Metabolic Regulation in Bacterial Continuous Cultures: II

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Received November 7, 1990/Accepted June 5, 1991

The transient behavior of a continuous culture of *Klebsiella pneumoniae* with mixed feed of glucose and xylose arising from step-up and step-down in dilution rates and from a feed-switching experiment is presented. The organism gradually switches from simultaneous utilization of the substrates at low growth rates to preferred utilization of the faster substrate (i.e., supporting a higher growth rate) at high dilution rates. The metabolic lags following a step increase in dilution rate and a significant accumulation of the slower substrate during the transient period result from the effects of metabolic regulation. The cybernetic modeling approach that successfully described the foregoing situations with single-substrate feeds is employed to describe mixed substrate behavior. The parameters in the mixed-substrate (glucose and xylose) model are the same as those in the single-substrate models with the singular exception of the rate constant for the xylose growth enzyme synthesis. The reason for this discrepancy is discussed in detail. It appears that the constitutive rate of enzyme synthesis for growth on a given substrate may be related to the past history of the organism in regard to whether or not the organism has been exposed to the particular substrate. Thus, the results further demonstrate the ability of the framework to effectively describe metabolic regulation in batch, fed-batch, and continuous microbial cultures.

Key words: *Klebsiella pneumoniae* • metabolic regulation • continuous cultures, cybernetic model

INTRODUCTION

In Part I, we developed a cybernetic model that described single-substrate microbial growth in a variety of situations: batch, perturbed fed-batch, steady-state, and transient continuous cultures. The existence of an explicit cellular resource required for enzyme synthesis was incorporated in the cybernetic model. The model also accounted for an increased maintenance demand for the substrate observed at very low growth rates. Further, cybernetic variables that modified the rates of synthesis of the growth and low-maintenance enzymes and the activity of the resource synthesis process were incorporated in the model formulation. These variables represented the increased interactions between growth and maintenance functions for the limited cellular resources especially in low growth rate situations.

The model described the long metabolic lags observed following a step-up in dilution rate by requiring that the cell first synthesize the resource and then the growth enzymes required to support the new higher growth rate. The undershoots in cell density and the near stoppage of growth in the transient period following a step-down in dilution rate were described by the model as a result of the preferential allocation of resources toward synthesis of the low-maintenance enzymes and the large maintenance demand for the limited substrate.

The objective of the present article is to extend the cybernetic model to describe the transient behavior of a mixed (substitutable) substrate continuous culture following step changes in the dilution rate. The model is compared with experimental results from continuous culture growth of *Klebsiella pneumoniae* on a mixture of glucose and xylose. The model is also compared with experimental results from a feed-switch experiment where the feed to the continuous culture growing solely on glucose was switched to xylose at the same dilution rate.

One of the significant features of the cybernetic framework is that it lends itself to describing microbial growth on mixed substitutable substrates in a very straightforward manner. Metabolic regulation controls the growth of the culture in a mixed substrate environment leading to varied behavior under different situations, such as sequential utilization of substrates in a batch culture and simultaneous utilization of the substrates in continuous culture. A standard kinetic model that describes single-substrate behavior needs to be expanded considerably to include all the extra elements of control that come into play in a mixed substrate environment. However, with a cybernetic model, the structure of the model remains the same as the effects of metabolic regulation are represented solely by the cybernetic variables that are calculated from optimal strategies. Therefore, no extra complexity in the form of additional kinetic constants is introduced. Hence, multiple-substrate growth is described by direct integration of single substrate models and the inclusion of the outcome of regulation in the form of cybernetic variables.

While in the single-substrate situation, the effects of regulation are represented by the allocation of resources between growth and maintenance functions and activity control of the rate processes, the cybernetic variables in the mixed-substrate situation account for regulation resulting from the presence of multiple substrates as well as the regulation among the different cellular processes. As a result, we need to include additional cybernetic
variables to account for the switching between the substrates. These variables do not increase the number of constants in the model. This approach is similar to that taken by others in developing cybernetic models to describe mixed substrate growth in batch and fed-batch situations.

MODEL DEVELOPMENT

Reaction Scheme and Rate Expressions

The reaction scheme for representing microbial processes in a mixed-substrate environment remains the same as in a single-substrate situation except that now the cells are able to utilize all of the substrates present. As a consequence, the cells possess growth, resource, low-maintenance, and basal-maintenance enzymes for each of the substrates and can grow, synthesize resource and maintain themselves on every substrate. Hence, we can represent the processes by:

Growth: \( B + S_i \cdot \frac{E_{G,i}}{e_i} (1 + Y_{G,i})B + \cdots \)
Basal maintenance: \( B + S_i \cdot \frac{E_{M,i}}{e_i} B + \cdots \)
Low maintenance: \( B + S_i \cdot \frac{E_{ML,i}}{e_i} B + \cdots \)
Resource: \( B \cdot \frac{S_i E_{R,i}}{e_i} B^{-} + R \)

where the subscript \( i \) refers to the \( i \)th substrate.

