Immobilized Cells: Principles and Applications

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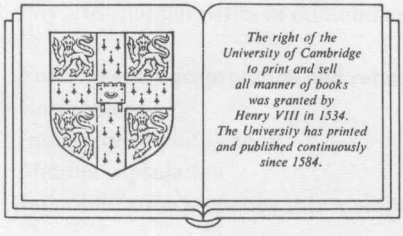

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Authors' note

In quoted work, the original authors' scientific names for organisms have been retained.

Introduction

Definition and scope

Although the word 'immobilized' is now part of the language of Biotechnology its scope is delineated more by practical examples than by precise definition. Bucke (1983) focuses attention upon the prevention of free cell movement within the liquid phases of a reactor system. Mattiasson (1983) gives greater emphasis to the matrix in, or upon, which the cells are immobilized, and also to the point that the cells may be at one extreme fully capable of division or at the other so debilitated as to possess only a single type of enzyme activity. For the purposes of the present book an immobilized cell is defined as a cell, or a remnant thereof, that by natural or artificial means is prevented from moving independently of its neighbours to all parts of the aqueous phase of the system under study. Such a definition is sufficiently broad as to include virtually all the commercially significant examples, but would also include many examples of natural immobilization, such as multicellular organisms and communities of microbial organisms. It is therefore necessary to add the further qualification that the purpose of such immobilization must be for use in the production or service industries, i.e. biotechnology (for definitions of which, see Bull, Holt & Lilly, 1982). It is easy to see why authors move rapidly to the details of the subject and avoid such wide-ranging definitions. It is, indeed, the economic exploitation of immobilized cells which is of special significance although there is no doubt that the enormous effort being devoted to them will lead to advances in many other, apparently unrelated, areas of scientific study.

Difficulty is sometimes experienced in distinguishing between conventional fermenters and immobilized cell reactors. A fermenter, regardless of its exact type, is a culture vessel designed to be used firstly to increase the biomass of the active organisms with which it has been inoculated. This growth may accompany or precede the conversion of growth substrate into some useful metabolic product. In the batch mode of operation the fermentation is terminated at a time considered appropriate and the cycle of inoculation, growth and harvesting repeated. Such a regime is satisfactory for many products, particularly those produced late in the growth cycle of the culture. Products of the latter type are often described as being non-growth-related, species-specific or

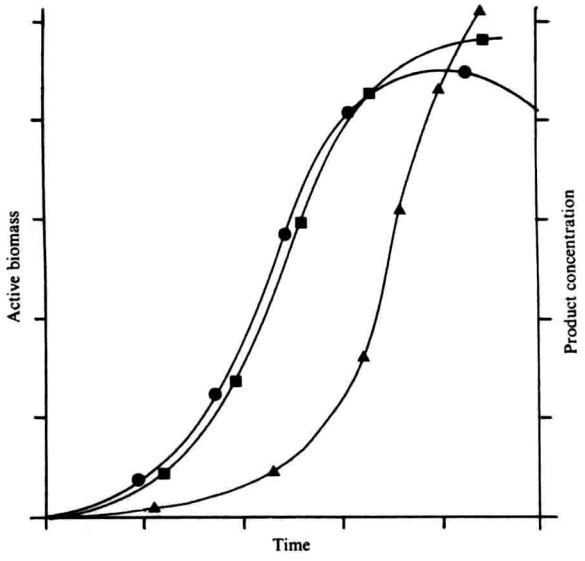

Figure 0.1. The time-course of growth (●) and the production of a typical growth-related (■) and non-growth-related (▲) fermentation product in a batch fermenter.

secondary in nature (Figure 0.1). In the continuous mode of operation growth-related substances or biomass itself can be readily produced but many metabolites of economic interest are often not produced in sufficient quantities. In either batch or continuous mode it may be possible to use the active biomass produced in the fermenter to carry out the biotransformation of a separately added precursor rather than the total *de novo* biosynthesis of a particular molecule. In some cases the cultured organism may not even be capable of the total synthesis. In such a situation it is clear that the living cells are being used simply as a biocatalyst. One of the major problems is, however, that the biocatalyst is generally not very stable and catalytic activity is often lost quite rapidly after the cessation of cell growth. Much of the industrial and academic research into enzyme and cell immobilization has been carried out in the search for 'extended-life biocatalysts'. It has been found by experiment that free cells grown up in a fermenter may have their useful biocatalytic life considerably extended by immobilization (Figure 0.2). The reasons for this increased stability are not entirely clear. Returned in the immobilized state to a vessel essentially similar to the original fermenter the system is an immobilized cell reactor. The boundaries between these uses of the two terms are, however, somewhat blurred. A fermenter may encourage the natural adsorption of living cells onto its internal surfaces,

