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A New Immobilization of Microbial Cells

Immobilized Growing Cells Using Carrageenan Gel and Their Properties

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Summary. A new immobilization of microbial cells based on the growth of cells in gel is presented. The cells grew very well in carrageenan gel when fed nutrients required for growth. The growing cells immobilized in gel formed a dense layer of cells near the gel surface. Because the cells were near the gel surface, they efficiently catalyzed single enzyme reactions. In addition, the immobilized growing cell system was applied to the complicated multienzyme reactions since large numbers of cells in the gel could constantly be maintained for long periods.

Introduction

A number of papers on enzyme reactions using immobilized microbial cells have been published (Chibata and Tosa, 1977). Generally, immobilized cells have been prepared by harvesting the cells from cultured broth and entrapping them into polymer matrices. In such cases, large numbers of cells were usually immobilized to obtain a preparation having high enzyme activity. The apparent activity of these immobilized cell preparations was not always satisfactory because the enzyme activity of homogeneously dispersed entrapped cells was not fully utilized.

Thus, the preparation of highly efficient immobilized growing cells using the κ-carrageenan gel method developed in this laboratory (Takata et al., 1977) was studied. Carrageenan, a polysaccharide isolated from seaweeds, can immobilize many kinds of enzymes and microbial cells under mild conditions using appropriate gel inducing agents. The enzyme activity of immobilized cells entrapped in carrageenan is generally high and stable. In addition, the immobilized cell matrix is readily converted to a cell suspension by removing the gel inducing agents. Thus, the number of living cells in gel can be easily counted. These characteristics are particularly suitable for studying immobilized growing cells.

In this paper, the preparation and properties of immobilized growing cells and their application in the production of useful compounds is described.

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Materials and Methods

Materials

κ-Carrageenan was obtained from Sansho Co. (Osaka, Japan). Alcohol dehydrogenase and NAD were obtained from Sigma Chemical Co. (Saint Louis, USA). All other chemicals and biochemicals were purchased from commercial sources.

Microorganisms and Immobilization

Escherichia coli ATCC 11303, *Flavobacterium arborescens* IAM 1100, *Serratia marcescens* Ar130-1 (Kisumi et al., 1970), *Acetobacter suboxydans* ATCC 621 and *Saccharomyces cerevisiae* IFO 2367 were used in this study. The cells were precultured under aerobic conditions for 18 h at 30°C in the following media:

E. coli, F. arborescens and *S. marcescens:* medium containing 0.5% glucose, 1.25% yeast extract, 1.0% peptone and 0.5% NaCl (pH 7.0).

A. suboxydans: medium of Yamada et al. (1978).

S. cerevisiae: medium containing 1% glucose, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract (pH 5.0).

Immobilization of cells was carried out under sterile conditions.

Each of immobilized growing cells was prepared using the carrageenan method (Takata et al., 1977) as follows: the precultured cells (1 ml of broth) were mixed with 4% carrageenan solution (50 ml) at 37°C. The mixture obtained was added dropwise to 2% KCl solution with gentle stirring at 20°C. Thus, carrageenan gel beads entrapping a small number of cells were formed (mean diameter of 4 mm). Entrapped cells (10 ml of beads) were incubated next on a rotary shaker at 30°C in the following media (100 ml):

Entrapped *E. coli* cells: medium of Chibata et al. (1974).

Entrapped *F. arborescens* cells: medium containing 0.5% glucose, 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.1% $(NH_4)_2SO_4$ and $MgSO_4 \cdot 7H_2O$ (pH 7.0).

Entrapped *S. marcescens* cells: medium A containing 3% glucose, 0.5% urea, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 1.5% D-threonine (pH 7.5).

Entrapped *A. suboxydans* cells: medium B containing 5% sorbitol, 0.1% corn steep liquor and 0.1% ammonium fumarate (pH 6.0).

Entrapped *S. cerevisiae* cells: medium C containing 10% glucose, 0.15% yeast extract, 0.25% NH_4Cl, 0.55% K_2HPO_4, 0.025% $MgSO_4 \cdot 7H_2O$, 0.1% NaCl, 0.001% $CaCl_2$ and 0.3% citric acid (pH 5.0).

