Influence of macromixing on plasmid stability during batch fermentation with recombinant bacteria

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Macromixing in a batch bioreactor has been analysed through an extended version of a two-region model proposed earlier. Two internal dilution rates characterise the degree of macromixing. Computations for an Escherichia coli culture containing the plasmid pBR Eco gap show that in order to maximise the average concentration (or mass fraction) of plasmid-containing cells, the region into which the inoculum is introduced should be poorly (or perfectly) mixed while the other region should be perfectly (or poorly) mixed. Time-domain profiles for the two regions suggest that plasmid-containing cells become homogeneously distributed faster than plasmid-free cells. Comparison of the regional profiles for the cases of the largest average concentration and the largest-average mass fraction of plasmid-containing cells reveal differences which indicate that a partially macromixed bioreactor may be an optimal choice.

Recent reviews attest the growing recognition of the importance of fluid mixing in determining the performance of bioreactors. Mixing occurs at two levels: (i) micromixing occurs at a local level and characterises the movements of fluid molecules and cell in a microscopic environment, (ii) macromixing describes phenomena in the large context of the bulk of the fermentation broth, and it is the more perceptible phenomenon. Neither complete micromixing nor complete macromixing may be desirable, and often there are optimum levels for both so as to achieve the best performance.

Macromixing normally gains significance as fermentation progresses because of heat effects and viscosity increases. Its influence increases with the size of the fermentation vessel and the growth of biomass. Macromixing can alter the product pattern, the rates of oxygen uptake, the relative growth rates of competing species in a batch bioreactor and the occurrence of in vitro oscillations. Moreover, since the relaxation times for cellular metabolism may be two to three orders of magnitude larger than those for fluid mixing, macromixing is often the more significant factor in determining bioreactor performance.

Studies by Ryu and associates, using an Escherichia coli strain harboring the plasmid pPLc23trpA1, suggest that fluid mixing in a bioreactor affects the stability of plasmid-containing cells and thus the formation of the recombinant enzyme, tryptophan synthetase. Using a dispersion model to characterise macromixing, it was shown for this system that the mass fraction of plasmid-containing cells increases with the Peclet number, Pe, until Pe is between 90 and 110, and then decreases. In a related study, macromixing was shown to influence the parametric region for the emergence of Hopf bifurcations in a continuous bioreactor; its importance is realised when we consider that macromixing is incomplete in large bioreactors and that induced oscillations favour the growth of plasmid-harboring cells relative to plasmid-free cells.

While productivity considerations might favour continuous fermentation, batch operation is preferred when the residence time required is large, as for penicillin G, and when sterility is a stringent consideration, as in fermentations for pharmaceutical and food products. Besides, kinetics and mixing characteristics are studied more conveniently in batch operation. Therefore many workers have chosen a batch bioreactor for fluid mixing studies.
Fig. 1—Two-region concept of a bioreactor.

Most models to describe macromixing use combinations of two or more well-mixed reactors with interconnecting streams. Unless the rheology is complex and heterogeneous, a linear train of two or three reactors, sometimes with recycle loops, adequately describes bioreactor dynamics. Sinclair and Brown were among the early users of a 'two-region model', and its continuing use underlines the validity and versatility of such an approach. The present study is based on an extended version of a two-region model proposed by Tanner et al.

Mathematical Development

Tanner et al. conceptualised the broth in the bioreactor to consist of two well-mixed regions with exchange of fluid (Fig. 1). The corresponding model is shown in Fig. 2, each region functions as a continuous flow stirred tank bioreactor (CFSTB), and the interconnecting streams represent internal circulation. Although the volume of broth in each CFSTB is constant, the two reactors may hold unequal volumes and, as shown below, this is related to the degree of macromixing in the corresponding regions. Since the overall flow is in a closed loop, the model simulates a batch environment. This model has a direct correspondence with the mixing pattern in a bioreactor equipped with an agitator having two sets of impellers; then the fluid in the region of each set of impellers is well-mixed but the two regions function differently. As the stirring speed is increased, the regions become more and more similar and the model approaches a perfectly mixed tank. This approach to homogeneity, i.e. the degree of macromixing, is expressed in the model through the (internal) dilution rate of each CFSTB. By varying the dilution rate from zero to infinity, any degree of macromixing from complete mixing to total segregation can be expressed. While providing close similarity to a bioreactor with a two-impeller agitator, the model adequately simulates macromixing with other types of agitators also.