The rate expressions for the above reactions are identical to those for the single-substrate model. The growth, maintenance, and low-maintenance process rates follow saturation kinetics and include the level of enzyme present that catalyzes the particular process. The enzyme synthesis expressions include the resource because the amount of resource in the cell affects the synthesis rates. The resource, although not substrate-specific, is synthesized in the presence of each of the substrates, and this synthesis is catalyzed by the substrate-specific resource synthesis enzymes. The rate expressions for the different processes are:

Growth: \( r_{G,i} = \mu_{G,i}^{max} \cdot \frac{e_i}{e_i^{max}} \cdot \frac{s_i}{K_{G,i} + s_i} \)
Basal maintenance: \( r_{M,i} = \mu_{M,i}^{max} \cdot \frac{e_i}{e_i^{max}} \cdot \frac{s_i}{K_{M,i} + s_i} \)
Low maintenance: \( r_{ML,i} = \mu_{ML,i}^{max} \cdot \frac{e_i}{e_i^{max}} \cdot \frac{s_i}{K_{ML,i} + s_i} \)
Enzyme synthesis: \( r_{E,G,i} = r_{E,M,i} = r_{E,R,i} = r_{E,i} \)

Resource: \( r_{R,i} = \alpha_{R,i}^{max} \cdot \frac{s_i}{e_i^{max} K_{R,i} + s_i} \)

The degradation of the growth, basal maintenance, and low-maintenance enzymes and resource are again first-order processes with rate constants \( \beta_{E,G,i}, \beta_{E,M,i}, \beta_{E,ML,i} \), and \( \beta_R \), respectively.

Basic Postulates and Formulation of the Cybernetic Variables

It now remains to introduce the effects of regulation to complete the model. The effects of metabolic regulation are more pronounced in a mixed substrate situation than in the single-substrate case. In addition to interactions between growth and maintenance processes, the cell is now faced with a choice of utilizing all or some of the substrates present in the environment. Ramkrishna et al.\(^5\) have listed and discussed characteristics apparent from observations of microbial growth on multiple substrates. These characteristics of mixed substrate growth are:

1. Given multiple substrates, bacteria prefer to utilize the substrate on which they can grow the fastest, commonly resulting in sequential utilization of the substrates in batch cultures.
2. The growth behavior ranges from simultaneous utilization of multiple substrates 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 the individual substrates.
4. If while growing on a slower substrate a faster substrate is added, growth on the slower substrate is quickly regulated through catabolite inhibition and repression of the enzymes involved.

Similar characteristics have been listed by Ingraham et al.\(^6\) The basic postulates enunciated previously about microbial metabolism should still hold for growth on multiple substrates. Therefore, in a mixed-substrate environment, both the mixed substrate characteristics and the postulates have to be accommodated by the multiple-substrate model. The implications of the postulates, however, must be reexamined in light of the additional competition between cellular processes in the presence of multiple substrates. Competition in a mixed-substrate environment exists among (1) the overall growth and maintenance processes, (2) the individual growth processes on each substrate, and (3) the individual maintenance processes on each substrate.

Postulate 1 states that growth is the primary goal of the cell. In a mixed-substrate environment, the cell is faced with the choice of growing on some or all of the substrates present. The cell in this situation allocates resources toward the synthesis of the different growth enzyme systems such that the growth rate of the culture is maximized. However, if the activity of the synthetase "sub"
sized growth enzymes were all equal to unity, mixed-substrate characteristic \( c \) would be violated because the growth rate of the culture would exceed the maximum growth rate on the fastest substrate. Hence, in a mixed-substrate situation, the activity of the \( i \)th growth enzyme is regulated by a cybernetic variable following the development of Kompala et al. Postulate 1 is not violated because the cell still grows at the fastest rate possible under the given circumstances, i.e., within the constraints of mixed-substrate characteristic \( c \). The expression for the variable is formulated by assuming that the activity is proportional to the growth rate.

Postulate 2, which takes a stand against constant maintenance activity, states that maintenance metabolism manifests itself at growth rates less than the maximum and becomes more active at lower growth rates. Although the cell can utilize all the substrates present in the mixed substrate environment for maintenance purposes, the implications of postulate 2 remain the same as in the single-substrate case because the postulate refers to maintenance metabolism in general.

Postulate 3 identifies growth and maintenance as competing processes for the limited cellular resources. This competition becomes more pronounced at low growth rates. Under low growth rate situations, the allocation of limited cellular resource between these two competing processes is accomplished such that substrate uptake is maximized.

Postulate 4 states that the activity of maintenance metabolism is such that the total rate of substrate consumption is maximized. The formulation of \( v_M \), the cybernetic variable controlling activity of maintenance metabolism, is derived from an understanding of this postulate and the assumption that the activity is proportional to the deficiency in growth rate. Further, \( v_M \) controls the activity of all maintenance processes because from postulate 2, no distinction is made between the different maintenance processes.

Postulate 5 states that the demand for internal resource depends on the growth rate with maximum resource demands being realized at the maximum growth rate. As in the single-substrate situation, this postulate identifies the formulation of the cybernetic variable \( v_R \) controlling the activity of the resource synthesis enzymes. Because no distinction is made between the different resource synthesis processes, all processes are controlled by the same activity variable.

The cybernetic variables that control the rates of the different cellular processes can be derived from the above discussion. Postulate 1 requires that the cell allocates its resource among the different growth enzyme systems such that the growth rate is maximized. The activities of the growth enzymes are also controlled (so that mixed-substrate characteristic \( c \) is not violated). Because the expressions for \( u_{G,i} \) and \( v_{G,i} \), the variables controlling the synthesis and activity of growth enzymes catalyzing competing growth processes, have been derived previously, only the final expression is presented. The expression for \( u_{G,i} \), the variable controlling the allocation of resource between the different growth enzymes, is

\[
u_{G,i} = \frac{r_{G,i}}{\sum_j r_{G,j}}
\]

while the expression for \( v_{G,i} \), which controls the activity of the \( i \)th growth process, is

\[
v_{G,i} = \frac{r_{G,i}}{\max_j (r_{G,j})}
\]

