by fitting the simulation of the model equations to the growth data. The value of e_0 thus fixed is 2.0×10^{-4} or ca. 18% of e_{max} for xylose utilization. Still lower values of e_0 show a relative insensitivity to the simulation results. At e_0 values ranging from 1.0×10^{-4} down to 1.0×10^{-7} (approximately the lower physical limit on the enzyme level), the initial lag period remained unchanged whereas higher values of e_0 reduced this lag period.

Figures 3 and 4 show the data and simulation for growth on arabinose and fructose, respectively, with inoculum grown on same sugars. Hence, the values of

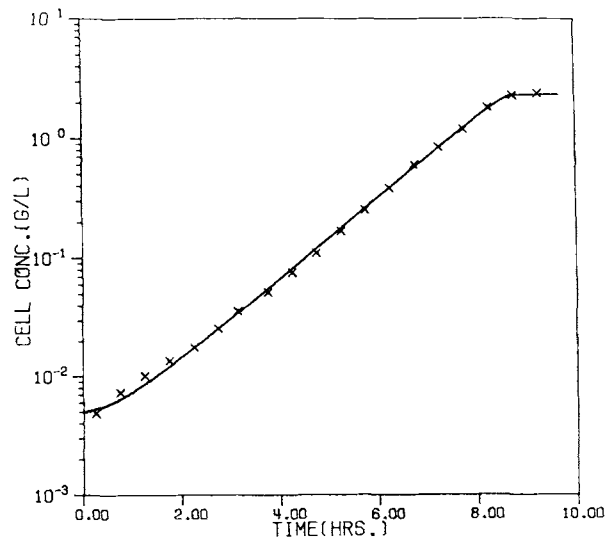

Figure 2. Estimation of model parameters for growth of *K. oxytoca* on xylose, with inoculum precultured on glucose: $e_{1,0} = 2 \times 10^{-4}$ = 18% $e_{1,max}$.

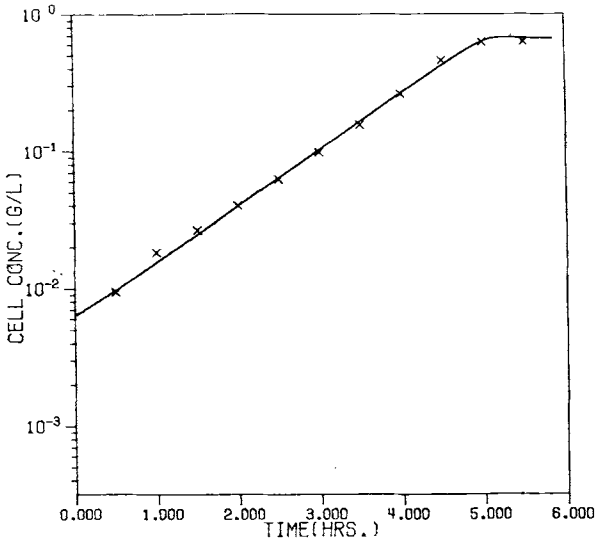

Figure 3. Estimation of model parameters for growth of *K. oxytoca* on arabinose, with inoculum precultured on arabinose: $e_{1,0} = 8.0 \times 10^{-4} = 84\% \, e_{1,max}$.

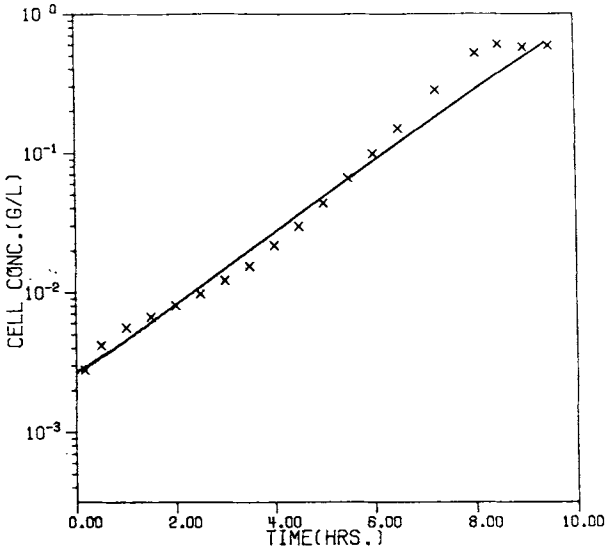

Figure 5. Batch growth data for *K. oxytoca* on lactose and model simulations with parameter values obtained from initial growth on lactose: $e_{1,0} = 8.0 \times 10^{-4} = 80\% \, e_{1,max}$.

