Biotechnology Letters Vol 10 No 9 619-624
Received August 4

EFFECT OF GROWING CONDITIONS OF RECOMBINANT *E. COLI* IN CARRAGEENAN GEL BEADS UPON BIOMASSE PRODUCTION AND PLASMID STABILITY

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SUMMARY

The biomass production and the plasmid stability of immobilized *E. coli* cells in K-carrageenan gel beads were investigated in continuous cultures. Several factors, such as inoculum size, gel bead volume and gel concentration were examined in order to increase the cell concentration inside the immobilized cell reactor, and therefore to increase the overall productivity.

INTRODUCTION

Commercial production of plasmid-coded gene products in continuous culture generally requires high recombinant plasmid stability and high cell densities.
Previous reports (Wada et al., 1979; Shinmyo et al., 1982; Inloes et al., 1983) have shown that with immobilization, a significantly higher cell concentration and consequently higher productivity of the plasmid coded gene product can be obtained in comparison to free-cell systems.
A variety of experimental approaches to overcome plasmid instability have been proposed (Uhlin et al., 1979; Meacock and Cohen, 1980; Skogman et al., 1983; Zurita et al., 1984).
We have previously reported the use of immobilized recombinant cells as a new strategy for maintaining a stable population of plasmid-carrying cells and demonstrated that in continuous cultures immobilized cells exhibit greater plasmid stability (Nasri et al., 1987; Sayadi et al., 1987). Other authors have also reported the effect of immobilization on plasmid stability (Joshi and Yamazaki, 1987; Oriel, P., 1988). The k-carrageenan method was used for immobilization, because this method is considered to be mild, inexpensive and easy to perform. However, an important disadvantage of immobilization based on entrapping cells, is diffusion limitations of oxygen and nutrients inside the gel beads (Hendrickx et al., 1986; Libiki et al., 1988). On the other hand we have reported that the cat-O_2-ase productivity of immobilized-cell continuous cultures are higher than those of free-cell cultures because of the high cell concentration in the support and presumably due also to the activation of metabolism caused by immobilization.
The main objective of this paper was to examine the effect of several factors on plasmid stability and biomass production.

MATERIALS AND METHODS

Bacterial strains and plasmids
Escherichia coli strains W3101 (recA$^-$, Nal^R, Trp$^-$) and B were used in this study. Plasmids used were pTG201 (Nasri et al., 1987) and pTG206 (Zukowski et al., 1983) (carrying the xylE gene coding for catechol 2,3-dioxygenase)
Media
The medium used throughout the experiments was L broth (LB : 1 % tryptone, 0.5 % yeast extract and 0.5 % NaCl). For immobilized cells, LB medium was supplemented with 0.1 M KCL to ensure mechanical stability of the gel bead. Agar (1.5 % w/v) was added to LB medium to prepare LB agar plates.
Cultivation conditions
Continuous culture experiments were conducted in 50 ml of medium inside 100 ml glass vessels that were

maintained at 37°C, with an aeration rate of 170 ml/min. Inocula for chemostat cultures were allowed to grow at 37°C in LB medium containing ampicillin (150 µg/ml). For immobilized cultures, gel beads were transfered to the chemostat, and immediately a continuous flow of LB medium containing 0.1 M KCL was initiated. The dilution rates were about 3.9/h for immobilized- cell system and 1.35/h for free-continuous culture.

Average cell density determination

The number of living cells in the carrageenan gel beads was determined using eight beads for each measurement. Gel beads removed from the reactor at regular intervals were first washed twice in 0.8 % NaCl, then dissolved in 1 % (w/v) sodium citrate by shaking at 42°C for 5 minutes.

Microscopic observations

Samples were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer pH8 containing 0.1 M KCl, for 2 h at 20°C and postfixed with 1 % osmium tetroxide for 2 h at 4°C. Specimens were then deshydrated and embedded in Epon 812 resin. Thin (for light microscopy) and ultrathin sections (for transmission electron microscopy) were prepared with an LKB ultrotome III. In the last case, sections were double stained with uranyl acetate and lead citrate and examined with an electron microscope.

