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REVIEW

Support-Bound Microbial Cells

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Index Entries: Immobilization; immobilized microbes; carriers; crosslinking; entrapment; biomass accumulation; encapsulation; continuous fermentation; covalent coupling; covalent binding; immobilized microbe technology; supports; continuous reactor; immobilization technology; adsorption; adsorptive forces; stationary phase; growth phase; sequential multi-enzyme reactions; synchronous growth; generation time; intracellular enzyme; enzyme; porous supports; polyacrylamide; collagen; carrageenan; multisequential enzyme reactions; microbe retention; periodicity.

I. Introduction

Early in their introduction to the subject of microbiology, students are indoctrinated in the techniques of streaking agar plates and colony counting with agar plates. Both of these techniques involve cells that are bound to supports. The support material in this case is a gel. These techniques may be classified as analytical procedures. Additionally, bound cells have been used for processing. A classical example of bound cells for this purpose is the trickling filter treatment of waste. More recently, bound or immobilized microbes have been used to produce products by either fermentation or enzyme-catalyzed conversions. It is this latter topic that is the subject of this review.

Bound microbial cells employed for the production of products may be engineered to efficiently utilize nutrients (carbon, nitrogen, oxygen, carbon dioxide) and energy. When a product is produced by a single enzyme within the cell, the utilization of a viable growing cell population represents a waste of both nutrients and energy. It would be appropriate, when a single enzyme within the cell is to be used, to find a condition that would kill the cell, but not affect the enzyme activity.

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When a product is produced only with a viable cell in either the stationary or the death phase, but not in the logarithmic growth phase, the nutrients would be wasted if they were utilized for growth. For this purpose, an appropriate approach might be to confine the cell by such techniques as encapsulation or entrapment to prevent reproduction while at the same time utilizing the nutrients for the production of the product.

Products such as single-cell proteins or yeast are most efficiently produced under logarithmic growth conditions. Effective delivery of nutrients at a rapid rate, and the subsequent efficient removal of metabolic waste materials are required to maximize cell growth and reproduction. Continuous reactors, such as the chemostat, fulfill these requirements; however, if the flow rates are too high, the cells are washed out. If the cells were retained by bonding them to a support, then high flow rate continuous reactor processes could be employed under what normally would be washout conditions. The bonding employed for this application would, of necessity, be quite different than that utilized for bonding cells that are to be used for a single enzyme reaction or for producing products in the stationary phase. Confinement by encapsulation or entrapment would minimize cell growth and reproduction.

Support-bound microbial cells or immobilized microbes have been the subject of a number of excellent reviews (*1–6*). The intent of this current review is to update prior literature and to assess the technology based on the current knowledge.

The technical interest in support-bound cells stems from the successes in applying immobilized enzymes to commercial production, which commenced in the late 1960s. The public awareness of recent advances in microbial technology originated with the controversy that evolved in the middle 1970s concerning genetic engineering. The excitement in this area has been further stimulated by the announcements in the newspapers of advances in genetic engineering. Public offerings of the stocks of the small biotechnology corporations have further stimulated the public awareness of this area.

II. Definition of Support-Bound Microbial Cells

The term, support-bound microbial cells, for the purpose of this review, is any microbe population, either alive or dead, that is bound to or within a support or to another cell by either adsorptive or covalent forces or a combination of those forces. The binding of these cells may be either permanent or temporary. Bound cells are synonymous with immobilized cells.

III. Bound Cells Employed for a Simple Enzyme Reaction

This is an alternative approach to immobilizing either a pure or purified enzyme. The advantages harvested by employing the whole cell rather than the purified enzyme are that isolation and purification are unnecessary and, in most instances, the enzyme is more stable in the cell environment than after isolation. The disadvan-

tages of employing the microbial cell are that an additional barrier to diffusion is encountered at the cell wall and/or membrane, and that less enzyme is immobilized per unit surface area or volume of reactor; this latter, in turn, implies the need for larger reactors, and, in addition, cells may contain unwanted extraneous reactions that may affect the product.