The expressions for the cybernetic variables, \( u_G \) and \( u_M \), which control the amount of resource allocated to the growth enzyme and the low-maintenance enzyme, respectively, are derived from postulate 3 by applying the Matching Law. In a mixed substrate situation they control the allocation of resource between the overall growth process and the overall maintenance process. Following the single substrate development, the return from the growth process in terms of substrate consumption is

\[
\sum_i \left( \frac{r_{G,i}v_{G,i}}{Y_{G,i}} \right)
\]

The return from the maintenance process, again in terms of substrate consumption, is

\[
\sum_i r_{TM,i}v_M
\]

where \( r_{TM,i} = r_{M,i} + r_{ML,i} \). Applying the Matching Law such that substrate consumption is maximized, we obtain

\[
u_G = \frac{\sum_i r_{G,i}v_{G,i}/Y_{G,i}}{\sum_i (r_{G,i}v_{G,i}/Y_{G,i} + r_{TM,i}v_M)}
\]

and

\[
u_M = \frac{\sum_i r_{TM,i}v_M}{\sum_i (r_{G,i}v_{G,i}/Y_{G,i} + r_{TM,i}v_M)}
\]

Competition for the resource allocated to the low-maintenance process still exists between the individual low-maintenance processes: The cybernetic variable controlling the allocation of resource to each of the low-maintenance enzyme synthesis systems is \( u_{M,i} \). The expression for \( u_{M,i} \) is obtained from the Matching Law such that substrate consumption is maximized. Hence, the expression for \( u_{M,i} \) is

\[
u_{M,i} = \frac{r_{ML,i}}{\sum_j r_{ML,j}}
\]
Before deriving the remaining cybernetic variables, we need to identify the maximum growth rate of a culture in a mixed-culture environment. From mixed substrate characteristic we know that it can never exceed the maximum growth rate on the fastest substrate. The growth rate in mixed substrates is

\[ \sum_i \left( \frac{\mu_{G,i}^{\max}}{e_i^{\max}} e_i \frac{s_i}{K_{E,i} + s_i} \right) \]

Under substrate saturating conditions and assuming that \( v_{G,i} = 1 \), the maximum growth rate, \( \mu^* \) is

\[ \mu^* = \sum_i \mu_{G,i}^{\max} \frac{e_i}{e_i^{\max}} \]

At maximum growth rate conditions we have \( R \gg K_{R,i}, s_i \gg K_{E,i} \) and \( u_G = 1 \). Therefore the enzyme level, calculated from the enzyme balance, is

\[ e_i = \frac{\alpha_{E,i}^* + \alpha_{E,i} u_{G,i}}{\beta_{E,i} + \mu^*} \]

The maximum enzyme level is given by

\[ e_i^{\max} = \frac{\alpha_{E,i}^* + \alpha_{E,i}}{\beta_{E,i} + \mu^*} \]

Typically, the constitutive rate of synthesis is much smaller than the inducible rate of enzyme synthesis: \( \alpha_{E,i}^* \ll \alpha_{E,i} \). The fractional enzyme level is therefore approximated by:

\[ \frac{e_i}{e_i^{\max}} = u_{G,i} \]

Hence, the maximum growth rate in a multiple substrate situation is

\[ \sum_i \mu_{G,i}^{\max} \mu_{G,i} \]

The above expression reduces to \( \mu_{G}^{\max} \) for single substrate growth as \( u_{G,i} \) is unity when only one substrate is present. As another check on the validity of the above expression, we consider the situation where a culture is placed in a batch situation with saturating amounts of two identical substrates, i.e., all constants defining growth on each individual substrate are equal. In such a situation, \( u_{G,i} \) for each substrate would be 0.5, and, therefore, the maximum growth rate would be \( \mu_{G,i}^{\max} \), which is the expected result from mixed-substrate characteristic.

The expression for the cybernetic variable \( v_M \), which controls the activity of all the maintenance processes, is formulated as in the single-substrate model. The deficiency in growth rate in a mixed substrate situation is

\[ \Sigma_i \mu_{G,i}^{\max} u_{G,i} - \Sigma_i r_{G,i} v_{G,i} \]

The assumption that the activity of the maintenance metabolism is proportional to the deficiency in growth rate leads to

\[ v_M = \lambda \left( \sum_i \mu_{G,i}^{\max} u_{G,i} - \sum_i r_{G,i} v_{G,i} \right) \]

Because \( 0 \leq v_M \leq 1 \) and from the requirement that substrate uptake be maximized, we obtain

\[ v_M = 1 - \frac{\sum_i r_{G,i} v_{G,i}}{\sum_i \mu_{G,i}^{\max} u_{G,i}} \]

Postulate 5 provides the expression for \( v_R \). Assuming that the activity of resource synthesis is proportional to the growth rate, we have

\[ v_R = \lambda \sum_i r_{G,i} v_{G,i} \]

Because \( 0 \leq v_R \leq 1 \), \( \lambda \) is also bounded

\[ 0 \leq \lambda \leq \frac{1}{\sum_i \mu_{G,i}^{\max} u_{G,i}} \]

We have now introduced three additional cybernetic variables (\( u_{G,i}, v_{G,i}, \) and \( u_{M,i} \)) to augment the variables (\( u_G, u_M, v_M, \) and \( v_R \)) present in the single-substrate model in order to account for the regulation in multiple substrates. These variables result from the competition between the individual growth enzymes and the individual low maintenance enzymes.

