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Industrial Fermentations with (Unstable) Recombinant Cultures [and Discussion]

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Industrial fermentations with (unstable) recombinant cultures

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Recombinant DNA technology is yielding organisms with virtually a complete range of genetic stability, i.e. from organisms that appear to be entirely stable over the course of many fermentations to those that are so unstable that their half-life as novel species may be appreciably less than a conventional fermentation time. As such, a novel time-dependent mixed culture community is created, containing at a minimum the original recombinant microbe and a second strain, which may be denoted as a revertant in some partial or full sense. These two strains will differ in one or more important characteristics such as (desired) product formation rates, substrate utilization abilities, morphology, biomass growth rates and oxygen demand. In this paper, I consider batch and continuous cultivation, and I use some plausible biological rate forms to explore implications in fermentor behaviour and product formation occasioned by the existence of this particular form of 'mixed culture'.

INTRODUCTION

'Plasmids are replicons which are stably inherited in an extrachromosomal state' (Old & Primrose 1980). If stability were of infinite duration for artificially recombinant plasmids, then the replicative nature of these blueprints for biological catalysts would guarantee an endless maintenance of active catalyst. However, given the finite stability of a number of artificially *recombinant* plasmid systems, we may expect catalyst 'deactivation' to occur routinely in the industrial development of recombinant DNA systems. In other industrial areas where catalytic conversions are common, such as the petroleum refining and petrochemical industries, catalyst deactivation is widely recognized as an important phenomenon, the understanding and prediction of which is critical to successful commercial operation (Butt 1972).

The consequence of plasmid, and thus biocatalyst, instability has been considered by several authors, including Hershfield *et al.* (1974), Hobom & Hogness (1974), Cohen *et al.* (1977) and Imanaka & Aiba (1981). Imanaka & Aiba (1981) examined the stability of plasmid RSF2124-trp in mutants of *Escherichia coli* W3110, noting maintenance of from 0 to 100% of plasmid in cells after 25 generations, depending on the environment (medium). These authors also noted that these *E. coli* W3110 plasmid-carrying mutants grew as fast as or faster than W3110 carrying recombinant plasmids. Similar reports of more rapid growth of plasmid-free populations than that of the plasmid-bearing species appear in table 1. Some variety or adaptation may arise, as Nagahari *et al.* (1977) noted that a recombinant-bearing cell (*E. coli* C600/pBR322-T-Leu) grew faster and exhibited a more stable plasmid after 10 days than when first prepared.

Some penalties associated with a slower-growing recombinant system in mixed culture with a faster-growing plasmid-free or plasma-inactivated system were noted by Imanaka & Aiba (1981). Thus, for cells in exponential growth, the plasmid-active and plasmid-inactive (free) species, designated here as X^+ and X^-, undergo two characteristic reactions:

$$\text{growth:} \qquad bS + X^+ \rightarrow 2X^+; \tag{1}$$

$$\text{plasmid loss:}\ aS + X^+ \rightarrow (2-p)\,X^+ + X^-\ (X^- = pX^+). \tag{2}$$

Table 1. Relative growth rates of plasmid (+) and plasmid (−) cells
(Adapted from table 4 of Imanaka & Aiba (1981).)

strain	plasmid	ratio of specific growth rates (μ^-/μ^+)	reference
E. coli C600	F′lac	0.99–1.10	Collins & Pritchard (1973)
E. coli K12EC1005	R1drd-19	1.05–1.12	Engberg & Nordstrom (1975)
E. coli JC7623	ColE1 (various)	1.06–1.29	Inselburg (1978)
P. aeruginosa PA01	TOL	2.00	Nakazawa (1978)
E. coli K12IR713	TP120	1.50–2.31	Godwin & Slater (1979)

The consequences of mixed-culture propagation here in exponential growth are well known, as treatment of recurrent mutations is established in genetics (we thank Professor Pontecorvo for the reminder at this symposium) (see Kimura & Ohta (1971), for example p. 49) In batch culture, the (+) fraction F will diminish continually with generation number n, since $\mu^- > \mu^+$ (Imanaka & Aiba 1981):

$$F = \frac{1-\alpha-p}{1-\alpha-2^{n(\alpha+p-1)}p}, \tag{3}$$

where $\alpha = \mu^-/\mu^+$. Accordingly, the number of generations needed to obtain a decrease to any *fraction* F of cells that are (+) is

$$n = \ln\left\{\frac{1}{F}+\left(\frac{1}{F}-1\right)\left(\frac{\alpha-1}{p}\right)\right\}\Big/(\alpha+p-1)\ln 2. \tag{4}$$

In continuous culture, when $\alpha(\equiv \mu^-/\mu^+) > 1$, the steady state requires that $X^+ = 0$, $X^- \neq 0$.

Plasmid stabilization techniques that may prove useful include those cited by Imanaka & Aiba (1981):

(*a*) the use of plasmids with temperature-dependent copy numbers (Uhlin *et al.* 1979);
(*b*) the use of environmental (selective) pressure to allow $\alpha(\equiv \mu^-/\mu^+) < 1.0$);
(*c*) the use of plasmids containing no transposable element;
(*d*) the use of recombination-deficient strains.

