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DEVELOPMENT OF CELL RECYCLE REACTOR FOR STUDYING CELL STARVATION

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Abstract—A cell recycle reactor was employed to study the rate of death in *E. coli* under glucose starvation. Hollow fibers were used for the microfiltration of the media. Oxygen was supplied through silicone tubing which was fabricated in the form of a heat exchanger. Cell broth was recycled through the inside of the hollow fibers and the silicone tubing. Model equations for oxygen transport were solved to predict the oxygen provision to the starved cells. Oxygenation experiments were performed to test this model. Batch culture of the starved cells was conducted and the results were compared with that of the recycled culture. As the difference in the rate of death in the batch culture and the recycled culture was insignificant, it was deduced the secretion from the dying cells did not influence the metabolism of the starved cells to any detectable extent.

INTRODUCTION

Starving bacteria are of interest in both an ecological and an applied context such as secondary metabolite production. Bacteria frequently undergo periods of starvation in their natural environment, making such studies important in the understanding of normal bacterial life cycles. Separating the growth and production phases of bacteria used in industrial fermentation processes gained the increasing recognition of cell starvation. In *E. coli* cells subjected to carbon starvation, the rate of protein synthesis drops during the first starvation period and remains roughly constant after that period [1].

To date, cell starvation studies have been conducted in batch culture [2]. The batch culture, however, may have a problem associated with secretion products from resting cells. The secretion products may accumulate in the medium of resting cells and consequently interfere with the growth rate of the population. This required a new type of culture method for the resting cells in order to eliminate the secreted material as well as to examine the various parameters affecting the resting cells. The cell recycle reactor was introduced so as to take advantage of the continuous removal of secreted materials. Microfiltration has been used for diluting the secreted materials in the bioreactor. The object of this study is to employ a closed cell recycle reactor for maintaining the culture volume at a constant level with sufficient oxygen tension in medium. The oxygen tension was maintained at a sufficient level through an oxygenator made of silicone tubes. A model of oxygen transfer was developed to predict the oxygen tension in medium, and then verified with an experiment.

THEORETICAL BACKGROUND

The conceptual design of a closed recycle reactor is shown in Figure 1. The pressure in the culture flask becomes lower than atmospheric pressure as microfiltration proceeds and consequently new medium is aspirated into the flask at the rate as microfiltrate is produces. In order to provide cells with oxygen without compromising the closed system, a membrane oxygenator was developed. Silicone polymers have a high gas permeability and can be extruded to form hollow fibers. The ability of this oxygenator to provide sufficient oxygen tension in the liquid media will be predicted using a mathematical model.

Referring to Figure 2, we employed a diffusion equation for oxygen transfer that is a modified version of the equation set in ref. 3. Resistance to gas transfer is contributed by both the silicone membrane and a water film, but the ultimate limit on the gas transfer is imposed by the properties of the membrane. In a membrane limiting situation, the gas diffusion equation for the membrane is:

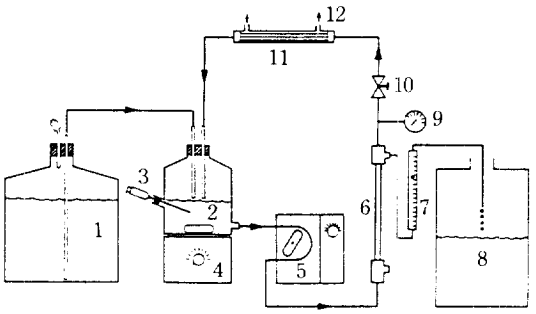

Fig. 1. Details of the cell recycle reactor.

1. M9 media lacking glucose for the starvation experiment
2. flask containing cell suspension
3. sterile syringe for sampling
4. magnetic stirrer
5. tubing pump
6. hollow fiber device for microfiltration
7. flowmeter
8. jar for collecting microfiltrate
9. pressure gauge
10. needle valve
11. oxygenator made of silicone tubing
12. air stream

$$S_m D_{O_2,m} \frac{1}{r} \frac{\partial}{\partial r} \left[r \frac{\partial P_{O_2}}{\partial r} \right] = 0 \qquad (1)$$

$$a < r < a+t$$

where $D_{O_2,m}$ is the diffusivity of oxygen in the membrane, P_{O_2} is the membrane partial oxygen pressure, and S_m is the oxygen solubility in the membrane.