e_0 assumed in both cases were 8.0×10^{-4}. For lactose the μ_{max} and K_s values could not be determined from batch growth data as its K_s value was large compared to that of the other sugars. There also appears to be some difficulty with reconciling the Monod model with the growth data on lactose. Hence, μ_{max} and K_s for lactose was determined from initial growth rate experiments. Figure 5 shows the comparison of the model simulation with these values to the actual batch growth data on the single substrate lactose. The experimental data exhibit a systematic and reproducible variation in growth rate, uncharacteristic of kinetics governed by the Monod model, whereas the model simulation shows an average and constant growth rate. The variations in the growth data may be attributed to an extra cellular breakage of the β-galactoside bond coupled with the utilization of the product sugars. A more accurate kinetic model for lactose utilization must include the mathematical description of such extracellular processes. However, for the purpose of testing our cybernetic framework, we will use the simple Monod model for the kinetics of utilization of each single substrate. Thus, for each substrate, the set of all parameter values used later in the prediction of bacterial growth behavior on mixed substrates is determined from the growth on single substrates alone. These values are listed in Table I.

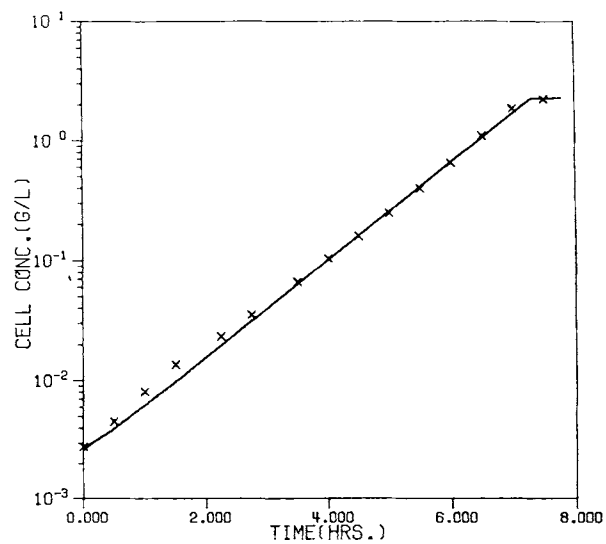

Figure 4. Estimation of model parameters for growth of *K. oxytoca* on fructose, with inoculum precultured on fructose: $e_{1,0} = 8.0 \times 10^{-4} = 79\% \, e_{1,max}$.

Multiple Substrate Data and Simulation

We have previously determined all the parameter values of, say, glucose and xylose. We have also quantified both the initial specific enzyme levels $e_{i,0}$ relative to $e_{i,max}$ for inoculum with a particular past history, namely, precultured on glucose. Hence all the constants used in the simulation are already determined. Figure 6 shows the experimental data for the growth of *Klebsiella oxytoca* on a mixed carbon source of glucose (0.5 g/L) and xylose (2.5 g/L) and the model simulation for the same. In a previous article,[4] we have detailed how the integration of the model equations results in sequential utilization of the two substrates. We have also shown the profiles of all the state and cybernetic variables during the batch growth on two substrates. Even with the new modification to the cybernetic variable, v_i, which models the actions of the regulatory processes of catabolite inhibition and activation, the behavior of all the variables through the

Table 1. Parameter values obtained from single substrate growth data.

Sugar	μ_{max} (h^{-1})	K_s (g/L)	Y (g dry wt/g)	α	β
Glucose	1.08	0.01	0.52	1.0×10^{-3}	0.05
Arabinose	1.00	0.05	0.5	1.0×10^{-3}	0.05
Fructose	0.94	0.01	0.52	1.0×10^{-3}	0.05
Xylose	0.82	0.2	0.5	1.0×10^{-3}	0.05
Lactose	0.95	4.5	0.45	1.0×10^{-3}	0.05

integration remains essentially the same. Again, the diauxic lag period predicted with the simulation is found to be insensitive to the lower values of $e_{2,0}$. However, higher values of $e_{2,0}$ change the nature of diauxic lag as shown in the simulation of Figure 7.