The mixed-culture biomass analysis of Imanaka & Aiba (1981) invites us to develop a fuller kinetic model, including aspects of product formation, substrate yield and catalyst recycling that may aid the maintenance of active cell species. In the following sections I consider three pertinent cases:

(*a*) the use of a Monod growth model for batch culture;
(*b*) the use of exponential and Monod models for continuous culture;
(*c*) the use of selective cell recycling to maintain an active (+) biomass in continuous culture.

Product formation and associated yields from substrate were not considered by Imanaka & Aiba (1981). Here I develop rate equations, in each of the three cases, for (+) and (−) biomass growth, product formation and substrate consumption. The consequent yield coefficients are also developed.

Batch culture

In order to have a model that is useful for the kinetic network in (1) and (2) at all substrate levels, and which allows for product formation in exponential, transition and/or stationary phases, I consider two general forms for growth and product formation, respectively.

(*a*) *Growth, Monod form*

$$\frac{\mathrm{d}X^+}{\mathrm{d}t} = \mu_\mathrm{m}^+ X^+ \frac{S}{K_\mathrm{S}+S}(1-p), \tag{5a}$$

and

$$\frac{\mathrm{d}X^-}{\mathrm{d}t} = \mu_\mathrm{m}^- X^- \frac{S}{K_\mathrm{S}+S} + p\mu_\mathrm{m}^+ X^+ \frac{S}{K_\mathrm{S}+S}. \tag{5b}$$

Here, the affinity or Monod constant, K_S, of each species $(+, -)$ for S is assumed to be identical, but the maximum specific growth rates, μ_m^+, μ_m^-, may differ.

(*b*) *Product formation*

This may be growth associated, or non-growth associated, or both. A kinetic form allowing for inclusion of all cases here is the form of Luedeking & Piret (1959):

$$\frac{\mathrm{d}P}{\mathrm{d}t} = nX^+ + m\frac{\mathrm{d}X^+}{\mathrm{d}t}, \tag{6}$$

where, for simplicity, we have assumed that X^- is inactive for the production of product P.

(*c*) *Substrate consumption*

This is obtained from a mass balance including exogenous maintenance (k_e):

$$\frac{\mathrm{d}S}{\mathrm{d}t} = -\frac{1}{Y_{\mathrm{X}^+}}\frac{\mathrm{d}X^+}{\mathrm{d}t} - \frac{1}{Y_{\mathrm{X}^-}}\frac{\mathrm{d}X^-}{\mathrm{d}t} - \frac{1}{Y_\mathrm{P}}\frac{\mathrm{d}P}{\mathrm{d}t} - k_\mathrm{e}^+ X^+ - k_\mathrm{e}^- X^-. \tag{7a}$$

The coupled equations (5*a*, 5*b*, 6, 7) can be conveniently examined. For convenience, we now neglect the maintenance terms k_e^+, k_e^-, so that

$$\frac{\mathrm{d}S}{\mathrm{d}t} = -\frac{1}{Y_{\mathrm{X}^+}}\frac{\mathrm{d}X^+}{\mathrm{d}t} + \frac{1}{Y_{\mathrm{X}^-}}\frac{\mathrm{d}X^-}{\mathrm{d}t} - \frac{1}{Y_\mathrm{P}}\frac{\mathrm{d}P}{\mathrm{d}t}. \tag{7b}$$

Dividing (5*b*) by (5*a*) provides a trajectory:

$$\frac{\mathrm{d}X^-}{\mathrm{d}X^+} = \frac{\mu_\mathrm{m}^- X^- + p\mu_\mathrm{m}^+ X^+}{(1-p)\,\mu_\mathrm{m}^+ X^+}. \tag{8}$$

Integration gives

$$\chi \equiv \frac{X^-}{X^+} = \frac{\{(\alpha+p-1)\,\chi_0 + p\}\,(X^+/X_0^+)^{(\alpha/1-p)-1} - P}{(\alpha+p-1)}. \tag{9}$$

The substrate utilization can now be similarly evaluated. The use of (6) in (7*b*) gives

$$\begin{aligned}\frac{\mathrm{d}S}{\mathrm{d}t} &= -\frac{1}{Y_{\mathrm{X}^+}}\frac{\mathrm{d}X^+}{\mathrm{d}t} - \frac{1}{Y_{\mathrm{X}^-}}\frac{\mathrm{d}X^-}{\mathrm{d}t} - \frac{1}{Y_\mathrm{P}}\left\{n + m\left(\frac{S}{K_\mathrm{S}+S}\right)\mu_\mathrm{m}^+(1-p)\right\}X^+ \\ &= -\frac{1}{Y_{\mathrm{X}^+}}\frac{\mathrm{d}X^+}{\mathrm{d}t} - \left\{\frac{\mu_\mathrm{m}^-\chi S}{Y_{\mathrm{X}^-}(K_\mathrm{S}+S)} + \frac{p\mu_\mathrm{m}^+ S}{Y_{\mathrm{X}^+}(K_\mathrm{S}+S)} + \frac{1}{Y_\mathrm{P}}\left(n + \frac{mS}{(K_\mathrm{S}+S)}\mu_\mathrm{m}^+(1-p)\right)\right\}X^+. \end{aligned} \tag{10}$$

