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Competitive Continuous Fermentations with Selective Recycle

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ABSTRACT

An inclined settler is used to partially separate desired cells from undesired cells in the product stream of a competitive continuous fermentation on the basis of differences in sedimentation velocities owing to differences in floc-forming capabilities. A stream that is enriched in the desired cells is then selectively recycled to the bioreactor. Experiments with nonsegregating yeast cultures and segregating bacterial cultures have demonstrated that maintenance of the slower-growing strains is achievable using selective recycle, whereas washout of these strains by the faster-growing competing strains occurs in the absence of selective recycle. These results are in good agreement with theoretical predictions.

Index Entries: Competitive fermentations; selective recycle; flocculation; sedimentation; plasmid instability.

INTRODUCTION

Selective recycle based on flocculation and sedimentation properties has been shown to successfully maintain slower-growing microorganisms in continuous bioreactors, despite competition from faster-growing organisms (*1,2*). This novel method for continuous fermentations can overcome problems of contamination and plasmid loss owing to segregation. The focus of this paper is to summarize work done in our laboratory with

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both yeast and bacterial cultures, present data that demonstrate that selective recycle can greatly increase cell mass in a continuous bioreactor, and briefly discuss the construction of a new bacterial plasmid that will allow for continuous maintenance of a plasmid-containing strain and the simultaneous production of a speciality chemical of interest.

Selective recycle for the maintenance of recombinant cells in a continuous reactor was first proposed by Ollis (*3*), who developed a theory that predicted the degree of selection necessary in a recycle stream. However, he did not comment extensively on a method for achieving the selectivity. Stephanopoulos et al. (*4*) noted that plasmid-bearing cells contain more protein and are larger than segregants and proposed that the two types of cells could be separated by differential sedimentation. Owing to the small sizes and relative densities of the cells, such a separation is expected to be difficult and perhaps impractical for large-scale continuous fermentations. However, they were able to successfully maintain a yeast culture in the presence of a bacterial culture, using inclined settling to achieve selective recycle (*5*), but they did not attempt to separate recombinant cells from host cells of the same species.

In our laboratory, we are selectively separating two phenotypes of the same microbial species by having one of the phenotypes be flocculent. When these cells flocculate, the resulting flocs or clumps of many cells settle much faster than the unflocculated cells. The two strains, therefore, may be separated on the basis of the differences in their sedimentation velocities. Yeast cells from flocs owing to cell surface interactions. Although these interactions depend on environmental factors, such as the pH and ionic strength of the medium, they are primarily under genetic control through the protein structure of the cell wall. One way that bacterial cells form flocs is owing to hydrophobic pili–pili interaction (*6*). Pili are proteinaceous surface appendages that are assembled by the polymerization of pilin, a protein that is rich in nonpolar amino acid side chains. When cells overproduce pili, they form aggregates or flocs as large as a few millimeters in diameter. Since pilus production is under direct genetic control, it may be exploited to control flocculation and, therefore, achieve selective separation and recycle based on flocculation and sedimentation.

SELECTIVE RECYCLE REACTOR THEORY

Selective recycle reactor theory for two competitive species, one faster-growing and nonflocculent, the other slower-growing and flocculent, was developed by Davis and Parnham (*1*) and is summarized here. A schematic of the reactor is shown in Fig. 1. The reactor is continuous with two product lines. One passes through an inclined settler (or other suitable separator), where the desired flocculent cells are separated from the undesired nonflocculent cells. A concentrated stream exits the bottom of the settler and is recycled back to the reactor, whereas a diluted stream is re-