The mechanism suggested by Imanaka and Aiba for plasmid loss through defective partitioning at the time of cell division has been used widely to model plasmid dynamics in fermentations. It may be represented schematically (and not stoichiometrically) as

\[ aS + X^+ \rightarrow (2 - p)X^+ + X^- \]
\[ bS + X^- \rightarrow 2X^- \]

where \( S \) is the substrate and \( X^+ \), \( X^- \) denote plasmid-bearing and plasmid-free cells respectively. The model assumes that \( X^+ \) cells degenerate to \( X^- \) cells with a probability \( p \) per generation, i.e. \( X^+ = pX^- \). Incorporation of the kinetic equations available from this mechanism in the material balances for the two bioreactors of the Tanner model (Fig. 2) leads to a set of equations,

\[
\frac{dx^-}{dt} = D_1(x^-_1 - x^-_2) + \mu^- x^- + p_i \mu^+ x^+ \\
\frac{dx^+}{dt} = D_1(x^+_1 - x^+_2) + (1 - p_i) \mu^+ x^+ 
\]
Table 1—Parameters and initial values

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Variable</th>
<th>Initial Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Y^-$</td>
<td>0.5</td>
<td>$x_i^-$</td>
<td>0.0</td>
</tr>
<tr>
<td>$Y^+$</td>
<td>0.5</td>
<td>$x_i^+$</td>
<td>0.0</td>
</tr>
<tr>
<td>$\mu^-_i$ (h$^{-1}$)</td>
<td>1.0</td>
<td>$s_i^-$</td>
<td>10.0</td>
</tr>
<tr>
<td>$\mu^+_i$ (h$^{-1}$)</td>
<td>0.9</td>
<td>$s_i^+$</td>
<td>0.0</td>
</tr>
<tr>
<td>$K$ (g l$^{-1}$)</td>
<td>0.1</td>
<td>$s_i^-$</td>
<td>0.1</td>
</tr>
<tr>
<td>$\alpha$ (h)</td>
<td>0.015</td>
<td>$s_2$</td>
<td>10.0</td>
</tr>
<tr>
<td>$\beta$ (h$^{-1}$)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>$\gamma$ (h$^{-1}$)</td>
<td>0.132</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$n$</td>
<td>1.78</td>
<td></td>
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</tbody>
</table>

\[
\frac{ds_i}{dt} = D_1(s_i - s^-_i) - \frac{\mu^-_ix_i^-}{Y^-} - \frac{\mu^+_ix_i^+}{Y^+} \quad \ldots (3)
\]
\[
\frac{dx_i^-}{dt} = D_1(x_i^- - x_i^+) + \mu^-_ix_i^- + p_2\mu^+_ix_i^+ \quad \ldots (4)
\]
\[
\frac{dx_i^+}{dt} = D_1(x_i^- - x_i^+) + (1 - p_2)\mu^+_ix_i^+ \quad \ldots (5)
\]
\[
\frac{ds_i}{dt} = D_2(s_i - s^-_i) - \frac{\mu^-_ix_i^-}{Y^-} - \frac{\mu^+_ix_i^+}{Y^+} \quad \ldots (6)
\]

In keeping with normal modelling practice,$^3,10$ the substrate consumption rates are considered proportional to the rates of formation of the two types of cells through their yield factors. This accounts for the last two terms in Eqs (3) and (6). The specific growth rates follow Monod kinetics,

\[
\mu_i^- = \frac{\mu^-_is_i}{K + s_i}; \quad i = 1, 2 \quad \ldots (7)
\]
\[
\mu_i^+ = \frac{\mu^+_is_i}{K + s_i}; \quad i = 1, 2 \quad \ldots (8)
\]

Previous studies$^{22,23}$ indicate that the equilibrium constants and yield factors for plasmid-harboring and plasmid-free cells are approximately equal. Hence the same $K$ has been used in Eqs (7) and (8). Although the original Imanaka-Aiba model and many applications of it$^{11,12,23}$ assumed the plasmid loss probability, $p_1$ or $p_2$, to be constant, there are reports$^{24,25}$ that it depends on the specific growth rate of plasmid-containing cells. This dependence may be expressed as$^{26}$

\[
p_i = \alpha \left[ \mu_i^+ - \frac{\beta(\mu_i^+ / \gamma)^n}{1 + (\mu_i^+ / \gamma)^n} \right]; \quad i = 1, 2 \quad \ldots (9)
\]

Thus $p_i$ is implicitly a function of time. Some implications of variable probability on cyclic stabilisation during continuous fermentation have been discussed recently$^{27}$. 