1. Bound Microbes for the Isomerization of Glucose

The immobilized enzyme to receive the greatest commercial attention is glucose isomerase. Glucose isomerase is an intracellular enzyme that converts less than one-half of the glucose in a solution exposed to it to fructose. The product defined as "high fructose corn syrup" is obtained by applying glucose isomerase to glucose derived from corn. High fructose corn syrup is employed as a substitute for sucrose in confectionary, food, and beverage applications.

The early efforts of Takasaki and Kanbayashi (*7*) and Tsumura (*8*) did not involve support-bound cells; however, their studies established that an enzyme could be fixed within the cell such that it would not leak out during the employment of those cells for enzyme conversions. Takasaki and Kanbayashi fixed the glucose isomerase within *Streptomyces sp.* by heating the cells to between 60° and 85°C for 10 min. Tsumura fixed the isomerase within *Streptomyces phaechromogenes* cells by β-ray irradiation. Tsumura et al. did bind the cells to a chitosan support (*9,10*).

Collagen was employed by Vieth and coworkers as the support for binding *Streptomyces phaeochromogenes* and *Streptomyces venezuelae*, and subsequently crosslinked with either formaldehyde or glutaraldehyde (*11–13*). Both of these microbes contain glucose isomerase. The support-bound cells were transferred to a column reactor that was operated at 70°C for 40 days on a continuous basis for the isomerization of glucose.

The support chosen by Chibata et al. (*14*) for the entrapment of *Streptomyces griseus*, which contains glucose isomerase, was polyacrylamide. Their studies led to the conclusion that magnesium ions were required for activation and that cobalt ions helped stabilize the enzyme activity within the bound cells.

A rather simple procedure for binding cells together by crosslinking with glutaraldehyde for 60 min at 25° was developed by Poulsen and Zittan (*15*) for *Bacillus coagulans* which contains glucose isomerase.

2. Other Bound Cells for Single Enzyme Reactions

An early effort of the Tanabe Seiyaku Company researchers was that of developing an immobilized system to convert fumaric acid to aspartic acid (*16–18*). *Escherichia coli* was the source of the enzyme, aspartase, employed for their studies. This enzyme is responsible for the addition of ammonia to the double bond in fumaric acid to form L-aspartic acid. Their first plan (*19*) was to develop a continuous system by entrapping the purified enzyme in polyacrylamide. Their efforts at these attempts led to the conclusion that the operational stability of this system was not satisfactory for industrial application. They were rewarded, subsequently, with success when they entrapped whole cells of *E. coli* in polyacrylamide gels by polymerizing the acrylamide monomer in the presence of *N,N'*-methylene-

bisacrylamide-β-dimethylaminopropionitrile and potassium sulfate for between 10 and 30 min at 40°C. They further noted that when the bound *E. coli* cells were suspended in a solution containing 1*M* ammonium fumarate and 1 m*M* Mg^{2+}, pH 8.5, and incubated at 37°C the activity increased 10-fold. This activation appeared to result from an increase in membrane permeability for the substrate and/or products owing to the autolysis of the cells within the gel. The enzyme did not leak from the entrapped preparation even with this lysis. This bound microbial cell system for the production of L-aspartic acid has been employed for commercial production at Tanabe Seiyaku Company since 1973. The overall production costs of the bound cell system are about 60% of the conventional batch process using intact cells. A new and improved system has been developed by this group, one that utilizes the κ-carrageenan for the entrapment of *E. coli*. The preparation is accomplished by cooling a κ-carrageenan gel containing *E. coli* to 10°C, soaking in 0.3*M* KCl, and then crosslinking with glutaraldehyde and hexamethylenediamine (*20*). The carrageenan-bound cells were demonstrated to be far more productive than those bound within polyacrylamide.

A support-bound microbial cell system pertinent to the medical industry is that for converting L-arginine to L-citrulline (*21*). L-Arginine deiminase found in *Pseudomonas putida* is responsible for converting the imine in arginine to the carbonyl in citrulline. These cells were entrapped in polyacrylamide. No surfactant is required for these entrapped cells, although the addition of cetyltrimethylammonium bromide is required for a high rate of citrulline formation in free cells. This implies that a change in the cell walls may have been induced during the entrapment process that readily allowed the penetration of the arginine to the active site of the enzyme or the removal of citrulline from that same site.