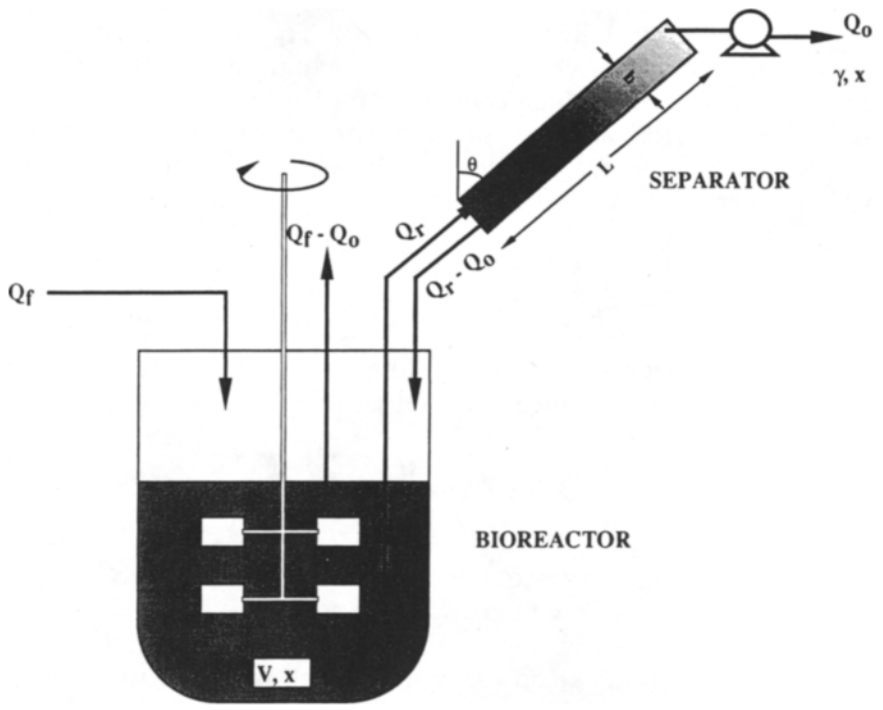

Fig. 1. Schematic of a selective recycle reactor and separator.

moved from the top of the settler and discarded. The other product line acts as a level controller and withdraws cell broth directly from the bioreactor.

Unsteady state mass balances about the entire system (reactor and cell separator) are:

$$dX^+ / dt = (\mu^+ \beta^+ D)X^+ - \mu^+ pX^+ \quad (1)$$

$$dX^- / dt = (\mu^- \beta^- D)X^- + \mu^+ pX^+ \quad (2)$$

where μ is the specific growth rate, X is the cell concentration in the fermentor, $D = Q_f/V$ is the dilution rate, Q_f is the volumetric feed rate, V is the fermentor volume, p is the segregation probability, and β is an effective cell dilution factor accounting for the direct product line and defined by:

$$\beta^+ = 1 - (1 - \gamma^+)\frac{Q_o}{Q_f} \qquad \beta^- = 1 - (1 - \gamma^-)\frac{Q_o}{Q_f} \quad (3)$$

The quantity, γ, is the cell dilution factor, defined as the ratio of the cell concentration of a given strain in the diluted stream exiting the inclined settler to that entering it (if the reactor is well stirred, the cell concentration entering the settler is the same as in the reactor). The desired strain is denoted as (+), whereas (−) denotes the undesired strain. Equations (1) and (2) assume sterile feed, negligible cell maintenance and death terms,

and negligible cell growth in the cell separator. The latter assumption is a reasonable approximation when the separator volume is small, compared to the fermentor volume, which is the case for the experiments described later in this paper. It is also assumed that there are no physiological differences between the recycled cells and those in the bioreactor. Monod kinetics were assumed, with μ^+ and μ^-, having the following forms

$$\mu^- = \frac{\mu_m^- S}{K^- + S} \qquad \mu^+ = \frac{\mu_m^+ S}{K^+ + S} \tag{4}$$

where S is the substrate concentration in the fermentor. The final equation needed is the mass balance on the limiting substrate:

$$\frac{dS}{dt} = D(S_O - S) - \frac{\mu^+ X^+}{Y^+} - \frac{\mu^- X^-}{Y^-} \tag{5}$$

where S_o is the limiting substrate concentration in the feed, and Y^+ and Y^- are the yield coefficients for cell growth.