When the entrapped cells of *E. coli, F. arborescens, S. marcescens, A. suboxydans,* and *S. cerevisiae* were incubated for 16, 18, 30, 40, and 60 h, respectively, the entrapped cells grew in beads and immobilized growing cells.

Production Using Immobilized Cells

The beads with the growing *E. coli* cells immobilized by incubation were used to study the production of L-aspartic acid. The production was carried out according to the method of Chibata et al. (1974).

The immobilized growing *S. marcescens* cell system was used to study the continuous production of L-isoleucine. The production in this system was carried out in a fluidized bed reactor (Fig. 1) containing the beads with immobilized growing cells (50 ml). The

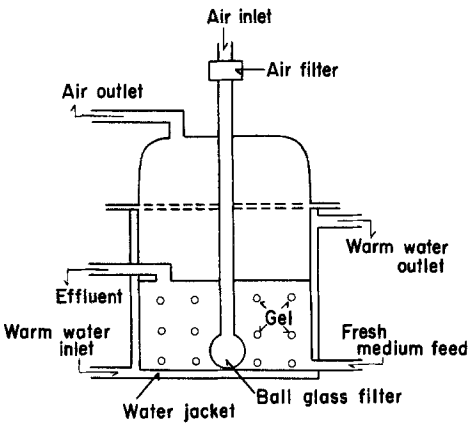

Fig. 1. Fluidized bed reactor. Working volume, 150 ml; diameter, 5 cm; air supply, 200 ml/min

system had a working volume of 150 ml, contained the medium A (100 ml), and ran at a feed rate of 15 ml/h. Dissolved oxygen (100% saturation) was maintained throughout the experiment. The temperature was maintained at 30°C.

The continuous production of L-sorbose using immobilized growing *A. suboxydans* cells was carried out under the same conditions as the immobilized growing *S. marcescens* system, except that the medium B was fed at a rate of 7.5 ml/h.

For continuous production of ethanol using immobilized growing *S. cerevisiae* cells, the beads (50 ml) were packed into a column and the medium C was passed through the column at a feed rate of 50 ml/h. The temperature was maintained at 30°C.

To prevent microbial contamination of the reactor, the production of L-isoleucine, L-sorbose, and ethanol was carried out under the similar sterile conditions as continuous culture. The production of L-aspartic acid was not carried out under sterile conditions.

Analytical Methods

The number of living cells was estimated by dissolving gel beads in sterile physiological saline. The beads (2 particles) isolated from medium were dissolved in 5 ml of physiological saline, gently shaken for 15 min at 37°C, and converted to a cell suspension. The cell suspension obtained was serially diluted and the number of viable cells was counted by drop-plate method (Postgate, 1969). The number of living cells in gel was calculated from the viable counts in cell suspension and was expressed in cells per ml of gel.

L-Aspartic acid and L-isoleucine were determined by microbioassay (Henderson and Snell, 1948). Sorbose was determined by the method of Somogyi (1952). Ethanol was determined using alcohol dehydrogenase (Bonnidisen, 1963).

Results and Discussion

Changes in cell population in carrageenan gel incubated in the nutrient medium were first investigated. The gel bead entrapping a small number of *E. coli* cells was incubated in the nutrient medium at 30°C on a rotary shaker (Fig. 2). As a control of cell growth, a small number of free cells was incubated under the same conditions. It was found that *E. coli* growth in gel was the same or even better than that of the free cells in liquid medium. *F. arborescens*, which is a strict aerobe, also showed better growth in gel as well as *E. coli*.

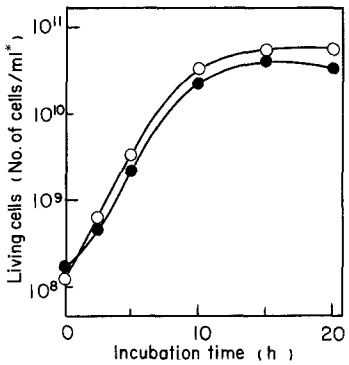

Fig. 2. Comparison of growth between *E. coli* cells in carrageenan gel and in liquid medium. *: Immobilized cells, number/ml of gel; free cells, number/ml of medium. Symbols: ○: immobilized cells, ●: free cells