The proper boundary conditions for this equation are

$$S_m P_{O_2} \big|_{r=a+t} = S_m P^{\infty}_{O_2} \qquad (2)$$

and

$$S_m P_{O_2} \big|_{r=a} = \frac{S_m}{S_w} S_w P^{w}_{O_2} \qquad (3)$$

where S_w is the oxygen solubility in water, $P^{\infty}_{O_2}$ is the oxygen partial pressure in the gas outside of the membrane, and $P^{w}_{O_2}$ is that in water.

The plug flow balance for oxygen concentration in the aqueous phase along the axial length z is

$$(\pi a^2 U) \frac{d}{dz} \bar{C}^{w}_{O_2} = 2\pi a D_{O_2} \frac{\partial C^{m}_{O_2}}{\partial r} \Big|_{r=a} \qquad (4)$$

Where $C^{m}_{O_2}$ is the oxygen concentration in the membrane and U is the mean velocity of fluid.

The boundary condition for the z coordinate is

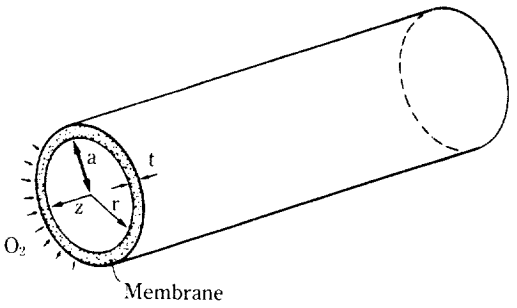

Fig. 2. Silicone tube model and coordinate system.

$$\bar{C}^{w}_{O_2} = C^{i}_{O_2} \quad \text{at } z=0. \qquad (5)$$

Finally the oxygen concentration in the water is a function of oxygen partial pressure and simply represented by the relation

$$\bar{C}^{w}_{O_2} = S_w P^{w}_{O_2}. \qquad (6)$$

Equations (1) through (6) are governing equations to describe the oxygen distribution along the silicone tubing.

The solution for the oxygen distribution is:

$$\bar{C}^{w}_{O_2} = [C^{i}_{O_2} - S_w P^{\infty}_{O_2}] \exp\left[\left(-\frac{S_m}{S_w}\alpha Z\right)\right] + S_w P^{\infty}_{O_2} \qquad (7)$$

where

$$\alpha = \frac{2D_{O_2,m}}{a^2 U} \frac{1}{\ln\left(1+\frac{t}{a}\right)}.$$

Using the physical data in Table 1, we can calculate the quantity of α and $\bar{C}^{w}_{O_2}$ at the outlet of the silicone tubing. The result is as follows:

$$\alpha = 2\times10^{-3}\ (\text{cm}^{-1})$$

$$\bar{C}^{w}_{O_2} = 2.3\times10^{-3}\ (\text{std cm}^3/\text{cm}^3\ H_2O).$$

The value of α is so small that the partial pressure of oxygen in water can be neglected when compared with that of air. Hence we can apply an equilibrium assumption to the transport of oxygen through the silicone membrane. Using the oxygenator having the dimmensions as described in Table 1, we calculated the transport rate of oxygen. The value was 3.8×10^{-3} std cm^3/sec.

EXPERIMENTAL

1. Oxygenation experiment

We designed an unsteady oxygen transfer experi-

Table 1. Data for the calculation of oxygen concentration along silicone tubing (ref. 4)

Oxygen diffusivity in membrane	$(D_{O_2,m})$	1.5×10^{-5}	cm^2/s
Oxygen solubility in membrane	S_m	4.1×10^{-3}	$\frac{std\ cm^3}{cm^3\cdot cmHg}$
Radius of silicone tubing	(a)	0.074	cm
Thickness of silicone tubing	(t)	0.024	cm
Oxygen solubility in water	(S_w)	3.8×10^{-4}	$\frac{std\ cm^3}{cm^3\cdot cmHg}$
Number of silicone tubes		10	
Length of silicone tubing		20	cm
Volumetric flow rate		100	cm^3/min
Oxygen concentration at inlet	(C')	0	

ment to test the silicone oxygenator. As in Figure 1, a flask [2] was connected to the oxygenator [11] and the pump [5]. Other instruments were removed for the oxygenation experiment. An oxygen probe was immersed through the sampling port [3] and connected to a oxygen meter (YSI model 58) from which the signal was recorded. Nitrogen gas was aerated to the flask in order to remove dissolved oxygen before we started the oxygenation experiment. Oxygenation was performed by pumping water through the oxygenator. The signal from the oxygen meter was recorded.