There exists a point of stable coexistence for the two strains, which can be found by assuming steady state and equating the dilution rates for the (+) and (−) strains, yielding:

$$\frac{\beta^-}{\beta^+}\frac{\mu^+}{\mu^-}(1-p) = 1 + p\frac{\mu^+}{\mu^-}\frac{X^+}{X^-} \tag{6}$$

The coexistence ratio, $G=\beta^-\mu^+(1-p)/\beta^+\mu^-$, determines whether a selective recycle reactor will fail (become 100% undesired cells). For nonsegregating cultures ($p=0$), complete washout of the desired (+) strain occurs if $G<1$, whereas complete washout of the undesired (−) strain occurs if $G>1$. Coexistence (metastable) is possible only if $G=1$. In contrast, stable coexistence for segregating cultures ($p\neq 0$) occurs for all $G>1$. The mass ratio, X^+/X^-, at steady state is given by Eq. (6) and increases with increasing G. For $G<1$, however, complete washout of the desired strain ($X^+=0$) occurs for all possible values of the segregation probability, p. Note that p is defined as the fraction of the daughter cells that are of the plasmid-free (−) phenotype when their parent cells are of the plamid-bearing (+) phenotype. Thus, p is a measure of the frequency of plasmid shedding, and it generally has a value of a few percent, or less.

INCLINED SETTLER THEORY

An inclined settler is used in this reactor scheme to selectively separate and recycle cells. This settler design, when compared to conventional settlers, enhances the sedimentation rate of particles or cells owing to an increase in available settling area. Not only can flocs of cells settle onto the

bottom of a vessel, but they can also settle onto the upward-facing inclined wall. These flocs then form a thin sediment layer that slides down to the bottom of the vessel because of gravity. Details of inclined settler theory and enhanced sedimentation are reviewed by Davis and Acrivos (*7*).

Figure 1 includes a schematic of a rectangular inclined settler. The equation for the volumetric rate of production of fluid that is free from particles or flocs of cells that settle with a verticle settling velocity, v, is

$$Q(v) = vw(L\sin\theta + b\cos\theta) \quad (7)$$

where L is the length of the inclined wall, w is the width of the inclined wall, b is the spacing between the plates of the settler, and θ is the angle of inclinaton of the settler walls from the vertical.

The inclined settler can be used continuously to separate two types of microbial organisms, provided that they have different sedimentation properties. In this work, flocs of cells settle at an average sedimentation velocity that is approximately 100-fold greater than single cells. This difference allows, for example, flocculent plasmid-bearing bacteria cells, which are on the order of 0.1–1 mm in diameter, to be separated from nonflocculent plasmid-free cells.

It is necessary to determine the distribution of settling velocities in order to design an inclined settler for the selective recycle reactor. Davis et al. (*8*) used Eq. (7), along with mass balances, to analyze inclined settlers operating continuously to separate faster-settling particles from slower-settling particles, predicting that

$$\gamma = \int_0^{v_o} \frac{Q_o - Q(v)}{Q_o} P(v)dv \quad (8)$$

The quantity v_o is the cutoff sedimentation for flocs or particles reaching the overflow and is given by $Q(v_o) = Q_o$. All flocs with settling velocities of $v \geq v_o$ will settle and be recycled to the reactor, whereas a portion of those with $v < v_o$ will be carried out in the overflow. P(v) is the normalized probability density function representing the distribution of settling velocities of particles fed into the settler. It can be determined experimentally using the sedimentation/light extinction apparatus designed by Davis and Hunt (*9*).

MATERIALS AND METHODS

Microbial Strains and Plasmids

Experiments were performed with nonsegregating yeast cultures and segregating bacterial cultures. Two strains of *S. cervisiae* were used for the yeast experiments. S646-1B (denoted as 1B and as the desired strain) is

Table 1
Experimentally Determined Parameters Needed for Reactor Design

Parameter	Yeast	Bacteria
μ^+ (h^{-1})	0.43±0.04	0.72±0.12
μ^- (h^{-1})	0.47±0.10	1.14±0.12
p	0	0.03±0.02
K^+ (g/L)	0.88±0.11	0.004
K^- (g/L)	0.27±0.05	0.004
Y^+ (g/g)	0.42	0.45
Y^- (g/g)	0.42	0.48

slower-growing and flocculent; it is also deficient for adenine. Fleischmann's baker's yeast (denoted as FY and as the undesired strain) is faster-growing and nonflocculent. *E. coli*, strain ORN103 (*recA lacU*169 derivative of P678-54), was the host bacterial strain for the plasmid, pORN108, used in this study. The plasmid, pORN108, is a 15.6 kb (kilobase) fragment and is a regulatory mutant of the *pil* operon inserted into the vector pACYC184 (*10*). It confers resistance to both chloramphenicol and kanamycin. Its isolation and characterization are described by Orndorff and Falkow (*6*). The average plasmid copy number for the plasmid, pORN108, when it is present in the strain, ORN103, was experimentally determined to be 3.8 copies/cell.

