Cell Immobilization in κ-Carrageenan with Tricalcium Phosphate

HENRY Y. WANG and DAVID J. HETTWER,
Department of Chemical Engineering,
The University of Michigan,
Ann Arbor, Michigan 48109

Summary

The objectives of this research were to investigate the growth of immobilized yeast cells in κ-carrageenan gel and study the effect of trapping hydroxyapatite (tricalcium phosphate) crystals into the matrix. Using κ-carrageenan, the final number of cells per milliliter of gel is at least an order of magnitude higher than free cells per milliliter of medium. A "cell retention" theory explaining this cell concentration difference was proposed. Coexistence of yeast cells and an additional agent such as tricalcium phosphate results in sustained viability through internal pH control, increased cell loading, greater settling velocity, and enhanced ethanol production.

INTRODUCTION

The current scarcity of petroleum in the world has stimulated the search for alternate sources of energy. Ethanol produced from renewable resources by microbial fermentation seems promising and has recently commanded a great deal of attention. Recent advances in immobilized whole-cell technology have suggested new ways of producing biochemicals and biofuels such as ethanol. Present industrial applications of immobilized cells include the conversion of glucose to fructose, and the production of amino acids and numerous organic acids.[1] Several methods can be employed for the immobilization of cells. Cells can be entrapped in a polymer matrix, covalently bonded to a support matrix, covalently crosslinked from cell to cell, adsorbed onto a solid support, or flocculated.[2] Immobilized cell systems involving entrapment offer several unique advantages over conventional free cell systems. The physical retention of the cells in the matrix facilitates the separation of the cells from the products. Also, high cell densities per reactor volume can be achieved. Since volumetric productivity generally depends on cell concentration, higher cell concentrations can lead to smaller reactor volumes and greater productivity. The immobilization matrix in bead form can also be used in packed columns or fluidized-bed reactors.[1,2] The benefits of these reactors include maximum reaction rates, minimum nutrient depletion, and

Biotechnology and Bioengineering, Vol. XXIV, Pp. 1827–1838 (1982)
 CCC 0006-0592/82/081827-12$02.20

product inhibition, and better mass transfer through decreased feed viscosity and increased differential velocities.[1]

Several researchers have demonstrated the feasibility of utilizing immobilized yeast cells to produce ethanol. Calcium alginate has been successfully employed as a matrix to entrap yeast cells.[3,4] κ-Carrageenan has also been used to develop an immobilized yeast cell system for the production of ethanol.[5] There are several problems associated with these immobilization systems that need to be investigated. First, there is mass-transfer limitation due to diffusion of substrates and products into and out of the gel. This has decreased productivity and in some cases, limited production to those cells existing near the outer surface of the matrix particle.[5] Second, maintaining an optimum pH in a packed column and minimizing any pH gradient between gel and solution may be difficult during scaleup. In general, maintaining optimum conditions within the gel cannot necessarily be achieved by controlling the bulk solution, as the environment within the matrix may differ significantly from the bulk solution.[4] Third, sustaining high cell viability and productivity with feeds other than complete medium has been difficult, indicating new cells need to be regenerated all the time. Also, the density of the hydrogel matrix is so close to that of water that the operation of a fluidized-type bed has been restricted. Finally, there are no readily available methods to separate beads that contain nonproducing cells from those containing producing cells so that prolonged operation is feasible.

The goal of our research is to more fully understand the behavior of yeast cells in a κ-carrageenan matrix and to investigate possible ways of solving some of the discussed problems encountered with immobilized yeast cell systems. The approach we took was to entrap hydroxyapatite or tricalcium phosphate crystals into the matrix. This system involves the coexistence of cells and an additional agent within the matrix structure.

Tricalcium phosphate is a white crystalline substance that reacts at low pH as follows:

$$Ca_{10}(PO_4)_6(OH)_2 \overset{H^+}{\rightleftharpoons} 10Ca^{2+} + 6H_3PO_4 + 2H_2O \qquad (1)$$

The solubility of this crystal in neutral pH is low and the undissolved crystals range in diameter from 2 to 30 μm. We hope to take advantage of these properties by incorporating tricalcium phosphate, and solve some of the problems associated with immobilized yeast cell systems. The dissolution of the crystals within the gel may act to reduce the pH gradient as well as to increase gel porosity, thus facilitating mass transfer into and out of the gel.