Immobilization procedure

Cells were immobilized using the method of Takata et al., (1977) as follows : 0.5 ml of precultured bacteria at a given concentration were mixed with 9.5 ml of 2.1 % (w/v) k-carrageenan solution (E407, CECA, France) at 42°C. Gel beads were formed by adding the mixture dropwise to 0.3 M KCL solution.

Plasmid stability test

The presence of cat-O_2-ase activity plasmid was detected by spraying bacterial colonies with 0.5 M catechol. A yellow color indicated a cat-O_2-ase producing colony (Zukowski et al., 1983).

RESULTS

1. Influence of gel bead volume

The experiment was first performed with 10 ml of gel beads, containing 5.7 X 10^8 *E. coli* W3101/pTG206 cells per ml, in 50 ml total bioreactor volume. The number of living cells in the gel beads reached the maximum, 1.3 $\cdot 10^{10}$ cells per ml of gel bead, after a 16 h incubation and then remained at a constant value during the course of the fermentations (Table 1). However, when cells were cultivated in a free-cell, continuous system, the steady state production of free cells was $1.0 \cdot 10^9$ cells per ml.

The production rate of *xylE* gene product was determined as previously described (Sayadi et al., 1987) and was found to be proportional to the cell mass (result not shown), therefore one strategy for increasing the volumetric productivity of a fermentor is to maintain a large mass of cells in the bioreactor. For this reason, we have performed additional experiments with 20 and 30 ml of the gel beads in 50 ml total bioreactor volume. In the three cases (10, 20 and 30 ml of 2 % k-carrageenan gel beads), the number of cells per ml of gel beads during steady state were similar, but the total cell concentration inside the immobilized cell bioreactor and the concentration of viable cells in the effluent increased with increasing gel bead volume (Table 1).

The pTG206 plasmid was found to be extremely stable under the three conditions tested. No loss of pTG206 was detected even after 300 generations.

Similar experiments were also performed with *E. coli* B/pTG201, showing that plasmid-coded gene product productivity increased with increasing gel bead volume (results not shown).

2. Influence of inoculume size

Previous studies have shown that immobilized cells grew near the surface of the gel beads, where they formed microcolonies (Wada et al., 1979; Shinmyo et al., 1982; Burill et al., 1982; Eikmeier et al., 1984) and were not present in the central part of the gel. In particular, it has been shown that the growth of cells was limited to the outer 50-150 µm of the gel beads.

With a small inoculum size and after 10 hours of incubation, the number of cells in the gel beads increased remarkably. Giant colonies near the gel surface and also in the centre of the gel beads were observed (Fig. 1). This indicates that with an initially very low cell content, the oxygen and nutrient mass transfer limitations are minimized.

We showed that recombinant cells grow in the gel beads for a limited number of generations before the clones escape from the gel matrix (Nasri et al., 1987). Thus, plasmid-free cells (P^-) that appear within the gel beads, compete with plasmid-containing cells (P^+) during only a few number of generations, while in a free-cell system and under non-selective growth conditions P^+ and P^- cells compete for many more generations. As plasmid containing cells had a lower maximum specific growth rate than plasmid-free cells, in a free-cell system the fraction of (P^+) decreases progressively during continuous culture.

In contrast to free cells, immobilized (P^+) cells exhibit a very high stability. As shown in figure (2), immediately after initiation of the chemostat and during the first 10 to 30 generations, the frequency of (P^+) cells decreased and than remained constant. According to the experimental data the length of the period corresponding to the decrease of P^+ cells appears to be a function of inoculum size. pTG201 was found to be more stable with a high inoculum size; that is, when the average number of cell divisions within the volume occupied by the active bacteria is lower. Only 2 % of the population lost the plasmid, whereas with a very low inoculum size, in which the average number of cell divisions is high (23-26), 15 % of *E. coli* had lost pTG201 after 110 generations.

The results presented in Table 2 show that the number of cell divisions increased by decreasing the inoculum size. The average number of bacteria per cavity and therefore the average number of cell divisions within the gel beads were determined according to the equation determined previously (Nasri et al., 1987): $x < X_n V_b / X_o V_v$ where X_o is the average number of cells immobilized before the onset of incubation, X_n is the average number of cells per bead after the n^{th} generations, V_b is the average per bead and V_v is the average volume actually occupied by active bacteria per bead. This last parameter is determined using optical microscopy. With low inoculum size ($< 6.00 \cdot 10^6$ cells/ml), the cellular growth was uniform throughout the gel beads. Therefore V_v is equal to V_b and $x < X_n / X_o$.