Experimental Determination of Reactor Design Parameters

The parameters needed to design and run a continuous fermentor, such as growth rate and yield coefficient, are summarized in Table 1 for both yeast and bacteria, where the confidence levels are 90%. The specific growth rates and bacterial segregation coefficient were determined in batch shake flask experiments. Minimal medium (0.5% NaOH, 0.0005% adenine, 1% succinic acid, 0.5% ammonium sulfate, 0.17% yeast nitrogen base, and 1% dextrose/L distilled water) was used for the yeast experiments, and M9CA medium (6g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4CL, 2 g casamino acids, 2 mL 1*M* $MgSO_4$, 0.1 ml 1*M* $CaCl_2$, 2 g glucose, 4.1 mL 1% L-leucine, 4.1 mL 4% L-proline, and 0.166 mL 0.1% Vitamin B1, in 1 L of water) was used for the bacteria experiments. Monod constants were experimentally determined from initial growth rate experiments for the yeast cells, and a value of 0.004 g/L was assumed for bacterial cells. This value is typical for *E. coli* strains growing in glucose-limited medium (*11*). The values of Y^+ and Y^- were also experimentally found by determining the dry cell mass and the concentration of cells in a batch cultue for a given limiting subtrate concentration, once the cells had reached the stationary phase.

Continuous Fermentations

All continuous experiments were performed in a 500 series LH fermentor that has dissolved oxygen, pH, temperature, and foam controls. For the yeast experiments, the fermentation vessel was 2 L in size with a 1.7 L working volume. The inclined settler was a rectangular glass tube with $L=40$ cm, $b=0.5$ cm, and $w=5.0$ cm. For the bacteria experiments, a 1 L vessel was used with a 0.6 L working volume, and the settler was 51 cm in length. The longer settler was needed because bacterial flocs are smaller than yeast flocs and, thus, require a greater area on which to settle. The angle of inclination from the vertical was varied between 30 and 45°. The design of the settler was based on Eq. (8) using experimentally determined settling velocity distributions, measured with a sedimentation/light extinction apparatus (*9*), with the design goal of γ^- close to unity (all undesired cells passing through the separator without settling) and γ^+ close to zero (all desired cells settling in the separator and subsequently recycled).

The yeast experiments were started as batch fermentations with growth from an inoculum of the slower-growing strain, 1B. When the stationary phase was reached, continuous operation was started. As steady state was approached, an inoculum of the faster-growing strain, FY, was added. Selective recycle, if used, was started at this time. Samples were withdrawn periodically from both the fermentor and settler overflow. The samples were diluted, and the flocs were dispersed with EDTA prior to cell counting. Cells counts were made by plating on minimal media with and without adenine and by hemocytometry. Values for γ^+ and γ^- were then calculated by dividing the measured values of X^+ and X^-, respectively, in the overflow from the settler by the corresponding values in the fermentor.

The procedure was slightly altered for the segregating bacterial cultures. The fermentor was initially run in batch mode, as described above, except antibiotics were added to the M9CA medium to maintain the plasmid-containing cells. The other difference is that the fraction of plasmid-bearing cells was determined by diluting the cells in mannose to disperse the flocs before plating them on LB medium. After the colonies had grown on the plates, they were transferred, using cotton velvet, to both an LB plate without antibiotics to determine the number of plasmid-containing cells and one with antibiotics to check the efficiency of the transfer.

