Analysis of the optimal model for substrate substitutability in continuous microbial cultures

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Abstract

A comprehensive model for microbial growth in multiple substrate environment developed by Venkatesh et al. (1997, Biotechnol. Bioengng, 56, 635–644) is applied to growth in continuous cultures and analyzed at steady state. Analytical expressions are derived for substrate concentrations in terms of cellular and reactor parameters. These expressions are seen to describe the substrate utilization pattern and to identify regions of steady state and wash-out for a given feed concentration. These expressions also give the nature of the isoclines just based on simple manipulation of the model equations. The model predictions were compared with experimental data and were found to match reasonably well. Simulations were also performed to get steady-state concentrations of substrates with varying dilution rate. It was seen that in mixed substrate cultures, the system washes out at higher dilution rates than when they were growing on single substrates. The model was extended for microbial growth on various substrate pairs and also on a mixture containing three substrates.

Keywords: E. coli K12; Consumer-resource relationship; Carbon sources; Chemostat

1. Introduction

The growth of microorganisms in the presence of multiple nutrients is commonly encountered in nature and in fermentation industry. Kinetic models that accurately describe such growth processes are imperative for successful bioreactor design and operation. These models also bring out the essential features of microbial growth and hence are very often mandatory for understanding the behavior of microorganisms.

When microorganisms are grown in a multi-substrate environment, they can either utilize all the substrates at same time (simultaneous utilization) or utilize one substrate completely before beginning to utilize another (sequential utilization). Naturally, cell growth and product formation rates are altered depending on which of the two ways the microorganism prefers to grow. The substrate utilization pattern is dependent upon the exact internal metabolism of the cell. Numerous models have been attempted to adequately describe these mechanisms with an ulterior motive of predicting cell growth, substrate consumption and product formation. One of the most important among these is the Cybernetic model developed by Kompala et al. (1984). The Cybernetic perspective of modeling growth views the microorganism as an optimal strategist with the primary goal as maximization of growth rate. While the early cybernetic models were able to predict only sequential utilization successfully, the recent work of Ramakrishna et al. (1997), through a new expanded kinetic structure, is able to predict both sequential and simultaneous patterns of substrate utilization. However this model also introduces a large number of new parameters that are difficult to evaluate.

The Optimal model (Venkatesh et al., 1997; Doshi and Venkatesh, 1998) was able to capture both sequential and simultaneous utilization of substrates by posing microbial growth in multiple substrates as a problem of multi-variable constraint optimization. The predictions of the model were shown to match very well with experimental studies on substrate pairs: glucose–fumarate (sequential) and pyruvate–fumarate (simultaneous). The model was also validated for the case where substrate utilization is regulated at the genetic level – for glucose–lactose substrate pair regulated by lac-operon. The advantage of the
model is the use of very few parameters and a simple scheme for representing the complex cellular metabolic processes.

In this article, the optimal model is applied to the case of chemostat growth of Escherichia coli in the presence of multiple substrates. The predictions of the model with regard to the identity of the preferred substrate and the pattern of substrate relationships are presented for the whole range of operating conditions. These predictions are found to match well with the results of a similar study by Ramakrishna et al. (1997) in some cases and differ in others. Most importantly these predictions are obtained purely from analytical solutions of the model equations without resorting to simulations. Multiple substrate growth experiments were conducted in a chemostat to validate the model predictions about the location of the isoclines. Further generalization of the model equations to the case of growth on three substrates yields interesting results in the shape of constant growth surfaces.

2. Optimal model for multiple-substrate growth

The optimal Model (Venkatesh et al., 1997) for microbial growth is based upon the basic postulate that every substrate in a multi-substrate environment is utilized by the microorganism through an independent pathway consisting of several reactions in series but all these pathways result in the formation of a common intermediate. The common intermediate formed is further utilized through a pathway that in reality represents a number of metabolic reactions put together. The maximum flux that can flow through the common pathway is represented by $\mu_{\text{max}}$ and this is characteristic of a particular microorganism. The fluxes in the independent pathways for the substrates get regulated so that the specific growth rate is maximized while the constraint of maximum flux is not violated in the common pathway. This is shown schematically in the resistance network model of a cell (Fig. 1).

The rate expression for the growth on the $i$th substrate is given by

$$r_i = \mu_i X$$

where

$$\mu_i = \frac{e_i k_i}{e_i \max + s_i}.$$  

(2)

In a multiple substrate environment, because of the control of the metabolic network inside the cell, the flux flowing towards cell growth through a branch is regulated. Mathematically this is manifested in the optimization variable $z_i$ which represents the fraction of the flux towards growth from the $i$th substrate. Similarly the enzyme synthesis rate is also assumed to be regulated and hence regulation is proportional to the control parameter $z_i$.