2. Bacterial strains, media and growth conditions

The *E. coli* K-12 was used in this study. M9 medium was used for the growth and starvation of cells. M9 medium contained the following (per liter): Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; $MgSO_4\cdot7H_2O$ 0.246 g; and $CaCl_2\cdot2H_2O$ 14.7 mg. Growth media were supplemented with 0.4% glucose as the carbon source. Cells were grown in Erlenmeyer flasks at 37C with shaking at 200 rpm. Growth was monitored by the increase in absorbance at 660 nm; 1 optical density unit was equivalent to 10^9 cells per ml.

3. Glucose starvation

Cells were grown in the appropriate medium to a density of 2×10^8 to 3×10^8 cells per ml (midlog phase). They were harvested asceptically by centrifugation at 10,000 g for 10 min, washed twice by suspension in the growth medium minus glucose, and suspended to the original density in the growth medium minus glucose. During starvation, cultures were shaken in Erlenmeyer flasks at 200 rpm at 37C.

4. Viability determination

The viability of starving cultures was determined by serial dilution and spreading of cells. At each time point, duplicate plates were counted. To make the agar medium, minimal salts media (1 mM $MgSO_4$ and 1 mM $CaCl_2$) were supplemented with 1.5% Difco Bacto-Agar and 0.02% glucose.

5. Cell recycle experiment

The cell recycle reactor (Fig. 1) used in this study consisted of a 1-liter culture flask, from which cells were constantly withdrawn by a pump (Sarns model Slok-II) and cycled through a hollow fiber filter unit (Accurel, Ghia Membrana, USA) and then back to the original culture flask. The filter unit consisted of three polypropylene fibers encased in a single shell. The fiber pore size was 0.2 μm, and the length of the fibers was 30 cm. Fresh starvation medium from a reservoir was drawn into the system at the same rate as used starvation medium was extruded through the filter.

The reactor and all the tubing were sterilized with a 5% formaldehyde solution. After purging the formaldehyde solution, 5 l of autoclaved distilled water was used to remove the remaining formaldehyde solution. The methods in Experimental 2 and 3 was used to prepare a starving cell suspension which was poured into a flask. At time zero, the tubing pump was started to recirculate the cell suspension as well as to induce filtration in the hollow fiber. In addition, a portion of the cell suspension was shaken in a flask as a control culture. Samples from the recycle reactor as well as from the shaker flask were periodically removed for viability determinations. Experiments were carried out in a warm room maintained 37C.

RESULTS AND DISCUSSION

1. Oxygenation experiment

Figure 3 shows a recorded signal from oxygen meter increasing with time. During two minutes the oxygen concentration increases at the rate of $\frac{16}{100}\ S_w$ (refer Table 1). The water volume is 600 ml, and so the rate of oxygen transport is calculated as

$$\frac{\frac{16}{100}\ S_w\times600\times\frac{76\ cmHg}{5}}{2\ min\times60\ sec/min}=4.6\times10^{-3}\ \frac{std\ cm^3}{sec}.$$

The calculated value was 3.8×10^{-3} std cm^3/sec, which approximately corresponds to the measured value of 4.6×10^{-3} std cm^3/sec despite of the uncertainty of the diffusivity through the silicone membrane. If we assume that the necessary oxygen uptake rate is 20

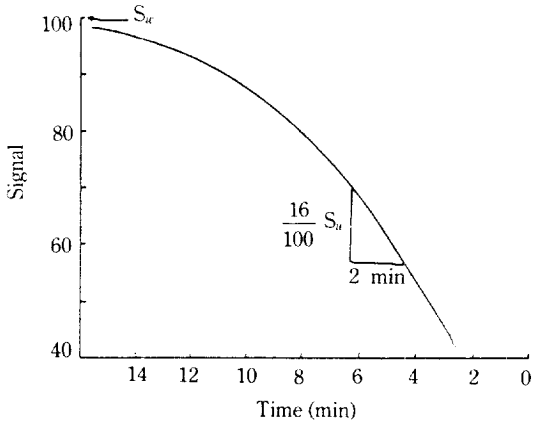

Fig. 3. Recorded signal from oxygen meter.