The results in Table 2 also show that cell concentrations in gel beads and in the effluent increased with increasing immobilized inoculum content. We also measured *XylE* activity and found that the activity increased with increasing inoculum content, as a result of increasing biomass concentration (result not shown).

2. Influence of k-caraggenan gel concentration

Recently, Furui and Yamashita, 1985 showed that the diffusion coefficients of solutes in immobilized *E. coli* cells, decreased with increasing gel concentration. Gosman and Rehm, 1986 reported that the specific oxygen uptake rate of immobilized microorganisms decreased with increasing alginate concentration. These results suggest that low gel concentrations minimize mass transfer limitations and maximize biomass production.

Growth and plasmid stability were compared at 1, 1.5, 2 and 3 % (w/v) k-carrageenan. The maximum number of living cells in the gel beads and in the effluent increased slightly by decreasing gel concentration. Furthermore, there was no significant difference in plasmid stability resulting from three conditions tested (result not shown).

DISCUSSION

Microscopic observations have shown that with a small inoculum size ($4.7 \cdot 10^3$ cells/ml) large microcolonies were observed even in the center of the gel beads (Fig. 1), and 24 to 26 generations of cells were required for the growth in the cavities of gel beads before an eventual disruption (Table 2). In contrast, use of very high inoculum size ($2.1 \cdot 10^{10}$ cells/ml) resulted in growth limited to the outer 50-150 µm of the gel beads and only 3-5 generations required for growth before cavity disruption (Table 2).

pTG201 plasmid which is very unstable in continuous cultures with free cells, was found to be stable in continuous cultures with immobilized cells. Higher stability is obtained with high inoculum content (Fig. 2). Cellular growth inside the gel beads may represent a period of gradually decreasing copy number. With high inoculum content this period is very short (3-5 generations). Thus, the plasmid copy number of these recombinant cells is high and equal to the original value. In contrast, with a very low inoculum content this period is long (20-26 generations) and the plasmid copy number decreases gradually to a level at which instability becomes apparent. Furthermore, plasmid-free cells that are generated compete with plasmid-containing cells for more generations than when a high inoculum content is used. These results are in accord with the hypothesis previously reported (Nasri et al., 1987) suggesting that the increased plasmid stability in immobilized cells is essentially due to the mechanical properties of the gel bead system, which may only allow a limited number of cell divisions to occur in each cavity before the clones escape from the gel beads. In immobilized system, pTG201-free cells appeared during growth inside cavities, the number of contamined cavities and the fraction of pTG201-free cells within cavities increased with the number of cell divisions within the gel cavities as a result of decreasing inoculum size.

Cells immobilized in gel beads exhibit the same behaviour as free cells during growth in continuous culture. The maximum specific growth rates in free and immobilized cells were similar. The fraction of P^+ cells in an immobilized system obeys to the equation defined by Imanaka and Aiba, 1981:

$$\frac{1 - \alpha - h}{1 - \alpha - h 2^{n(2+h-1)}}$$

only during the first generations, corresponding to the phase of growth within gel bead cavities until an equilibrium between cellular growth within the matrix and cell leakage is reached. α is the relative growth rate between P^- and P^+ ($\alpha = \mu P^+ / \mu P^-$), and h is the probability of plasmid loss per cell generation. Maximum n (number of generations) is measured from the maximum cavity size which is determined using electron microscopy.

An advantage obtained by using cell immobilization is an increased volumetric productivity, due to the high cell concentration obtained even at a high dilution rate in a continuous culture. The biomass concentration within the gel beads and in the effluent may be increased by using : (a) high inoculum content : > $1.0 \cdot 10^{10}$ cells per ml of gel bead, (b) high gel bead volume : 40-60 % of the total bioreactor volume, (c) low gel concentration (2 % w/v).
There are other possibilities for increasing biomass concentration and consequently production, in immobilized cells : (a) use of an anaerobic bacteria or (b) smaller beads . The former is not practical because many of the products made industrially require oxygen to participate in the synthesis; however, making the beads smaller will allow for maximum use of the beads by ollowing occupation of all the accessible gel volume and will minimize mass transfer limitations (Hulst et al., 1985).
In conclusion, the results reported here indicate that we can cultivate recombinant cells for at least 200 generations in an immobilized-cell system with complete stability i.e. high plasmid stability, high and constant biomass production and *xylE* expression throughout the experiment. These results indicate that cell immobilization might be suitable for recombinant plasmid stability and for the production of recombinant proteins.