RESULTS

The results of two continuous fermentations, performed by Davis and Parnham (*1*) with yeast cells, are reproduced in Fig. 2. The *y-axis*, F^+, is the fraction of desired cells and is defined as $X^+/(X^+ + X^-)$. For both experiments, the dilution rate was set at $D=0.1\ h^{-1}$. Recycle was not used

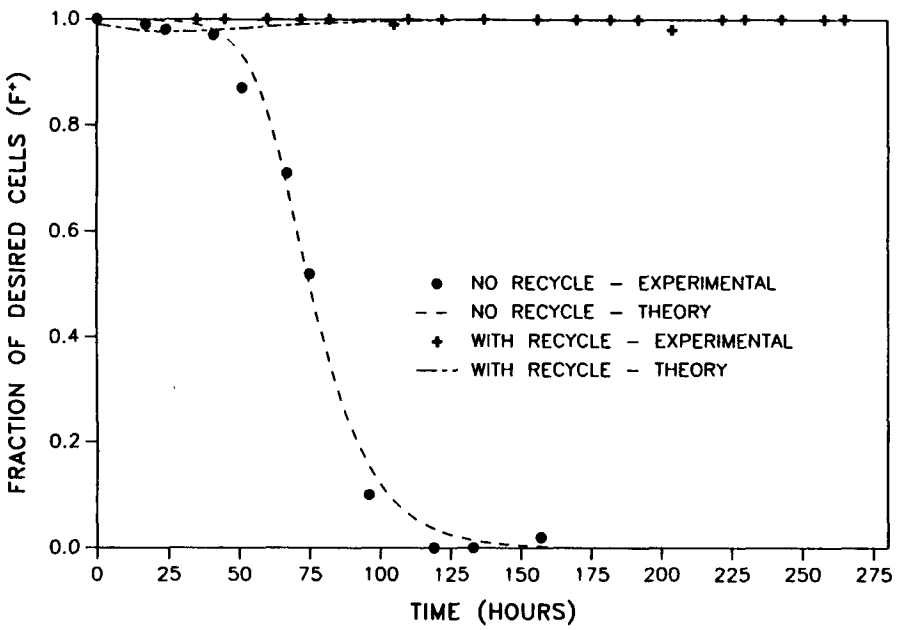

Fig. 2. Yeast experiments: washout of the slower-growing strain when recycle is not used and maintenance of the slower-growing strain when recycle is used (from Davis and Parnham (*1*)).

for one experiment, and the slower-growing stain was indeed washed out of the reactor. The value of G for this experiment was calculated to be 0.9, which correctly predicts the washout of the slower-growing strain. The second experiment was a continuous fermentation with recycle, and the slower-growing strain was maintained as dominant for 270 h, at which time fermentation was discontinued. For this experiment, the measured values of the dilution factor are $\gamma^- = 0.33$ and $\gamma^+ = 0.002$, in close agreement with those predicted by Eq. (8). The value of G for this experiment was calculated to be 6.5, which correctly predicts the maintenance of the slower-growing strain. In both cases, the theory and experiment are in very close agreement.

Figure 3 shows similar experiments performed with the segregating bacterial culture. Not only are the plasmid-containing cells slower-growing, but they also have a finite possibility of losing a plasmid, which results in an increased amount of segregant (–) cells. The first experiment depicted was without recycle and demonstrated that the plasmid-bearing strain is washed out of the reactor. The dilution rate was set at 0.45 h^{-1} and the value of G was calculated to be 0.61, which again correctly predicts the washout of the desired strain. The second experiment shown in the figure is with recycle, and the plasmid-bearing strain was maintained as dominant in the reactor for a period of about 180 h, after which fermentation was stopped. For this experiment, the dilution rate was 0.16 h^{-1}, and the value of G was calculated to be 1.33. The dilution factors were measured to