In the next two figures are plotted the growth data of *Klebsiella oxytoca* on a mixture of glucose (0.33 g/L) and xylose (2.0 g/L) with the inocula precultured on glucose (Fig. 7) or on xylose (Fig. 8). In both cases, glucose is consumed during the first growth phase and xylose is consumed during the second growth phase. The essential difference between the two sets of data is during the diauxic lag periods. In the first set, there is a sudden stoppage of growth after glucose gets exhausted, and after a period of time, the growth on xylose begins. This shows that there has been a repression on the synthesis of enzymes of xylose utilization and only after glucose gets fully consumed the synthesis of xylose enzymes is induced. Preculturing the inoculum on the less preferred substrate brings out the nature of regulatory process even more clearly. As the inoculum has been growing previously on xylose, all the enzymes necessary for the utilization of xylose are already present in the cells near their maximum levels. However, in the presence of a higher growth-supporting substrate, namely glucose, the activities of these enzymes are inhibited. Enzymes for growth on glucose are synthesized and the xylose enzymes are repressed, and the bacteria grows preferentially on glucose. When glucose is virtually exhausted, the already existing enzymes for xylose utilization are activated, resulting in a reduced diauxic lag period. To observe this effect clearly in the experiments, it is necessary that the growth in the first growth phase be short; otherwise, the xylose enzymes that are present in near maximum levels at the beginning of the batch growth will get diluted by the excessive number of cell divisions. In this experiment, we have used a large inoculum size, so that the glucose supplied will be consumed by the time the inoculum multiplies to about 6 times the initial concentration. Simulations for both these figures are done with the same parameter values as for Figure 6. The initial enzymes levels, however, have been chosen to reflect the difference in their past histories. The values for $e_{1,0}$ and $e_{2,0}$ for the simulation in Figure 7 are the same as those used in Figure 6 and for the simulation in Figure 8, $e_{1,0}$ and $e_{2,0}$ are chosen as 3.0×10^{-4} and 6.0×10^{-4}, respectively, as the inoculum has been precultured on S_2. Here also the cybernetic model predicts that the growth will be preferentially on glucose

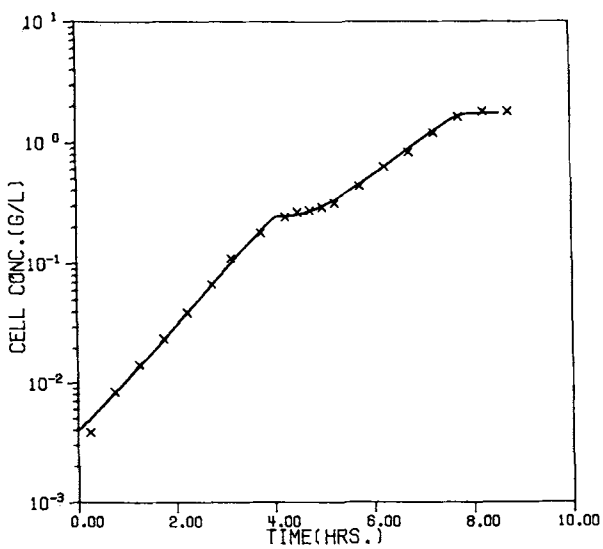

Figure 6. Experimental data and model prediction for growth of *K. oxytoca* on glucose (0.5 g/L) and xylose (2.5 g/L), with inoculum precultured on glucose: $e_{1,0}/e_{1,max} = 0.90$, $e_{2,0}/e_{2,max} = 0.18$.

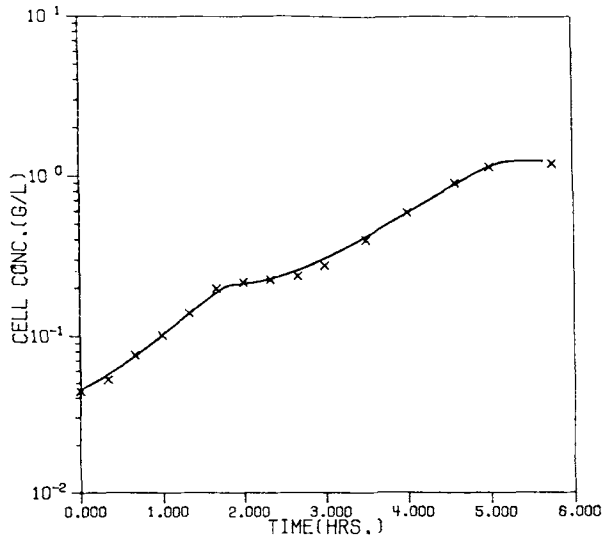

Figure 7. Experimental data and model prediction for growth on glucose (0.33 g/L) and xylose (2.0 g/L), with high initial cell concentration. The inoculum was precultured on glucose: $e_{1,0}/e_{1,max} = 0.90$, $e_{2,0}/e_{2,max} = 0.18$.