Based on these basic postulates, the balance equations for substrates, enzyme levels and biomass for a continuous culture are as follows:

$$\frac{ds_i}{dt} = -\frac{z_i \mu_i X}{Y_i} + D(s_{i, f} - s_i),$$  

(3)

$$\frac{d}{dt} \left( \frac{e_i}{e_i \max} \right) = \frac{x_i (\mu_{m, i} + \beta) s_i}{k_i + s_i} - \frac{e_i}{e_i \max} (\mu + \beta),$$  

(4)

$$1 \frac{dX}{dt} = \mu - D,$$  

(5)

where

$$\mu = \sum z_i \mu_i.$$  

(6)

In the above equations $s_i$ represents substrate concentrations, $\mu_i$ is the specific growth rate on individual substrates, $z_i$ is the optimization variable for the $i$th pathway, $X$ is the total biomass, $D$ is the dilution rate, $s_{i, f}$ is the feed concentration of $i$th substrate, $e_i/e_i \max$ is the intracellular key enzyme level and $\beta$ is the rate of enzyme degradation.

The maximization criterion is given as follows:

$$\max(\mu) = \max \left( \sum z_i \mu_i \right)$$

such that, $0 \leq z_i \leq 1$ and $\sum z_i \mu_i \leq \mu_{\text{max}}$.

For the case of growth on two substrates the optimization procedure yields the following values for the control variables:

$$\mu_1 + \mu_2 \leq \mu_{\text{max}}$$

if $\mu_1 + \mu_2 \leq \mu_{\text{max}}$

then $z_1 = z_2 = 1$,
while if \( \mu_1 + \mu_2 \geq \mu^\text{max} \) and \( \mu_1 > \mu_2 \) \( \text{(10)} \)
then \( \alpha_1 = 1 \) and \( \alpha_2 = \frac{(\mu^\text{max} - \mu_1)}{\mu_2} \) \( \text{(11)} \)

This represents that the cell would prefer a substrate with a higher flux towards growth and would proportionately reduce the flux in the other branch by metabolic control.

3. Materials and methods

_E. coli_ K12 (MTCC Catalogue #1302) was obtained from the Microbial Type Culture Collection, IMTECH, Chandigarh, India, and was used in all the experiments. The detailed protocol for medium and inoculum preparation is given by Venkatesh et al. (1997).

Fermentations were conducted in a 1 l fermenter with an initial culture volume of 500 ml. The pH of the medium was adjusted to 6.0. For the low concentration of carbon medium and cell concentration used, it was not necessary to control pH. The fermenter was aerated with enriched air. Fermentations were conducted in an aerobic environment with pre-culturing on glucose. The data from these experiments was used to characterize parameters for single substrate growth as given in Table 1 (Venkatesh et al., 1997).

Chemostat experiments were also conducted in the same fermentor with varying inlet feed rates to fix different values of dilution rates. The feed medium contained 5 g/l of glucose. At each dilution rate, the chemostat was allowed to reach a steady state and after which the feed was switched to a medium containing known quantity of the substrates (glucose, lactate, acetate and pyruvate taken two at a time). The sample was withdrawn for analysis to ascertain if the system reaches steady state or washes out. Chemostat experiments for steady-state analysis were conducted twice. It was also noted that the system washes out beyond a dilution rate of 0.76 and hence all the experiments were done for dilution rates lower than this value. The chemostat experiments were not analyzed for exact substrate concentrations at steady state since the substrate concentration would be too low. Instead, the attainment of steady state was established by measuring the cell concentration as OD.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glucose</th>
<th>Acetate</th>
<th>Pyruvate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \mu^\text{a} ) (h⁻¹)</td>
<td>0.72</td>
<td>0.32</td>
<td>0.27</td>
<td>0.68</td>
</tr>
<tr>
<td>( K_i ) (g/l)</td>
<td>0.04</td>
<td>0.1</td>
<td>0.01</td>
<td>0.43</td>
</tr>
<tr>
<td>( Y_{X/g} ) (gm X/gm S)</td>
<td>0.446</td>
<td>0.12</td>
<td>0.155</td>
<td>0.8</td>
</tr>
<tr>
<td>( \beta ) (h⁻¹)</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

4. Analysis of the model at steady state

4.1. Optimization variables

During growth, the optimization variables \( \alpha_i \)'s either all take the value of unity or adjust in such a way so as to make the overall growth rate \( \mu \) match the maximum growth rate possible for the microbe. However in the case of a chemostat there is an additional constraint imposed that the steady state \( \mu \) should also match the dilution rate \( D \). Mathematically at steady state (for two substrates),

\[ \alpha_{1ss} \mu_{1ss} + \alpha_{2ss} \mu_{2ss} = D. \]  

(10)

There are two possible ranges of dilution rates. Considering first the case when \( D < \mu^\text{max} \), two possibilities arise

1. If \( \mu_{1ss} + \mu_{2ss} \leq \mu^\text{max} \), \( \alpha_1 = \alpha_2 = 1 \)
2. If \( \mu_{1ss} + \mu_{2ss} > \mu^\text{max} \), \( \mu^\text{max} = D \), which is not true as \( \mu^\text{max} > D \) is assumed.

Hence, for all dilution rates \( D < \mu^\text{max} \), \( \alpha_{1ss} = 1 \).