The actual rate expressions to describe growth in the presence of multiple substrates are:

**Growth:**

\[ r_G = \sum_i r_{G,i} v_{G,i} \]

**Maintenance:**

\[ r_{TM,i} = (r_{M,i} + r_{ML,i}) v_M \]

**Enzyme synthesis:**

\[ r_{E,i} = \alpha_{E,i}^* + \alpha_{E,i} \frac{s_i}{K_{E,i} + s_i} R \]

\[ r_{E,i} = \alpha_{E,i}^* + \alpha_{E,i} \frac{s_i}{K_{E,i} + s_i} R \]

\[ r_{EML,i} = \alpha_{EML,i}^* + \alpha_{EML,i} \frac{s_i}{K_{EML,i} + s_i} u_{M,i} \]

**Resource:**

\[ r_R = \sum_i \frac{e_{R,i}^{\max}}{e_{R,i}^{\max}} \frac{s_i}{K_{RS,i} + s_i} v_R \]

where the rates of growth and maintenance on the individual substrates and the cybernetic variables are calculated from expressions listed earlier. The rate expressions for the growth enzyme and the maintenance enzyme synthesis are multiplied by two cybernetic variables to obtain the actual rates of enzyme synthesis.
This is because the fraction of critical resource that is allocated to either the overall growth or overall maintenance process is then further allocated among the individual growth and maintenance processes on each of the substrates present.

The rates of the cellular processes are not purely additive, because the enzyme levels and activity of the enzymes involved in the processes of growth, maintenance, and resource synthesis alter the actual rate observed. We consider the case of a culture growing at its maximum rate in a batch system containing saturating amounts of glucose and xylose to point out the workings of the model. Under these conditions, the cell grows exclusively on glucose. The expression for \( u_{G,1} \) (eq. (1)) causes all of the resource to be allocated to synthesizing only the growth enzyme for glucose: \( u_{G,1} = 1 \) and \( u_{G,2} = 0 \). This results in \( e_{G,1} = e_{G,1}^{\max} \) and \( e_{G,2} = 0 \) and consequently \( r_G = \mu_{G,1}^{\max} \). Also \( v_M = 0 \), and, therefore, the rate of maintenance processes is zero. This indicates that all the glucose is used in growth, and maintenance consumption is negligible. Further, because \( e_{G,i} = e_{R,i} \) and \( v_M = 1 \), all the resource is synthesized only from glucose metabolism. When the glucose is exhausted, the cell grows exclusively on xylose, and the reverse is true. Under low growth rate situations, the culture can grow on both glucose and xylose simultaneously. Both substrates are capable of supporting this low growth rate. Further, maintenance functions consume a significant portion of the substrate.

The allocation of resource to growth and maintenance functions is governed by eqs. 3 and 4, respectively. The resource allocated to growth is further allocated to the glucose growth enzyme and xylose growth enzyme according to eq. 1, while the resource allocated to maintenance is allocated to glucose and xylose low-maintenance enzymes according to eq. 5. This results in lower \( E_{G,i} \) and \( E_{M,i} \) values than if only one substrate were present. The rates of growth and maintenance on each of the substrates, therefore, are, correspondingly lower. Because the enzyme level affects the rate of resource synthesis and \( E_{G,i} = E_{R,i} \), resource is now synthesized from the metabolism of both sugars, and the resulting rate is lower than that from a strictly additive process. A similar argument holds for the rate of maintenance.

The mixed substrate model is essentially the composite of single substrate models for each of the substrates present together with cybernetic variables accounting for regulations. Hence, the parameters for the mixed substrate model are the same as those obtained from the single-substrate growth on each of the substrates present in the mixed environment. The set of kinetic constants connected with describing growth on a particular substrate identifies the substrate and the organism, and because all the regulation is embedded in the cybernetic variables, the single-substrate parameters are used in the mixed-substrate model. However, because the formulation of the rate of enzyme synthesis was altered to include a constitutive synthesis term due to inconsistencies in using a purely inducible enzyme synthesis term to describe mixed substrate transients, the exact value of this parameter \( a_{k,i}^{C} \) is fixed from mixed substrate experiments.

**MATERIALS AND METHODS**

The microorganism *K. pneumoniae* (ATCC 13832), obtained from the American Type Culture Collection (Rockville, MD), was used in all experiments. The preparation of the growth medium and inoculum, fermenter setup, and growth and sugar measurements are described in an earlier article.

**RESULTS AND DISCUSSION**

**Batch Culture**

The cybernetic model for growth on multiple substitutable substrates can be applied to describe growth in a batch reactor. The description of growth on two substrates requires balances on the growth enzyme, the low-maintenance enzyme, and the substrate for each individual substrate, and only a single balance for the substrate-independent resource. Because the parameter values for growth on a single substrate characterize that substrate and the organism, there are no extra or adjustable parameters in the earlier description of mixed substrate growth.

The results of a batch growth experiment on 0.9 g/L glucose and 0.7 g/L xylose are plotted in Figure 1 as are the predictions of the cybernetic model with the single substrate parameters for growth on glucose and xylose (Table I). The initial conditions for cell density and substrate concentration were specified by inoculum...
Table 1. Model parameter values for growth on glucose and xylose.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Glucose</th>
<th>Xylose</th>
</tr>
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<tr>
<td>( \mu_{\text{max}} )</td>
<td>h(^{-1})</td>
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<td>0.56</td>
</tr>
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<td>( \mu_{\text{max}}' )</td>
<td>h(^{-1})</td>
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<td>( \mu_{\text{max}}'' )</td>
<td>h(^{-1})</td>
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<td>0.40</td>
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<tr>
<td>( Y_{G} )</td>
<td>gdw/g</td>
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<td>0.39</td>
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<tr>
<td>( K_{G} )</td>
<td>g/L</td>
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<td>0.013</td>
</tr>
<tr>
<td>( K_{M} )</td>
<td>g/L</td>
<td>0.003</td>
<td>0.013</td>
</tr>
<tr>
<td>( K_{E} )</td>
<td>g/L</td>
<td>0.003</td>
<td>0.013</td>
</tr>
<tr>
<td>( K_{R} )</td>
<td>g/L</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>( K_{E,M} )</td>
<td>g/L</td>
<td>( 1 \times 10^{-6} )</td>
<td>( 1 \times 10^{-6} )</td>
</tr>
<tr>
<td>( K_{M} )</td>
<td>g/L</td>
<td>( 1 \times 10^{-6} )</td>
<td>( 1 \times 10^{-6} )</td>
</tr>
<tr>
<td>( a_{G}^{E} )</td>
<td>h(^{-1})</td>
<td>( 8 \times 10^{-5} )</td>
<td>( 5 \times 10^{-5} )</td>
</tr>
<tr>
<td>( a_{E} )</td>
<td>h(^{-1})</td>
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<td>0.001</td>
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<tr>
<td>( \alpha_{E,M} )</td>
<td>h(^{-1})</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>( \beta_{E} )</td>
<td>h(^{-1})</td>
<td>0.05</td>
<td>0.05</td>
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<td>( \beta_{E,M} )</td>
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<tr>
<td>( \beta_{R} )</td>
<td>h(^{-1})</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