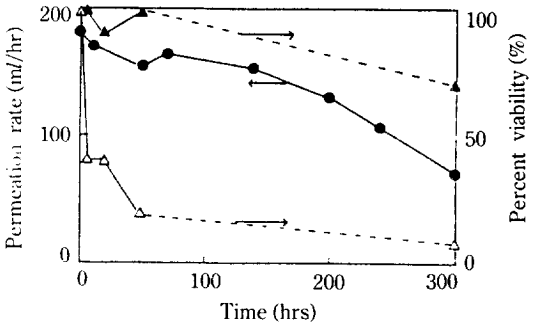

Fig. 4. Glucose starvation of *E. coli* in a cell recycle reactor; recirculation rate=100 ml/min; ▲: shaker culture; △: cell recycle reactor; temp.: room temperature.

nmol/ml min [5], the membrane oxygenator is able to maintain an oxygen level in a 500 ml culture at a fully saturated level.

2. Cell starvation experiment

The results of the first starvation experiment shown in Figure 4 revealed several operational problems:

1) Continuous decrease in the permeation rate

2) Rapid decline of cell number in the feed flask (#2 in Fig. 1) due to the trapped cells in a dead zone somewhere in the lines.

The reduction of cell number in the feed flask (#2 in Fig. 1) was observed to be a reversible phenomenon. Flushing of the lines by temporarily increasing the recirculation rate led to a restoration of the turbidity of the cell suspension. The open triangular points at the left side of the lower dotted line in Fig. 4 are from the samples taken when the recycle reactor was operated without flushing. The dotted lines represent missing data due to contamination of the dilution media before obtaining the last data point. The last point indicates that although the flush reduces the decline rate of viability counts, the number of viable cells in the recycle reactor is less than that in a batch culture after the same length of time. From this observation, we presumed that an irreversible adsorption of cells to flow channel had occurred and consequently brought about a coating on the fiber surfaces which led to the decrease in microfiltration rate.

In the second experiment, we increased the recirculation rate to as high as 300 ml/min to eliminate cell adhesion and to achieve the concomitant turbidity increase of the cell suspension [6, 7]. The shear rate at the above flow rate was calculated to 6400 sec^{-1}, which is sufficient not to adsorb cells on membrane surface. The experiment was performed at 37℃ in order to observe the decrease in viable cells in both

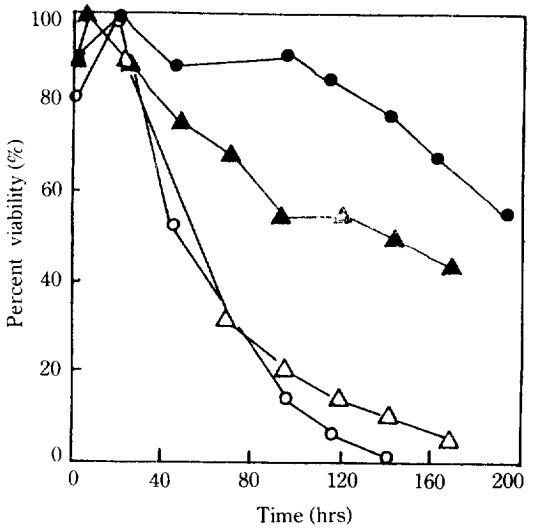

Fig. 5. Glucose starvation with and without microfiltration; recirculation rate=300 ml/min; ▲: shaker culture control; △: cell recycle reactor without microfiltration; ●: shaker culture control; ○: cell recycle reactor with microfiltration; temp.=37℃; microfiltration rate=100 ml/hr.