As mentioned earlier, \( \alpha_i \) is the fraction of the maximum flux that flows through the path \( i \). If \( \alpha_i \) is unity, the flux in that pathway is equal to the instantaneous maximum flux allowable in that particular pathway. Physically this means that, at steady state, the cell wants to utilize the substrates at the maximum possible rate and prefers an unconstrained state. This result can also be interpreted as a state wherein the cells would rather not produce enzymes and deactivate it (that is \( \alpha_i < 1 \)). Similar behavior is not possible for batch growth as in a batch system the conditions change continuously with time. This indicates that in a chemostat fermentation one need not apply the optimal strategy. At steady state, the equality between the dilution rate and the specific growth rate itself would fix the enzyme concentrations.

If the dilution rate is such that it exceeds the maximum possible flux for that cell (\( D > \mu^\text{max} \)) then again two cases might exist

1. If \( \mu_{1ss} + \mu_{2ss} < \mu^\text{max} \)
   then, \( \mu_{1ss} + \mu_{2ss} = D \)
   and, \( \alpha_{1ss} \mu_{1ss} + \alpha_{2ss} \mu_{2ss} < D \)
   But for this case it has been assumed that \( D > \mu^\text{max} \)
   and so this condition cannot arise.

2. If \( \mu_{1ss} + \mu_{2ss} > \mu^\text{max} \)
   then, again \( D = \mu^\text{max} \)
   which is again not permitted according to the assumption made (\( D > \mu^\text{max} \)).

Thus the case of \( D > \mu^\text{max} \) is not permitted at all indicating that dilution rates beyond the maximum possible limiting flux through the common pathway cannot be sustained. This is an easily foreseeable conclusion that follows immediately from the model assumptions and the
above discussion is just a mathematical formalism for expressing it.

4.2. Analytical expressions for substrate relationship patterns

Having proved that the optimization variables achieve the value of unity for all $D < \mu_{\text{max}}$, we now proceed to derive analytical expressions which yield directly the substrate relationship patterns.

At steady state the enzyme and cell mass balance equations yield:

$$
\frac{d}{dt} \frac{e_i}{e_{i,\text{max}}} = \frac{g_i(\mu_{m,i} + \beta)s_i}{k_i + s_i} - \frac{e_i}{e_{i,\text{max}}(\mu + \beta)} = 0,
$$

(13)

$$
\frac{1}{X} \frac{dX}{dt} = \mu - D = 0.
$$

(14)

Putting the expression from Eq. (14) for $\mu$ into the above equations yields:

$$
\frac{e_i}{e_{i,\text{max}}} = \frac{(\mu_{m,i} + \beta)s_i}{(k_i + s_i)(\beta + D)}.
$$

(15)

Also,

$$
D = \sum_i k_i \mu_i = \sum_i \frac{e_i}{e_{i,\text{max}} s_i}.
$$

(16)

Using Eqs. (15) and (16) in Eq. (14) gives

$$
\sum_i \frac{\mu_i(\mu + \beta)s_i^2}{(k_i + s_i)^2} = D(\beta + \beta).
$$

(17)

Eq. (17) is the basic equation of the substrate relationship between the multiple substrates $s_i$'s. It holds in itself information about the pattern of substrate utilization, the identity of the preferred substrate and the range of sustainable dilution rates for the multiple substrate growth. For the two substrate case it becomes

$$
\frac{\mu_{m,1}(\mu_{m,1} + \beta)s_1^2}{(k_1 + s_1)^2} + \frac{\mu_{m,2}(\mu_{m,2} + \beta)s_2^2}{(k_2 + s_2)^2} = D(\beta + \beta).
$$

(18)

Plots of residual substrate concentrations, $s_1$ vs. $s_2$, at a fixed dilution rate obtained from Eq. (18) are the substrate relationship curves for the substrate pair. These have been named Zero Net Growth Isoclines (ZNGIs) by Ramakrishna et al. (1997) and represent the pattern of steady-state substrate concentrations at any sustainable dilution rate. A brief description of the significance of different isoclines is presented in the next section.

5. Representation of substrate utilization through isoclines

5.1. The concept of isoclines

The concept of ZNGIs is based on the idea of indifference curves in micro-economic theory that applies to the case of a consumer having two resources to choose from. A given indifference curve represents a particular level of satisfaction for a consumer and anywhere along it the consumer derives the same benefit. When applied to a microbe growing in the presence of a substrate pair an isocline represents a constant growth rate curve. The microbe is seen as a consumer making a conscious choice between the various available substrates that are its resources. Thus the same growth rate might be maintained at several different substrate pair combinations. In practice, this can be achieved in a chemostat as at steady state, the specific growth rate can be maintained at a constant value.