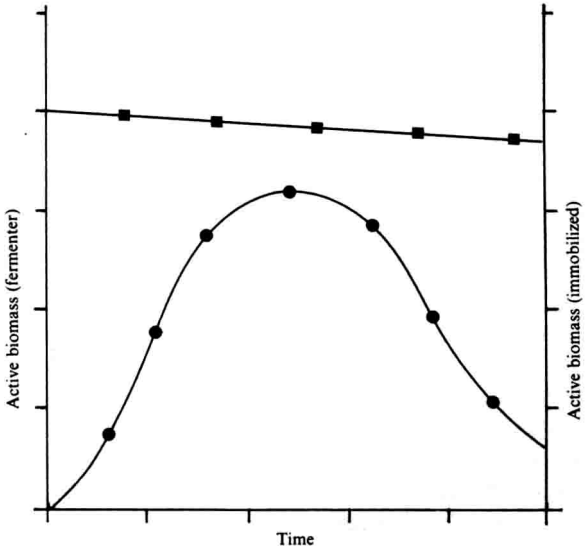

Figure 0.2. Time-course of the change in the amount of biocatalytically active biomass present in a typical batch fermenter (●) and an immobilized cell reactor (■).

or additional support material may be intentionally added, either in sheet or particulate form. Active biomass is then retained if the fermenter is operated continuously. An alternative strategy might be to recycle the active biomass after it has been separated from the liquid culture medium outside the fermenter. The use of flocculent strains of micro-organism may allow the liquid medium to be run off without the cells. Conversely, it is possible to reactivate a debilitated living immobilized cell system by adding fresh culture medium to allow regrowth, *in situ*, of the immobilized cells.

Another viewpoint on immobilization is to regard it as a strategy for process intensification (Atkinson, Black & Pinches, 1980). It is generally accepted that processes using biocatalysts suffer from severe disadvantages compared to simple inorganic catalysis. Chief amongst these are the low concentrations of active biomass and the enormous quantity of water generally present. These lead to low rates of reaction (per unit of reactor volume) and high product recovery costs. Immobilization can pack more active biocatalyst per unit of reactor volume and hence reduce reactor size and cost (Figure 0.3).

The development of techniques for cell immobilization has followed that of enzyme immobilization. In the latter case the enzyme is first separated from cells or cell debris and then, in a crude or purified form,

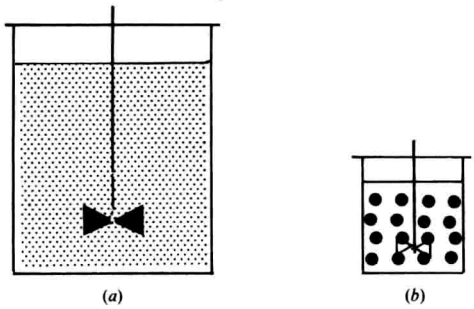

Figure 0.3. Diagram illustrating the principle of process intensification by immobilization, resulting in higher volumetric productivity for the same quantity of active biomass. (*a*) Free cell reactor; (*b*) immobilized cell reactor.

immobilized. The literature on immobilized enzymes is very extensive and falls outside the scope of the present book. Only in a few special cases, where the more advanced state of knowledge is relevant, will reference be made to work on immobilized cell-free enzymes. The interest in immobilized cells draws attention to the fact that due to economic or scientific reasons there are drawbacks to the use of the comparable immobilized enzyme systems. Those with a knowledge of enzyme immobilization techniques will note that many of the methods can be used also for cell immobilization. This is no more than is to be expected from the nature of the reactive groups involved. In general, however, techniques for cell immobilization are cheaper and easier to carry out. This is because the enzyme separation stage is entirely avoided and the functional activity of the enzymes is protected from damage by a multitude of other substances present, together with the enzyme, in a cell. In addition, many alternative and often physiologically mild techniques are available for cell immobilization.