Further, the population of *E. coli* cells immobilized by growing in gel was found to be different from that of cells immobilized homogeneously by conventional methods (immobilized cells-homogeneous). Namely, *E. coli* cells grown in gel formed a dense layer of cells near the surface of gel beads (immobilized cell-layer) and the cells were not observed at the central part of gel bead as shown in Fig. 3. The cell layer was probably formed because nutrient availability due to diffusion was greatest near the gel surface. In other words, the cell layer was formed where the cells found the most suitable environmental conditions for growth. This new immobilization of growing cells was also found to be applicable to many other microorganisms such as *Acetobacter, Serratia, Leuconostoc*, and *Saccharomyces* species. As described above, microbial cells may grow

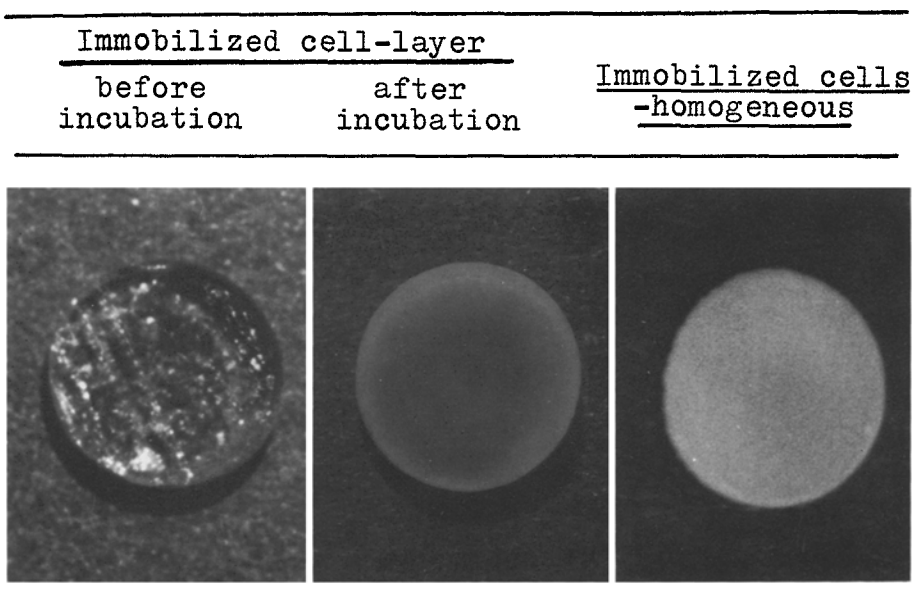

Fig. 3. Photographs of gel bead slices entrapping *E. coli* cells. The immobilized *E. coli* cell-layer before and after incubation were compared with homogeneous immobilized *E. coli* cells

in response to a suitable environment in the gel and may even be protected from environmental changes, resulting in better growth.

It was expected that the new immobilized cell-layer system would have efficient catalytic activity in enzyme reactions because the cell layer was present near the gel surface where substrate availability would be high. Thus, the application of this new immobilized *E. coli* cell-layer system to the production of L-aspartic acid by the method of Chibata et al. (1974) was tried. The efficiency of aspartic acid production using the new system was compared with that using immobilized *E. coli* cells-homogeneous system (Fig. 4). The aspartase activity of immobilized cell-layer (VI) was about 1.5 fold higher than that of immobilized cells-homogeneous (IV). In the new system, the activity of immobilized cell-layer (VI) was almost the same after being homogenized (VII) and it was also equivalent to those of free cells (II) and homogenized cells (III). These facts suggest that the immobilized cell-layer did not show any apparent loss of enzyme activity caused by diffusion limitation of substrate into gel, and the intrinsic catalytic activity of cells in gel was fully utilized because the cell layer was near the surface of gel. The activity of immobilized cells, homogeneous (IV), was 60% of that of the homogenized gel (V). This result indicates that the intrinsic activity of cells in gel (IV) is not fully utilized after being immobilized.