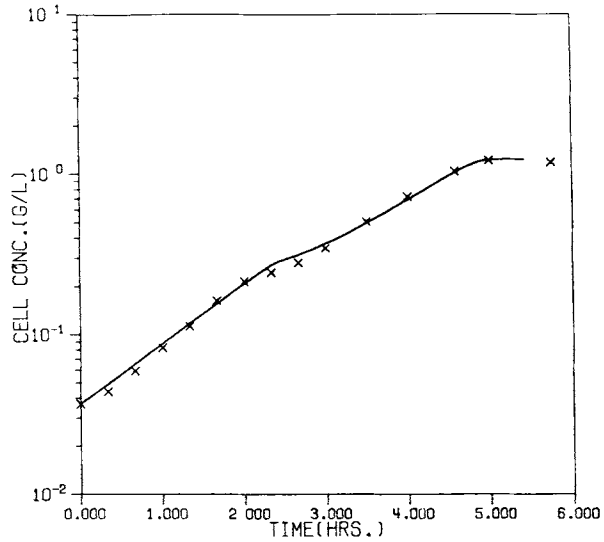

Figure 8. Experimental data and model prediction for growth on glucose (0.33 g/L) and xylose (2.0 g/L), with high initial cell concentration. The inoculum was precultured on xylose: $e_{1,0}/e_{1,max}$ = 0.34, $e_{2,0}/e_{2,max}$ = 0.52.

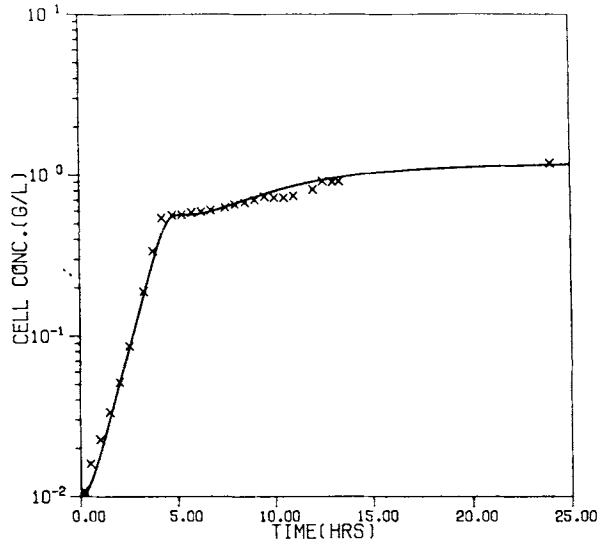

Figure 10. Experimental data and model prediction for growth on glucose (1.1 g/L) and lactose (1.5 g/L): $e_{1,0}/e_{1,max}$ = 0.90, $e_{2,0}/e_{2,max}$ = 0.20.

in the first growth phase and that the diauxic lag is reduced due to the activation of the already present enzymes for xylose at the end of the first growth phase.

The mixture of glucose and lactose has been a celebrated combination of sugars for many previous observations of the diauxie. A special feature of growth of many bacteria on this combination is the observation of a pronounced intermediate lag phase of no apparent growth before a second growth phase, particularly when small lactose concentrations are used.[10] These long lags have been attributed to the existence of a very small proportion of mutants constitutive for β-galactosidase in normal inducible populations.[11] Careful measurements of optical density in our experiments yielded noticeable growth of cells during the period after the exhaustion of glucose. In fact, as the lactose concentrations were increased in the batch fermentations, the growth became faster and as the concentration neared the K_s value (4.5 g/L) for lactose, the intermediate lag was only of a few hours duration rather than the 15–25-h duration reported for fermentations with lactose concentration of 0.15 g/L. This observation suggests that the induction of β-galactosidase is proportional to the lactose concentration in the medium and that the presence, if any, of a very small fraction of constitutive mutants plays an insignificant

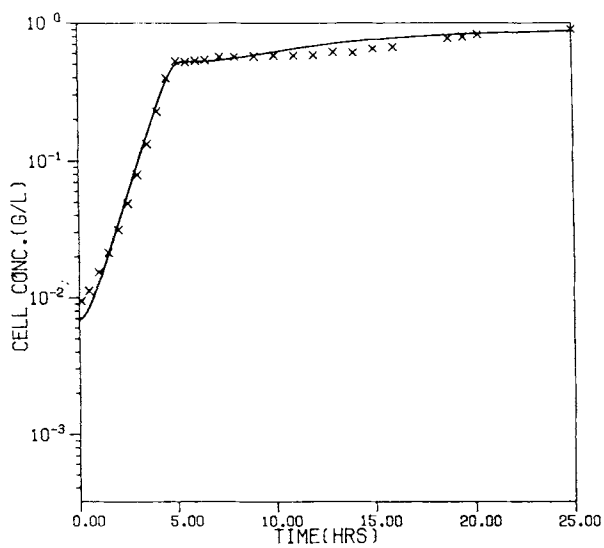

Figure 9. Experimental data and model prediction for growth on glucose (1.0 g/L) and lactose (1.0 g/L): $e_{1,0}/e_{1,max}$ = 0.90 and $e_{2,0}/e_{2,max}$ = 0.20.