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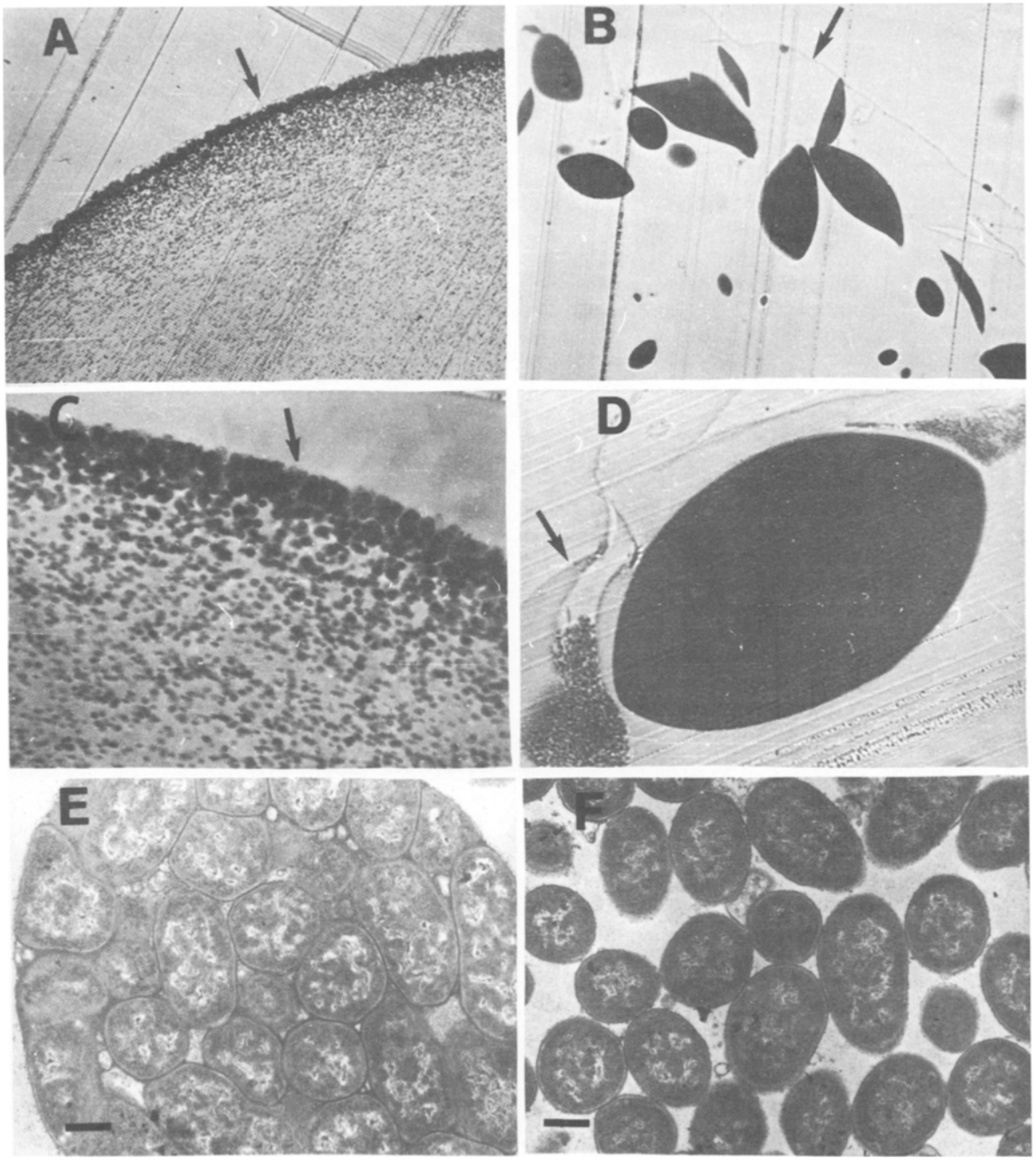