the shaker culture and in the recycle reactor more rapidly. Figure 5 shows that cell viability in the recycle reactor is again less than that in the shaker culture. Comparing the second experiment with the first one, however, we can see that the decrease in viability in the second recycle experiment is not as great as before. This would suggest that increasing the recirculation rate reduced cell adhesion to tubing and fibers as expected but may have caused additional loss of cell viability due to the higher hydrodynamic shear rates. Therefore, another effect other than adsorption

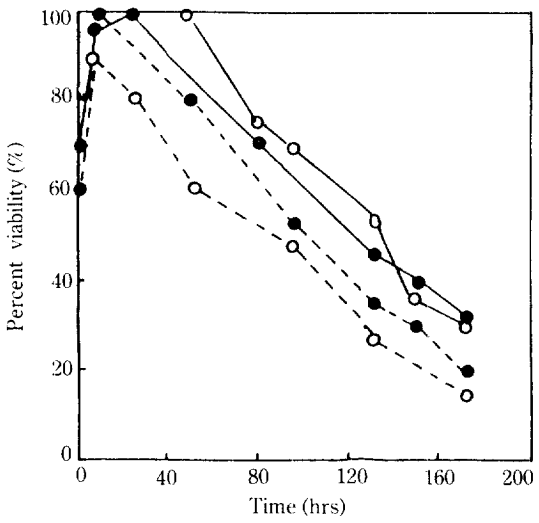

Fig. 6. Cell recycle of *E. coli* K. 12; results for two separate experiments; ●: batch control; ○ : recycled cells; —: first run; ---: second run.

is responsible for the decrease of cell viability. Also, the microfiltration rate remained essentially constant throughout the experiment, an observation that tends to confirm our conclusion that cell adhesion had been minimized or eliminated. As mentioned above, hydrodynamic shear may cause cell lysis and therefore cell loss in the recycle reactor. To observe the shear effect without the interference of microfiltration, a third experiment was carried out. In this experiment the experimental conditions were the same except that the microfiltrate line was blocked. The results are represented by triangular points. The viability behaviour observed in the cell recycle experiments with and without microfiltration shows a similarity that implies the importance of the hydrodynamic shear. Another implication is that replenishing the media in the flask with filtration had no effect on cell viability. The findings presented in the third experiment suggests that nutrients or toxic material released by dying or lysing cells was not taken up by other starving cells.

In order to eliminate the possible high-shear components in the recycle reactor, the configuration of the reactor was modified. The magnetic stirrer [4], pressure gauge [9], and needle valve [10] were removed from the system. The stirrer was removed because it appeared that heating of the stirring motor over long periods of time caused slight heating of the culture to a temperature as high as 41℃. And sufficient mixing of the culture was already afforded by the high flow rate of the culture through the system. The pressure gauge and needle valve were removed because sufficiently high filtration rates were obtainable without any back pressure on the filter unit and these two fixtures represented possible points where shear might have occurred. The resistance of *E. coli* against shear is reported as high as 10^5 sec^{-1} [7]. This value is much larger than the calculated value of 6400 sec^{-1} on the surface of hollow fiber where the main shear occurs.

As shown in Figure 6, the modifications of the cell recycle system discussed above yielded encouraging results. Basically, we may conclude that carbon-starved *E. coli* cells whose medium is recycled about once every 100 minutes exhibit the same viability behavior as cells starved in batch culture. Thus, we may conclude that the batch culture starving cells do not carry out cryptic growth, i.e., growth at the expense of metabolites released by lysing cells in the culture, to any marked extent. If they did, we would have expected the recycled cells to be less stable than the batch cells. As it now stands, the cell recycle system promises to be an ideal model system for determining the effects of varying concentrations of medium components on starving cells. For example, the effects of continuous low levels of glucose or amino acids, levels which do not allow growth but do allow energy maintenance, on culture stability can be studied using the cell recycle apparatus [8].

NOMENCLATURE

a : inner radius of silicon tube [cm]
C : oxygen concentration [std cm^3/cm^3]
D : diffusivity of oxygen [cm^2/s]
P : partial pressure of oxygen [cmHg]
r : radial coordinate [cm]
S : oxygen solubility [std $cm^3/cm^3 \cdot cmHg$]
U : mean velocity fluid [cm/s]
Z : axial coordinate [cm]

Superscripts

i : inlet
∞ : gas phase
m : membrane
w : water

Subscripts

m : membrane
w : water

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