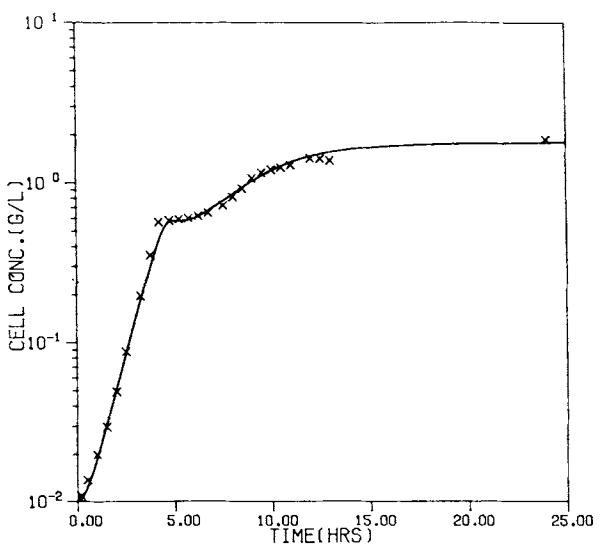

Figure 11. Experimental data and model prediction for growth on glucose (1.1 g/L) and lactose (2.8 g/L): $e_{1,0}/e_{1,max}$ = 0.90, $e_{2,0}/e_{2,max}$ = 0.20.

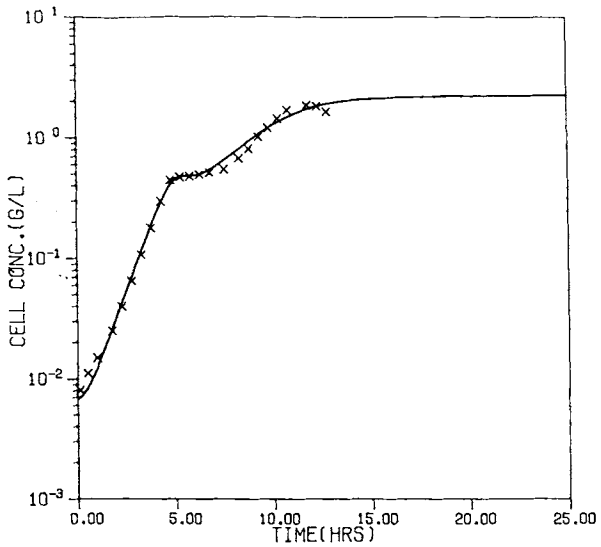

Figure 12. Experimental data and model prediction for growth on glucose (1.0 g/L) and lactose (4.0 g/L): $e_{1,0}/e_{1,\max} = 0.90$, $e_{2,0}/e_{2,\max} = 0.20$.

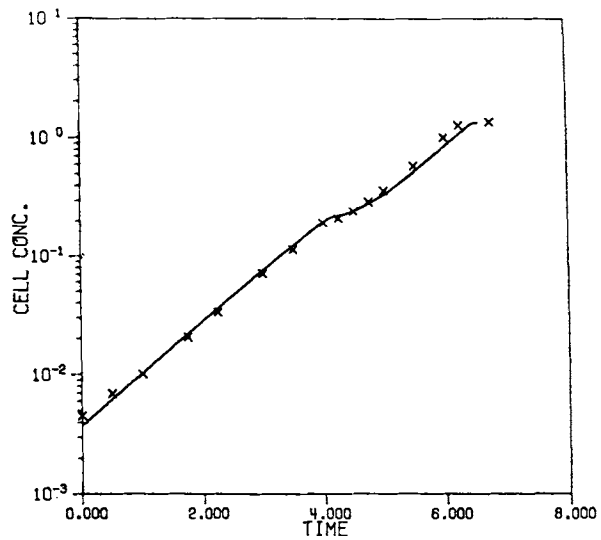

Figure 14. Experimental data and model prediction for growth on glucose (0.5 g/L) and arabinose (2.0 g/L); $e_{1,0}/e_{1,\max} = 0.90$, $e_{2,0}/e_{2,\max} = 0.21$.

role in the intermediate lag period when lactose concentrations are above the starvation levels. Figures 9, 10, 11, and 12 show the growth data as well as the model simulations for mixtures of glucose and lactose with increasing concentrations of lactose. The simulations are in surprisingly good agreement with the experimental data, particularly with respect to the increasing rates during the second growth phase as the lactose concentration is increased. The minor disagreements between the data and predictions may have been inherited from the essential inadequacy of the Monod model in describing growth on lactose.