MATERIALS AND METHODS

Materials

The κ-carrageenan was obtained from FMC Corporation, Marine Colloids Division (Springfield, NJ). Tricalcium phosphate (hydroxyapatite) was ob-

tained from Stauffer Chemical Company (Westport, CT). Other chemicals were purchased from other commercial sources.

Microorganism

Saccharomyces cerevisiae NRRL Y-132 was used in these studies (Northern Regional Research Laboratory, Peoria, IL).

Medium

The medium used to grow yeast cultures and incubate the beads had the following composition: 5% glucose, 0.5% yeast extract (which may change as indicated in the **Results** section), 0.4% $MgSO_4 \cdot 7H_2O$, 1.3% $(NH_4)_2SO_4$, and 0.26% $CaCl_2 \cdot 2H_2O$. A Ringer salt solution was used in the batch ethanol production studies: 0.9% NaCl, 0.042% KCl, 0.032% $CaCl_2 \cdot 2H_2O$, 0.02% $NaHCO_3$, 0.04% $MgSO_4 \cdot 7H_2O$, and 20% glucose (with no nitrogen source).

Immobilization of Yeast Cells

A 4% (w/v) κ-carrageenan solution with/without tricalcium phosphate was prepared using physiological saline (0.9% NaCl). The solution was heated to 60°C to completely dissolve the κ-carrageenan. After cooling to 40°C, a sample of yeast culture broth was added, and the resulting mixture was pumped into a 2% KCl solution to induce gelation. The resulting bead size depended on the tube diameter—typical bead diameters were 5.0–6.0 mm. Beads with tricalcium phosphate can also be placed in an acidic 1% KCl solution to dissolve either all or part of the crystals out of the gel to enhance porosity of the beads.

Assays

Ethanol was determined be gas chromatography (model 5840A, Hewlett Packard, CA). The residual sugar concentration of the fermentation broth was measured by means of the Somogyi method. The exact glucose content was measured by a Worthington Glucostat kit.

RESULTS AND DISCUSSION

Immobilized versus Free Cell Growth

Figure 1 shows the growth curves of immobilized and free cells. The inoculum was the same in each case (6×10^5 cells/mL gel or solution). Growth in the medium surrounding the beads was the result of cell leakage and the subsequent growth of these cells.

The growth curves exhibit similar behavior but reach different peak cell concentrations. The initial lag phase is followed by exponential growth and a

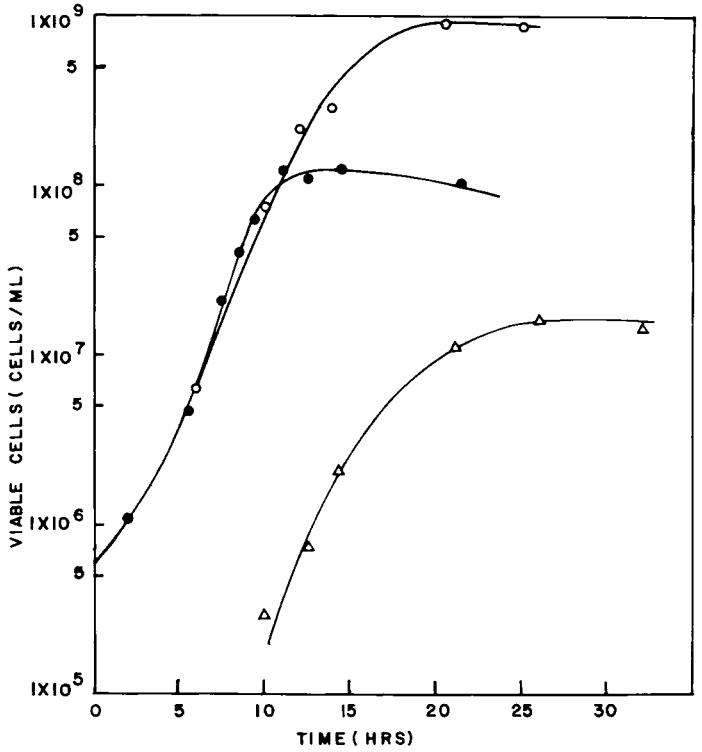


Fig. 1. Immobilized vs. free cell growth in batch reactors (5 mL beads and 50 mL medium, 0.5% yeast extract): (●) culture, (△) plain beads in solution, and (○) plain beads in gel.