level and substrate concentration used in the experiment. The initial level of the glucose growth enzyme was chosen to be 85% of the maximum glucose enzyme level, because the organism was precultured on glucose. The initial xylose growth enzyme level was taken to be 3% of the maximum value, given by the constitutive enzyme synthesis rate.

Results of the batch simulations were not sensitive to the initial values of the other components. The model predicts the initial growth on glucose until the glucose is completely consumed, followed by a lag period during which the xylose growth enzymes are synthesized, and then growth on xylose until all the substrate is exhausted. We see that the present model retains the ability of the previous cybernetic model to predict microbial growth on mixed substitutable substrates.

Continuous Culture

The general balance equations for the different components of the cybernetic model to describe growth on mixed substrates in a continuous culture system are:

\[
\frac{dc}{dt} = \left( \sum \frac{r_{G,i}}{v_{G,i} - D} \right) c \tag{13}
\]

\[
\frac{dc}{dt} = r_{E,i} - e \left( \beta_{E,i} + \frac{1}{c} \frac{dc}{dt} \right) \tag{14}
\]

\[
\frac{dc}{dt} = r_{E,M,i} - e_{M,i} \left( \beta_{E,M,i} + \frac{1}{c} \frac{dc}{dt} \right) \tag{15}
\]

\[
\frac{dR}{dt} = r_{R} - R \left( \beta_{R} + \frac{1}{c} \frac{dc}{dt} \right) \tag{16}
\]

\[
\frac{ds_{i}}{dt} = D(s_{E,i} - s_{i}) - \left( \frac{r_{G,i}v_{G,i}}{Y_{G,i} + r_{T,i}v_{M}} \right) c \tag{17}
\]

The rates of the individual reactions and the expressions for the cybernetic variables are calculated from the

Feed-Switching Experiment

The growth-limiting nutrient supply to a continuous culture of *K. pneumoniae* was changed from pure glucose \( (s_{G} = 0.5 \text{ g/L}) \) to pure xylose \( (s_{X} = 0.5 \text{ g/L}) \), while the dilution rate was kept constant. The behavior of the culture during the transient period of adaptation to the new environment was recorded and the results are plotted in Figure 2. The culture, which is initially growing on glucose at a constant rate of 0.38 h\(^{-1}\), is suddenly subjected to a change in growth-limiting substrate by a changing to a pure xylose feed. The cells, which have a high level of the enzymes required to catabolize glucose, quickly exhaust the residual glucose in the fermenter. When the glucose is exhausted, the culture, which lacks enzymes to catabolize the incoming xylose, essentially stops growing. Because the dilution rate is much larger than the growth rate, there is a washout of the culture from the fermenter vessel and a concomitant buildup of xylose concentration in the fermentor.

The cells, on encountering xylose in the environment, begin to synthesize the necessary enzymes to catabolize xylose. When the xylose enzymes buildup to a level that can support growth on xylose, the cells grow rapidly on xylose. There is a corresponding increase in the cell density that continues until the residual xylose concentration drops, and the culture grows at a rate equal to the dilution rate. The transient period, during which the culture adapts to xylose, lasts over 12 h. These results are similar to those reported by Standing et al.\(^8\)
The cybernetic model provides a reasonable prediction of the transient behavior of the culture following the switch in the growth-limiting nutrient, although it predicts a slightly faster recovery from washout and a slightly slower attainment of the new steady state than the experimental results. An explanation of the working of the cybernetic model is as follows: The cells, growing on glucose, possess a high level of the glucose growth enzyme that permits the residual glucose to be quickly consumed following the change in feed substrate. Once the glucose is exhausted, the cells stop growing, and consequently they are washed out of the fermenter. At the same time, the resource level in the cells decreases.

The presence of xylose induces the synthesis of the xylose enzymes required to catalyze the consumption of xylose by the low-maintenance and growth processes. However, because the cell is not growing, it allocates most of the resource toward the low-maintenance enzymes; they are, therefore, synthesized rapidly. The incoming substrate is primarily used for maintenance because maintenance requirements of the cell increase as the deficiency between the specific growth rate and its maximum value increases. During this period, there is a buildup of substrate in the environment and a gradual increase in the xylose growth enzyme and resource levels. This results in an increase in the growth rate and a reallocation of resources toward growth as opposed to maintenance.

The interaction among the enzyme levels, growth rate, and cybernetic variables is essentially autocatalytic, resulting in a rapid increase in the growth rate. Once the growth rate is higher than the dilution rate, the cells accumulate in the fermenter. This increase in cell density continues until the residual xylose level in the fermenter drops to low levels. The growth rate of the culture then decreases, and a steady cell density is reached. At this point, the growth rate of the culture is equal to the dilution rate. The cybernetic model, with a representation of distinct enzymes for the catabolism of different sugars and the interactions of metabolic regulation, is thus able to predict the transient behavior following a feed-switch.