Example calculations from these equations are shown in figures 1, 2 and 3 for a 'standard' set of values given in table 2. These calculations include both growth and *non-growth* terms for product formation. This last condition requires that product formation does not depend explicitly on the particular substrate S that limits biomass growth (e.g. S may be a nitrogen (N) source, but P may not contain N). Thus, we drop the term $(\mathrm{d}P/\mathrm{d}t)/Y_\mathrm{P}$ in (7*b*) above.

For a fixed X_0^+ value, the consumption of S predicted from (5*a*), (5*b*), (9) and (10) is shown in figure 1.

The variation of X with time depends on the initial value, χ_0 (equation (9)), for the inoculum, as seen in figure 2, where $\chi_0 = 0$, 0.25, 0.5 and 1.0. The plateau values achieved in all cases at values of dimensionless time of from 11 to 12 units corresponds to substrate exhaustion (figure 1).

The influence of inoculum purity, represented by χ_0, on product formation (equation (6)) is shown for three cases in figure 3*a*, *b* and *c*, corresponding to $p = 0.01$ and growth associated (figure 3*a*), non-growth associated (figure 3*b*), and simultaneous growth and non-growth associated (figure 3*c*) product formation.

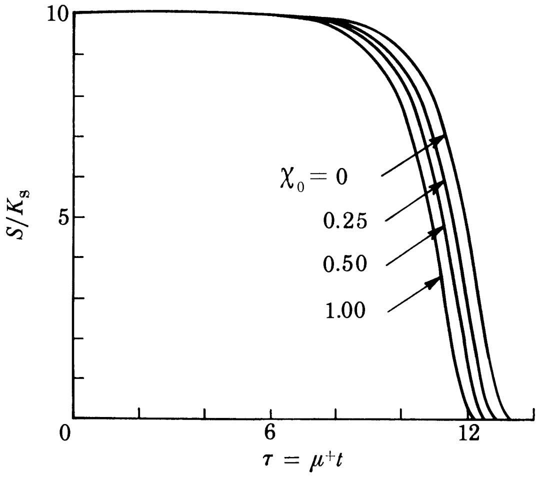

FIGURE 1. Substrate–time plot: batch fermentation (see table 2 parameter values). (From Chang & Ollis (1981).)

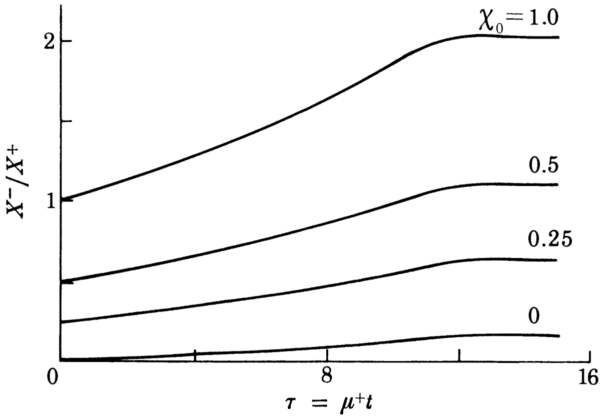

FIGURE 2. Ratio X^-/X^+ ($\equiv \chi$) with time: batch fermentation (see table 2 parameter values). (From Chang & Ollis (1981).)

CONTINUOUS CULTURE

Exponential growth

The tradition in fermentation is batch operation, the main exception being non-sterile mixed cultures involved in biological waste treatment. None the less, continuous culture is of major interest. I here consider some implications for continuous culture of this novel mixed culture.

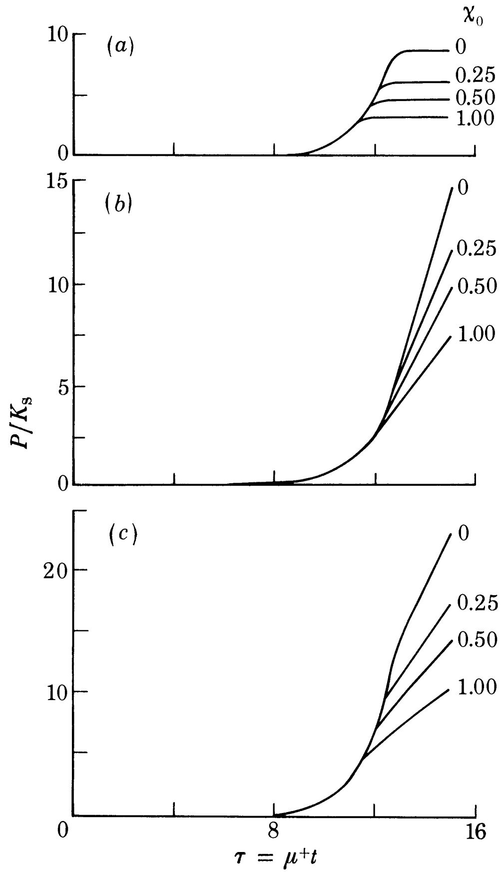

FIGURE 3. Product P plotted against time: batch fermentation (see table 2 parameter values): (*a*) growth associated, (*b*) non-growth associated, (*c*) mixed.