Figures 13 and 14 show the growth on glucose (0.5 g/L) and arabinose (0.5 and 2.0 g/L) at two different concentrations. At the lower concentration of arabinose, the diauxic lag is pronounced and at the higher concentration, it is shortened. The experimental data and the model simulation show reasonable agreement in this trend.

Figure 15 shows the growth on three different sugars, glucose (0.5 g/L), xylose (1.5 g/L), and lactose (5.0 g/L). This results in a triauxic growth, which is a sequential utilization of the substrates with two intermediate lag phases. The model described above can be readily extended to any number of substrates with sufficiently different pathways of assimilation. The

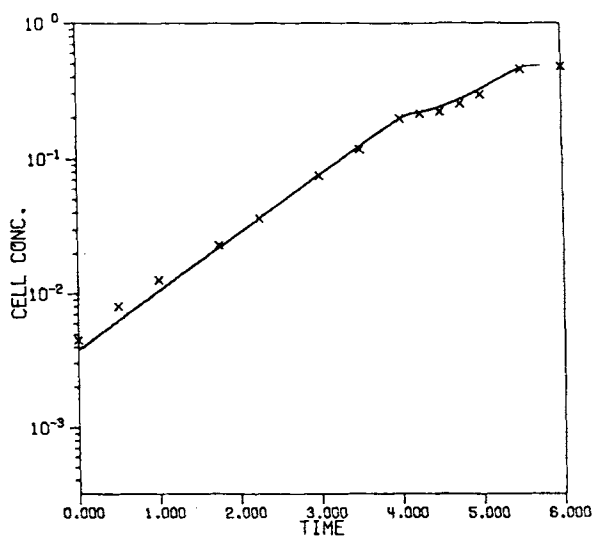

Figure 13. Experimental data and model prediction for growth on glucose (0.5 g/L) and arabinose (0.5 g/L): $e_{1,0}/e_{1,\max} = 0.90$, $e_{2,0}/e_{2,\max} = 0.21$.

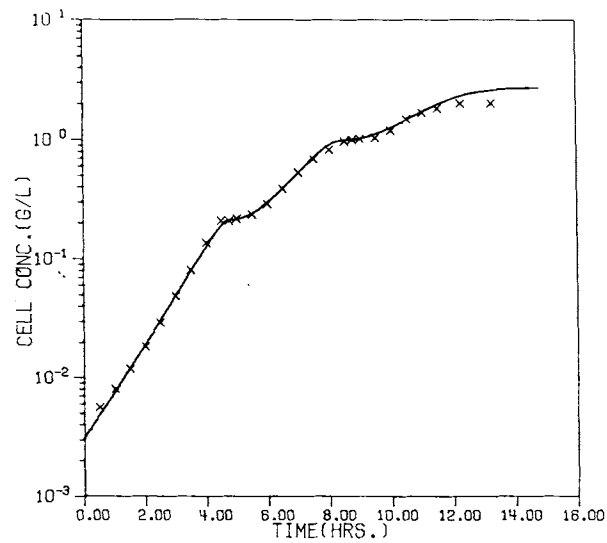

Figure 15. Experimental data and model prediction for triauxic growth on glucose (0.5 g/L), xylose (1.5 g/L), and lactose (5.0 g/L): $e_{1,0}/e_{1,\max} = 0.90$, $e_{2,0}/e_{2,\max} = 0.17$, $e_{3,0}/e_{3,\max} = 0.20$.

framework of the model remains the same and the extension is in the form of one extra differential equation each, for the third substrate and the third key enzyme and one set of cybernetic variables, u_3 and v_3, for the regulatory actions on this key enzyme. The model parameters are the same as those obtained from single substrate growth experiments and listed in Table I for these sugars. The values of the initial enzymes are the same as those used in the glucose–xylose diauxie and $e_{3,0}$ was assumed to be the same as $e_{2,0}$. Even lower values of $e_{3,0}$ produced the same simulation results.

The experimental data indicate that, the presence of an additional slower sugar, namely lactose, does not alter the nature of the bacterial growth on glucose and xylose as well as the kinetics of such growth. This is contrary to the assumptions in some previous modeling efforts, in which equations specifically include the concentrations of all other substrates in the growth kinetics of a particular substrate as inhibitory effects. (Yoon and co-workers[12] point out the observation[13] of additive growth on multiple sugars as a basis for such an equation. However, this additive growth was observed in an uncharacterized heterogeneous population. Such instances of additive growth could easily arise, in a mixture of just two organisms growing on a mixture of two substrates, when one organism prefers one substrate and the other organism prefers the other substrate). The cybernetic model here does not have any such interactions of other substrates on the kinetics of growth on any substrate and interactions due to the presence of other substrates are in the form of cybernetic variables representing the regulatory processes of catabolite repression and inhibition. Indeed addition of a faster (higher growth-supporting) substrate does invoke these regulatory actions.