stationary phase where the cell concentration levels off. In both cases, the maximum specific growth rate of the yeast cells is approximately the same. An order of magnitude difference in peak cell concentration exists between the immobilized and free cells. The immobilized cells in this case reached a maximum around 1×10^9 cells/mL gel (ca. 25 g/L) while the free solution peaked at 1×10^8 cells/mL solution. A much lower concentration, 1.5×10^7 cells/mL solution, was reached in the medium containing the immobilized cell beads.

The stationary phase of the yeast growth is a result of nutrient depletion. The growth in the beads and the medium containing the beads leveled off at the same time. As shown in Table I, higher yeast extract concentrations in the medium lead to more dense cell populations in the gel. Even higher cell concentrations (2×10^9 cells /mL) in the beads can be achieved by reducing cell leakage and growth of these leaked cells. A number of different bead sizes were used to investigate whether cell loading would be affected. No significant difference in maximum cell growth and cell density was observed for beads with diameters between 3.5 and 5.5 mm (Table II).

TABLE I
Nutrient Concentration versus Cell Loading[a]

Yeast extract (%)	Peak cell concentration (cells/mL gel)($\times 10^9$)
0.1	1.5
0.5	2.1
1.0	2.6
1.5	2.9

[a]1 mL of gel, 50 mL of medium.

Cell Retention Theory

Several suggestions have been proposed to explain the cell density differences between immobilized and free cell systems. One suggestion is that the partition coefficient of glucose between the gel and solution is greater than that of ethanol.[5] This favors cell growth in the gel because glucose is available while the inhibitory end product (ethanol) escapes. Some speculate that the matrix may concentrate nutrients at the liquid-solid interface, thus allowing dense cell populations to develop.[1] However, our initial experiments to estimate the partition coefficients of yeast extract and other nutrients between the gel and medium have found them to be close to 1.0. There is also the possibility that an immobilized cell grows better than a free cell due to the limited motion it experiences, even though there is no existing data to demonstrate this.

We found that the major reason for the order of magnitude difference in cell concentration between immobilized and free cell systems (Fig. 1) is a physical phenomenon resulting from the retention of cells in the matrix. Immobilized growth can be described as the process whereby nutrients diffuse into the matrix and are converted to cells which are retained in the matrix. Since cell leakage and the subsequent growth of these leaked cells is insignifi-

TABLE II
Bead Diameter versus Peak Cell Concentration[a]

Bead diameter (mm)	Bead volume (mL)	Peak cell concentration (cells/mL gel)($\times 10^9$)
3.5	0.022	1.3
4.5	0.048	1.4
5.5	0.087	1.2

[a]4 mL of gel, 50 mL of 0.5% yeast extract medium.

cant, the nutrients from the entire volume of medium have essentially been converted to cell mass that is confined to a much smaller volume. Using the same inoculum as in the immobilized cell system, free cells exhibited similar growth behavior and the final cell population was the same (mass balance between nutrients and cell mass). However, because the volumes in which these cells were distributed varied in these two cases, the concentration of free cells was only one-tenth of that for the immobilized cells.

Table III indicates that the volume of gel added to a given volume of medium is critical to the final cell density that will be achieved in the gel because cell leakage depends on cell concentration and other environmental factors. A simple inverse relationship between gel volume and cell concentration does not hold.

The final cell concentration achieved in the gel is also a function of the difference in inoculum between the gel and the surrounding medium. The peak cell concentrations for two cases were compared. Table IV shows the different cell distribution that resulted when a) only the gel was inoculated, and b) the gel and surrounding medium were inoculated equally. When only the gel is inoculated, a cell concentration difference between gel and medium is observed. When the immobilized cells must compete with an equal concentration of free cells as in case b, this difference in cell density is not seen. The lower cell concentration in the gel in case b is probably the result of some cell death during immobilization (i.e., the immobilized cells may have started at a lower concentration than the medium).