TABLE 2. BATCH FERMENTATION PARAMETER VALUES FOR FIGURES 1, 2 AND 3

$\mu_m^+ = 0.15\ \mathrm{h}^{-1}$
$K_S = 2\ \mathrm{g\ S\ l^{-1}}$
$p = 0.01$
$m = 1.85$
$n = 0.155\ \mathrm{h}^{-1}$

$Y_{X^+} = Y_{X^-} = 0.5\ \mathrm{g\ X\ g^{-1}\ S}$
time units × 6.6 = 1 h

figure 3

	a	*b*	*c*
$n =$	0.155	0	0.155
$m =$	0	1.85	1.85

We return first to a result of Imanaka & Aiba (1981). For exponential growth, with $\alpha = 1 (\mu_m^- = \mu_m^+ = \mu)$,

$$\mathrm{d}X^+/\mathrm{d}t = (1-p)\,\mu X^+ - DX^+ \tag{11a}$$

and

$$\mathrm{d}X^-/\mathrm{d}t = p\mu X^+ + \mu X^- - DX^-, \tag{11b}$$

where D is the dilution rate.

Addition gives $\mathrm{d}(X^+ + X^-)/\mathrm{d}t = (\mu - D)\,(X^+ + X^-)$, so that

$$(X^+ + X^-) = (X^+ + X^-)_0\, \mathrm{e}^{(\mu-D)\,t}. \tag{12}$$

Here, $D = \mu$ provides the only steady state. But (11*a*) and (11*b*) become, if $D \equiv \mu$,

$$\mathrm{d}X^+/\mathrm{d}t = -p\mu X^+ \tag{11a'}$$

and

$$\mathrm{d}X^-/\mathrm{d}t = +p\mu^+X^+. \tag{11b'}$$

The solutions are

$$X^+(t) = X^+(0)\,\mathrm{e}^{-p\mu t} \tag{13}$$

and

$$X^-(t) = X^+(0)\,(1-\mathrm{e}^{-p\mu t})+X^-(0). \tag{14}$$

As noted by Imanaka & Aiba (1981), the steady-state solution is $X^+(t) = 0$, $X^-(t) \neq 0$. However, when values of p are small, e.g. $p < 0.01$, we see that the 'deactivation' time constant, $k_\mathrm{d} \equiv 1/p\mu$, is much longer than a characteristic growth time. Consider an example: let $\mu = 0.3\ \mathrm{h}^{-1}$, $p = 0.01$, and $F = \frac{1}{2}$ when $X^- = X^+$; then if $X^-(0) = 0$, at $\mathrm{e}^{-p\mu t} = \frac{1}{2}$, $t = (1/p\mu)$ $(\ln 2) = 0.693/(0.01)\,(0.3) = 23.1$ h.

Similarly, $p = 0.001$ and $p = 0.0001$ correspond to $F = \frac{1}{2}$ at 231 h (9.6 days) and 2310 h (96 days). Hence the 'deactivating' culture may be operated continuously for appreciable lengths of time, even when some deactivation may be unavoidable. Thus, while 'steady state' is never achieved, a sufficiently slowly deactivating catalyst may still be interesting. Indeed, in heterogenous catalysis involving petroleum refining and petrochemical manufacture, such slow deactivations are the rule rather than the exception.

Thus, in 'continuous' culture, the question is not whether to run, but how long to run!

Let us first continue, then, the exponential growth example, enlarged now to include substrate consumption and product formation rates as well.

As before, we take

$$\begin{aligned} DP &= nX^+ + m(\mathrm{d}X^+/\mathrm{d}t)_{\mathrm{growth}} \\ &= nX^+ + m\{(1-p)\,\mu X^+\} \\ &= \{n+m(1-p)\,\mu\}\,X^+. \end{aligned}$$

Thus the instantaneous volumetric productivity of the reaction, DP, is given by

$$DP = \{n+m(1-p)\,\mu\}X^+(0)\,\mathrm{e}^{-p\mu t}. \tag{15}$$

The substrate consumption rate is then

$$D(S_0-S) = \frac{1}{Y_{\mathrm{X}^+}}\,DX^+ + \frac{1}{Y_{\mathrm{X}^-}}\,DX^- + \frac{1}{Y_\mathrm{P}}\,DP; \tag{16}$$

thus the change in S with time is given by

$$S(t) = \left\{S_0 - \frac{X^+(0)}{Y_{\mathrm{X}^-}}\right\} - \left\{\frac{1}{Y_{\mathrm{X}^+}} - \frac{1}{Y_{\mathrm{X}^-}} + \frac{(n+m(1-p)\,\mu)}{Y_\mathrm{P}}\right\}X^+(0)\,\mathrm{e}^{-p\mu t}. \tag{17}$$