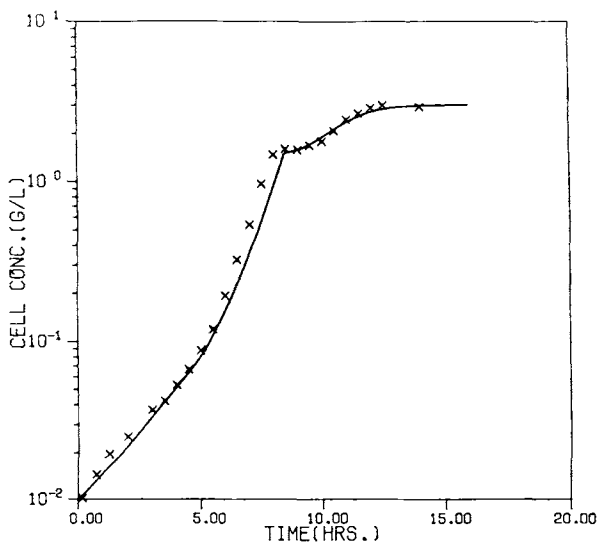

Figure 16. Experimental data and model prediction for batch growth on lactose (4.0 g/L) perturbed with addition of 2.0 g/L glucose at 4.5 h: $e_{l,0}/e_{l,\max} = 0.80$, $e_{g,0}/e_{g,\max} = 0.23$.

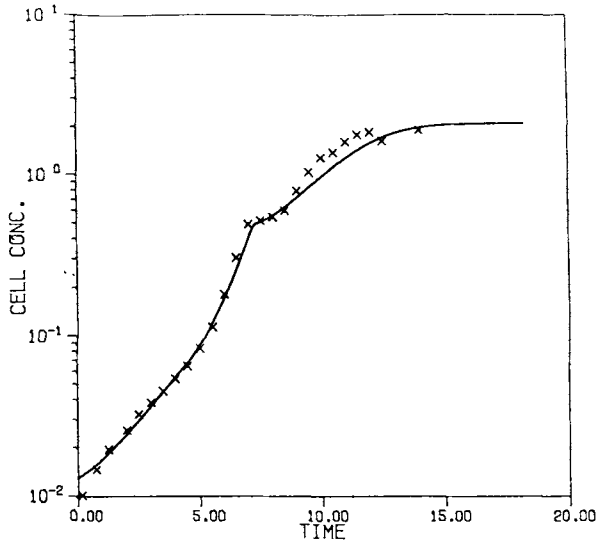

Figure 17. Experimental data and model prediction for batch growth on lactose (4.0 g/L) perturbed with addition of 0.5 g/L glucose at 4.5 h: $e_{l,0}/e_{l,\max} = 0.80$, $e_{g,0}/e_{g,\max} = 0.23$.

In Figures 16 and 17 are shown the bacterial growth on lactose, perturbed with the addition of a faster substrate, namely glucose, while growth on lactose is under way. After $t = 4.5$ h when the batch cultures are perturbed, by addition of glucose, the actions of catabolite repression and inhibition come into play immediately and the growth rate accelerates to that on glucose. After glucose has been consumed, the bacteria revert to the consumption of lactose, after a clear intermediate lag period, characteristic of catabolic repression. The simulations of the cybernetic model do indeed predict these consequences of regulatory processes with reasonable quantitative precision. Again, the disagreements may be attributed to the problems in simulating growth on lastose with Monod kinetics.

The experimental data and model simulation discussed so far pertain to the most common type of bacterial growth behavior on mixed substrates, namely the diauxic growth. A less common type of growth behavior, known as biphasic growth,[14] is the sequential utilization of two substrates without any diauxic lag period. This type of growth is usually observed when the two substrates are close isomers, with their metabolic pathways identical to a large extent. Examples of this type are the growth of *E. Coli* on mixtures of glucose and galactose,[15] saccharose, and mannose[5] and growth of *K. oxytoca* on glucose and fructose.[16] Such growth data show a clear reduction in the logarithmic slope at the end of the first growth cycle, but no diauxic lag phase. This indicates that the enzymes for the utilization of the second substrate are already present in maximum levels at the end of first growth phase and that this utilization is totally inhibited until the first substrate is exhausted. Here also, the growth in the first phase is always higher than the second phase, in

the same optimal nature as expressed in the diauxic growth phenomenon. Hence the cybernetic approach is equally applicable for modeling the biphasic growth phenomenon.