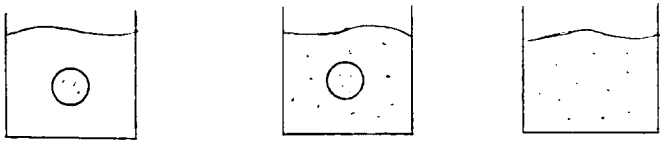
Microscopic Observation of Immobilized Cells

After incubation, the cross section of κ-carrageenan beads containing immobilized cells were examined under a microscope. Figure 2(a) is a thin section of the inner region of a bead where growth has reached a concentration of 1.5×10^9 cells/mL gel. Cells exist throughout the gel, an indication that nutrients can diffuse to the inner regions of the beads. Most of the immobilized cells exist in dense localized colonies. Although cells exist throughout the gel, it can be seen from Figure 2(a) that they are more dense near the surface. Using Toluidine blue stain and higher magnification (320×), we can

TABLE III
Volume of Gel versus Peak Cell Concentration

Volume of gel (mL)	Peak cell concentration (cells/mL gel)
1	1.9×10^9
5	1.0×10^9
10	7.0×10^8

TABLE IV
Cell Recycle Theory



(a) (b) (c)

	Peak gel concentration (cells/mL^{-1} gel)	Peak solution concentration (cells/mL sol.)	Total (cells)($\times 10^9$)
(a) Gel inoculated	9.5×10^8	2.8×10^7	4.8
(b) Gel and medium inoculated	5.8×10^7	1.0×10^8	5.2
(c) Only medium inoculated	—	1.0×10^8	5.0

see clearly that individual dual localized colonies exist in the thinly sliced gel [Fig. 2(b)].

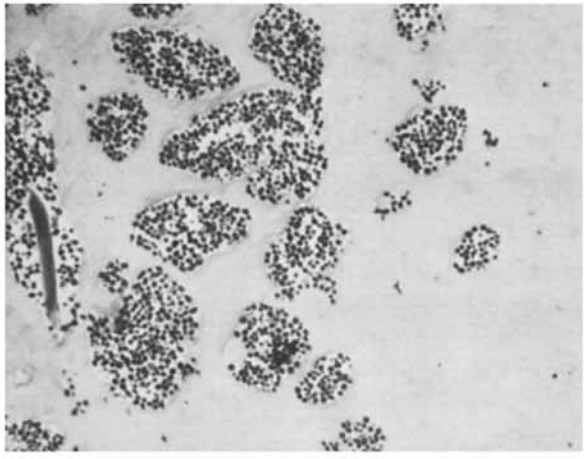
Effect of Entrapping Hydroxyapatite into the Matrix

Sustained viability

Five percent (w/v) tricalcium phosphate was incorporated into the κ-carrageenan gel as described in the **Materials and Methods** section. Figure 3 shows that the immobilized cells in these tricalcium phosphate enriched beads remained viable for a longer time. The viability of the cells in plain beads dropped sharply soon after the peak concentration was reached. Sustaining the cells in a viable state is advantageous because ethanol production is usually related to cell viability.[6]

To determine the reason for this sustained viability behavior, some possible causes were examined. Calcium sulfate crystals instead of tricalcium phosphate were entrapped in the gel. As Figure 4 indicates, the sustained viability is not a result of excess Ca^{2+} ions introduced by the dissolution of the crystals. The final pH of the medium containing plain beads was observed to drop to 2.5–3.0 during growth. This low pH in normally observed at the end of the batch ethanol fermentation. Adjusting the pH of the fermenting broth to 4.0 using NaOH during growth would show a higher cell viability, similar to those obtained with the tricalcium phosphate entrapment. This indicates that the buffering capacity of the crystals is maintaining the pH at a level more favorable to the cells.

(a)



(b)

Fig. 2. Microscopic observation of immobilized yeast cells in κ-carrageenan matrix: (a) a thin section of the immobilized cell bead (100×) and (b) organization of the localized colonies in thin sliced gel (320×).