Ramakrishna et al. (1997) have classified ZNGIs into six distinct patterns (Fig. 2) which are described below briefly:

1. **Perfectly substitutable** — Growth may be supported on either substrate ($s_1$ or $s_2$) alone and $s_1$ and $s_2$ are replaceable in direct proportion.
2. **Complementary** — Growth is again sustainable on either substrate alone but here lesser amounts of $s_1$ and $s_2$ can sustain the same growth as compared to the perfectly substitutable case. Hence there is a synergistic effect.
3. **Switching** — Though $s_1$ and $s_2$ can individually sustain the growth rate, both are limiting substrates in different regions.
4. **Active** essential, (F) hemi-essential.

![Fig. 2](Image)

Fig. 2. Schematic representation of consumer-resource relationships in a multiple resource scenario (Ramakrishna et al., 1997). (A) Perfectly substitutable, (B) complementary, (C) switching, (D) essential, (E) interactive essential, (F) hemi-essential.
4. **Essential** – Both $s_1$ and $s_2$ are required for sustaining the growth rate but there are distinct regions where each is the limiting substrate.

5. **Interactive essential** – This is the same as essential except for substitutability between $s_1$ and $s_2$ leading to a synergistic effect again.

6. **Hemi-essential** – Growth is sustainable on one substrate ($s_1$) alone but not on the other ($s_2$)

Classification of the behavior of any substrate pair under the given cases yields a lot of information about the relationship between the substrates.

5.2. **Optimal model predictions**

As already discussed, Eq. (18) is the basis for the isoclines between two substrates in a chemostat. We now explore the behavior of this equation for the boundary values of $s_1$ and $s_2$. There is no loss of generality in assuming that $\mu_{m,1} > \mu_{m,2}$.

When $s_2 \to 0$, Eq. (18) yields

$$s_{10} = \frac{k_1}{1 - \frac{\sqrt{D(D + \beta)}}{\mu_{m,1}(\mu_{m,1} + \beta)}}$$  \hspace{1cm} (19)

$s_{10}$ represents the intercept of the isocline on the 1st substrate axis and its existence signifies that the substrate $s_1$ will be independently able to sustain growth for the given dilution rate. If the condition that $s_{10}$ can take only non-negative values is imposed on Eq. (18), we get for $s_1$ vs. $s_2$ curve to touch the $s_1$ axis ($s_2 = 0$),

$$\mu_{m,1}(\mu_{m,1} + \beta) > D(D + \beta).$$  \hspace{1cm} (20)

It can be shown that this condition implies $\mu_{m,1} > D$. A similar analysis for the case when $s_2 \to 0$ gives

$$\mu_{m,2} > D.$$  \hspace{1cm} (21)

So for the perfectly substitutable/complementary case in which either substrate can individually support growth we need to have

$$\mu_{m,1} > \mu_{m,2} > D.$$  \hspace{1cm} (22)

Another extreme case is that of one substrate, $s_2$, achieving very high values as compared to the other, $s_1$. In other words the isocline is asymptotic to the $s_2$ axis. For this case we have $s_2 \gg k_2$ which yields,

$$s_1^* = \frac{k_1}{\sqrt{\mu_{m,1}(\mu_{m,1} + \beta) - \mu_{m,2}(\mu_{m,2} + \beta)}}.$$  \hspace{1cm} (23)

Again if $s_1^*$ exists, the isocline is asymptotic to the $s_2$ axis and for this case

$$D(D + \beta) > \mu_{m,2}(\mu_{m,2} + \beta).$$  \hspace{1cm} (24)

It can be shown with some algebra that this implies $D > \mu_{m,2}$. Thus for this case, both $s_1^*$ and $s_2^*$ exist and the curve is asymptotic to both the axis showing the essential nature of the substrates. Hence, by similar arguments, it can be shown that if $\mu_{m,1} > D > \mu_{m,2}$ the curve will be hemi-essential type touching the $s_1$ axis but asymptotic to the $s_2$ axis.

Fig. 3 shows the experimental verification of the optimal model predictions for acetate–pyruvate and glucose–acetate substrate pairs. As discussed above, the indifference curves demarcate regions of attainment of a steady-state and of wash-out. If the feed concentration lies above the curve, the system attains a steady state with steady state concentrations defined somewhere along the isocline and if the feed concentration lies below the curve, the substrate concentrations are not sufficient to sustain growth and result in wash-out. In all the cases, $\bigcirc$ denotes the feed concentration that attains a steady state and $\bigtriangleup$ denotes the feed concentration that results in wash-out. It was observed that the system washes out for dilution rates more than 0.76 h$^{-1}$, indicating that the maximum capacity of the cell is indeed the maximum growth rate on glucose (Table 1).