Results and Discussion

Eqs (1)-(9) were solved for the system studied by Mosrati et al.$^{26}$. They used an E. coli C600 gal K, ATCC 23724, strain modified by insertion of the plasmid pBR Eco gap· encoding for glyceraldehyde-3-phosphate dehydrogenase. Experimental details are available in their papers$^{21,26}$. The values of the parameters and initial conditions were the same as in a recent analysis$^{27}$ of this system, reproduced in Table 1. To promote the preferential growth of plasmid-containing cells, the inoculum had no wild type cells, and, as suggested by previous work on macromixing$^{17,28}$, it was introduced in the bottom half of the bioreactor near the impeller blades. Translated to Fig. 2, the method of inoculation implies zero initial values for plasmid-free cells in both CFSTBs and for plasmid-containing cells in the first CFSTB.

Tanner et al.$^{15}$ equated $D_1$ and $D_2$ a priori and varied the single dilution rate. In the present analysis this restriction has been removed and four combinations of $D_1$ and $D_2$ were studied, (i) $D_1=0.1$ h$^{-1}$, $D_2=0.1$ h$^{-1}$; (ii) $D_1=0.1$ h$^{-1}$, $D_2=10$ h$^{-1}$; (iii) $D_1=10$ h$^{-1}$, $D_2=0.1$ h$^{-1}$; (iv) $D_1=10$ h$^{-1}$, $D_2=10$ h$^{-1}$. These combinations were chosen from two considerations. First, one value of each dilution rate is smaller than the two maximum
specific growth rates (1.0 and 0.9 h⁻¹) while the other value is larger than both. The larger dilution rate would normally correspond to a situation where the cells are flushed out of a CFSTB; however, such washout does not happen in batch fermentation and neither does it in the model because the total flow is in a closed loop. Secondly, computations showed that a dilution rate of 10 h⁻¹ was large enough to provide a good approximation of perfect macromixing; larger rates produced only marginal changes in the CFSTB performance.

To compare the four cases, the overall concentration and mass fraction of plasmid-containing cells in the batch bioreactor were calculated as the volumetric averages of their values in the two CFSTBs at each instant of time. Since the flowrates through the two CFSTBs have to be equal in order to maintain closed loop mass balance (Fig. 2), the average values may be expressed in terms of dilution rates as,

\[ x_{av} = \frac{x^+_1 D_2 + x^+_2 D_1}{D_1 + D_2} \quad \ldots (10) \]
\[ \phi_{av} = \frac{\phi_1 D_2 + \phi_2 D_1}{D_1 + D_2} \quad \ldots (11) \]

where
\[ \phi_i = \frac{x_i^+}{(x_i^+ + x_i^-)}; i = 1, 2 \quad \ldots (12) \]

The variations of \( x_{av} \) and \( \phi_{av} \) with time for all four cases have been plotted in Figs 3 and 4. Their general trends agree with previous observations based on different \( E. coli \) strains. In a study ²² of a single CFSTB and experiments therein with two CFSTBs in series ²¹, the concentration of plasmid-harboring cells increased with time whereas their mass fraction decreased, which is consistent with the fact that the additional metabolic requirement of the foreign plasmid slows down the growth rate of the cell. However, between Figs 3 and 4 there is an interesting difference. While the average concentration of plasmid-bearing cells remains highest when the inoculated (lower) region is poorly mixed and the upper region is perfectly mixed \((D_1=10 \text{ h}^{-1}, D_2=0.1 \text{ h}^{-1})\), their mass fraction is consistently highest in the converse situation, i.e. the inoculated region is perfectly mixed and the upper region poorly mixed \((D_1=0.1 \text{ h}^{-1}, D_2=10 \text{ h}^{-1})\). This suggests that (a) an intermediate degree of mixing may be optimal and (b) inoculating both regions might improve the performance. The existence of an optimum degree of macromixing for the recombinant \( E. coli \) strain studied here is consistent with similar reports for micromixing ⁶ and for macromixing in fermenters using bacteria which have not been genetically modified ⁴ ⁵. A study comparing bioreactor performance for inoculations in one region and in both regions is nearing completion but since it does not pertain to a recombinant strain, the results have not been mentioned here.

To understand how the two regions differ, the time-domain profiles of the concentrations and
increased cAMP production and the secretion of proteins in recombinant cells may be linked with the synthesis of flagellae and modification of cell shape, which help their motion and thus favor rapid homogenisation.