Cellular material for immobilization

Virtually any cell type or subcellular organelle is a possible candidate for immobilization. The limiting factor in the first instance is likely to be the availability of sufficient cells of the required type. Provided they can be conveniently cultured microbial cells present few problems and many are very readily available and, for that reason alone, have been extensively studied. The ease of supply of conventional brewer's or baker's yeast (*Saccharomyces cerevisiae*) has made it one of the most common organisms for the purely academic investigator. Industrialists have, naturally enough, chosen to work on organisms of direct relevance to some already existing or proposed processes. Certain service industries,

such as wastewater treatment, have always based their biological processes upon mixed cultures of micro-organisms which show a natural immobilization characteristic. The natural flocculation of yeasts has been of fundamental significance to the production of potable alcohol for centuries. Many micro-organisms attach themselves to the walls of culture vessels and several types of fermenters have been specifically designed to encourage this tendency, allowing simple biomass retention and the development of continuous processes so that the fermenter used for initial growth becomes an immobilized cell reactor by mere change of operational procedure.

As the artificial techniques for cell immobilization developed attention turned to the cells of animals and plants, where the problems of availability are much greater. Techniques for the culture of plant cells are well advanced and suitably selected cell lines of many species are already grown in fermenters. The preparation of large quantities of plant cells is, however, by no means easy. Because of the slow rate of cell division and the need for exceptional care in maintaining sterility, progress has not been as rapid as with microbial immobilization, but the possible rewards of success have interested many academic and industrial laboratories (Brodelius & Mosbach, 1982). One of the major considerations, however, is the extent to which the plant cells grown in culture are able to carry out the metabolism of the differentiated cells typical of the original plant (Fuller & Bartlett, 1985). It may well be that the process of immobilization, simulating more closely the conditions existing in an intact plant, can help solve this difficult problem. Plant cells, by their possession of a cell wall, are comparable to microbial cells and similar techniques of immobilization may be used. Due to the absence of a cell wall, animal cells are inherently more fragile. The problem of the bulk culture of many normal animal cells is further aggravated by the possession of two attributes: the need for a support surface to grow on and contact inhibition. Some typical forms of cell are given in Figure 0.4. When a normal animal cell in culture contacts another cell its further division is inhibited, leading to low cell yields from culture. The development of microcarrier systems (see, for example, Pharmacia, 1981; Hirtenstein & Clark, 1983) has allowed natural immobilization to be exploited as a means of obtaining high yields. Abnormal cells, such as those in cancerous cell lines, may be grown more readily in bulk but are incapable of producing many of the important substances of the anchorage-dependent cell lines. Few of those working with immobilized cells have the necessary skills to cope with the difficulties of animal cell culture.

When the cell wall is removed from a plant or microbial cell the protoplast, surrounded by the plasma membrane, is left as a functional unit. This is comparable to an animal cell in terms of its response to

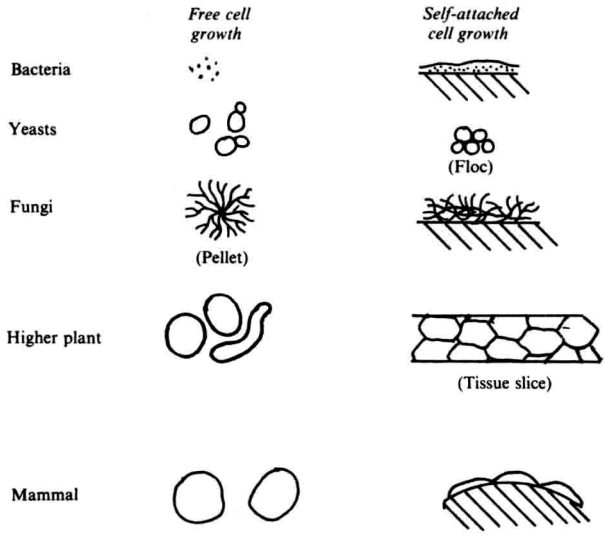

Figure 0.4. Some typical physical forms of cells used in immobilization studies.

osmotic shock and has occasionally been the subject of specialized immobilization studies. The continued integrity of the plasma membrane of any cell is essential if it is to remain capable of growth and division. It is possible to alter the selective permeability of the plasma membrane to allow the freer diffusion of substrates into the cell or metabolites out of it. This process is described as permeabilization and may be achieved by a wide variety of different techniques. With mild treatments only a relatively slight change in the selective permeability of the cells will occur. This will generally be sufficient to prevent further cell division but still allows the majority of the cell's metabolism to continue and may even stimulate the activity of certain metabolic pathways. With harsh treatments the metabolic integrity of the cell will be destroyed and only certain facets of enzymatic activity will remain. These remnants are, however, often considerably stabilized and even activated, by the treatment. The process of permeabilization may be carried out before, during or after immobilization.