Fig. 3a–c represent the three different regimes for acetate–pyruvate substrate pair. When the dilution rate, $D$ (=0.22 h$^{-1}$) is lower than $\mu_{m}$, of pyruvate (=0.27 h$^{-1}$) and acetate (=0.32 h$^{-1}$), the substrate pair is complementary (Eq. (22) and Table 2) with both the substrates capable of sustaining growth independently (Fig. 3a). By substituting appropriate values in Eq. (19), it can be shown that if for acetate to sustain growth independently, a minimum feed concentration of 0.243 g/l is required while this value for pyruvate is 0.048 g/l. When the dilution rate $D$ (=0.3 h$^{-1}$) is between the $\mu_{m}$ of acetate and pyruvate, the substrate pair is hemi-essential (Table 2) with acetate capable of sustaining growth independently but not pyruvate. Eq. (23) predicts the minimum feed concentration of acetate required for the system to attain steady state. This value, $s_1^*$ is 0.066 h$^{-1}$ (Fig. 3b). As the dilution rate is increased further to values more than $\mu_{m}$ of both the substrates (Fig. 3c), the system shifts to interactive-essential case wherein both the substrates are needed for sustaining growth. The minimum feed concentrations required to sustain growth are respectively 0.559 and 0.036 g/l for acetate and pyruvate. The mixed substrates wash-out together at a higher dilution rate than either of the substrates alone. This value is determined by the feed substrate concentrations and the substrate pair will continue to remain essential till this dilution rate is reached.

An interesting observation is noted for glucose–acetate pair wherein the maximum allowable dilution rate is the same as the maximum growth rate on one of the substrates namely glucose. In this case for $D < \mu_{m,2}$, expectedly, both the substrates can independently sustain growth (complementary). When dilution rate is increased beyond
Fig. 3. Isoclines for representative dilution rates. ○ and △ represent feed concentration that result in steady state and wash-out, respectively. (a) acetate–pyruvate pair at $D = 0.22 \, \text{h}^{-1}$ (b) acetate–pyruvate pair at $D = 0.3 \, \text{h}^{-1}$ (c) acetate–pyruvate pair at $D = 0.39 \, \text{h}^{-1}$ (d) acetate–glucose pair at $D = 0.46 \, \text{h}^{-1}$.

<table>
<thead>
<tr>
<th>Dilution rate</th>
<th>Nature of isocline</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D &lt; \mu_{m,2} &lt; \mu_{m,1}$</td>
<td>Complementary</td>
</tr>
<tr>
<td>$\mu_{m,2} &lt; D &lt; \mu_{m,1}$</td>
<td>Interactive-essential</td>
</tr>
<tr>
<td>$\mu_{m,2} &lt; \mu_{m,1} &lt; D$</td>
<td>Washout</td>
</tr>
<tr>
<td>$\mu_{m,2} &lt; \mu_{m,1} &lt; D^{*} &lt; D$</td>
<td>Washout</td>
</tr>
</tbody>
</table>

The values for the boundary concentrations of the two substrates are obtained by use of the parameter values obtained by batch experiments on *E. coli* presented in Table 1. These are listed along with the representative ranges of dilution rates in Table 3 for two substrate pairs: acetate–pyruvate and glucose–acetate.

6. Prediction of wash-out from model equations

In a chemostat there is a limit to the dilution rate sustainable by the growing microbe in the presence of multiple substrates. This limit is dependent on the substrate pair sustaining growth and their feed concentrations. Of the substrate pairs considered the pair acetate–pyruvate is analyzed first. The occurrence of
rate is given by significantly high (rate is found to be \( E \) for washout of the pair acetate—pyruvate. Mathematically (for wash-out. Mathematically (for initial substrate concentrations become simply the feed concentrations. Substitution of these steady-state substrate concentrations in Eq. (18) yields the dilution rate at wash-out. Mathematically (for \( D < \mu_{\text{max}} \))

\[
\frac{\mu_{m,1}(\mu_{m,1} + \beta) s^2_f}{(k_1 + s_{1f})^2} + \frac{\mu_{m,2}(\mu_{m,2} + \beta) s^2_f}{(k_2 + s_{2f})^2} = D^{\text{cr}}(D^{\text{cr}} + \beta).
\]

(25)

Solution of the above equation yields the dilution rate for washout of the pair acetate—pyruvate. On substituting the parameter values for growth on \( E. coli \) this dilution rate is found to be \( D = 0.403 \text{ h}^{-1} \) for \( s_{1f} = 1 \text{ g/l} \). For the limiting case of the substrate feed concentrations being significantly high (\( s_{1f} \gg k_1 \)) the maximum limiting dilution rate is given by

\[
\mu_{m,1}(\mu_{m,1} + \beta) + \mu_{m,2}(\mu_{m,2} + \beta) = D^{\text{cr}}(D^{\text{cr}} + \beta).
\]

(26)

which yields the value of \( D = 0.428 \text{ h}^{-1} \) on substituting the parameters. Thus the maximum possible sustainable dilution rate on acetate—pyruvate pair is obtained as above by simple manipulation of the model equations. An important observation for this substrate pair is that the individual fluxes in the two parallel metabolic pathways are the limiting quantities that determine this maximum sustainable dilution rate.

For the pair glucose—acetate the flux that limits the maximum sustainable dilution rate is that through the common metabolic pathway (\( \mu_{\text{max}} \)). This is because the individual fluxes in the two branches are large enough due to the large specific growth rates on the individual substrates. Mathematically, for initial substrate concentrations \( s_{1f} = 1 \text{ g/l} \), the flux limited dilution rate comes out to be \( D = 0.784 \text{ h}^{-1} \). Eq. (25) is valid only for \( D < \mu_{\text{cr}} \), and for \( D > \mu_{\text{max}} \), irrespective of the combination of substrates that is used, there will be a wash-out.

