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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[1-3] 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[4-6].

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[2,3]. Macromixing can alter the product pattern[5], the rates of oxygen uptake[7], the relative growth rates of competing species in a batch bioreactor[8] and the occurrence of *in vitro* oscillations[9]. Moreover, since the relaxation times for cellular metabolism may be two to three orders of magnitude larger than those for fluid mixing[10], macromixing is often the more significant factor in determining bioreactor performance[5,9].

Studies by Ryu and associates[11,12], 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[13] 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[9], 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[14].

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[15-17] have chosen a batch bioreactor for fluid mixing studies.

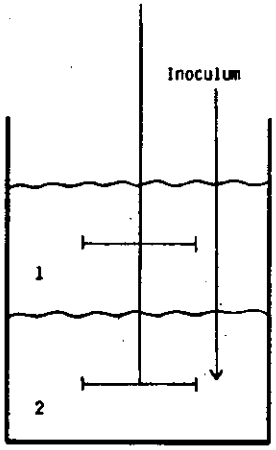


Fig. 1—Two-region concept of a bioreactor.

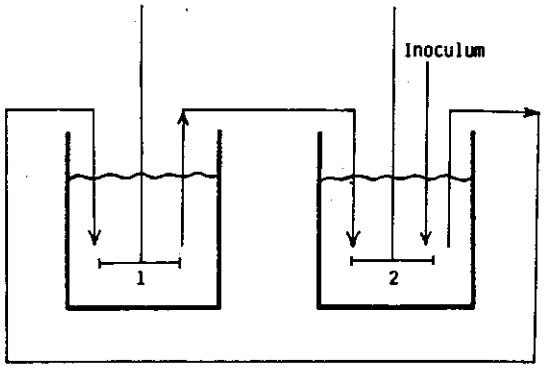


Fig. 2—Schematic representation of the model of Tanner *et al.*[15]. The inoculum is fed as a single dose and not continuously.

st models[2] to describe macromixing use nations of two or more well-mixed reactors nterconnecting streams. Unless the rheology plex and heterogeneous, a linear train of two e reactors, sometimes with recycle loops, tely describes bioreactor dynamics. Sinclair own[18] were among the early users of a 'two- model', and its continuing use[4,9,15,16] ines the validity and versatility of such an ch. The present study is based on an ed version of a two-region model proposed ner *et al.*[15].

matical Development

ner *et al.*[15] conceptualised the broth in the tor to consist of two well-mixed regions change of fluid (Fig. 1). The corresponding is shown in Fig. 2, each region functions as nuous flow stirred tank bioreactor (CFSTB), e interconnecting streams represent internal tion. Although the volume of broth in each 3 is constant, the two reactors may hold l volumes and, as shown below, this is to the degree of macromixing in the onding regions. Since the overall flow is in ed loop, the model simulates a batch ment. This model has a direct ondence with the mixing pattern in a tor equipped with an agitator having two impellers; then the fluid in the region of et of impellers is well-mixed but the two function differently[2,19]. 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[19].

The mechanism suggested by Imanaka and Aiba[20] 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[21],

$$aS + X^+ \rightarrow (2-p)X^+ + X^-$$

$$bS + X^- \rightarrow 2X^-$$

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[21] from this mechanism in the material balances for the two bioreactors of the Tanner model (Fig. 2) leads to a set of equations,

$$\frac{dx_1^-}{dt} = D_1(x_2^- - x_1^-) + \mu_1^- x_1^- + p_1\mu_1^+ x_1^+ \quad \ldots (1)$$

$$\frac{dx_1^+}{dt} = D_1(x_2^+ - x_1^+) + (1-p_1)\mu_1^+ x_1^+ \quad \ldots (2)$$