Quite distinct from the process of permeabilization is the prior disruption of living cells by an appropriate technique of cellular homogenization and the recovery of separated organelles, or fragments thereof. Conventional techniques for purification of the fractions can be applied and the resulting preparation immobilized. Much of this work is carried out for purely academic investigations, albeit of great scientific

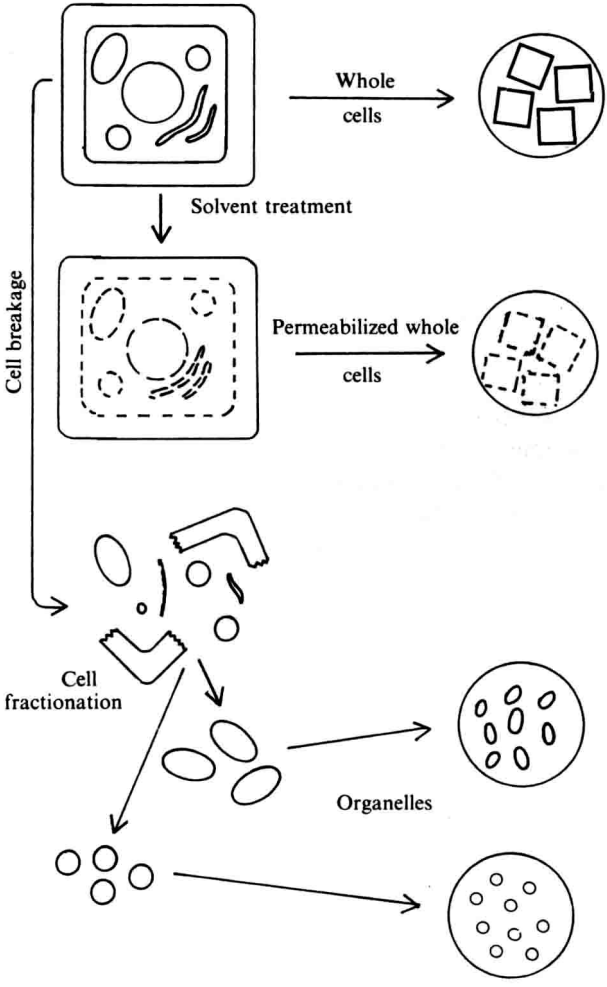

Figure 0.5. Some possible approaches to the immobilization of the biocatalytic activity of cells by entrapment.

merit, with industrial application a long way in the future. Chloroplasts, mitochondria, microsomes, peroxisomes, chromatophores and other membrane fragments have all been investigated (see, for example, the review article by Tanaka & Fukui, 1983). Various combinations of immobilized enzymes, subcellular fragments, organelles and intact cells have also been tried, often with components taken from different species (Figure 0.5).

Immobilization methods

A simplified classification of immobilization methods is given in Table 0.1. Adsorption of cells onto a solid surface is probably the mildest of cell immobilization techniques. In its simplest form it is also one of the cheapest methods and therefore of particular use where an industrial process does not result in a 'high added-value' product. The success of the technique depends, in the first instance, upon the properties of the cells themselves. The natural evolution of species has produced many organisms that are capable of adhering to surfaces. Some of these cause problems to man, as in the microbial fouling within pipes and on all manner of other surfaces exposed to an aqueous environment. An important method of wastewater treatment, the trickling filter system, has made use of this property for decades. In adsorption there is generally an initial weak attachment of the cells, which can be easily reversed. This is followed by the development of stronger (multiple attachment) binding. Often extracellular material produced by the cells is important in fixing the cells to the adsorption substrate and this may be followed by a natural entrapment of the cells in a biopolymer matrix. In the older industrial processes the selection of the appropriate support material has been largely fortuitous and has relied upon the organism's own ability to attach to the surface. The more recent active interest in the adsorption process has led to the study and development of all manner of support materials, ranging from simple crude mineral substances to complex ion-exchange derivatives of organic polymers. In addition, the physical form of the support has received considerable attention, particularly with regard to porosity and the shape of the material. Coating support materials with other substances or mixing the cells with various polyelectrolytes (often natural polymers) can increase cell adsorption. One of the interesting features of adsorption is that it is not only microbial cells which adhere. Cultured cells from higher plants and animals also show this property, often to a remarkable degree. Many of the newer supports for adsorption are essentially non-selective. Indeed, this very fact makes them useful for a wide range of applications. An alternative strategy, however, is to make the support extremely selective. This may be considered as analogous to the process of affinity chromatography, now widely used by biochemists. As far as the cells are concerned it is very mild. Only those with the appropriate surface reactive groups will be bound, but the binding can be strong under operational conditions.