That is, as soon as dilution rate exceeds \( D^{\text{cr}} = \mu_{\text{max}} \) (= 0.76 h⁻¹) the flux through the common metabolic pathway becomes limiting and there is a wash-out. Hence in mixed culture the substrates glucose—acetate washout at \( D^{\text{cr}} > \mu_{\text{max}} \).

Simulations were also done to get the variation of residual steady-state concentrations of the two substrates with dilution rate. Fig. 4 shows the variation of steady state substrate concentrations with variation in dilution rate for the substrate pair glucose and acetate. The wash-out of acetate is shifted from a value of around 0.29 h⁻¹ in single growth to a value of 0.76 h⁻¹ in the presence of glucose. This shows that the presence of glucose is able to sustain acetate at higher dilution rates in a chemostat mixed culture. However the residual concentration of acetate at any dilution rate is more than that for glucose indicating that glucose is the preferred substrate. The values of wash-out dilution rates obtained from this plot are seen to be the same as predicted from the initial theoretical analysis. This was verified by carrying out experiments at \( D = 0.29 \text{ h}^{-1} \) for acetate alone as the feed substrate, which resulted in a washout. Whereas experiments at \( D = 0.65 \text{ h}^{-1} \) with glucose and acetate in the medium resulted in a steady state. This demonstrates that acetate can sustain higher dilution rates in presence of glucose.

6.1. Constant growth circles and spheres

A new representation of substrate relationships is possible based on the optimal model equations which incorporates not only the pattern of the isoclines but also clearly outlines the permissible range of dilution rates for a given substrate pair. Proceeding from Eq. (18), if enzyme degradation, \( \beta \), is neglected considering its small

<table>
<thead>
<tr>
<th>Dilution rate (h⁻¹)</th>
<th>( s_{10} )</th>
<th>( s_{20} )</th>
<th>( s^*_1 )</th>
<th>( s^*_2 )</th>
<th>Isocline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate—pyruvate</td>
<td></td>
<td></td>
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<tr>
<td>( D = 0.22 )</td>
<td>0.2428</td>
<td>0.0485</td>
<td>–</td>
<td>–</td>
<td>Complementary</td>
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<td>( D = 0.30 )</td>
<td>1.6156</td>
<td>–</td>
<td>0.0656</td>
<td>–</td>
<td>Hemi-essential</td>
</tr>
<tr>
<td>( D = 0.39 )</td>
<td>–</td>
<td>–</td>
<td>0.5591</td>
<td>0.0364</td>
<td>Interactive-essential</td>
</tr>
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<td>Glucose—acetate</td>
<td></td>
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<td>( D = 0.46 )</td>
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<td>0.0307</td>
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<td>Glucose—lactose</td>
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<td>( D = 0.52 )</td>
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<td>Hemi-essential</td>
</tr>
<tr>
<td>( D = 0.72 )</td>
<td>0.0744</td>
<td>–</td>
<td>0.2678</td>
<td>–</td>
<td>Hemi-essential</td>
</tr>
</tbody>
</table>

Table 3
Model predictions for Isocline Behaviour at Limiting Conditions

Fig. 4. Variation of steady-state concentration with dilution rate (\( D \)) for acetate and glucose – in single substrate and multiple substrate cultures.

[Image 312x538 to 545x732]
magnitude when compared with the operating dilution rates this equation yields
\[
\frac{\mu_{m,1}s_1^2}{(k_1 + s_1)^2} + \frac{\mu_{m,2}s_2^2}{(k_2 + s_2)^2} = D^2
\] (27)
or
\[
\mu_1^2 + \mu_2^2 = D^2
\] (28)
where, \( \mu_i = \mu_{m,i} s_i / (k_i + s_i) \) are new specific growth rates defined without the enzyme concentrations.

It can be noted from Eq. (15) (neglecting \( \beta \)) that
\[
e_i / e_i^{max} = \mu_i / D.
\] (29)
By multiplying \( e_i / e_i^{max} \) on both sides of the above equations,
\[
(e_i / e_i^{max})^2 = \mu_i / D.
\] (30)
This indicates that the square of the specific enzyme concentration is equal to the ratio of growth rate on that specific enzyme to the overall growth rate. The enzyme pool synthesized at steady state is to the extent of its contribution to the overall growth rate. Summing over all the enzymes, Eq. (30) will simplify to
\[
\sum_i (e_i / e_i^{max})^2 = \sum_i \mu_i / D = 1.
\] (31)
Eq. (31) is in terms of the specific enzyme concentrations and is another form of Eq. (28).