Table 1—Parameters and initial values

Parameter	Value	Variable	Initial Value
Y^-	0.5	x_1^- (g l^{-1})	0.0
Y^-	0.5	x_1^+ (g l^{-1})	0.0
μ_m^- (h^{-1})	1.0	s_1 (g l^{-1})	10.0
μ_m^+ (h^{-1})	0.9	x_2^- (g l^{-1})	0.0
K (g l^{-1})	0.1	x_2^+ (g l^{-1})	0.1
α (h)	0.015	s_2 (g l^{-1})	10.0
β (h^{-1})	0.215		
γ (h^{-1})	0.132		
n	1.78		

$$\frac{ds_1}{dt} = D_1(s_2 - s_1) - \frac{\mu_1^- x_1^-}{Y^-} - \frac{\mu_1^+ x_1^+}{Y^+} \quad \ldots (3)$$

$$\frac{dx_2^-}{dt} = D_2(x_1^- - x_2^-) + \mu_2^- x_2^- + p_2\mu_2^+ x_2^+ \quad \ldots (4)$$

$$\frac{dx_2^+}{dt} = D_2(x_1^+ - x_2^+) + (1-p_2)\mu_2^+ x_2^+ \quad \ldots (5)$$

$$\frac{ds_2}{dt} = D_2(s_1 - s_2) - \frac{\mu_2^- x_2^-}{Y^-} - \frac{\mu_2^+ x_2^+}{Y^+} \quad \ldots (6)$$

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,

$$\mu_i^- = \frac{\mu_m^- s_i}{K + s_i};\ i = 1,2 \quad \ldots (7)$$

$$\mu_i^+ = \frac{\mu_m^+ s_i}{K + s_i};\ i = 1,2 \quad \ldots (8)$$

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],

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

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].

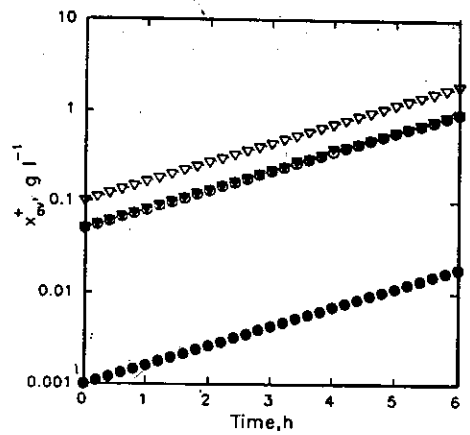


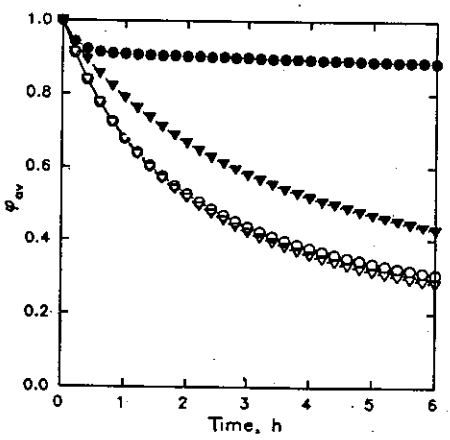
Fig. 3—Variation of the average concentration of plasmid-containing cells with time.

○ D_1=0.1 h^{-1}, D_2=0.1 h^{-1},
● D_1=0.1 h^{-1}, D_2=10 h^{-1},
▽ D_1=10 h^{-1}, D_2=0.1 h^{-1},
▼ D_1=10 h^{-1}, D_2=10 h^{-1},

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



-Variation of the average mass fraction of plasmid-ng cells with time. The symbols have been same ion as in Fig. 3.

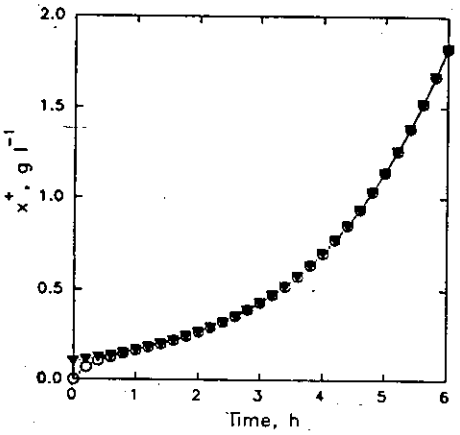


Fig. 5—Time-domain profiles of the regional concentrations of plasmid-containing cells for the case of the highest x_{av}^{+} profile, i.e. D_1=10 h^{-1}, D_2=0.1 h^{-1}.