In the production system for L-aspartic acid using immobilized *E. coli* cells, the reaction is catalyzed by a single enzyme and the aspartase is maintained in an active and stable state even though the cells entrapped in gel are not viable. However, many useful compounds produced by fermentation methods are usually formed by multi-step reactions catalyzed with many kinds of enzymes in living microbial cells. These reactions often require the regeneration systems of ATP and of other coenzymes. Recently, several investigators described studies on multi-step reactions using immobilized

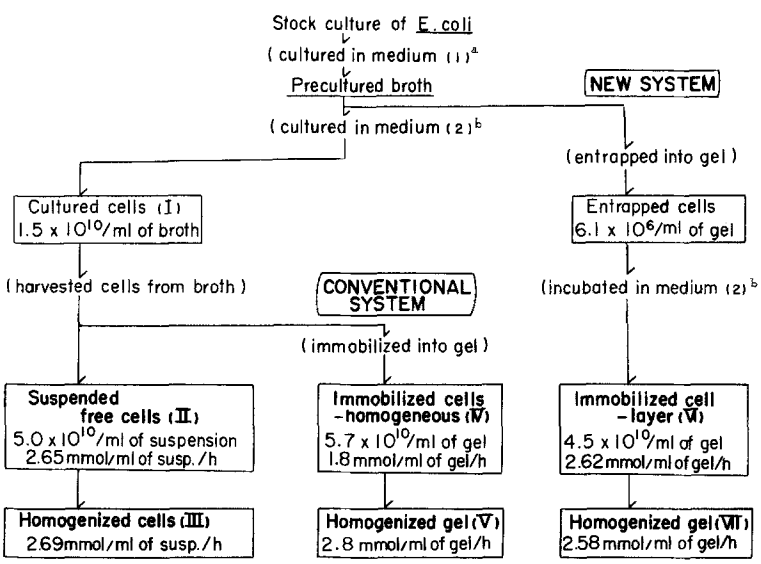

Fig. 4. Scheme of immobilization of *E. coli* cells and comparison of aspartase activities of various preparations. Aspartase activities were determined by the method of Chibata et al. (1974). a The preculture medium of *E. coli* cells. b The incubation medium of entrapped *E. coli* cells

cell systems (Kennedy et al., 1975; Weetal and Bennet, 1976). Activation or reactivation of enzyme activities of immobilized cells was observed by incubating with nutrients (Hackel et al., 1975; Kierstan and Bucke, 1977; Larsson et al., 1976; Somerville et al., 1977). Little is known about the viability of immobilized cells and their behavior in the reaction systems. As described above, the cells grow well in gel by incubating in the nutrient medium, and it was expected that the immobilized cell-layer would be useful for multi-step reactions by maintaining the viability of cells in gel. Thus, the production of L-isoleucine from D-threonine, L-sorbose from D-sorbitol and ethanol from glucose using new immobilized cell-layer systems of *S. marcescens*, *A. suboxydans*, and *S. cerevisiae*, respectively was investigated. Table 1 shows the conditions for operation and the results of continuous production in each system. The supply of nutrient medium for growth was essential for continuous production using immobilized growing cell systems. In these systems, the cell numbers in gel and the productivities of gel were kept at steady state by cells growing on the nutrient supply. The depletion of nutrients caused death of cells and decrease of productivity. Oxygen supply also was essential for the production of L-isoleucine and L-sorbose. These facts suggest that the multi-enzyme systems responsible for the formation of products are constantly regenerated as cells grow.

In continuous culture, cells are also reproduced. The amounts of cells in reactor, however, may be smaller than those of immobilized growing cell system. The productivity of free cells was similar to that of immobilized growing cells. The immobilized growing cell system, therefore, might be more useful for production compared to continuous culture system.

As described above, immobilized growing cells can be applied to complicated multi-step reaction systems as well as to the simple enzyme reaction systems. The practical applications of these immobilized growing cell systems are now being studied.

Table 1. Application of new immobilized growing cell system to continuous multi-step reactions

Product	L-Isoleucine	L-Sorbose	Ethanol
Microorganisms	*Serratia marcescens*	*Acetobacter suboxydans*	*Saccharomyces cerevisiae*
Operational conditions			
Reactor	Fluidized bed	Fluidized bed	Packed column
Working volume (ml)	150	150	50
Feed rate (ml/h)	15	7.5	50
Aeration rate (ml/min)	200	200	
Living cells in gel (No. of cells/ml of gel)			
Initial state	6×10^7	2×10^7	3×10^6
Steady state	4×10^{10}	7×10^9	5×10^9
Productivity of gel (mg/ml of gel/h)	0.5	0.2	43
Operational stability			
Steady state (day)	> 30	> 15	> 30

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