The main objective of this experiment was to validate the inclusion of the constitutive rate of enzyme synthesis in the model formulation. Turner and Ramkrishna introduced the constitutive rate after they found that a purely inductive enzyme synthesis term was not capable of describing the utilization of xylose in a continuous culture following a switch from a pure glucose feed to a glucose/xylose feed. The model, without the constitutive term, would predict an extraordinarily long time for the cell to synthesize the xylose enzymes, because of the autocatalytic nature of the interactions between regulation and the cellular components. It was, therefore, argued that cells always maintain a low level of all enzymes, even those that are not immediately required.

The present formulation of the rate of enzyme synthesis essentially implies that the constitutive portion is not regulated while the inductive portion is subject to regulation. The feed-switching experiment described earlier does in fact show that the model, without the constitutive term, predicts that the culture would take a very long time to attain the new steady state. The model simulations are very sensitive to changes in the value of the constitutive rate of xylose enzyme synthesis ($\alpha_{x^+}$). Because the culture in the feed-switching experiment has never experienced xylose in its environment prior to the switch to xylose, the response time of the culture is indicative of the level of xylose growth enzyme present in a cell that has never grown on xylose before.

**Mixed-Feed Experiments**

The steady state behavior of *K. pneumoniae* growing in a continuous culture on a mixture of glucose and xylose was studied. The feed stream concentrations of glucose and xylose in the experiments were 0.31 g/L and 0.25 g/L, respectively. The steady-state cell density and residual xylose concentration at different dilution rates of such mixed-feed experiments are plotted in Figure 3. The residual glucose concentration was always below the detection limit of 10 mg/L of the glucose assay. The results show a simultaneous utilization of glucose and xylose at the low growth rates. At high dilution rates, the cells grow preferentially on glucose, and xylose leaves the fermentor without being utilized as seen by the high residual xylose concentrations. However, wall growth proved to be problematic in these mixed substrate experiments, and, therefore, it was not possible to exactly identify the dilution rate at which the culture switched from growing on both glucose and xylose to growing only on xylose. Measurements of xylose concentration in the fermentor indicated that above a dilution rate of 0.85 h⁻¹, no xylose was used by the culture.

The simulations of the cybernetic model are performed by setting the time derivative of the continuous

![Figure 3](image-url)  
**Figure 3.** Steady-state behavior of *K. pneumoniae* growing on 0.31 g/L glucose and 0.25 g/L xylose. Experimental results and cybernetic model simulations.
culture balance equations [eqs. (13-17)] equal to zero and solving for the levels of the different cellular components. The simulation results describe the observed experimental behavior (Fig. 3). The value of \( \alpha_{G,2}^* \), the constitutive rate of xylose growth enzyme synthesis, was chosen to be \( 5.0 \times 10^{-1} \) h\(^{-1} \). This value corresponds to 20% of the enzyme for growth on xylose being synthesized by the constitutive portion of enzyme synthesis compared to the 3% used in the single substrate model and the batch and feed-switching simulations. However, on accounting for the effect of wall growth within the cybernetic framework, the value of \( \alpha_{G,2}^* \) dropped to \( 2 \times 10^{-4} \) h\(^{-1} \), because the cells coming off the wall act as a source of enzymes, corresponding to 10% of the xylose enzyme being synthesized constitutively. Possible reasons for the discrepancy in the values used for \( \alpha_{G,2}^* \) in the model, i.e., 3% and 10%, are discussed in the last section. The values of all the other parameters were those obtained from the single substrate experiments (Table I). This parameter set was used in all the mixed-feed experiment simulations presented subsequently.

The model describes the simultaneous utilization of both glucose and xylose at the low growth rates and the preferential utilization of glucose at the high dilution rates. Simultaneous utilization at the low growth rates results in a higher cell density than at the high growth rates when preferential utilization of glucose is observed, even though substrate consumption for maintenance is high at low growth rates. The model also describes the experimental observation of less xylose utilization at all growth rates, as indicated by the higher residual xylose concentration in the fermenter. Either substrate is capable of supporting the low growth rates imposed on the cell by the low dilution rates; the cell, in maximizing the rate of substrate uptake, therefore uses both substrates. However, only glucose is capable of supporting growth at high dilution rates and, therefore, the cell grows exclusively on glucose at the higher growth rates.

Mixed substrate transient behavior of *K. pneumoniae* was studied by changing the dilution rate and recording the changes in cell density and residual substrate concentration until the next steady state was attained. The concentrations of glucose and xylose in the feed stream were 0.31 g/L and 0.25 g/L, respectively. The results of one such transient, in which the dilution rate was changed from 0.352 to 1.04 h\(^{-1} \), are plotted in Figure 4. The transient experiment shows the cell switching from simultaneous utilization of glucose and xylose at the initial growth rate of 0.352 h\(^{-1} \) to growing only on glucose at the high dilution rate of 1.04 h\(^{-1} \). The residual xylose concentration in the fermenter increases to 0.25 g/L, the feed concentration, indicating that no xylose is used by the cell at the high growth rate. The model simulation results that are plotted on the same figure show good agreement with the experimental behavior.