It is a simple observation in the preparation of alcoholic beverages that yeasts are capable of flocculating. In this process, the individual cells are, in effect, adsorbed onto one another. A similar process occurs during the settling of sewage sludge. The process has been extensively studied in the brewing industry and, by strain selection, it is possible to control the time

Table 0.1. *A simple classification of cell immobilization techniques*

Technique	Some typical Advantages	Disadvantages
Adsorption		
Neutral supports	Cheap Mild Reusable Simple	Cell leakage Sensitive to pH changes
Charged supports	Mild Reusable Simple	
Flocculation	Simple Mild	Cell leakage Diffusional limitations
Entrapment		
Natural polymers	Mild Simple	Diffusional limitations
Synthetic polymers	May be simple	Toxicity Expensive Diffusional limitations
Covalent coupling	Permanent	Toxicity Expensive
Containment	Mild Simple Reusable	Diffusional limitations Expensive

at which this occurs and so influence the properties of the product. Under continuous culture conditions, by means of relatively simple design features of the vessel, it is possible to select flocculating strains of organisms which are not generally considered to show this phenomenon. A further extension is to add polyelectrolytes or other suitably active material, and initiate flocculation artificially. Again there is a long-standing and extensive literature on this topic from the traditional brewing and wastewater treatment industries. The chief interest in the present context is its application to the development of new industrial processes. Although fundamentally different in its origin it is convenient to mention at this point the process of pelletization. It has been found by experiments that certain filamentous micro-organisms can be encouraged to grow as discrete, spherical mycelial masses (pellets) rather than as a single mat. This is usually achieved by using a spore inoculum and carefully controlling the cultural conditions. In particular, a relatively strong agitation and the absence of 'dead-spots' in the liquid flow pattern may be favourable. This cannot really be considered as an immobilization technique but may lend itself to process intensification and the development of processes based on extended biocatalytic activity.

Gel entrapment, because of its mildness, ease of operation and wide applicability, has been one of the most studied of all immobilization techniques. As the name implies, it is merely the trapping of cells within a three-dimensional matrix such that the pores in the matrix are smaller than the cells. It follows, therefore, that the matrix (or lattice) must be constructed *de novo* around the cells rather than the cells adsorbed into a preformed porous material. A wide range of materials is available for entrapping cells, ranging from natural polysaccharides and proteins to purely synthetic polymers such as polyacrylamide. In general, it may be said that the cells are trapped within a gel and that the gel may be permanent or reversible. Soft and reversible gels may have poor functional properties while the permanent ones may involve potentially toxic stages in their preparation. In some techniques the gels are further stabilized after preparation. Obviously, where full cell viability needs to be retained the milder methods must be used, but for many potential applications it may be adequate to retain only a very limited amount of enzymic diversity in the cells. Where full viability is retained it will be possible to increase the active biomass within the gel by supplying appropriate nutrients and growth conditions, thus promoting process intensification. Excessive growth in a weak gel may, however, disrupt the matrix and allow cell leakage.

One problem encountered in using cells entrapped in a gel is the diffusional resistance of the gel to substrate and products. A logical means of reducing this is to have only a thin layer of material around groups of cells, which are free to move within their own particular capsule. The advantages of larger particle size are retained by this encapsulation, but still with some diffusional problems. Two major categories may be distinguished. In the first are those where the retaining capsule barrier separates two essentially similar liquid phases. The barrier may be of a lipophilic material, simulating the type of membrane which already surrounds a cell, but with much simpler permeability properties. Alternatively the capsule could, perhaps, be of synthetic or natural polymers. A second approach uses liquid two-phase systems, made by separately preparing two solutions of different polymers, such as a dextran and a PEG (polyethylene glycol). When mixed they are able to form an emulsion of one aqueous phase within another. By adjusting the properties of the two phases very interesting partition effects may be achieved so that products, which might cause inhibition of the catalytic activity, may be effectively removed from the reaction environment into the other phase.

An extension of the membrane separation principle is to have two compartments to the system. There are a number of different methods of achieving this. Obviously, the surface area of the membrane, in relation to the mass of active biocatalyst present, and the length of the average diffusion path from one compartment to another, are important. The