An antidote for hyperammoniemia and a component for amino acid infusion, malic acid, may be produced by the addition of water to the double bond of fumaric acid. This reaction catalyzed by the enzyme, fumarase, was a subject of the Tanabe Seiyaku group. The objective of the program was to convert a batch process into a continuous reactor operation (*22–24*). *Brevibacterium ammoniagenes*, a cell that has high fumarase activity, was entrapped in polyacrylamide. It was found that these bound cells formed succinic acid as a product. The separation of this acid from malic acid is very difficult. Succinic acid production could be suppressed by treatment of the bound cells with deoxycholic acid, bile acid, or bile extract. These detergents also enhance the formation of L-malic acid by the entrapped cells. One of their studies (*24*) demonstrated that *Brevibacterium flavum* had a high fumarase activity and a high operational stability after entrapment. A more recent procedure (*25*) involving entrapment in carrageenan was utilized on this latter microbe. The carrageenan bound *B. flavum* was demonstrated to be 5.2 times more productive than the polyacrylamide entrapped *B. ammoniagenes*.

The sunscreen agent, urocanic acid, produced from L-histidine through the catalytic removal of the amine and the formation of the double bond adjacent to the carboxyl group, is governed by the enzyme L-histidine ammonia-lyase. The batch process that employs either the extracted enzyme or the microbial broth of a *Achromobacter liquidium* was converted to a continuous packed-bed process by bonding the cells (*26*). This microbe also contains an adverse enzyme activity,

urocanase, that converts the desired product urocanic acid to imidazolone propionic acid. This undesired activity can be eliminated by heat treating the cells at 70°C for 30 min prior to the immobilization (*27*). The heat-treated cells are then entrapped in polyacrylamide. The half-life of the enzyme activity in a continuous packed-bed reactor and in the presence of magnesium ions at 37°C was estimated to be approximately 180 days.

L-Sorbosone, an intermediate in the synthesis of vitamin C, may be produced by the oxidation of sorbose in the presence of L-sorbose dehydrogenase. The enzyme found in *Gluconobacter melanogenus* was studied by Martin and Perlman (*28*). After entrapping the cells in polyacrylamide, they encountered a major problem in its application, that of oxygen delivery. When pure oxygen was delivered to the bound cells, the enzyme was rapidly destroyed.

6-Aminopenicillanic acid (6-APA) is required for the production of synthetic penicillin. Penicillin amidase found in *Escherichia coli* deacylates penicillin to produce 6-APA. Although polyacrylamide entrapped *E. coli* cell exhibits high penicillin amidase activity (*29*), they also contain penicillinase activity, which decomposes both penicillin and 6-APA. It was found that the penicillinase inactivation was exceedingly difficult. The penicillinase activity is much lower than the penicillin amidase activity; thus this preparation may have some utility. In a case of this nature, however, it may be judicious to employ the purified enzyme rather than the whole cell.

The entrapment of cells in polyacrylamide gel was first practiced by Mosbach and Mosbach in 1966. They bound the lichen, *Umbilicaria pustulata*, and demonstrated that the entrapped cells retain both esterase and decarboxylase activity over a 3-month period at 20°C with periodic testing (*30*). Subsequently, *Curvularia lunata* was bound by this procedure (*31*). These latter cells contain the enzyme, 11-β-hydroxylase, which converts Reichstein compound S to cortisol. The entrapped cells lose part of their activity after storage; however, the enzyme activity may be restored by incubating the preparation in a nutrient medium containing cortisone. This apparent reactivation may be a result of cell growth.

An immobilization process involving the crosslinking of cells by glutaraldehyde in the presence of polyamine is described in a British patent application (*32*). It should be noted that the intracellular enzymes were glutaraldehyde-sensitive; thus the polyamine presence is of great significance. According to this application, *Bacillus pasteurii* containing urease, *Kluyveromyces fragilis* containing lactase, and *leuconostoc oenos* containing malolactic enzyme were also bound successfully by this process.