Increased cell concentration in the beads

Figure 3 also shows that the cell concentration in the 5% tricalcium phosphate beads was 20% higher than that in the plain beads, possibly due to the crystals maintaining a pH level that is more favorable to growth. We did not attain maximum cell concentration due to the low yeast extract con-

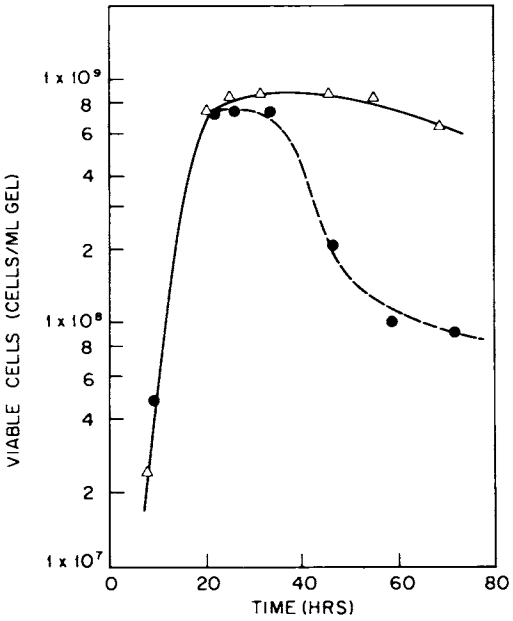


Fig. 3. Immobilized cell growth in κ-carrageenan with tricalcium phosphate entrapment: (●) plain κ-carrageenan, (△) plus 5% hydroxyapetite.

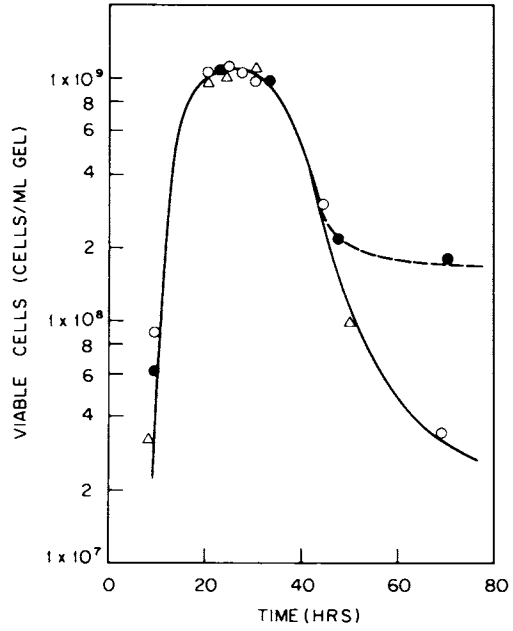


Fig. 4. Immobilized cell growth in κ-carrageenan with calcium phosphate entrapment: (●) plain beads, (○) with 5% $CaSO_4 \cdot 2H_2O$, (△) with 10% $CaSO_4 \cdot 2H_2O$.

centration used in this experiment. The effect of crystal dissolution on cell density was also investigated. Figure 5 shows three different types of beads: plain beads, 5% undissolved tricalcium phosphate beads, and 5% tricalcium phosphate beads with 25% of the crystals dissolved from the gel before incubation. Plain beads showed their typical pattern of a rapid loss in viability following their peak cell concentration. Beads with 5% tricalcium phosphate attained higher cell concentrations while maintaining cell viability. The beads where 25% of the crystals were dissolved showed the greatest cell density. This increase may be the result of increased porosity in the gel, facilitating an increased diffusion of nutrients. The viability remained high, indicating that enough crystals were left in the gel to buffer the medium.

Density effect

Another result of trapping tricalcium phosphate into the matrix is an increase in bead density. Table V shows the densities and settling velocities of different beads. A bead with 10% tricalcium phosphate crystals has twice the settling velocity of plain beads. This characteristic may be utilized for the operation of a fluidized bed reactor to prevent washout, thus permitting the use of increased feed rates. Increased feed rates increase the differential velocity of substrates and consequently enhance mass transfer.[1] The density change that occurs as the crystals dissolve may also be utilized in the separation of new and old beads in a fluidized reactor. The slower settling rate of old beads, which contain fewer crystals, could conceivably allow for the removal of these nonproducing beads in the overflow.