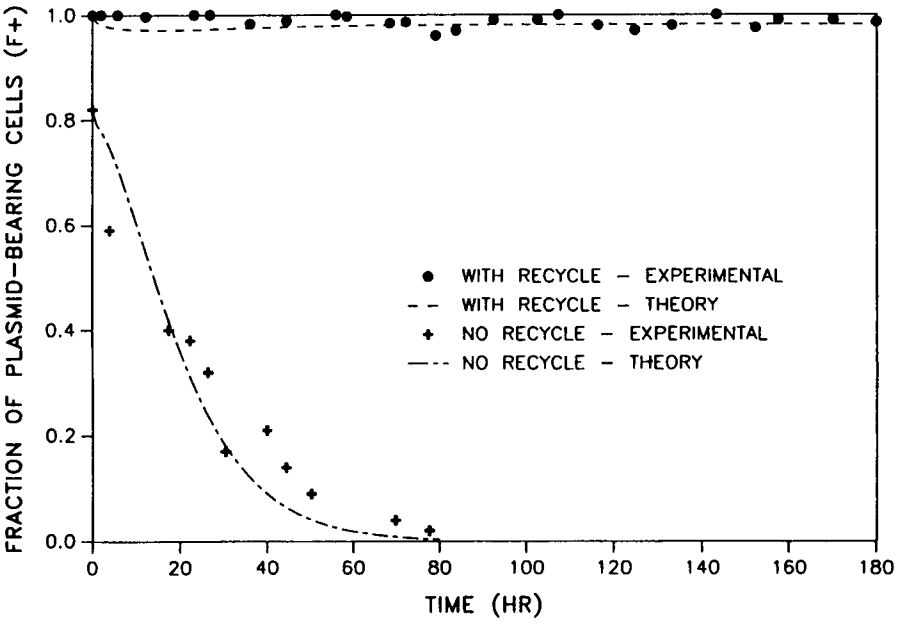

Fig. 3. Bacteria experiments: washout of the plasmid-bearing strain without recycle and maintenance of the plasmid-bearing strain using selective cycle.

be $\gamma^+ = 0.45$ and $\gamma^- = 1.0$. Again, there is very good agreement between experiment and theory. Both the yeast and bacteria experiments demonstrate that slower-growing microorganisms can be maintained in a continuous fermentation by selective recycle despite competition from faster-growing microorganisms and that this strategy can even overcome problems, such as plasmid instability.

A third experiment with bacterial cultures demonstrates the power of the selective recycle reactor (Fig. 4). The reactor was allowed to fail ($G < 1$) to the point where it contained about 20% plasmid-bearing cells and 80% segregants. At this time ($t = 0$ on the graph), the dilution rate was lowered, which caused the value of γ^+ to decrease owing to the increased holdup time in the settler, placing the system in favorable conditions for recovery ($G > 1$). This shows that selective recycle can be used to recover a failing reactor that would otherwise have to be shut down. The theoretical curve and data, again, agree well.

Selective recycle also has the added advantage of increasing cell mass in the fermentation vessel. Figure 5 shows experimental data and the corresponding theoretical predictions for the total dry cell mass in the yeast fermentations depicted in Fig. 2. The experimental values were determined by first dispersing the flocs and then performing cell counts on a hemacytometer slide under a microscope. The resulting number concentrations were multiplied by the average dry cell weight determined by filtering, drying, and weighing a sample with a known cell concentration.

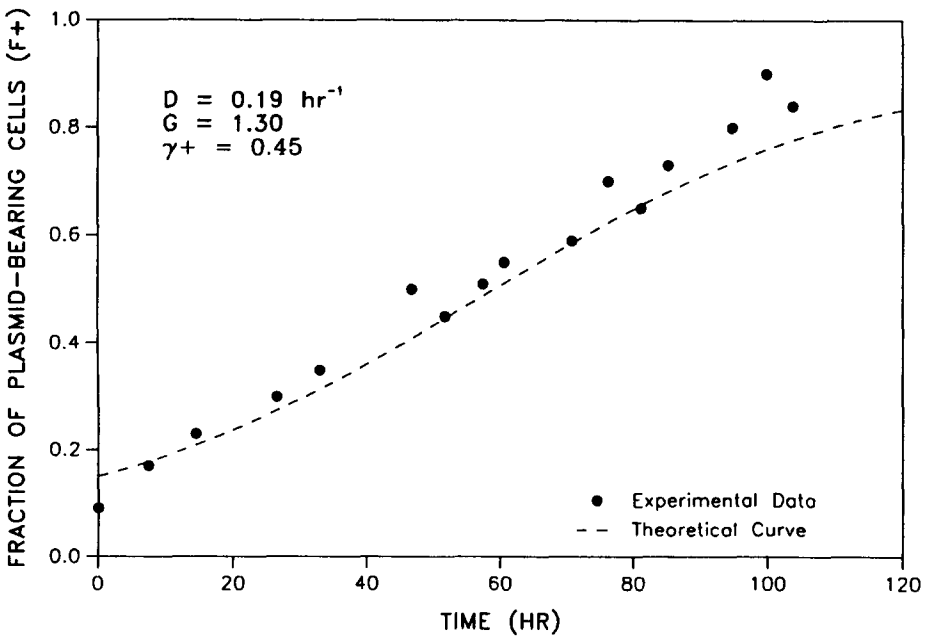

Fig. 4. Recovery of the plasmid-bearing bacterial strain using selective recycle.