IV. Bound Microbes for Sequential Enzyme Reactions and Cofactors

Coenyzme A produced by *Brevibacterium ammoniagenes* involves five sequential steps: Pantothenic acid → phosphopantothenic acid → phosphopantothenoylcysteine → phosphopantetheine → dephosphocoenzyme A → coenyzme A. For this purpose, the microbial cells were entrapped in polyacrylamide gel. En-

hanced productivity was noted upon the addition of sodium lauryl sulfate to the reaction mixture containing the entrapped cells. A 5-day half-life at 37°C was noted in a continuous column reactor (*33*).

Micrococcus denitrificans can reduce nitrate to nitrite and subsequently to ammonia. These cells were encapsulated in a liquid-surfactant membrane by Mohan and Li (*34,35*). They demonstrated the continuous reduction of nitrate by its disappearance without an additional supply of cofactors. The viability of this multienzyme system and the liquid membrane encapsulation approach was demonstrated by this study.

Another multisequential enzyme reaction is that which converts glucose to glutamic acid performed by *Corynebacterium glutamicum*. This microbe was again entrapped in polyacrylamide gel (*36*). The bound cells were incubated at 30°C with stirring in a medium containing glucose. The glutamic acid produced after 144 h was determined. The productivity of the bound cells was greater than that of the free cells. Difficulties in supplying oxygen to the entrapped cells in a continuous column reactor were encountered, indicating that an improvement upon this approach was necessary.

The continuous production of ethanol employing calcium alginate entrapped *Saccharomyces cerevisiae* with a solution of glucose delivered to a packed-bed column was examined (*37*). The conversion of glucose to ethanol was 90% of the theoretical yield. Although the half-life of this system is approximately 10 days, it was employed for the continuous production of ethanol over a 24-day period.

Agar bound *Rhodospirillium rubrum*, a photobacterium was found to be capable of producing hydrogen from malate and water in the presence of light by Bennet and Weetall (*38*).

V. Bound Cells for Stationary Phase Products

It is generally considered that cells must be viable to produce stationary phase products. Suzuki and Karube studied the production of antibiotics from entrapped microbes. These investigators compared collagen, alginate, and polyacrylamide for the entrapment of *Penicillium chrysogenum* to produce penicillin. The cells entrapped in alginate contained the highest activity; however, this preparation was found to be too fragile in the presence of phosphate ions to be used in a reactor. The mycelium bound within collagen was relatively inactive. The reagent used for tanning the collagen, glutaraldehyde, may have caused this inactivation. The polyacrylamide-bound cells were chosen to compare the productivity of the bound mycelia to that of the free microbe. The free mycelium decreased in production with repeated use while the entrapped mycelium initially increased in productivity and subsequently decreased gradually. The overall productivity of the bound cells was substantially greater than that of the free microbes. The half-life of the entrapped cells was estimated to be 6 times longer than that of the free microbes. The oxygen uptake of the entrapped cells was about 30% of that of the free microbes. The entrapped cells produced only a small quantity of penicillin under anaerobic conditions; therefore, oxygen was required for the system synthesizing

penicillin. It was further noted that upon grinding the entrapped cells, viable mycelium could be recovered.

Bacillus sp. was entrapped in polyacrylamide gel (*39*) with the intent of producing the antibiotic bacitracin. The productivity of the entrapped cells was found to be approximately 20–25% of the free cells. The lower rate of antibiotic production appeared to be caused by the inactivation of enzymes by the polymerizing reagents and the diffusion limitations of the substrates and/or products. Initial experiments with the entrapped cells demonstrated that the bacitracin production was reduced upon reuse when a fermentation medium was employed. Further, the bacteria were released from the entrapped preparation, and growth was noted in the fermentation medium. The effect of the medium composition on the productivity of bacitracin was then explored. Bacitracin productivity in a medium containing meat extract or peptone was greater than that in a medium containing carbohydrate. The productivity upon reuse was retained when peptone or meat extract was utilized. The maintenance of a constant level of bacitracin production with the reemployment of free cells was also noted when peptone medium was used. Bacitracin productivity in peptone medium from free cells was compared to that of entrapped cells with repeated batch productions. The productivity of the free cells diminished gradually after the third reuse and precipitously after the fourth reuse. The productivity of the entrapped cells increased with successive utilization until about the 7th reuse. After that, the productivity of the entrapped cells appeared to remain constant through about the 14th run. The increased activity of the entrapped preparation was attributed to the synthesis of additional cells within the gel.