Eq. (28) shows that a plot of \( \mu_1 \) vs. \( \mu_2 \) for a certain dilution rate is an arc of a circle with radius as \( D \). The limit of permissibility of range for this curve is determined by two factors. Firstly, as is obvious, only the first quadrant is allowed as the values of the specific growth rates have to be non-negative. Secondly, the span of the arc is further limited by proceeding to higher dilution rates as the substitutable nature of first one and then the other substrate is lost and eventually both of them become essential. The span of the arc diminishes to zero when the maximum sustainable dilution rate is reached and beyond this there is a wash-out.

Constant growth arcs obtained for the pair acetate vs. pyruvate (Fig. 5a) illustrate the above points. At low dilution rates, the plot of \( \mu_1 \) vs. \( \mu_2 \) is a complete arc touching both the axis indicating the complementary nature of the substrate pair. As dilution is increased beyond \( \mu_m \) of pyruvate (\( D = 0.27\ h^{-1} \)), the arc detaches itself from the pyruvate axis and on further increase in dilution rate (\( D = 0.32\ h^{-1} \)), the arc detaches from the acetate axis also. The former case represents the hemi-essential nature of the substrate pair while the latter, the essential nature. On substituting \( \beta = 0 \) in Eq. (18), it can be seen that the critical dilution rate at which wash out occurs is given by
\[
D^{cr} = \sqrt{\mu_1^2 + \mu_2^2}.
\] (32)

On substituting appropriate values, we get \( D^{cr} \) to be 0.42 \( h^{-1} \). This is same as the length of the diagonal of the rectangle with sides as \( \mu_m \)'s of the two substrates in which the arcs are bounded.

Similar analysis for glucose–acetate substrate pair was done and the results are shown in Fig. 5b. The arcs in this case are bounded on one side by the \( \mu_m \) of pyruvate and on the other side by the arc \( D = 0.76 \). In this case, at low dilution rates, the curves touch both the axis showing the complementary nature. As the dilution rates are increased, the curves detach from the acetate axis but remain attached to glucose axis indicating that glucose alone can sustain growth but not acetate. So, till \( D^{cr} \), the substrate pairs are just hemi-essential.

The analysis of the case of three substrates is exactly analogous to that for two substrates. For the three substrate case the optimization variables are again all equal to unity at steady state. Analogous to Eq. (28) we get
\[
\mu_1^2 + \mu_2^2 + \mu_3^2 = D^2.
\] (33)

Thus plots of \( \mu_1, \mu_2, \mu_3 \) for different \( D \) are spheres with radii being given by \( D \). Again the range of the permissible surface segment keeps decreasing with increasing dilution rates.
7. Substrate relationships for glucose–lactose

It has been experimentally observed in a number of studies (von Hippel et al., 1974) that for the growth on the substrate pair glucose–lactose, the presence of glucose catabolically represses synthesis of lactose metabolizing enzymes by lac-operon. As a result, the inducible part of enzyme synthesis for lactose is completely switched off. However there is constitutive enzyme synthesis for lactose which causes enzyme growth at a constant rate \( \mu_0 \) which is small compared to the rate of inducible enzyme synthesis. For glucose however the irreducible part of enzyme synthesis is much larger than its constitutive part (Baloo et al., 1991). It is thus reasonable to assume only the inducible part of enzyme synthesis operating for glucose. These assumptions can be represented in the form of enzyme synthesis equations as follows:

\[
\frac{d e_1}{dt} = \frac{\beta s_1}{k_{s,1} + s_1} - \frac{e_1}{e_{1,\text{max}}} (\mu + \beta), \quad (34)
\]

\[
\frac{d e_2}{dt} = \frac{\mu_0}{\frac{\mu_0}{e_{2,\text{max}}} (\mu + \beta)}. \quad (35)
\]

The steady-state equations are solved by equating the time derivatives to zero. On rearranging, as in the previous case, we get the equation of isocline to be

\[
\frac{\mu_{m,1}(\mu_{m,1} + \beta)s_1^2}{(k_{s,1} + s_1)^2} + \frac{\mu_{m,2}s_2}{k_{s,2} + s_2} \mu_0 = D(D + \beta). \quad (36)
\]

Experiments were performed at different dilution rates with glucose–lactose substrate pair. The \( s_1 \) and \( s_2 \) values so measured and other parameter values were substituted in the above equation and a best fit for \( \mu_0 \) was obtained. This value was found to be 0.13 h\(^{-1}\).

Neglecting \( \beta \), Eq. (36) reduces to

\[
\mu_{1,\text{max}}^2 + \mu_0 \mu_{2,\text{max}}^2 = D^2. \quad (37)
\]

Inspection the above equation clearly shows that a plot of \( \mu_1 \) vs. \( \mu_2 \) is a parabola at a given dilution rate for sequential utilization. This is in contrast to the previous case, that is for simultaneous utilization wherein the plot of \( \mu_1 \) vs. \( \mu_2 \) is a circle. The isoclines (defined by Eq. (36)) show two distinct patterns depending upon the dilution rate. As in the previous cases, these regions can be classified as

for \( D < \sqrt{\mu_{m,2}\mu_0} < \mu_{m,1} \), complementary,

for \( \sqrt{\mu_{m,2}\mu_0} < D < \mu_{m,1} \), hemi-essential.