The model we have developed for the diauxic growth phenomenon is unsuitable for simulating the biphasic growth as the catabolic enzymes are common for both substrates and there is no catabolite repression. The only regulatory action that is apparent in the growth data is that of the activity of the enzyme which is found to be completely active for S_1 and inactive for S_2 as long as S_1 is present. Thus, the regulatory processes are acting at the level of enzyme activity to optimize the instantaneous growth rate. We develop below an alternate cybernetic formulation that may be used for simulating biphasic growth.

As both S_1 and S_2 are close isomers, they are competing for the same active sites on the key enzyme E. The sharing of the same sites translates into a constraint on the cybernetic variable v_i as $\Sigma_i v_i = 1$. The optimal strategy to maximize the total growth rate $\Sigma_i r_i v_i$ is:

$$v_i = \begin{cases} 1 \text{ if } r_i > r_j \\ 0 \text{ if } r_i < r_j \end{cases} \tag{18}$$

where r_i is the instantaneous growth rate on S_i. As the key enzyme is common for both substrates and at its maximum level throughout the batch growth, r_i can be simplified to:

$$r_i = \frac{\mu_{\max,i}\, s_i C}{k_i + s_i} \tag{19}$$

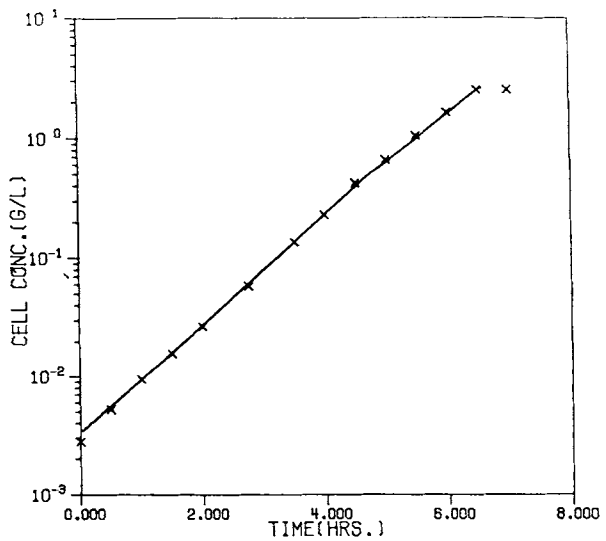

Figure 18. Experimental data and model prediction for sequential utilization of glucose (1 g/L) and fructose (4 g/L) without any diauxic lag.

The complete model equations for biphasic growth are given by:

$$\frac{ds_i}{dt} = -\frac{1}{Y_i} r_i v_i \tag{20}$$

$$\frac{dc}{dt} = \Sigma_i\, r_i v_i \tag{21}$$

Figure 18 shows the experimental data for the biphasic growth of *Klebsiella oxytoca* on glucose (1 g/L) and fructose (4 g/L) and simulations of the equations given above with parameter values from the single substrate growth data. The growth curve on the logarithmic plot shows two distinct growth rates, the first phase at the rate of growth on glucose alone and changing sharply to the growth rate on fructose alone, when glucose gets consumed. The simulations of the simplified model are in excellent agreement with the experimental results.

CONCLUSIONS

In this article, we have been able to show that growth of *Klebsiella oxytoca* on single substrates alone can lead to information on growth on mixed substrates via the cybernetic models proposed here and elsewhere.[1–4] In establishing this, we have used different combinations of substrates among glucose, xylose, arabinose, fructose, and lactose. With the glucose–fructose system, it was necessary to modify the model by proposing that, with isomers with broadly coinciding metabolic pathways, the same key enzyme may be involved in the growth process on both substrates. The modeling of glucose–lactose system leaves some features to be desired in that growth on lactose alone needs to be described by a model more suitable than that of Monod. However, inspite of this inadequacy the diauxic curves obtained with different ratios of glucose–lactose mixtures agree remarkably with model predictions.

The models also perform well with describing the effect of perturbing growth on the less preferred substrate with additions of the preferred substrates. Also, the triauxic curve with glucose, xylose, and lactose mixture is described well by the cybernetic model with parameters obtained entirely from single substrate growth experiments.

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NOMENCLATURE

B	biomass
c	biomass concentration
e_i	specific enzyme level
E_i	ith key enzyme

K_s, K_i	Michaelis constant
r_i	rate of biomass production through consumption of S_i
r_{Ei}	rate of E_i synthesis
s_i	substrate concentration
S_i	ith substrate
t	time
u_i, v_i	cybernetic variables
Y_i	yield coefficient
α_i	enzyme synthesis rate constant
β_i	enzyme decay rate constant
λ	proportionality constant in eq. (14)
μ_i	growth rate coefficient
$\mu_{max,i}$	maximum specific growth rate

Subscripts

0 (zero)	initial
max	maximum

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