Polyacrylamide entrapped *Clostridium butyricum* was employed for sensing biochemical oxygen demand (BOD) in wastewater (*40*). These entrapped cells remained active for long periods of time under specific conditions. This technique could be employed for the rapid estimation of BOD.

VI. Bound Cells Employed Under Growth Conditions

The bound cells to this point were either not reproducing or they were reproducing at a very low rate. The studies of Kennedy et al. (*41*) appeared to be a transition between the previous discussions and the rapid growth of cells. These authors entrapped *Saccharomyces cerevisiae* and *E. coli* in hydrated titania or zirconia gels, and then examined them for viability and oxygen uptake. They found the rate of oxygen uptake of the bound cells was about 30% of that of the same number of free cells. The reduced rate of oxygen uptake may have resulted from the restriction by the metal hydroxides of access to the aerated buffer and a decrease of cell surface area available for oxygen diffusion. Another investigation utilizing this entrapment procedure for *Serratia marcescens* demonstrated that the growth of cells was associated with the matrix when fresh culture was added.

A continuous fermentation system that employed bound cells under growth conditions, the anaerobic filter for waste treatment, was developed by McCarty and coworkers (*42,43*). Facultative and anaerobic microbes are adsorbed on the surface

of stones. The bound film of cells produces progeny that are then elaborated into the flowing stream. The process time for reducing BOD and producing methane in this packed-bed reactor is markedly reduced compared to the traditional batch reactors as a result of the elaboration of the progeny cells from the film of bound cells on the stone surfaces.

Durand and coworkers at the National Institute of Applied Sciences (INSA) in Toulouse, France investigated the relative retention of microorganisms by various supports (*6,44*). Wood, polyvinylchloride, pouzzolan (a finely ground, burnt clay or shale), resins, and silica were studied on a comparative basis. They found that wood chips retained between 6 and 120 times more *Saccharomyces carlsbergensis* cells than any other of the supports studied. They also noted in this study the adverse effect of glutaraldehyde on these cells in a comparison of untreated silica and silica treated with glutaraldehyde. The conclusion of their study was that retention, simultaneously, depends on the type of adsorbant and the type of microbe.

Earlier studies of Harttori and Furusaka (*45,46*) demonstrated that *E. coli* bound by adsorption on ion exchange resins displayed a periodicity in the release of free cells. It was further noted that these cells would divide synchronously in the growth media. The release of progeny from the resin surface proceeded according to the age distribution of the adsorbed cells. Helmstetter and Cooper (*47,48*) demonstrated that *E. coli* adsorbed on filter membrane and exposed to a flowing nutrient medium exhibited shorter doubling times than the freely suspended microbes. Hattori et al. (*49–51*) observed that both in batch studies and under continuous cultivation adsorbed cells demonstrated diminished generation times.

A pilot plant for producing fermenented beverages based upon the retention of microbes on a solid support and which consists of two columns, the first of which is used for changing wort into beer and the second, which is termed the storage column, is used for the maturation of the beer, was constructed by the INSA group (*52*). PVC and porous bricks were the supports evaluated in this plant. The equilibrium state was obtained very rapidly in the reactor. It was also noted that if the reactor was stopped it could be started again without any lag time. Wort was fermented with a residence time of 90 min. Fermentations without the addition of yeast have proceeded over an 8 month period. Since the progeny cells are removed from the reactor by the flowing liquid, no plugging was encountered during this period.

A study comparing bound and free forms of the yeast, *Saccharomyces uvarum*, was conducted by Navarro and Durand (*53*). In this study, several supports and several techniques for bonding were compared. γ-Aminopropyltrimethoxysilane-treated porous silica was used to adsorb the cells and to covalently couple the same cells through glutaraldehyde. In addition, adsorption of this microbe to granules of porous brick was evaluated. These studies were performed in column reactors. The generation time for the free cells was found to be 8 h. The cells adsorbed on the γ-aminopropyltrimethoxysilane-treated porous silica were found to have a generation time of about 4 h under the same conditions. The cells adsorbed on the porous brick were found to have a generation time of 2 h and 45 min, while those covalently coupled through glutaraldehyde to the silane-treated porous silica were found to have a generation time of 1 h and 45 min; all generation times were determined by nephelometry. The column effluent was monitored at 600 nm and