The instantaneous ratio of product formed per unit of S consumed is obtained by dividing (15) by (16):

$$\frac{P}{S_0-S} = \frac{\{n+m(1-p)\,\mu\,\mathrm{e}\}^{-p\mu t}}{\left\{\dfrac{X^+(0)}{Y_{\mathrm{X}^-}}\right\} - \left\{\dfrac{1}{Y_{\mathrm{X}^+}} - \dfrac{1}{Y_{\mathrm{X}^-}} + \dfrac{n+m(1-p)\,\mu}{Y_\mathrm{P}}\right\}X^+(0)\,\mathrm{e}^{-p\mu t}}, \tag{18}$$

which is seen to diminish continually (when S_0 is constant).

Monod model

The Monod model allows growth rate variation by operation over a range of dilution rates. The unsteady-state equations for X^+, X^-, P, and S are:

$$\frac{\mathrm{d}X^+}{\mathrm{d}t} = \mu_{\mathrm{m}}^{+}\frac{X^+S(1-p)}{K_{\mathrm{S}}+S} - DX^+, \tag{19a}$$

$$\frac{\mathrm{d}X^-}{\mathrm{d}t} = \mu_{\mathrm{m}}^{-}X^-\frac{S}{K_{\mathrm{S}}+S} + \frac{p\mu_{\mathrm{m}}^{+}X^+S}{(K_{\mathrm{S}}+S)} - DX^-, \tag{19b}$$

$$\frac{\mathrm{d}P}{\mathrm{d}t} = \frac{1}{Y_{\mathrm{P}}}\left\{nX^+ + m\mu_{\mathrm{m}}^{+}X^+\left(\frac{S}{K_{\mathrm{S}}+S}\right)\right\} - DP, \tag{20}$$

$$\begin{aligned}\frac{\mathrm{d}S}{\mathrm{d}t} &= -D(S_0-S) + \left(-\frac{DX^+}{Y_{\mathrm{X}^+}} - \frac{DX^-}{Y_{\mathrm{X}^-}} - \frac{DP}{Y_{\mathrm{P}}}\right)\\ &= D\left\{S - S_0 - \frac{X^+}{Y_{\mathrm{X}^+}} - \frac{X^-}{Y_{\mathrm{X}^-}} - \frac{P}{Y_{\mathrm{P}}}\right\}.\end{aligned} \tag{21}$$

From (19a) and (19b), if $\alpha = 1$ again ($\mu_{\mathrm{m}}^{+} = \mu_{\mathrm{m}}^{-} = \mu$),

$$\begin{aligned}\mathrm{d}(X^+ + X^-)/\mathrm{d}X &= \mu\{S/(K_{\mathrm{S}}+S)\}(X^+ + X^-) - D(X^+ + X^-)\\ &= [\mu\{S/(K_{\mathrm{S}}+S)\} - D](X^+ + X^-).\end{aligned} \tag{22}$$

We may presume here that, if chemostat operation is being used, the plasmid characteristic loss time is long compared with the residence time of material in the fermenter, i.e.

$$1/D \ll 1/p\mu. \tag{23}$$

This situation applies to many deactivation problems in catalysis; it is the case where the two relaxation times of the system differ widely. Here we can use steady-state concentration solutions, then introduce a time decay for the active biomass. Setting the derivative in (12) equal to zero,

$$S = DK_{\mathrm{S}}/(\mu_{\mathrm{m}} - D). \tag{24}$$

The transient solution is then obtained from (19a) and (19b) individually:

$$\begin{aligned}\mathrm{d}X^+/\mathrm{d}t &= \mu_{\mathrm{m}}X^+S/(K_{\mathrm{S}}+S)(1-p) - DX^+\\ &= -pDX^+\end{aligned} \tag{11a'}$$

and

$$\begin{aligned}\mathrm{d}X^-/\mathrm{d}t &= DX^- + pDX^+ - DX^-\\ &= pDX^+.\end{aligned} \tag{11b'}$$

The solutions are again

$$X^+(t) = X^+(0)\,\mathrm{e}^{-pDt} \tag{12a}$$

and

$$X^-(t) = X^+(0)(1-\mathrm{e}^{-pDt}). \tag{12b}$$

With these solutions, (20) becomes

$$\mathrm{d}P/\mathrm{d}t = (1/Y_{\mathrm{P}})[n + mD]X^+(0)\,\mathrm{e}^{-pDt} - DP.$$

Integration gives

$$P(t) = P(0)\,\mathrm{e}^{-Dt} + \frac{(n+mD)X^+(0)}{Y_{\mathrm{P}}(1-p)D}(\mathrm{e}^{-pDt} - \mathrm{e}^{-Dt}). \tag{25}$$

Since (24) forces S (and thus $S_0 - S$) to be constant, the instantaneous molar yield of product per mole of substrate consumed is given by

$$\frac{P}{S_0 - S} = \frac{P(0)\,\mathrm{e}^{-Dt} + \dfrac{[n + mD]\,X^+(0)}{Y_\mathrm{P}(1-p)\,D}\,[\mathrm{e}^{-pDt} - \mathrm{e}^{-Dt}]}{S_0 - DK_\mathrm{S}/(\mu_\mathrm{m}^+ - D)} \tag{26}$$

which decreases continuously at long times when $\mathrm{e}^{-Dt} \approx 0$.