These predictions were validated by experiments and the results are shown in Fig. 6a and b. The experimental dilution rates fall in the hemi-essential region and this is brought out in the figures. Fig. 7 shows the simulation results for the steady-state substrate concentrations with variation in dilution rate for glucose–lactose pair. In mixed substrate culture, it can be seen that glucose is consumed preferentially over lactose for entire range of dilution rates. The residual concentration of lactose in mixed culture is much more compared to that of glucose (Table 3). It can also be seen that lactose in mixed substrate growth washes out along with glucose at \( D = 0.76 \) h\(^{-1}\) as against growth on lactose alone. It is to be noted that at low dilution rates, maintenance plays an important role and the actual substrate concentrations will be expected to be lower than that of the present simulated values.

8. Substrate relationships for three substrates

Proceeding on lines analogous to the case of two substrate growth, substrate relationship curves for growth in the presence of three substrates can be represented by an
analytical equation given by

\[
\frac{\mu_{m,1}(\mu_{m,1} + \beta)s_1^2}{(k_1 + s_1)^2} + \frac{\mu_{m,2}(\mu_{m,2} + \beta)s_2^2}{(k_2 + s_2)^2} + \frac{\mu_{m,3}(\mu_{m,3} + \beta)s_3^2}{(k_3 + s_3)^2} = D(D + \beta).
\]

Eq. (38) represents a surface which is the three-dimensional manifestation of an isocline. Such surfaces are christined Zero Net Growth Surfaces (ZNGSs) and represent the locus of steady states for three substrate growth in chemostat. Plots of Eq. (38) for the substrate trio glucose–acetate–pyruvate were plotted for three representative dilution rates and these gave the expected surface topology. For a dilution rate that is lower than the maximum sustainable growth rate on either of the three substrates, any one of them can sustain growth on its own and the ZNGS is seen to touch all the three axes (Fig. 8). Since the \( D \) value was maintained at \( 0.22 \, h^{-1} \), from Table 1 it is clear that the cells can sustain on all three substrates. Chemostat experiments on feeds with three substrates have shown to match with the Zero Net Growth Surfaces. Feed points below the surface results in wash-out, while points above reaches a steady-state point on the surface. As the dilution rate is increased the substitutable nature of first pyruvate and then acetate is lost. As argued earlier, beyond a dilution rate of \( D = \mu_{\text{max}} \) there is a washout and no growth can be sustained.

9. Conclusions

The present work has looked at some aspects of substrate utilization in multiple-substrate chemostat growth. Through steady-state analysis of the model equations, analytical expressions have been derived for substrate relationship curves. Based on these expressions, predictions of the pattern of the substrate relationship isoclines have been made. The representative regions of the dilution rates where a particular isocline persists have also been identified by looking at the boundary values of the isoclines. The trends found match to a great extent with those of the cybernetic model (Ramakrishna et al., 1997). It is seen that for a given substrate pair the nature of the isocline varies with dilution rate. Further, even for a given dilution rate it is seen that the identity of the rate-limiting substrate is dependent on the substrate feed concentrations. Some trends that were demonstrated
through *cybernetic* model (for example substitutability of glucose–fumarate pair) did not perfectly match with our results, although similar trends were obtained. A new representation for demonstrating trends in chemostat growth has been cited. From the model equations, plots of specific growth rates without enzyme levels are shown to represent effectively the substrate relationships. These constant growth circles and spheres not only characterize the patterns of substrate relationships in different dilution rate regimes but also objectively show the dilution rates where wash-out occurs. Another important part of the study has been to extend the concept of isoclines to the case of growth on three substrates. Analytical equations for Zero Net Growth Surfaces have been obtained in this regard and plots of these are shown to yield expected trends. Thus the simple nature of the *optimal* model is exploited to theoretically derive a number of important results.

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**Notation**

- $a$: ratio of specific growth rate
- $D$: dilution rate, h$^{-1}$
- $e$: key enzyme level
- $k_i$: half-saturation constant for substrate $i$, g/l
- $s$: substrate concentration, g/l
- $s_{i0}$: isocline intercept substrate concentration for substrate $i$, g/l
- $s_i^*$: isocline asymptotic substrate concentration for substrate $i$, g/l
- $s_{if}$: feed concentration of substrate $i$, g/l
- $t$: time, h
- $X$: biomass concentration, g/l
- $Y$: biomass yield concentration, g/g

**Greek letters**

- $\alpha$: optimization variable of optimal model
- $\beta$: enzyme decay constant, h$^{-1}$
- $\mu$: specific growth rate, h$^{-1}$
- $\mu_0$: specific growth rate on constitutive enzymes, h$^{-1}$
- $\mu_i$: specific growth rate neglecting enzyme degradation rate, h$^{-1}$

**Superscripts**

- $cr$: critical value
- $max$: maximum
- $ss$: value at steady state
- $i$: constant substrate concentration

**Subscripts**

- $i$: $i$th subsurface
- $m$: maximum
- $ss$: steady state

**References**