A third feature that distinguishes Figs 7 and 8 from Figs 5 and 6 is the occurrence of minima in the inoculated region. To explain this, we recall that the flowrate is the same through both CFSTBs; hence a large/small dilution rate implies a small/large volume. Since $D_2 \gg D_1$, the volume of the inoculated region is small therefore less substrate is available initially than is required for complete cell growth in this region. This favors plasmid-free cells because their specific growth rates are larger. Together with the faster migration (homogenisation) of plasmid-harboring cells as explained before, this leads to a short-term fall in the concentration of plasmid-harboring cells. However, as mixing proceeds, more substrate becomes available in the inoculated region and consequently both $x$ and $\phi$ increase. Read against the (desirable) absence of this feature and the (undesirable) rapid decline of $\phi$ in Fig. 6, the foregoing explanation from a CFSTB analog of a batch bioreactor complements previous observations of optimum mixing conditions and optimum dilution rates in continuous
fermentations with recombinant bacteria.

Conclusions

Macromixing in a batch bioreactor may be modeled by means of two CFSTBs with interconnecting flows in a closed loop. Each CFSTB represents a mixed region in the bioreactor, and the degree of mixing in each is expressed through its dilution rate. A pure culture containing only recombinant cells of an E. coli strain harboring the plasmid pBR Eco gap was introduced as the inoculum. The volumetric average concentration of plasmid-bearing cells was largest when the inoculated region was poorly mixed and the other region well-mixed. On the contrary, the mass fraction of these cells was highest at all times in the converse situation. This difference suggests an optimum degree of macromixing and the possibility of benefiting from introducing the inoculum in both regions simultaneously.

Time-domain profiles of the concentrations and mass fractions of plasmid-containing cells in each CFSTB indicated that these cells distributed themselves homogeneously in the broth sooner than plasmid-free cells. While the profiles for the situation where the average concentration was largest (inoculated region poorly mixed) increased or decreased monotonically as fermentation progressed, those for the case when their mass fraction was largest (perfectly mixed inoculated region) decreased briefly and then increased (in the inoculated region only). The latter profiles may be explained in terms of limited availability of substrate initially in the small inoculated region and the rapid migration of plasmid-containing cells. Comparison of the two sets of profiles indicates the importance of optimum macromixing in batch fermentation, similar to its role in continuous culture.

Nomenclature

\[ D_i = \text{dilution rate in } i\text{-th CFSTB in Fig. 1, h}^{-1} \]
\[ K = \text{equilibrium constant, g L}^{-1} \]
\[ n = \text{constant in Eq. (9), dimensionless} \]
\[ p_i = \text{plasmid loss probability in } i\text{-th CFSTB, dimensionless} \]
\[ S_i = \text{concentration of substrate in } i\text{-th CFSTB, g L}^{-1} \]
\[ x_i = \text{concentration of plasmid-free cells in } i\text{-th CFSTB, g L}^{-1} \]
\[ x_i^* = \text{concentration of plasmid-containing cells in } i\text{-th CFSTB, g L}^{-1} \]
\[ x_{av} = \text{average concentration of plasmid-containing cells in bioreactor, g L}^{-1} \]
\[ Y = \text{yield factor for plasmid-free cell, g g}^{-1} \]
\[ Y^* = \text{yield factor for plasmid-containing cells, g g}^{-1} \]
\[ \alpha = \text{constant in Eq. (9), h} \]
\[ \beta = \text{constant in Eq. (9), h}^{-1} \]
\[ \gamma = \text{constant in Eq. (9), h}^{-1} \]
\[ \mu_i = \text{specific growth rate of plasmid-free cells in } i\text{-th CFSTB, h}^{-1} \]
\[ \mu_i^* = \text{specific growth rate of plasmid-containing cells in } i\text{-th CFSTB, h}^{-1} \]
\[ \mu_{av} = \text{maximum specific growth rate of plasmid-free cells, h}^{-1} \]
\[ \mu_{av}^* = \text{maximum specific growth rate of plasmid-containing cells, h}^{-1} \]
\[ \phi_i = \text{mass fraction of plasmid-containing cells in } i\text{-th CFSTB, dimensionless} \]
\[ \phi_{av} = \text{average mass fraction of plasmid-containing cells in bioreactor, dimensionless} \]
1 = upper (non-inoculated) region of bioreactor
2 = lower (inoculated) region of bioreactor

References