The models discussed above both predict product yields $(P/(S_0 - S))$ that decline with time, as indicated by the characteristic term $\mathrm{e}^{-p\mu t}$. Process strategies for operation with deactivating catalysts are evident:

(i) continue the run until P is too small to recover, then shut down, reinoculate, and start up again;

(ii) alter D (continuously or stepwise) to maintain either a given value of P (concentration) or of $P/(S_0 - S)$ (yield), halting when DP falls to a minimal acceptable productivity;

(iii) alter S_0 slowly to maintain a desired objective, e.g. constant P, continuing until the yield falls to a minimum acceptable value;

(iv) alter the temperature to maintain desired rate (while temperature variations are established traditions in non-biological catalytic processes, this mode of control is likely to be less useful here, owing to thermal deactivation of cells in general).

Selective cell recycling

Recombinant vector–host systems may produce (+) cells with physical characteristics different from the (−) strain. Examples may include cells with an altered shape or density due to the formation of internal storage products, cells with different net external surface charge, size, etc. Under some of these circumstances, we may inquire about the effect of selective cell recycling on chemostat stability and operation. While the difference in properties between (+) and (−) cells may not be large, a modest selective cell recycle may suffice, i.e. we simply note that an obvious process goal is only the maintenance of the desired culture in chemostat, rather than the achievement of full resolution of (+)/(−) mixtures. Since table 1 indicates that $\alpha (\equiv \mu^-/\mu^+)$ is often only 10–20% larger than unity, it follows that a minor (+) enrichment in the recycling stream may compensate sufficiently to produce a desired result, i.e. (+) maintenance.

Motivations for process biological recycling have been examined by a number of authors, from Herbert (1961) to Hamer (1981). My prime focus here will be enrichment of the fermenter in the concentration of desired (+) cells by the use of partial cell separation and recycling (figure 4).

I consider exponential growth first. The dilution rate required to maintain a single exponentially growing population with recycling is given by

$$D = \mu/\{1 - a(b-1)\}, \tag{27}$$

where a is the ratio of recycling to feed stream (F_r/F_0) and b is cell enrichment in the recycling stream (X_r/X).

Equation (27) above does not contain S, since the cells are growing exponentially at an assumed constant specific growth rate. Under this circumstance, the (+) population behaves independently of the (–) species (the effect of joint competition for S is considered later in the Monod model). Thus, for a fixed value of a, and a separator that produces $b^+ (\equiv X_r^+/X^+)$ and $b^- (\equiv X_r^-/X^-)$, the dilution rates D^+ and D^- at which each population can be *separately* maintained are given (for $p \approx 0$) by

$$D^+ = \mu^+/\{1-a(b^+-1)\} \tag{28a}$$

and

$$D^- = \mu^-/\{1-a(b^--1)\}. \tag{28b}$$

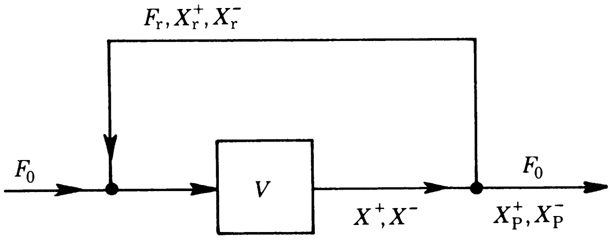

FIGURE 4. Chemostat with selective cell recycling.

The corresponding chemostat plus recycle balances for the two species system of (1)–(3) are given by

$$F_r X_r^+ + \mu^+ X^+ (1-p)\,V - (F_0 + F_r)\,X^+ = 0,$$

$$F_r X_r^- + (p\mu^+ X^+ + \mu^- X^-)\,V - (F_0 + F_r)\,X^- = 0,$$

or

$$aDb^+X^+ + \mu^+(1-p)\,X^+ - (a+1)\,DX^+ = 0, \tag{29a}$$

$$aDb^-X^- + (p\mu^+X^+ + \mu^-X^-) - (a+1)\,DX^- = 0. \tag{29b}$$

From (29a), a steady-state, finite value of X^+ is obtained only if

$$D^+ = \{\mu^+(1-p)\}/\{1-a(b^+-1)\}. \tag{30a}$$

Similarly, if $X^+ = 0$, a finite non-zero value for X^- occurs only if

$$D^- = \mu^-/\{1+a(b^--1)\}. \tag{30b}$$

The steady-state problem gives rise to a competitive exclusion if $b^- = b^+$, since we will usually have $\mu^- > \mu^+$, i.e. $D^- > D^+$, as in the earlier case cited above (no recycling) by Imanaka & Aiba (1981). However, if selective cell recycling operates to enrich the reactor in (+) relative to (–), i.e. if $b^+ > b^-$, a stabilization of the slower growing culture, X^+, may be achieved. The criterion is seen by setting $D^- = D^+$, corresponding to marginal stability of X^+. Thus