▼ Inoculated region
○ Non-inoculated region

c growth rates (1.0 and 0.9 h^{-1}) while the alue is larger than both. The larger dilution ould normally correspond to a situation the cells are flushed out of a CFSTB; er, such washout does not happen in batch tation and neither does it in the model e the total flow is in a closed loop. ly, computations showed that a dilution rate h^{-1} was large enough to provide a good imation of perfect macromixing; larger rates ed only marginal changes in the CFSTB iance.

compare the four cases, the overall tration and mass fraction of plasmid-ing cells in the batch bioreactor were ted as the volumetric averages of their in the two CFSTBs at each instant of time. he flowrates through the two CFSTBs have qual in order to maintain closed loop mass (Fig. 2), the average values may be ed in terms of dilution rates as,

$$\frac{x_1^{+}D_2 + x_2^{+}D_1}{D_1 + D_2} \quad \ldots (10)$$

$$\frac{\phi_1 D_2 + \phi_2 D_1}{D_1 + D_2} \quad \ldots (11)$$

$$/(x_i^{+} + x_i^{-});\ i = 1,2 \quad \ldots (12)$$

variations of x_{av}^{+} and ϕ_{av} with time for all es have been plotted in Figs 3 and 4. Their trends agree with previous observations n different *E. coli* strains. In a study[22] of a single CFSTB and experiments therein with two CFSTBs in series[23], 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 h^{-1}, D_2=0.1 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 h^{-1}, D_2=10 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

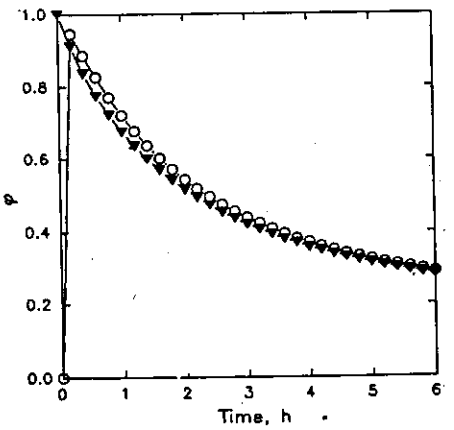


Fig. 6—Time-domain profiles of the regional mass fractions of plasmid-containing cells for the case of the highest x_{av}^{+} profile, i.e. D_1 =10 h^{-1}, D_2 =0.1 h^{-1}. The explanation of the symbols may be seen in Fig. 5.

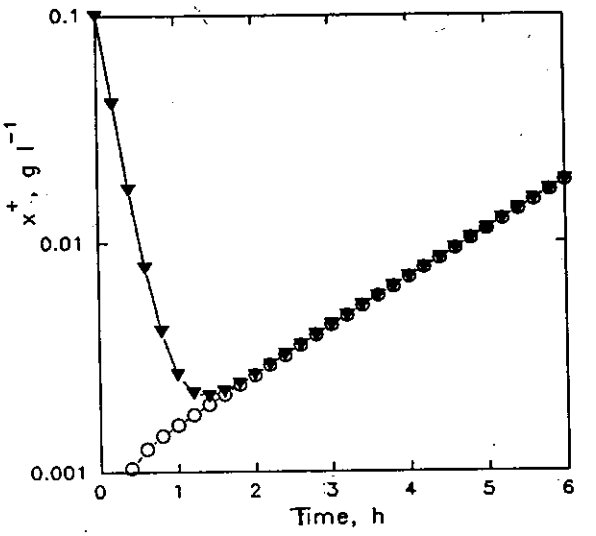
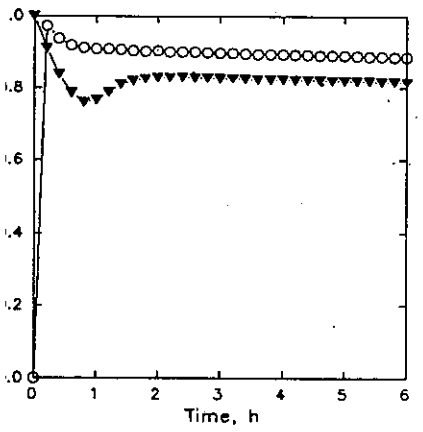


Fig. 7—Time-domain profiles of the regional concentrations of plasmid-containing cells for the case of the highest ϕ_{av} profile, i.e. D_1 =0.1 h^{-1}, D_2 =10 h^{-1}. Symbols are as explained in Fig. 5.