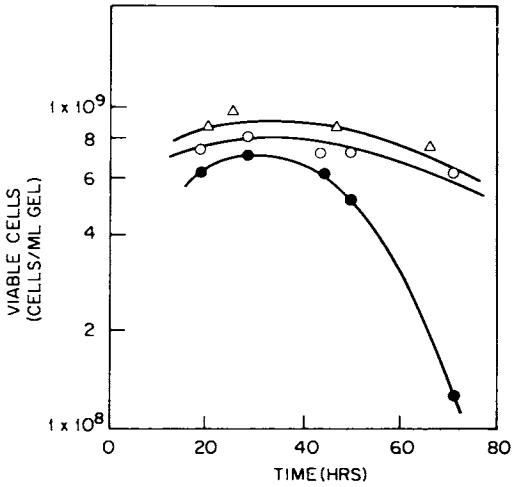


Fig. 5. Effect of porosity of the gel (after crystal dissolution) on yeast growth: (●) plain beads, (○) with 5% hydroxyapetite, (△) with 5% hydroxyapatite (25% crystal dissolved).

TABLE V
Density and Settling Velocity of Different Beads

Type of bead	Density (g/cm^3)	Settling velocity (cm/s)
Plain beads	1.03	42.6
5% Tricalcium phosphate	1.06	65.8
10% Tricalcium phosphate	1.08	89.8

Ethanol productivity of immobilized and free cells

Table VI shows the ethanol productivity of immobilized and free cells. The specific ethanol productivity for cells immobilized in plain beads is almost the same as for free cells, indicating that the immobilized cells are being fully utilized. The productivity of cells in a force addition homogeneously inoculated bead at similar cell densities is actually lower than the productivity of cells that exist due to immobilized growth. Diffusion of substrate to the inner region of the gel is probably limiting in this case. The specific ethanol productivity for cells immobilized in a matrix with tricalcium phosphate crystals is the highest of the four cases. This is a result of a favorable internal environment maintained by the trapped crystals.

CONCLUSIONS

The order of magnitude difference between immobilized and free yeast cell concentrations is a result of physical retention of the growing cells. Nutrients

TABLE VI
Ethanol Productivity of Immobilized and Free Cells

	Specific ethanol productivity (mg / 10^8cells^{-1} h^{-1})
Free cells	1.7
Immobilized cells in plain beads	1.8
Immobilized cells with tricalcium phosphate	2.5
Homogeneously inoculated immobilized cells	1.2

from a large volume are converted into cells that are confined to a smaller volume. The cell concentration difference is not observed when immobilized cells must compete with free cells on an equal basis.

Tricalcium phosphate entrapped in the matrix can a) maintain pH at a favorable level, b) sustain cell viability, c) increase gel density, and d) enhance ethanol productivity.

The coexistence of the crystals with the yeast cells within the gel (either κ-carrageenan or calcium alginate) produced encouraging results that warrant us to initiate larger-scale reactor studies (packed-column and fluidized-bed) in order to determine if similar results can be achieved in these systems.

This work was supported in part by a DOE-SERI grant. The authors acknowledge the help by Mr. S. S. Lee in the ethanol fermentation studies and Mr. G. F. Payne's help in preparing this article.

References

1. B. J. Abbott, *Annu. Rep. Ferment. Processes*, **1**, 205 (1977).
2. C. Bucke and A. Wiseman, *Chem. Ind.*, **4**, 234 (1981).
3. M. Kierstan and C. Bucke, *Biotechnol. Bioeng.*, **19**, 387 (1977).
4. D. Williams and D. M. Dynnecke, *Biotechnol. Bioeng.*, **23**, 1813 (1981).
5. M. Wada, J. Kato, and I. Chibata, *Eur. J. Appl. Microbiol. Biotechnol.*, **10**, 275 (1980).
6. S. S. Lee, F. M. Robinson, and H. Y. Wang, *Biotechnol. Bioeng. Symp.*, **11**, 641 (1981).

Accepted for Publication April 9, 1982