$$\frac{\mu^+(1-p)}{1-a(b^+-1)} = D^+ = D^- = \frac{\mu^-}{1+a(b^--1)}.$$

Rearranging,

$$b^+ - 1 = \frac{\alpha + p - 1}{\alpha a} + \frac{1-p}{\alpha}\,(b^- - 1), \tag{31}$$

where $\alpha \equiv \mu^-/\mu^+ > 1.0$ as before (table 1). This equality would give a marginally stable situation: here the disadvantage that species (+) has in slower specific growth rate ($\mu^+ < \mu^-$) is just balanced by a more selective recycling in favour of X^+ ($b^+ > b^-$).

As an example, let $\alpha = 1.1$ (table 1), $p = 0.01$ (relatively unstable) and $a = 0.1$. Then the marginal stability equation is

$$(b^{+}-1) = 1.0+0.9(b^{-}-1);$$
$$b^{+} = 1.1+0.9b^{-}. \tag{32}$$

The value of b^{+} needed to just balance a given b^{-} value is shown in table 3.

The maximum b^{+} enrichment noted in table 3 arises because $a \equiv F_{\mathrm{r}}/F_0 = 0.1$. As we recycle one-tenth of the liquid phase in this example, we can only enrich the recycling liquid up to $10X^{+}$, i.e. $b^{+}_{\mathrm{max}} = 10$. The key point demonstrated by this simple analysis is that, for major cell recycle fractions, it is possible with only modest selectivity in cell recycling to obtain a chemostat operating stably with a slower growing (+) strain in a non-washout steady state. This example is the only steady-state example among all the cases presented above, i.e. it is the only true *chemically* static case.

TABLE 3. RECYCLING ENRICHMENTS FOR (−) AND (+) CELLS

b^{-} ($\equiv X_{\mathrm{r}}^{-}/X^{-}$)	minimum value of b^{+} ($\equiv X_{\mathrm{r}}^{+}/X^{+}$) needed to maintain (+)
2.0	2.9
3.0	3.8
4.0	4.7
5.0	5.6
6.0	6.5
7.0	7.4
8.0	8.3
9.0	9.2
9.9	10.0 (max. possible if $a = 0.1$)

The mixed culture example just presented illustrates conveniently the notion that selective recycling can stabilize a slower exponentially growing population. While stabilization is possible, the particular nature of an exponential growth rate under condition (29*a*) means that X^{+} is indeterminate. A case exists that verifies such behaviour (Finn & Wilson 1954); however, in the more general case, the specific growth rates are substrate-dependent functions.

Consider a chemostat (figure 4) with selective cell recycling and two populations (+, −) growing according to Monod's model. The steady-state equations for X^{+}, X^{-}, S, and P are given by the following four statements:

$$Dab^{+}X^{+}+\frac{\mu^{+}_{\mathrm{m}}(1-p)\,X^{+}S}{K_{\mathrm{S}}+S}-(a+1)\,DX^{+} = 0; \tag{33a}$$

$$Dab^{-}X^{-}+\frac{p\mu^{+}_{\mathrm{m}}SK^{+}}{K_{\mathrm{S}}+S}+\frac{\mu^{-}_{\mathrm{m}}SX^{-}}{K_{\mathrm{S}}+S}-(a+1)\,DX^{-} = 0; \tag{33b}$$

$$DP = \left(\frac{nS}{K_{\mathrm{S}}+S}+m\right)X^{+}; \tag{34}$$

$$D(S_0-S) = \frac{D}{Y_{\mathrm{X}}}\,(X^{+}_{\mathrm{P}}+X^{-}_{\mathrm{P}})+\frac{DP}{Y_{\mathrm{P}}}$$
$$= D\,\frac{\{1-a(b^{+}-1)\}X^{+}+\{1-a(b^{-}-1)\}X^{-}}{Y_{\mathrm{X}}}+\frac{DP}{Y_{\mathrm{P}}}. \tag{35}$$

At the steady state for population $(+)$, $X^+ \neq 0$ requires from (33*a*) that

$$D = \frac{\mu_m^+(1-p)\,S}{\{1-a(b^+-1)\}(K_S+S)} = \mu_m' \frac{S}{K_S+S} \tag{36a}$$

or

$$S = \frac{DK_S}{\mu_m'-D}, \tag{36b}$$

the analogue to (24) for the case without recycling. Again, the existence of this steady-state solution requires that D be large enough to wash out $(-)$ cells in the absence of $(+)$, i.e.

$$D > (\mu_m^-)'\,S/(K_S+S). \tag{37}$$

In the presence of $(+)$ cells the $(-)$ strain is continuously reinoculated by virtue of the transformation in (2).