The working of the model and the effect of metabolic regulation in determining the observed behavior is understood by following the profiles of the internal components and the cybernetic variables. The culture, which is initially growing at 0.352 h\(^{-1} \), uses both glucose and xylose for growth and maintenance. The residual substrate concentrations are therefore low, and the glucose and xylose growth enzyme levels are high. The resource level in the cell at the low growth rate is also low. Because both substrates can support the low growth rate, the cell allocates the available resources toward the synthesis of both growth enzymes, although glucose enzyme synthesis is slightly favored over the xylose enzyme (due to the lower \( K_E \) value for glucose). Therefore, the values of the cybernetic variables, \( u_{G,1} \) and \( u_{G,2} \), are both approximately 0.5. The activities of the growth enzymes, \( v_{G,1} \) and \( v_{G,2} \), are also high, because the cell grows at the maximum rate possible under the given environmental conditions.

When the dilution rate is increased to 1.04 h\(^{-1} \), the residual concentration of the sugars in the fermenter increases. However, because the resource takes time to be synthesized, the enzyme levels increase slowly as does the growth rate. The cells wash out of the fermentor because the growth rate is less than the dilution rate. As the growth rate increases, glucose, which supports a higher growth rate than xylose, becomes the preferred substrate. Therefore, the value of \( u_{G,2} \) decreases, while the value of \( u_{G,1} \) increases. The activity of the glucose enzyme (\( v_{G,1} \)) remains high, and the activity of the xylose enzyme decreases. The resource level in the cell also increases. Because less of the xylose is used for growth, the residual xylose concentration in the fermentor increases. This sequence of interactions continues until the cell reaches the growth rate dictated by the imposed dilution rate. The cell density settles at the steady-state value, and the residual xylose concentration is equal to the feed concentration. Under these conditions, the cells grow only on glucose. The entire transient period lasted 4 h.
The cybernetic model also provides a good description of the transient behavior of a mixed-feed continuous culture following a shift-down experiment in which the dilution rate was decreased from 1.0 to 0.281 h\(^{-1}\) (Fig. 5). The culture during this experiment proceeds from a condition of growing exclusively on glucose to utilizing both glucose and xylose at the dilution rate of 0.281 h\(^{-1}\). There is an initial sharp rise in the cell density for about half an hour followed by a more gradual increase, until the biomass reaches a maximum level of 0.20 gdw/L in approximately 4 h. The cell density then decreases to the final value of 0.17 gdw/L in another 4 h. The xylose concentration, which is initially at the feed concentration of 0.25 g/L, drops to a value of 0.004 g/L over the first 4 h of the transient.

Following the changes in cell density, glucose and xylose concentrations in the fermentor, resource, and values of the cybernetic variables help explain the working of the cybernetic model during a step-down. The culture at the dilution rate of 1.0 h\(^{-1}\) is growing exclusively on glucose. The glucose growth enzyme level, the allocation of resource toward its synthesis, given by \(u_{G,1}\), and the enzyme activity, given by \(v_{G,1}\), are all high. In contrast, the xylose enzyme level and the cybernetic variables, \(u_{G,2}\) and \(v_{G,2}\), are low.

When the dilution rate is decreased to 0.281 h\(^{-1}\), the cells accumulate in the fermentor as the dilution rate is now less than the growth rate. The cells immediately use up the residual glucose in the fermentor, resulting in a sharp drop in the glucose concentration and a corresponding increase in cell density. As a result, there is a drop in the growth rate of the culture and the resource level in the cell. The drop in growth rate permits the cells to utilize the less-preferred substrate, xylose, which is present at high concentrations. The cell diverts resources toward the synthesis of the xylose growth enzymes as indicated by increasing \(u_{G,2}\) value, and the promotes the usage of the existing enzymes as indicated by the increased value of \(v_{G,2}\).

The culture now begins growing rapidly on xylose; this translates into an accumulation of cells in the fermentor, a leveling of the resource in the cell, and a decrease in the xylose concentration. The resource allocation toward the glucose enzyme, \(u_{G,1}\), and the activity of glucose enzyme, \(v_{G,1}\), drops. When the xylose level decreases to low values, the incoming feed of glucose and xylose is not sufficient to support the high cell density in the fermentor. The growth rate of the culture drops further, as seen by the reduced glucose concentration and reduced resource level. There is a concomitant washout of the culture followed by a stabilization at the new steady state. The cells completely utilize the incoming glucose and xylose for growth and maintenance functions.

The results of another set of step-up and step-down mixed substrate transient experiments are plotted in Figures 6 and 7. The model simulations show reasonable agreement with the experimental data. The step-up simulations describe the switch from simultaneous utilization of both substrates to growing preferential utilization of glucose after the step in dilution rate. Because only glucose supports the growth rate dictated by the high dilution rate (Fig. 6). The step-down simulations point to the cell synthesizing the xylose enzymes after the step-down and then growing on both glucose and xylose. The values of the model parameters used in all the simulations are the single-substrate growth parameters, except for the constitutive rate of xylose growth enzyme synthesis, which was changed to 5.0 \(\times\) 10\(^{-4}\) h\(^{-1}\). Additional results are presented by Baloo.\(^{1}\)

### Regulation of Enzyme Synthesis

The nature of metabolic regulation in cells subject to mixed substitutable substrates in the environment is

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**Figure 5.** Experimental data and cybernetic model simulations of a continuous culture with a mixed feed of glucose and xylose following a step-down in dilution rate from 1 to 0.281 h\(^{-1}\).

**Figure 6.** Experimental data and cybernetic model simulations of a continuous culture with a mixed feed of glucose and xylose following a step-up in dilution rate from 0.469 to 1.05 h\(^{-1}\).
quite complex. This regulation can occur at any or all of the following levels: (1) regulation of substrate uptake following a step-down in dilution rate from 0.869 to 0.296 h⁻¹, (3) regulation of enzyme activity. A detailed discussion of regulation at each of these levels has been presented by Harder and Dijkhuizen; therefore, we will restrict ourselves to discussing possible reasons for the very different response observed in the two types of mixed substrate continuous culture experiments resulting in different values of $aE_{2}$ in the model.