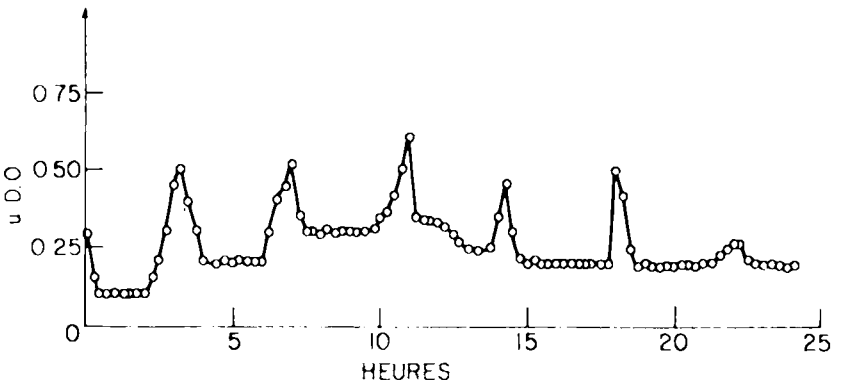

Fig. 1. Optical density plotted as a function of time (hours). Synchronous growth exhibited by cells adsorbed on γ-aminopropyltrimethoxysilane-treated silica. The generation time was determined by the time between the peak values. Reproduced with the permission of G. Durand from *C. R. Acad. Sci.* **290** Serie D, 455 (1980).

the optical density was plotted as a function of time. Peak values (see Fig. 1) were noted every 4 h in the case of the cells adsorbed on the γ-aminopropyltrimethoxysilane-treated porous silica. These peaks represented synchronous growth and the generation time was determined by the time between the peaks. This synchronous growth was maintained for four to five generations (4–5 peaks in Fig. 1) in the case of cells adsorbed to the silane-treated silica. The synchronous growth was maintained for 5–7 generations when the cells were bound to brick, and for 10–12 generations when the cells were covalently coupled to the silica surface. The authors attribute this synchronous growth to the fact that the daughter cells, in the form of the buds, are released from the surface while the parent cell is retained on the support.

VII. Optimization of Porous Supports for Binding Cells and Growth

The accumulation of viable microbes may be related to the pore morphology of a dimensionally stable inorganic support (*54–56*). The critical factors involved in this relationship are the cell or spore dimensions, the mode of reproduction, and the pore diameter of the support. High quantities of cells are bound to the support when at least 70% of the pores of an inorganic support have pore diameters in the range of one times the smallest major dimension through five times the largest major dimension of a cell that reproduces by fission. High accumulations of microbes that reproduce by budding are achieved when 70% of the pores have pore diameters that are greater than one times the smallest dimension of the cell and less than four times the largest cell dimension. High binding and growth are experienced in the case of microbes that form spores and exhibit mycelial growth when at least 70% of the pores of the inorganic support have pore diameters that are greater than one times the smallest spore dimension, but less than 16 times the largest dimension of the spore. These correlations were confirmed by varying the physical parameters of

supports as well as their chemical composition. It was further indicated in these studies that the accumulation of cells and growth was also dependent upon the surface contributions of the support material. This was demonstrated in comparative studies of *Streptomyces olivochromogenes* and *Penicillium chrysogenum*. The initial growth of the *Streptomyces olivochromogenes* was substantially greater on the more negative surfaces of glass than on cordierite. The opposite occurred in that *Penicillium chrysogenum* indicated greater growth on the more positively charged cordierite than on the glass.

VIII. Conclusion

Support-bound microbial cell technology offers the opportunity to convert traditional batch processes that require extensive plant facilities to the more economical approach of the continuous reactor. The advantages gained by employing the bound cells are to be found not only in the reduction in plant size, but also in the conservation of energy. By employing the appropriate binding technique with respect to whether a single enzyme within the cell is required or whether growth is required for the production of products, conservation of energy may be achieved in the form of the nutrients utilized and the heat required for either the enzyme reaction or the growth of cells.

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