The biomass concentrations are determined as follows. The dilution rate is eliminated by using (33*a*), (33*b*) and (36*a*) to give

$$\begin{aligned}\frac{X^-}{X^+} &= \frac{(a+1)\,(\mu_m'/\mu_m^+)-p}{ab^-(\mu_m'/\mu_m^+)+(\mu_m^-/\mu_m^+)} \\ &\equiv \chi.\end{aligned} \tag{38}$$

The use of (34) and (38) in the substrate balance gives

$$(S_0-S) = \frac{\{1-a(b^+-1)\}+\{1-a(b^--1)\}\chi}{Y_X}\,X^+ + \left(\frac{nS}{K_S+S}+m\right)X^+. \tag{39}$$

With (36*b*) and (39), the solution of X^+ becomes

$$X^+ = \left(S-\frac{DK_S}{\mu_m'-D}\right)\Big/\left[\frac{\{1-a(b^+-1)\}+[1-a(b^--1)]\,X}{Y_X}+\left(\frac{nD}{\mu_m'}+m\right)\frac{1}{Y_P}\right].$$

The apparent yield of product per unit of substrate consumed is given by

$$\begin{aligned}\frac{P}{S_0-S} &= \left(\frac{nD}{\mu_m'}+m\right)\Big/\left[\frac{\{1-a(b^+-1)\}+\{1-a(b^--1)\}\chi}{Y_X}+\frac{1}{Y_P}\left(\frac{nD}{\mu_m'}+m\right)\right] \\ &= Y_{app} = f(D).\end{aligned}$$

Thus, as D becomes large, $Y_{app} \to Y_P$. As D diminishes, the product yield diminishes to a smaller value, reaching, in the limit as $D \to 0$,

$$\frac{P}{S_0-S} = Y_{app} = Y_P\Big/\left[\frac{\{1-a(b^+-1)\}+\{1-a(b^--1)\}\chi Y_P}{mY_X}+1\right].$$

Thus

$$Y_{app} < Y_P.$$

If a product is exclusively non-growth associated, $n = 0$, and the apparent product yield is again that of the last equation above, for all values of dilution rate.

The dilution rate cannot be widely varied independently of other operating conditions, however. In particular, D must always satisfy (36*a*) for $(+)$ to be stably maintained; otherwise, the $(-)$ population will outgrow the $(+)$ population and result in a competitive exclusion.

OTHER CASES

Many other examples suggest themselves. Further kinetic modelling will be most interesting when specific examples are available. The following additional cases are noted here.

1. *Wall growth* is not subject to washout. Thus, provided that a stably attached (+) colony can continuously reinoculate the bulk reactor, X^+ can be stably maintained.

2. When (+) overproduces some product that stresses the cell, we may expect (+) to pay a higher *maintenance* price than (−).

3. Selective recycling is just one example of an *artificial selection pressure*. Others include the use of plasmids coded for antibiotic resistance, so that a trace antibiotic addition to the feed, or a periodic dosage, could maintain the desired space–time niche for (+).

4. Immobilized whole cell reactors may also be of interest. Here, if no further cell growth is occurring, the situation is analogous to that of a deactivating enzyme catalyst, except that each species (+) and (−) may continue to consume some S for maintenance. The maintenance requirements for (+) and (−) may differ (especially if the (+) cells are stressed by their production of P).

Still other situations may arise, including the design of extraordinarily stable recombinant plasmid systems. None the less, three-quarters of a century of experience in chemical and petroleum refining industries with continuous flow catalytic reactors indicates that catalyst deactivation *is* a major phenomenon of central importance in process economics. Table 1 suggests that the most typical form of deactivating catalyst that we may expect with industrially interesting recombinant systems is a mixed culture characterized symbolically by the very simple system given in (1) and (2). This discussion has indicated, by analysis of parameter magnitudes and typical rates, that slowly deactivating systems may still be of interest in batch fermentations, and that even stable chemostat operation of a desired population (+) with $\mu^+ < \mu^-$ is possible with modestly selective cell recycling.

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Discussion

D. C. Ellwood & A. Robinson (*P.H.L.S./C.A.M.R., Porton Down, U.K.*). We wish to report some of our results on the stability of plasmid-carrying strains in long-term continuous cultures because our experimental data do not fully support the theoretical analysis of Professor Ollis. (1) R^+ strains carrying natural plasmids were generally stable in the chemostat but the high copy numbers of small plasmids were not usually maintained. (2) Small constructed plasmids (e.g. pBR322) were rapidly lost from chemostat cultures after a fall in copy number. (3) A small residual population of R^+ cells (0.1–1 %) was always maintained in the continuous cultures. This was not related to wall growth. (4) It is possible to stabilize constructed plasmids by the introduction of specific DNA sequences derived from plasmid ColE1.

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