mass fractions of plasmid-harboring cells in the regions have been plotted separately. Figs 5 and 6 pertain to the case where the average concentration is the highest (D_1=10 h^{-1}, D_2=0.1 h^{-1} in Fig. 3); it is seen that the two regions approach each other very quickly and the broth becomes homogeneous in less than half an hour. The abrupt jump in ϕ for the upper region (the first CFSTB) occurs because initially there are no cells here and therefore the transport of even a small fraction of the inoculum causes a sharp increase in the mass fraction to nearly unity. The profiles for the case where the average mass fraction is highest (D_1=0.1 h^{-1}, D_2=10 h^{-1} in Fig. 4) are significantly different (Figs 7 and 8) from the corresponding profiles in Figs 5 and 6. Firstly, they are not monotonic. Secondly, even though the concentrations of plasmid-containing cells coincide after about 1.5 h, their mass fractions ,differ throughout the fermentation. It may be recognised, however, that the mass fractions in the regions stabilise after the same length of time. These observations suggest two inferences: (i) the growth rates of both kinds of cells become constant, but remain different, after 1.5 h and (ii) the circulation rate of plasmid-containing cells is faster than that of plasmid-free cells; consequently plasmid-containing cells become homogeneously distributed by the time their growth rate stabilises. Although no direct information is available for the rates of locomotion of wild type *vis-a-vis* recombinant *E. coli* cells, the increased cAMP production and the secretion of proteins in recombinant cells may be linked with the synthesis of flagellae[29] and modification of cell shape[30], 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 ϕ increase. Read against the (desirable) absence of this feature and the (undesirable) rapid decline of ϕ in Fig. 6, the foregoing explanation from a CFSTB analog of a batch bioreactor complements previous observations of optimum mixing conditions[6,13] and optimum dilution rates[22,23] in continuous



ne-domain profiles of the regional mass fractions containing cells for the case of the highest ϕ_{av}, D_1 =0.1 h^{-1}, D_2 =10 h^{-1}. Symbols are as explained

ions with recombinant bacteria.

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mixing in a batch bioreactor may be by means of two CFSTBs with ecting flows in a closed loop. Each represents a mixed region in the r, and the degree of mixing in each is through its dilution rate. A pure culture g only recombinant cells of an *E. coli* boring the plasmid pBR Eco gap was d as the inoculum. The volumetric oncentration of plasmid-bearing cells was hen the inoculated region was poorly d the other region well-mixed. On the the mass fraction of these cells was all times in the converse situation. This suggests an optimum degree of ing and the possibility of benefiting from ig the inoculum in both regions ously.

omain profiles of the concentrations and tions of plasmid-containing cells in each indicated that these cells distributed s homogeneously in the broth sooner nid-free cells. While the profiles for the where the average concentration was oculated region poorly mixed) increased ased monotonically as fermentation d, those for the case when their mass vas 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	=	dilution rate in i-th CFSTB in Fig. 1, h^{-1}
K	=	equilibrium constant, g l^{-1}
n	=	constant in Eq. (9), dimensionless
p_i	=	plasmid loss probability in i-th CFSTB, dimensionless
Sl_i	=	concentration of substrate in i-th CFSTB, g L^{-1}
x_i^-	=	concentration of plasmid-free cells in i-th CFSTB, g L^{-1}
x_i^+	=	concentration of plasmid-containing cells in i-th CFSTB, g L^{-1}
x_{av}^+	=	average concentration of plasmid-containing cells in bioreactor, g L^{-1}
Y^-	=	yield factor for plasmid-free cell, g g^{-1}
Y^+	=	yield factor for plasmid-containing cells, g g^{-1}
α	=	constant in Eq. (9), h
β	=	constant in Eq. (9), h^{-1}
γ	=	constant in Eq. (9), h^{-1}
μ_i^-	=	specific growth rate of plasmid-free cells in i-th CFSTB, h^{-1}
μ_i^+	=	specific growth rate of plasmid-containing cells in i-th CFSTB, h^{-1}
μ_m^-	=	maximum specific growth rate of plasmid-free cells, h^{-1}
μ_m^+	=	maximum specific growth rate of plasmid-containing cells, h^{-1}
ϕ_i	=	mass fraction of plasmid-containing cells in i-th CFSTB, dimensionless
ϕ_{av}	=	average mass fraction of plasmid-containing cells in bioreactor, dimensionless
1	=	upper (non-inoculated) region of bioreactor
2	=	lower (inoculated) region of bioreactor

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