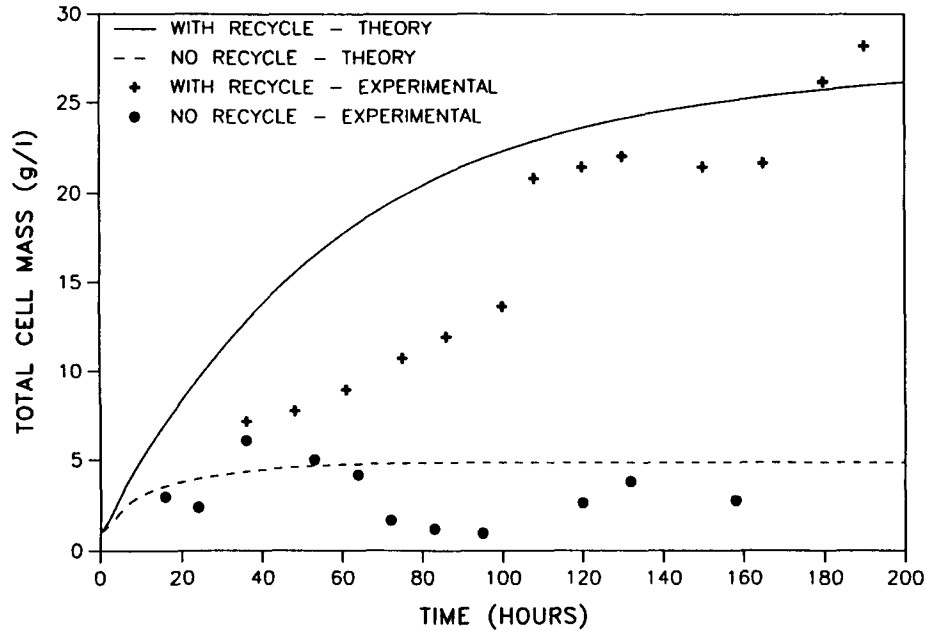

Fig. 5. Total dry cell mass vs time in continuous yeast fermentations with and without recycle.

Both theory and experiment show that a greater than fivefold increase in total cell mass was attained when recycle was used, compared to when it was not used. The cell mass measurements that are lower than the theoretical predictions may have resulted from the fact that a biofilm of yeast cells formed in a ring on the bioreactor wall near the foam level, and these cells would not be present in the samples taken.

CONCLUDING REMARKS

A selective recycle reactor has been shown to continuously maintain a flocculent, slower-growing yeast strain, despite competition from a faster-growing, nonflocculent yeast strain. This novel reactor system has also been shown to maintain unstable plasmid-bearing bacterial cells in a continuous reactor. Selective recycle reactor theory agrees well with the experiments, and the utility of the stable maintenance ratio, *G*, has been demonstrated. The value of this ratio allows a researcher to determine in advance whether a selective recycle reactor will be able to maintain a slower-growing microorganism as dominant when in competition with a faster-growing organism. The added advantage of using selective recycle, that of obtaining higher cell densities in continuous reactors, has also been demonstrated.

Current research involves developing the capability to artificially control the genes responsible for flocculation in bacteria. This would allow the cells to grow without overproducing pili in the fermentor. They would then enter the inclined settler where the *pil* operon would be induced, causing the formation of flocs and allowing the separation of plasmid-bearing cells from segregant cells. The advantage of this strategy is that the plasmid-bearing cells in the main fermentor would then not be subject to the extra metabolic burden of overproducing pili. As seen in Table 1, this burden causes a significant reduction in the growth rate. At present, the *pil* operon has been placed under lactose control. That is, the cells can grow in the absence of lactose, and the *pil* operon is repressed. When lactose or IPTG (a more efficient inducer) is added to the bacteria, they begin to produce pili. The problem with this operator/promoter system is that it is inhibited by glucose. Therefore, current research involves placing the *pil* operon behind either a *tac* promoter or a temperature sensitive promoter, neither of which is catabolically repressed.

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