In the feed-switching experiment, the cells have never experienced xylose in the environment before the switch to xylose. In the mixed-feed experiment, however, the cells have previously grown on xylose and in fact encounter xylose in the environment continuously. They do not utilize the xylose at the high dilution rate because of metabolic regulation. In this case the specific mechanisms of regulation are catabolite repression and/or inducer exclusion. Even if xylose enzyme synthesis is not shut off by the above two regulatory mechanisms, the xylose catabolizing enzymes should be inhibited, because the cells do not use xylose at high dilution rates in the mixed-feed experiment. The cell typically stops synthesizing unused enzymes because the expensive nature of protein synthesis dictates that redundant protein synthesis is counterproductive, especially at high growth rates. We would, therefore, expect the level of the xylose enzymes to decrease over the time frame of a few generations in a continuous culture. However, the response of mixed-feed culture to a step-down in dilution rate indicates that the culture uses the xylose within a short period (1 h). This implies that cells possess a sufficiently high level of xylose enzymes at the high growth rate at the time of the shift.

The response of the feed-switching experiment indicates that the cells possess very low amounts of the xylose enzymes before the switch and therefore take a long time to synthesize sufficient quantities and then grow on xylose (6 h). It is this discrepancy in the xylose enzyme levels within the cells in the two situations that translates into different values for the constitutive rate of xylose enzyme synthesis from the modeling perspective.

The reasoning behind the very different response observed in the feed-switching experiment as compared to the mixed-feed experiment may be because the cell has grown previously on xylose and is continually encountering xylose in the environment in the mixed-feed experiment while it experiences xylose for the first time in the feed-switching experiment. The history of the cell could influence the cell to augment its xylose-enzyme-synthesizing machinery and therefore be able to synthesize the enzymes required to catabolize xylose the second time around.

Although there is no direct evidence of cells altering their enzyme-synthesizing capacity in the literature, there are examples of microorganisms regulating the synthesizing capacity of other cellular components such as the carbon overcompensation and phosphate overcompensation phenomena. These examples show that the cell is able to alter the synthesis rate of a cellular component after exposure to a period during which that particular component is required. A similar situation could be taking place in the case of xylose enzyme synthesis. The cells in the mixed-feed experiment have previously grown on xylose. Hence, when conditions become favorable for xylose utilization, following the step-down in dilution rate, the cell is able to synthesize the xylose enzymes faster than the low growth rate prior to the first dilution rate shift. From a modeling standpoint, an augmented enzyme synthesis capability would result in an increase in the growth enzyme synthesis rates.

The discrepancy in the values of $aE_{2}$ between the mixed-feed and feed-switching experiment could also result from an incomplete description of metabolic regulation in the cybernetic model. The regulatory mechanisms of inducer exclusion and catabolite repression are highly dependent on the concentration of glucose, the preferred substrate in the environment. In a batch culture, the initial glucose and xylose concentrations are typically high. Glucose represses the synthesis of the xylose enzyme system, and the cell grows exclusively on glucose.

When glucose levels drop to very low values (the glucose is essentially exhausted), repression of the xylose system is lifted, and the cell grows on xylose. The residual glucose concentration in a continuous culture is low except near the maximum dilution rate. This low concentration of glucose in the fermentor possibly may not be sufficient to cause complete inducer exclusion, i.e., prevent any xylose molecules from being transported into the cell. However, because the cAMP levels are usually very low at high growth rates, the xylose enzymes could be completely repressed by catabolite repression.
This interaction between the two regulatory mechanisms could allow for a partial synthesis of the xylose enzymes. The cell could have an intermediate level of xylose enzymes (rather than a very low level) at the high growth rates and respond quickly to the drop in dilution rate. The cybernetic framework with a low rate of constitutive enzyme synthesis (3%) would predict that the cell will not use xylose past the maximum growth rate on xylose. It does not allow for a partial synthesis of xylose enzyme at high growth rates. The xylose enzyme level is therefore very low, and the model predicts a slow response to a step-down in dilution rate in a mixed-feed experiment. A high constitutive rate of xylose enzyme synthesis (10%), however, corrects this problem by requiring an intermediate level of xylose enzymes to be present even at high growth rates.

CONCLUSIONS

In this article we have shown that transient mixed substrate continuous culture behavior is described by a cybernetic model that is direct extension of the single-substrate formulation. No new kinetic parameters are added in the mixed substrate formulation because all the effects of regulation arising from the presence of multiple substrates in the environment are represented by cybernetic variables. The values of all parameters in the mixed substrate model except for the constitutive rate of enzyme synthesis are the same as in the single-substrate model. We have identified two possible reasons for the difference in the \( \alpha_{E,2} \) value between the feed-switching and mixed-feed experiments: (1) a cell, grown previously on xylose, could augment the rate of xylose enzyme synthesis, and (2) an incomplete representation of the effects of regulation during a step-down in dilution rate in mixed-feed experiments could change the xylose enzyme level.

Only a better understanding of the phenomena leading to the observed transient response in the mixed-feed experiment and the exact nature of enzyme synthesis can identify the reason for the discrepancy. However, the cybernetic model does provide an accurate representation of the effects of regulation in all the other mixed-substrate situations studied both in this work and previously.\(^7\)\(^12\) Furthermore, only a small change in one parameter (from 3% to 10%) results in a quantitative agreement between the experiments and model simulations. The cybernetic model developed describes microbial growth on single and mixed substitutable substrates in batch, fed-batch, perturbed fed-batch, and continuous cultures.

References