Fig.1: Optical (A,B,C,D) and electron (E,F) micrographs of sections of carrageenan gel beads containing *E. coli* B/pTG201 microcolonies after 48h of incubation. The concentration of inoculum were $2.1 \cdot 10^{10}$ cells/ml (A, C, E) and $4.7 \cdot 10^{3}$ cells/ml (B, D, F). The arrow indicates the surface of the gel bead.
Magnification: A, B: X33 and C, D: X132 and Bar indicates 500 nm.

Table 1 : Effect of gel bead volume on biomass production

Gel bead volume	10 ml	20 ml	30 ml
Liquid volume	40 ml	30 ml	20 ml
Immobilized cells[a]	$1.30 \cdot 10^{10}$	$1.17 \cdot 10^{10}$	$1.17 \cdot 10^{10}$
Released cells [b]	$2.05 \cdot 10^{8}$	$2.40 \cdot 10^{8}$	$3.00 \cdot 10^{8}$
Bioreactor cells[c]	$2.75 \cdot 10^{9}$	$4.82 \cdot 10^{9}$	$7.10 \cdot 10^{9}$

a : Corresponding to the cell number per ml of gel bead after a 16 hours incubation.
b : Corresponding to the number of released cells per ml of culture medium at the steady-state production of biomass.
c : Corresponding to the cell number per ml of bioreactor volume.
Total bioreactor volume = gel bead volume + liquid volume = 50 ml.

Table 2 : Effect of inoculum size on biomass production

Inoculum size[a]	$2.10 \cdot 10^{10}$	$4.70 \cdot 10^{8}$	$6.00 \cdot 10^{6}$	$4.7 \cdot 10^{3}$
Cell divisions	4-5 [b]	9-10[b]	11-15[c]	26[c]
Immobilized cells[d]	$3.80 \cdot 10^{10}$	$2.89 \cdot 10^{10}$	$2.32 \cdot 10^{10}$	$2.00 \cdot 10^{10}$
Released cells[d]	$3.70 \cdot 10^{8}$	$2.05 \cdot 10^{8}$	$2.11 \cdot 10^{8}$	n.d.

a : Number of viable immobilized cells per ml of gel bead before the onset of incubation.
b : Average number of cell divisions within the volume actually occupied by active bacteria.
c : Average number of cell divisions within the gel beads assuming a uniform growth of cells throughout the gel beads.
d : Viable cell number of immobilized cells is expressed per ml of gel bead, and that of released cells per ml of liquid medium.
n.d.: not determined.

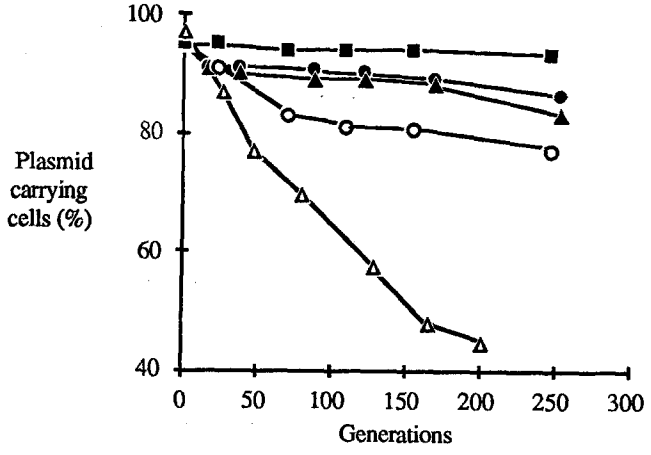

Fig. 2 : Effect of inoculum size on the stability of pTG201 plasmid in continuous cultures with immobilized *E. coli* B cells.

(■) $2.10 \cdot 10^{10}$ (●) $4.70 \cdot 10^{8}$ (▲) $6.00 \cdot 10^{6}$ (○) $4.70 \cdot 10^{3}$ (△) free cells

Each point represents the mean value of at least two independent experiments.